The Effects O-1602, O-1918 or Different Dietary Fatty Acids have on Whole body and Skeletal Muscle Energy Homeostasis: A Focus on Putative Cannabinoid Receptors, Adiponectin and Fatty Acid Signalling.

Doctor of Philosophy

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DECLARATION OF AUTHENTICITY

I, Anna Roy, declare that the PhD thesis entitled 'The effect that O-1602, O-1918 and different dietary fatty acids have on whole body and skeletal muscle homeostasis, with a focus on putative cannabinoid receptors and adiponectin signalling' is no more than 100,000 words in length including quotes and exclusive tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature: …… Date: 10 /11 / 2017

ABSTRACT

The prevalence and incidence of obesity and related co-morbidities such as insulin resistance and type two diabetes mellitus (T2DM) are rapidly increasing world-wide. These health burdens negatively impact individuals, families, the community and the government, for a multitude of reasons. The search for suitable therapeutic targets for obesity, in combination with a healthy diet and increased physical activity is a strategy for weight management and associated co-morbidities such as T2DM.

The endocannabinoid system is a lipid derived signalling system that is modulated by different dietary fatty acids and has a role in regulating energy homeostasis. Modulation of cannabinoid receptor 1 (CB_1) reduces whole body adiposity and increases oxidative capacity within the skeletal muscle. The skeletal muscle is a major contributor to whole body energy metabolism through the oxidation of fatty acids, insulin signalling and glucose uptake, therefore understanding the impact that endocannabinoid pharmaceutics have on this tissue is essential. Putative endocannabinoid receptors including G Protein-Coupled Receptor (GPR55) and G Protein-Coupled Receptor 18 (GPR18) may potentially be beneficial pharmaceutical targets for obesity and associated co-morbidities. GPR55 expression is up-regulated in visceral adipose tissue in obesity and T2DM. Surprisingly *GPR55 knockout* mice have increased adiposity and reduced physical activity. While GPR18 has been shown to be expressed in adipose tissue, its role in obesity is unknown. Furthermore the role that atypical cannabinoid compounds, O-1602 or O-1918 (which have/ are hypothesised to have affinities for these receptors and therefore modulate these putative cannabinoid receptors) have in obesity and skeletal muscle homeostasis, following chronic treatment has yet to be determined.

The dietary intake of specific fatty acids can also alter circulating endocannabinoid concentrations depending on the type of fatty acid consumed. The effect that different dietary fatty acids have on the putative cannabinoid receptors GPR55 and GPR18 and whole body energy homeostasis in obesity is unknown.

Therefore the overall focus for this thesis was to determine the role that atypical cannabinoids have on homeostatic skeletal muscle signalling and whole body energy metabolism in obesity. In addition to this, the effect that different dietary fatty acids have in obesity and whole body energy metabolism were also determined, which may partially be attributed to GPR55 and GPR18 signalling.

The results from this thesis demonstrate that O-1602 does not alter markers of oxidative capacity or adiponectin signalling: NFATc1, PGC1α and APPL1, which are involved in skeletal muscle homeostasis *in vitro* in C_2C_{12} myotubes. However, in the *in vivo* model of diet induced obesity (DIO), chronic administration (for six weeks) of O-1602 beneficially reduced adipose tissue mass and two appetite regulating hormones, leptin and gherlin. Despite this, O-1602 administration also had negative side-effects for the liver, as the liver was enlarged in weight (liver weight/ body weight %) and there were increased concentrations of circulating aspartate aminotransferase (AST) and regulated upon activation of normal T-Cells expressed and secreted (RANTES), in light of these findings there may also potentially be detrimental effects in other organs such as heart, brain, liver, gastric mucosa, adipose tissue and kidneys based on these increased circulating markers.

In addition, this thesis demonstrated that the chronic administration of O-1918 in a DIO rodent model did not alter body weight or body composition, however, brown adipose tissue mass was decreased and circulating concentrations of both gherlin and leptin were also decreased. Unfortunately some circulating pro-inflammatory cytokines IL-1α, IL-2, IL-17α, IL-18 and RANTES (some of these markers are related to insulin resistance and T2DM development) were increased, as well as markers of organ damage including circulating AST and RANTES.

The final aim of this thesis, was to determine the effect that the consumption of high fat different dietary fatty acid diets; either saturated (SFA), monounsaturated (MUFA) or omega-6 polyunsaturated fatty acids (PUFA), or the effect that energy restriction (by reintroducing the SCD) has on whole body energy metabolism in a rodent model of obesity *in vivo*. The energy restricted group had reduced body

fat composition compared to some of the high fat different dietary fatty acids groups. In the DIO rats fed the high fat different dietary fatty acids there were no differences in fat pad mass between groups, however, the DIO MUFA had increased epididymal fat pad mass compared to the SCD and DIO SCD groups. Additionally, the perirenal fat pad mass was increased in the SFA, MUFA and PUFA groups compared to the SCD and DIO SCD groups. The blood glucose response to synthetic insulin in the DIO SFA group compared to DIO PUFA was increased at 120 and 180 minutes. Additionally the circulating concentration of IL-10 was decreased in the DIO MUFA groups compared to the SCD and DIO SCD groups, the remaining circulating concentrations of cytokines and hormones were not altered between the different dietary feeding regimes.

Therefore, the outcomes of this thesis highlight that the chronic administration of either O-1602 or O-1918 in DIO rodents does impact markers of obesity, which could potentially be due to their effects on the putative cannabinoid receptors GPR55 and GPR18. While O-1602 reduces adiposity, O-1918 does not. Nonetheless, biochemically O-1918 increases pro-inflammatory cytokines, some of which are involved in the pathogenesis of T2DM, this diminishes this compounds suitability as a future obesity therapeutic. Furthermore, the different dietary fatty acids or energy resistriction in DIO did not appear to have an effect on whole body markers of energy homeostasis in this current study. Further molecular work is required to look at tissue specific effects in both the pharmaceutical and different dietary fatty acid projects detailed in the current thesis.

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PUBLISHED MANUSCRIPTS ARISING FROM THIS THESIS

Simcocks AC, O'Keefe L, Jenkin KA,Mathai ML, Hryciw DH & McAinch AJ 2014, A potential role for GPR55 in the regulation of energy homeostasis, *Drug Discovery Today*, vol. 19, no. 8, pp. 1145 - 1151.

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Slattery C, Jenkin KA, Lee A, **Simcocks AC**, McAinch AJ, Poronnik P, Hryciw DH, 2011, 'Na+ H+ exchanger regulatory factor 1 (NHERF 1) PDZ scaffold binds an internal binding site in the scavenger receptor megalin', *Cell Physiology & Biochemistry* vol. 27, no. 2, pp.171-178.

Hryciw DH, Jenkin KA, **Simcocks AC**, Grinfeld E, McAinch AJ, Poronnik P 2012, 'The interaction between megalin and ClC-5 is scaffolded by the Na^+-H^+ exchanger regulatory factor 2 (NHERF2) in proximal tubule cells', *International Journal of Biochemistry and Cell Biology*, vol.44, no.5, pp.815-23.

Cornall LM, Mathai ML, Hryciw DH, **Simcocks AC**, O'Brien PE, Wentworth JM, McAinch AJ 2013, 'GPR119 regulates genetic markers of fatty acid oxidation in cultured skeletal muscle myotubes', *Molecular Cell Endocrinology*, vol.365, no.1, pp.108-18.

O'Keefe L, **Simcocks AC**, Hryciw DH, Mathai ML, McAinch AJ 2013, 'The cannabinoid receptor 1 and its role in influencing peripheral metabolism, *Diabetes, Obesity & Metabolism*, vol.16, no.4, pp.294-304.

Jenkin KA, O'Keefe L, **Simcocks AC**, Grinfeld E, Mathai ML, McAinch AJ, Hryciw DH 2015, 'Chronic administration of AM251 improves albuminuria and renal tubular structure in obese rats', *Journal of Endocrinology*, vol.225, no.2, pp.113-24.

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ABSTRACTS, ORAL & POSTER PRESENTATIONS

International Conference – Oral Presentation

XII International Congress on Obesity, Kuluar Lumpar, Malaysia.

Anna Simcocks, Lannie O'Keefe, Kayte Jenkin, Michael Mathai, Deanne Hryciw, Andrew McAinch 2014, 'O-1602 reduces adiposity, but increases liver mass in a diet induced obese rat model'.

University Conference – Oral Presentation

University College of Health & Biomedicine Student Conference, Victoria University, St Albans, Australia.

Anna C. Simcocks, Lannie O'Keefe, Kayte A. Jenkin, Michael L. Mathai, Deanne H. Hryciw, Andrew J. McAinch 2013, 'O-1602 reduces adiposity, but increases liver mass in a diet induced obese rat model'.

National Conference – Oral Presentation and Published Abstract

Australian & New Zealand Obesity Society (ANZOS) 2013, Melbourne, Australia.

Anna Simcocks, Lannie O'Keefe, Kayte Jenkin, Michael Mathai, Deanne Hryciw, Andrew McAinch 2013, 'Chronic administration of O-1602 diminishes adiposity in diet induced obese rat model', *Obesity Research & Clinical Practice*, Vol. 7, pp. e45.

Local University Presentation – Oral Presentation

Biomedical & Lifestyle Diseases Unit (BioLED) Scientific Meeting October, Western CHRE, Sunshine Hospital.

Anna Simcocks, Lannie O'Keefe, Kayte Jenkin, Michael Mathai, Deanne Hryciw, Andrew McAinch 2013, 'Chronic administration of O-1602 diminishes adiposity in diet induced obese rat model'.

National Conference – Oral Presentation and Published Abstract

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A.C. Simcocks, L.M. Cornall, E. Grinfeld, M.L. Mathai, D.H. Hryciw, A.J. McAinch 2011,'Putative endocannabinoid receptor GPR55 is expressed in skeletal muscle', *Obesity Research & Clinical Practice*, Vol. 5, p. 63–64.

Oral Presentation – Presentation to an international audience

Victoria University and University of Texas El Paso (UTEP) health research symposium, presented using satellite technology.

A.C Simcocks, D.H. Hryciw & A.J McAinch 2011, The effect of GPR55 on adiponectin signalling and oxidative capacity in skeletal muscle.

COLLABORATIVE ABSTRACTS, ORAL & POSTER PRESENTATIONS

Poster Presentation – (Generated and presented by Shaan Naughton)

Metabolic Diseases: break through discoveries in diabetes and obesity Conference, St Kilda, Melbourne, Australia.

Shaan Naughton, Cesar Meza, **Anna Simcocks**, Cynthia Montenegro, Lannie O'Keefe, Catalina De La Pena, Michael Mathai, Deanne Hryciw, Sudip Bajpeyi, Andrew McAinch 2016, Adiponectin is capable of sparing skeletal muscle glycogen content with a monounsaturated fatty acid high fat diet.

Poster Presentation – (Presented by Supervisor Professor Andrew McAinch)

Metabolic Diseases: break through discoveries in diabetes and obesity Conference, St Kilda, Melbourne, Australia.

Anna C Simcocks, Lannie O'Keefe, Kayte A. Jenkin, Michael L. Mathai, Deanne H. Hryciw, Andrew J. McAinch.

'O-1918 does not alter food intake, body weight or adiposity but reduces appetite hormones and increases certain pro-inflammatory cytokines in a diet induced obesity model'.

Oral/ Poster Presentation – (Presented by Supervisor Professor Andrew McAinch)

Australia & New Zealand Obesity Society (ANZOS) 2016, Brisbane, Australia.

Anna C. Simcocks, Lannie O'Keefe, Kayte A. Jenkin, Michael L. Mathai, Deanne H. Hryciw, Andrew J. McAinch.

'O-1918 does not alter food intake, body weight or adiposity but reduces appetite hormones and increases certain pro-inflammatory cytokines in a diet induced obesity model', *Obesity Research & Clinical Practice*.

Oral Presentation – (Presented by Supervisor Professor Andrew McAinch)

Australian & New Zealand Obesity Society (ANZOS) 2014, Sydney, Australia.

Abstract

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Abstract

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Abstract

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Abstract

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Abstract

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International Conference (abstract submitted but did not attend) – Poster Presentation (Presented by student collaborator Lannie O'Keefe)

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A. Simcocks, L.M Cornall, D. Hryciw, A. McAinch 2012,'O-1602 does not regulate mRNA expression of markers involved in oxidative capacity and adiponectin sensitivity in C_2C_{12} myotubes', *Obesity Research & Clinical Practice*, Vol. 6, p. 70.

Abstract

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Abstract

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Abstract

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ABBREVIATIONS

2-AG	2 Arachidonylglycerol
3T3-L1	Continuous pre-adipocyte cell line derived from mouse
5'AMPK	5' Adenosine Monophosphate Kinase
Δ9 THC	Delta 9 Tetrahydrocannabinol
µL	Microlitre
µg/ Kg	Micrograms per kilogram
x g	Times Gravity
AA	Arachidonic Acid
Abn-CBD	Abnormal Cannabidiol
AdipoR1	Adiponectin Receptor1
AdipoR2	Adiponectin Receptor2
AEA	Anandamide
AEC	Animal Ethics Committee
AEEC	Animal Experimentation Ethics Committee
AIHW	Australian Institute of Health & Welfare
Akt	Serine/Threonine Kinase
ALT	Alanine Transaminase
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Kinase
AMPKα2	Adenosine Monophosphate Kinase alpha 2
AM251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide

ANOVA	Analysis Of Variance
ApoE	Apolipoprotein E
APPL1	Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1
APPL2	Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 2
ARC	Animal Resource Centre
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
AUC	Area Under the Curve
BAT	Brown Adipose Tissue
βActin	Beta Actin
βHAD	Beta-Hydroxyacyl-CoA Dehydrogenase
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
BRIN-BD11	Insulin Secreting Pancreatic Islet Beta Cell Line
BSA	Bovine Serum Albumin
BV-2	Microglial cell line derived from mouse
C2C12	Continuous myoblast cell line derived from CH3 mouse
Ca2+	Intracellular calcium
CART	Cocaine and Amphetamine Regulated Transcript
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBD	Cannabidiol

Cdc42	Cell division control protein 42
CEBPα	CCAAT/ enhancer-binding protein alpha
CH3	A cross between a Bagg female and a DAB male mouse
CHO	Carbohydrate
CHO cells	Chinese Hamster Ovary Cells
CID2440433	3-[[4-(2,3-dimethylphenyl)-1-piperazinyl]carbonyl]-N,N-dimethyl-4-(1-pyrrolidinyl)-benzenesulfonamide
CO2	Carbon Dioxide
CREB	cAMP Response Element Binding Protein
CVD	Cardiovascular Disease
DIO	Diet Induced Obese
cDNA	Complementary Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate Treated Water
DIO	Diet Induced Obese
DMAB	Dimethylaminoborane
D-MEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonucleic Acidase
EC50	Effective Concentration 50
Echo MRI	Echo Magnetic Resonance Imaging
EDL	Extensor Digitorum Longus
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
EPO	Erythropoietin

ER	Endoplasmic Reticulum
ERK	Extracellular Signal Related Kinase
ERK 1/2	Extracellular Signal Related Kinase 1/2
ERRα	Estrogen Related Receptor α
FA	Fatty Acid
FAT/CD36	Fatty Acid Translocase/ Cluster Differentiation 36
FOXO	Forkhead Box Protein
FOXO1	Forkhead Box Protein 1
g	Grams
g/ kg	Grams/ kilogram
g/ mL	Grams/ millilitre
G-SCF	Granulocyte Colony Stimulating Factor
GLP-1	Glucagon Like Peptide 1
GLUT4	Glucose Transporter Type 4
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPR18	G Protein Coupled Receptor 18
GPR55	G Protein Coupled Receptor 55
GPR119	G Protein Coupled Receptor 119
GPCR	G Protein Coupled Receptor
GRO/KC	Growth Regulated α Protein/ Keratinocyte Chemoattractant
GSK494581A	[4-[2-fluoro-4-(1-methoxyethyl)phenyl]piperazin-1-yl]-[2-(4-fluorophenyl)-5-methylsulfonylphenyl]methanone
GSK319197A	[4-(3,4-dichloro-phenyl)-piperazin-1-yl]-(4′-fluoro-4-methanesulfonyl-biphenyl-2-yl)-methanone
GTPγS	Guanosine Triphosphate gamma S

HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
HEC-1B	Human Endometrial Adenocarcinoma Cells
HEK293	Human Embryonic Kidney 293 Cells
HFD	High Fat Diet
HMW	High Molecular Weight
HPRT1	Hypoxanthine Phosphoribosyltransferase
IFN-γ	Interferon Gamma
ICa	L type calcium channel current
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin-1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-10	Interleukin 10
IL-12p70	Interleukin 12p70
IL-13	Interleukin 13
IL-17α	Interleukin 17 alpha
IL-18	Interleukin 18
IMCL	Intramyocellular Lipids
ip	Intraperitoneal
ip. GTT	Intraperitoneal Glucose Tolerance Test
ip. IST	Intraperitoneal Insulin Sensitivity Test

IU	International Units
IU/ Kg	International Unit per Kilogram
IUPHAR	International Union of Basic and Clinical Pharmacology
i.c.v.	Intracerebroventricular
JAK	Janus Kinase
Kg	Kilogram
LA	Linoleic Acid
LDL	Low Density Lipoprotein
LMW	Low Molecular Weight
LPI	L α-lysophosphatidylinositol
LPS	Lipopolysaccharide
mins	Minutes
mmol/ L	Millimoles per litre
mRNA	Messenger Ribonucleic Acid
m2	Metre squared
MAPK	Mitogen Activated Protein Kinase
mg	Milligram
mg/ daily	Milligrams/ daily
mg/ kg	Milligram/ kilogram
mg/ mL	Milligrams/ millilitre
MCP-1	Monocyte Chemotactic Protein 1
MCSF	Macrophage Colony Stimulating Factor
MIP-3	Macrophage Inflammatory Protein-3
MJ/ Kg	Megajoules/ Kilogram
mL	Millilitre

mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MUFA	Monounsaturated Fatty Acid
n	Number
NADH	Nicotinamide Adenine Dinucleotide
NAGly	N-Arachidonylglycine
nM	Nanomolar
NFκβ	Nuclear Factor Kappa Beta
NFAT	Nuclear Factor of Activated T-cells
NFATc1	Nuclear Factor of Activated T-cells calcineurin dependent 1
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Respiratory Factor 2
O-1602	Methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol
O-1918	1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene
ob/ ob	Obese mouse with a genetic leptin mutation
OCT	Optimal Cutting Temperature Compound
OEA	Oleoylethanolamide
p	Probability
p38 MAPK	p38 Mitogen Activated Protein Kinase
p44/ 42	phospho 44/ 42
PAI-1	Plasminogen Activator Inhibitor 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDC	Pyruvate Dehydrogenase Complex

PDK	Pyruvate Dehydrogenase Kinase
PDK4	Pyruvate Dehydrogenase Kinase isoform 4
PEA	Palmitoylethanolamide
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PhD	Doctor of Philosophy
PI3K	Phosphoinositide 3-Kinase
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
p.o	Per os
PUFA	Polyunsaturated Fatty Acid
RANTES	Regulated upon Activation of Normal T-Cells Expressed and Secreted
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homologue gene family member A
Rimonabant	N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
RNA	Ribonucleic Acid
SAT	Subcutaneous Adipose Tissue
SCD	Standard Chow Diet
SCG	Superior Cervical Ganglion
SFA	Saturated Fatty Acid
SRE	Serum response element
SR141716A	N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
THCV	Tetrahydrocannabivarin
TNFα	Tumour Necrosis Factor alpha
TRPV	Transient receptor potential cation channel subfamily V
TRPV1	Transient receptor potential cation channel subfamily V member 1
UCP-1	Uncoupling Protein 1
VAT	Visceral Adipose Tissue
VEGF	Vascular Endothelial Growth Factor
WAT	White Adipose Tissue
WIN55212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
WHO	World Health Organisation

CHAPTER ONE

1.0 Literature Review

1.1 Obesity and Type 2 Diabetes Mellitus

The prevalence and incidence of obesity and related co-morbidities such as type two diabetes mellitus (T2DM) are dramatically increasing world-wide (WHO 2016). Overweight and obesity is defined by the Word Health Organisation (WHO 2016) as 'abnormal or excessive fat accumulation that may impair health'. Body mass index (BMI) is calculated using the equation 'weight (kg) divided by height (m^2)', and is commonly used to predict overweight and obesity. In the adult population being overweight is considered at value of 25 kg/ m^2 or greater, while obesity is considered at a value of 30 kg/ m^2 or greater (WHO 2016). The World Health Organisation (WHO) estimated that in 2016, a concerning 1.9 billion adults were overweight and 600 million adults were obese globally (WHO 2016). Although, caution does need to be taken as BMI does not take into account total body composition and therefore does not differentiate between total body fat and lean tissue mass.

The Australian Bureau of Statistics Australian Health Survey from 2011-12 found that an estimated 35% of adults were overweight and an additional 28% of adults were considered to be obese, which has been increasing over the past few decades (ABS 2013). According to the (ABS 2011) the total cost of obesity to the Australian society and government was estimated at an overall $58.2 billion including direct financial costs ($8.3 billion, which included productivity costs as well as health system and carers costs) and burden of disease costs ($49.9 billion, which included the cost of disability, loss of well-being and premature death as a result of obesity and/ or related co morbidities). It is therefore not surprising that with the impact that obesity has both at an individual and family level as well as a community and government level that in 2008 obesity was considered to be one of the nine now national health priority areas for Australia (AIHW 2016).

Obesity has a number of associated co-morbidities one of which is T2DM (Guh, Zhang et al. 2009), which is a metabolic disorder accompanied by hyperglycaemia (Lin and Sun 2010). The development of hyperglycaemia in T2DM can involve increased production of glucose through gluconeogenesis by the liver (Stumvoll, Goldstein et al. 2005) stimulated by a hormone in the α islets of the pancreas called glucagon (Taborsky 2010) which still appear to function during the hyperglycaemic state (Lee, Berglund et al. 2012), reduced secretion from the β islets of the pancreas of a blood glucose lowering hormone insulin, as well as impaired insulin action in insulin sensitive cells such as the liver (Stumvoll, Goldstein et al. 2005). The development of insulin resistance can encompass a reduced or delayed response to the action of insulin in insulin sensitive tissues such as the skeletal muscle and this is generally as a result of a defect of insulin signalling within the cell which can be due to the accumulation of ectopic lipids (Guilherme, Virbasius et al. 2008). If a defect in insulin secretion is present in T2DM, it is thought to be due to a decline in secretion rate in individual β cells that secrete insulin and/ or decreased β-cell mass, which take into account both the number of cells and size (Cantley and Ashcroft 2015). Further it appears in T2DM that only in severe insulin insufficiency is there an apparent increase in glucose production. There are some inconsistencies/ disagreements in the literature as to whether gluconeogenesis or glycogenolysis (which is the release of glucose from stored glycogen, and the liver is a major body store for glycogen) is responsible for this increase in hepatic output of glucose (Nuttall, Ngo et al. 2008).

Hyperglycaemia can also be managed in combination with increased physical activity (Toledo, Menshikova et al. 2008), which increases energy expenditure and increased muscle contractions can cause glucose uptake by insulin-independent mechanisms (Merry and McConell 2009) which may involve intracellular calcium (Ca^{2+}), adenosine monophosphate kinase (AMPK), reactive oxygen species and nitric oxide (Merry and McConell 2009). A healthy well balanced diet high in fibre, can also help with the management of hyperglycaemia.

In addition to lifestyle modifications, hyperglycaemia can also be managed pharmaceutically using oral anti-hyperglycaemia agents such as metformin which decreases hepatic glucose output, thiazolidinediones which improves insulin sensitivity and dyslipidaemia or sulfonylurea derivatives which enhance β cell insulin secretion (Stumvoll, Goldstein et al. 2005). Uncontrolled or poorly controlled T2DM, where chronic hyperglycaemia is event, can result in a number of health complications such as neuropathy, nephropathy and cardiovascular disease which can impact the quality of life for an individual and can result in an increased risk of mortality (AIHW 2013).

1.2 White Adipose Tissue

Obesity primarily results from an increased energy intake accompanied by reduced energy expenditure over a sustained or chronic period of time (AIHW 2016). This energy imbalance contributes to an increased storage of triglycerides as an energy store within the white adipose tissue (WAT), resulting in adipocyte hypertrophy (Hausman, DiGirolamo et al. 2001). WAT not only acts as a fat storage, it also has been shown to function as an endocrine organ which produce and secrete adipokines (Kershaw and Flier 2004). Some adipokines secreted from mature adipocytes include leptin, adiponectin (described in section 1.3.2 in more detail), tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6) (Kershaw and Flier 2004). Changes in either the production of these adipokines and/ or modulation in downstream signalling pathways are implicated in the obese state (Greenberg and Obin 2006). There is also evidence to suggest that obesity is associated with chronic low grade inflammation (Weisberg, McCann et al. 2003), whereby there can be an increased production of pro-inflammatory circulating cytokines and chemokines, which are involved in increasing inflammatory processes and this has a number of negative multi-organ systemic effects (Guri and Bassaganya-Riera 2011).

1.3 Skeletal Muscle

The skeletal muscle is a metabolically active tissue involved in regulating whole body energy expenditure (Zurlo, Larson et al. 1990). Skeletal muscle signalling is altered in obese, insulin resistant and T2DM states (Abdul-Ghani and DeFronzo 2008; Abdul-Ghani and DeFronzo 2010). The skeletal muscle is a highly plastic and adaptable organ that is influenced by its' environment (Stewart and Rittweger 2006). Nutrients are stored such that they are readily available for the skeletal muscle to fuel muscle contractions in the form of glycogen (Griffiths and Rahim 1978) as well as intramyocellular lipids (with the main type being intramuscular triglycerides) (Li, Xu et al. 2015).

The skeletal muscle is a heterogeneous organ which is comprised of different fibre types (Saltin, Henriksson et al. 1977). Initially skeletal muscles were divided into different muscle types based on colour either red or white skeletal muscle (Needham 1926). At a histochemical level, muscle fibres can be divided into slow and fast twitch fibres. Using ATPase histological staining human skeletal muscle fibre types include: Type I, Type IIa, and IIb fibres (Brooke and Kaiser 1970). While using immunohistochemical staining (which focuses on thedifferent myosin heavy-chain isoforms) indicate that human IIb fibres are IIx fibres as they contain MyHCIIx which is similar to the Type IIx fibres found in rat and mouse skeletal muscle.

Type I fibres contain higher amounts of mitochondria and capillaries compared to Type II fibres. Type IIa fibres have a greater mitochondrial and capillary density than Type IIb fibres. Type I and Type IIa fibres therefore have a greater oxidative phenotype, Type IIa fibres have properties which resemble type I fibres, although they are not as fatigue resistant. While Type IIb fibres have more of a glycolytic phenotype. Obese and T2DM individuals tend to have a higher proportion of the glycolytic IIb muscle fibre phenotypes, (Tanner, Barakat et al. 2002; Oberbach, Bossenz et al. 2006). Type IIx is a mixture between the characteristics of IIa and IIb fibre types. In rodents, skeletal muscle fibre types appear to more homogenous (Delp and Duan 1996).

Insulin sensitivity can be influenced by the fibre phenotype of the skeletal muscle (Lillioja, Young et al. 1987; Henriksen, Bourey et al. 1990; Song, Ryder et al. 1999). In which slow twitch oxidative fibres and capillary density is correlated with insulin action in insulin resistant men, further fasting plasma glucose and insulin were negatively correlated with capillary density (Lillioja, Young et al. 1987). Insulin-stimulated glucose transport is enhanced in slow twitch muscle fibres (Henriksen, Bourey et al. 1990; Song, Ryder et al. 1999). While physical inactivity has been shown to have negative implications to insulin sensitivity, as there is a reduction in oxidative capacity and insulin sensitivity within the skeletal muscle (Papa 1996). Furthermore, the ratio between glycolytic and oxidative enzyme activity in the skeletal muscle of T2DM or obese individuals has also been shown to be related to insulin resistance (Simoneau, Colberg et al. 1995; Simoneau and Kelley 1997). In addition to this, in obesity, insulin resistance and T2DM there is a defect in skeletal muscle metabolism, which appears to involve increased fatty acid uptake into the skeletal muscle, combined with the reduction in oxidative capacity and is thought to result in intramyocellular triglyceride accumulation (Phielix and Mensink 2008). This process has a negative impact on skeletal muscle homeostasis and potentially contributes to the development of insulin resistance and T2DM (Phielix and Mensink 2008).

At a biochemical level, skeletal muscle fibre phenotype are intricately regulated by a number of different signalling pathways, some pathways include calcineurin/ NFAT pathway (Chen, Glover et al. 1998; Naya, Mercer et al. 2000), and through peroxisome proliferator γ co-activator 1α (PGC-1α) (Lin, Wu et al. 2002). NFATc1 and PGC1α are discussed in more detail in section (1.3.1.1 and 1.3.1.2).

1.3.1 Biochemical markers involved in skeletal muscle metabolism

As the skeletal muscle is a highly adaptable and plastic organ, genetic predetermining factors, as well as environment and lifestyle influences can impact signalling pathways within this tissue (Argiles, Campos et al. 2016). High fat diets (HFD) are energy dense and therefore contribute to excess energy intake and have low satiety inducing properties which have been shown to induce obesity (Golay and Bobbioni 1997). Chronic consumption of a HFD can negatively alter metabolic signalling within the skeletal muscle in obesity, insulin resistance and T2DM (Abdul-Ghani and DeFronzo 2008; Abdul-Ghani and DeFronzo 2010).

A number of proteins involved in the signalling for oxidative capacity, fatty acid oxidation and metabolism (including adiponectin signalling) are altered (or hypothesised to be altered) in the obese, insulin resistant and/ or T2DM states. Some key proteins involved in these processes are discussed in further detail below (in sections 1.3.1.1 – 1.3.1.6 and 1.3.2).

1.3.1.1 Nuclear Factor of Activated T Cells (NFATc1)

NFATc is from the family of Nuclear Factor of Activated T Cells (Hogan, Chen et al. 2003). NFATc is highly expressed in skeletal muscle (Hoey, Sun et al. 1995) and could be involved in the transformation of fast-twitch fibres to slow-twitch fibre (Liu, Youn et al. 2001), as *in vitro* work shows that upon nerve stimulation NFATc localises to the nucleus of the cell (Liu, Youn et al. 2001). NFATc initiates the transcription of genes that are involved in the cellular remodelling in the skeletal muscle to a fibre type that has a greater oxidative capacity (Chin, Olson et al. 1998; Jordan, Jiang et al. 2005). In states of increased intracellular calcium, such as during muscle contraction Ca^{2+}-dependent phosphatase calcineurin dephosphorylates NFAT (Hogan, Chen et al. 2003), which in turn causes NFAT to translocates to the nucleus of the cell, here this transcription factor regulates a number of genes (Timmerman, Clipstone et al. 1996; Rao, Luo et al. 1997). NFAT is rephosphorylated when intracellular calcium concentrations are back to resting concentrations and NFAT translocates back to the cytosol of the cell (Timmerman, Clipstone et al. 1996; Hogan, Chen et al. 2003).

NFATc1 is expressed in rat skeletal muscle (Mutungi 2008), however this protein's expression appears to be dependent on fibre type, as NFATc1 is expressed in both type I and type IIa muscle fibres, but not type IIb fibres (Mutungi 2008). The expression of NFATc1 was more abundant in slow twitch soleus muscle than the fast twitch extensor digitorium longus (EDL) muscle (Mutungi 2008). In the soleus tissue, NFATc1 was located mainly in the nuclei of the cell, while in EDL NFATc1 was located in both the nuclei and cytoplasm of the cell (Mutungi 2008). Currently the role of NFAT in obesity, insulin resistance and T2DM is unclear, however it could be hypothesised that as there are greater amounts of glycolytic muscle fibres and less oxidative muscle fibres in these states (Tanner, Barakat et al. 2002) NFATc1 expression could be decreased among these individuals, or greater amounts of phosphorylated NFAT could be located in the cytosol of the cell, which could be due to a decreased translocation of NFATc1 to the nucleus, however this still remains unknown and further research into this is required.

1.3.1.2 Peroxisome proliferator activator receptor gamma co-activator 1 alpha (PGC1α)

PGC1α is an isoform of Peroxisome proliferator activator receptor gamma co-activator 1 (PGC1), which is a transcriptional co-activator that controls the transcription of genes involved in regulating fibre type and mitochondrial biogenesis (Sparks, Xie et al. 2005; Handschin, Choi et al. 2007). The physiological role of this transcription factor in the skeletal muscle is to increase the transcription of myosin heavy chain type I and type II a, as well as myoglobin resulting in the remodelling of muscle fibres which have a greater oxidative capacity (Scherer, Williams et al. 1995). Furthermore, PGC1α promotes mitochondrial biogenesis by increasing the transcription of key proteins involved in this process, including: Estrogen Related Receptor α (ERRα), Nuclear Respiratory Factor 1 (NRF1) and Nuclear Respiratory Factor 2 (NRF2) which are involved in the regulation of mitochondrial DNA transcription and replication (Mootha, Lindgren et al. 2003; Patti, Butte et al. 2003).

A downstream signalling target of PGC1α is glucose transport type 4 (GLUT4) (Michael, Wu et al. 2001). Up-regulation of PGC1α has also positively been associated with GLUT4 mRNA expression

(Michael, Wu et al. 2001; Park, Scheffler et al. 2009). GLUT4 is an important protein involved in insulin signalling and the uptake of glucose into insulin sensitive tissues. Dysfunction in the phosphorylation of this protein can result in the development of T2DM (Michael, Wu et al. 2001) due to the association with insulin resistance. PGC1α may also be altered as a result of the reduced adiponectin signalling, as adiponectin receptor 1 (AdipoR1) knockout mice have reduced PGC1α mRNA and protein expression (Iwabu, Yamauchi et al. 2010).

Skeletal muscle fibre phenotypes that have a greater oxidative capacity express significantly higher amounts of PGC1α when compared to fibre phenotypes that are more glycolytic (Lin, Wu et al. 2002). PGC1α is down regulated as a result of consuming a diet high in fat (Sparks, Xie et al. 2005) and is up-regulated during exercise (Russell, Feilchenfeldt et al. 2003). PGC1α is down regulated in obesity and T2DM (Nagatomo, Gu et al. 2009) and in conditions associated with lower physical activity and a higher fat diet (Mootha, Lindgren et al. 2003). Gain of function for PGC-1α in a transgenic mouse model also provides some insight into the role that this transcription factor has when it is over-expressed in the skeletal muscle (Lin, Wu et al. 2002). In this gain of function model there is 20% and 10% conversion of fast-twitch type IIb muscle fibres to type IIa and slow-twitch type I fibres respectively. Additionally there is also an activation of mitochondrial genes that are involved in oxidative metabolism and isolated muscle obtained from the PGC1α transgenic mice also have an increased resistance to fatigue when the muscle is electrically stimulated (Lin, Wu et al. 2002). Taken together the current evidence suggests that the up-regulation of PGC1α in the skeletal muscle maybe beneficial to the obese and T2DM states.

1.3.1.3 Fatty Acid Transporter Cluster of Differentiation36 (FAT/CD36)

FAT/CD36 is a plasma membrane receptor that facilitates the transport of long chain fatty acid uptake into the skeletal muscle (Glatz, Luiken et al. 2010). FAT/CD36 over-expression in metabolically stable C_2C_{12} myotubes, results in the up-regulation of markers involved in fatty acid oxidation, including FOXO1 and PDK4 (Nahle, Hsieh et al. 2008). However, interestingly the rate in which long chain

fatty acids are taken up into the skeletal muscle does not correlate to the rate in which they are oxidised, which subsequently results in increased fatty acid synthesis and intramyocellular lipid (IMCL) storage, as observed in conditions like obesity (Luiken, Arumugam et al. 2001). In obesity the abundance of FAT/CD36 is increased and transport capacity of this protein and triglyceride content are also increased, while fatty acid oxidation is decreased (Li, Paran et al. 2011). Furthermore, in the presence of insulin and/ or skeletal muscle contraction there is an up-regulation of FAT/CD36 protein expression in the plasmmlemma (Jain, Chabowski et al. 2009). This seems to show that there is a compensatory mechanism for the increased circulating fatty acids present is these states, therefore if there is to be further up-regulation of this receptor in the skeletal muscle, there would also need to be an up-regulation in the fatty oxidation to prevent the accumulation of IMCL in the skeletal muscle and exacerbate the defective skeletal muscle signalling present in these pathophysiological states.

1.3.1.4 βHydroxyacyl-CoA Dehydrogenase (βHAD)

βHAD is a key enzyme located in the inner mitochondrial membrane and is a regulator of the metabolic pathway β oxidation, this enzyme targets medium and long chain fatty acids substrates (Eaton, Bartlett et al. 1996). Deficiency of βHAD, results in inhibition of β oxidation which in turn causes long chain 3-hydroxyacyl- and 2-enoyl-CoA and carnitine esters to accumulate (Kler, Jackson et al. 1991). High fat feeding alters βHAD activity, McAinch et al. (2003) demonstrated that βHAD mRNA in slow twitch skeletal muscle was up-regulated when compared to fast twitch skeletal muscle (represented as change from high carbohydrate diet) in rats fed a HFD for eight weeks. Furthermore, βHAD activity was also up-regulated in both soleus and EDL skeletal muscle as a result of high fat feeding in the same rats (McAinch, Lee et al. 2003). While in humans the ratio of NADH oxidase/ βHAD in *vastus lateralis* skeletal muscle are significantly increased in both obesity and T2DM when compared with lean skeletal muscle (Ritov, Menshikova et al. 2010) and a combination of weight loss plus exercise intervention in obese or T2DM individuals also significantly increased the content of βHAD in the skeletal muscle (Ritov, Menshikova et al. 2010). This demonstrates that a HFD, obesity, T2DM,

weight loss and physical activity are all factors which alter the amount of βHAD present in skeletal muscle.

Therefore, the up regulation of βHAD in combination with the up-regulation of FAT/CD36 would be beneficial in the obese, insulin resistant and T2DM state, providing that the fatty acids do actually undergo the process of fatty acid oxidation and conversion of energy to ATP.

1.3.1.5 Pyruvate Dehydrogenase Kinase (PDK4)

Pyruvate Dehydrogenase Kinase (PDK) is a family of proteins and regulates the pyruvate complex, in which there are four isozymes (PDK1–4) (Sugden and Holness 2006). PDK is a mitochondrial protein involved in the regulation of fatty acid metabolism as this protein inhibits pyruvate dehydrogenase complex (PDC), which is involved in the process of glucose oxidation and controlling the conversion of pyruvate to acetyl-CoA and therefore the entry of this substrate into the mitochondria (Sugden and Holness 2006). In cellular oxidative metabolism PDK phosphorylates PDC, this subsequently inactivates PDC and in turn increases fatty acid metabolism while reducing glucose metabolism. PDK4 knockout mice have been shown to have greater PDC activity (Jeoung, Wu et al. 2006) and elevated circulating concentrations of non-esterified fatty acids (Jeoung, Wu et al. 2006).

PDK4 activity is altered by the fasted state (Wu, Inskeep et al. 1999; Jeoung, Wu et al. 2006). PDK4 gastrocnemius mRNA and protein expression is up-regulated during the fasted state (Wu, Inskeep et al. 1999), which would be expected given fatty acids are a major substrate source for skeletal muscle when glycogen stores and oral ingestion of carbohydrate are depleted. Furthermore, starvation of C_2C_{12} cells mediated the up regulation of FOXO gene expression and caused an up-regulation of PDK4 (Furuyama, Kitayama et al. 2003). These studies support the role of PDK4 in fatty acid metabolism and regulation.

PDK4 is regulated by fatty acids, in rats fed a HFD for eight weeks the mRNA expression of PDK4 is up-regulated in fast twitch EDL skeletal muscle when compared to slow twitch soleus skeletal muscle

(analysed as fold change from high carbohydrate diet) (McAinch, Lee et al. 2003). Furthermore, lipid infusion has also been shown to up-regulate the mRNA expression levels of this protein (Tunstall and Cameron-Smith 2005).

PDK expression is altered in the diabetic state. In the liver (rather than in the skeletal muscle) PDK4 expression is inhibited by insulin, which is mediated by FOXO1 phosphorylation and PI3K/ Akt signalling in a liver cell cancer line (Kwon, Huang et al. 2004), this may however be tissue specific and would be useful to determine if this occurs in the skeletal muscle also. PDK4 is up-regulated in insulin resistant skeletal muscle in humans (Rosa, Di Rocco et al. 2003) and STZ-induced Type 1 Diabetes Mellitus (T1DM) rats have been shown to have increased mRNA expression PDK4 (Wu, Inskeep et al. 1999). Additionally the insulin sensitising hormone, globular adiponectin and pharmacological activation of AMPK decreased PDK4 mRNA expression in obese and T2DM human primary myotubes (McAinch and Cameron-Smith 2009).

PDK4 is regulated by a number of transcription factors and co-activators some include FOXO1 (regulator of enhanced fatty acid oxidation and negative regulator of insulin sensitivity) and PGC-1α (regulator of mitochondria biogenesis and marker of oxidative capacity) and both are discussed further in section (1.3.1.2 and 1.3.1.6) (Bastie, Nahle et al. 2005; Wende, Huss et al. 2005). Therefore it could be hypothesised that the up-regulation of PDK4 could assist in promoting fatty acid oxidation and assist in reducing the IMCL accumulation that is apparent in obesity, insulin resistance and T2DM, providing that the fatty acids do actually undergo the process of fatty acid oxidation and conversion of energy to ATP.

1.3.1.6 Forkhead Box Protein 1 (FOXO1)

FOXO1 is a member of FOXO protein family that have a number of cellular functions such as cellular proliferation and differentiation, apoptosis, metabolism, inflammation and immunity and regulation of cytokine expression to name a few (Wang, Zhou et al. 2014). FOXO1 is expressed in a number of

other insulin sensitive tissues such as adipose tissue, skeletal muscle, liver and heart (Choi, Yoon et al. 2011) and is a major transcriptional regulator of energy metabolism. FOXO1 is up regulated in states of skeletal muscle energy deprivation including starvation, streptozotocin (STZ) induced T1DM and post exercise (Kamei, Mizukami et al. 2003). In the state of starvation, FOXO1 expression is involved in the switch from carbohydrate oxidation to fatty acid oxidation (Bastie, Nahle et al. 2005). The ability for FOXO1 to regulate fatty acid oxidation is further supported in a C_2C_{12} myotubes model, in which an over expression of FOXO1 promotes increased enzyme lipoprotein lipase expression (Kamei, Mizukami et al. 2003) this enzyme cleaves triglycerides into fatty acids and glycerol. FOXO1 over expression also increases PDK4 (Bastie, Nahle et al. 2005). Additionally the gene expression profile obtained from mice over expressing FOXO1 indicates that genes for a type I skeletal muscle fibre phenotype were reduced (Kamei, Mizukami et al. 2003) as well as a reduced physical activity capacity, which could suggest that FOXO1 reduces mitochondrial activity. In support of this hypothesis, another group found that the deletion of FOXO1 in the liver restores mitochondrial function in a hepatic insulin resistant model using insulin receptor substrate-1 and insulin receptor substrate-2 double knockout mice (Bueno, Oyama et al. 2008). FOXO1 also has a role in skeletal muscle insulin and glucose homeostasis (Sugiishi, Kimura et al. 2013), whereby, over expression of FOXO1 has been shown to impair glycaemic control as a result of reduced skeletal muscle mass (Sugiishi, Kimura et al. 2013).

FOXO1 could also have a role in adiponectin signalling as it is involved in the regulation of AdipoR1 expression (Hill, Peters et al. 1993). The hormone adiponectin and its receptors are discussed below in more detail in section 1.3.2. Insulin negatively regulates AdipoR1 expression via the inactivation of FOXO1 (Tsuchida, Yamauchi et al. 2004). Insulin has been shown to inhibit skeletal muscle and myocardial AdipoR1 expression in hyperinsulinaemia rats (Hill, Peters et al. 1993). The same study shows that FOXO1 has a role in the transcription of AdipoR1 mediated via insulin in cardiomyocytes by binding directly to the AdipoR1 promoter (Hill, Peters et al. 1993). Furthermore, silencing FOXO1

resulted in reduced AdipoR1 expression and AMPK activation in the same study (Hill, Peters et al. 1993).

As there is an apparent increased systemic inflammation accompanied by an up regulation of inflammatory markers in T2DM (Weisberg, McCann et al. 2003). FOXO1 total protein expression has been shown to be increased in obese older aged T2DM individuals (Perry, Caldow et al. 2016). Skeletal muscle FOXO1 is involved in ubiquitin proteolysis pathway (Rosenbaum, Nicolson et al. 1996) which in turn is involved in T2DM skeletal muscle atrophy. In combination, FOXO1 and NFκβ are thought to be involved in the inflammatory process present in obesity and insulin resistance (Considine, Sinha et al. 1996) and therefore in the development of T2DM. The combination of these proteins can induce the transcription of pro-inflammatory cytokine interleukin 1β (IL-1β), whereby detection of the IL-1β protein is associated with greater risk of developing T2DM and is up-regulated in VAT of obese individuals (Considine, Sinha et al. 1996).

It is clear that FOXO1 has been extensively researched given that it has a major role in energy homeostasis. Taken together it may be feasible to hypothesise that as FOXO1 is a key regulator in fatty acid oxidation, up-regulation of FOXO1 could assist in promoting fatty acid oxidation and reduce the IMCL that is apparent in obesity, insulin resistance and T2DM, providing that the fatty acids do actually undergo the process of fatty acid oxidation and conversion of energy to ATP. Furthermore, FOXO1 positively regulates AdipoR1 expression, which in the skeletal muscle promotes fatty acid oxidation.

However, given the role of FOXO1 in regulating fibre types that are more glycolytic and less oxidative and that over expression of FOXO1 leads to reduced muscle mass and impaired glycaemic control, up regulation of this protein may not be entirely beneficial and caution may need to be taken into account regarding this. Figure 1.3A summarises some of the markers involved in fatty acid oxidation and oxidative capacity as discussed earlier in section 1.3.1.1 – 6.

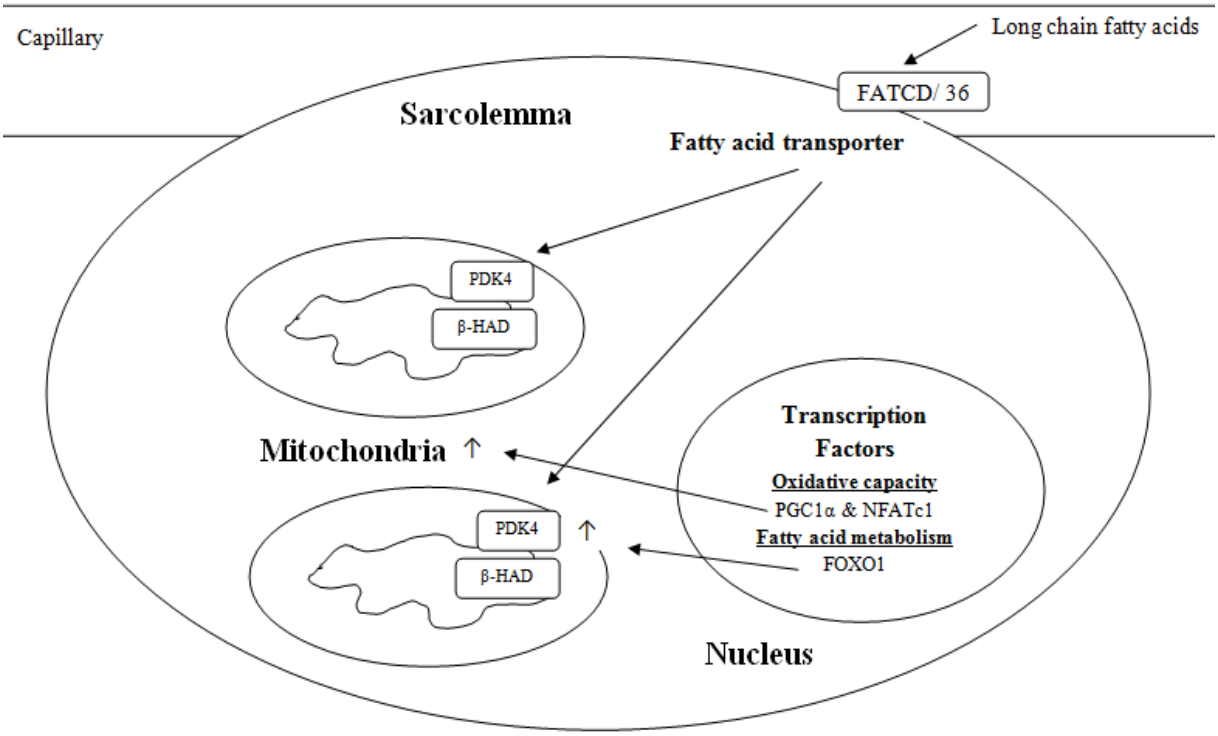


Figure 1.3A – Markers of fatty acid oxidation & oxidation

This is a schematic diagram which highlights some of the cellular proteins involved in the process of fatty acid oxidation (FATCD/36, PDK4 and βHAD) as well as the transcription factors that regulate fatty acid metabolism (FOXO1) and oxidative capacity (PGC1α and NFATc1) in the skeletal muscle, in a homeostatic environment.

1.3.2 Adiponectin

One adipokine that has been shown to have anti-inflammatory properties is adiponectin (Ouchi, Kihara et al. 2003; Devaraj, Swarbrick et al. 2008). As mentioned earlier, this hormone is synthesized from the adipose tissue (Kershaw and Flier 2004). Full length adiponectin is a 30 kilodalton protein consisting of 247 amino acids (Scherer, Williams et al. 1995). Cleavage of the N-terminal collagen-like domain of adiponectin by the enzyme leukocyte elastase, a fragmented globular domain adiponectin is formed (Waki, Yamauchi et al. 2005). Circulating adiponectin is comprised of different multimeric complexes whereby a number of adiponectin molecules are bound together, these complexes include: high molecular weight (HMW) adiponectin consisting of a multimeric adiponectin complexes and low molecular weight (LMW) adiponectin consisting of either trimer or hexomer adiponectin complexes (Lara-Castro, Luo et al. 2006). The different multimeric complexes appear to have varying physiological roles (Bobbert, Rochlitz et al. 2005) and it appears that HMW adiponectin as well as the HMW adiponectin to total adiponectin ratio has a strong association with insulin sensitivity (Lara-Castro, Luo et al. 2006; Kizer, Arnold et al. 2012).

1.3.2.1 Adiponectin Signalling

Adiponectin has an affinity to the two receptor isoforms which include: AdipoR1 and adiponectin receptor 2 (AdipoR2). AdipoR1 is abundantly expressed in the skeletal muscle and AdipoR2 is expressed predominately in the liver, which highlights that there is a relationship between substances secreted from adipose tissue that signal to the skeletal muscle as well as the liver. The AdipoR2 is also expressed in the human primary skeletal muscle myotubes, but is less abundant when compared to the AdipoR1 receptor (Chen, McAinch et al. 2005). Globular adiponectin has a high affinity for AdipoR1 receptor (Yamauchi, Kamon et al. 2003). In mouse derived myocytes and hepatocytes, 5’ Adenosine Monophosphate Activated Protein Kinase (5’AMPK) is stimulated by adiponectin (Yamauchi, Kamon et al. 2002) In the skeletal muscle AMPK is involved in regulating fatty acid oxidation (Cannon and Nedergaard 2010), while in the liver AMPK is involved in decreasing the process of gluconeogenesis

(Rui 2014). AdipoR1 has also been found to at least partially regulate mitochondrial biogenesis, in which AdipoR1 knockout mice have reduced mRNA expression and protein expression of the transcription factor and regulator of mitochondrial biogenesis PGC1α (Iwabu, Yamauchi et al. 2010).

In tissues other than the liver and skeletal muscle both AdipoR1 and AdipoR2 have also been shown to be expressed in pancreatic islets derived from mice and a cultured mouse beta cell-line (MIN6 cells) (Wijesekara, Krishnamurthy et al. 2010). This study suggests that adiponectin has tissue specific signalling properties as it can signal independently of AMPK, and possibly via Akt and ERK pathways in pancreatic cells in mice (Wijesekara, Krishnamurthy et al. 2010).

Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1 (APPL1) is a positive mediator of adiponectin signalling in muscle cells (Wang, Xin et al. 2009). In human *vastus lateralis* skeletal muscle, APPL1 mRNA expression is increased in obese and T2DM skeletal muscle when compared to lean skeletal muscle, while protein expression for APPL1 was increased in T2DM skeletal muscle when compared to the lean and obese skeletal muscle (Holmes, Yi et al. 2011). The authors discuss that the increase in APPL1 could potentially be a compensatory mechanism to try to enhance adiponectin signalling in skeletal muscle in obesity and T2DM, due to the decreased circulating concentrations present in these states (Holmes, Yi et al. 2011). APPL1 knockout mice have impaired glucose tolerance and insulin resistance (Ryu, Galan et al. 2014), and decreased AMPK activity (Mao, Kikani et al. 2006), respectively, while over expression of APPL1 increases AMPK activity (Mao, Kikani et al. 2006). The downstream signalling of adiponectin is also compromised when APPL1 is blocked, thereby resulting in reduced glucose uptake, fatty acid oxidation as well as the translocation of GLUT4 (Mao, Kikani et al. 2006). In mouse, APPL1 has also been shown to beneficially promote the inhibition of insulin-mediated glucose production in the liver (Cheng, Iglesias et al. 2009).

Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 2 (APPL2) is an isoform of APPL1, which is thought to be a negative mediator of adiponectin signalling (Wang, Xin et al. 2009). APPL1 and APPL2 share only a 54% identical protein sequence (Miaczynska, Christoforidis et al. 2004). APPL2 interacts with APPL1 and this dimer negatively regulates adiponectin signalling by binding with AdipoR1 and AdipoR2 (Wang, Xin et al. 2009). Over expression of APPL2 in C_2C_{12} skeletal muscle cells inhibited adiponectin mediated p38 MAPK signalling (Wang, Xin et al. 2009), while treatment with either adiponectin or metformin in C_2C_{12} skeletal muscle cells breaks the connection between APPL1 and APPL2 dimer (Wang, Xin et al. 2009).

Physiologically adiponectin is an anti-inflammatory (as mentioned previously) (Ouchi, Kihara et al. 2003; Devaraj, Swarbrick et al. 2008), anti-fibrotic hormone (Adachi and Brenner 2008) that promotes insulin sensitivity (Fruebis, Tsao et al. 2001; Yamauchi, Kamon et al. 2001; Yamauchi, Kamon et al. 2002). Evidence so far suggests that this hormone plays a role in regulating energy homeostasis (Lee and Shao 2014). Much research has been conducted over the past few decades and it is clear that this hormone is implicated in a number of pathophysiological conditions including obesity, insulin resistance and T2DM as there is altered signalling within these states. See figure 1.3.2B.

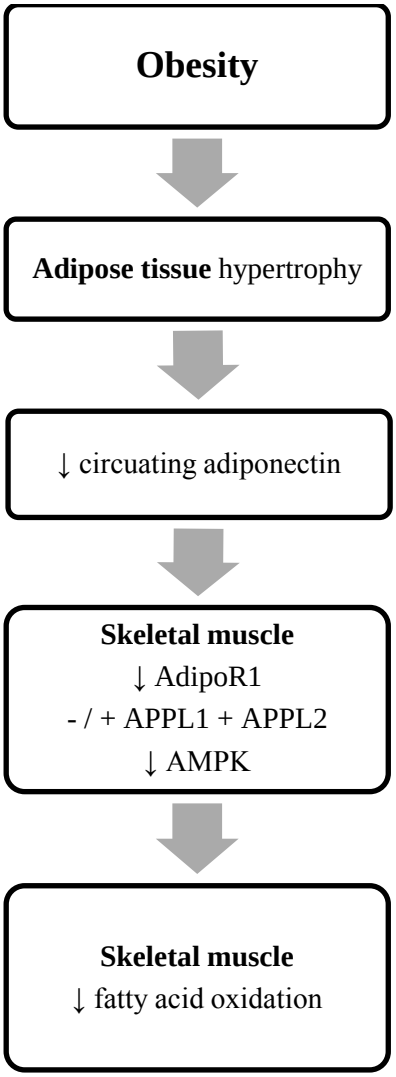


Figure 1.3B: The influence of skeletal muscle adiponectin signalling in obesity

The obese state is accompanied by a reduction in circulating adiponectin, reduced activity of AMPK, which results in reduced fatty acid oxidation in the skeletal muscle, adapted and modified from (Aguilera, Gil-Campos et al. 2008).

1.3.2.2 Adiponectin, Obesity & T2DM

Adiponectin skeletal muscle cellular signalling is dysregulated in obesity and T2DM. Despite an increase in adipose tissue mass (where adiponectin is synthesised) as previously mentioned in the obese state, circulating concentrations of adiponectin are decreased in obesity (Arita, Kihara et al. 1999), as well as in insulin resistance (Abbasi, Chu et al. 2004) and T2DM (Hotta, Funahashi et al. 2000). While adiponectin is abundantly present in healthy humans, there is a negative association between adiponectin concentrations in plasma and BMI (Arita, Kihara et al. 1999). Individuals with

high concentrations of serum adiponectin also have a reduced risk of developing T2DM (Yamamoto, Matsushita et al. 2014). Furthermore loss of weight in obese and T2DM individuals has been shown to increase circulating adiponectin concentrations (Hotta, Funahashi et al. 2000).

The mRNA receptor expression of AdipoR1 and AdipoR2 appears to be down regulated in the obese state (Tsuchida, Yamauchi et al. 2004). In insulin resistant *ob/ ob* mice there is a reduction in the mRNA receptor expression of AdipoR1 and AdipoR2 in both the adipose tissue and skeletal muscle of these animals (Tsuchida, Yamauchi et al. 2004), accompanied by an impairment of AMPK activation through adiponectin mediated pathways (Tsuchida, Yamauchi et al. 2004). Interestingly, this was further supported by the fact that both receptors were decreased during the fed state as well as after re-feeding, but increased in the fasted state in both the skeletal muscle and liver tissues (Tsuchida, Yamauchi et al. 2004).

The circulating adiponectin concentration is influenced by the site in which this hormone is stored in the adipose tissue (Turer, Khera et al. 2011). The locations of fat storage in humans can be further categorised as VAT (the adipose tissue that surrounds internal organs within the abdomen) whereas the subcutaneous adipose tissue (SAT) (the adipose tissue that underlies the skin). Studies have indicated that VAT is associated with a higher risk of insulin resistance and T2DM (Fox, Massaro et al. 2007). There is a reduced production of adiponectin in VAT when compared with the SAT (Turer, Khera et al. 2011) therefore sites in which triglycerides are stored within the adipose tissue also impact on circulating adiponectin concentrations (Turer, Khera et al. 2011). Furthermore cellular size of the adipocytes also impacts circulating adiponectin, as hypertrophic adipocytes seem to express less adiponectin (Michaud, Boulet et al. 2014).

1.4 The endocannabinoid system as a pharmaceutical target

The endocannabinoid system is a physiological signalling system, in which fatty acid derived molecules referred to as endocannabinoids signal to the G Protein Coupled Receptors, the two identified receptors Cannabinoid Receptor 1 (CB_1) and Cannabinoid Receptor 2 (CB_2) have been extensively reviewed in the literature (Louheranta, Turpeinen et al. 1998). Endocannabinoids include anandamide, and 2 arachidonyl glycerol (2-AG) (Louheranta, Turpeinen et al. 1998). This system is involved in both central and peripheral energy expenditure predominately through the activation of CB_1 and CB_2 (Louheranta, Turpeinen et al. 1998). The endocannabinoid signalling system has been implicated in obesity and T2DM (Louheranta, Turpeinen et al. 1998). CB_1 (Cavuoto, McAinch et al. 2007) and CB_2 (Cavuoto, McAinch et al. 2007) are both expressed in skeletal muscle and blockade of CB_1 has been shown to improve metabolic adaptations within this tissue (Crespillo, Suarez et al. 2011). Pharmacologically targeting the endocannabinoid system has shown promising results for weight control (Salmeron, Hu et al. 2001) mediated through both central and peripheral pathways, however, unfortunately the side-effect of increased depressive mood states and suicidal tendencies outweighs the benefits of weight loss. Therefore, in addition to pharmacologically targeting the other known cannabinoid receptor CB_2, other potential receptors within this signalling system may be suitable targets. It is proposed that other putative cannabinoid receptors exist (Johns, Behm et al. 2007; Ryberg, Larsson et al. 2007; Pertwee, Howlett et al. 2010), some potential candidates referred to in the literature include; G Protein-Coupled Receptors 55 (GPR55) and G Protein-Coupled Receptor 18 (GPR18).

Recent research indicates that atypical cannabinoids Cannabidiol (CBD), Abnormal Cannabidiol (Abn-CBD), O-1602 and O-1918 have affinities (or putative affinities) to GPR55 and GPR18. These receptors and ligands are reviewed in more detail below (in section 1.4.1 - 1.4.4 and 1.5.1 - 1.5.2).

1.4.1 Cannabidiol (CBD)

CBD is an atypical cannabinoid derived from the *Cannabis Savita* plant and this compound was discovered in 1940 (Adams 1940), while much research has focused on THC, this compound also appears to have a number of roles in pathophysiological states in humans which are reviewed in detail (Zhornitsky and Potvin 2012). Refer to Table 1.4A for chemical structure of CBD.

CBD is an antagonist for both the putative cannabinoid receptors GPR55 and GPR18 (Ryberg, Larsson et al. 2007; Whyte, Ryberg et al. 2009; McHugh, Page et al. 2012) and has also been shown to act as a partial agonist for GPR18 (McHugh, Page et al. 2012). CBD like most other ligands has off targeted effects on receptors such as TRPV (Gaetani, Fu et al. 2010).

CBD appears to elicit beneficial effects in a number of pathophysiological conditions. Studies have found that the compound CBD has neuro-protective properties including anxiolytic and anti-depressive activity (de Mello Schier, de Oliveira Ribeiro et al. 2014), as well as anticonvulsant properties in epilepsy (Devinsky, Marsh et al. 2016) and anti-psychotic effects in schizophrenia (Leweke, Piomelli et al. 2012). CBD also has anti-inflammatory properties in arthritis as this compound reduces pro-inflammatory cytokines (Malfait, Gallily et al. 2000).

The role of CBD in energy metabolism has been investigated by a number of studies. However, there is conflicting data when investigating food intake and the administration of CBD. Farrimond et al. (2012) found a reduction in food intake with acute oral administration of 4.4 mg/ kg CBD (Farrimond, Whalley et al. 2012). Conversely, other research groups have found that CBD administered using an ip. injection (1.0 – 100 mg/ daily dose range) did not alter mouse or rat feeding behaviour (Wiley, Burston et al. 2005; Scopinho, Guimaraes et al. 2011).

CBD has also been shown to have protective effects in diabetes models and disease associated complications (El-Remessy, Al-Shabrawey et al. 2006; Rajesh, Mukhopadhyay et al. 2007). CBD has been shown to have a role in energy metabolism as this compound induces a browning phenotype in a

cell culture model using 3T3-L1 adipocytes, further this compound promotes lipolysis, thermogenesis and reduces lipogenesis in this cell type (Parray and Yun 2016).

However, recently Jadoon et al. (2016) published a randomised doubled blinded placebo controlled parallel group pilot study that focused on the effect of CBD and tetrahydrocannabivarin (THCV) in non-insulin treated T2DM individuals. This study unfortunately did not appear to have the desired effects that CBD was observed to have in the animal and cell culture models. This trial concluded that 5 mg of THCV could be a potential therapeutic agent for glycaemic control in T2DM, but 100 mg of CBD did not appear to have many significant effects while the compound did decrease resistin (where circulating concentrations of this hormone are increased with adiposity) and increased glucose-dependent insulin trophic peptide when compared with baseline but not the placebo group (Jadoon, Ratcliffe et al. 2016).

While there appears to be quite a lot of research focusing on the role of CBD in pathophysiological conditions including T2DM, the role of structurally related compounds including Abn-CBD, O-1602 and O-1918 (which are reviewed in sections 1.4.2, 1.4.3 and 1.4.4 respectively) in obesity and T2DM have not fully been characterised or research is limited in the case of the compounds O-1602 and O-1918.

1.4.2 Abnormal cannabidiol (Abn-CBD)

Abn-CBD (−)-4-(3-3,4-trans-p-menthadien-1,8)-yl-olivetol) is a synthetic isomer of CBD (Kreutz, Koch et al. 2009). Refer to Table 1.4A: for chemical structure of Abn-CBD. This compound is thought to be an agonist for GPR55 (Ryberg, Larsson et al. 2007), although as previously mentioned some studies have shown no effect from Abn-CBD at GPR55 (Oka, Nakajima et al. 2007; Kapur, Zhao et al. 2009). It is generally considered in the literature that Abn-CBD is an agonist/ biased agonist for GPR18 (McHugh, Hu et al. 2010; McHugh, Page et al. 2012; Console-Bram, Brailoiu et al. 2014).

Abn-CBD does not bind to the cannabinoid receptors CB_1 or CB_2 (Offertaler, Mo et al. 2003), and in mice deficient of the CB_1 and CB_2 receptor, this compound has been shown to cause endothelial-dependent vasodilation (Jarai, Wagner et al. 1999). This lead to the hypothesis of the CBx receptor, which is an unknown receptor of the cannabinoid system and potential candidates include GPR55 and/or GPR18. CBx is sensitive to Abn-CBD and a number of other atypical cannabinoid compounds, further research into determining the CBx receptor(s) is therefore required.

Abn-CBD has a role in diabetes, treating insulin secreting BRIN-BD11 βcells with this compound resulted in stimulated insulin secretion mediated through GPR55 (McKillop, Moran et al. 2013; McKillop, Moran et al. 2016). Interestingly and very recently McKillop et al. (2016) found that Abn-CBD has beneficial properties for diabetes using a diabetes model (low dose administration of STZ in mice), Abn-CBD was found to reduce food intake, polydipsia and plasma glucose and increase plasma insulin (McKillop, Moran et al. 2016). Furthermore following four weeks of treatment with Abn-CBD, this compound enhanced glucose tolerance and reduced plasma glucose concentration, while insulin resistance, triacylglycerols and total cholesterol was decreased. This study also found that Abn-CBD was able to enhance βcell proliferation but not alter islet area (McKillop, Moran et al. 2016).

Studies so far indicate that Abn-CBD is beneficial in the treatment of diabetes mellitus, however, further research is required to help support these findings and provide further characterisation. It would be interesting to determine this compound's role in obesity and T2DM, however, this is not within the scope of this thesis. Structurally similar compounds O-1602 and O-1918 (which are reviewed in sections 1.4.3 and 1.4.4 respectively) have not been characterised in obesity or T2DM, therefore it would be interesting to determine whether these compounds show similar promising effects as observed with the structurally similar compound Abn-CBD.

1.4.3 O-1602

O-1602 (5-Methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol) is an analogue of the synthetic compound Abn-CBD (Handschin, Choi et al. 2007). Refer to Table 1.4A for chemical structure of O-1602. O-1602 is an agonist for the putative cannabinoid receptor GPR55 (Johns, Behm et al. 2007) and a biased agonist for the putative cannabinoid receptor GPR18 (Console-Bram, Brailoiu et al. 2014) (both GPR55 and GPR18 are discussed in more detail in sections 1.5.1 - 1.5.2). There is some conflicting pharmacology surrounding O-1602 and GPR18 in the literature, with one study finding that O-1602 initiated cell migration in both GPR18 transfected HEK293 cells and microglial cells known to express GPR18 (McHugh, Page et al. 2012). Conversely, another study found heterologously expressed GPR18 in superior ganglion from rats failed to elicit a response (Lu, Puhl et al. 2013). Recently Console-Bram et al. (2014) concluded that O-1602 is a biased agonist for GPR18 which helps to provide a reason for the differences observed.

Regardless of the receptors this compound has an affinity for, O-1602 appears to be an anti-inflammatory molecule in some studies and has been show to improve pathological changes in a pancreatitis model in mice (Li, Feng et al. 2013), reduce joint afferent mechanosensitivity in an acutely induced arthritis model (Handschin, Choi et al. 2007), as well as reduce inflammation in experimental colitis model (Schicho, Bashashati et al. 2011). Furthermore, in a colon cancer model, there was a decrease in tumour growth by reducing tumour cell viability, proliferation and promoting apoptosis (Kargl, Andersen et al. 2016).

On the other hand, O-1602 has been shown to have pro-inflammatory and pro-atherogenic effects thought to be mediated by GPR55 (Lanuti, Talamonti et al. 2015). Using human macrophages and foam cells, O-1602 promoted lipid accumulation, in which the effect was blunted by CBD. The lipid accumulation observed by O-1602 was mediated through the up-regulation of protein expression of CD36- and Scavenger Receptor Class B I (SR-BI) (Lanuti, Talamonti et al. 2015). Furthermore, this

study also showed that human foam cells treated with O-1602 up-regulates TNFα, while this compound down regulates anti-inflammatory cytokine IL-10 (Lanuti, Talamonti et al. 2015).

O-1602 has also been shown to impact energy homeostasis. A study by Diaz-Arteaga et al. (2012) showed that direct administration of O-1602 into the brain of rodents increased food intake and acute administration of O-1602 via ip. injection caused a transient increase in food intake at six hours in rats following an ip. injection and this is likely due to cocaine amphetamine regulated transcript (CART) down-regulation. Whereas, sub-chronic administration (seven days) of O-1602 showed no significant change in food intake or body weight, however there was an increase in fat mass (Diaz-Arteaga, Vazquez et al. 2012). Taken together, these findings suggest that O-1602 acutely affects food intake rather than having chronic effects in the non-obese state However to date, the role that this compound has in obesity is not known and understanding whether it is a suitable pharmacotherapy would be beneficial or at least to provide some understanding into its effect in the obese state, given that O-1602 has been shown to influence food intake and fat mass in previous research.

1.4.4 O-1918

O-1918 (1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene) is a synthetic derivative of plant constitute cannabidiol (CBD) (Michael, Wu et al. 2001). Refer to Table 1.4A for chemical structure of O-1918. As mentioned earlier, O-1918 is an antagonist for the CBx receptor (Pertwee, Howlett et al. 2010), while in the literature it is alluded to be a putative antagonist for GPR55 (Henstridge, Balenga et al. 2011; Kremshofer, Siwetz et al. 2015), whereas, this compound has been shown to act as either a biased agonist (Console-Bram, Brailoiu et al. 2014) or an antagonist (McHugh, Hu et al. 2010) for GPR18.

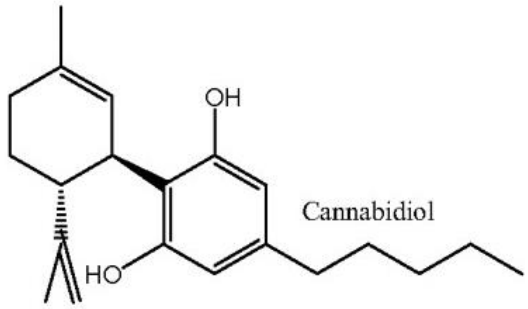
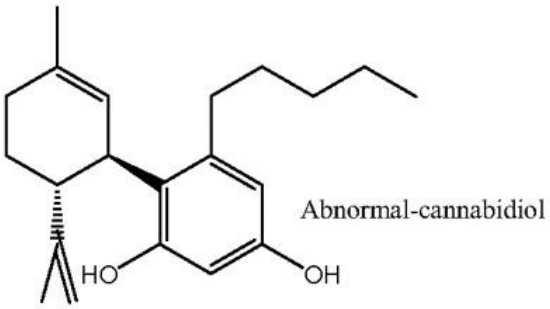
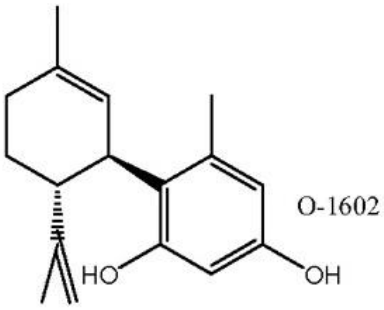
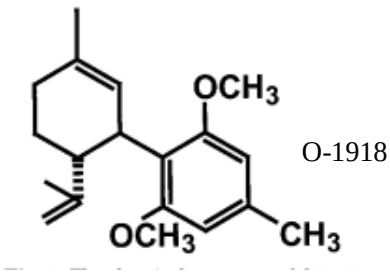
In the literature, there is limited published research investigating the actions of O-1918. Much research focuses on the effect that this compound has in the vasculature in which O-1918 blocks the vasodilatory effect of Abn-CBD (Johns, Behm et al. 2007), blood pressure regulation (Park, Scheffler et al. 2009)

and intraocular pressure (Caldwell, Hu et al. 2013). In anaesthetised rats the hypotensive effect of anandamide is enhanced by O-1918 (Park, Scheffler et al. 2009). While in murine eye, O-1918 blocked the beneficial effects that both Abn-CBD and NAGly had on reducing intraocular pressure and this study suggests the signalling to be mediated through GPR18 (Caldwell, Hu et al. 2013).

There does not appear to be any research focusing on the role of O-1918 in skeletal muscle or obesity. However, unpublished observations from our lab *Simcocks et al* (produced during my final year of undergraduate studies and summer research scholarship) show that C_2C_{12} myotubes serum starved for six hours and treated for 24 hours with 100 nM of O-1918, caused an increase in mRNA expression of NFATc1, PGC1α and PDK4, while APPL1 appeared increased, it was not significant and APPL2 and AMPKα2 mRNA expression were both not altered (See Appendix Two).

O-1602 or O-1918 may have varying roles in obesity and associated co-morbidities such as insulin resistance and T2DM. Due to the need for further therapeutic targets to assist in the treatment of obesity and T2DM, these compounds therefore maybe suitable targets in combination with reduced energy intake and increased physical activity.

Table 1.4A: Chemical structure of atypical cannabinoids: cannabidiol and associated compound derivatives.

Cannabidiol **(Pertwee, Howlett et al. 2010)**	
Abnormal-Cannabidiol **(Pertwee, Howlett et al. 2010)**	
O-1602 **(Pertwee, Howlett et al. 2010)**	
O-1918 **(Offertaler, Mo et al. 2003)**	

1.5 Putative Cannabinoid Receptors GPR55 and GPR18

1.5.1 A potential role for GPR55 in the regulation of energy homeostasis

The following is a direct excerpt from the published manuscript Simcocks AC, O'Keefe L, Jenkin KA, Mathai ML, Hryciw DH & McAinch AJ 2014, 'A potential role for GPR55 in the regulation of energy homeostasis', *Drug Discovery Today*, vol. 19, no. 8, pp. 1145 - 1151., and has been edited for formatting to be consistent with this thesis. In addition to this manusctipt please refer to Appendix One for a more up to date discussion of the literature surrounding GPR55.

1.5.1.1 GPR55: a putative endocannabinoid receptor?

Although GPR55 has recently been reported as a novel member of the cannabinoid family (Ryberg, Larsson et al. 2007) and has been termed the possible 'third' cannabinoid receptor, inconsistencies exist in the literature, with some studies showing reduced or no specificity of GPR55 to these ligands (Oka, Nakajima et al. 2007; Kapur, Zhao et al. 2009; Oka, Toshida et al. 2009). These inconsistencies might be linked to the fact that the ligands were not tested for a dose response before the assays (Henstridge, Balenga et al. 2010). There is also the possibility that there was a toxic effect of these compounds on the cells, owing to the high dose of the compounds as well as the duration of exposure to these ligands (Henstridge, Balenga et al. 2010) or as a result of biased agonism. For a more comprehensive review on the inconsistent findings for the pharmacology of GPR55, see (Pertwee, Howlett et al. 2010). Despite this debate around the classification of GPR55 as a novel member of the endocannabinoid system, the role of this receptor in human physiology is still of interest.

1.5.1.2 Tissue distribution

GPR55 has been shown to be expressed in several tissues throughout the body, with a similar distribution between mouse and rat and with some limited investigation of GPR55 expression in human tissue (Sawzdargo, Nguyen et al. 1999; Ryberg, Larsson et al. 2007; Moreno, Andradas et al. 2014). GPR55 mRNA is most abundantly expressed in the adrenal tissue, ileum, jejunum, frontal cortex and striatum in mice (Ryberg, Larsson et al. 2007). In addition, GPR55 is expressed in tissue that is

involved in regulating energy intake and expenditure. Localisation studies in different organisms have shown that this receptor has been identified in the hypothalamus in mice (Ryberg, Larsson et al. 2007) and in different regions of the gastrointestinal tract, including the oesophagus, stomach, jejunum and colon in mouse (Ryberg, Larsson et al. 2007), and jejunum, ileum and colon in rat (Lin, Yuece et al. 2011; Schicho, Bashashati et al. 2011). In the rat small intestine, GPR55 was localised mainly in the submucosa and myenteric plexus (Lin, Yuece et al. 2011) GPR55 mRNA and protein expression have also been located in the liver in rats (Sawzdargo, Nguyen et al. 1999) mice (Romero-Zerbo, Rafacho et al. 2011) and humans (Moreno-Navarrete, Catalan et al. 2012), in adipose tissue from rats (Romero-Zerbo, Rafacho et al. 2011), in visceral and subcutaneous white adipose tissue in humans (Moreno-Navarrete, Catalan et al. 2012) and in pancreas from rat, specifically the islets of Langerhans (Romero-Zerbo, Rafacho et al. 2011). GPR55 tissue expression in brown adipose tissue has yet to be determined. Importantly, the localisation of GPR55 in several tissues involved in regulating energy intake and expenditure suggests a role for this GPCR in the maintenance of energy homeostasis. Interestingly, the localisation of GPR55 in metabolically active skeletal muscle is largely overlooked in the current literature. Despite this, unpublished observations from our laboratory have confirmed the protein expression of GPR55 in rat skeletal muscle (*A. Simcocks et al., unpublished*).

1.5.1.3 Endogenous and synthetic ligands of GPR55

There are currently limited commercially available synthetic agonists and antagonists that are specific for GPR55. A large number of studies have investigated the pharmacology of GPR55 (Johns, Behm et al. 2007; Lauckner, Jensen et al. 2008; Waldeck-Weiermair, Zoratti et al. 2008; Henstridge, Balenga et al. 2009; Ross 2009; Whyte, Ryberg et al. 2009), with contradictory findings reported in the pharmacology and signalling of this receptor (Ross 2009). Despite these inconsistencies, there is general agreement that specific cannabinoid ligands interact with GPR55 with high affinity (Johns, Behm et al. 2007; Ryberg, Larsson et al. 2007; Lauckner, Jensen et al. 2008), which are different to those reported for CB_1 or CB_2 receptors. For example, HU210, a potent synthetic agonist of CB_1 and

CB_2 receptors, and JWH015, a selective CB_2 agonist, are both potent agonists of GPR55, but at different concentrations (Ryberg, Larsson et al. 2007; Lauckner, Jensen et al. 2008). Conversely, WIN55212-2, a synthetic cannabinoid that is a more potent agonist of CB_2 than of CB1 receptors, was ineffective in the modulation of GPR55 (Ryberg, Larsson et al. 2007; Lauckner, Jensen et al. 2008). A more recent study indicated that synthetic compounds AM251 and SR141716A, which are both antagonists for CB_1, act as agonists for GPR55. Furthermore, AM281, another antagonist for CB_1, is also an agonist for GPR55, albeit less potently than AM251 and SR141716A (Henstridge, Balenga et al. 2010). In addition to typical cannabinoids, such as delta 9-tetrahydrocannabinol (THC) (Anavi-Goffer, Baillie et al. 2012), GPR55 is also activated by abnormal cannabinoids, specifically abnormal cannabidiol (Johns, Behm et al. 2007) and its synthetic analogue O-1602 (Johns, Behm et al. 2007). Furthermore, GPR55 is inhibited by cannabidiol (Ryberg, Larsson et al. 2007) and its synthetic analogue O-1918. O-1602 and O-1918 are not specific modulators of GPR55 because they can also act on GPR18 (McHugh, Hu et al. 2010). In addition, the structural analogues of AEA, namely the fatty acids palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), activate GPR55 via GTPγS binding (Ryberg, Larsson et al. 2007). Importantly, PEA has low affinity and OEA has no affinity for CB_1 and CB_2, although both are shown to activate GPR55. Conversely, other researchers found that neither ligand had an affinity for GPR55 (Oka, Nakajima et al. 2007). A study looking at high-potency selective ligands for GPR55 found three compounds that are selective agonists, namely CID1792197, CID1172084 and CID2440433 (Kotsikorou, Madrigal et al. 2011), because these compounds did not interact with CB_1 or CB_2. A more recent study used an analogue of CID2440433 and GSK494581A, GSK319197A (GlaxoSmithKline) as a selective agonist for GPR55 (Kargl, Balenga et al. 2012). A selective antagonist for GPR55, CID16020046, was also recently used to determine the role of GPR55 in platelet and endothelial function (Kargl, Balenga et al. 2012), in which this compound blocked the GPR55 potent agonist L α-lysophosphatidylinositol (LPI) by inhibiting the signalling of downstream targets, such as nuclear factor of activated T cells (NFAT), serum response element (SRE) and nuclear

factor (NF)- kB, as well as GPR55 internalisation. In addition to GPR55 being activated by cannabinoids, LPI, which is a lipid that is a constituent of the plasma membrane and is involved as a signalling molecule in metabolic processes, also regulates the receptor and appears to be one of the most potent endogenous agonists for GPR55 (Lauckner, Jensen et al. 2008; Waldeck-Weiermair, Zoratti et al. 2008).

1.5.1.4 Signalling pathways for GPR55

Experiments using HEK293 cells overexpressing GPR55 have shown that GPR55-mediated signalling is facilitated by the coupling of the receptor to Gα13, which leads to the activation of small GTPases, including Ras homologue gene family member A (RhoA), cell division control protein 42 (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) (Ryberg, Larsson et al. 2007). This results in oscillatory release of calcium from the endoplasmic reticulum, (Henstridge, Balenga et al. 2009) which triggers several metabolic pathways (Henstridge, Balenga et al. 2010). Activation of GPR55 using LPI triggers NFAT through the calcineurin–NFAT pathway (Henstridge, Balenga et al. 2009) as well as rapid phosphorylation of p38 mitogen activated protein kinase (MAPK) (Oka, Kimura et al. 2010), phosphorylation of the extracellular signal regulated kinase 1/2 (ERK1/2) pathways (Henstridge, Balenga et al. 2010) as well as activation of 30 –50 -cyclic adenosine monophosphate response binding protein (CREB), NF-κβ (Henstridge, Balenga et al. 2010) and SRE (Kargl, Brown et al. 2013) (Fig. 1.5A).

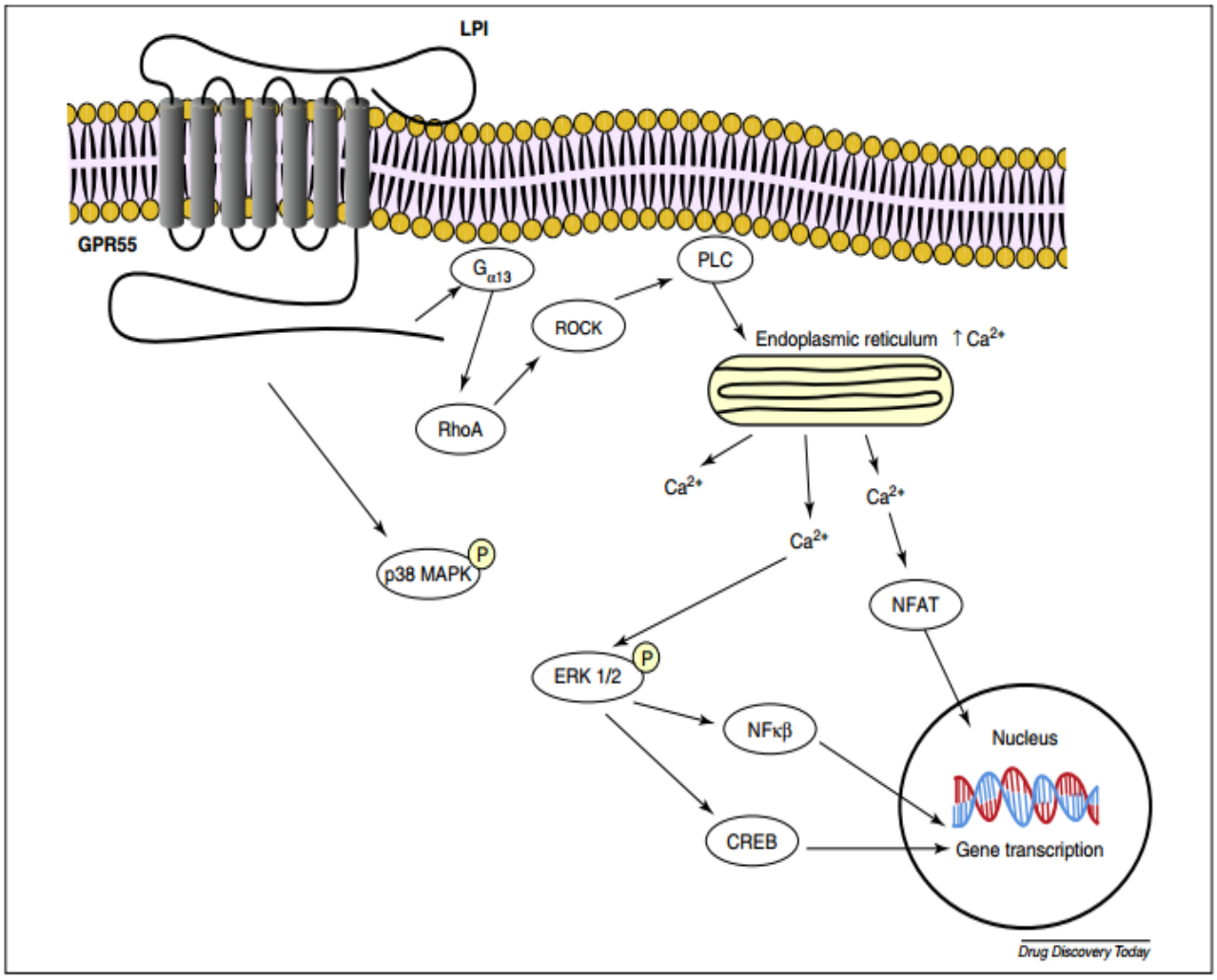


Figure 1.5A: G protein-coupled receptor 55 (GPR55) cellular signalling

This diagram illustrates the downstream signalling pathways initiated when GPR55 is activated by the potent endogenous agonist L-a-lysophosphatidylinositol (LPI) in human embryonic kidney (HEK293) cells overexpressing GPR55. Initiating the activation of Ga13 triggers Ras homologue gene family member A (RhoA)-dependent release of oscillatory calcium (Ryberg, Larsson et al. 2007) from the endoplasmic reticulum (ER) (Henstridge, Balenga et al. 2009), resulting in extracellular signal-regulated kinase 1/2 (ERK1/2) (Kapur, Zhao et al. 2009; Oka, Toshida et al. 2009; Whyte, Ryberg et al. 2009; Henstridge, Balenga et al. 2010) phosphorylation; in addition, nuclear factor kappa light polypeptide gene (NKκβ) enhances B cells (Henstridge, Balenga et al. 2010) and 30–50 -cyclic adenosine monophosphate response binding protein (CREB) activation (Henstridge, Balenga et al. 2010). Increased calcium release through the ER also activates nuclear factor of activated T cells (NFAT) (Henstridge, Balenga et al. 2009), which results in translocation from the cytosol to the nucleus. GPR55 also results in p38 mitogen activated protein kinase (p38 MAPK) phosphorylation activation (Oka, Kimura et al. 2010) through LPI.

It appears that different agonists for GPR55 activate different signalling pathways (Henstridge, Balenga et al. 2010). GPR55 activation through LPI has been shown to trigger ERK 1/2 phosphorylation (Kapur, Zhao et al. 2009). Conversely, endogenous cannabinoid compounds AEA and 2-AG have been shown to have no effect on ERK 1/2 signalling (Oka, Nakajima et al. 2007; Kapur, Zhao et al. 2009; Oka, Kimura et al. 2010; Anavi-Goffer, Baillie et al. 2012), except at a supraphysiological dose (Anavi-Goffer, Baillie et al. 2012), in which GPR55 activation using AEA (10 mM) stimulated ERK1/2 (Whyte, Ryberg et al. 2009). Alternatively, synthetic cannabinoid agonists for GPR55, AM251 and SR141716A have both also been shown to activate ERK1/2 signalling (Henstridge, Balenga et al. 2010). GPCRs have the ability to form heteromers (Smith and Milligan 2010), which can result in changes to the subsequent downstream signalling pathways stimulated compared with homomers of the GPCRs (Smith and Milligan 2010). The concept that GPCRs can form heteromers has further implications pharmacologically, because modulating a specific receptor might influence other receptor(s) and, therefore, result in altered cellular signalling (Smith and Milligan 2010). A recent study indicated that CB_1 can form a heteromer with GPR55 in HEK293 cells (Kargl, Balenga et al. 2012). In isolation, GPR55 activation using both LPI and SR141716A in vitro induced NFAT and SRE stimulation. However, in the presence of CB_1, the activation of NFAT and SRE by LPI was significantly reduced (Kargl, Balenga et al. 2012). Furthermore, in the presence of CB_1, treatment with either SR141716A (a known antagonist and/or inverse agonist for CB_1 (Landsman, Burkey et al. 1997) and agonist for GPR55 (Kapur, Zhao et al. 2009)), or the selective GPR55 agonist GSK319197A, failed to activate NFAT and SRE (Kargl, Balenga et al. 2012). In addition, with in vitro expression of both CB_1 and GPR55, GPR55-mediated ERK1/2 phosphorylation was inhibited but CB_1-mediated ERK1/2 phosphorylation was enhanced (Kargl, Balenga et al. 2012). This suggests that, when CB_1 is in the presence of GPR55, there is enhanced CB_1 signalling (Kargl, Balenga et al. 2012). Interestingly, when CB1 and GPR55 coexist, WIN55,212-2 and AEA (agonists for both GPR55 and CB1) reinstate GPR55 signalling. This study

suggests that, when CB_1 is blocked in vitro, GPR55 signalling is inhibited; however, when CB_1 is activated, there is no effect on GPR55-mediated signalling. Investigation is required to determine whether the same signalling pattern mediated by CB_1 and GPR55 occurs in vivo. Nonetheless, the finding that CB_1 and GPR55 form a heteromer in vitro should be taken into consideration when describing the role of these receptors in obesity and related states of disordered metabolism. The endocannabinoid system is overactive in the obese state. 2- AG concentrations in plasma and visceral adipose tissue are elevated in obese individuals (Bluher, Engeli et al. 2006; Cote, Matias et al. 2007). Both AEA and 2-AG have been demonstrated to activate CB_1 (Di Marzo, Ligresti et al. 2009). It could be hypothesised that, if there is co expression of the receptors in the presence of an overactive endocannabinoid system, GPR55-mediated signalling should not be altered because CB_1 is also activated (Kargl, Balenga et al. 2012). Further investigation into the ability of CB_1 and GPR55 to form heteromers in vivo is needed to demonstrate the significance of this interaction in homeostasis, as well as in disease states. GPR55 has also been demonstrated to interact with CB_2 (Balenga, Aflaki et al. 2011). Both GPR55 and CB_2 are co-expressed in human neutrophils and, when both receptors are activated, there is enhanced cellular signalling of RhoA and Cdc42 and reduced signalling of Ras related C3 botulinum toxic substrate 2 (Rac2) (Balenga, Aflaki et al. 2011). This crosstalk between GPR55 and CB_2 signalling might occur in other tissues and could have implications for other cellular functions, such as cellular energy metabolism. Importantly, this is likely to occur in tissues where both CB_2 and GPR55 are co expressed, such as metabolically active tissues, including adipose tissue, liver, pancreas and skeletal muscle. Taken together, these studies suggest that GPR55 signalling is influenced by CB_1 and CB_2.

1.5.1.4 Pathophysiological role of GPR55

Several studies have also identified a role for GPR55 in the development of non-metabolic disease. Primarily, this has been driven by research on the potent endogenous agonist for GPR55, LPI. GPR55 expression has been detected in numerous human cancer cell line models, including cancer models of

the breast (Ford, Roelofs et al. 2010; Andradas, Caffarel et al. 2011), pancreas (Andradas, Caffarel et al. 2011), ovarian (Pineiro, Maffucci et al. 2011), prostate (Pineiro, Maffucci et al. 2011) and bile ducts (Huang, Ramirez et al. 2011). Recently, GPR55 was identified in a cell line of squamous cell carcinoma (Perez-Gomez, Andradas et al. 2013). There is also a positive correlation between the aggressiveness of the cancer and the expression of GPR55 (Andradas, Caffarel et al. 2011). Furthermore, overexpression of GPR55 increased cancer cell proliferation through ERK activation, whereas silencing GPR55 had the opposite effect and reduced ERK activation and, therefore, cancer cell proliferation (Andradas, Caffarel et al. 2011). Significantly, GPR55 expression and LPI are associated with cellular proliferation of prostate (Pineiro, Maffucci et al. 2011), ovarian (Pineiro, Maffucci et al. 2011), breast (Andradas, Caffarel et al. 2011), glioblastoma (Andradas, Caffarel et al. 2011) and migration in breast cancer (Ford, Roelofs et al. 2010). Thus, it appears that GPR55 is involved in the progression of this disease state. In addition, GPR55 has been demonstrated to be involved in inflammatory and neuropathic pain (Staton, Hatcher et al. 2008). GPR55 might have a pro inflammatory role in gastrointestinal inflammation, because the severity of colitis in GPR55-knockout mice was significantly lower compared with wild-type mice (Schicho, Bashashati et al. 2011). Therefore, blocking this receptor might be beneficial for the treatment of pro inflammatory conditions, such as inflammatory bowel disease (IBD). One research group demonstrated that rats with experimentally induced septic ileus [using lipopolysaccharide (LPS)] had a greater expression of GPR55 in the small intestine compared with the control rats. In mice, LPS slowed upper gastrointestinal transit time; however, when treated with the GPR55 antagonist cannabidiol, this transit time was improved, whereas O-1602, an agonist for GPR55, had no effect (Lin, Yuece et al. 2011). Furthermore, in control mice, cannabidiol did not elicit an effect on upper gastric transit time, which had been observed in the experimental group treated with LPS (Lin, Yuece et al. 2011). These data suggest that cannabidiol would be beneficial for pro inflammatory intestinal and pathophysiological conditions. Given that cannabidiol is an antagonist for GPR55, this receptor could be involved in

gastrointestinal transit in IBD. Further research is required to determine whether these findings are GPR55 mediated; in addition, knockout models or specific agonists and/or antagonists are needed to confirm the role of GPR55 in inflammatory bowel conditions. GPR55 also appears to have a role in bone turnover (Whyte, Ryberg et al. 2009), because both in vivo and in vitro experiments looking at GPR55-knockout mice show that blocking GPR55 might be beneficial for the treatment of arthritis and other metabolic bone-associated disorders. Further studies, from animals and cell culture models, are required to investigate the role of this receptor in such systems.

1.5.1.5 Physiological role of GPR55 in obesity and T2DM

Emerging research has identified a potential role for GPR55 in obesity and T2DM (Romero-Zerbo, Rafacho et al. 2011; Moreno-Navarrete, Catalan et al. 2012). This receptor has been identified in several metabolically active organs (Fig. 1.5B) and, therefore, might be a beneficial therapeutic target in such diseases.

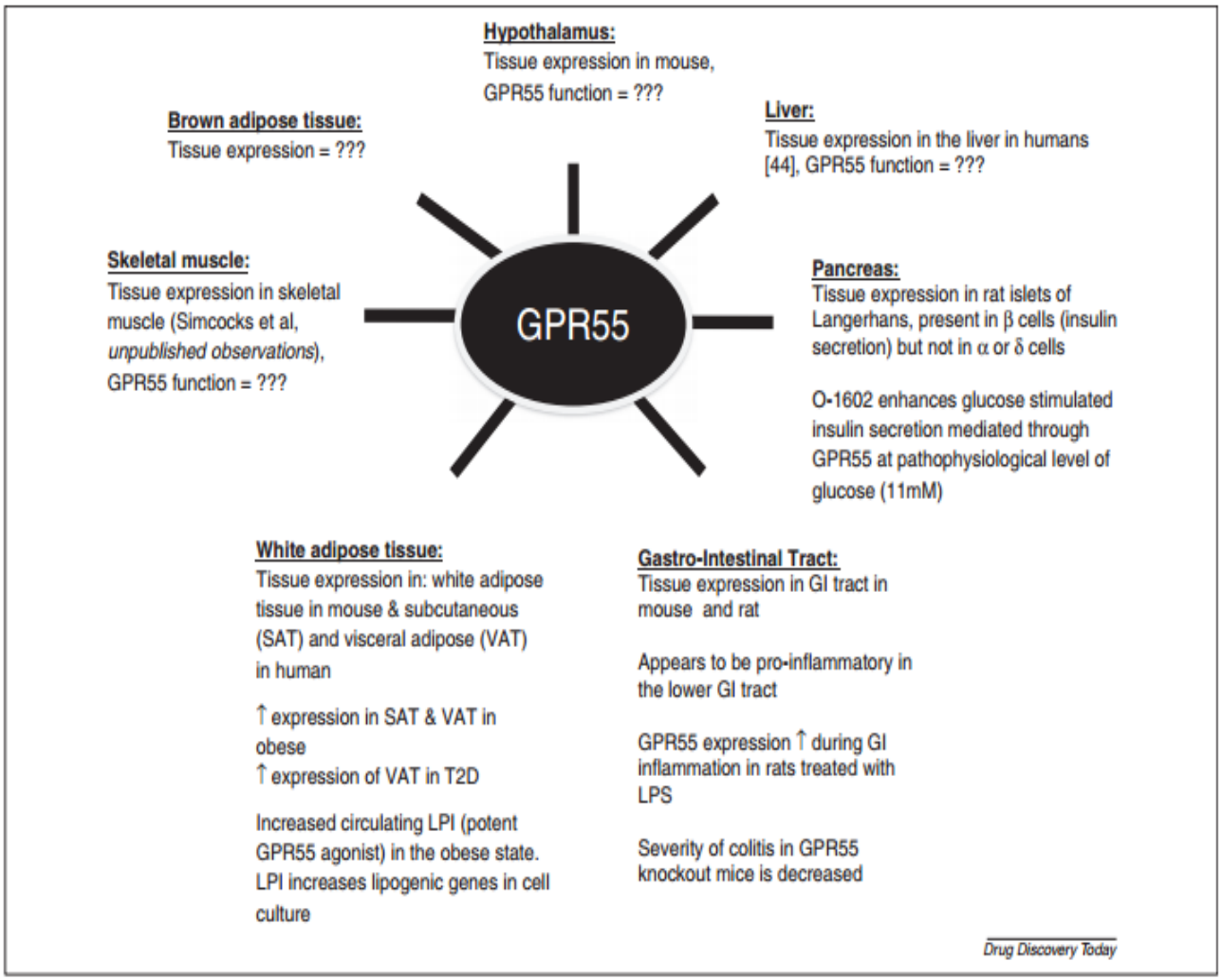


Figure 1.5B: G protein-coupled receptor 55 (GPR55) expression and its postulated role

This figure summarises the currently known tissue distribution of GPR55 in metabolically active organs that are involved in the regulation of energy homeostasis; including the hypothalamus (Ryberg, Larsson et al. 2007), gastrointestinal tract (Ryberg, Larsson et al. 2007; Lin, Yuece et al. 2011; Schicho, Bashashati et al. 2011), pancreas (Romero-Zerbo, Rafacho et al. 2011), liver, skeletal muscle (A. Simcocks et al., unpublished), white (Moreno-Navarrete, Catalan et al. 2012) and brown adipose tissue and the presently known physiological role that GPR55 has in these tissues. All these tissues are affected by obesity and related metabolic states.

A recent study by Moreno-Navarrete et al. 2012 demonstrated that GPR55 expression was increased in both visceral and subcutaneous adipose tissue in obese and T2DM (Moreno-Navarrete, Catalan et al. 2012). In addition to this, LPI is also increased in obesity in humans, with LPI increasing [Ca^{2+}] and the expression of lipogenic genes in a cell culture model obtained from human adipose tissue (Moreno-Navarrete, Catalan et al. 2012). The GPR55 agonist, O-1602, has also been shown to influence obesity, because this compound increased food intake and adiposity in Sprague-Dawley rats (Diaz-Arteaga, Vazquez et al. 2012). However, the increase in food intake was still evident in GPR55-knockout mice (Diaz-Arteaga, Vazquez et al. 2012), indicating that this compound was also acting on other receptor(s). GPR55 might also have a role in improving diabetic complications via the modulation of glucose homeostasis through targeting of the pancreas. GPR55 mRNA and protein were identified in rat islets of Langerhans (Romero-Zerbo, Rafacho et al. 2011). Specifically, GPR55 is expressed in b cells, which secrete insulin, whereas neither a cells, which secrete glucagon, nor d cells, which secrete somatostatin (Elayat, el-Naggar et al. 1995), express GPR55 (Romero-Zerbo, Rafacho et al. 2011). This cellular localisation indicates that GPR55 is involved in the endocrine function of the pancreas, but only through insulin secretion and possibly the maintenance of blood glucose concentrations (Romero-Zerbo, Rafacho et al. 2011). Treatment with O-1602 in the presence of 11 mM glucose, resulted in an increase in intracellular Ca^{2+} (Romero-Zerbo, Rafacho et al. 2011) and an almost twofold increase in glucose-stimulated insulin secretion (Romero-Zerbo, Rafacho et al. 2011). However, lower concentrations of O-1602 and glucose did not alter insulin secretion, compared with glucose alone (Romero-Zerbo, Rafacho et al. 2011). This was supported by analysis of GPR55-knockout mice, where this effect was abolished (Romero-Zerbo, Rafacho et al. 2011). Furthermore, in lean Wistar rats, the administration of O-1602 (0.2 mg/ kg) resulted in a significant decrease in blood glucose concentrations at 15, 30 and 60 minutes after the administration of a glucose load when compared with the vehicle (Romero-Zerbo, Rafacho et al. 2011). O-1602 caused an increase in plasma insulin concentrations 30 minutes after glucose administration (Romero-Zerbo, Rafacho et al. 2011).

In obese and T2DM states, there can be a reduced insulin secretion response as a result of damage to b cells and apoptosis (Butler, Janson et al. 2003), as well as insulin resistance in insulin-sensitive tissues, which together result in hyperglycaemia (Weyer, Bogardus et al. 1999). Given that O-1602 enhances glucose-stimulated insulin secretion (mediated through GPR55), this compound could improve β cell function and, therefore, could be a potential therapeutic target for treating T2DM. Further investigation into this compound is required to look specifically at T2DM models that display β cell apoptosis and insulin resistance. The study by Romero-Zerbo et al. 2011 used lean Wistar rats and, therefore, indicated the role that this compound has in normal glucose homeostasis. T2DM is associated with insulin resistance in target tissues, including the skeletal muscle and adipose tissue. However, the functional role of GPR55 in skeletal muscle in obesity and T2DM is poorly understood.

1.5.1.6 Physiological role for GPR55 in skeletal muscle?

The skeletal muscle is an essential organ for the regulation of whole-body energy metabolism. Dysfunction in this organ in obese and T2DM states is characterised by impaired glucose and lipid oxidation. Furthermore, muscle dysfunction can also result in changes to mitochondria that negatively impact insulin sensitivity and glucose metabolism (Kim, Wei et al. 2008). To improve nutrient handling for glucose uptake through enhancing insulin sensitivity and fatty acid oxidation, these pathways in the skeletal muscle can be modulated pharmacologically (Carey and Kingwell 2009). GPR55 has been shown to induce phosphorylation of p38 MAPK and ERK1/2 in HEK293 cells that overexpress GPR55 (Henstridge, Balenga et al. 2010; Oka, Kimura et al. 2010). Activation of p38 MAPK in the skeletal muscle is involved in stimulating mitochondrial biogenesis and oxidative capacity through activating the downstream target Peroxisome proliferator-activated receptor gamma co activator 1-alpha (Akimoto, Pohnert et al. 2005) and glucose transport through the translocation of glucose transporter 4 (GLUT 4) (Somwar, Kim et al. 2001). ERK1/2 triggers cluster differentiation 36 (CD36), which stimulates fatty acid oxidation and fatty acid uptake (Turcotte, Raney et al. 2005) and is also thought to be partly involved in glucose uptake (Chen, Bandyopadhyay et al. 2002). Together,

activation of these pathways in the skeletal muscle can promote signalling that enhances nutrient utilisation (Leng, Steiler et al. 2004) and might be beneficial for treatment of obesity and associated disease states. Given that the presence of GPR55 in skeletal muscle has been determined (*A. Simcocks et al., unpublished observations*), GPR55 has also been demonstrated to be expressed in smooth muscle in mice (Schicho, Bashashati et al. 2011) and cardiac muscle in neonatal rat cardiomyocytes (Yu, Deliu et al. 2013). Observations in skeletal muscle for GPR55 are in line with the observation that other GPCRs are expressed in the skeletal muscle (Cavuoto, McAinch et al. 2007). Therefore, it can be hypothesised that pharmacological activation of GPR55 in the skeletal muscle might up regulate phosphorylation of both p38 MAPK and ERK1/2 and be beneficial for the treatment of obesity and associated comorbidities. Stimulation of GPR55 using LPI also results in activation of NFAT in HEK293 cells overexpressing GPR55 (Henstridge, Balenga et al. 2009). In the skeletal muscle, NFAT is involved in fibre-type switching to enhance oxidative capacity (Mutungi 2008). Therefore, activation of GPR55 in skeletal muscle might result in activation of NFAT, and this could be a potential therapeutic target for obesity and T2DM. Thus, this novel receptor might have a physiological role in skeletal muscle homeostasis and its activation might be a useful target for metabolic disorders, such as obesity and T2DM.

1.5.1.7 Concluding Remarks

GPR55 is a putative cannabinoid receptor that has been demonstrated to be involved in numerous physiological processes in several tissues. Although it might be a potential drug target for obesity and T2DM, most research in this area has utilised two synthetic compounds, O-1602 and O-1918, which are nonspecific and are thought to act on other receptors, such as GPR18. Therefore, the development and experimental investigation of new and selective GPR55 agonists and antagonists are essential. Thus, further research into the physiological role of GPR55 is important to gain a better understanding of the role that this receptor might have in pathophysiological conditions, including obesity and T2DM.

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This ends the extract of the manuscript Simcocks AC, O'Keefe L, Jenkin KA, Mathai ML, Hryciw DH & McAinch AJ 2014, 'A potential role for GPR55 in the regulation of energy homeostasis', Drug Discovery Today, vol. 19, no. 8, pp. 1145 - 1151.

1.5.2 G Protein-Coupled Receptor 18 (GPR18)

G protein-coupled receptor 18 (GPR18) is a 331 amino acid, seven transmembrane G protein-coupled receptor, that was first cloned in humans in 1997 (Gantz, Muraoka et al. 1997), and is mapped to chromosome 13q32 (Gantz, Muraoka et al. 1997). Orthologues for GPR18 have also been found in rodents and canines (Gantz, Muraoka et al. 1997; Vassilatis, Hohmann et al. 2003).

GPR18 is highly expressed in testes, but is also abundant in tissues involved in immune regulation, these tissues include; the thymus, spleen, lymph nodes, appendiceal tissue, peripheral blood leukocytes in humans (Gantz, Muraoka et al. 1997).

1.5.2.1 The Pharmacology of GPR18

As mentioned previously GPR18 is a putative cannabinoid receptor (McHugh, Hu et al. 2010; McHugh, Page et al. 2012). It has been suggested that GPR18 could be the Abn-CBD receptor or CBx (McHugh, Hu et al. 2010), although, the protein sequences only meet 13% and 8% similarity to the other cannabinoid receptors CB_1 and CB_2 receptors, respectively (Rajaraman, Simcocks et al. 2016). Like GPR55, currently GPR18 does not meet the IUPHAR cannabinoid receptor classification criteria, so can only be deemed a putative cannabinoid receptor (Pertwee, Howlett et al. 2010). There are a number of non-specific agonists and antagonists for GPR18, many of these compounds are cannabinoid derived or have a similar structure to cannabinoids (Alexander 2012). The pharmacology for GPR18 appears to be quite complex, like GPR55 has also been shown. A potent agonist for GPR18 seems to be *N* arachidonyl glycine (NAGly), which is an endogenous metabolite of the endocannabinoid AEA, this finding is supported by a number of studies (Kohno, Hasegawa et al. 2006; McHugh, Hu et al. 2010; McHugh, Page et al. 2012; Console-Bram, Brailoiu et al. 2014). However, studies by Lu et al. (2013) and Yin et al. (2009) contradict the fact that NAGly is an agonist for GPR18. Neuronal cells injected with GPR18 treated with NAGly did not activate GPR18, at least by the pathways that this type of G Protein-Coupled Receptor usually would signal through (Lu, Puhl et al. 2013). The study by Yin et al, (2009) reported NAGly did not activate GPR18 when a lipid screen was

completed (Yin, Chu et al. 2009). Additional GPR18 cannabinoid agonists appear to include: Δ^{-9} THC, Abn-CBD, O-1602 and AEA (Yin, Chu et al. 2009). Although the same scenario occurred whereby, Lu et al. (2013) found that Abn-CBD, AEA and O-1602 did not appear to activate GPR18 at least through the expected signalling pathways. While antagonists for GPR18 include AM251, CBD (which both also appear to act as partial agonists) and O-1918 (McHugh, Hu et al. 2010), although O-1918 has recently also been shown to act as a biased agonist (Console-Bram, Brailoiu et al. 2014). These conflicting finding indicate the complexity surrounding cannabinoid signalling and given that these compounds are known to act on other receptors such as GPR55 and that G Protein-Coupled Receptors can form heteromers complexes in tissues may attribute to this complexity. It is clear that further research into understanding these complexities is required.

1.5.2.2 The possible role for GPR18 in energy homeostasis

The role of GPR18 is largely unknown, with limited research conducted into this receptors' (patho) physiological role. Currently to the best of my knowledge the role of GPR18 in energy metabolism is unknown. While this receptor is expressed in tissues involved in energy metabolism such as, adipose tissue in human (Amisten, Neville et al. 2015) and is highly expressed in the hypothalamus of the brain of mice (although does not appear to be expressed in whole brain in human) (Gantz, Muraoka et al. 1997; Vassilatis, Hohmann et al. 2003). Interestingly, O-1602 induced feeding behaviour in GPR55 knockout mice, it may be feasible to hypotheses that GPR18 could have some involvement in feeding behaviour, especially given that the receptor is expressed in mice hypothalamus (Vassilatis, Hohmann et al. 2003), however this is just speculation. Studies have shown that this receptor is not expressed in other organs involved in regulating energy homeostasis such as skeletal muscle, pancreas, liver in either/ or both human and mouse depending on the tissue type (Gantz, Muraoka et al. 1997; Vassilatis, Hohmann et al. 2003). *Simcocks et al. unpublished observations*, do however indicate that GPR18 is expressed in the gastrocnemius skeletal muscle obtained from rat (data shown in *chapter seven*). In the gastrointestinal system which is involved in food/ nutrient digestion and absorption, GPR18 appears

to be expressed in the stomach, intestine and colon of mice and small intestine of humans in which this receptor may also have some involvement. As with GPR55, the expression of GPR18 in brown adipose tissue is yet to be determined.

As mentioned previously, GPR18 is abundant in tissues of the immune system, so it maybe hypothesised that GPR18 could have a role in conditions that involve immune system regulation such as obesity whereby chronic low grade inflammation is present and is a risk factor for insulin resistance and T2DM (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003).

While some of the atypical cannabinoid compounds act as agonists, biased agonist, partial agonists or antagonists have been used in a number of studies looking at GPR18, the effects that these compounds have may not necessarily be fully be attributed to GPR18 alone. Given the conflicting reports surrounding the pharmacology of GPR18 and cannabinoid compounds, the development of GPR18 selective agonists and antagonists may also help to provide more (patho) physiological information when this receptor is ablated, which could help in areas of treatment for obesity, insulin resistance and/ or T2DM.

Despite all the conflicting pharmacological reports in the literature regarding which receptors the compounds O-1602 or O-1918 target, the role that these compounds have in energy homeostasis in obesity is unclear. O-1602 clearly has a role in energy homeostasis and adiposity in lean rats (Diaz-Arteaga, Vazquez et al. 2012), and reports from our laboratory show that O-1918 treatment up-regulates the mRNA expression of markers involved in fatty acid oxidation, fibre type switching and mitochondrial biogenesis in C_2C_{12} myotubes (see Appendix Two). Therefore it would be of benefit to determine whether these pharmacological compounds would be a suitable modality for obesity and associated co-morbidities such as insulin resistance and T2DM.

1.6 Different dietary fatty acids

Not all fatty acids have the same physiological role. Dietary fatty acids can be classified into different groups based on their chemical structure. The carbon atoms in saturated fatty acids (SFA) are bound by single hydrogen bounds, while monounsaturated fatty acids (MUFA) have one single double bond and polyunsaturated fatty acids (PUFA) have more than one double bond. PUFA can be further grouped as omega-3 or omega-6 PUFA depending on the location of the double bond from the methyl end of the fatty acid. Omega-3 PUFA have a double bond three carbons away from the methyl end, while omega-6 PUFA have a double bond six carbons away from the methyl end. A high SFA intake is associated with an increased risk of cardiovascular disease, due to the ability for these FAs to increase circulating low density lipoproteins (LDL) (Kromhout, Menotti et al. 1995). While recommendations for the prevention of CVD indicate that MUFA and PUFA are beneficial to help reduce the risk of heart disease, as MUFA increase high density lipoproteins (HDL) and PUFA increase HDL and decrease LDL (Eilat-Adar, Sinai et al. 2013).

As a result of these dietary recommendations there has been an increase in vegetable oils consumption, as these oils are high in PUFAs. While fatty acids can be classified based on their chemical structure, not all fatty acids have the same physiological implications even within this grouping classification. An example of this is when comparing lauric acid and palmitic acid, both of these molecules are classified as SFA but appear to have different physiological roles. The palmitic acid associated fatty acid palmitate is a long chain fatty acid and has been found to promote insulin resistance within the skeletal muscle (Hommelberg, Plat et al. 2011), which is mediated by increased inflammation, reduced mitochondrial function and oxidative capacity and also increased oxidative stress (Martins, Nachbar et al. 2012). On the other hand, the medium chain lauric acid has been shown to have the potential to decrease adiposity, increase energy expenditure and oxidative capacity in the skeletal muscle (Montgomery, Osborne et al. 2013).

Another example of this is when comparing PUFA which are further broken omega-6 fatty acids and omega-3 fatty acids. In quite broad terms, omega-6 fatty acids can be classed as pro-inflammatory molecules, while omega-3 long chain fatty acids can be classed as anti-inflammatory molecules (Wall, Ross et al. 2010). Linoleic acid, an essential omega-6 dietary fatty acid is hypothesised to partially be associated with the increased obesity rates as well as the associated co-morbidities (Naughton, Mathai et al. 2016). The consumption of linoleic acid has increased largely over recent decades as a result of nutrition transition to a 'Western Diet' resulting in plant based vegetable oils that are high in linoleic acid as they are convenient to produce and cost effective (Naughton, Mathai et al. 2016) which is also likely contributing to this observed increased consumption. This essential fatty acid has the ability to convert to arachidonic acid, and to the endocannabinoids AEA and 2-AG. Not surprisingly both increased dietary linoleic acid and arachidonic acid are associated with increased circulating endocannabinoid concentrations (Alvheim, Malde et al. 2012). As mentioned earlier negative health consequences associated with an over-active endocannabinoid system, include: weight gain, increased adipose storage and insulin resistance which all contribute to the pathogenesis of obesity and T2DM.

In addition to endocannbinoids, two other relevant compounds that are structural analogues of AEA and that have been found to have a role in energy homeostasis are OEA and PEA. OEA plays a role in inducing satiety and studies have shown that short term exposure to high fat feeding as seen in obesity in a rodent model lowers the amount of OEA in the small intestine (Diep, Madsen et al. 2011). Furthermore, the production of OEA is altered in the small intestine of diet induced rats and mice (Igarashi, DiPatrizio et al. 2015). While PEA is proposed to have an anti-inflammatory role, in ApoE knockout mice, a model used to induce atherosclerotic plaque (even when animals are fed a standard chow diet (SCD)), the VAT concentrations of PEA are decreased (Montecucco, Matias et al. 2009). Furthermore, PEA down regulates TNFα in human primary adipocytes induced inflammation (with treatment of LPS) and reduced concentrations of leptin (Hoareau, Buyse et al. 2009).

OEA is derived from the MUFA, oleic acid while paradoxically PEA is derived from the SFA, palmitic acid. For molecular structure of the dietary fatty acids see Table 1.6A. While fatty acids are not receptor specific, OEA has been reported to activate GPR55 (Ryberg, Larsson et al. 2007) in addition to being a potent agonist of GPR119 (Overton, Fyfe et al. 2008). While one study showed that PEA does activate GPR55 (Ryberg, Larsson et al. 2007), another found that this compound did not elicit a response (Kapur, Zhao et al. 2009). The roles that these fatty acidss have on GPR18 do not appear to have been assessed. For molecular structures of endocannabinoids and acylethanolamides see Table 1.6B.

Taken together it would be beneficial to determine the role that different dietary fatty acids have in obesity with a focus on whole body energy homeostasis and how this could be hypothetically attributed to putative cannabinoid receptors such as GPR55 and GPR18.

Table 1.6A: Chemical structure of certain dietary fatty acids which act as precursors to certain endocannabinoids and other acylethanolamines

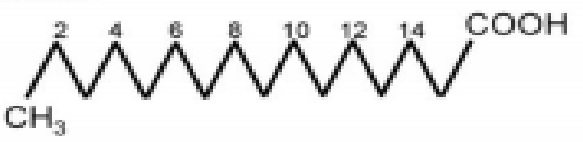
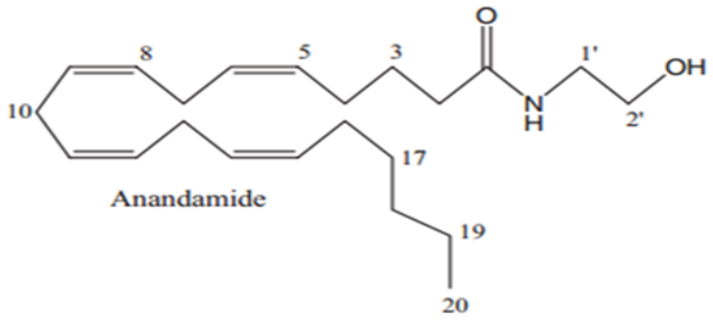
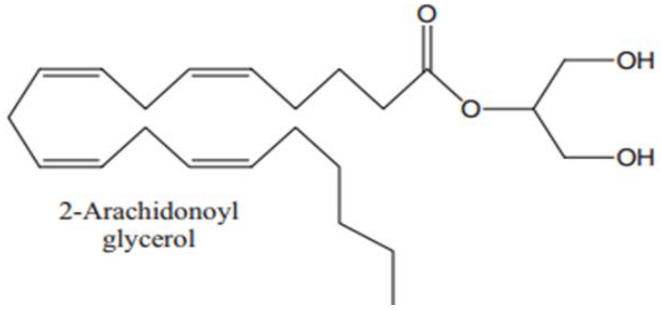
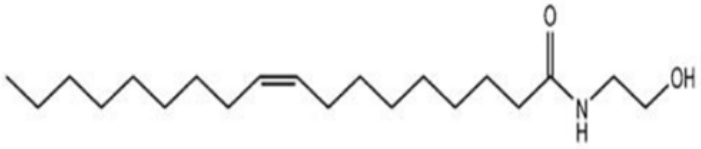
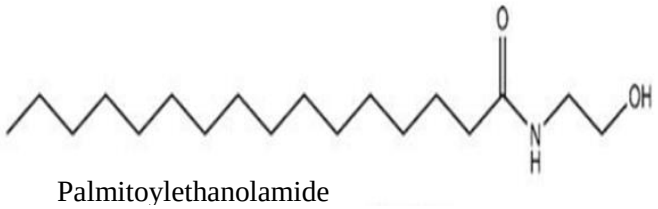
Linoleic Acid (Rennison and Van Wagoner 2009)	
Arachidonic Acid (Rennison and Van Wagoner 2009)	
Oleic Acid (Rennison and Van Wagoner 2009)	
Palmitic Acid (Rennison and Van Wagoner 2009)	

Table 1.6B: Chemical structure of certain endocannabinoids and other acylethanolamines

Anandamide (Pertwee, Howlett et al. 2010)	
2 Arachidonyl Glycerol (Pertwee, Howlett et al. 2010)	
OEA (Hansen and Diep 2009)	
PEA (Hansen and Diep 2009)	

1.7 General Aims

The general aim of this thesis was to determine the roles atypical cannabinoid compounds O-1602 or O-1918 have on whole body energy homeostasis or skeletal muscle homeostasis in DIO. In addition the general aim of this thesis was to also to determine the effect that HFDs predominately composed of different dietary fatty acids have on DIO by specifically determining their role on whole body energy homeostasis and circulating hormones and cytokines.

1.8 Specific Aims

1.8.1 Chapter Three

To determine the effect that O-1602 has on homeostatic skeletal muscle homeostasis (including oxidative capacity, fatty acid metabolism and adiponectin signalling).

1.8.2 Chapter Four

To determine the effect that O-1602 has on whole body energy homeostasis in a DIO rat model.

1.8.3 Chapter Five

To determine the effect that O-1918 has on whole body energy homeostasis in a DIO rat model.

1.8.4 Chapter Six

To determine the effect that either O-1602 or O-1918 have on peripheral skeletal muscle homeostasis in a DIO rat model.

1.8.5 Chapter Seven

To determine the effect that the consumption of different dietary fatty acids (SFA, MUFA and omega-6 PUFA fatty acids) have on whole body energy homeostasis in a DIO rat model.

Hypothesis

Chapter Three

As O-1602 is associated with increased adiposity in rodents (Diaz-Arteaga, Vazquez et al. 2012) and C_2C_{12} myotubes treated with O-1918 significantly up-regulate markers of oxidative capacity and fatty acid metabolism and there was a trend for a mediator of adiponectin signalling to be up-regulated (*Simcocks et al, unpublished observations see Appendix Two*), it is hypothesised that O-1602 will down regulate markers of oxidative capacity and adiponectin signalling in C_2C_{12} myotubes.

Chapter Four

As O-1602 is associated with increased adiposity in rodents (Diaz-Arteaga, Vazquez et al. 2012) and C_2C_{12} myotubes treated with O-1918 significantly up-regulate markers of oxidative capacity and fatty acid metabolism and there was a trend for a mediator of adiponectin signalling to be up-regulated (*Simcocks et al, unpublished observations see Appendix Two*), it is hypothesised that in DIO rats O-1602 will cause weight gain, increased body fat percentage, increased fat pad mass (epididymal and peri-renal), increased circulating leptin, increased circulating ghrelin, decreased adiponectin, increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines.

Chapter Five

As C_2C_{12} myotubes treated with O-1918 significantly up-regulate markers of oxidative capacity and fatty acid metabolism with a trend to increase mediators of adiponectin signalling (Simcocks et al, unpublished observations see Appendix Two), it is hypothesised that in DIO rats that O-1918 will cause weight loss, decreased fat mass and body fat percentage, decreased fat pad mass (epididymal and perirenal), decreased circulating leptin, decreased circulating ghrelin and increased adiponectin, decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines.

Chapter Six

Given that GPR55 and GPR18 are expressed in a number of tissues involved in energy homeostasis, it is hypothesised that both receptors will be expressed in the skeletal muscle obtained from the DIO rats.

In addition to the hypotheses in *Chapters Three* and *Four*, it is hypothesised that O-1602 will down regulate markers of oxidative metabolism, fatty acid metabolism and adiponectin signalling, particularly in slow twitch red gastrocnemius skeletal muscle obtained from the DIO rats.

In addition to the hypotheses in *Chapter Five*, it is hypothesised that O-1918 will up-regulate markers of oxidative metabolism, fatty acid metabolism and positive mediators of adiponectin signalling, particularly in slow twitch red gastrocnemius skeletal muscle obtained from the DIO rats.

Chapter Seven

It is hypothesised that DIO rats fed a high fat SFA diet will result in impaired glucose tolerance and insulin sensitivity, increased pro-inflammatory cytokines and decrease anti-inflammatory cytokines. Due to the duration of the diet only being six weeks it is not hypothesised that there will be a significant change in body fat pad mass or weight during this time.

It is hypothesised that DIO rats fed a high fat MUFA diet will result in improvements in glucose tolerance and insulin sensitivity, it is also hypothesised that there would be a reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines. Due to the duration of the diet only being six weeks it is not hypothesised that there will be a significant change in body fat pad mass or weight during this time.

It is hypothesised that DIO rats fed a high fat PUFA diet (linoleic being a precursor to endocannabinoids) will result in further impairment in glucose tolerance and insulin sensitivity, it is also hypothesised that there would be an increase in pro-inflammatory cytokines and a decrease in

anti-inflammatory cytokines. Due to the duration of the diet only being six weeks it is not hypothesised that there will be a significant change in body fat pad mass or weight during this time.

It is hypothesised that DIO rats that recommence a SCD diet will have a reduction in body weight, body fat mass improvements in glucose tolerance and insulin sensitivity, reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines.

CHAPTER TWO

2.0 Detailed methodology for all experiments

2.1 Cell Culture

2.1.1 C_2C_{12} Myoblast Proliferation

C_2C_{12} myoblasts (a kind gift from Professor David Cameron-Smith, Deakin University, Melbourne, Australia) are a continuous immortalised cell line which are derived from CH3 mouse thigh muscle satellite cells (Yaffe and Saxel 1977). The passage nine cells were obtained from the liquid nitrogen storage system (in which they were stored and suspended in freezing medium). The cells were immediately thawed and added to growth medium and centrifuged at room temperature at 500 x g for five minutes. Supernatant containing the freezing medium (10% Dimethyl Sulfoxide (DMSO)) was discarded and the pellet of C_2C_{12} myoblasts were resuspended and maintained in Dulbecco's Modified Eagle Medium (D-MEM) high glucose growth medium (Invitrogen, Victoria, Australia, Victoria, Australia) which contains 10% foetal bovine serum (vol/ vol) (Invitrogen, Victoria, Australia, Victoria, Australia), 1% penicillin streptomycin (vol/ vol) (Invitrogen, Victoria, Australia, Victoria, Australia), 0.5% Amphotericin B (vol/ vol) (Invitrogen, Victoria, Australia, Victoria, Australia) and maintained in a cell culture incubator at 37°C, 5% CO_2. C_2C_{12} myoblast were cultured according to previously established methods Cornall et al. (2013) which is briefly detailed in this section (2.1.1) and section 2.1.2. Cells were washed two times with phosphate buffered saline (PBS) and the growth medium was changed every second day.

2.1.2 Differentiation of C_2C_{12} Myoblasts into C_2C_{12}Myotubes

For the differentiation process once the cells had reached ~70% confluence they were detached from flask using trypsin (Invitrogen, Victoria, Australia, Victoria, Australia), centrifuged and resuspended immediately in differentiation medium (D-MEM high glucose media containing 2% horse serum (vol/

vol), 1% penicillin streptomycin (vol/ vol) and 0.5% Amphotericin B (vol/ vol)) and cells were seeded into six well plates for future treatment and subsequent mRNA expression analysis. The C_2C_{12} cells were differentiated for 72 hours with the media changed every second day.

2.1.3 Serum Starving

Following the differentiation period, C_2C_{12} myotubes were washed three times with PBS and then serum starved in D-MEM medium containing 0.1% bovine serum albumin (BSA) for six hours prior to treatment. Serum starvation is a widely used process in cellular experiments as it maintains the cells in a quiescent state, as the serum provides conditions for optimal growth.

2.1.4 Treatment

Following the serum starving process, the C_2C_{12} myotubes were treated for 24 hours with either control (0.1% ethanol) or a dosage range of 10 – 1000 nM of the atypical cannabinoid compound, O-1602 and 0.1% ethanol (Cayman Chemicals, Michigan, USA) in D-MEM containing 0.1% BSA. The concentrations selected for the dose response were based around the effective concentration 50 (EC_{50}) for O-1602, which has previously been shown to be 13 nM ± 2 in a GTPγS binding assay in human embryonic kidney (HEK293) cells transiently transfected with human GPR55 (Ryberg, Larsson et al. 2007) and was also the dose range that I have previously used to treat obese and diabetic human primary myotubes (*Simcocks et al. Unpublished Observations*). The stock solution of O-1602 was suspended in 100% ethanol, the treatment doses were then added to D-MEM medium containing only 0.1% BSA and the ethanol concentration was standardised to 0.1% ethanol. 1 mL of the treatment was added to the cells in the corresponding well. See Figure 2.1A for a visual representation of the treatments dosages prepared for the O-1602 C_2C_{12} myotube cell culture experiment.

The myotubes were treated n = nine per treatment group, the same dosage of the compound or vehicle was spread across the nine separate six well plates, this was to take into account the plate to plate variation of cells, as well as minimising the risk of the majority of one treatment group being lost due

to one of the plates becoming contaminated. See Figure 2.1B, which highlights the treatments for the O-1602 C_2C_{12} cell culture experiment across the 9 x 6 well plates.

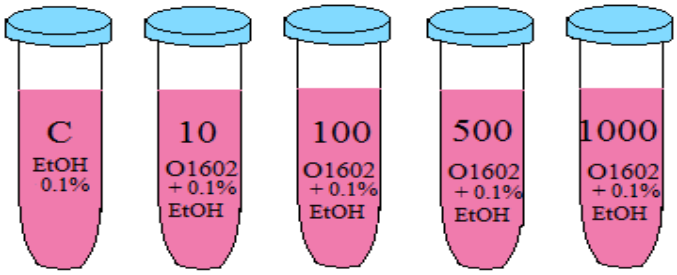


Figure 2.1A: A visual representation of the treatments for the O-1602 C_2C_{12} cell culture experiment. Control = D-MEM 0.1% BSA 0.1% EtOH; Treatment Groups = D-MEM 0.1% BSA and 0.1% EtOH 10nM, 100nM, 500nM and 1000nM.

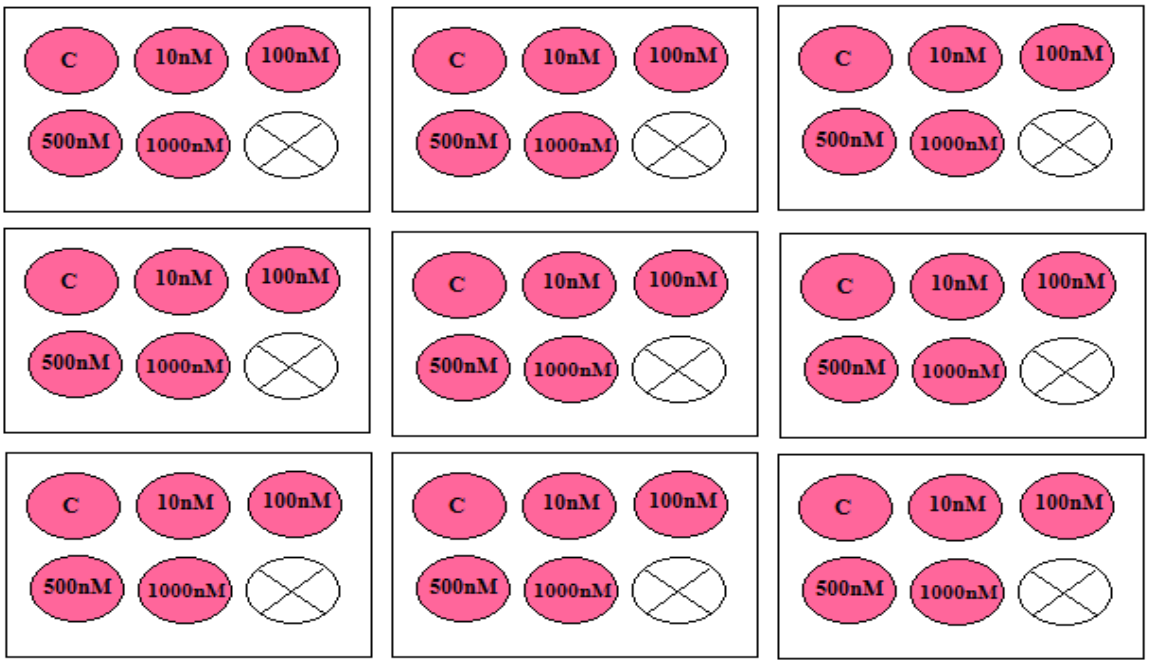


Figure 2.1B: A visual representation of treatments for the O-1602 C_2C_{12} cell culture experiment across the 9 x 6 well plates.

Following the 24 hour treatment period, the cells were washed three times with ice cold PBS, excess liquid was removed from the wells with a pasture pipette. The cells were transferred on ice from the Class 2 Biological Safety Cabinet into a fume hood, and were then lysed using 800 µL of TRIzol reagent (Invitrogen, Victoria, Australia). The cells were then swirled gently and the TRIzol reagent

containing cell lysate was then transferred into a 1.5 mL Eppendorf tube and stored at – 80°C for subsequent RNA extraction (refer to section 2.7.1 for details).

2.2 Animals

In *Chapters Four – Seven*, seven week old male Sprague Dawley rats were utilised, with a starting body weight between 280 – 400 grams. All of the rats were all purchased from the Animal Resources Centre (ARC), (Canning Vale, WA, Australia).

Sprague Dawley rats were selected to be utilised due to their ability to gain weight on a HFD (Farley, Cook et al. 2003). This strain of rat also shows a diverse response in weight gain to a HFD with some Sprague Dawley rats being obese resistant (lean phenotype) and some being predisposed to obesity (obese phenotype) (Levin, Dunn-Meynell et al. 1997; Farley, Cook et al. 2003) which is a similar response that is observed in humans that consume a long term HFD.

In *Chapters Four – Six* the rats were housed at Howard Florey Institute, Melbourne University, Parkville, Australia. Ethical approval was obtained for this study by the Howard Florey Animal Ethics Committee (AEC) # 11-036. In *Chapter Seven*, rats were housed at the Werribee Animal House, Victoria University, Australia. Ethical approval was obtained for this study by the Victoria University Animal Experimental Ethics Committee (VU AEEC) # 13/ 051.

In both establishments the rats were housed at a temperature of 22 – 24 °C and a twelve hour light/ dark cycle (07:00 lights on, 19:00 lights off). Rats were housed separately to assess food intake over the duration of both studies. The rats had *ad libitum* access to food and water. Prior to commencement of the experimental procedures the animals were acclimatised into their new environment for at least one week.

2.3 Chapters Four – Six the effect that either O-1602 or O-1918 administration has on whole body and skeletal muscle energy homeostasis in a DIO rat model

This study was part of a larger study that was completed in collaboration with two other PhD students at the time (Ms Lannie O'Keefe and Dr Kayte Jenkin), which involved the investigation of different compounds not detailed in this thesis, as well as the effects of O-1602 or O-1918 on the kidney.

2.3.1 Dietary Intervention

The Sprague Dawley rats were randomly allocated into either a HFD group to induce obesity or a SCD group for the duration of the fifteen week study. The rats that were maintained on either ahigh fat western diet (21% fat, by weight; SF00-219) purchased from Speciality Feeds (Glen Forrest, WA, Australia), or the rat and mouse SCD (5% fat) which was supplied from Barastoc (Ridley Agriproducts, Pakenham, Victoria, Australia). See Tables 2.3A – 2.3D for the ingredients listed for both of the diets and the nutritional composition.

Table 2.3A: Ingredients list for the high fat Western diet (SF00-219).

Ingredients	Western Diet SF00-219
Casein (Acid)	195 g/ Kg
Sucrose	341 g/ Kg
Clarified Butter (Ghee)	210 g/ Kg
Cellulose	50 g/ Kg
Wheat Starch	154 g/ Kg
DL Methionine	3.0 g/ Kg
Calcium Carbonate	17.1 g/ Kg
Sodium Chloride	2.6 g/ Kg
AIN93 Trace Minerals	1.4 g/ Kg
Potassium Citrate	2.6 g/ Kg
Potassium Dihydrogen Phosphate	6.9 g/ Kg
Potassium Sulphate	1.6 g/ Kg
Choline Chloride (75%)	2.5 g/ Kg
SF00-219 Vitamins or AIN93 Vitamins	10 g/ Kg
Cholesterol	1.5 g/ Kg
Oxicap E2	0.04 g/ Kg

Table 2.3B: Ingredients list for Barastoc Rat and Mouse SCD (Quantities of the diet ingredients are unknown)

Ingredients
Cereal grains and cereal by-products
Legumes and legume by-products
Vegetable protein meals
Fats and oil
Vitamins
Minerals
Yucca Schidigera extracts

Table 2.3C: The nutritional composition of the Speciality Feeds high fat Western diet (SF00-219).

Nutritional Composition	Western Diet (SF00-219)
Protein	19.0%
Total Fat	21.0%
Crude Fibre	4.7%
AD Fibre	4.7%
Digestible Energy	19.4 MJ/ Kg
% Total calculated digestible energy from lipids	40.0%
% Total calculated digestible energy from proteins	17.0%
Amino Acids Composition	
Valine	1.20%
Leucine	1.80%
Isoleucine	0.80%
Threonine	0.80%
Methionine	0.80%
Cystine	0.06%
Lysine	1.50%
Phenylanine	1.00%
Tyrosine	1.00%
Tryptophan	0.30%
Histidine	0.59%

Calculated Total Minerals	
Calcium	0.60%
Phosphorous	0.30%
Magnesium	0.10%
Sodium	0.12%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	80 mg/ Kg
Copper	7.0 mg/ Kg
Iodine	0.2 mg/ Kg
Manganese	20 mg/ Kg
Zinc	52 mg/ Kg
Molybdenum	0.15 mg/ Kg
Selenium	0.3 mg/ Kg
Chromium	1.0 mg/ Kg
Fluoride	1.0 mg/ Kg
Lithium	0.1 mg/ Kg
Boron	2.3 mg/ Kg
Nickel	0.5 mg/ Kg
Vanadium	0.1 mg/ Kg
Calculated Total Vitamins	
Vitamin A (Retinol)	11650 IU/ Kg
Vitamin D (Cholecalciferol)	1100 IU/ Kg
Vitamin E (α Tocopherol acetate)	64 mg/ Kg
Vitamin K (Menadione)	12.5 mg/ Kg
Vitamin C (Ascorbic acid)	700 mg/ Kg
Vitamin B1 (Thiamine)	11 mg/ Kg
Vitamin (Riboflavin)	11 mg/ Kg
Niacin (Nicotinic acid)	50 mg/ Kg
Vitamin B6 (Pryridoxine)	11 mg/ Kg
Pantothenic Acid	34 mg/ Kg
Biotin	200 µg /Kg
Folic Acid	1 mg/ Kg
Inositol	55 mg/ Kg
Vitamin B12 (Cyancobalamin)	18 µg/ Kg
Choline	3860 mg/ Kg

Calculated Fatty Acid Composition	
Saturated Fats C12:0 or less	1.80%
Myristic Acid 14:0	2.6%
Palmitic Acid 16:0	7.00%
Stearic Acid 18:0	2.40%
Palmitoleic Acid 16:1	0.40%
Oleic Acid 18:1	5.50%
Linoleic Acid 18:2 n6	0.40%
α Linolenic Acid 18:3 n3	0.20%
Arachadonic Acid 20:4 n6	Trace
Total n3	0.35%
Total n6	0.41%
Cholesterol	0.15%
Total Monounsaturated Fats	6.23%
Total Polyunsaturated Fats	0.77%
Total Saturated Fats	13.99%

Table 2.3D: The nutritional Composition of the Barastoc SCD

Nutritional Composition	Standard Chow Diet
Crude Protein (minimum)	20%
Crude Fat (minimum)	5.0%
Crude Fibre	5.0%
Digestible Energy	17.25 MJ/ Kg
% Total calculated digestible energy from lipids	10.7%
% Total calculated digestible energy from proteins	19.7%
Salt (maximum added)	0.5%
Copper (added)	7.5 mg/ kg
Selenium (added)	0.1 mg/ kg
Calcium (added)	0.8%
Phosphorous (minimum)	0.45%

2.3.2 Pharmacological intervention

The rats that were maintained on the HFD to induce obesity were allocated into one of three groups after the nine week feeding regime to induce obesity. For a further six weeks the rats were administered daily treatment via intraperitoneal (ip.) injection which consisted of 0.9% saline solution (Mcfarlane Medical, Surry Hills, Victoria, Australia), containing either the vehicle (Tween 80), O-1602 and vehicle or O-1918 and vehicle, and are detailed further in the paragraph below. Both O-1602 and O-1918 are insoluble in saline and therefore needed to be dissolved in a medium that is both hydrophobic and hydrophilic, such that the compounds can be suspended in and therefore delivered to the animals in a saline solution. Tween 80 (polyethylene sorbitol ester) is composed of fatty acid esters and can be used as an emulsifier/ or to disperse substances as it has both hydrophobic and hydrophilic properties and is commonly used as a vehicle for pharmaceutical purposes (Zhang, Yao et al. 2003).

The DIO control rats were administered a vehicle containing 0.75% Tween 80 in a 0.9% saline solution, the DIO O-1602 rats were administered O-1602 (Tocris Bioscience, Bristol, United Kingdom) at a dose of 5 mg/ kg of body weight, dissolved in 0.75% Tween 80 in a 0.9% saline solution, and the DIO O-1918 rats were administered O-1918 (Tocris Bioscience, Bristol, United Kingdom) at a dose of 1 mg/ kg of body weight (Offertaler, Mo et al. 2003) dissolved in 0.75% Tween 80 in a 0.9% saline solution. The lean control rats maintained on the SCD for fifteen weeks were also administered a vehicle containing 0.75% Tween 80 dissolved in a 0.9% saline solution. See Figure 2.3A for a flow chart that visually represents the treatment groups used in this study.

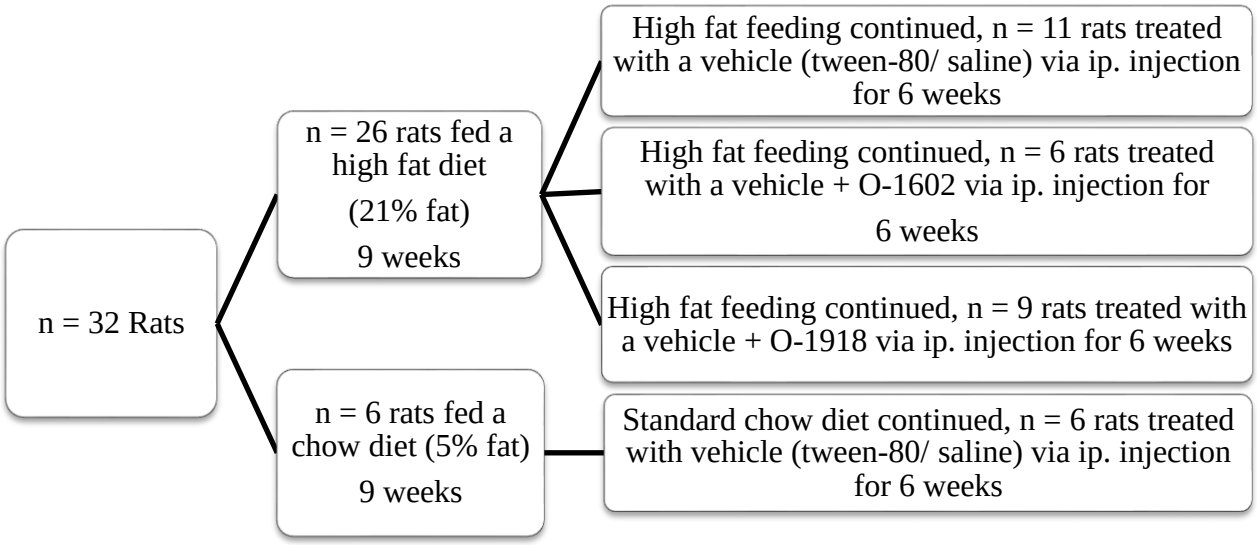


Figure 2.3A: A flow chart illustrating the treatment groups for *Chapters Four – Six* to determine the effect that the administration of either O-1602 or O-1918 have on whole body and skeletal muscle energy homeostasis in a DIO rat model.

2.3.2.1 Measurements taken over the experimental period

As previously mentioned Sprague Dawley rats have mixed phenotypes for obesity, which is similar to humans, therefore to ensure that all of the groups had evenly matched metabolic profiles at baseline prior to commencing the pharmacological treatment, a 'ranking criteria' was developed to evaluate the profiles for each group. This 'ranking criteria' takes into account the animal's body weight, body composition including both body fat percentage as well as lean body mass percentage, blood pressure including both systolic and diastolic (however, blood pressure measurements are not within the scope of this thesis, and have previously been reported in Jenkin et al (*Unpublished Observations*) and therefore will not be reported in this thesis), insulin sensitivity and glucose tolerance. The measurements as described above were all obtained during week nine prior to commencing the pharmacological treatment.

2.3.2.2 Food Intake and Weight

Food intake was measured via weighing the remaining pellets from both on top of the cage (where the food was presented daily) as well as in amongst the cage for any remaining food that hadn't been consumed. Bodyweight was measured in which rats were placed in a bucket and weighed on tared scales. Food intake and body weight were monitored daily during the beginning of the light cycle, and a weekly average for both measurements was calculated.

2.3.2.3 Echo Magnetic Resonance Imaging (MRI)

Echo MRI^{TM} system (Echo-MRI^{TM} 900, Houston, TX, United States of America) was utilised to determine whole body composition for the purpose of this study to differentiate between lean tissue and fat mass. Echo MRI is a validated method to obtain body composition in rats and mice (Taicher, Tinsley et al. 2003). Echo MRI is a non-invasive method used to measure live rats and this procedure does not required anaesthesia. This process involved firstly calibrating the machine using canola oil, following this the animal was then placed into a plastic tube (containing holes for breathing) and scanned in the Echo MRI to determine body composition. Rats were acclimatised to this procedure at week five and then measurements were taken at week nine (baseline), week twelve (mid-treatment) and then at week fifteen (end of treatment). See Figure 2.3B which highlights the Echo MRI measurements over the treatment period.

2.3.2.4 Intraperitoneal Glucose Tolerance Test (Ip. GTT)

An ip. GTT was performed to determine the effect of a glucose load on blood glucose concentrations over the treatment period. Without providing the mechanism of cause, a GTT helps to provide an overview of changes to glucose tolerance as a result of a treatment (Nagatomo, Gu et al. 2009; Bowe, Franklin et al. 2014). This procedure was completed at week nine (baseline) and the beginning of week fourteen (towards the end of the treatment period). In accordance with previously published methods (Jenkin, O'Keefe et al. 2016) rats were fasted overnight for approximately fifteen-sixteen hours, with adequate access to water. A tail snip was performed and drop of blood was gently obtained and

measured to calculate the baseline blood glucose concentration using a glucometer (Optimum, Xceed, Abbott, USA) and accompanying blood glucose strips. Following this the rats were administered a dose of 2 mg/ kg body weight of glucose/ 0.9% sterile saline solution (Mcfarlane Medical, Surry Hills, Victoria, Australia) the solution was made under sterile conditions. A 0.5 mg/ mL working solution was used, because 2 mg/ mL of glucose is insoluble in saline, (as the saturation point for glucose is approximately 0.9 mg/ mL, therefore 0.5 mg/ mL was selected due to ease of calculation), therefore the dosage administered was multiplied by four, to give the desired concentration compared to body weight. A 25 gauge needle and 3 mL syringe (McFarelene Medical, Surry Hills, Victoria, Australia) was utilised, due to the larger volume and viscosity of the liquid administered. Following the delivery of the desired glucose load, subsequent blood glucose readings were taken by carefully brushing the scab of the previously performed tail snip to produce a droplet of blood.

The blood glucose concentrations were measured at baseline, fifteen minutes, thirty minutes, sixty minutes and one hundred and twenty minutes. Throughout this procedure the rats had access to water, once this procedure was finished the rats were then given access to food again. See Figure 2.3B which highlights the ip. GTT measurements obtained over the treatment period.

2.3.2.5 Intraperitoneal Insulin Sensitivity Test (Ip. IST):

An ip. IST was performed to determine the effect that the pharmocological compounds had on blood glucose concentrations following the administration of insulin. This procedure was completed at week nine (baseline) and the end of week fourteen (towards the end of the treatment period). In accordance with previously published methods (Jenkin, O'Keefe et al. 2016) rats were fasted for only two hours prior to the test (to help reduce the risk of hypoglycaemia following administration of the hormone in insulin sensitive rats), with adequate access to water. A tail snip was performed to take the baseline blood glucose concentration using a droplet of blood. A blood glucose reading was obtained using a glucometer and glucose strips (Optimum, Xceed, Abbott, USA). Following this the rats were administered a dose of 0.75 IU of Humalog (Eli Lilly, Indianapolis, Indiana, United States of America)

/ 0.9% saline solution (the working solution used was a 0.75 IU/ mL solution) using a 27 gauge 1 mL insulin syringe with needle (Mcfarlane Medical, Surry Hills, Victoria, Australia). Blood glucose readings were taken by carefully brushing the scab to produce a droplet of blood, following the baseline measurement blood glucose concentrations were also recorded at fifteen minutes, thirty minutes, sixty minutes and one hundred and twenty minutes time points. Throughout this procedure rats had access to water, once this procedure was finished the rats were given access to food again. See Figure 2.3B which highlights the ip. IST measurements obtained over the treatment period.

2.3.2.6 Anaesthesia &Tissue Collection:

To determine the effects that the pharmacological compounds had on a number of organs involved in energy storage and energy utilisation, blood and a number of other tissues were collected for future analysis. When rats were deeply anaesthetised using isoflurane, skeletal muscle was surgically collected, immediately following this while rats remained deeply anaesthetised, the rats were administered 100 mg/ kg of sodium pentabarbitone (Virbank, Milperra, NSW, Australia) using an ip. injection and killed via a cardiac puncture.

2.3.2.7 Blood Collection

Blood was immediately collected via cardiac puncture using an eighteen gauge needle (to help reduce red blood cell lysis) and a 10 mL syringe (Mcfarlane Medical, Surry Hills, Victoria, Australia), then transferred into 10 mL coated ethylenediaminetetraacetic acid (EDTA) BD coated vacutainer (Macfarlane Medical, Surry Hills, Victoria, Australia), and subsequently placed on ice. Following this the blood tube was then centrifuged at 4°C at 4000 x g for 10 minutes to separate the plasma from the red blood cells. The plasma was then transferred to eppendorff tubes and stored at – 80°C for subsequent analysis of circulating hormones, cytokines, adiponectin and liver function parameters. The red blood cells were also stored at – 80°C for part of the larger study which was not within the scope of this thesis.

2.3.2.8 Skeletal Muscle Collection

Once the rats were anaesthetised the skeletal muscle was collected from tendon to tendon. Left hind limb Extensor Digitorium Longus (EDL) and soleus skeletal muscle was removed and transferred immediately to an oxygenated organ bath as part of a larger project (as this is not within the scope of this thesis the organ bath will not be described in any further detail). The same process was followed for the right hind leg. Following this the gastrocnemius skeletal muscle was also dissected and superated into sections of red gastrocnemius and white gastrocnemius, which were immediately transferred into a cryotube and snap frozen in liquid nitrogen. The red and white gastrocnemius skeletal muscles were stored for future analysis of mRNA expression (discussed in further detail in section 2.7.2). A cross section of the tip of gastrocnemius muscle (which is a mixture of red and white muscle fibres), was also collected and placed into a cryomold containing OCT embedding solution (Tissue Tek, Torrance, California, United States of America) for subsequent sectioning and analysis (tissue sectioning and analysis was also not within the scope of this thesis, so will not be discussed in any further detail).

2.3.2.10 Other Organ Collection

As this study was part of a larger study the following organs were collected post-mortem including: the pancreas, heart, fat pads (epididymal, peri renal and brown adipose tissue (BAT)), liver, kidneys and hypothalamus. The fat pads, heart, kidney and liver were weighed and the mass of these organs was recorded in grams. A portion of the tissues were then transferred to cryotubes and immediately snap frozen in liquid nitrogen for future analysis of the tissue. For the purpose of this thesis the post modem extracted tissues that will be discussed in C*hapters Four & Five* are epididymal adipose tissue, perirenal adipose tissue, BAT and the liver.

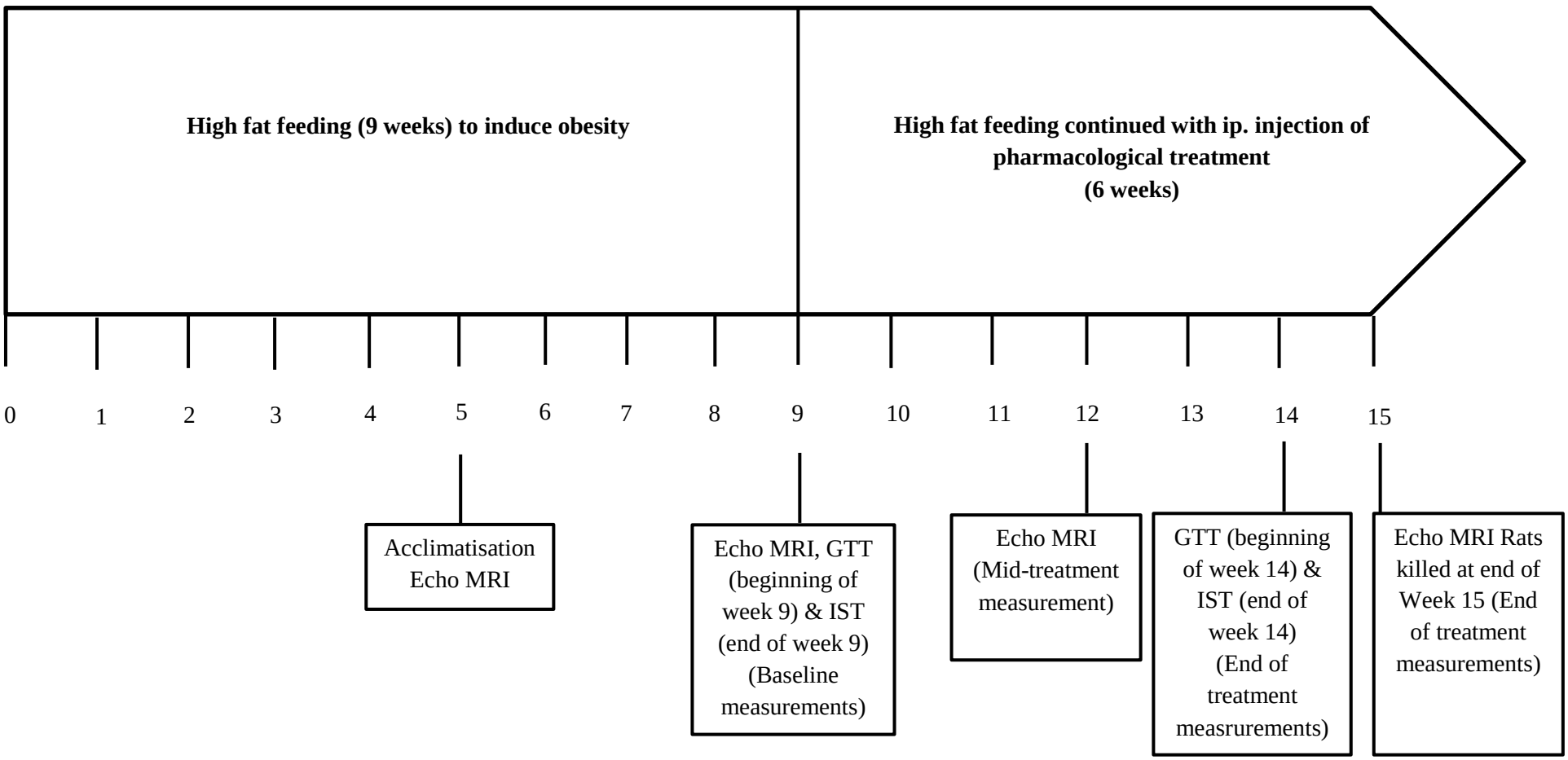


Figure 2.3B: A timeline of the measurements taken during the fifteen week treatment period for the project that focuses on the effect that the administration of either O-1602 or O-1918 have on whole body and skeletal muscle energy homeostasis in a DIO rat model (*Chapter Four – Six*).

2.4 The effect that different dietary fatty acids have on whole body energy homeostasis in a DIO rat model

This study was completed in collaboration with two other PhD students at the time (Ms Shaan Naughton and Ms Lannie O'Keefe).

2.4.1 Dietary Intervention

The Sprague Dawley rats were randomly allocated to either a HFD group or a SCD lean control group for the duration of the fifteen week study. The Sprague Dawley rats that were maintained on a high fat western diet (21% fat by weight; SF13-115) to induce obesity, the diet was purchased from Speciality Feeds (Glen Forrest, WA, Australia). The other group of rats were maintained on a meat free Rat and Mouse SCD (4.8% fat) that was also purchased from Speciality Feeds (Glen Forrest, WA, Australia) for fifteen weeks as a lean control group. See Tables 2.3A – 2.3C for the ingredients listed for both diets and the nutritional composition of the diets.

HFD have been shown to induce obesity in rats that are susceptible to obesity (Buettner, Scholmerich et al. 2007), the Western HFD (SF13-115) which contains lard as the main fat source was used as the HFD for *Chapter Seven*, in place of the Western HFD (SF00-219) which was previously used in *Chapters Four - Six*. In which the main fat source used for the SF00-219 diet was clarified butter. This means that the diet used in C*hapter Seven* was slightly higher in PUFAs and slightly lower in MUFAs and MUFAs but both HFDs used between the two studies overall have the same fatcontent of 21% fat (by weight). The different diet was selected in an attempt to induce glucose intolerance/ insulin resistance which did not occur on the diet SF00-219 as detailed in *Chapters Four – Six*. See Table 2.4A for the comparison in nutritional composition between the two obesity inducing diets.

Table 2.4A: A comparison of the ingredients between theWestern diets HFD SF00-219 and SF13-115 used to induce obesity.

This table highlights the difference in ingredients between the Western HFD used in *Chapters Four – Six.* The effect of either O-1602 or O-1918 administration on whole body and skeletal muscle energy homeostasis in a diet induced in obesity model and the Western HFD used in *Chapter Seven* the effect of different dietary fatty acids on whole body energy homeostasis in a DIO rat model.

Ingredients	Western HFD Diet SF00-219	Western HFD Diet SF13-115	Comparison of ingredients between the SF00-219 to SF13-115 diet
Casein (Acid)	195 g/ Kg	233 g/ Kg	↑
Sucrose	341 g/ Kg	201 g/ Kg	↓
Clarified Butter (Ghee)	210 g/ Kg	-	Not present in SF13-115
Lard	-	210g/ Kg	Not present in SF00-219
Cellulose	50 g/ Kg	58 g/ Kg	↑
Wheat Starch	154 g/ Kg	118 g/ Kg	↓
Dextrinised Starch	-	117 g/ Kg	Not present in SF00-219
DL Methionine	3.0 g/ Kg	3.5 g/ Kg	↑
Calcium Carbonate	17.1 g/ Kg	6.4 g/ Kg	↓
Sodium Chloride	2.6 g/ Kg	2.6 g/ Kg	-
AIN93 Trace Minerals	1.4 g/ Kg	1.6 g/ Kg	↑
Potassium Citrate	2.6 g/ Kg	19.2 g/ Kg	↑
Potassium Dihydrogen Phosphate	6.9 g/ Kg	15.1 g/ Kg	↑
Potassium Sulphate	1.6 g/ Kg	1.6 g/ Kg	-
Choline Chloride (75%)	2.5 g/ Kg	1.3 g/ Kg	↓
SF00-219 Vitamins or AIN93 Vitamins	10 g/ Kg	12 g/ Kg	↑
Cholesterol	1.5 g/ Kg	-	Not present in SF13-115
Oxicap E2	0.04 g/ Kg	-	Not present in SF13-115

 # Note that the same amount of fat (%) was used in both diets however the fat was from a different ingredient source.

Upon concluding the 9 weeks of high fat feeding, the DIO rats were allocated into 1 of 4 diet groups. Like with the previous *Chapters Four – Six,* the same 'ranking criteria' was utilised to evaluate the metabolic profiles of the rats, to ensure that all of the groups had evenly matched metabolic profiles at baseline prior to commensing the different fatty acid feeding regimes (as previously detailed in section 2.3.2.1).

Rats were allocated to 1 of the 4 dietary feeding groups that were composed of varying compositions of dietary fatty acids or a SCD for an additional six week period. The first diet group continued on the Western HFD (SF13-115) predominately composed of SFAs, the second group continued on the HFD predominately composed of MUFA (SF13-113), the third group continued of the HFD predominately composed of PUFA (SF13-114) and the fourth group were provided with the SCD (Meat free rat & mouse SCD). See Figure 2.4A for a flow chart that visually represents the treatment groups used in this study. See Tables 2.4B and 2.4C for the ingredients used in each of the diets and see Table 2.4D for the Nutritional composition of each of the diets.

Table 2.4B: A comparison of the nutritional composition between the three types of different fatty acids high fat diets.

Ingredients	Western High SFA Diet SF13-115	High MUFA Diet SF13-113	High PUFA Diet SF13-114
Casein (Acid)	233 g/ Kg	233 g/ Kg	233 g/ Kg
Sucrose	201 g/ Kg	201 g/ Kg	201 g/ Kg
Linseed (Flax) Oil	-	4 g/ Kg	4.8 g/ Kg
Canola Oil	-	-	2.6 g/ Kg
Lard	210 g/ Kg	-	-
Olive Oil	-	192 g/ Kg	-
Safflower Oil (High Linoleic)	-	-	200 g/ Kg
Sunflower Oil	-	-	2.2 g/ Kg
Sunola Oil	-	14 g/ Kg	-
Cellulose	58 g/ Kg	58 g/ Kg	58 g/ Kg
Wheat Starch	118 g/ Kg	118 g/ Kg	118 g/ Kg
Dextrinised Starch	117 g/ Kg	117 g/ Kg	117 g/ Kg
DL Methionine	3.5 g/ Kg	3.5 g/ Kg	3.5 g/ Kg
Calcium Carbonate	6.4 g/ Kg	6.4 g/ Kg	6.4 g/ Kg
Sodium Chloride	2.6 g/ Kg	2.6 g/ Kg	2.6 g/ Kg
AIN93 Trace Minerals	1.6 g/ Kg	1.6 g/ Kg	1.6 g/ Kg
Potassium Citrate	19.2 g/ Kg	19.2 g/ Kg	19.2 g/ Kg
Dicalcium Phosphate	15.1 g/ Kg	15.1 g/ Kg	15.1 g/ Kg
Potassium Sulphate	1.6 g/ Kg	1.6 g/ Kg	1.6 g/ Kg
Choline Chloride (75%)	1.3 g/ Kg	1.3 g/ Kg	1.3 g/ Kg
AIN93 Vitamins	12 g/ Kg	12 g/ Kg	12 g/ Kg

Table 2.4C: Ingredients list for Speciality Feed Meat Free Rat and Mouse SCD (Quantities of the diet ingredients are unknown)

Ingredients
Wheat
Barley
Lupins
Soya Meal
Fish Meal
Mixed Vegetable Oils
Canola Oil
Salt
Calcium Carbonate
Dicalcium phosphate
Magnesium Oxide
Vitamin and trace mineral premix

Table 2.4D: A comparison of the nutritional composition between all of the diets utilised in the different dietary fatty acids study.

Nutrient Composition	CHOW	HIGH SFA SF13-115	HIGH MUFA Diet SF13-113	HIGH PUFA Diet SF13-114
Protein	20.00%	22.60%	22.60%	22.60%
Total Fat	4.80%	21.00%	21.00%	21.00%
Crude Fibre	4.80%	5.40%	5.40%	5.40%
AD Fibre	7.60%	5.40%	5.40%	5.40%
Total Carbohydrate	59.40%	No data	No data	No data
Digestible Energy	14.0 MJ / Kg	18.8MJ / Kg	18.8 MJ / Kg	18.8 MJ / Kg
% Total calculated digestible energy from lipids	12.00%	41.00%	41.00%	41.00%
Amino Acid Composition				
Valine	0.87%	1.50%	1.50%	1.50%
Leucine	1.40%	2.10%	2.10%	2.10%
Isoleucine	0.80%	1.00%	1.00%	1.00%
Threonine	0.70%	0.90%	0.90%	0.90%
Methionine	0.30%	1.00%	1.00%	1.00%
Cystine	0.30%	0.07%	0.07%	0.07%
Lysine	0.90%	1.70%	1.70%	1.70%
Phenylanine	0.90%	1.20%	1.20%	1.20%
Tyrosine	0.50%	1.20%	1.20%	1.20%
Tryptophan	0.20%	0.30%	0.30%	0.30%
Histidine	0.53%	0.70%	0.70%	0.70%
Calculated Total Minerals				
Calcium	0.80%	0.71%	0.71%	0.71%
Phosphorous	0.70%	0.47%	0.47%	0.47%
Magnesium	0.20%	0.07%	0.07%	0.07%
Sodium	0.18%	0.14%	0.14%	0.14%
Chloride	No data	0.16%	0.16%	0.16%
Potassium	0.82%	0.83%	0.83%	0.83%
Sulphur	0.20%	0.25%	0.25%	0.25%
Iron	200 mg/ Kg	65 mg/ Kg	65 mg/ Kg	65 mg/ Kg
Copper	23 mg/ Kg	9.3 mg/ Kg	9.3 mg/ Kg	9.3 mg/ Kg
Iodine	0.5 mg/ Kg	0.23 mg/ Kg	0.23 mg/ Kg	0.23 mg/ Kg
Manganese	104 mg/ Kg	21 mg/ Kg	21 mg/ Kg	21 mg/ Kg
Cobalt	0.7 mg/ Kg	No data	No data	No data
Zinc	90 mg/ Kg	55 mg/ Kg	55 mg/ Kg	55 mg/ Kg
Molybdenum	1.2 mg/ Kg	0.18 mg/ Kg	0.18 mg/ Kg	0.18 mg/ Kg
Selenium	0.4 mg/ Kg	0.4 mg/ Kg	0.4 mg/ Kg	0.4 mg/ Kg
Cadmium	0.05 mg/ Kg	No data	No data	No data
Chromium	No data	1.2 mg/ Kg	1.2 mg/ Kg	1.2 mg/ Kg
Fluoride	No data	1.2 mg/ Kg	1.2 mg/ Kg	1.2 mg/ Kg
Lithium	No data	0.1 mg/ Kg	0.1 mg/ Kg	0.1 mg/ Kg
Boron	No data	2.3 mg/ Kg	2.3 mg/ Kg	2.3 mg/ Kg
Nickel	No data	0.6 mg/ Kg	0.6 mg/ Kg	0.6 mg/ Kg
Vanadium	No data	0.1 mg/ Kg	0.1 mg/ Kg	0.1 mg/ Kg

Calculated Total Vitamins				
Vitamin A (Retinol)	10950 IU/ Kg	4660 IU/ Kg	4660 IU/ Kg	4660 IU/ Kg
Vitamin D (Cholecalciferol)	2000 IU/ Kg	1170 IU/ Kg	1170 IU/ Kg	1170 IU/ Kg
Vitamin E (a Tocopherol acetate)	110 mg/ Kg	87 mg/ Kg	90 mg/ Kg	96 mg/ Kg
Vitamin K (Menadione)	20 mg/ Kg	1.2 mg/ Kg	1.2 mg/ Kg	1.2 mg/ Kg
Vitamin C (Ascorbic acid)	No data	None added	None added	None added
Vitamin B1 (Thiamine)	80 mg/ Kg	7.1 mg/ Kg	7.1 mg/ Kg	7.1 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/ Kg	7.3 mg/ Kg	7.3 mg/ Kg	7.3 mg/Kg
Niacin (Nicotinic acid)	145 mg/ Kg	35 mg/ Kg	35 mg/ Kg	35 mg/ Kg
Vitamin B6 (Pryridoxine)	28 mg/ Kg	8 mg/ Kg	8 mg/ Kg	8 mg/ Kg
Pantothenic Acid	60 mg/ Kg	19 mg/ Kg	19 mg/ Kg	19 mg/ Kg
Biotin	410 ug/ Kg	233 ug/ Kg	233 ug/ Kg	233 ug/ Kg
Folic Acid	5 mg/ Kg	2.4 mg/ Kg	2.4 mg/ Kg	2.4 mg/ Kg
Inositol	No data	None added	None added	None added
Vitamin B12 (Cyancobalamin)	150 ug/ Kg	120 ug/ Kg	120 ug/ Kg	120 ug/ Kg
Choline	1640 mg/ Kg	790 mg/ Kg	790 mg/ Kg	790 mg/ Kg
Calculated Total Fatty Acids				
Saturated Fats C12:0 or less	No data	0.07%	Trace	Trace
Myristic Acid 14:0	0.03%	0.32%	Trace	0.03%
Palmitic Acid 16:0	0.50%	5.57%	2.29%	1.31%
Stearic Acid 18:0	0.14%	3.61%	0.53%	0.49%
Other Saturated Fats	No data	0.20%	Trace	0.17%
Palmitoleic Acid 16:1	0.01%	0.36%	0.17%	0.11%
Oleic Acid 18:1	1.90%	7.10%	15.76%	2.72%
Gadoleic Acid 20:1	0.03%	0.15%	0.08%	0.06%
Linoleic Acid 18:2 n6	1.30%	3.02%	1.80%	15.65%
α-Linolenic Acid 18:3 n3	0.30%	0.29%	0.36%	0.35%
Arachadonic Acid 20:4 n6	0.01%	No data	No data	No data
EPA 20:5 n3	0.02%	No data	No data	No data
DHA 22:6 n3	0.05%	No data	No data	No data
Total n3	0.37%	0.33%	0.37%	0.35%
Total n6	1.31%	3.05%	1.82%	15.65%
Total Mono Unsaturated Fats	2.00%	7.68%	16.01%	2.97%
Total Poly Unsaturated Fats	1.77%	3.48%	2.18%	16.00%
Total Saturated Fats	0.74%	9.76%	2.82%	2.00%

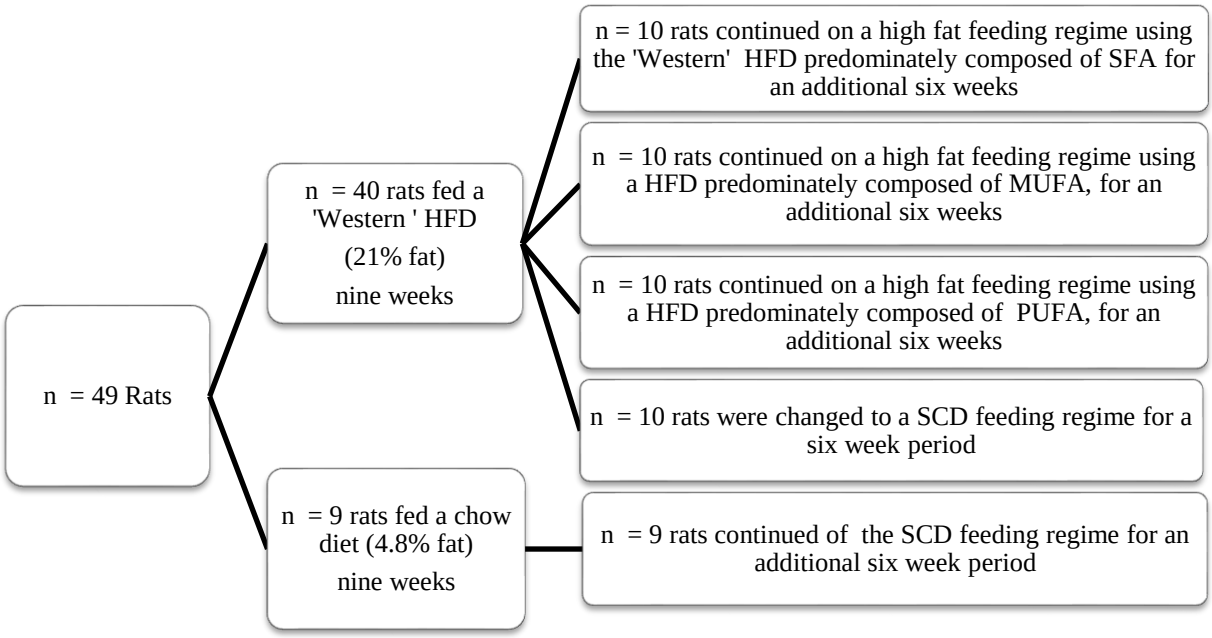


Figure 2.4A: A flow chart illustrating the treatment groups for *Chapter Seven* to determine the effect that different dietary fatty acids have on whole body energy homeostasis in a DIO rat model.

2.4.1.1 Measurements taken over the experimental period

The methodology for the collection of data for food intake, body weight, Echo MRI and ip. GTT was the same as described in sections 2.3.2.2 – 2.3.2.4. The ip. IST methodology was slightly modified and therefore is described below. See Figure 2.4B which highlights the Echo MRI, ip. GTT and ip. IST measurements over the entire fifteen week period.

2.4.1.2 Ip. Insulin Sensitivity Test (Ip. IST)

This procedure was used in place of the previous ip IST methodology described in C*hapters Four & Five* detailed in section 2.3.2.5. Due to variable results obtained in the previous chapters, a slower acting insulin was utilised, requiring the measurement of blood glucose concentrations over a longer duration of time, furthermore, there was reduced risk of the animal developing hypoglycaemia. The methodology for this procedure was previously published by Stengel et al. (2013). The ip. IST was

completed at week nine (baseline) and the end of week fourteen (towards the end of the treatment period) (Russell, Feilchenfeldt et al. 2003). Rats were fasted for two hours, with adequate access to water. A tail snip was performed to take the baseline blood glucose concentration reading and subsequent readings were taken by carefully brushing the scab to produce a droplet of blood. Following this the rats were administered a dose of 1.0 IU/ kg of Humalin-R (Eli Lilly, Indianapolis, Indiana, United States of America) / 0.9% saline solution (Mcfarlane Medical, Surry Hills, Victoria, Australia) the working solution used to administer the insulin was 1.0 IU/ mL using a 27 gauge 1 mL insulin syringe needle. There was also a longer duration between the time points of the blood glucose readings which included: baseline, fifteen minutes, thirty minutes, sixty minutes, one hundred and twenty minutes, one hundred and eighty minutes and two hundred and forty minutes. Throughout this procedure rats had access to water, once this procedure was finished rats were immediately given access to food.

2.4.1.3 Anaesthesia:

To determine the effects that the different types of dietary fatty acids had on a number of organs involved in energy storage and energy utilisation, a number of tissues were collected for future analysis. Isoflurane was administered and rats were deeply anaesthetised. Skeletal muscle was collected as part of a larger study and as such is not detailed within this thesis.

2.4.1.5 Blood Collection

Following skeletal muscle collection and while remaining deeply anaesthetised the rats were immediately killed via a cardiac puncture. Blood was removed using a 18 gauge needle and 10 mL syringe (to help reduce red blood cell lysis), then transferred into 10 mL EDTA BD coated vacutainer (Macfarlane Medical, Surry Hills, Victoria), then placed on ice. The blood tube was then subsequently centrifuged at 4°C at 4000 x g for 10 minutes to separate the plasma from the red blood cells. The plasma was then transferred to eppendorff tubes and stored at – 80°C for subsequent analysis of circulating hormones and cytokines.

2.4.1.6 Other Organ Collection

The same methodology for organ collection was utilised as per *Chapters Four – Six*. The organ weights for white adipose tissue, BAT and the liver were measured at the time of collection.

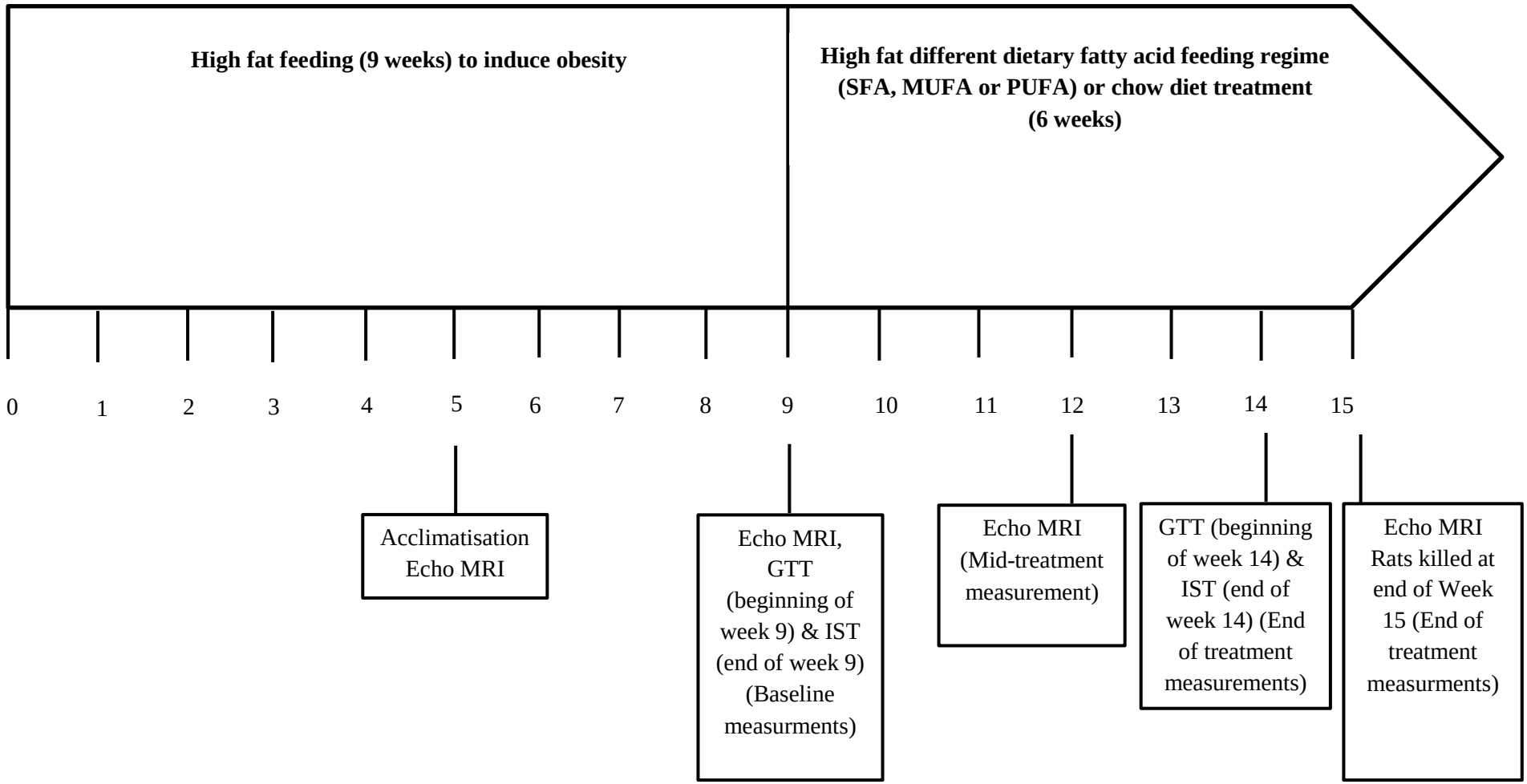


Figure 2.4B: A timeline of the measurements taken during the fifteen week treatment period for the project that focuses on the effect that different dietary fatty acids have on whole body energy homeostasis in a DIO rat model (*Chapter Seven*).

2.5 Plasma Analysis

To determine the effects that either the pharmacological compounds or different dietary fatty acids (*Chapters Four – Seven*) have on plasma hormones (involved in appetite regulation and blood glucose regulation) as well as the pro-inflammatory and anti-inflammatory cytokines. A number of different assays were utilised to analyse the plasma, these tests include: Bioplex assay, adiponectin assay, and liver function tests which are described in more detail below in sections 2.5.1 - 2.5.3.

2.5.1 Bioplex

A Bioplex hormone immunoassay (171K1001M, Bio-Rad Laboratories, Munich, Germany) was utilised to analyse the plasma obtained in both C*hapters Four – Seven.* The hormones analysed from this immunoassay are highlighted in Table 2.5A.

The Bioplex cytokine immunoassay (YL0000006Q, Bio-Rad Laboratories, Munich, Germany) was utilised to analyse the plasma obtained in both C*hapters Four - Seven*. The cytokines analysed from this immunoassay are highlighted in Table 2.5B.

Both the Bioplex Hormone and Cytokine Immunoassays (Bio-Rad Laboratories, Munich, Germany) were conducted according to the manufacturer's instructions.

2.5.2 Adiponectin Assay

To determine the effects that pharmacological compounds and different dietary fatty acids had on total adiponectin concentrations in *Chapters Four – Seven* an adiponectin ELISA assay was conducted. Total plasma adiponectin concentrations were measured using AdipoGen - Adiponectin Rat enzyme-linked immunosorbent assay (ELISA) assay, AG-45A-0005TP-KI01 (Adipogen, Liestal, Switzerland). Plasma samples were diluted 1: 1000 using the diluent that was provided with the assay. The procedure was followed in accordance to the manufacturer's instructions. The physiological role of adiponectin and its role in obesity have previously been detailed in sections 1.3.2 – 1.3.2.2

2.5.3 Liver Function Test

To determine the effects that pharmacological compounds had on plasma liver function parameters a plasma liver function test was assessed in C*hapters Four* and *Five*. This test was conducted by the University of Melbourne's Veterinary Hospital. A 200 µL aliquot of plasma was transferred on dry ice from Victoria University St Albans Campus to University of Melbourne Veterinary Hospital for analysis of aspartate aminotransferase (AST), alanine transaminase (ALT) and albumin. Analysis was conducted at room temperature using the bench top clinical analyser Randox Daytona (Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom).The samples were analysed according to manufacturer's instructions for the following analytes: AST (AS3804, Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom), ALT (AL3875, Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom), and albumin (AB3800, Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom). The liver function analytes physiological role and their role in obesity are highlighted in Table 2.5C.

Table 2.5A: Analytes from Bioplex hormone immunoassay

Analyte	Physiological Role	Role in obesity
Leptin	Secreted predominately from the adipose tissue (Zhang, Proenca et al. 1994). Involved in appetite regulation, induces satiety (Halaas, Gajiwala et al. 1995).	Increased circulating plasma leptin in obesity (Nehete, Magden et al. 2014), there is thought to be a resistance to leptin at the hypothalamus (de Git and Adan 2015).
Glucagon	Produced by the alpha islets of pancreas, this hormone reduces food intake (Schulman, Carleton et al. 1957).	Increased circulating plasma glucagon in obesity (Nehete, Magden et al. 2014).
Ghrelin	Secreted predominately from the stomach (Ariyasu, Takaya et al. 2001). Acutely secreted hormone that causes hunger (Wren, Seal et al. 2001). Increased circulating concentrations of ghrelin preprandial (Cummings, Purnell et al. 2001) and decreased circulating ghrelin concentrations postprandial (Tschop, Wawarta et al. 2001).	There is decreased circulating ghrelin concentrations in human obesity (Tschop, Weyer et al. 2001).
Glucagon-Like Peptide (GLP-1)	Induces satiety (Naslund, Gutniak et al. 1998), inhibits food intake (Gutzwiller, Goke et al. 1999), slows gastric emptying and induces weight loss (Naslund, Gutniak et al. 1998).	Increased in circulating plasma in obesity when compared to lean and overweight groups (Nehete, Magden et al. 2014).

Table 2.5B: Analytes from Bioplex cytokine immunoassay

Analyte	Function	Role in obesity and related comorbidities
Erythropoietin (EPO)	Anti-inflammatory cytokine that is synthesised by kidney and regulates red blood cell development and survival (Borissova, Djambazova et al. 1993).	Improves insulin sensitivity (Caillaud, Mechta et al. 2015). Improves glucose tolerance and blood glucose concentrations in glucose intolerant DIO rats fed a HFD (Caillaud, Mechta et al. 2015). EPO treatment also reduced systemic TNFα, oxidative stress and reinstated HSP72 expression in Soleus skeletal muscle (Caillaud, Mechta et al. 2015).
Interleukin 1α (IL-1α)	Pro-inflammatory cytokine that regulates transcription factors such as nuclear factor-B (NF-κβ) (Wolf, Chen et al. 2001). IL-1α promotes the expression of genes involved in cell survival, proliferation, and angiogenesis (Wolf, Chen et al. 2001).	IL-1rα is increased in obesity (Meier, Bobbioni et al. 2002).
Interleukin 1β (IL-1β)	Pro-inflammatory cytokine (Pickup 2004).	Increase circulating plasma IL-1β in obesity (Nehete, Magden et al. 2014). Promotes insulin resistance, Down regulates PPARγ (Tanaka, Itoh et al. 1999).
Monocyte Chemotactic Protein -1 (MCP-1)	Regulates the migration and infiltration of immune cells including monocytes and macrophages (Hill, Lin et al. 1992).	MCP-1 over expression in adipose tissue results in insulin resistance mediated by macrophage recruitment (Kamei, Tobe et al. 2006).
Interleukin 2 (IL-2)	Involved in cell proliferation, differentiation and lymphocyte cell defence (Abbasi, Chu et al. 2004).	Serum concentrations IL-2 are reduced in obesity (Vargas, Ryder et al. 2016), insulin from the obese individuals was negatively correlated with IL-2 in insulin resistant obese individuals (Vargas, Ryder et al. 2016).
Interleukin 4 (IL-4)	An inhibitor of pro-inflammatory cytokines: TNFα, IL-6, IFN-γ and IL-1β (Lee, Rhoades et al. 1995).	Increased IL-4 in circulating plasma (Nehete, Magden et al. 2014). Inhibits adipogenesis and promotes lipogensis (Tsao, Shiau et al. 2014).
Interleukin 5 (IL-5)	IL-5 exerts its effects for proliferation and differentiation (Takatsu 2011).	IL-5 is increased in general and central obesity (Schmidt, Weschenfelder et al. 2015).

Interleukin 6 (IL-6)	Pro-inflammatory cytokine secreted predominately from adipose tissue, also secreted from the skeletal muscle and liver (Carrero, Park et al. 2009). Increases inflammatory protein C-Reactive Protein (Tanaka, Narazaki et al. 2014).	IL-6 is correlated with BMI, waste circumference, VAT and total body fat in humans (Hermsdorff, Zulet et al. 2011). Increased circulating plasma IL-6 concentrations in obesity (Nehete, Magden et al. 2014).
Interleukin 10 (IL-10)	Anti-inflammatory cytokine, that is an inhibitor of cytokine synthesis, supresses macrophage function and inhibits the production of pro-inflammatory cytokines (Tedgui and Mallat 2001).	Increased circulating concentrations of IL-10 in obese woman while low serum concentrations are linked to the development of metabolic syndrome (Esposito, Pontillo et al. 2003).
Interleukin 17α (IL-17α)	Pro-inflammatory cytokine involved in a number of autoimmune disorders and cancer (Afzali, Lombardi et al. 2007). Regulates chemokines that are involved in the infiltration of tissue leukocytes such as MCP-1 (Gaffen, Kramer et al. 2006).	mRNA expression of IL-17α is increased in SAT and VAT in morbidly obese women when compared to women with the healthy BMI range, however, the circulating concentration of this cytokine is reduced in women with a healthy BMI range when compared to the morbidly obese women (Zapata-Gonzalez, Auguet et al. 2015). IL-17 is negatively involved in systemic glucose homeostasis, IL-17 deficient mice have increased serum adiponectin (Zuniga, Shen et al. 2010). Conversely, another study found that circulating plasma concentrations of IL-17A were not altered in obesity in Chimpanzees (Nehete, Magden et al. 2014).
Interleukin 18 (IL-18)	Is a pro-inflammatory cytokine that synergistically interacts with other cytokines to produce IFN-γ (Srivastava, Salim et al. 2010).	Expressed in adipocytes (Skurk, Kolb et al. 2005) but predominately expressed in a number of different immune cells. IL-18 is linked with obesity, insulin resistance and T2DM (Cota, Marsicano et al. 2003; Bosch, Lopez-Bermejo et al. 2005; Bruun, Stallknecht et al. 2007).
Interferon gamma (IFN-γ)	Is a pro-inflammatory cytokine that is secreted from natural killer cells (Fauriat, Long et al. 2010).	Increased circulating plasma IFN-γ in obesity (Nehete, Magden et al. 2014). Obese IFN-γ knockout mice have improved insulin sensitivity and decreased adipocyte size (O'Rourke, White et al. 2012).

Regulated on Activation Normal T-Cells Expressed and Secreted (RANTES)	Pro-inflammatory cytokine that promotes cell accumulation and activation and is involved in atherosclerosis, arthritis, liver disease (Schall 1991; Appay, Brown et al. 1999; Veillard, Kwak et al. 2004)	Circulating plasma RANTES is not altered in obesity (Nehete, Magden et al. 2014). Neither, systemic, VAT or SAT concentrations of RANTES is a reliable marker of adiposity in humans (Madani, Karastergiou et al. 2009). RANTES expression is elevated in the adipose tissue in DIO mice and this is associated with an increased T-cell infiltration (Madani, Karastergiou et al. 2009).
Tumour Necrosis Factor α (TNFα)	Pro-inflammatory cytokine produced by monocytes and macrophages that promotes the release of inflammatory cytokines including IL-1β and IL-6.	Increased circulating plasma TNFα in obesity (Nehete, Magden et al. 2014)
Vascular Endothelial Growth Factor (VEGF)	VEGF is a signalling cytokine that is involved in angiogenesis including arteries, veins and lymphatic vessels (Conn, Bayne et al. 1990; Alvheim, Malde et al. 2012).	Positive correlation between VEGF and BMI in healthy males (Loebig, Klement et al. 2010). Blockade of VEGF can reduce the expansion adipose tissue in DIO (Tam, Duda et al. 2009). Circulating unbound VEGF is increased in overweight/ obese women compared with lean woman (Montecucco, Bondarenko et al. 2016)
Interleukin-12p70 (IL-12p70)	Interleukin 12 is involved in inhibition of angiogenesis (Sgadari, Angiolillo et al. 1996).	Increased circulating plasma concentrations in obesity (Nehete, Magden et al. 2014). Elevated circulating IL-12p70 in pre-obese individuals compared to non-obese and normal weight obese individuals (De Lorenzo, Del Gobbo et al. 2007).
Interleukin 13 (IL-13)	An anti-inflammatory cytokine that inhibits the synthesis of proinflammatory cytokines (de Waal Malefyt, Figdor et al. 1993). Modulates functions of human neutorphils (Girard, Paquin et al. 1996).	Over expression of IL-13 prevented weight gain as a result of high fat feeding and did not affect food consumption. IL-13 over expression also inhibited macrophage infiltration in adipose tissue (Darkhal, Gao et al. 2015).

Table 2.5C: Analytes from liver function test

Analyte	Physiological Role
AST	Is an enzyme that catalyses amino acids (asaprate) allowing entry into the citric acid cycle (Giannini, Testa et al. 2005). The enzyme activity of AST is localised to both the cytosol and the mitochondria. Highly expressed in the liver also expressed in heart, skeletal muscle, kidneys, brain and red blood cells (Giannini, Testa et al. 2005)
ALT	Is an enzyme that catalyses amino acids (alanine) allowing entry into the citric acid cycle (Giannini, Testa et al. 2005). The enzyme activity is localised to cytoplasm (Giannini, Testa et al. 2005).
Albumin	Albumin concentrations are reflective of dietary intake or liver as albumin is syntheised by the liver (Giannini, Testa et al. 2005). Albumin concentrations can also be decreased in patients with kidney disease or protein losing enteropathy (Giannini, Testa et al. 2005).

2.6 Hydroxyproline Analysis

Hydroxyproline is marker of collagen and is used to determine fibrosis. In C*hapter Four* a hydroxyproline colorimetric assay was used to determine total collagen content and concentration in liver and heart tissue. 100 – 200 mg of liver and heart (left ventricle) samples were obtained from the liquid nitrogen storage and cut on dry ice using a sterile petri dish and scalpel blade. The samples were transferred on dry ice to Monash University; Clayton Campus for subsequent analysis, this procedure was undertaken with the guidance and in collaboration with Dr Chrishan Samuel from Monash University.

To prepare the liver and heart samples for the hydroxyproline assay the tissues were freeze dried using lysophilizer to obtain a dry weight (milligrams) measurement. The previously developed methods which are described in Samuel et al. (2009) which appear to be a modified methodology from Bergman & Loxley (1963) were utilised to complete this assay (Samuel 2009). The procedure involved freeze drying the tissue samples, then subsequently rehydrating in an acetate/ citrate buffer (5.7g sodium acetate, 3.75g tri-sodium citrate and 0.55g citric acid in 38.5 mL isopropyl alcohol and made up to 100 mL of total volume with distilled water) for approximately twenty four hours at 4 °C. The samples were then transferred to Kimax^{TM} screw capped glass tubes, immersed in 6 M hydrochloric acid (HCl) and hydrolysed at 110 °C for approximately eighteen to twenty four hours. The samples were subsequently then cooled to 4 °C and dried by evaporation using sodium hydroxide overnight. The residual matter obtained from the samples were then utilised for the hydroxyproline assay as detailed below.

Each sample was then dissolved in 0.1M HCl (as the hydroxyproline standard is suspended in this acid), using Kimax^{TM} screw capped glass tubes either 10 µL of the tissue residue/ HCl mixture was then added to 90 µL of distilled water. To determine the samples hydroxyproline concentration a 1mg/mL hydroxyproline standard (dissolved in 0.1M HCl) was used to generate a linear standard curve ranging from 2 µL, 4 µL, 6 µL, 8 µL or 10 µL or water (as blank) and the difference to 100 µL was added in distilled water (in duplicate), in Kimax^{TM} screw capped glass tubes. Therefore all of the samples, standards and blanks had equal volumes of 100 µL.

200 µL of isopropanol was added to the tube and immediately vortexed. Subsequently 100 µL of 7% Cholamine T solution (composed of Cholamine T (Sigma, St Louise, Missouri, United States of America) and distilled water), which is a light sensitive solution that acts as an oxidation buffer, was freshly made on the day of the assay immediately prior to commensing this process. Following this 1.3 mL of analytical isopropanol solution (3 parts Ehrlich's Reagent and 13 parts isopropanol) was

added to each tube and immediately vortexed and then the cap was screwed on tightly. Ehrlich's Reagent (2.0 g para-dimethylaminobenzaldehyde (DMAB, Sigma, St Louise, Missouri, United States of America) was dissolved in 3.0 mL of 60% (vol/ vol) perchloric acid in a dark bottle as this is light sensitive). Next, the samples were placed at 60°Cand shaken for twenty five minutes, then subsequently cooled at 4°C for a further five to ten minutes.

Following this an additional 3.3 mL of isopropanol was added to the tube, providing a total tube volume of 5.5 mL, then vortexed and the contents transferred to disposal cuvettes (Lake Charles Manufacturing, Lake Charles, Louisiana, United States of America) for subsequent spectrophotometry. Then the hydroxyproline content of the blank, standards and samples was measured using a spectrophotometer (Beckman, DU-640) at an absorbance wave length of 558 nm. The unknown sample concentrations were determined using the generated concentration curve and a line of best fit.

2.7 RNA extraction

2.7.1 Cell Culture

RNA was extracted according to the methods previously described by McAinch et al. (2006). This involved using C_2C_{12} myotube TRIzol lysate that was previously stored at –80°C (as detailed in section 2.1.4) which was thawed on ice in a RNA specific fume hood and 200 μL of chloroform (Sigma Aldrich, St Louise, Missouri, United States of America) was then added, and mixed vigorously using vortex. The samples were placed on ice for 5 minutes and then centrifuged (Beckman Avanti ^{TM} 30 Centrifuge, CEN-GS15 [874145]) at 12,000 x g for 15 minutes at 4°C. This process caused the sample to be separated into 2 distinct phases, the lower phase was pink in colour and contains the TRIzol, cellular protein and debris, while the clear upper phase contains the chloroform and RNA. The upper phase was then transferred to a sterile Eppendorf containing 600 μL of isopropanol (Sigma Aldrich,

St Louise, Missouri, United States of America) and 10 µL of 5 M sodium chloride, which was then precipitated over night at – 20°C.

Pellets of RNA were formed when the precipitated samples were centrifuged for 20 minutes at 12,000 x g at 4°C. The supernatant was removed from the RNA pellet and the pellet was washed with 400 µL of 75% ethanol solution (composed of 100% absolute ethanol (Sigma Aldrich, St Louise, Missouri, United States of America)) and diethylpyrocarbonate (DEPC) treated water (Invitrogen, Victoria, Australia). DEPC treated water is used in the RNA extraction process as it inhibits RNAases/ nucleases (Solymosy, Fedorcsak et al. 1968) which may be present in the water and this reduces degradation of the RNA sample.

Following the washing of the pellet the samples were then centrifuged for 8 minutes at 8000 x g, the ethanol solution was removed and the pellets of RNA were left to air dry for 5 minutes. Subsequently the pellets were then dissolved in 10 µL in DEPC treated water heated to 60°C. Following this RNA was diluted 1: 20 in which 1 µL of diluted RNA was removed and further diluted in 19 µL of DEPC treated water and stored at – 80°C. RNA was quantified using a Nano Drop 2000 Spectrophotometer (Thermo Scientific, Scoresby, Victoria, Australia), which enabled the RNA concentration to be measured for each sample.

2.7.2 Rat Skeletal Muscle

Red and white gastrocnemius rat skeletal muscle which was previously obtained (as detailed in section 2.3.2.8) was removed from the liquid nitrogen storage system and then cut on dry ice into ~ 25 - 35 mg portions using petri dish and scalpel blade (Swann Morton, Sheffield, England). The portions of tissue were then placed in a sterile Eppendorf tube, and stored at -80°C.

Initially a column RNA extraction kit had been trialled to extract RNA from skeletal muscle in place of the TRIzol method. However, it was then established that additional chemicals were required for

this method of RNA extraction when using this kit on skeletal muscle tissue. Due to the time constraints associated with the extraction process it was decided that TRIzol extraction would be used. In addition to the occupation health and safety (OH&S) procedures surrounding the use of TRIzol, due to potential adverse foetal effects associated with this chemical and while it was not imperative, as I was in the early stages of pregnancy it was decided that my PhD supervisor would complete only up to the completion of the RNA extraction process as an additional precaution.

800 μL of TRIzol was added to each previously sterilised 2 mL lysis matrix tubes (MP Biomedicals, California, United States of America) containing 1000 mg of sterilised ceramic beads then the tubes were subsequently placed on ice. Following this, the previously cut portions of red or white gastrocnemius skeletal muscle were then added to the corresponding 2 mL lysis matrix tubes (MP Biomedicals, California, United States of America) containing the sterile ceramic beads and TRIzol.

The tubes were then transferred on ice to the Fast Prep (FP120, cell disruptor, Electron Corporation, Milford, United States of America) and processed for 20 seconds, the samples were then placed on ice and homogenised again. Following this the samples were centrifuged at 12,000 x g for 15 minutes at 4 °C such that the cellular debris became a pellet at the bottom of the tube. The TRIzol and RNA containing supernatant was subsequently transferred to a sterile 1.5 mL Eppendorf tube containing 250 μL of chloroform, which was then vigorously mixed using a vortex and left to stand on ice for 5 minutes. From this step forward the same process was followed for the cell culture RNA extraction procedure as detailed in section 2.7.1.

2.8 Reverse Transcription of RNA into complimentary DNA (cDNA)

2.8.1 DNAse treatment

As some of the primers selected such as GPR18 do not span an intron, prior to performing the reverse transcription step, the samples were DNAse treated. DNAse is an enzyme that degrades any DNA products (either double or single stranded) in the sample and helps to ensure that the sample is not contaminated by DNA. This process involved 1 – 2 μg of RNA (per sample) and 7 – 8 μL of DEPC treated water being added to 0.2 mL 8 strip PCR tubes (Astral Scientific, New South Wales, Australia), then following the manufacturer's instructions for the Rq1 RNAse-Free DNAse kit (Promega Corporation, Madison, United States of America), 1 μL of RNAse Free DNAse x 10 and RQ1 1 μL of RNAse free DNAse per μg was added to the same PCR tubes. Following this the samples were incubated in the PCR Express Thermal Cycler at 37 °C for 30 minutes, then 1 μL of DNAse stop solution was added to the tube to terminate the reaction. Samples were subsequently incubated for a further 10 minutes at 65°C (to inactivate the DNAse) in the PCR Express Thermal Cycler (BioRad Laboratories, Hercules, California, United States of America). Then the sample's RNA concentration was quantified using Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Victoria, Australia), ready for the subsequent Reverse Transcription step.

2.8.2 Reverse Transcription

Reverse transcription is a process in which RNA is converted to complementary DNA (cDNA) using an enzyme call reverse transcriptase. The cDNA can then be used for 'Real Time' Polymerase Chain Reaction (PCR) quantification. During this process the RNA samples, previously stored at – 80 °C were made up to concentrations of 0.5 μg. The RNA was then reverse transcribed into cDNA using the iScript^{TM} cDNA Synthesis Kit (BioRad Laboratories, Hercules, California, United States of America), a master mix of the reaction mix and enzyme was added to each sample, in which 2 μL of iScript reaction mix and 0.5 μL of the enzyme reverse transcriptase was added to 0.5 μg of RNA

sample. Internal controls (using a mixture of some of the concentrated samples) were also required to determine plate to plate variation during the 'Real Time' PCR quantification as well as a negative reverse transcription sample to ensure that the reverse transcription step was successful, in which 0.5 µL of DEPC treated water was added in place of the enzyme. Samples were then incubated in PCR Express Thermal Cycler (BioRad Laboratories, Hercules, California, United States of America) for 5 minutes at 25 ˚C; 30 minutes at 42 ˚C; 5 minutes at 85˚C; holding at 4 ˚C until storage.

2.8.3 Dilution of cDNA from Cell Culture

The 10 µL of cDNA was diluted 1: 20 (190 µL of DEPC treated water was added to each sample) and stored at -20 °C for subsequent 'Real Time' PCR mRNA quantification.

2.8.4 Dilution of cDNA from Rat Skeletal Muscle

To determine the receptor expression of GPR18, the samples were required to be more concentrated (as GPCR tend to be in less abundant in tissues compared to other proteins), some cDNA was diluted 1: 5 with DEPC treated water to allow for this. The remaining cDNA was then diluted 1: 20 to quantify other intracellular analytes. The diluted cDNA was then stored at –20 °C for subsequent 'Real Time' PCR mRNA quantification.

2.9 Mouse and Rat Oligonucleotide Primer Sequences

An oligonucleotide primer is a short nucleic acid sequence that is the initial point of DNA synthesis. Primers were designed using Oligoperfect Suite (Invitrogen, Victoria, Australia, Victoria, Australia) and were purchased from Geneworks Pty Ltd (Adelaide, Australia). The primer sequences used for both *Chapters Three* and *Six* are detailed in Table 2.9B: Mouse Primer Sequences used for 'Real Time' PCR analysis in C_2C_{12} myotubes and Table 2.9C: Rat Primer Sequences used for 'Real Time' PCR analysis red and white gastrocnemius from rat. A BLAST search was conducted for each primer to determine homologous binding to the target mRNA sequence.

Primer optimisation was undertaken to determine the optimal concentration for primers utilised in both experiments for the 'Real Time' PCR analysis. This process involves different combinations of primer concentrations for forward and reverse primers ran at 50 cycles. See Table 2.9A.

Table 2.9A: Concentration combinations used for primer optimisations

Primer Concentration Combinations	
Forward	**Reverse**
3 µM	3 µM
3 µM	9 µM
9 µM	3 µM
9 µM	9 µM

Table 2.9B: Mouse Primer Sequences used for 'Real Time' PCR analysis C_2C_{12} myotubes

Primer	Accession Number	Direction	Sequence
HPRT1	NM_013556.2	Forward ('5 '3)	GCAAACTTTGCTTTCCCTGG
		Reverse ('5 '3)	ACTTCGAGAGGTCCTTTTCACC
NFATc1	NM_016791.3	Forward ('5 '3)	TCCAAAGTCATTTTCGTGGA
		Reverse ('5 '3)	GTTGCGGAAAGGTGGTATCT
PGC1α	NM_008904.1	Forward ('5 '3)	CACCCACACGATCAGAACAA
		Reverse ('5 '3)	GGTCATCGTTTGTGGTCAGA
APPL1	NM_145221.2	Forward ('5 '3)	ATCAGGCGGAAGAAGTGAGA
		Reverse ('5 '3)	TTTCTGATGCCCTACGATCC

APPL1; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, HPRTI; Hypoxanthine guanine phosphoribosyltransferase, NFATc1; Nuclear Factor of Activated T Cells calcineurin dependent 1 and PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha.

Table 2.9C: Rat Primer Sequences used for 'Real Time' PCR analysis red and white gastrocnemius skeletal muscle

Primer	Accession Number	Direction	Sequence
Cyclophilin	NM_017101.1	Forward ('5 '3)	CTG ATG GCG AGC CCT TG
		Reverse ('5 '3)	TCT GCT GTC TTT GGA ACT TTG TC
βActin	NM_031144	Forward ('5 '3)	CTA AGG CCA ACC GTG AAA TGA
		Reverse ('5 '3)	CCA GAG GCA TAC AGG GAC AAC
GPR18	NM_001079710.1	Forward ('5 '3)	GTG GGG GTC TGG ATA ATG AC
		Reverse ('5 '3)	CGC GTG AAG TTA AGC ACA TT
GPR55	XM_006226918	Forward ('5 '3)	TCG CCA TCC AGT ACC CTC TTC
		Reverse ('5 '3)	ATG CAG CAG ATC CCA AAG GTT
AdipoR1	NM_207587.1	Forward ('5 '3)	TGA GGT ACC AGC CAG ATG TC
		Reverse ('5 '3)	CGT GTC CGC TTC TCT GTT AC
APPL1	XM_008771023.1	Forward ('5 '3)	TCA CTC CTT CCC CAT CTT TC
		Reverse ('5 '3)	TAG AGA GAG GGC AGC CAA AT
APPL2	NM_001108741.1	Forward ('5 '3)	TGC TCG GGC TAT TCA CAA
		Reverse ('5 '3)	AAA CAG GCC CGT GAC ACT
PGC1α	NM_031347.1	Forward ('5 '3)	ACC CAC AGG ATC AGA ACA ACC
		Reverse ('5 '3)	GAC AAA TGC TCT TTG CTT TAT TGC
FOXO1	NM_001191846.2	Forward ('5 '3)	CTC GGC GGG CTG GAA
		Reverse ('5 '3)	TCA TTC TGT ACT CGA ATA AAC TTG
PDK4	NM_053551.1	Forward ('5'3)	GGG ATC TCG CCT GGC ACT TT
		Reverse ('5 '3)	CAC ACA TTC ACG AAG CAG CA
βHAD	AF095449.1	Forward ('5 '3)	TCG TGA CCA GGC AAT TCG T
		Reverse ('5 '3)	CCG ATG ACC GTC ACA TGC T
FAT/CD 36	NM_031561.2	Forward ('5 '3)	GAC CAT CGG CGA TGA GAA A
		Reverse ('5 '3)	CCA GGC CCA GGA GCT TTA TT

AdipoR1; Adiponectin Receptor 1, APPL1; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, APPL2; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2, βActin; beta actin, βHAD; beta-hydroxyacyl-CoA dehydrogenase, FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, FOXO1; Forkhead box protein O1, GPR18; G-Protein Coupled Receptor 18, GPR55; G Protein-Receptor 55, PDK4; Pyruvate Dehydrogenase Kinase 4, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha.

2.10 'Real Time' Polymerase Chain Reaction (PCR)

'Real Time' PCR is an analytical procedure used to quantify the amount of mRNA in a sample of tissue (Ponchel, Toomes et al. 2003). SYBR Green florescence 'Real Time' PCR is a validated method used to quantify mRNA and is less expensive than other approaches such as Taq Man 'Real Time' PCR, and still is reliably accurate (Ponchel, Toomes et al. 2003).

IQ SYBR™ Green (BioRad Laboratories, Hercules, California, United States of America) is a dye that binds to double stranded cDNA as it fluoresces and is used to amplify and visualise mRNA expression. A master mix containing sterile DEPC treated water and the optimal concentration of forward and reverse primers was prepared. See Table 2.9D Procedure for 'Real Time' PCR master mix. Using a 96 well plate (BioRad Laboratories, Hercules, California, United States of America) the master mix was added to each well, following this 2 µL of cDNA was then added to each specified well. Each sample was analysed in triplicate. The plate was then covered using Microseal 'B' Film (BioRad Laboratories, Hercules, California, United States of America) and the plate centrifuged to remove bubbles using Centrifuge (HD Scientific supplies Pty Ltd, NSW, Australia, model # D-78532 0001788) at 4°C until 650 x g was reached. Following this the 96 well plate was then transferred into the BioRad MY iQ® Real-Time PCR detection system (model # My iQ optics model, serial # 569BR/0611).

Table 2.9D: Procedure for 'Real Time' PCR master mix

Master Mix	
Component	**Amount per well**
IQ SYBR green	8 µL
DEPC treated water	6 µL
Forward Primer (optimised concentration)	2 µL
Reverse Primer (optimised concentration)	2 µL

The 'Real Time' PCR protocol included: either 40 or 50 cycles of 95 °C for 15 s and 60 °C for 60 s. The temperature increase causes the products to undergo a denaturation process, which involves

separation of the cDNA to single strands, promoting annealing of the primers and the target sequence and polymerisation in which more dye binds to the newly synthesised DNA and the fluorescence is monitored in real time (Bustin 2000).

A housekeeping gene is one that is required for the basal functioning of a cell and required for the existence of a cell irrespective of tissue type, developmental stage, cell cycle state, or external signal (for example pharmacological treatment) (Eisenberg and Levanon 2013). Therefore the house keeping gene(s) selected in this study should not be affected by treatment with the pharmacological compounds O-1602 or O-1918. The housekeeping gene utilised in C*hapter Three* was HPRT1 (Yamaoka, Kondo et al. 1997) and the house keeping gene utilised in *Chapter Six* was the average of two housekeeping genes, Cyclophilin and βActin . The genes of interest were then normalised to housekeeping gene (or average) and data analysed calculated using 2 Delta Ct method (Livak and Schmittgen 2001).

CHAPTER THREE:

3.0 The effect that O-1602 has on C_2C_{12} myotubes and the regulation of markers involved in skeletal muscle energy homeostasis.

3.1 Abstract

Background: Atypical cannabinoid, O-1602 has an affinity for the putative cannabinoid receptors GPR55 and GPR18. Treating C_2C_{12} myotubes with O-1918, (a compound which also has an affinity for the two cannabinoid receptors GPR55 and GPR18) results in an increase in markers of oxidative capacity (NFATc1 and PGC1α) and fatty acid oxidation (PDK4) with a trend for APPL1 to be up-regulated (*Simcocks et al. 2010 Unpublished Observations see Appendix Two*). Currently the role of O-1602 in skeletal muscle metabolism is unclear. Therefore the aim of this study is to determine the effect that O-1602 has on C_2C_{12} myotubes and markers that regulate oxidative capacity and adiponectin signalling.

Materials & Methods: C_2C_{12} myoblasts were proliferated in growth medium then differentiated into myotubes using differentiation medium. The myotubes were then serum starved (0.1% bovine serum albumin DMEM) for two hours, then treated for 24 hours with O-1602 (with a dose range of 10 – 1000 nM) in 0.1% bovine serum albumin DMEM. Myotubes were lysed, mRNA was extracted, and analysed using 'Real Time' PCR.

Results, Discussion & Conclusion: O-1602 does not appear to play a role in regulating mRNA expression of markers involved in regulating oxidative capacity (NFATc1 and PGC1α) and the positive regulator of adiponectin signalling (APPL 1) in C_2C_{12} myotubes within the dose range of 10 – 1000 nM. Further investigation into the mRNA expression of the putative cannabinoid receptors GPR55 and GPR18 within C_2C_{12} myotubes is required to determine the receptors expression in this tissue.

3.2 Background

O-1602 is an atypical synthetic cannabinoid that is structurally similar to Abn-CBD a derivative of CBD from the plant *Cannabis Savita*. O-1918 is also a compound structurally similar to CBD (Michael, Wu et al. 2001). Treating C_2C_{12} myotubes with O-1918 results in increased mRNA expression of markers involved in oxidative capacity including NFATc1, PGC1α, PDK4 and a trend to increase APPL1 (*Simcocks et al. Unpublished Observations see Appendix Two*). Therefore, suggesting that O-1918 may positively regulate oxidative capacity, fatty acid oxidation and potentially adiponectin signalling in the skeletal muscle and that exposing C_2C_{12} myotubes to O-1602 may modulate downstream targets in the adiponectin signalling pathway.

Previously, O-1602 has been identified for playing a role in regulating energy homeostasis (Diaz-Arteaga, Vazquez et al. 2012). In rats fed a SCD, sub-chronic administration of this compound via i.c.v. for seven days resulted in increased fat mass (Diaz-Arteaga, Vazquez et al. 2012). Mechanistically, O-1602 induces GTPγS to GPR55 in a homologous HEK293 cell-line (Ryberg, Larsson et al. 2007), indicating that this compound has an affinity for GPR55. In humans, GPR55 expression is increased in VAT and SAT in obesity (Moreno-Navarrete, Catalan et al. 2012). Conversley, GPR55 knockout mice have increased adiposity and impaired insulin sensitivity (Meadows, Lee et al. 2016).

O-1602 also has an affinity for GPR18 and the role that this receptor has in energy homeostasis is unknown, but as this receptor is abundantly expressed in tissues and cells from the immune system and considering that obesity is a state of chronic low grade inflammation (Weisberg, McCann et al. 2003) this receptor may have a role in obesity (Rajaraman, Simcocks et al. 2016). In support of a possible role that GPR18 has in energy homeostasis, GPR18 is expressed in organs involved in the regulation of energy homeostasis including adipose tissue and the hypothalamus (Vassilatis, Hohmann et al. 2003) but the receptors function is not clear. Regardless, the ability for O-1602 to regulate energy homeostasis in the skeletal muscle is unknown. Therefore this experiment aimed to determine the

effect that the compound O-1602, has on the mRNA expression of markers that regulate oxidative capacity and adiponectin signalling in a metabolically stable skeletal muscle model.

Hypothesis

As O-1602 is associated with increased adiposity in rodent models (Diaz-Arteaga, Vazquez et al. 2012) and C_2C_{12} myotubes treated with O-1918 significantly up-regulates markers of oxidative capacity and mediators of adiponectin signalling (*Simcocks et al, unpublished observations see Appendix Two*), it is hypothesised that O-1602 will down regulate markers of oxidative capacity in C_2C_{12} myotubes.

3.3 Material & Methods

C_2C_{12} myoblasts, a kind gift from Professor David Cameron-Smith (Deakin University, Melbourne, Australia) were utilised for this experiment. C_2C_{12} myoblasts were proliferated in D-MEM high glucose growth medium, the cells were then seeded into 6 wells plates. The C_2C_{12} myoblasts were differentiated into myotubes (within ~72 hours). Detailed methods are included in section 2.1.1 – 2.1.2

C_2C_{12} were serum starved (0.1% BSA and D-MEM) for six hours prior to treatment commencement. The C_2C_{12} myotubes were treated for 24 hours with vehicle (0.1% ethanol; Sigma Aldrich, St Louise, Missouri, United States of America) or O-1602 (10 nM - 1000 nM; Cayman Chemical, Michigan, United States of America) in 0.1% BSA and D-MEM. Detailed methods are included in 2.1.3 and 2.1.4. The dose response was based around the EC_{50} for GPR55 in O-1602, where 13 nM ± 2 induces GTPγS binding in HEK293 cells transiently transfected with human GPR55 (Ryberg, Larsson et al. 2007).

Following the treatment, cells were washed; excess liquid was removed from the six well plates with a pasture pipette, cells were lysed, on ice, using TRIzol Reagent® (Invitrogen, Victoria, Australia) and the lysate stored at -80°C for subsequent RNA extraction.

RNA was extracted according to previously established methods (McAinch, Steinberg et al. 2006) and is detailed further in Section 2.7.1. cDNA was generated from 0.5 µg of RNA using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, California), cDNA was then diluted 1: 20 and stored at – 20 °C for subsequent analysis. Further detail about reverse transcription and dilution of cDNA is described in sections 2.8.2 and 2.8.3.

Oligonucleotide primers were developed for the genes of interest and designed using Oligoperfect Suite (Invitrogen, Victoria, Australia, Victoria, Australia), the primers were purchased from Geneworks Pty Ltd (Adelaide, Australia). A BLAST search was conducted for each primer to determine homologous binding to the target mRNA sequence. The forward and reverse oligonucleotide primer sequences are included in Table 3.3A and a description of the markers role in skeletal muscle metabolism is highlighted in Table 3.3B.

'Real Time' PCR was utilised to determine mRNA expression of markers involved in the regulation of oxidative capacity and adiponectin signalling. The SYBR green method was utilised during this procedure (Ponchel, Toomes et al. 2003) to visualise the mRNA expression, IQ SYBR^{TM} Green and BioRad MY iQ® Real-Time PCR detection system (model # My iQ optics model, serial # 569BR/ 0611) were utilised to perform 'Real Time' PCR. Changes in mRNA abundance were normalised to house-keeping gene HPRT1 and calculated using $2^{-\Delta\Delta CT}$ method. This is further described in sections 2.9 and 2.10.

Table 3.3A: Forward and Reverse Oligonucleotide Primer Sequences for 'Real Time' PCR

Primer	Accession Number	Direction	Sequence
HPRT1	NM_013556.2	Forward ('5 '3)	GCAAACTTTGCTTTCCCTGG
		Reverse ('5 '3)	GCAAACTTTGCTTTCCCTGG
NFATc1	NM_016791.3	Forward ('5 '3)	TCCAAAGTCATTTTCGTGGA
		Reverse ('5 '3)	GTTGCGGAAAGGTGGTATCT
PGC1α	NM_008904.1	Forward ('5 '3)	CACCCACACGATCAGAACAA
		Reverse ('5 '3)	GGTCATCGTTTGTGGTCAGA
APPL 1	NM_145221.2	Forward ('5 '3)	ATCAGGCGGAAGAAGTGAGA
		Reverse ('5 '3)	TTTCTGATGCCCTACGATCC

Table 3.3B: Selected markers role in skeletal muscle metabolism

Marker	Function in the skeletal muscle
HPRT1	HPRT1 is an enzyme that generates purine nucleotides (Yamaoka, Kondo et al. 1997), HPRT1 protein is stably expressed across tissues including spleen, kidney, cerebrum, cerebellum, ventricle and skeletal muscle in rats (Kim, Na et al. 2014). This gene has been selected to be used as a housekeeping gene and normalised to the genes of interest using the $2^{-\Delta\Delta ct}$ method.
NFATc1	A transcription factor involved in the cellular remodelling in skeletal muscle to a fibre phenotype that has a greater oxidative capacity (Chin, Olson et al. 1998; Jordan, Jiang et al. 2005). Detailed description in *Chapter One* Section 1.3.1.2.
PGC1α	A transcription factor involved in oxidative capacity regulation and is a marker of mitochondrial biogenesis (Sparks, Xie et al. 2005). PGC-1 α abundantly expressed in oxidative skeletal muscle fibre types (Lin, Wu et al. 2002). Detailed description in *Chapter One* Section 1.3.1.3.
APPL1	Positive mediator of adiponectin signalling (Wang, Xin et al. 2009). Detailed description in *Chapter One* Section 1.3.2.1.

APPL1: Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1; HPRT1: Hypoxanthine Phosphoribosyltransferase; NFATc1: Nuclear Factor of Activated T-cells calcineurin dependent 1; PGC-1α: Peroxisome proliferator-activated receptor gamma co-activator 1-alpha.

3.3.1 Statistical Analysis

The statistical analysis was completed using Graph Pad Prism Software 7.0. Normality of data sets was analysed using Sharipo-Wilk normality test. In parametric data a one way ANOVA, with a Dunnett's Post Hoc test was utilised to determine whether there was a significant difference between dose treatments compared to the control group. In non-parametric data a Kruskal Walis Test was conducted with a Dunnett's Post Hoc test to determine whether there was a significant difference between dose treatments compared to control group.

3.4 Results

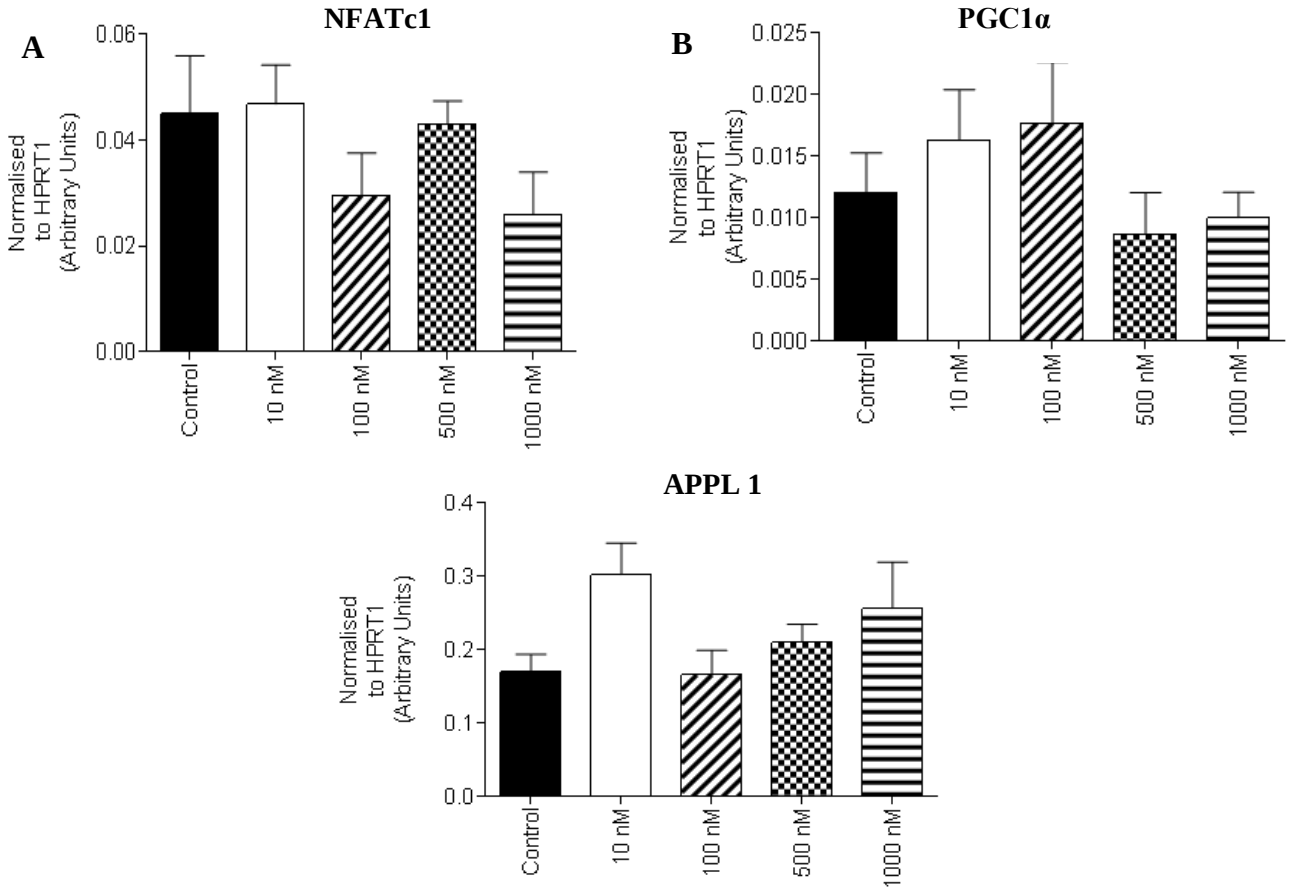


Figure 3.4A The effect that O-1602 has on markers of oxidative capacity and adiponectin signalling in C_2C_{12} myotubes.

The effect that treatment for 24 hours with O-1602 (10 - 1000 nM) has on C_2C_{12} myotubes, on markers involved in oxidative capacity and adiponectin signalling regulation. The abundance in mRNA expression of these markers were normalised to housekeeping gene Hypoxanthine Phosphoribosyltransferase (HPRT1) and expressed as mean ± SEM (n = 6 – 9). (A) Nuclear Factor of Activated T- Cells calcineurin dependent 1 (NFATc1), (B) Peroxisome proliferator-activated receptor gamma co activator 1-alpha (PGC1α) and (C) Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1 (APPL1). ■ Control, vehicle 0.1% EtOH, ▭ 10 nM O-1602, ▨ 100 nM O-1602, ▩ 0 500 nM O-1602, ▤ 1000 nM O-1602.

O-1602 does not alter markers of oxidative capacity or adiponectin signalling

Using a metabolically stable skeletal muscle model, C_2C_{12} myotubes that were treated with O-1602 (10 - 1000 nM) for 24 hours did not have altered mRNA expression for markers of oxidative capacity or adiponectin signalling (Figure 3.4A); namely NFATc1, PGC1α or APPL1.

3.5 Discussion

I have previously treated C_2C_{12} myotubes with a different atypical cannabinoid compound O-1918 and have found that there was an increase in markers involved in oxidative capacity, fatty acid metabolism and adiponectin signalling (*Simcocks et al, unpublished observations see Appendix Two*) following 24 hours of treatment. However, the key finding from this study shows that treating C_2C_{12} myotubes with O-1602 within the dose range of 10 nM - 1000 nM for 24 hours does not alter mRNA expression of oxidative capacity markers NFATc1, PGC1α or the positive adiponectin signalling regulator APPL1 when normalised to house-keeping gene HPRT1.

O-1602 has previously been demonstrated to be involved in energy metabolism regulation (Diaz-Arteaga, Vazquez et al. 2012), with Sprague Dawley rats treated with this compound for seven days having increased adiposity (Diaz-Arteaga, Vazquez et al. 2012). Additionally, cultured adipocytes treated with O-1602 had increased intracellular calcium which may be linked to increased lipogenesis (Diaz-Arteaga, Vazquez et al. 2012). In cultured 3T3-L1 adipocytes O-1602 also induced lipid accumulation and increased the regulator or adipocyte differentiation CCAAT/ enhancer-binding protein alpha (CEBPα) mRNA expression (Diaz-Arteaga, Vazquez et al. 2012). Therefore as this compound has a role in energy homeostasis in adipose tissue it was hypothesised it would also have a role in the skeletal muscle.

As it is now established that O-1602 is a non-specific ligand and has an affinity for both GPR55 (agonist) and GPR18 (biased agonist) and the role this compound has in the obese state is unknown. I have previously found that GPR55 is expressed in C_2C_{12} myotubes (*Simcocks et al, Unpublished Observations*), however the protein expression of this receptor was determined using western blotting and G Protein-Coupled Receptors antibodies can lack selectivity (Michel, Wieland et al. 2009); therefore conformation from GPR55 knockout mouse skeletal muscle is required to confirm antibody reliability, and validate my findings. While the dosage selected in this study was based around the EC_{50} for GPR55 in HEK293 cells transiently expressing GPR55 (Ryberg, Larsson et al. 2007), it

cannot be discounted that GPR18 also has an affinity for this compound. Furthermore, G Protein-Coupled Receptors have been shown to form heteromers with other G Protein-Coupled Receptors (Igarashi, DiPatrizio et al. 2015), for example GPR55 signalling has been shown to be influenced by CB_1 and CB_2 as they are able to form heteromers with GPR55 (Balenga, Aflaki et al. 2011; Kargl, Balenga et al. 2012), so therefore it maybe hypothesised that GPR55 could potentially form a heteromers with GPR18.

Taken together these findings show that using metabolically stable C_2C_{12} myotubes treated with O-1602 does not effect NFATc1, PGC1α or APPL1 mRNA expression. Notwithstanding these data, the role that O-1602 has in obesity remains unclear, as skeletal muscle homeostasis in the obese state is dysregulated, and while the data from this study does not highlight O-1602 having a role, the compounds role in a DIO state is worth investigating.

CHAPTER FOUR:

4.0 The effect that O-1602 administration has on whole body energy homeostasis in a diet induced obesity model

4.1 Abstract

Background: O-1602 has an affinity for the putative cannabinoid receptors GPR55 and GPR18. This compound has been shown to have anti-inflammatory effects that may be beneficial to the obese state which is considered to be a chronic state of low-grade inflammation. The aim of this study is to determine the effect that chronic administration of O-1602 has in a DIO model focusing on the compound's effect on weight, food intake, body composition, blood glucose control, plasma hormone and cytokine concentrations as well as liver function.

Materials & Methods: DIO rats (maintained on a 21% HFD) by weight for nine weeks, were then administrated O-1602 (5 mg/ kg body weight) ip. injection for six weeks. Food consumption and body weight were monitored daily, body composition was monitored using Echo MRI at baseline, weeks three and six of the treatment period. An ip. GTT and an ip. IST were performed at weeks eight and fourteen. Blood was collected at time of death via cardiac puncture which was then centrifuged to separate the plasma. Adipose and liver tissues were collected and immediately snap frozen for subsequent analysis. Circulating hormones, cytokines and liver function markers in the plasma were assessed using commercially available products.

Results: In DIO rats, O-1602 caused a reduction in body fat (weeks three and six of treatment) and increased lean body mass percentage (week six treatment only) as well as a reduction in epididymal fat pad size. While body weight (grams) was not altered between the control and treatment group, there was a reduction in body weight (% weight change from baseline) from weeks two through to six of treatment with O-1602 in the DIO rats. Food intake transiently decreased at week one only of the

treatment period. Whole body insulin sensitivity was not altered by the compound following five weeks of treatment for either ip. GTT or ip. IST. O-1602 also caused a reduction in plasma leptin and ghrelin. However, treatment with this compound caused plasma RANTES to increase and the rats had abnormal and dark appearing regions on their liver as well as raised plasma AST. Other plasma liver function markers ALT and albumin were not altered, further hydroxyproline which is the fibrotic marker of collagen content in both the liver and heart were not changed.

Discussion & Conclusion: Chronic administration of O-1602 in the obese state partly plays a role in energy metabolism and body composition by reducing body fat percentage and increasing lean tissue mass percentage. However, this compound appears to have negative consequences in relation to liver, including both its morphology as well as elevated plasma AST concentrations. This compound also increased the chemokine RANTES. The side effects that this compound had on the liver in addition to increasing RANTES appear to outweigh the benefits seen with the change in body composition in these rats. Further investigation into understanding the mechanisms involved as a result of the observational and biochemical data is therefore required.

4.2 Background

The endocannabinoid system is involved in a number of physiological functions including partially regulating energy homeostasis both centrally and peripherally through appetite control, hedonic feeding behaviours as well as glucose and lipid metabolism (Di Marzo, Ligresti et al. 2009). While obesity and related comorbidities are increasing, there is a need to find novel compounds which may be beneficial for the treatment of these conditions, in conjunction with appropriate dietary and physical activity modifications. O-1602 is a synthetic analogue of Abn-CBD, in which Abn-CBD is a synthetic isomer of the phytochemical CBD (Kreutz, Koch et al. 2009) found in *Cannabis Sativa* (Zhornitsky and Potvin 2012).

O-1602 is an atypical cannabinoid and does not have a high affinity for the cannabinoid receptors CB_1 or CB_2 (Jarai, Wagner et al. 1999; Ryberg, Larsson et al. 2007), therefore this compound may not have undesired psychotropic effects that was observed with Rimonbant (Salmeron, Hu et al. 2001; Despres, Golay et al. 2005).

Previous research has demonstrated that both Abn-CBD and O-1602 are agonists for putative cannabinoid receptor; GPR55 (Johns, Behm et al. 2007; Ryberg, Larsson et al. 2007), and a biased agonist for GPR18 (Console-Bram, Brailoiu et al. 2014). There has been conflicting pharmacology surrounding O-1602 and Abn-CBD in relation to GPR18 in the literature. One study identified that both compounds were potent initiators of cell migration in both GPR18 transfected HEK293 cells and microglial cells shown to endogenously express GPR18 (McHugh, Hu et al. 2010). Whereas, another study concluded that O-1602 and Abn-CBD were not agonists for GPR18; this experiment looked at heterologous expressed GPR18 in superior ganglion from rats, which failed to elicit the hypothesised response of I_{Ca} inhibition when treated with these compounds (Lu, Puhl et al. 2013). However, a recent study demonstrated that both compounds are biased agonists for GPR18 which helps to validate the differences observed between the studies described (Console-Bram, Brailoiu et al. 2014).

O-1602 has been identified to have an anti-inflammatory role (Li, Feng et al. 2013). As such, research has focused on its role in a number of different inflammatory mediated pathophysiological conditions. For example O-1602 has improved pathological changes in a cerulin induced acute pancreatitis mouse model (Li, Feng et al. 2013), reduced joint afferent mechanosensitivity in an acutely induced arthritis model (Handschin, Choi et al. 2007), reduced inflammation in experimental colitis (Schicho, Bashashati et al. 2011) as well as decreased tumour growth by reducing tumour cell viability, proliferation and promoted apoptosis in a colitis induced colon cancer model (Kargl, Haybaeck et al. 2013).

O-1602 has also been shown to have a role in energy homeostasis (Diaz-Arteaga, Vazquez et al. 2012). Currently there is one study published which found that acute administration of O-1602 directly into the brain of rodents caused an increase in food intake (Diaz-Arteaga, Vazquez et al. 2012), while a single ip. injection of the same compound in rats caused a transient increase in food intake six hours following the injection which may be as a result of the down-regulation of the neuropeptide Cocaine and Amphetamine Regulated Transcript (CART) (Diaz-Arteaga, Vazquez et al. 2012). However, seven days of treatment with O-1602 (either centrally via i.c.v. at a dose of (0.4 μg/ hour) or peripherally ip. at a dose of 0.1 mg/ kg) showed no change in food intake or body weight, but there was an increase in fat mass in both the centrally and the peripherally treated rats (Diaz-Arteaga, Vazquez et al. 2012). Taken together, the current literature suggests that O-1602 has more of an acute effect on food intake rather than a chronic effect, however the previous research investigated the effect that this compound had under physiological conditions, as such it is unclear of the compounds role in obesity.

Obesity is often accompanied by a state of chronic low grade inflammation (Hotamisligil, Shargill et al. 1993; Kim, Park et al. 2006; Calder, Ahluwalia et al. 2011; Fernandez-Real and Pickup 2012) which can be partially involved in the development of T2DM (van Greevenbroek, Schalkwijk et al. 2013). Therefore the anti-inflammatory effects that O-1602 has been shown to elicit in the other conditions

may be of benefit for the obese state. The aim of this study was to therefore determine the effect that chronic administration of O-1602 has on whole body energy homeostasis in a DIO model. In particular the aim of this study was to examine the effect that this compound had on weight, food intake, body composition including fat depots, blood glucose control, plasma hormone and cytokine concentrations as well as its effects on the liver.

Hypothesis

O-1602 is an agonist for GPR55, as O-1602 is associated with increased adiposity in rodents (Diaz-Arteaga, Vazquez et al. 2012) and C_2C_{12} myotubes treated with O-1918 (a structurally different compound, that is an antagonist for GPR55) significantly up-regulated markers of oxidative capacity and fatty acid metabolism with a trend for a positive mediator of adiponectin signalling to be up-regulated (*Simcocks et al, Unpublished Observations*), while C_2C_{12} myotubes treated with O-1602 indicated no change in the same markers (measured in the O-1918 C_2C_{12} experiment) that regulate oxidative capacity or adiponectin signalling (described previously in *Chapter Three*), it is hypothesised that in DIO rats, the compound O-1602 will cause increased weight gain, increased body fat percentage, increased white adipose tissue fat pad mass (epididymal and peri-renal), increased circulating leptin, increased circulating ghrelin, decreased adiponectin, increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines.

4.3 Material & Methods

4.3.1 Animals & Housing

Seventeen, seven week old male Sprague Dawley rats were purchased from ARC (Canning Vale, WA, Australia), the rats were acclimatised for at least seven days and then housed singly for the duration of this study. The rats were maintained on a HFD (21% fat diet by weight) (Cornall, Mathai et al. 2011; Jenkin, O'Keefe et al. 2016) for a total period of fifteen weeks. The first nine weeks of high fat feeding was to induce obesity, then the DIO rats were allocated into groups based on their metabolic characteristics and for a further six weeks were chronically treated with either: (1) Vehicle 0.75% Tween 80 and saline or (2) 5 mg/ kg O-1602 dissolved in 0.75% Tween-80 and saline.

The dosage of O-1602 was selected as previously mice have been treated sub-chronically with this compound in experimentally induced colitis in mice (Schicho, Bashashati et al. 2011). Animal ethics was obtained from the AEC at the Howard Florey Institute (Parkville, Melbourne, Australia) approval number 11-036. For further detail refer to sections 2.2, 2.3.1 and 2.3.2.

4.3.1.1 Food Intake and Body Weight

Both food intake (in grams) and body weight (in grams) were measured and recorded on a daily basis and the weekly average was calculated and represented. For further detail refer to section 2.3.2.2.

4.3.1.2 Echo MRI

To determine body fat and lean tissue mass the previously validated (Taicher, Tinsley et al. 2003) Echo MRI scan was completed using Echo MRI^{TM} system (Echo-MRI 900 Houston, United States of America) was utilised. Measurements were obtained week nine (at baseline of DIO), week twelve (during treatment) and week fifteen (at the end of the treatment period). For further detail refer to section 2.3.2.3.

4.3.1.3 Ip. GTT

To determine the effect a glucose load had on blood glucose concentrations, an ip. GTT was conducted using previously published methods (Jenkin, O'Keefe et al. 2016). This procedure was conducted at week eight and week fourteen. For further detail refer to section 2.3.2.4

4.3.1.4 Ip. IST

To determine the effect a bolus of insulin had on blood glucose concentrations, an ip. IST was conducted using previously published methods (Jenkin, O'Keefe et al. 2016). Measurements were obtained at week eight and week fourteen. For further detail refer to section 2.3.2.5.

4.3.1.5 Anaesthesia, Blood and Tissue Collection

At the end of the treatment period, rats were deeply anesthetised with isoflurane, then administered 100 mg/ kg of sodium pentaborbitone via an ip. injection and then killed via cardiac puncture. Blood was removed, transferred into EDTA BD coated vacutainer (Macfarlane Medical, Surry Hills, Victoria), and placed on ice, then subsequently centrifuged to obtain plasma. Plasma was then transferred to Eppendorf tubes and stored at -80°C for subsequent analysis. Whole adipose tissue depots, liver and heart were immediately harvested and the weight (in grams) of the organs were recorded, tissues then transferred to cryotubes and snap frozen in liquid nitrogen for subsequent analysis. For further detail refer to section 2.3.2.6.

4.3.2 Biochemical Analysis

4.3.2.1 Adiponectin Assay

Total circulating adiponectin concentrations were determined utilising Adiponectin ELISA Kit (AG-45A-0005TP-KI01) (Adipogen, Liestal, Switzerland) in accordance with the manufacturer's instructions. For further detail refer to section 2.5.2.

4.3.2.2 Bioplex Assay

Both the Bioplex hormone immunoassay (171K1001M) and cytokine assay (YL0000006Q) were used to analyse circulating plasma hormone and cytokine concentrations using the Bioplex 200 System (Bio-Rad Laboratories, Munich, Germany) in accordance with the manufacturer's instructions. For further detail refer to section 2.5.1.

4.3.2.3 Liver Function Test

To determine the effects on plasma markers relating to liver function, a plasma liver function test was conducted by the University of Melbourne's Veterinary Hospital. AST (AS3804), ALT (AL3875), and albumin (AB3800) were all analysed using Randox Daytona (Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom) in accordance with the manufacturer's instructions. For further detail refer to section 2.5.3.

4.3.2.4 Hydroxyproline Assay

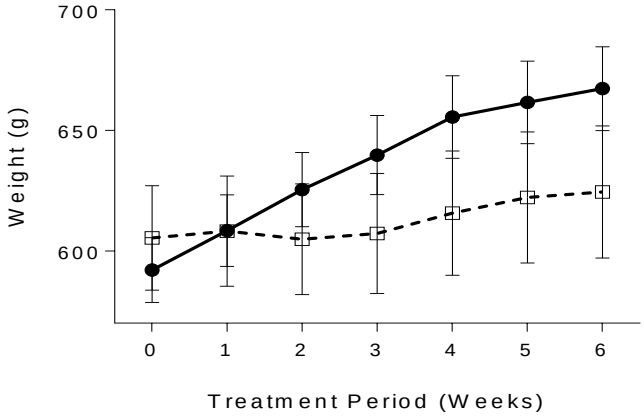
To determine total collagen content and concentration in both liver and heart tissue, a colorimetric based hydroxyproline assay was utilised. Hydroxyproline is a key marker of fibrosis and was assessed using a previously published methodolgy (Samuel 2009). For further detail refer to section 2.6.

4.3.3 Statistical Analysis

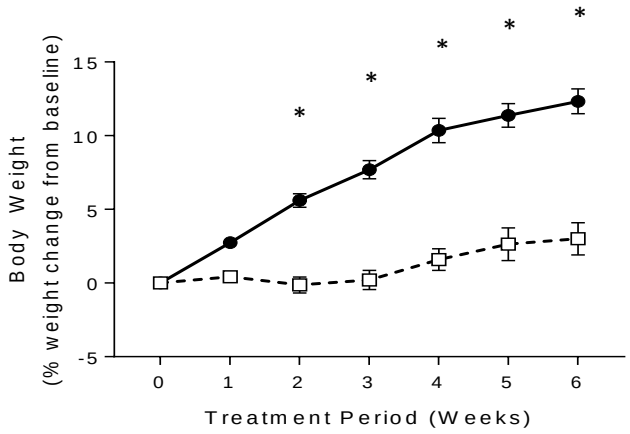
All statistical analysis was completed using Graph Pad Prism Software 7.0. Normality of data sets were analysed using Sharipo-Wilk normality test, when data was normally distributed a parametric unpaired t-test was utilised, whereas Mann-Whitney test was utilised for any nonparametric data sets. When comparing multiple time points a 2-way ANOVA, with a Bonferroni Post Hoc test was utilised to determine significant difference between time points and treatment type.

4.4 Results

A



B



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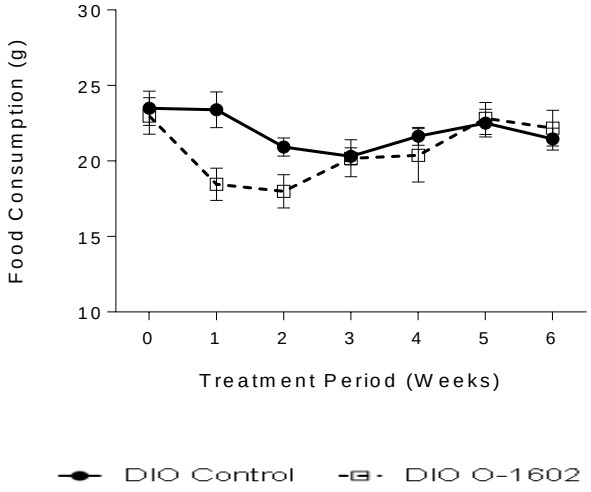


Figure 4.4A: Body Weight & Food Consumption

(A) Body weight (grams) (B) Body Weight (% weight change from baseline) and (C) Food Consumption (grams) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as

grouped average ± SEM. Black circles: DIO Control rats treated with saline (n=11). White boxes DIO rats treated with O-1602 (n=6). * indicates significance ($p < 0.05$).

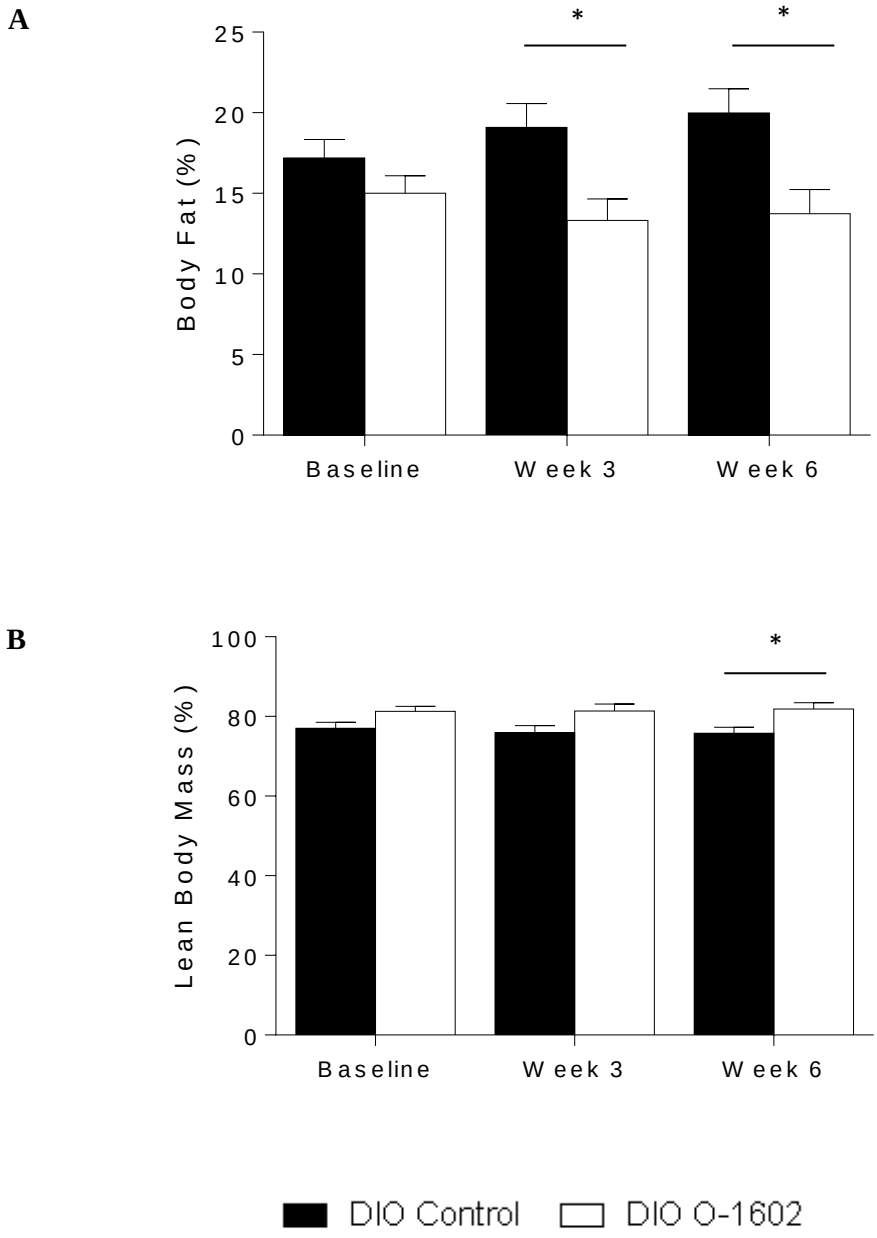


Figure 4.4B: Body Composition

(A) Body fat (%) (B) Lean body mass (%) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5mg/ kg O-1602/ 0.75% tween 80/ saline) for six weeks. Data is reported as grouped average ± SEM. Closed bars - DIO Control rats treated with saline (n = 11). Open bars - DIO rats treated with O-1602 (n = 6).

* indicates significance ($p < 0.05$).

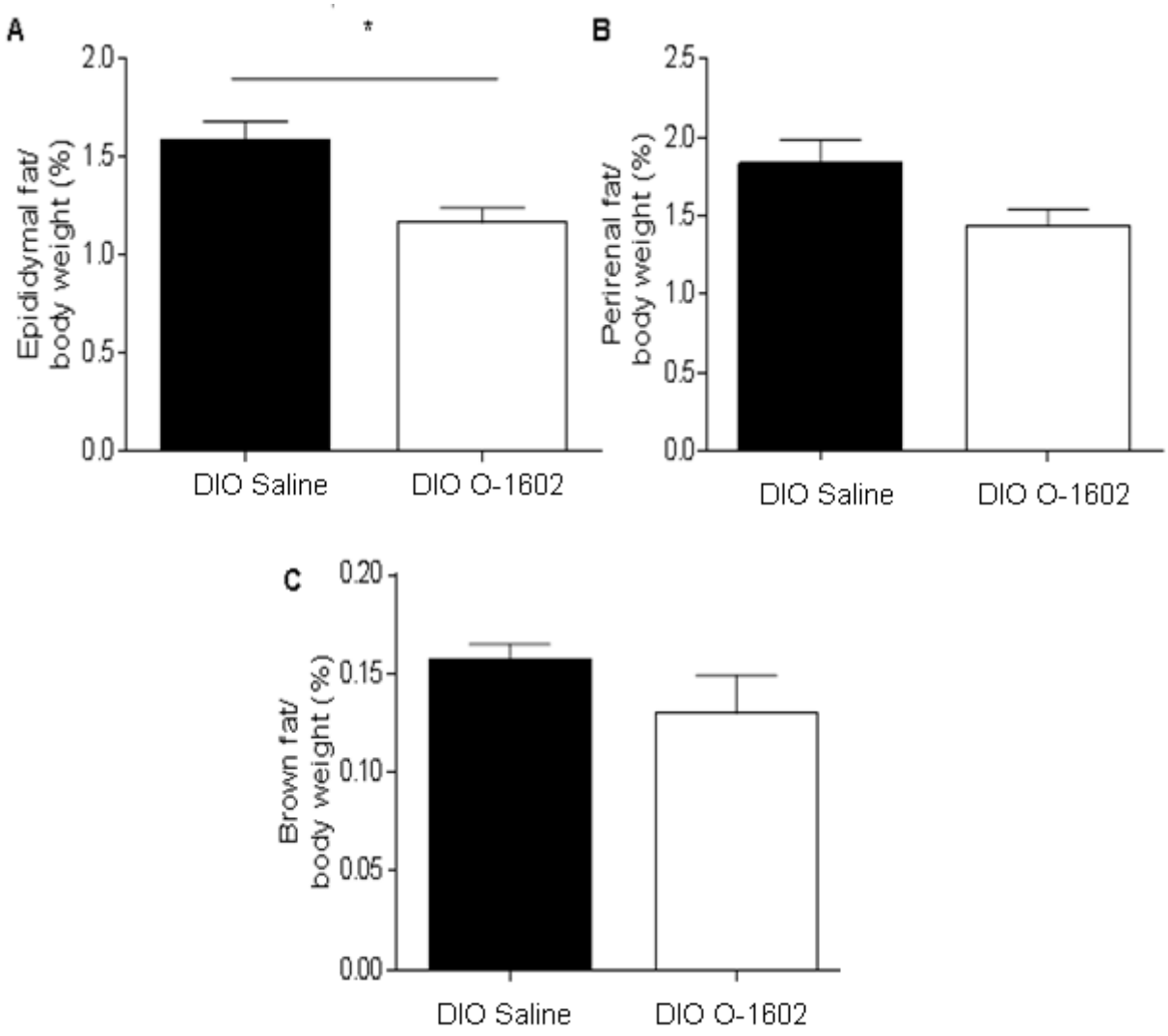


Figure 4.4C: Fat Pad Weights

(A) Epididymal fat weight/ body weight (%) (B) Perirenal fat weight/ body weight (%) (C) Brown fat weight/ body weight (%) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as grouped average ± SEM. Closed bars - DIO Control rats treated with saline (n = 11). Open bars - DIO rats treated with O-1602 (n = 6). * indicates significance ($p < 0.05$).

Food Intake

DIO O-1602 treated rats had a transient reduction in food intake at week one only ($p < 0.05$) during the six week treatment period when compared to the DIO Control group (Figure 4.4A C).

Body Weight and Body Composition

The body weight (in grams) of DIO O-1602 treated rats was not significantly altered when compared to DIO Control group (Figure 4.4A A). However, when data was represented as Body Weight (% weight change from baseline) the DIO O-1602 treated rats had significantly reduced weight from weeks two to six when compared to the DIO Control group (Figure 4.4A B). To further support this finding, when looking at the body composition of the DIO O-1602 treated rats, these rats had decreased body fat (%) at week three and week six of the treatment period when compared with the DIO Control group ($p < 0.05$). While treatment with O-1602 in these DIO rats significantly increased lean body mass (%) at the end of the treatment period (week six) when compared with DIO Control group (Figure 4.4B A & B) ($p < 0.05$).

When observing the WAT and BAT fat depots collected, the DIO O-1602 treated rats had reduced epididymal fat depots ($p < 0.05$), while perirenal and BAT depots remained unchanged when compared to the DIO Control group (Figure 4.4C A - C).

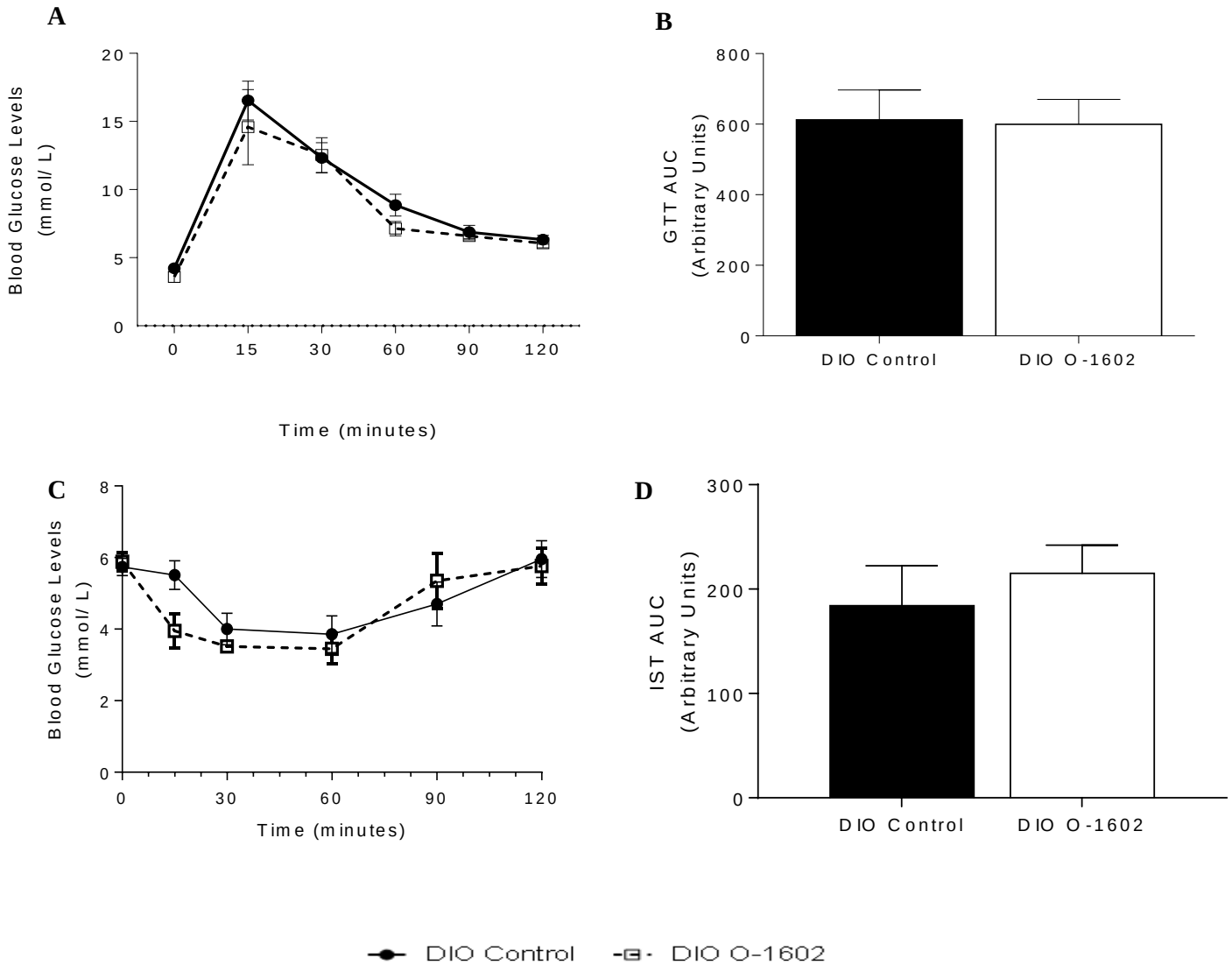


Figure 4.4D: Glucose Tolerance & Insulin Sensitivity

(A) Ip. GTT blood glucose concentrations (mmol/ L) (B) Ip. GTT Area Under the Curve (AUC) (arbitrary units) (C) Ip. IST blood glucose concentrations (mmol/ L) (D) Ip. IST AUC (arbitrary units) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as grouped average ± SEM. Black circles - DIO Control rats treated with saline (n = 11). White boxes - DIO rats treated with O-1602 (n = 6). Closed bars - DIO Control rats treated with saline (n = 11). Open bars - DIO rats treated with O-1602 (n = 6).

Glucose Tolerance & Insulin Sensitivity

Whole body glucose handling and the response to insulin was assessed using an ip. GTT and an ip. IST in rats fed a HFD to induce obesity at both baseline and following five weeks of treatment with O-1602. The blood glucose concentrations and AUC (arbitrary units) obtained from the DIO O-1602 treated rats for either the ip. GTT or the ip. IST were not significantly altered when compared to DIO Control group (Figure 4.4D A-D).

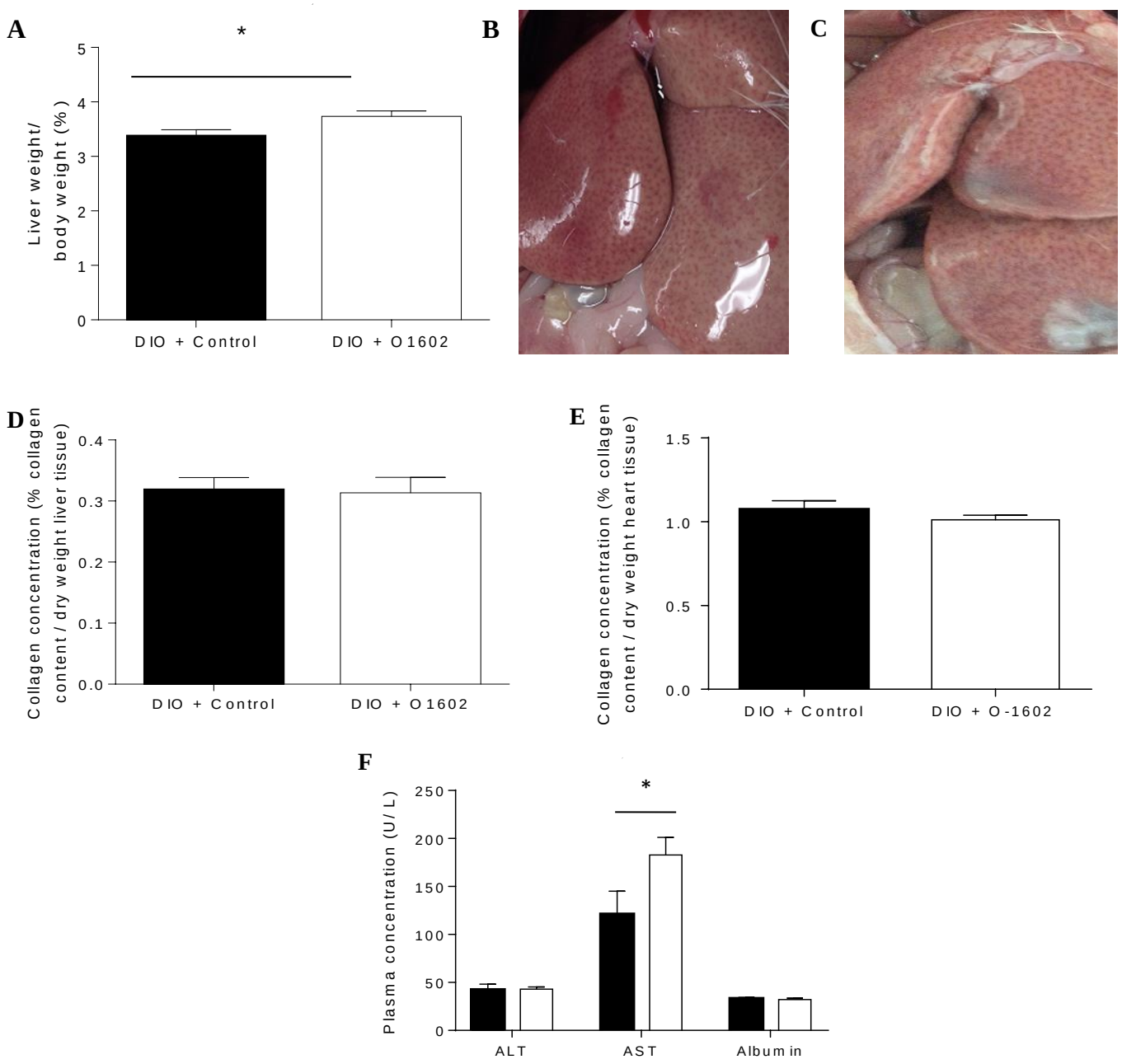


Figure 4.4E: Liver Function Parameters

(A) Liver weight/ body weight (%) (B) Representative photo of whole liver obtained from the DIO Control group (n = 1) (C) Representative photo of liver obtained from DIO O-1602 group (n = 1) (D) Liver collagen content (E) Heart collagen content (F) Plasma liver function test obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg / kg O-1602/ 0.75% tween 80/ saline) for six weeks. Data is reported as group average ± SEM. Closed bars - DIO Control rats treated with saline (Figure A n = 11, Figure D – F n = 8). Open bars - DIO rats treated with O-1602 (Figure A, D – F n = 6). * indicates significance ($p < 0.05$).

Liver Function Parameters

DIO O-1602 treated rats had significantly enlarged livers ($p < 0.05$) with abnormal and dark appearing regions when compared to the DIO Control group (Figure 4.4E A - C). Fibrotic marker hydroxyproline, which indicates collagen content in both the liver and heart obtained from the DIO O-1602 group were however not altered (Figure 4.4E D - E). Liver function tests indicates an increase in AST in the DIO O-1602 treated group compared to DIO control group, while ALT and albumin were not altered (Figure 4.4E F).

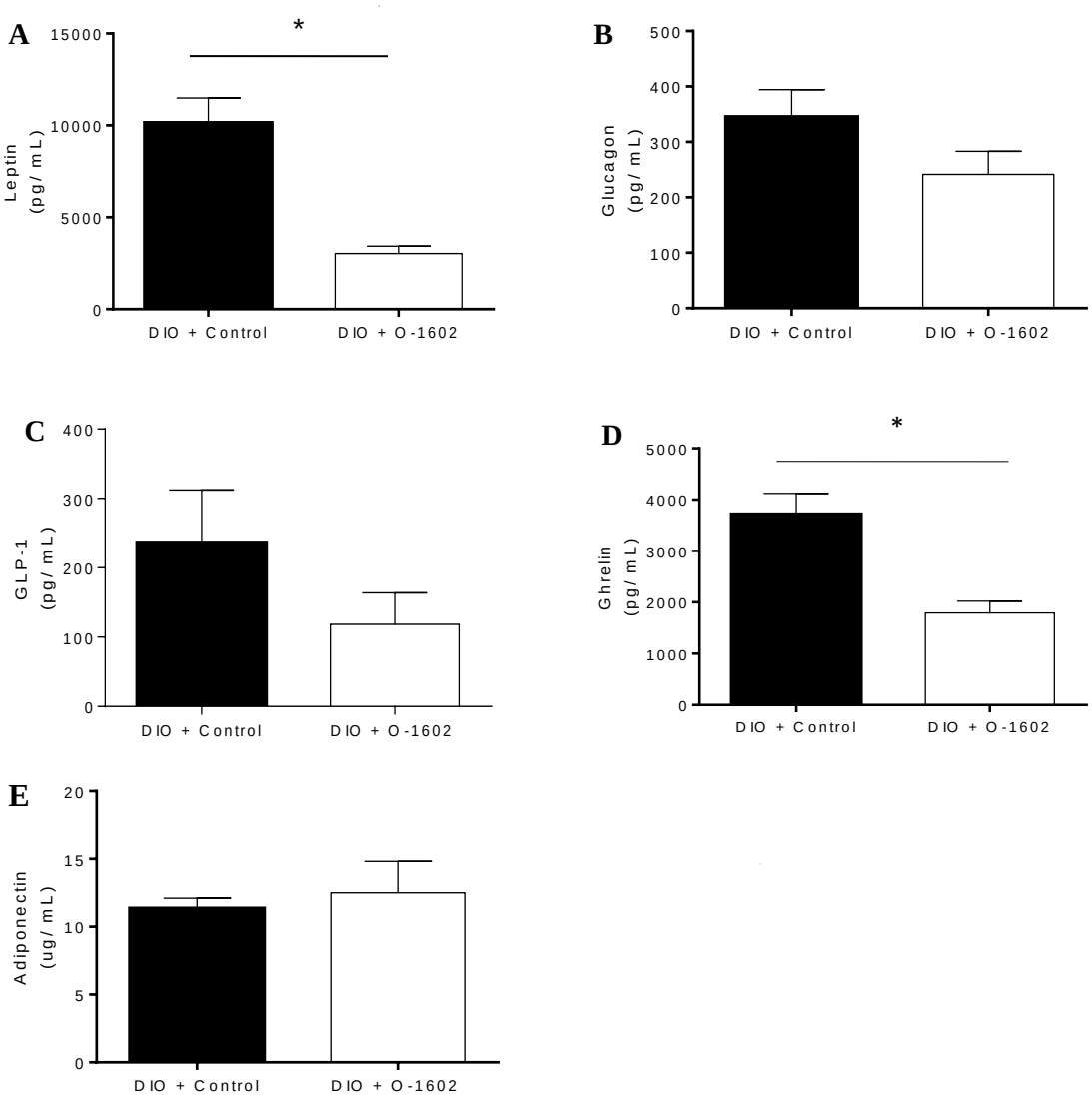


Figure 4.4F: Plasma Hormone Profiles

Plasma concentrations of hormones including; (A) Leptin, (B) Glucagon; (C) Glucagon like peptide-1 (GLP-1); (D) Ghrelin and (E) Adiponectin obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with an vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as group average ± SEM. Closed bars - DIO Control rats treated with saline (n = 11). Open bars - DIO rats treated with O-1602 (n = 6). * indicates significance ($p < 0.05$).

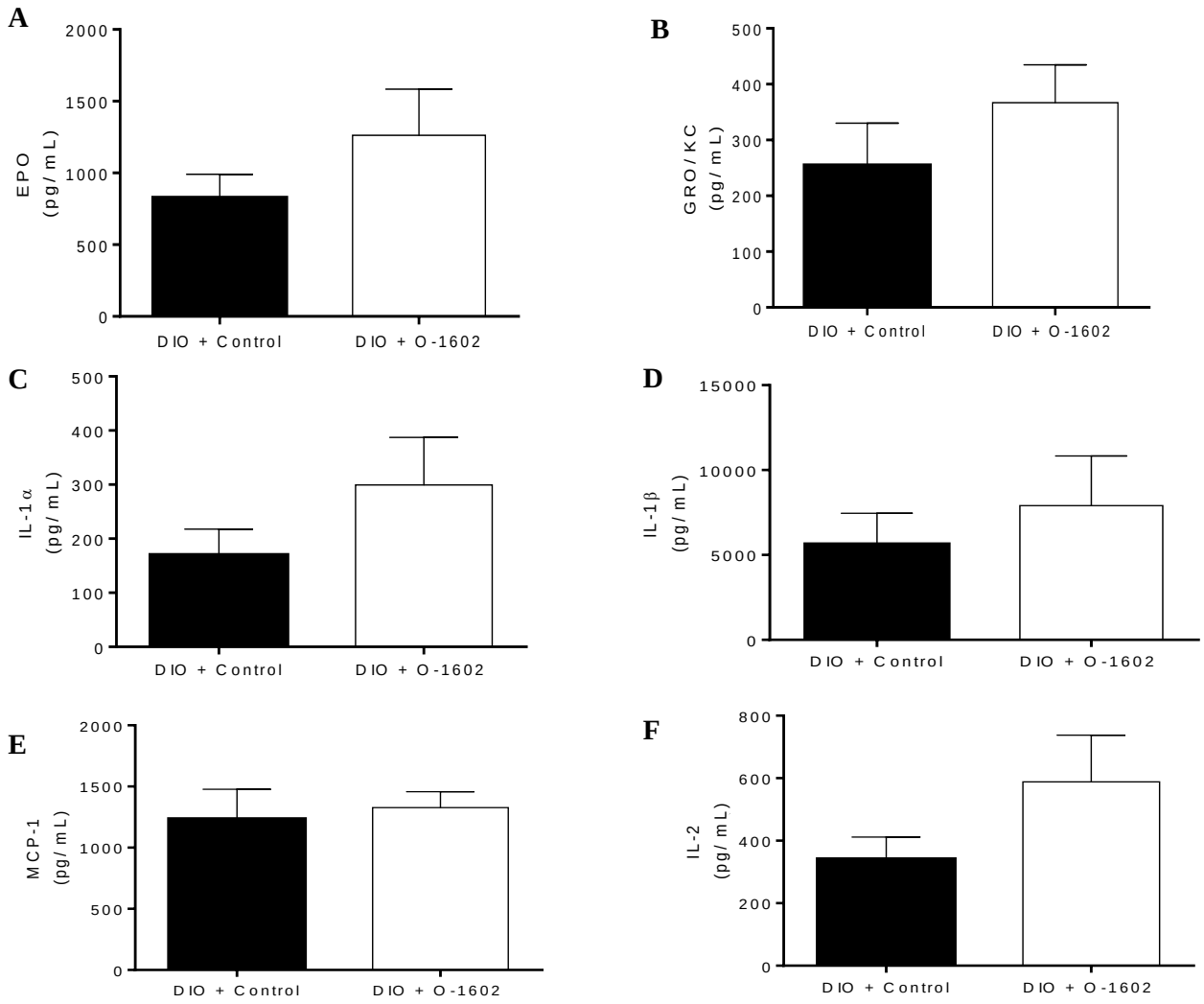


Figure 4.4G: Plasma Cytokine Profile

Plasma concentrations of cytokines including; (A) Erythropoietin (EPO); (B) Growth Regulated α Protein/ Keratinocyte Chemoattractant (GRO/ KC); (C) Interleukin-1α (IL-1α); (D) Interleukin-1β (IL 1β); (E) Monocyte Chemoattractant Protein-1 (MCP-1); (F) Interleukin-2 (IL-2) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as group average ± SEM. Closed bars - DIO Control rats treated with saline (n = 9 - 11). Open bars - DIO rats treated with O-1602 (n = 6).

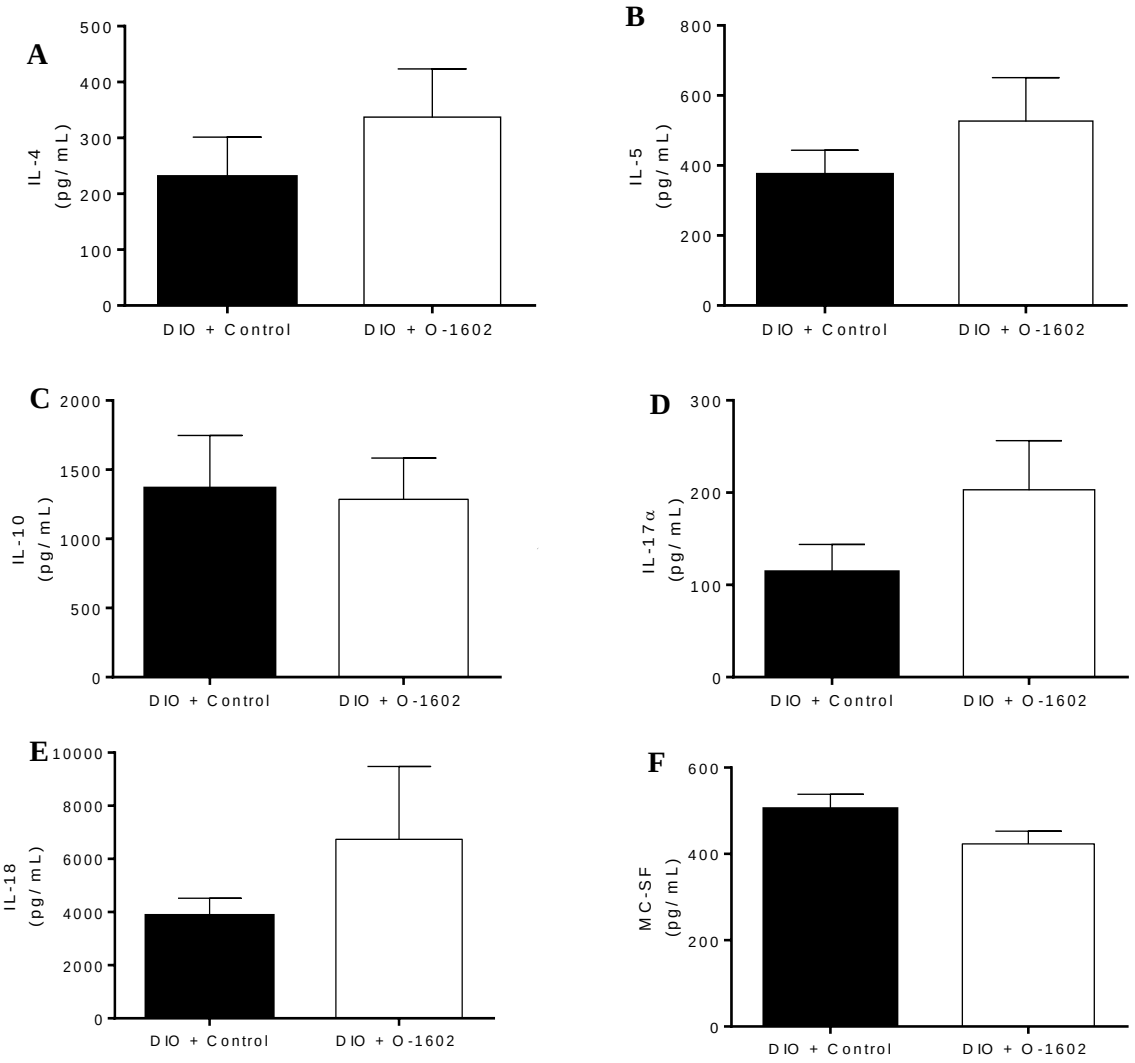


Figure 4.4H: Plasma Cytokine Profile

Plasma concentrations of cytokines including; (A) Interleukin-4 (IL-4); (B) Interleukin-5 (IL-5) (C) Interleukin 10 (IL-10); (D) Interleukin-17α (IL-17α); (E) Interleukin-18 (IL-18); (F) Monocyte Colony Stimulating Factor (MC-SF) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as group average ± SEM. Closed bars - DIO Control rats treated with saline (n = 9 - 11). Open bars - DIO rats treated with O-1602 (n = 6). * indicates significance ($p < 0.05$).

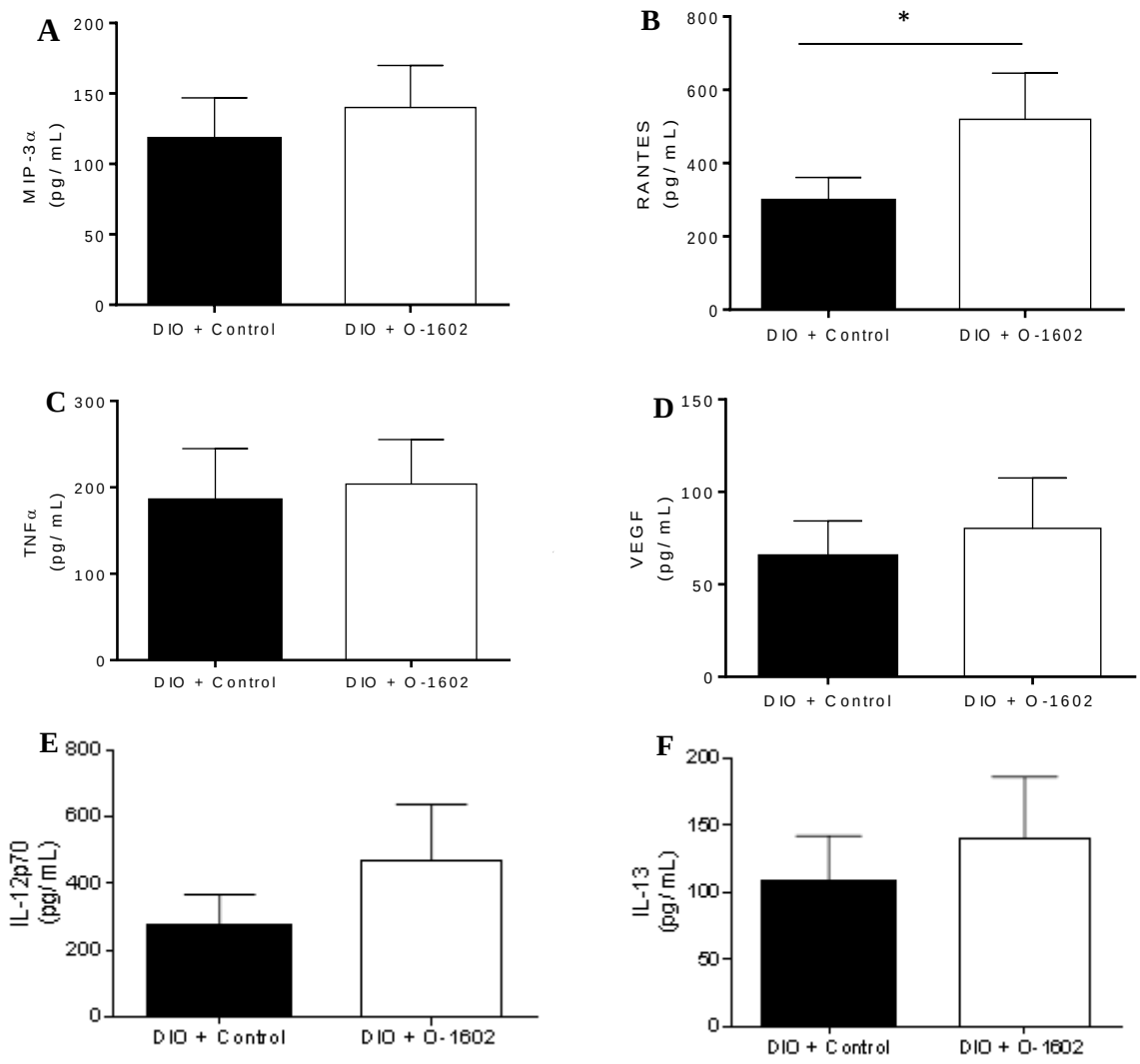


Figure 4.4I: Plasma Cytokine Profile

(A) Macrophage Inflammatory Protein 3α (MIP-3α); (B) Regulated on Activation Normal T Cells Expressed and Secreted (RANTES); (C) Tumour Necrosis Factor α (TNFα); (D) Vascular Endothelial Growth Factor (VEGF); (E) Interleukin 12p70 (IL-12p70); (F) Interleukin 13 (IL-13) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1602 rats were treated with the pharmacological compound O-1602 (5 mg/ kg O-1602/ 0.75% tween 80/ saline) for a further six weeks. Data is reported as group average ± SEM. Closed bars - DIO Control rats treated with saline (n = 9 - 11). Open bars - DIO rats treated with O-1602 (n = 6). * indicates significance ($p < 0.05$).

Plasma Hormones & Cytokines

The plasma hormone profile shows that DIO O-1602 treated rats had reduced leptin ($p < 0.05$) and ghrelin ($p < 0.05$) (Figure 4.4F A & D) when compared to the DIO Control group, but the other hormones measured were not altered (Figure 4.4F B, C & E). The plasma cytokine profiles shows that DIO O-1602 treated rats had significantly increased RANTES ($p < 0.05$) (Figure 4.4I B), while all of the other cytokines were not altered (Figures 4.4G A-I, 4.4H A-I and 4.4I A& C-I).

It should also be noted that some of the analytes measured from the hormone and cytokine plates were excluded from analysis due to a number of samples being out of range either from the DIO Control and/ or the DIO O-1602 group(s) and therefore the statistical analysis was unable to be completed as a result of this. These analytes include PAI-1, G-SCF, GM-CSF, IFN-γ, IL-6 and IL-7.

4.5 Discussion

This study is the first to look at an alternative cannabinoid derivative, O-1602, and the effect that chronically administrating this compound has on whole body energy homeostasis in a DIO rat model. The key findings from this study are that chronic treatment with O-1602 (at a dose of 5 mg / kg) in DIO rats resulted in reduced total body fat percentage and a reduction in WAT epididymal fat depots but not the fat depots for perirenal or BAT. There was also an increase in lean tissue mass. Interestingly total body weight (in grams) was not altered but when represented as Body Weight (% weight change from baseline) the DIO O-1602 treated rats weight was significantly reduced from weeks two to six of treatment when compared with the DIO control group. Food intake was transiently decreased within the first week of treatment only in the DIO O-1602 treated group, there was also a reduction in circulating plasma hormones leptin and ghrelin, which are involved in appetite regulation. Glucose tolerance and insulin sensitivity were not altered by treatment with O-1602 in obesity. The livers obtained from the DIO O-1602 treated rats were increased in weight, and appeared to have abnormal darker regions, while fibrotic marker hydroxyproline was not altered in the liver or heart tissue there was an increase in circulating plasma RANTES and the liver function marker AST, but not ALT or albumin.

The reduction in food intake within the first week of treatment with O-1602 is not unexpected given that another cannabinoid compound SR141716 has been shown to transiently reduce food intake in mice and rats with in the first week of treatment (Colombo, Agabio et al. 1998; Ravinet Trillou, Arnone et al. 2003). Wister rats have been shown to develop a tolerance to the anorexic effect of SR141716 after five days of administration with this compound (Colombo, Agabio et al. 1998), which given that O-1602 has an affinity for other putative cannabinoid receptors, a similar tolerance to O-1602 could be occurring at the targeted receptors. The acute reduction in food intake observed in the DIO O-1602 treatment group was not sustained enough to cause a reduction in body weight (in grams) during the treatment period. The reduction in body fat in particular the epididymal fat depots and the increase in

lean tissue mass however, provides some evidence that this compound has a role in metabolic homeostasis. Other research in Sprague Dawley rats maintained on a SCD and administered a lower dose of O-1602 (0.1 - 1 mg/ kg), showed that the compound did not elicit an effect on food intake or body weight, although the dosage 0.1 mg/ kg did have the opposite effect to our results, in which body fat mass was increased (Diaz-Arteaga, Vazquez et al. 2012). However, conflicting findings are likely due to differing study design, and that the rats were not obese and the dose and duration of administration of the compound was at a much lower concentration and for a much shortly time period. As O-1602 acts as both an agonist/ biased agonist for GPR55 and GPR18, respectively, this may also account for the differences observed, as different dosages of O-1602 could result in varying pharmacological responses and therefore different physiological effects. Diaz-Artega *et al.* (2012) acutely administered 200 µg/ kg of O-1602 to *GPR55* knockout mice and found that food intake of the animals increased, indicating that this compound may elicit an effect on receptors other than GPR55, which is not necessarily surprising given that other research groups have found that O-1602 has an affinity for GPR18 (McHugh, Page et al. 2012; Console-Bram, Brailoiu et al. 2014).

While the reduction in body fat and epididymal fat pad depots from this study can be seen as beneficial for obesity, the observations of the liver morphology and the elevated plasma AST indicates that this compound also elicited undesirable effects. There are currently no studies in the literature looking at the effect that O-1602 has in the liver and metabolism. One study looked at the role that O-1602 has in the pathophysiological condition cholangiocarcinoma (Huang, Ramirez et al. 2011), cholangiocarcinoma is a neoplasm of the bile duct, which can occur in the liver or extra hepatic bile ducts (Ebata, Ercolani et al. 2016), however this is in a pathophysiological state that can occur in the liver rather than a physiological state. Experimentally induced cholangiocarcinoma in mice as well as cell culture models of this cancer type were used, and this study found that O-1602 caused anti-proliferative effects in both the *in vivo* and *in vitro* models of cholangiocarcinoma and this was mediated through GPR55 (Huang, Ramirez et al. 2011). As this study by Huang et al. (2011) was

focusing on the compounds role in cancer, it did not look at the metabolic effects that O-1602 had in the hepatic system. Further investigation into the role that this compound has on the liver in the obese state is required as the current results show that a 5 mg/ kg administration of O-1602 over a six week period in DIO rats causes adverse effects including; enlarged liver size, abnormal appearing regions on the liver and elevated plasma AST concentrations, while the other liver function markers including both ALT and albumin were not altered. An enlarged liver or hepatomegaly, can be indicative of the following reasons including; (1) drug induced hepatitis as compounds such as paracetamol (acetaminophen) can cause enlarged livers as a result of hepatic injury/ inflammation and O-1602 could be having a similar effects, or (2) the compound could be causing an increased storage of glycogen in the liver but further analysis is required to determine the actual cause. An increase in fatty acid storage in the liver would be unlikely given that the whole body composition Echo MRI results indicate that the total body fat (regardless of the area that the fat is stored) compared to the DIO control group was reduced in the DIO O-1602 treated group. Therefore further biochemical analysis of the liver would be required to confirm the actual cause of the enlarged liver. AST and ALT are both markers of hepatocyte integrity (Giannini, Testa et al. 2005), while AST also is found in other tissues including the skeletal muscle, kidney, brain and red blood cells, it is highly concentrated in the hepatocytes, while ALT is specifically found in hepatocytes (Giannini, Testa et al. 2005). Albumin is also a marker of liver function, apart from dietary intake influencing albumin concentrations the liver is involved in albumin synthesis (Giannini, Testa et al. 2005). Given that the fibrotic marker hydroxyproline in both the liver and heart tissue was not affected in the DIO O-1602 treated rats, further investigation into the causes of this observations occurring in the liver tissue is required to gain understanding into the abnormalities that were observed as a result of the O-1602 treatment.

This study is the first to investigate the effect that O-1602 has on a number of hormones which are known to be altered in obesity and T2DM. The results from this study showed that two hormones involved in regulating food intake including leptin and ghrelin were both decreased as a result of

treatment with the compound O-1602. Other hormones, namely, GLP-1, glucagon and adiponectin were not altered with this treatment. Both leptin and ghrelin have opposing role in relation to food intake and appetite control and therefore the impact in obesity, while leptin induces satiety, ghrelin increases appetite. The obese state is also accompanied by a dysregulation in leptin signalling in which hyperleptinameia (Maffei, Halaas et al. 1995) and leptin resistance (Enriori, Evans et al. 2007) is observed. Both leptin and ghrelin concentrations can be influenced by food intake (Friedman and Halaas 1998; Kojima, Hosoda et al. 1999), as the release of ghrelin is produced during the fasted state and inhibited during the fed state (Kojima, Hosoda et al. 1999), whereas leptin secretion is promoted during food intake and the fed state and is decreased during starvation (Friedman and Halaas 1998). Plasma leptin concentration is reflective of total adipose tissue stores (Schwartz, Peskind et al. 1996) and as the rats from this study had a reduction in epididymal fat pad mass as well as reduced total body fat percentage this could help explain the results observed for the decreased circulating leptin concentrations. Given that the food intake was not altered towards the end of the treatment period and the time of death from the rats was during the light cycle (which is towards the end of their sleeping period) these factors could have contributed to the reduced circulating ghrelin concentrations that were observed in the DIO O-1602 treated group.

This study is also the first to look at the role that O-1602 has in the obese state for a number of different circulating cytokines and chemokines. Given the anti-inflammatory role that O-1602 has been shown to have in a number of other pathophysiological conditions (Handschin, Choi et al. 2007; Kargl, Haybaeck et al. 2013; Li, Feng et al. 2013), it was hypothesised that this compound may have an anti-inflammatory effects. However, the majority of the cytokines and chemokines were not altered following the treatment period despite reductions in body fat (%) and epididymal fat depots (epididymal fat weight/ body weight (%)). O-1602 treatment in contrast however, did in fact increase the chemokine RANTES (CCL5). RANTES has been shown to be increased in obesity in humans (Baturcam, Abubaker et al. 2014) and is also involved in the development of atherosclerosis in mice

(Braunersreuther, Zernecke et al. 2007), as well as being involved in glucose tolerance impairment and the development of T2DM in humans (Herder, Peltonen et al. 2006). Furthermore, RANTES has also been shown to promote the development of hepatic fibrosis in mice (Seki, De Minicis et al. 2009), while antagonising its receptor in the liver was shown to improve the experimentally induced fibrosis (Berres, Koenen et al. 2010). Although the hydroxyproline results did not indicate fibrosis in these animals, it could indicate the early stages changes of fibrosis before the actual process of fibrosis has occurred.

The results from this study highlight that chronic administration of O-1602 plays a role in energy metabolism. However, this compound appears to have negative consequences in relation to liver including both its morphology as well as elevated plasma AST concentrations. This compound also increased RANTES a chemokine which is increased in obesity and involved in the development of a number of associated co-morbidities including; arthrosclerosis, impairing glucose tolerance, T2DM as well as promoting fibrosis in the liver. While O-1602 appears to have positive effects on body composition with a reduction in body fat accompanied by increased lean tissue mass the side effects that this compound has on the liver including increased biochemical plasma analytes AST and RANTES appear to out-weight the benefits seen with the body composition in this experimental model. Further investigation into understanding the mechanisms involved from the observational and biochemical results regarding the liver function parameters is therefore required.

CHAPTER FIVE:

5.0 The effect that O-1918 has on whole body energy homeostasis in a diet induced obesity model

5.1 Abstract

Background: O-1918 is a putative antagonist for GPR55 and is an antagonist/ biased agonist depending on the experimental conditions used for GPR18. I have previously demonstrated that O-1918 up-regulates mRNA expression of markers involved in oxidative capacity and fatty acid metabolism with a trend for a positive mediator of adiponectin signalling to be up-regulated in C_2C_{12} myotubes (*Simcocks et al. Unpublished Observations, see Appendix Two*), however the role that this compound has in whole body energy homeostasis as well as obesity is currently unknown. Therefore the aim of this study was to determine the effect that chronic administration of O-1918 has on whole body energy homestasis in a DIO model focusing on the compound's effect on weight, food intake, body composition, blood glucose control, plasma hormone and cytokine concentrations as well as liver function.

Materials & Methods: DIO rats were maintained on a 21% (weight) HFD for nine weeks, the rats were then administrated O-1918 (1 mg/ kg body weight) via ip. injection for an additional six weeks. Measurements obtained include body weight (monitored daily), food consumption (monitored daily), body composition (obtained at baseline, week three and week six of treatment), ip. GTT and ip. IST (obtained at baseline and week five of treatment). Blood was collected at time of death via cardiac puncture and adipose and liver tissue were collected and immediately weighed post mortem and snap frozen for subsequent analysis. Circulating hormones and cytokines and liver function markers were determined using commercially available products.

Results, Discussion & Conclusion: In DIO rats, O-1918 treatment did not alter food intake, body weight, body composition or white adipose tissue weight. However the brown adipose tissue (BAT) weight (brown fat/ body weight %) was decreased compared to the DIO control group. As would be expected with no change in weight or body composition, whole body insulin sensitivity and glucose tolerance was not altered by the compound following the treatment for either ip. GTT or ip. IST. Interestingly, O-1918 treatment reduced both plasma leptin and ghrelin which have opposing effects on appetite control and may help to justify why there was no change in food intake in the rats treated with this compound. A number of pro-inflammatory cytokines some which are linked with the development of insulin resistance and T2DM were increased with treatment of O-1918, these cytokines included IL-1α, IL-2, IL-17α, IL18 and RANTES. While EPO had a trend to be increased (p 0.056) and a number of other hormones and cytokines were not altered with treatment of O-1918 which include: GRO/ KC, IL-1β, MCP-1, IL-4, IL-5, IL-6, IL-10, MC-SF, MIP-3α, TNFα, VEGF, IL-12p70 and IL-13. Some analytes were excluded from analysis due to a number of samples being out of range, these included: PAI-1, G-CSF, GM-CSF, IFN-γ and IL-7. The appearance of the livers obtained from the rats treated with O-1918 were similar to the DIO control group in appearance, however, liver function markers plasma AST was elevated in the DIO rats treated with O-1918, while not altering plasma albumin or ALT. Taken together these findings suggest that O-1918 is not an effective anti-obesity therapeutic in the DIO model used for this study. The changes observed require further investigation into the tissue specific effects, especially considering that some of the pro-inflammatory cytokines are involved in the development of insulin resistance and T2DM.

5.2 Background

O-1918 (1,3-dimethoxy-5-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene) is a synthetic derivative of plant constitute CBD (Offertaler, Mo et al. 2003). CBD is an antagonist for the putative cannabinoid receptors GPR55 and GPR18 (Ryberg, Larsson et al. 2007; Whyte, Ryberg et al. 2009), and has also been described as a partial agonist for GPR18 (McHugh, Page et al. 2012). CBD has also been shown to have an affinity to other receptors such as TRPV receptors (Gaetani, Fu et al. 2010). While O-1918 is a putative antagonist for GPR55 (Henstridge, Balenga et al. 2011; Kremshofer, Siwetz et al. 2015), this compound has been shown to act as either a biased agonist (Console-Bram, Brailoiu et al. 2014) or antagonist (McHugh, Hu et al. 2010) for GPR18 (McHugh, Hu et al. 2010; Console-Bram, Brailoiu et al. 2014).

Research investigating the role of CBD in energy homeostasis has shown that this compound has protective effects in diabetes models and complications resulting from diabetes (El-Remessy, Al-Shabrawey et al. 2006; Rajesh, Mukhopadhyay et al. 2007). Although, a recent pilot study in humans using 100 mg dose of CBD did not appear to have the desired effect in for glycaemic control in T2DM (Jadoon, Ratcliffe et al. 2016). When investigating the role that CBD has in food intake in animal models the results are conflicting, some studies have found a reduction in food intake with administration of CBD (Farrimond, Whalley et al. 2012) while others found a reduction in weight gain in young rats. Conversely, other research groups have found that CBD administered ip. (1.0 - 100 mg/ daily dose range) did not alter feeding behaviour in mice, or in fed or fasted rats (Wiley, Burston et al. 2005; Scopinho, Guimaraes et al. 2011). Interestingly, CBD has been shown to induce a browning phenotype in a cell culture model using 3T3-L1 adipocytes, as well as promoting lipolysis, thermogenesis and reducing lipogenesis in these cells (Parray and Yun 2016).

There currently is limited research investigating the efficacy of O-1918 in obesity, with previous studies characterising the compounds role in blood pressure regulation (Park, Scheffler et al. 2009), the vasculature (Johns, Behm et al. 2007) and intraocular pressure (Caldwell, Hu et al. 2013). The role

that O-1918 has in energy metabolism is unknown. I have previously treated C_2C_{12} myotubes with O-1918 for six hours, which resulted in an increase in mRNA expression of NFATc1, PGC1α and PDK4 (*Simcocks et al. Unpublished Observations*), while APPL1 also did not increase there was a trend for this the mRNA expression to be up-regulated, while APPL2 and AMPKα2 were also not altered.

Hypothesis

Given that O-1918 is structurally similar to CBD, and the potential role that CBD has in energy homeostasis, it may be hypothesised that the compound O-1918 is a pharmacological candidate for the treatment of obesity and associated comorbidities such as insulin resistance and T2DM. Additionally, C_2C_{12} myotubes treated with O-1918 up-regulate markers of oxidative capacity and fatty acid metabolism with a trend for a positive mediator of adiponectin signalling to be up-regulated (*Simcocks et al, Unpublished Observations*) which can be seen as beneficial for skeletal muscle energy homeostasis.It is therefore hypothesised that O-1918 would have a beneficial role in DIO and on whole body energy homeostasis. It is hypothesised that in DIO O-1918 will cause weight loss, decreased body fat percentage, decreased fat pad mass (epididymal and peri-renal), decreased circulating leptin, decreased circulating ghrelin, increased adiponectin, decreased pro-inflammatory cytokines and decreased anti-inflammatory cytokines

5.3 Materials & Methods

5.3.1 Animals & Housing

Twenty, male Sprague Dawley rats were purchased from ARC (Canning Vale, WA, Australia) at seven weeks of age and were acclimatised for at least seven days and then housed singly for the duration of this study. The rats were fed a HFD (21% fat diet by weight) (Cornall, Mathai et al. 2011; Jenkin, O'Keefe et al. 2016) purchased from Speciality Feeds (Glen Forrest, WA, Australia) for 15 weeks. The first nine weeks of HFD feeding was to induce obesity, then the rats were subsequently ranked based on metabolic characteristics and the allocated into groups at the end of week nine. For a further six weeks the DIO rats were chronically treated with either: (1) a vehicle of 0.75% Tween-80/ saline solution or (2) 1 mg/ kg O-1918 dissolved in 0.75% Tween-80/ saline.

The dosage of O-1918 was selected due to the compounds ability to block the hypotensive response of Abn-CBD in anesthetised mice (Offertaler, Mo et al. 2003). Animal ethics was obtained from the AEC at the Howard Florey Institute (Parkville, Melbourne, Australia) approval number 11-036. For further detail refer to sections 2.2, 2.3.1 and 2.3.2.

5.3.1.1 Food Intake and Body Weight

Food intake (grams) and body weight (grams) were measured and recorded daily and the weekly average was calculated and reported. For further detail refer to section 2.3.2.2.

5.3.1.2 Echo MRI

Whole body composition (body fat and lean tissue mass) was analysed utilising Echo MRI^{TM} system (Echo-MRI 900 Houston, United States of America) which is a previously validated method (Taicher, Tinsley et al. 2003). Measurements were obtained at week nine (baseline of DIO), week twelve (during treatment) and week fifteen (at the end of the treatment period). For further detail refer to section 2.3.2.3.

5.3.1.3 Ip. GTT

To determine the effect a glucose load had on blood glucose concentrations an ip. GTT was conducted using previously published methods (Jenkin, O'Keefe et al. 2016). This test was conducted at the beginning of week eight (baseline) and at the beginning of week 14 (at the end of the treatment period). For further detail refer to section 2.3.2.4.

5.3.1.4 Ip. IST

To determine the effect a bolus of insulin had on blood glucose concentrations an ip. IST was conducted using previously published methods (Jenkin, O'Keefe et al. 2016). This test was conducted at the beginning of week eight (baseline) and at the end of week 14 (at the end of the treatment period). For further detail refer to section 2.3.2.5.

5.3.1.5 Anaesthesia, Blood and Tissue Collection

At the end of the treatment period, rats were deeply anesthetised using isoflurane, then administered an ip. injection of 100 mg/ kg of sodium pentabarbitone and killed via cardiac puncture. Blood was transferred to EDTA BD coated vacutainer (Macfarlane Medical, Surry Hills, Victoria), then subsequently placed on ice. Blood tubes were centrifuged at 4°C at 4000 x g for 10 minutes to separate the plasma from the red blood cells. Plasma was transferred to Eppendorf tubes and stored at -80°C for future analysis. Whole adipose tissue depots and the liver were harvested and the weight (in grams) of the organs were recorded, following this portions of the tissue were transferred to cryotubes and snap frozen in liquid nitrogen for subsequent analysis. For further detail refer to section 2.3.2.6.

5.3.2 Biochemical Analysis

5.3.2.1 Adiponectin Assay

Total circulating concentrations of adiponectin were determined using Adiponectin ELISA Kit (AG-45A-0005TP-KI01) (Adipogen, Liestal, Switzerland). Plasma was analysed according to the manufacturer’s instructions. For further detail refer to section 2.5.2.

5.3.2.2 Bioplex Assay

Bioplex hormone immunoassay (171K1001M) and cytokine assay (YL0000006Q) were used to analyse circulating hormone and cytokine concentrations using the Bioplex 200 System (Bio-Rad Laboratories, Munich, Germany). Plasma was analysed according to the manufacturer's instructions. For further detail refer to section 2.5.1.

5.3.2.3 Liver Function Test

To determine the effects on plasma markers relating to liver function, University of Melbourne's Veterinary Hospital analysed plasma AST (AS3804), ALT (AL3875), and albumin (AB3800) (Randox Laboratories Ltd, Crumlin, County Antrem, United Kingdom) according to the manufacturer's instructions. For further detail refer to section 2.5.3.

5.3.3 Statistics Analysis

All statistical analysis was completed using Graph Pad Prism Software 7.0. DIO Control group was compared to DIO O-1918 group. Normality of data sets were analysed using Sharipo-Wilk normality test, when data was normally distributed a parametric unpaired two tailed t-test was utilised, whereas Mann-Whitney two tailed test was utilised for nonparametric data sets. When comparing multiple time points a 2-way ANOVA, with a Bonferroni Post Hoc test was utilised to determine significant difference between time points and the treatment type.

5.4 Results

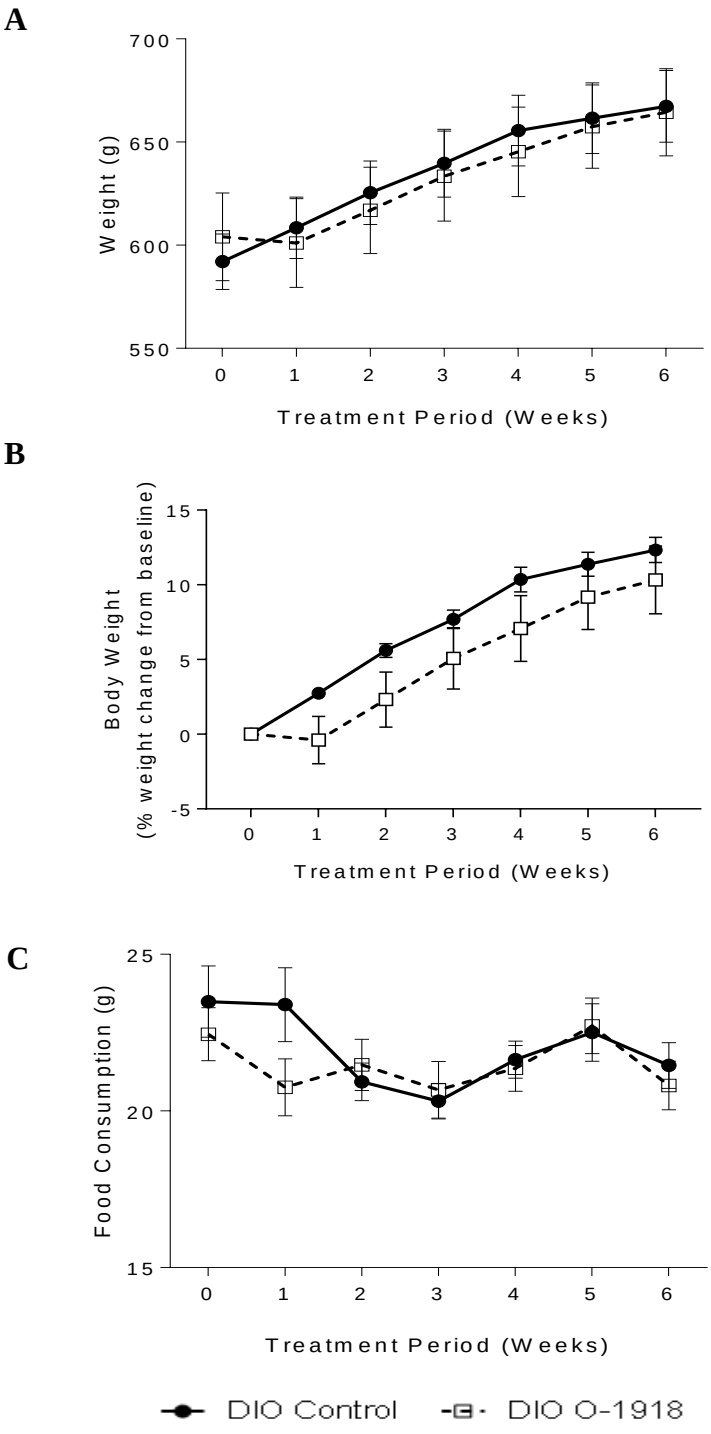


Figure 5.4A: Body Weight & Food Consumption

(A) Body weight (grams) and (B) Body Weight (% weight change from baseline) (C) Change in food consumption (grams) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.075% tween 80/ saline) or DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween/ saline) for a further six

weeks. Black circles: DIO control rats treated with saline (n = 11). White boxes DIO rats treated with O-1918 (n = 9).

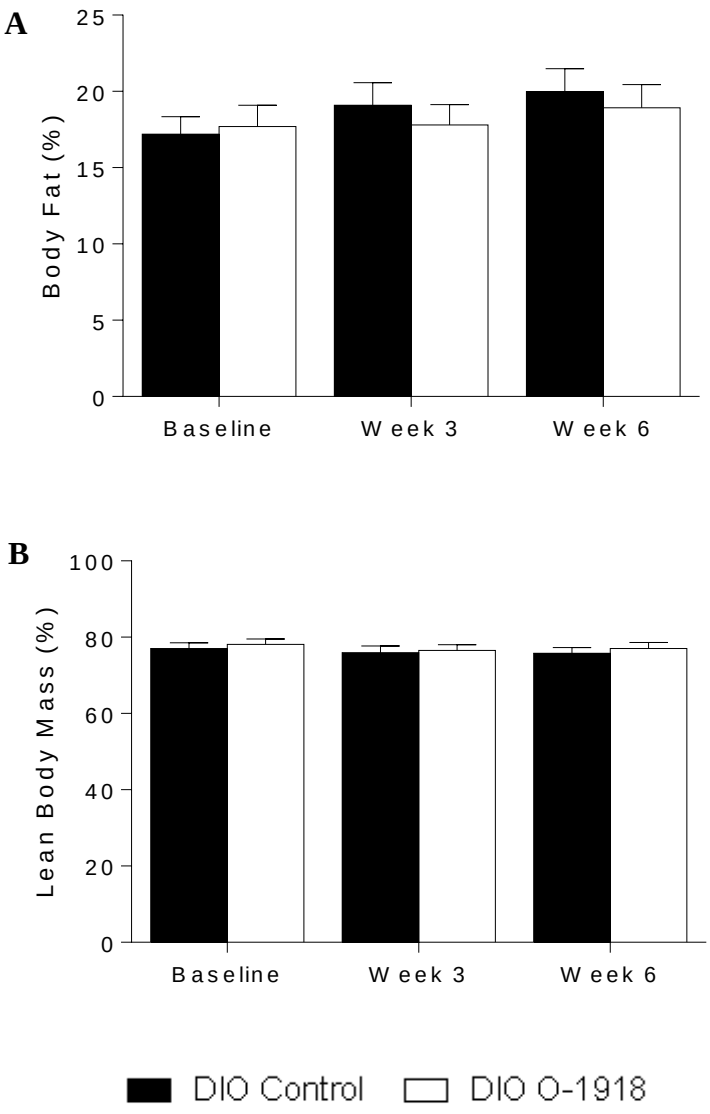


Figure 5.4B: Body Composition

(A) Body fat (%) and (B) Lean body mass (%) obtained from rats fed a HFD for nine weeks to induce obesity, the DIO Control rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars - DIO Control rats treated with saline (n= 11). Open bars - DIO rats treated with O-1918 (n = 9).

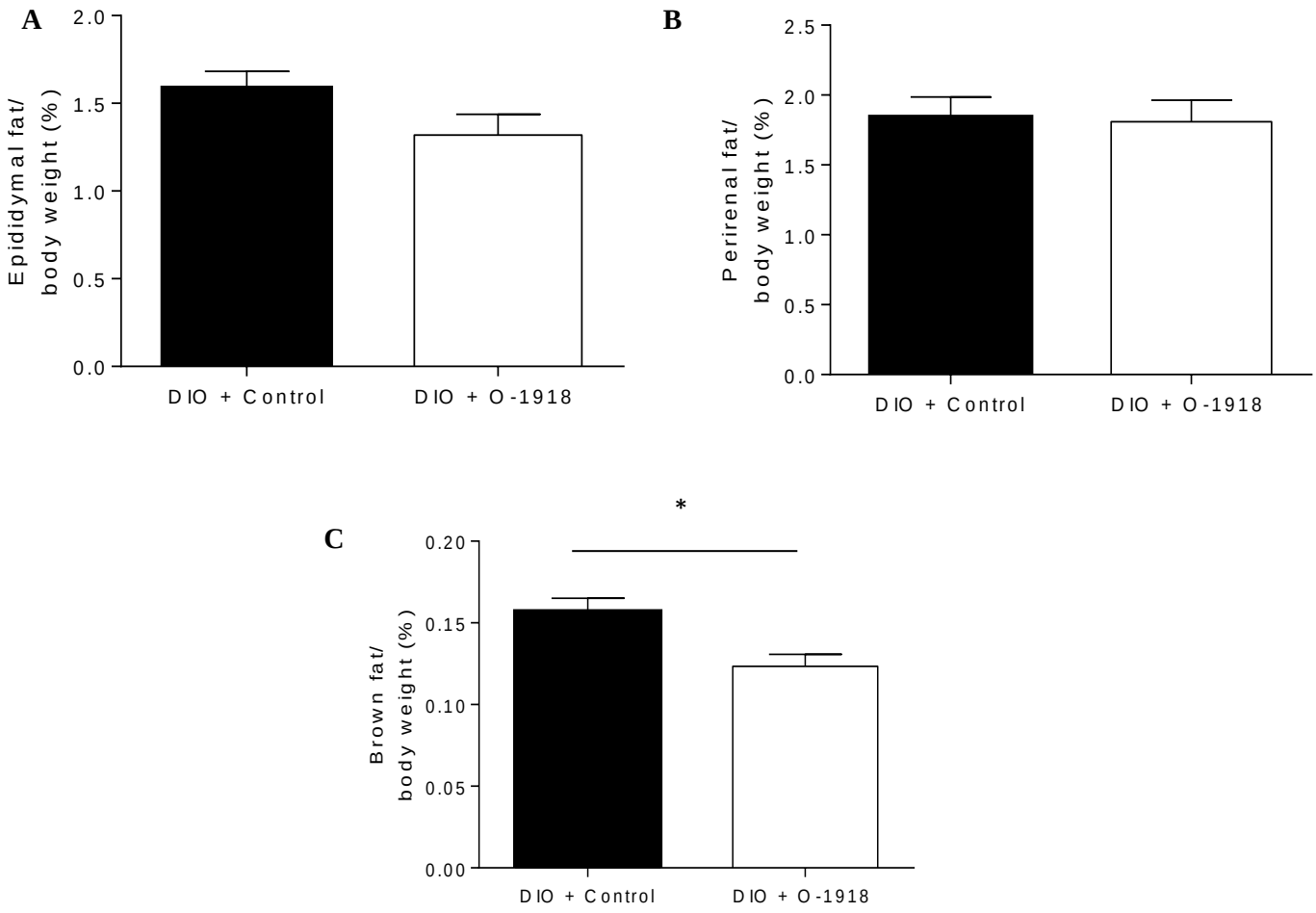


Figure 5.4C: Fat Pad Weights

(A) Epididymal fat weight/ body weight (%), (B) Peri-renal fat weight/ body weight (%) and (C) Brown fat weight/ body weight (%) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars - DIO control rats treated with saline (n = 11). Open bars - DIO rats treated with O-1918 (n = 9). * indicates significance ($p<0.05$).

Body Weight and Body Composition

The body weight (both (grams and % weight change from baseline)) and the body composition (including both fat and lean body mass percentage) of the DIO O-1918 treated rats were not significantly altered when compared to the DIO Control group (Figure 5.4A A & B, Figure 5.4B A & B).

DIO O-1918 treated rats had significantly reduced brown fat/ body weight (%) ($p < 0.05$) while WAT depots including epididymal and perirenal fat pads/ body weight % remained unchanged when compared to the DIO group (Figure 5.4C).

Food Intake

DIO O-1918 treated rats had no change in food intake over the six week pharmocological treatment period when compared with the DIO Control group (5.4A C).

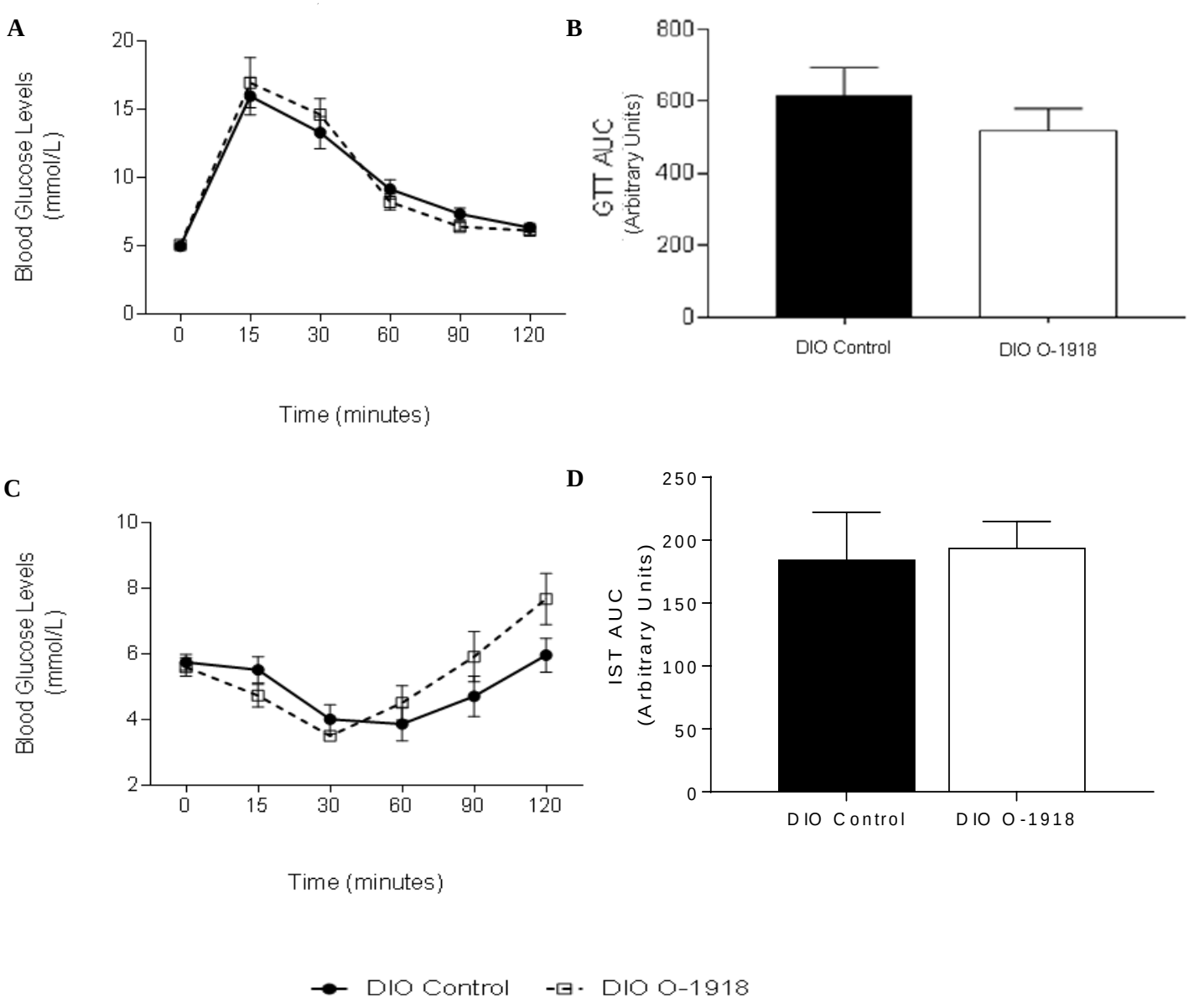


Figure 5.4D: Intraperitoneal Glucose Tolerance Test (ip. GTT) & Intraperitoneal Insulin Sensitivity Test (ip. IST)

(A) ip. GTT blood glucose concentrations (mmol/ L), (B) ip. GTT Area Under the Curve (AUC) (arbitrary units), (C) ip. IST blood glucose concentrations (mmol/L) and (D) ip. IST AUC (arbitrary units) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1918 rats were treated with the pharmacological compound O-1918 (1mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Black circles - DIO control rats treated with saline (n = 11). White boxes DIO rats treated with O-1918 (n = 9).Closed bars DIO control rats treated with saline (n = 11). Open bars DIO rats treated with O-1918 (n = 9).

Glucose Tolerance & Insulin Sensitivity

Whole body glucose handling and insulin sensitivity was assessed using both an ip. GTT and ip. IST, following five weeks of treatment with the pharmacological compound O-1918. Both the DIO O-1918 treated rat's blood glucose concentrations (mmol/ L) as well as AUC (arbitrary units) for both ip. GTT and ip. IST were not significantly different when compared to the DIO Control group (Figure 5.4D A-D).

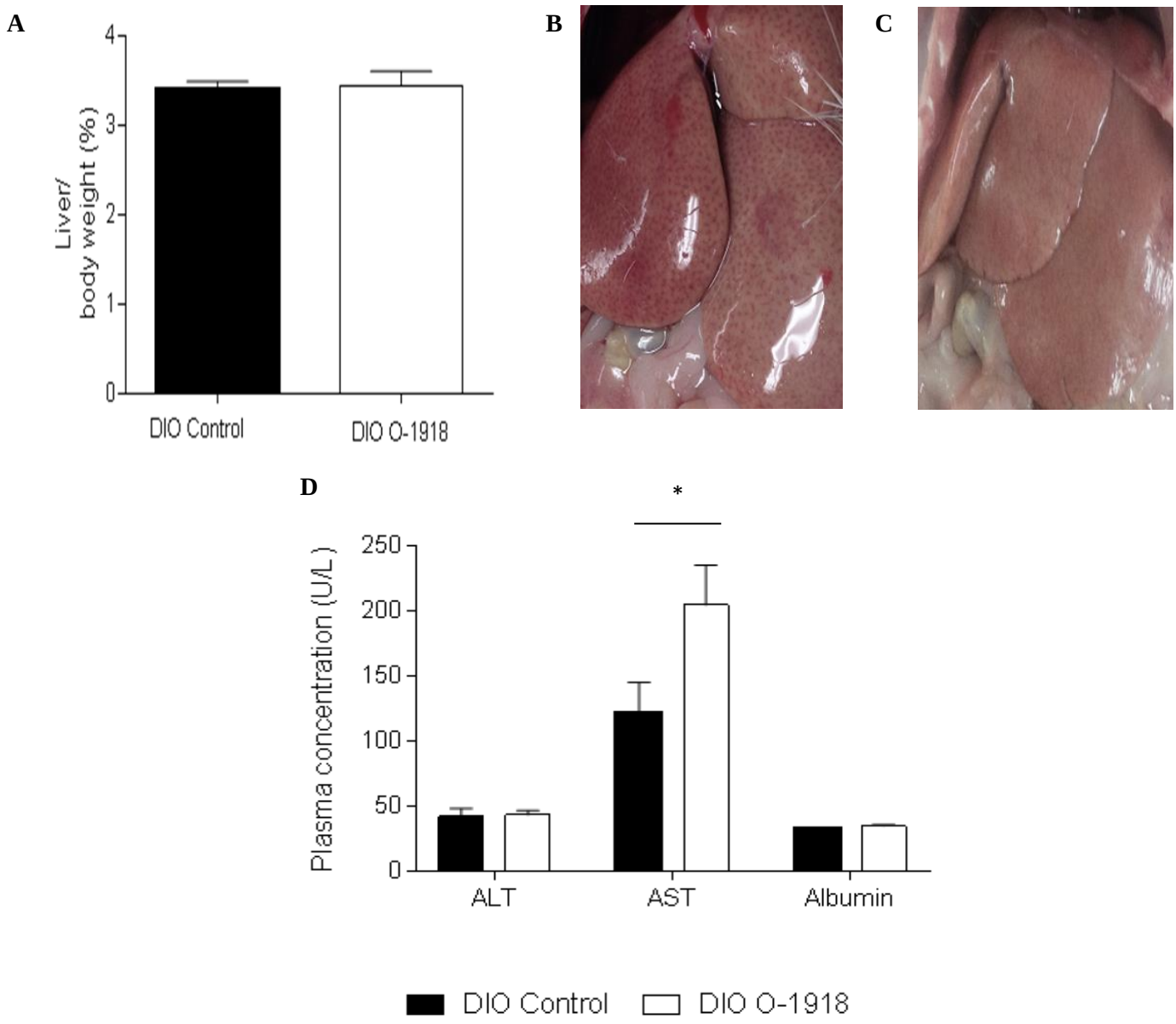


Figure 5.4E: Liver Function Parameters

(A) Liver weight/ body weight (%), (B) Representative photo of whole liver obtained from the DIO Control group (n = 1), (C) Representative photo of whole liver obtained from the DIO O-1918 group (n = 1) and (D) Plasma liver function test obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars - DIO control rats treated with saline (Figure A n = 11, Figure D n =8). Open bars DIO rats treated with O-1918 (n =9). * indicates significance ($p < 0.05$).

Liver Function Parameters

DIO O-1918 treated rats had no significant difference in liver/ body weight % or visual appearance of the liver when compared to the DIO Control group (Figure 5.4E A - C). Circulating plasma concentrations of AST were increased ($p < 0.05$) while ALT and albumin were not altered (Figure 5.4E D).

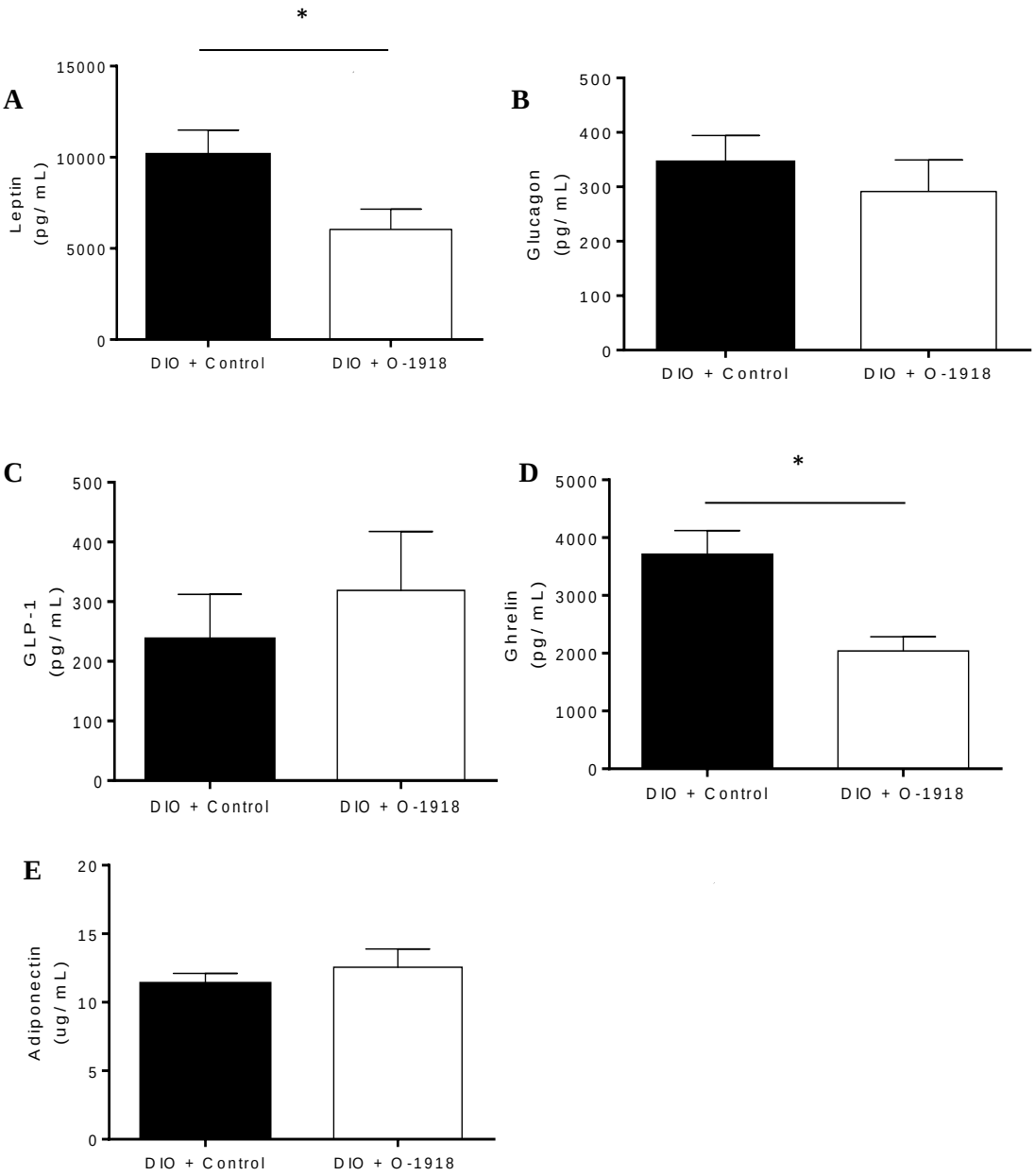


Figure 5.4F: Plasma Hormone Profiles

Plasma concentrations of hormone including; (A) Leptin; (B) Glucagon; (C) Glucagon Like Peptide-1 (GLP-1); (D) Ghrelin; (E) Adiponectin obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or the DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars - DIO Control rats treated with saline (n = 8 - 11). Open bars - DIO rats treated with O-1918 (n = 9). * indicates significance ($p < 0.05$).

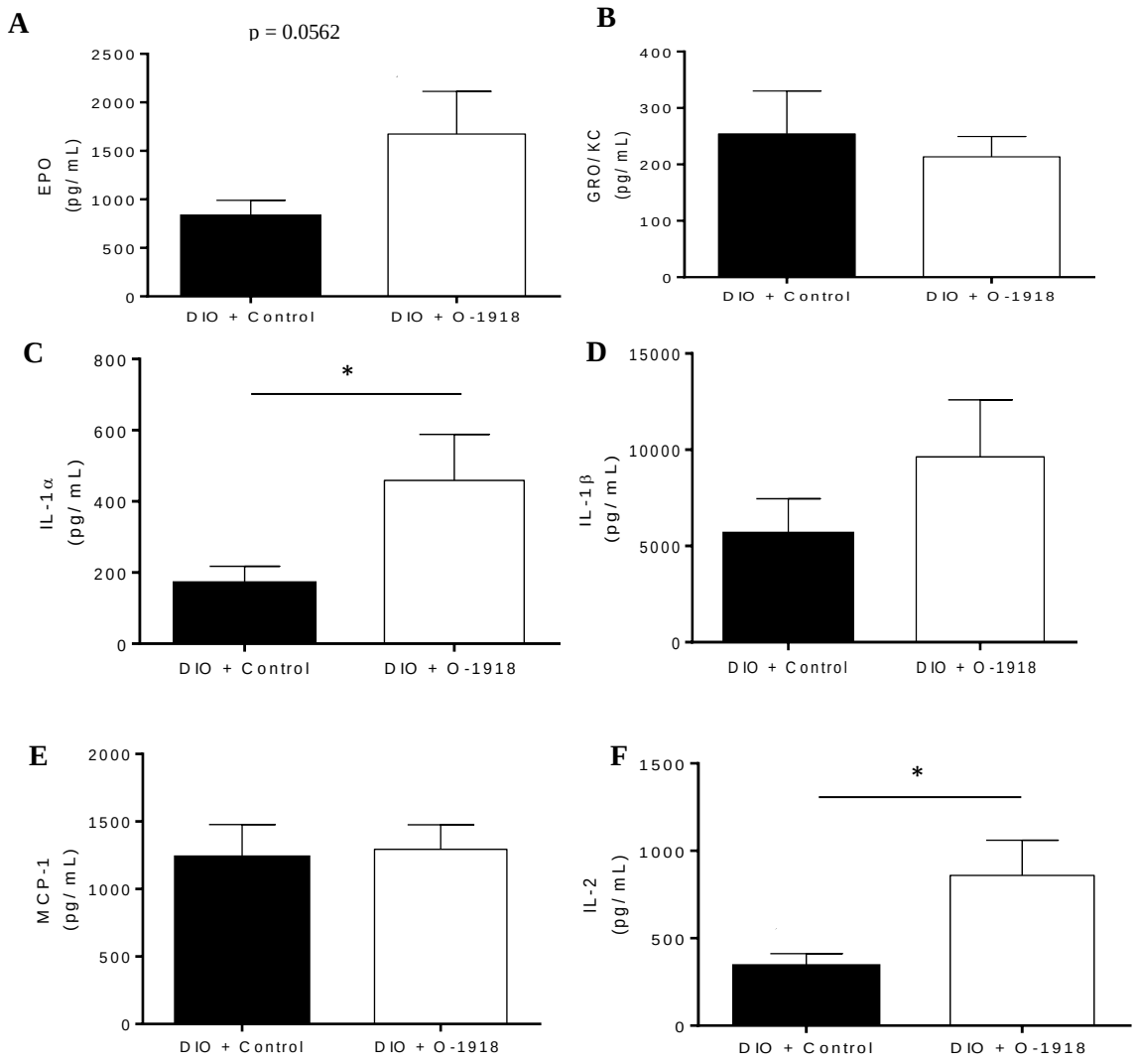


Figure 5.4G: Plasma Cytokine Profiles

Plasma Concentrations of cytokines including: (A) Erythropoietin (EPO); (B) Growth Regulated Oncogene/ Keratinocyte Chemo-attractant (GRO/KC); (C) Interleukin-1α (IL-1α); (D) Interleukin-1β (IL 1β); (E) Monocyte Chemo-attractant Protein 1 (MCP-1); (F) Interleukin-2 (IL-2); obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) or the DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars DIO control rats treated with saline (n = 9 – 11). Open bars DIO rats treated with O-1918 (n = 9). * indicates significance ($p < 0.05$).

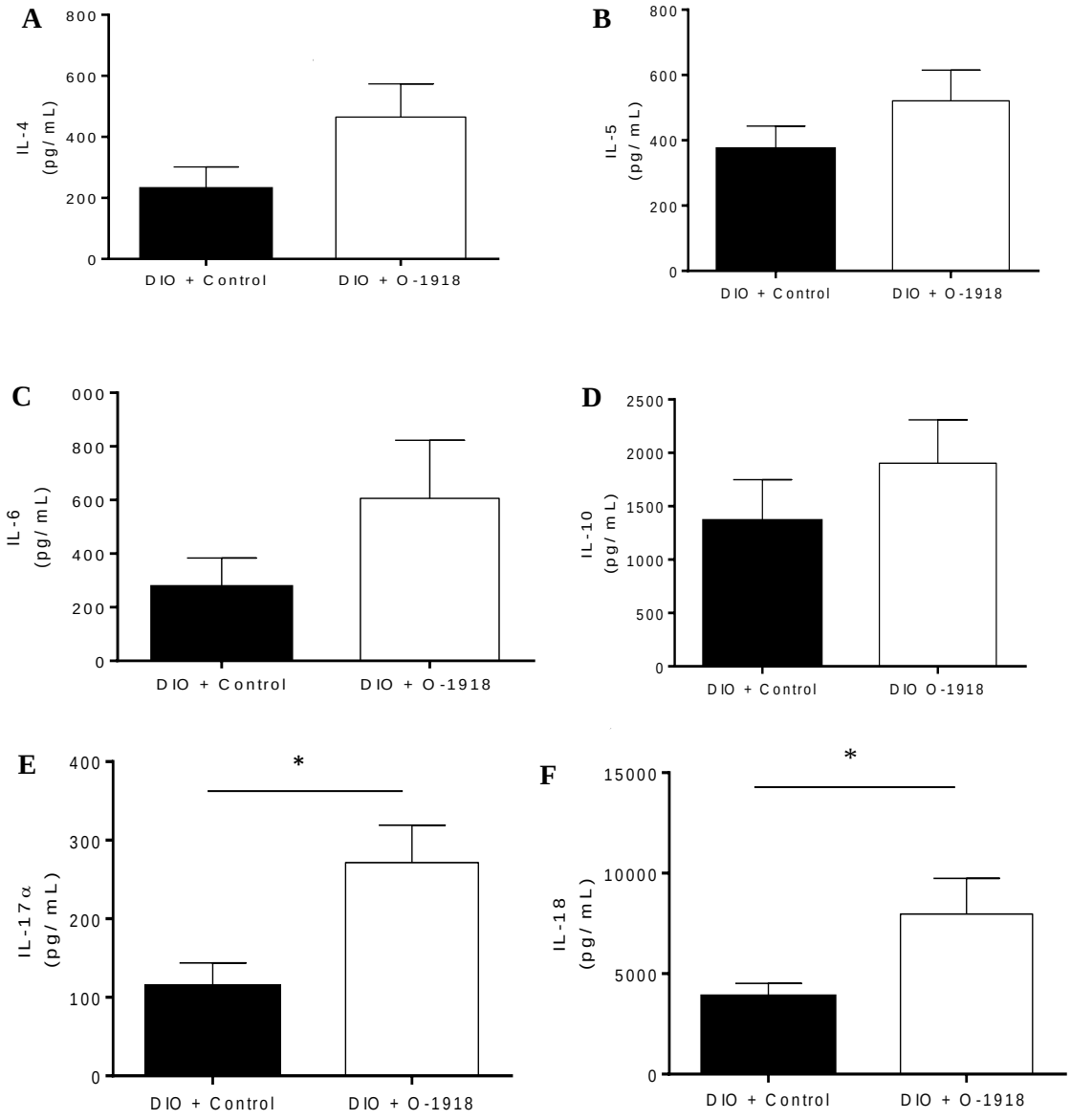


Figure 5.4H: Plasma Cytokine Profiles

Plasma Concentrations of cytokines including: (A) Interleukin 4 (IL-4); (B) Interleukin 5 (IL-5); (C) Interleukin 6 (IL-6); (D) Interleukin 10 (IL-10) (E) Interleukin 17α (IL-17α); (F) Interleukin 18 (IL-18) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) and the DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars DIO control rats treated with saline (n = 9 – 11). Open bars DIO rats treated with O-1918 (n = 9). * indicates significance ($p < 0.05$).

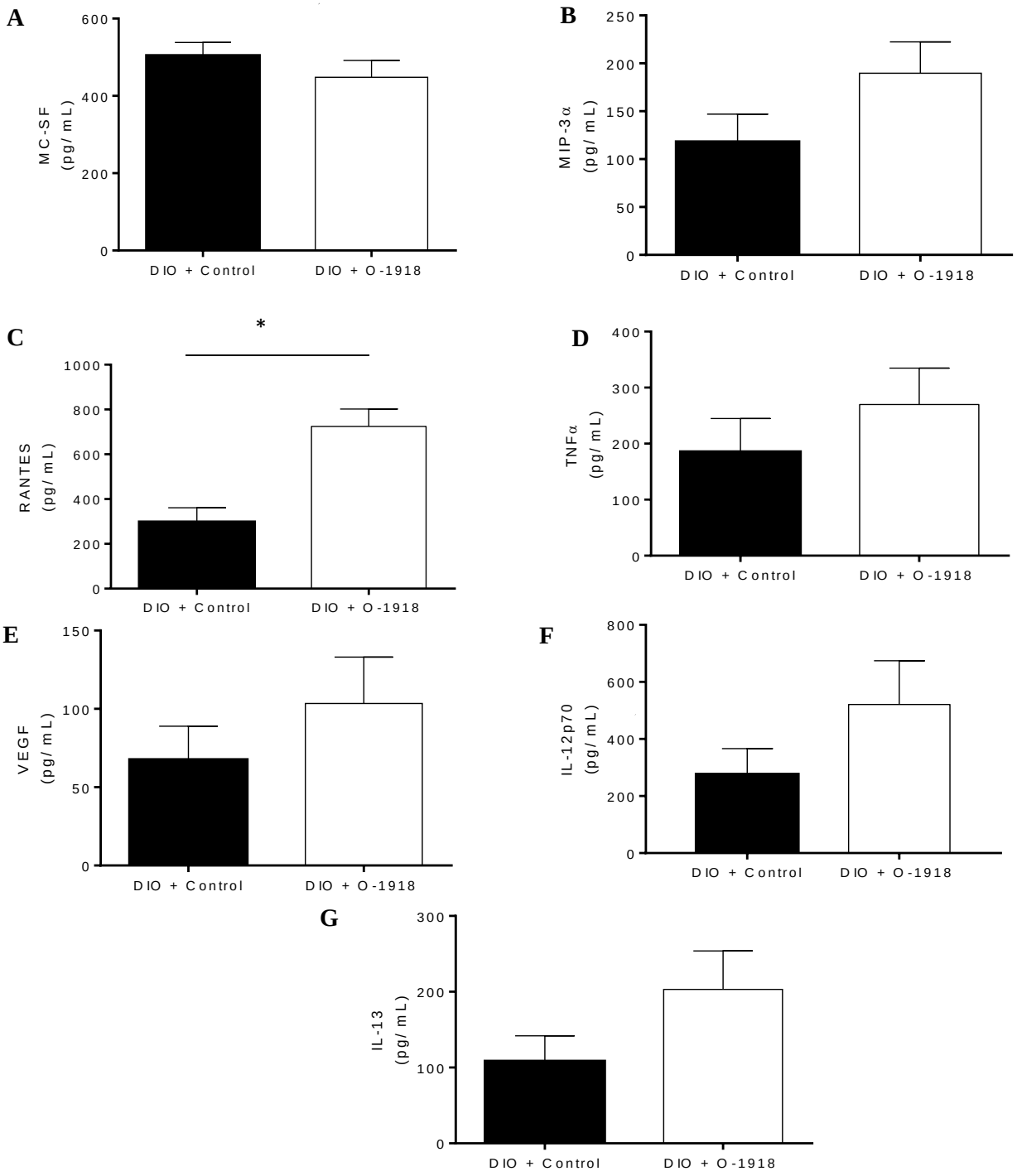


Figure 5.4I: Plasma Cytokine Profiles

Plasma concentration of cytokines including: (A) Monocyte Colony Stimulating Factor (MC-SF); (B) Macrophage Inflammatory Protein 3α (MIP-3α); (C) Regulated on Activation Normal T Cells Expressed and Secreted (RANTES); (D) Tumour Necrosis Factor α (TNFα); (E) Vascular Endothelial Growth Factor (VEGF); (F) Interleukin12p70 (IL-12p70); (G) Interleukin 13 (IL-13) obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats were treated with a vehicle (0.75% tween 80/ saline) and the DIO O-1918 rats were treated with the pharmacological compound O-1918 (1 mg/ kg O-1918/ 0.75% tween 80/ saline) for a further six weeks. Closed bars DIO control rats treated with saline (n = 9 - 11).

Open bars DIO rats treated with O-1602 (n= 8 – 9). * indicates significance ($p < 0.05$).

Plasma Hormone & Cytokines

The plasma hormone profile shows that DIO O-1918 rats had reduced leptin and ghrelin ($p < 0.05$) (Figure 5.4F) when compared to the DIO Control group, while other hormones including glucagon, GLP-1 and adiponectin plasma concentrations were not altered (Figure 5.4F A - E).

The plasma cytokine profile shows that DIO O-1918 treated rats had increased plasma pro-inflammatory cytokines when compared to the DIO Control group, these analytes included: IL-1α, IL-2, IL-17α, IL-18 and RANTES ($p < 0.05$), there was also a non-significant trend for EPO to be increased ($p = 0.0562$). A number of other cytokine plasma concentrations were also not significantly altered namely: GRO/ KC, IL-1β, MCP-1, IL-4, IL-5, IL-6, IL-10, MC-SF, MIP-3α, TNFα, VEGF, IL-12p70 and IL-13 (Figure 5.4G, 5.4H 5.4I).

A number of samples from either the DIO Control and/ or the DIO O-1918 group were out of range for some of the analytes tested from the hormone and cytokine plates and statistical analysis was unable to be conducted as a result. The analytes excluded from the statistical analysis include: Plasminogen Activated Inhibitor-1 (PAI-1), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) Interferon-gamma (IFN-γ) and Interleukin 7 (IL-7).

5.5 Discussion

This study is the first to look at the CBD analogue O-1918 and the effect that chronically administering this compound has on whole body energy homeostasis in a DIO rat model. Key findings from this study show that chronic treatment with O-1918 (1 mg/ kg) in DIO rats did not alter food intake, body weight or WAT weight when compared to the DIO control group. While BAT weight was reduced there were no alterations in glucose tolerance or insulin sensitivity. Circulating plasma leptin and ghrelin concentrations were reduced and pro-inflammatory cytokines were increased namely, IL-1α, IL-2, IL-17α, IL-18 and RANTES in the DIO O-1918 treated group, circulating plasma AST was also elevated, while albumin and ALT were not altered.

The DIO O-1918 treated rats had reduced BAT depots when compared to the DIO Control rats. BAT is an organ that is involved in thermogenesis and partly involved in energy expenditure regulation (Cannon and Nedergaard 2004). Additionally this organ is also involved in glucose disposal and clearance of triglycerides (Potter and Heller 2010). At a molecular level this type of adipose tissue differs from WAT, for example BAT contains a large amount of mitochondria and expresses UCP-1 (Cannon and Nedergaard 2010), which is involved in oxidative metabolism and ATP generation. Active BAT is beneficial to obesity (Townsend and Tseng 2012) and therefore associated co-morbidities such as insulin resistance and T2DM. In DIO rodents, blocking CB_1 results in increased temperature of BAT and the up-regulation of UCP-1 (Bajzer, Olivieri et al. 2011). While in the current study we measured BAT depots and did not investigate the change in function (if any) with the treatment of O-1918 in this tissue, the increase in pro-inflammatory cytokines some of which are involved in the development of insulin resistance, accompanied by the decrease in BAT could suggest these findings are inter-related. Pro-inflammatory cytokines lead to dysfunction and apoptosis in brown adipocytes, using a murine cell culture model, in which the effect was suggested to be mediated through a suppression of UCP-1 and β-Klotho (Rebiger, Lenzen et al. 2016). The pro-inflammatory cytokines used in the study by Rebiger et al. (2016) include IL-1β, TNFα and IFN-γ, which is in

contrast to the results in this thesis which demonstrate that IL-β and TNFα were not altered and IFN-γ was excluded due to multiple samples being out of range. This study is also the first to look at the relationship between O-1918, obesity and the effects on circulating anti-inflammatory and pro-inflammatory cytokines. While O-1918 in the DIO state increased pro-inflammatory cytokines: IL-1α, IL-2, IL-17α, IL-18 and RANTES, it may be feasible to hypothesis that this increase in pro-inflammatory cytokines may have contributed to the reduction in BAT mass as a result of apoptosis, however, further investigation into the cause and effect is required to fully understand the link between increased circulating pro-inflammatory cytokines and reduction in BAT mass.

O-1918 did not alter ip. GTT and ip. IST, however, the rats in this thesis were not glucose intolerant or insulin resistant as a result of the HFD diet used (data not shown). Therefore the effect that O-1918 has on impaired glucose homeostasis could not be assessed in this model. This is in line with previous research that has found that nine weeks of high fat feeding (25% fat, derived from soybean oil) does not induce glucose intolerance or insulin insensitivity (Stark, Timar et al. 2000), this could be due to their genetic variability to gain weight and store fat as a result of HFD. Therefore this data suggests that O-1918 does not alter glucose tolerance and sensitivity in obesity. Despite the DIO O-1918 treated rats not having insulin resistance or glucose intolerance, circulating cytokines IL-1α, IL-2, IL-17α, IL-18 and RANTES were increased in this model. Moreover, GPR55 knockout mice have increased adiposity and insulin resistance (Meadows, Lee et al. 2016) and given that O-1918 is a putative antagonist for GPR55 at a molecular level this compound could be inducing pro-inflammatory cytokines that are involved in the pathogenesis of insulin resistance and T2DM.

This study is the first to look at the effect that O-1918 has on a number of hormones, some of which are altered in obesity and T2DM. The results from this study show a reduction in both circulating leptin and ghrelin with the treatment of O-1918 in the DIO state. The other hormones tested which include: GLP-1, glucagon and adiponectin were not altered with treatment of O-1918. Leptin is a hormone that

promotes satiety, and obesity is a state of hyperleptinaemia, and is accompanied by leptin resistance (de Git and Adan 2015), as expected in our study the DIO Control rats had increased circulating concentrations of plasma leptin when compared to SCD fed rats (data not shown). Previous research has shown that there is a relationship established between cannabinoids and the hormone leptin (Buettner, Scholmerich et al. 2007). O-1918 is a cannabinoid derivative and is a putative antagonist for GPR55 (Henstridge, Balenga et al. 2011; Kremshofer, Siwetz et al. 2015), leptin is involved in GPR55 regulation, as in leptin deficient *ob/ ob* mice GPR55 gene expression in WAT is decreased (Moreno-Navarrete, Catalan et al. 2012). Further a 48 hour starvation period in rats increased mRNA expression of GPR55 and leptin administration in starved rats reverts the GPR55 mRNA expression back to the same concentrations as rats with *ad libitum* access to a SCD (Imbernon, Whyte et al. 2014). Therefore as there is an interaction between leptin and GPR55, it is not surprising that treatment with O-1918 altered plasma concentrations of this hormone. In further support, BAT has also been shown to produce leptin (Tsuruo, Sato et al. 1996; Margetic, Gazzola et al. 2002) and given that our data shows that O-1918 reduced BAT mass this could account for the reduction in leptin production.

The effect that O-1602 (discussed in *Chapter four*) or O-1918 have *in vivo* in DIO rats have a number of similarities and differences, O-1602 caused a reduction in body fat percentage, and an enlarged liver and abnormal appearing liver, while O-1918 did not alter weight or body fat percentage it did cause a reduction in brown fat pad mass. Both O-1602 or O-1918 caused an increase in circulating RANTES and AST and decreased circulating leptin and ghrelin concentrations, as these atypical compounds are not selective they could be having off targeted effects on either or both GPR55 and GPR18. Further investigation into the targeted effects that is occurring with treatment of these compounds is therefore required.

Taken together the findings from this study suggest that O-1918 is not an effective anti-obesity therapeutic in the obesity model used. The changes observed including the BAT weight reduction as well as the increase in circulating pro-inflammatory cytokines and AST require further investigation

into the tissue specific effects. Especially considering that some of the pro-inflammatory cytokines are involved in the development of insulin resistance and T2DM and that BAT is involved in the regulation of energy homeostasis.

CHAPTER SIX:

6.0 The effect that administration of either O-1602 or O-1918 have on skeletal muscle homeostasis in a DIO model.

6.1 Abstract

Background: O-1602 and O-1918 are both atypical cannabinoid compounds that have an affinity for the putative cannabinoid receptors GPR55 and GPR18. In the literature O-1602 has a role in energy homeostasis while it is unclear whether O-1918 has an effect. *Chapters Four and Five* of this thesis indicates that either O-1602 or O-1918 have varying effects on energy homeostasis in a DIO rat model. As skeletal muscle is a regulator of whole body energy expenditure it is therefore a suitable target to modulate in obese and T2DM states. The skeletal muscle is a heterogeneous organ that is composed of different fibre phenotypes that have varying roles in nutrient utilisation. Currently the role that either O-1602 or O-1918 have on skeletal muscle homeostasis in obesity is unknown. Therefore the aim of this study was to investigate the effects that either O-1602 or O-1918 have on mRNA expression of markers involved in skeletal muscle metabolism and the putative cannabinoid receptors GPR18 and GPR55 in the red gastrocnemius skeletal muscle or white gastrocnemius skeletal muscle in a DIO rat model.

Material & Methods: DIO rats maintained on 21% HFD by weight for nine weeks, were then administered either O-1602 or O-1918 for a further six weeks via ip. injection. C*hapters Four and Five* provide detailed characteristics of the DIO rats following treatment with either compound. The rats were deeply anaesthetised using isoflurane, red and white gastrocnemius skeletal muscle were surgically collected and immediately snap frozen in liquid nitrogen. Following this the rats were immediately killed via administration of sodium pentabarbitone and cardiac puncture. Further, RNA was extracted from both muscle types and analysed using 'Real Time' PCR.

Results, Discussion & Conclusion: GPR18 mRNA was confirmed to be expressed in DIO rat skeletal muscle. GPR18 mRNA expression was increased in abundance in the red gastrocnemius skeletal muscle when compared to the white gastrocnemius skeletal muscle in the control group, the O-1602 group and the O-1918 group. The fact that GPR18 is expressed in both red and white gastrocnemius skeletal muscle suggests that this receptor may have a role in skeletal muscle homeostasis. Treatment with either O-1602 or O-1918 however, did not significantly alter mRNA expression of GPR18 compared to the control group for either muscle type. O-1602 did not alter the mRNA expression of markers involved in the regulation of adiponectin signalling, oxidative capacity and fatty acid oxidation in the red or white gastrocnemius skeletal muscle in DIO. O-1918 down regulated the mRNA expression of AdipoR1 in the red gastrocnemius skeletal muscle of DIO rats, while in the white gastrocnemius skeletal muscle there was an up regulation of APPL2 mRNA expression. Other markers involved in the regulation of adiponectin signalling, oxidative capacity and fatty acid oxidation were not altered in either tissue type in DIO with the treatment of O-1918. The effects observed from the treatment with chronic administration of O-1602 or O-1918 in this DIO model indicates that O-1602 does not appear to have an effect in oxidative capacity, fatty acid oxidation or adiponectin signalling pathways in the skeletal muscle in obesity. O-1918 may however regulate mRNA expression of proteins involved in adiponectin signalling, in a skeletal muscle fibre type-specific fashion. These changes may be related to the reduced circulating plasma leptin concentration apparent in this DIO model following treatment, however, further investigation into the O-1918 treated skeletal muscle tissue is required.

6.2 Background

With the rise in obesity rates world-wide and associated co-morbidities such as T2DM (WHO 2016), targeting these conditions pharmaceutically may be required in combination with a healthy diet and increased physical activity to help reduce the health related costs and burden to the individual, as well as the community. The synthetic cannabinoid derivatives O-1602 and O-1918 have been shown to have an affinity or hypothesised to have an affinity to the putative cannabinoid receptors GPR55 and GPR18 (Ryberg, Larsson et al. 2007; McHugh, Hu et al. 2010; Henstridge, Balenga et al. 2011; Console-Bram, Brailoiu et al. 2014; Kremshofer, Siwetz et al. 2015). O-1602 is reported to be an agonist for GPR55 (Ryberg, Larsson et al. 2007) and a biased agonist for GPR18 (Console-Bram, Brailoiu et al. 2014), whereas O-1918 is a putative antagonist for GPR55 (Henstridge, Balenga et al. 2011; Kremshofer, Siwetz et al. 2015) and either a biased agonist or antagonist for GPR18 (Console-Bram, Brailoiu et al. 2014; Kremshofer, Siwetz et al. 2015). These compounds may have a role in regulating energy homeostasis, as O-1602 has been shown to induce adiposity (Diaz-Arteaga, Vazquez et al. 2012) and O-1918 up-regulates mRNA expression of markers that regulate oxidative capacity, fatty acid oxidation and a tendency to up-regulate the positive mediator of adiponectin signalling APPL1 (*Simcocks et al, Unpublished Observations*).

The skeletal muscle regulates whole body energy expenditure (Zurlo, Larson et al. 1990), therefore targeting this organ pharmaceutically for the treatment of obesity and T2DM may be of benefit (Carey and Kingwell 2009). Skeletal muscle is a heterogeneous organ that is composed of different fibre types (Saltin, Henriksson et al. 1977). Oxidative fibre types utilise more ATP than glycolytic fibre types and individuals that are obese or T2DM have a greater proportion of glycolytic fibre types when compared with lean individuals (Tanner, Barakat et al. 2002; Oberbach, Bossenz et al. 2006).

Currently in the literature the role that either O-1602 or O-1918 have on skeletal muscle homeostasis and in obesity is unknown. Therefore the aim of this study was to determine the role that the two atypical cannabinoid compounds either O-1602 or O-1918 have on mRNA expression of markers

involved in skeletal muscle metabolism in the red gastrocnemius skeletal muscle and white gastrocnemius skeletal muscle in a DIO rat model. The markers selected in this study were involved in adiponectin signalling, oxidative capacity, insulin signalling, and fatty acid transport. Changes in expression of these markers may be beneficial in obesity and associated co morbidities such as T2DM.

Hypothesis

It is hypothesised that GPR18 will be expressed in the skeletal muscle, given the effects described in *Chapters Four and Five* following treatment with either O-1602 or O-1918. In light of the findings observed in *Chapter Four*, in which the DIO rats treated with O-1602 had a decrease in body fat percentage and epididymal fat mass (epididymal fat (g)/ body weight (%)), it is also hypothesised that the markers of adiponectin signalling, oxidative capacity and fatty acid oxidation would be up regulated particularly in the red gastrocnemius skeletal muscle, which has a higher oxidative capacity. Prior to commencing this study it was hypothesised that O-1918 would be beneficial to obesity and associated co-morbidities such as T2DM, as the mRNA expression of NFATc1, PGC1α and PDK4 were up-regulated, with a tendency to up-regulate APPL1 in C_2C_{12} myotubes (*Simcocks et al. Unpublished Observations*). However, given the findings observed in *Chapter Five*, in which the DIO rats treated with O-1918 had no alteration in weight or body fat percentage but did have a decrease in BAT mass, leptin and ghrelin and an increase in pro-inflammatory cytokines IL-1α, IL-2, IL-17α, IL18 and RANTES, it would be hypothesised that O-1918 would have negligible effects or down regulate the mRNA expression of markers involved in oxidative capacity, adiponectin signalling and fatty acid oxidation but not enough to cause increased adiposity or body weight.

6.3 Material & Methods

6.3.1 Animals & Housing

Twenty six, seven week old male Sprague Dawley rats were purchased from ARC (Canning Vale, WA, Australia), and acclimatised for at least seven days. Rats were then singly housed for the duration of this study and fed a HFD (21% fat diet by weight) (Cornall, Mathai et al. 2011; Jenkin, O'Keefe et al. 2016) purchased from Speciality Feeds (Glen Forrest, WA, Australia) for a total period of 15 weeks. The first nine weeks of high fat feeding was to induce obesity, the rats were then allocated into three groups based on their metabolic characteristics for even distribution amongst groups. For a further six weeks the DIO rats were chronically treated with either: (1) DIO Control: 0.75% Tween-80/ saline solution, (2) 5 mg/ kg O-1602 dissolved in 0.75% Tween-80/ saline (3) 1 mg/ kg O-1918 0.75% Tween-80/ saline. Animal ethics was obtained from the AEC at the Howard Florey Institute (Parkville, Melbourne, Australia) approval number 11-036. For further detail refer to sections 2.2, 2.3.1 and 2.3.2.

The rats underwent a number of tests to determine metabolic parameters throughout the duration of the study which have previously been detailed in *Chapters Two, Four & Five*. Following the six week treatment period and upon the completion of the study the rats were deeply anaesthetised using isoflurane. During this time red and white gastrocnemius skeletal muscle was surgically removed and a portion of red and white gastrocnemius skeletal muscle was snap frozen in LN_2 for subsequent analysis (See below in Section 6.3.2 – 6.3.3). Immediately while the rats remained deeply anesthetised they were administered 100 mg/ kg sodium pentabarbitone and then immediately killed using cardiac puncture. For further detail refer to section 2.3.2.6.

6.3.2 Skeletal Muscle Analysis

~25 - 35 mg portions of either the red or white gastrocnemius skeletal muscle obtained from the rats were utilised to extract RNA according to previously published methods (Cavuoto, McAinch et al. 2007; Cornall, Mathai et al. 2013). Samples were treated using RQ1 RNAse-free DNAse kit (Promega Corporation, Madison, United States of America) according to manufacturer's instructions. Then 0.5

µg of RNA was reverse transcribed into cDNA using the iScript™ cDNA synthesis kit (BioRad Laboratories, Hercules, California, United States of America) according to manufacturer's instructions. cDNA was diluted 1: 5 for analysis of GPR18 mRNA expression and 1: 20 for analysis of all other genes measured and stored at – 20°C.

Oligonucleotide primers were developed for the genes selected using Oligoperfect Suite (Invitrogen, Victoria, Australia). The primers were purchased from Geneworks Pty Ltd (Adelaide, Australia). Then a BLAST search was conducted to confirm homologous binding for the target mRNA sequence. The forward and reverse oligonucleotide primer sequences for the genes of interest are detailed in Table 6.3A and a description of the markers role in skeletal muscle metabolism is highlighted in Table 6.3B.

To quantify mRNA expression in the red and white gastrocnemius skeletal muscle, 'Real Time' PCR was utilised using previously validated SYBR Green method (Ponchel, Toomes et al. 2003). In which SYBR™ Green (BioRad Laboratories, Hercules, California, United States of America) and the BioRad MY iQ® Real-Time PCR detection system (model # My iQ optics model, serial # 569BR/0611) were used. The samples were run for 40 – 50 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. Changes in mRNA expression was normalised to the average of housekeeping genes (Cyclophilin and βActin) and quantified using the previously validated $2^{-\Delta\Delta}$ct method (Livak and Schmittgen 2001).

Table 6.3A: Forward and Reverse Oligonucleotide Primer Sequences for 'Real Time' PCR

Primer	Accession Number	Direction	Sequence
Cyclophilin	NM_017101.1	Forward ('5 '3)	CTG ATG GCG AGC CCT TG
		Reverse ('5 '3)	TCT GCT GTC TTT GGA ACT TTG TC
βActin	NM_031144	Forward ('5 '3)	CTA AGG CCA ACC GTG AAA TGA
		Reverse ('5 '3)	CCA GAG GCA TAC AGG GAC AAC
GPR18	NM_001079710.1	Forward ('5 '3)	GTG GGG GTC TGG ATA ATG AC
		Reverse ('5 '3)	CGC GTG AAG TTA AGC ACA TT
GPR55	XM_006226918	Forward ('5 '3)	TCG CCA TCC AGT ACC CTC TTC
		Reverse ('5 '3)	ATG CAG CAG ATC CCA AAG GTT
AdipoR1	NM_207587.1	Forward ('5 '3)	TGA GGT ACC AGC CAG ATG TC
		Reverse ('5 '3)	CGT GTC CGC TTC TCT GTT AC
APPL1	XM_008771023.1	Forward ('5 '3)	TCA CTC CTT CCC CAT CTT TC
		Reverse ('5 '3)	TAG AGA GAG GGC AGC CAA AT
APPL2	NM_001108741.1	Forward ('5 '3)	TGC TCG GGC TAT TCA CAA
		Reverse ('5 '3)	AAA CAG GCC CGT GAC ACT
PGC1α	NM_031347.1	Forward ('5 '3)	ACC CAC AGG ATC AGA ACA ACC
		Reverse ('5 '3)	GAC AAA TGC TCT TTG CTT TAT TGC
FOXO1	NM_001191846.2	Forward ('5 '3)	CTC GGC GGG CTG GAA
		Reverse ('5 '3)	TCA TTC TGT ACT CGA ATA AAC TTG
PDK4	NM_053551.1	Forward ('5'3)	GGG ATC TCG CCT GGC ACT TT
		Reverse ('5 '3)	CAC ACA TTC ACG AAG CAG CA
βHAD	AF095449.1	Forward ('5 '3)	TCG TGA CCA GGC AAT TCG T
		Reverse ('5 '3)	CCG ATG ACC GTC ACA TGC T
FAT/CD 36	NM_031561.2	Forward ('5 '3)	GAC CAT CGG CGA TGA GAA A
		Reverse ('5 '3)	CCA GGC CCA GGA GCT TTA TT

AdipoR1; Adiponectin Receptor 1, APPL1; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, APPL2; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2, βActin; beta actin, βHAD; beta-hydroxyacyl-CoA dehydrogenase, FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, FOXO1; Forkhead box protein O1, GPR18; G-Protein Coupled Receptor 18, GPR55; G Protein-Receptor 55, PDK4; Pyruvate Dehydrogenase Kinase 4, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha.

Table 6.3B: Selected markers role in skeletal muscle metabolism

Marker	Function in the skeletal muscle
Cyclophilin	Present in all cells and is involved in cellular processes such as protein maturation and trafficking used as a house keeping gene (Diep, Madsen et al. 2011) normalise genes of interest using the $2^{-\Delta\Delta}$ct method.
βActin	Component of cytoskeletal and involved in function of cell structure and has been used as a house keeping gene (Diep, Madsen et al. 2011) normalised to the gene of interest using the $2^{-\Delta\Delta}$ct method.
GPR18	Putative cannabinoid receptor, currently unknown if expressed in skeletal muscle and the role is unknown. Detailed description in *Chapter One* Section 1.5.2.
GPR55	Putative cannabinoid receptor, currently unknown if expressed in skeletal muscle and role unknown. Detailed description in *Chapter One* Section 1.5.1
AdipoR1	Abundantly expressed in the skeletal muscle (Chen, McAinch et al. 2005) mediates adiponectin signalling such as partially regulating mitochondrial biogenesis through PGC1α (Iwabu, Yamauchi et al. 2010). Promotes fatty acid oxidation and glucose uptake in skeletal muscle. Detailed description in *Chapter One* Section 1.3.2.
APPL1	Positive mediator of adiponectin signalling (Wang, Xin et al. 2009). Detailed description in *Chapter One* Section 1.3.2.
APPL2	Proposed negative mediator of adiponectin signalling (Wang, Xin et al. 2009). Detailed description in *Chapter One* Section 1.3.2.
PGC1α	A transcription factor involved in oxidative capacity regulation and a marker of mitochondrial biogenesis (Sparks, Xie et al. 2005) that is abundantly expressed in oxidative skeletal muscle fibre types (Lin, Wu et al. 2002). Detailed description in *Chapter One* Section 1.3.1.2
FOXO1	Transcriptional regulator of energy metabolism (Gross, van den Heuvel et al. 2008) that promotes fatty acid oxidation (Kamei, Mizukami et al. 2003). Detailed description in *Chapter One* 1.3.1.6
PDK4	Promotes fatty acid metabolism and is regulated by FOXO1 (Bastie, Nahle et al. 2005) and PGC1α (Wende, Huss et al. 2005). Detailed description in *Chapter One* Section 1.3.1.5
βHAD	A mitochondrial enzyme from the inner mitochondrial membrane that promote medium and long chain fatty acid metabolism through the β oxidation pathway (Eaton, Bartlett et al. 1996). Detailed description in *Chapter* One Section 1.3.1.4
FAT/CD 36	Plasma membrane receptor that facilitates long chain fatty acid transport and uptake into the skeletal muscle (Glatz, Luiken et al. 2010). Detailed description in *Chapter One* Section 1.3.1.3

AdipoR1; Adiponectin Receptor1, APPL1; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, APPL2; Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2, βActin; betaactin, βHAD; beta-hydroxyacyl-CoA dehydrogenase, FAT/ CD 36; Fatty Acid Translocase/ Cluster of Differentiation 36, FOXO1; Forkhead box protein O1, GPR18; G-Protein Coupled Receptor 18, GPR55; G-Protein Receptor 55, PDK4; Pyruvate Dehydrogenase Kinase 4, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha.

6.3.3 Statistical Analysis

Graph Pad Prism Software 7.0 was utilised to generate all of the figures as well as perform statistical analysis. Data is reported as mean ± SEM. Normality of the data was assessed using either the Kolmogorov Sminov-Test or Sharpiro-Wilk Test. When data was considered normally distributed a parametric independent two tailed t-test was utilised and when data was not considered normally distributed the non-parametric Mann Whitney two tailed test was performed to determine differences between groups. Statistical significance is indicated at * $p < 0.05$.

6.4 Results

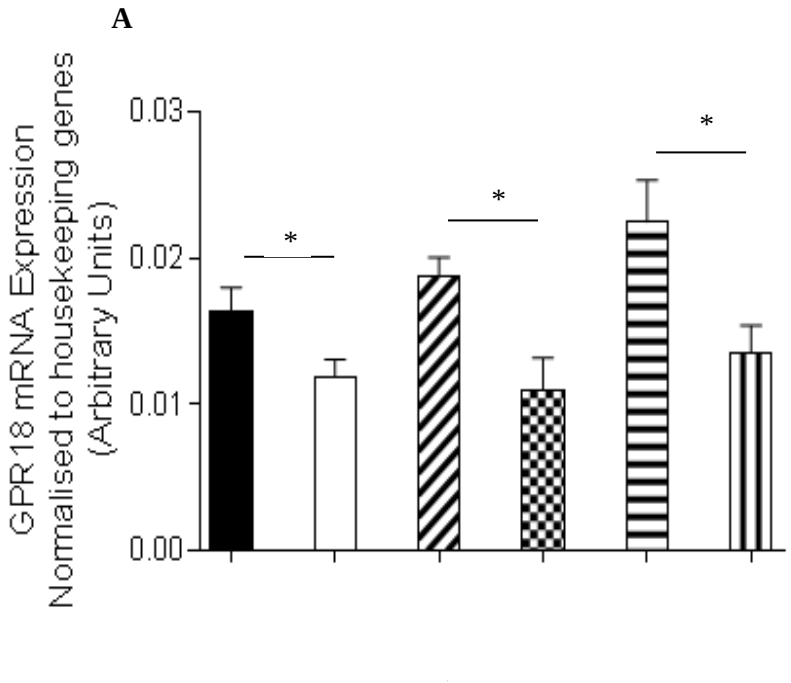


Figure 6.4A: Comparison of G-Protein Coupled Receptor (GPR18) mRNA expression in the presence or absence of cannabinoid compounds O-1602 or O-1918 in red gastrocnemius and white gastrocnemius skeletal muscle.

GPR18 mRNA expression in red and white gastrocnemius obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline), DIO O-1602 rats (5mg/ kg O-1602/ 0.75% tween 80/ saline) and the DIO O-1918 rats (1 mg/ kg O-1918/ 0.75% tween 80/ saline) were treated for a further six weeks. GPR18 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units ± SEM. ■ DIO Control red gastrocnemius (n = 10), ▭ DIO Control white gastrocnemius (n = 10), ▨ DIO O-1602 red gastrocnemius (n = 6), ▩ DIO O-1602 white gastrocnemius (n = 6), ▤ DIO O-1918 red gastrocnemius (n = 9) and ▥ DIO O-1918 white gastrocnemius (n = 7). * Indicates a significant difference between red gastrocnemius skeletal muscle and white gastrocnemius skeletal muscle for each of the treatment types ($p < 0.05$).

GPR18 mRNA expression in both red and white gastrocnemius skeletal muscle:

In DIO rats treated with either vehicle, O-1602 or O-1918 there was an increased abundance of GPR18 mRNA expression in red gastrocnemius skeletal muscle when compared to white gastrocnemius skeletal muscle for all three treatment groups ($p <0.05$) (Figure 6.4A A)

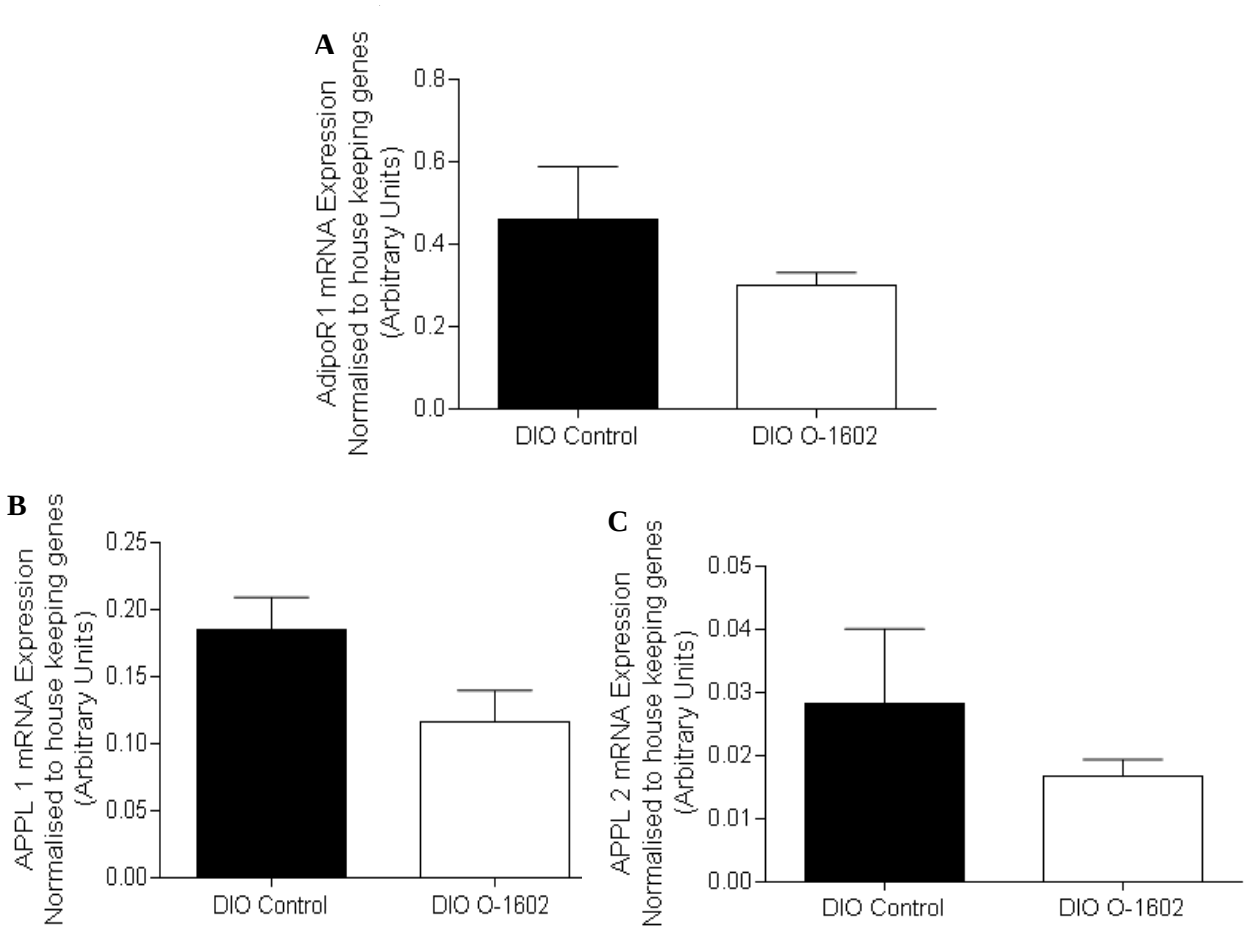


Figure 6.4B: Comparison of mRNA expression for markers upstream of adiponectin signalling in red gastrocnemius skeletal muscle obtained from DIO rats treated with either vehicle or O-1602 for six weeks.

(A) AdipoR1 (B) APPL1 and (C) APPL2 mRNA expression in red gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline) and the DIO O-1602 rats (5mg/ kg O-1602/ 0.75% tween 80/ saline) were treated for a further six weeks. AdipoR1, APPL1 and APPL2 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units average ± SEM. Closed bars DIO control rats treated with saline (n = 9 - 10). Open bars DIO rats treated with O-1602 (n= 6).

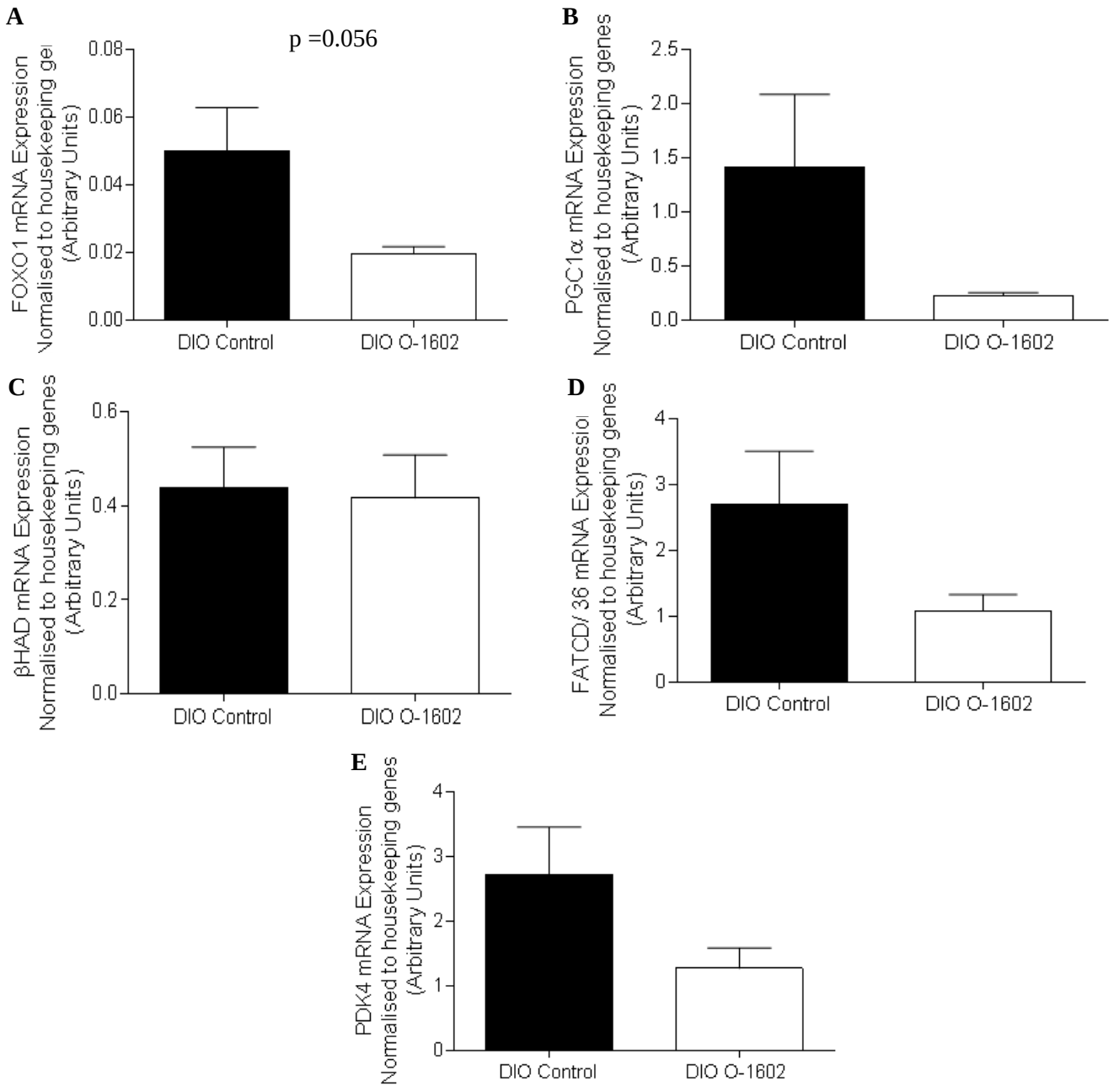


Figure 6.4C: Comparison of mRNA expression for markers involved in fatty acid metabolism and oxidative capacity in red gastrocnemius skeletal muscle obtained from DIO rats treated with either the vehicle or O-1602 for six weeks.

(A) FOXO1 (B) PGC1α (C) βHAD (D) FATCD/ 36 (E) PDK4 mRNA expression in red gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline) and the DIO O-1602 rats (5mg/ kg O-1602/ 0.75% tween 80/ saline) were treated for a further six weeks. FOXO1, PGC1α, βHAD, FATCD/ 36 and PDK4 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units ± SEM. Closed bars DIO control rats treated with saline (n = 10). Open bars DIO rats treated with O-1602 (n= 6).

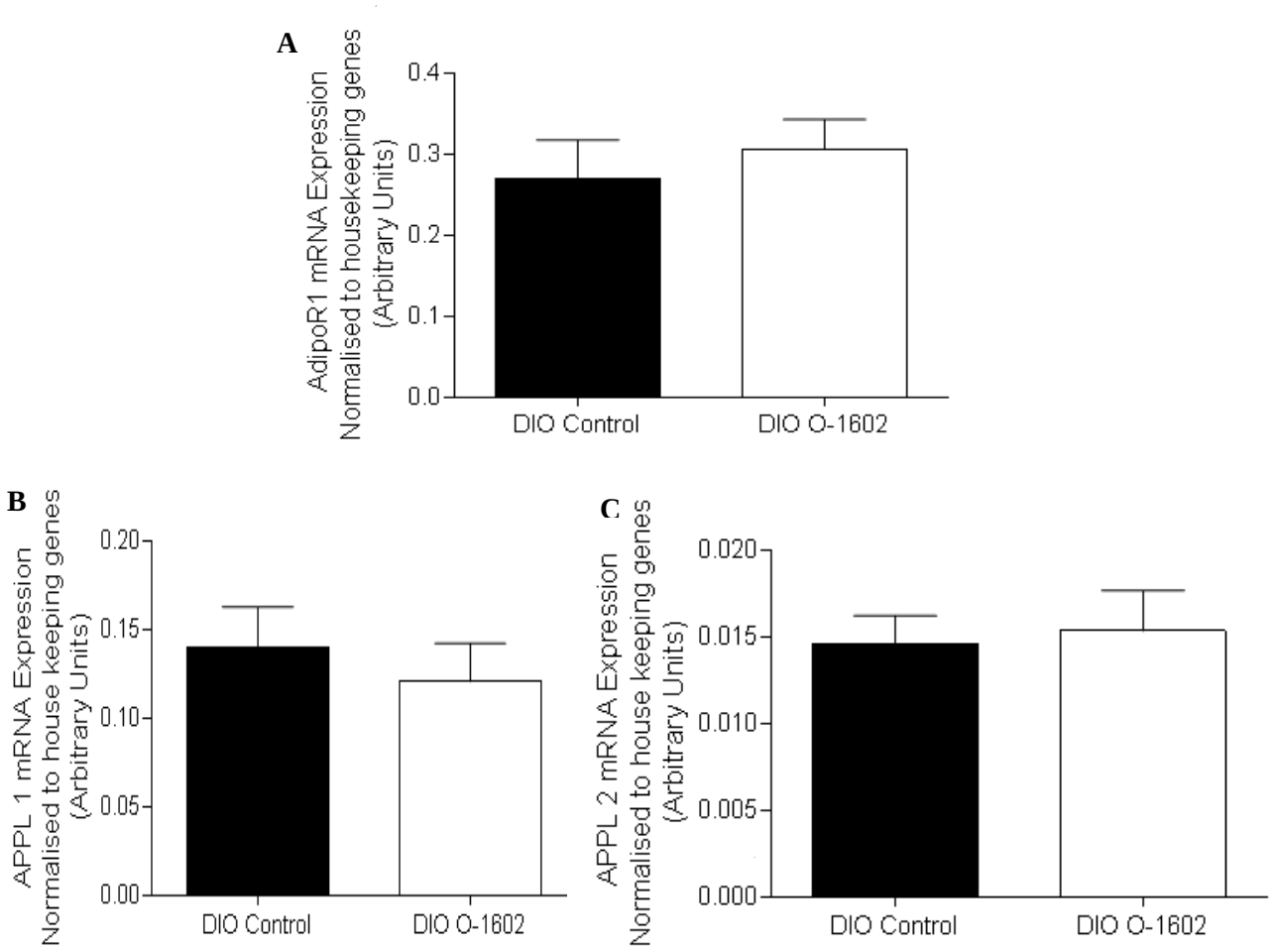


Figure 6.4D: Comparison of mRNA expression for markers upstream of adiponectin signalling in white gastrocnemius skeletal muscle obtained from DIO rats treated with either the vehicle or O-1602 for six weeks.

(A) AdipoR1 (B) APPL1 and (C) APPL2 mRNA expression in white gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control (0.75% tween 80/ saline) and the DIO O-1602 rats (5mg/ kg O-1602/ 0.75% tween 80/ saline) were treated for a further six weeks. AdipoR1, APPL1 and APPL2 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units average ± SEM. Closed bars DIO control rats treated with saline (n = 10 - 11). Open bars DIO rats treated with O-1602 (n= 6).

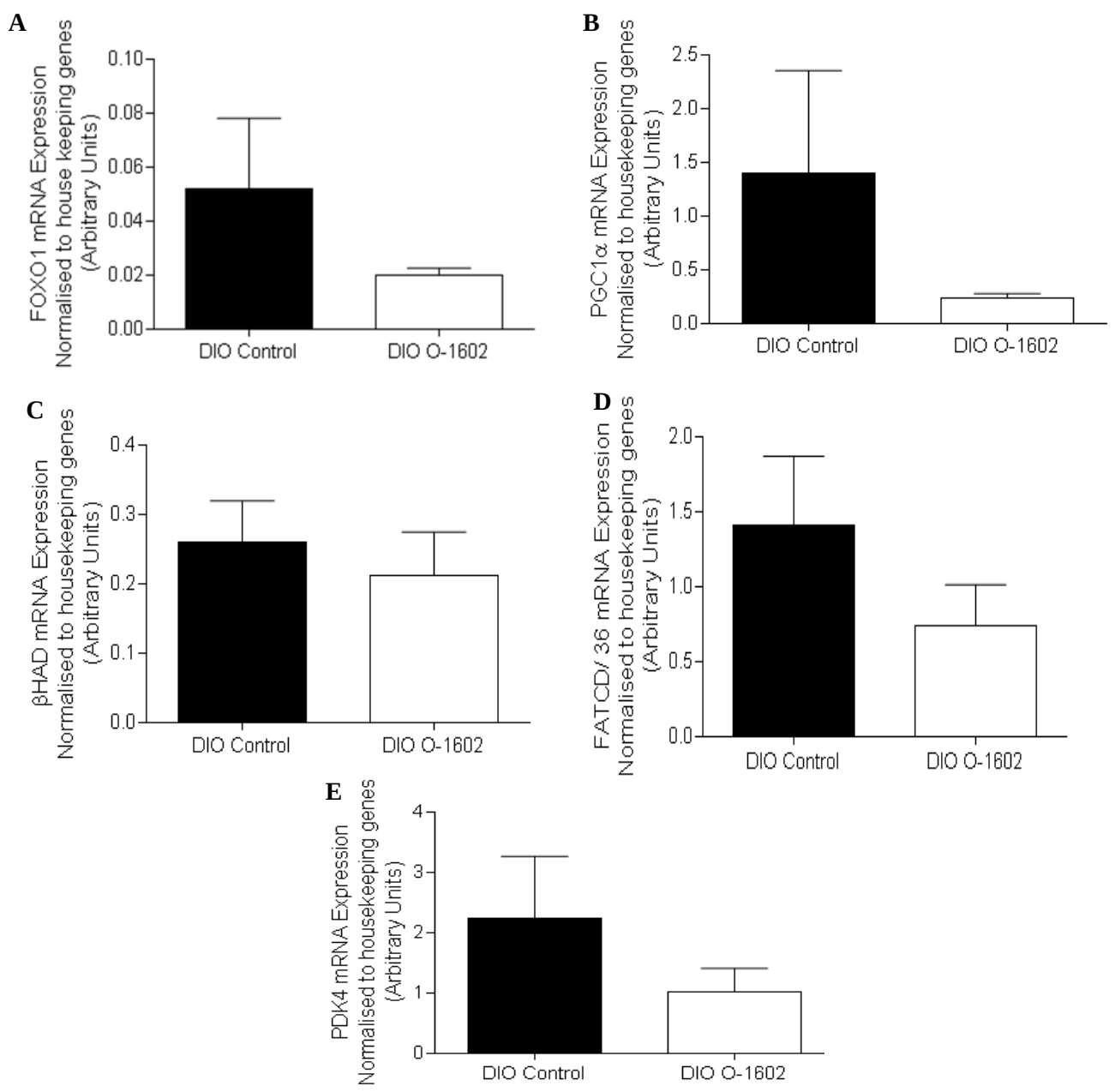


Figure 6.4E: Comparison of mRNA expression for markers involved in fatty acid metabolism and oxidative capacity in white gastrocnemius skeletal muscle obtained from DIO rats treated with either the vehicle or O-1602 for six weeks.

(a) FOXO1 (b) PGC1α (c) βHAD (d) FATCD/36 (e) PDK4 mRNA expression in white gastrocnemius skeletal muscle from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline) and DIO O-1602 rats (5 mg/ kg O-1602/ 0.75% tween 80/ saline) were treated for six weeks. FOXO1, PGC1α, βHAD, FATCD/ 36 and PDK4 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units ± SEM. Closed bars DIO control rats treated with saline (n = 11). Open bars DIO rats treated with O-1602 (n = 6).

The effect O-1602 has on mRNA expression of markers upstream of adiponectin signalling, fatty acid metabolism and oxidative capacity in either red or white gastrocnemius skeletal muscle in DIO:

In DIO rats treated with either vehicle or O-1602 there was no alteration in the mRNA expression of AdipoR1, APPL1, APPL2, FOXO1 (p 0.056 red gastrocnemius), PGC1α, βHAD, FATCD/ 36, PDK4 in either the red or white gastrocnemius skeletal muscle (Figures 6.4B - 6.4D).

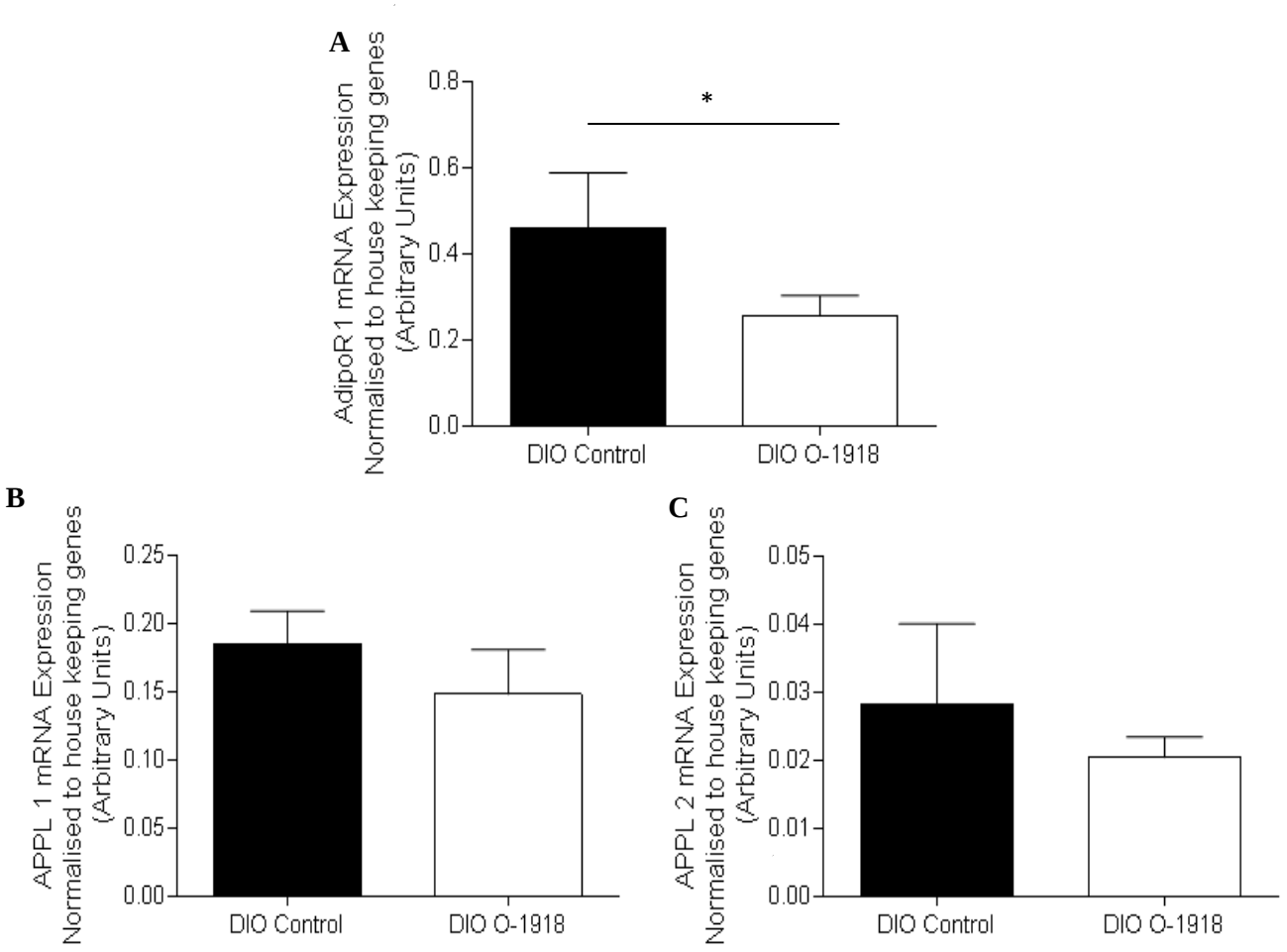


Figure 6.4F: Comparison of mRNA expression for markers upstream of adiponectin signalling in red gastrocnemius skeletal muscle obtained from DIO rats treated with either the vehicle or O-1918 for six weeks.

(A) AdipoR1 (B) APPL1 and (C) APPL2 mRNA expression in red gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline) and DIO O-1918 rats (1 mg/ kg O-1918/ 0.75% tween 80/ saline) were treated for a further six weeks. AdipoR1, APPL1 and APPL2 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units average ± SEM. Closed bars DIO control rats treated with saline (n = 9 - 10). Open bars DIO rats treated with O-1918 (n = 9). * Indicates a significant difference ($p < 0.05$).

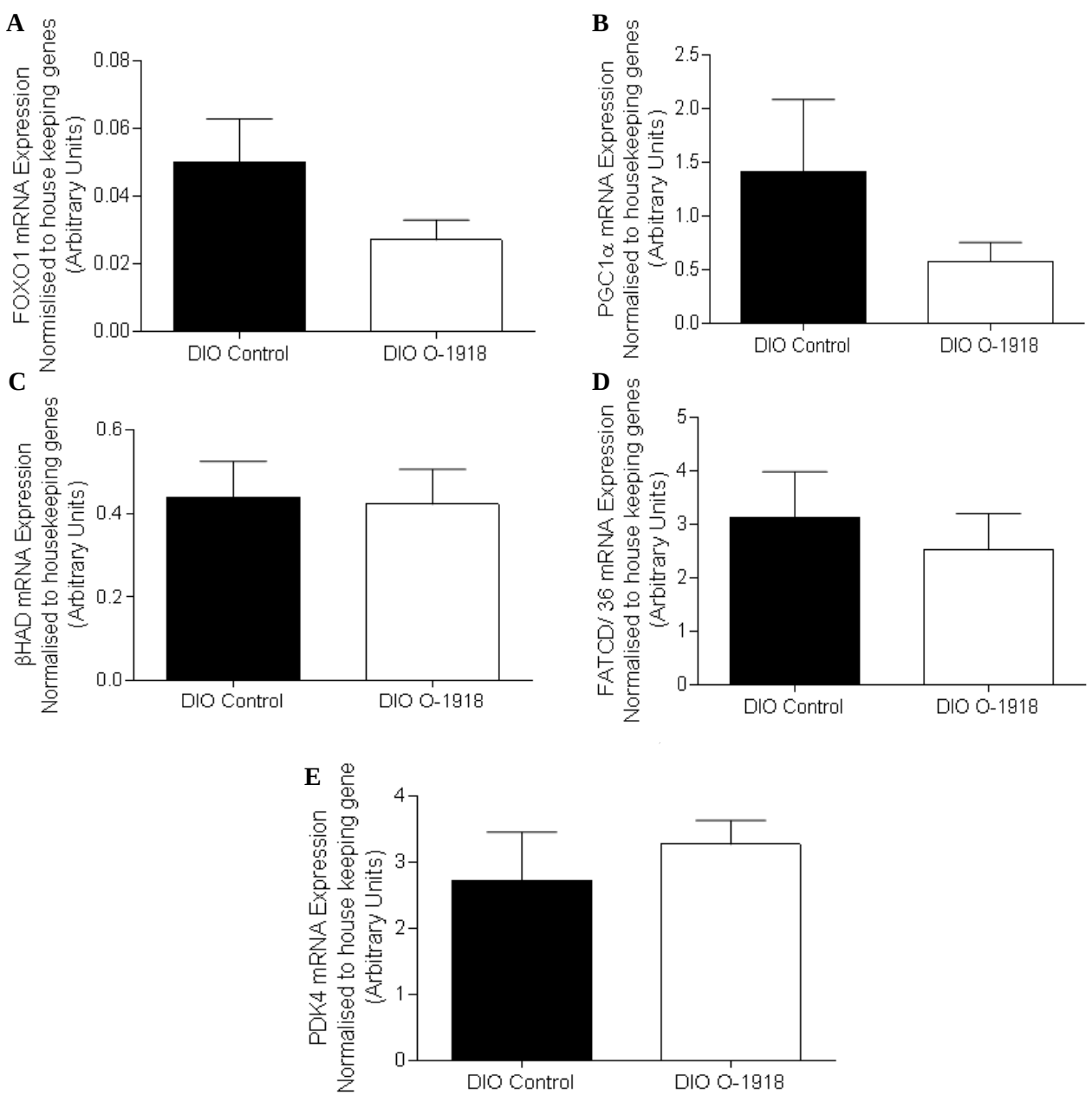


Figure 6.4G: Comparison of mRNA expression for markers involved in fatty acid metabolism and oxidative capacity in red gastrocnemius skeletal muscle obtained from DIO rats treated with either vehicle or O-1918 for six weeks.

(A) FOXO1 (B) PGC1α (C) βHAD (D) FATCD/36 (E) PDK4 mRNA expression in red gastrocnemius skeletal muscle from rats fed a HFD for nine weeks to induce obesity, then DIO Control rats (0.75% tween 80/ saline) and the DIO O-1918 rats (1 mg/ kg O-1918/ 0.75% tween 80/ saline) were treated for a further six weeks. FOXO1, PGC1α, βHAD, FATCD/36 and PDK4 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units ± SEM. Closed bars DIO control rats treated with saline (n = 10). Open bars DIO rats treated with O-1918 (n = 9).

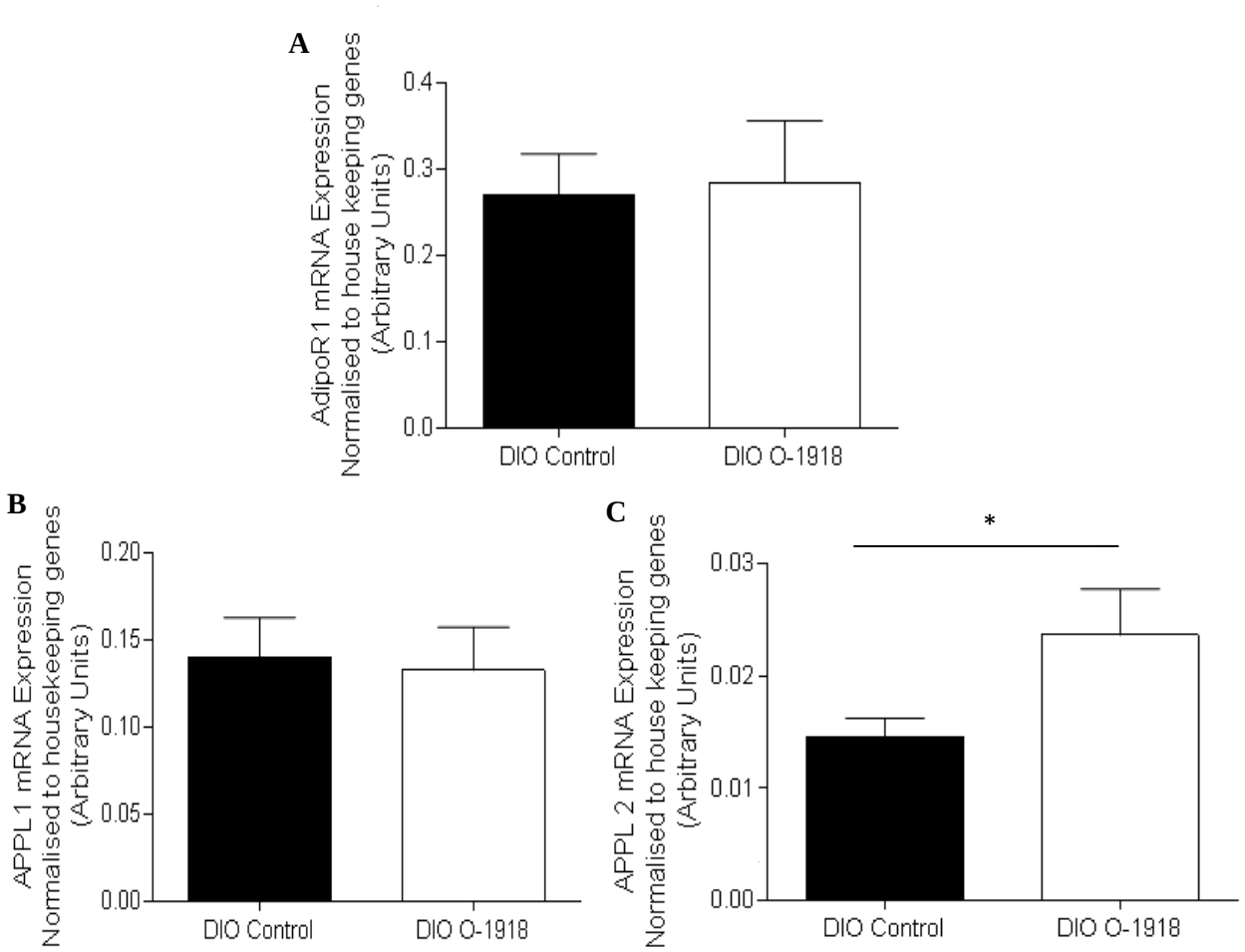


Figure 6.4H: Comparison of mRNA expression for markers upstream of adiponectin signalling in white gastrocnemius skeletal muscle obtained from DIO rats treated with either the vehicle or O-1918 for six weeks.

(A) AdipoR1 (B) APPL1 and (C) APPL2 mRNA expression in white gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control (0.75% tween 80/ saline) and DIO O-1918 rats (1 mg/ kg O-1918/ 0.75% tween 80/ saline) were treated for a further six weeks. AdipoR1, APPL1 and APPL2 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units average ± SEM. Closed bars DIO control rats treated with saline (n = 10 - 11). Open bars DIO rats treated with O-1918 (n= 8 - 9). * Indicates a significant difference ($p < 0.05$).

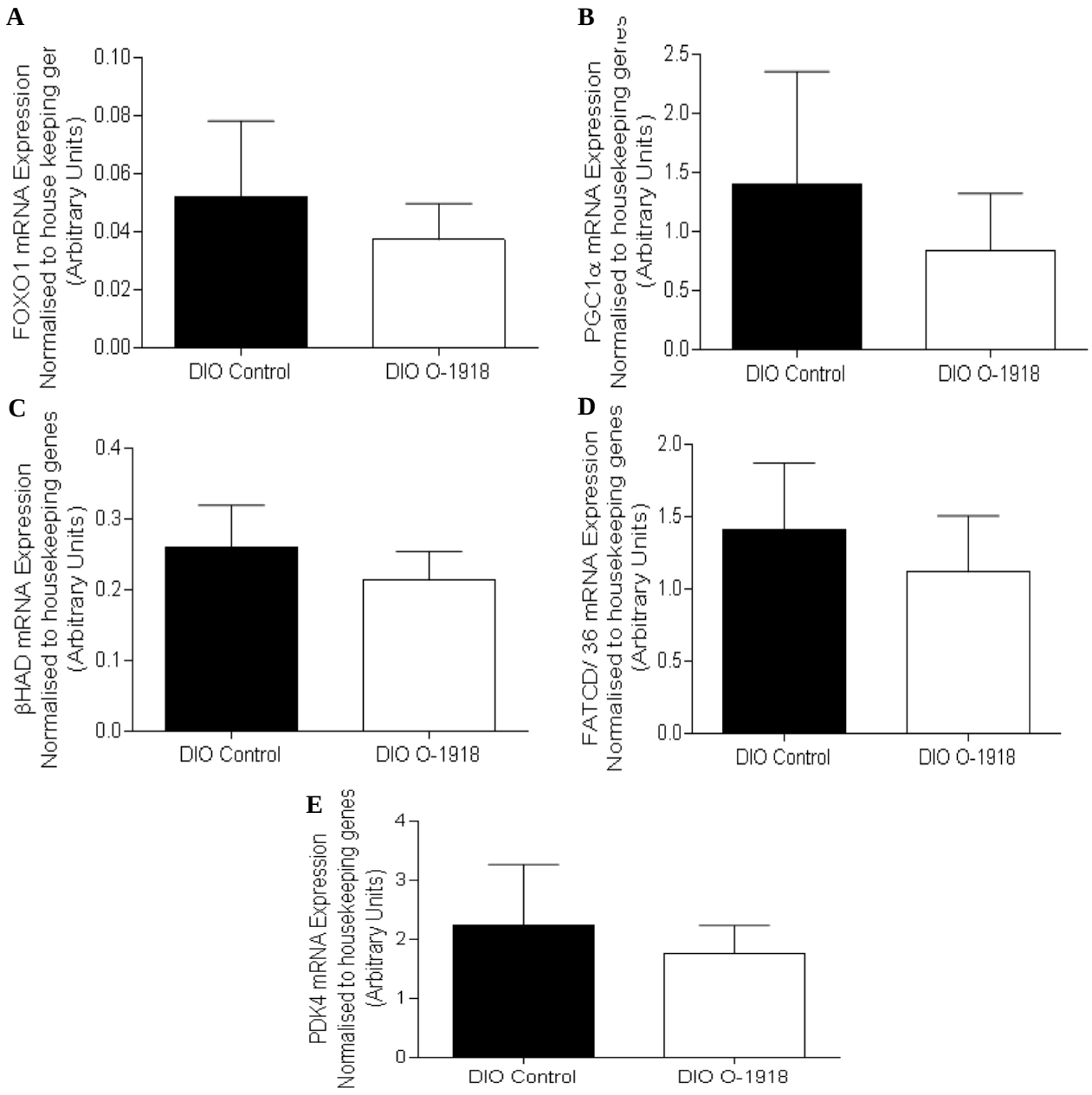


Figure 6.4I: Comparison of mRNA expression for markers involved in fatty acid metabolism and oxidative capacity in white gastrocnemius skeletal muscle obtained from DIO rats treated with either a vehicle or O-1918 for six weeks.

(A) FOXO1 (B) PGC1α (C) βHAD (D) FATCD/36 (E) PDK4 mRNA expression in white gastrocnemius skeletal muscle obtained from rats fed a HFD for nine weeks to induce obesity, then the DIO Control rats (0.75% tween 80/ saline) and the DIO O-1918 rats (1 mg/ kg O-1918/ 0.75% tween 80/ saline) were treated for a further six weeks. FOXO1, PGC1α, βHAD, FATCD/36 and PDK4 mRNA expression was normalised to the average of housekeeping genes Cyclophilin and βActin and group data is reported as arbitrary units ± SEM. Closed bars DIO control rats treated with saline (n = 11). Open bars DIO rats treated with O-1918 (n= 9).

The effect that O-1918 has on mRNA expression of markers upstream of adiponectin signalling, fatty acid metabolism and oxidative capacity in red or white gastrocnemius skeletal muscle in DIO:

In DIO rats treated with O-1918, there was a down regulation in the mRNA expression of AdipoR1 in the red gastrocnemius skeletal muscle ($p < 0.05$), but APPL1, APPL2, FOXO1, PGC1α, β-HAD, FATCD/ 36 and PDK4 were not altered (Figures 6.4E and 6.4F). While in the white gastrocnemius skeletal muscle there was an up regulation of APPL2 mRNA expression ($p <0.05$) but APPL1, FOXO1, PGC1α, β-HAD, FATCD/ 36 and PDK4 were not altered (Figures 6.4G and 6.4H).

6.5 Discussion

This study is the first to look at the effect that either O-1602 or O-1918 have on skeletal muscle homeostasis, focusing on quantifying changes to markers of adiponectin signalling, oxidative capacity and fatty acid oxidation in oxidative or glycolytic skeletal muscle obtained from DIO rodents. Furthermore, this study is the first to investigate whether GPR18 is expressed in skeletal muscle, and to quantify the difference in expression between the different tissue fibre phenotypes in DIO rats.

Findings from this study show that firstly the putative cannabinoid receptor GPR18 is in fact expressed in DIO rat skeletal muscle, further this receptor is up-regulated in red gastrocnemius skeletal muscle which has a greater oxidative capacity when compared with the white gastrocnemius skeletal muscle which has a lower oxidative capacity. The putative cannabinoid receptor GPR55 (*Simcocks et al, Unpublished Observations*) as well as the traditional cannabinoid receptors CB_1 and CB_2 (Cavuoto, McAinch et al. 2007) are expressed in skeletal muscle and have a putative role or role in energy homeostasis, therefore it was hypothesised that GPR18 would also be expressed in the skeletal muscle. The function that GPR18 has in rat skeletal muscle remains unclear, however an up-regulation in red gastrocnemius skeletal muscle may indicate that this receptor has a role in oxidative metabolism, but further analysis is required. Treatment with either of the compounds O-1602 or O-1918 that have an affinity for GPR18, did not alter mRNA expression of GPR18 in either the red or the white gastrocnemius skeletal muscle, indicating that chronic administration of these compounds in DIO does not alter GPR18 expression, however this does not discount alterations in the signalling mediated from GPR18 with the treatment of either O-1602 or O-1918.

GPR55 was unable to be detected in skeletal muscle in this experiment, the GPR55 primer (Imbernon, Whyte et al. 2014) was unable to detect mRNA expression of GPR55 in cDNA diluted 1:5 from either the red or white gastrocnemius skeletal muscle. This indicates that either the receptor is not expressed in skeletal muscle or that there is a very low abundance of GPR55 in rat gastrocnemius skeletal muscle..

In contrast to the hypothesised role of GPR18 in the skeletal muscle in DIO rats, following six weeks treatment of O-1602 (that is a biased agonist for for GPR18 as well as an agonist for GPR55) there were no alterations in the mRNA expression of AdipoR1, APPL1, APPL2, FOXO1 ($p = 0.056$), PGC1α, β-HAD, FATCD/ 36, PDK4 in either the red or white gastrocnemius skeletal muscle. While in *Chapter Four* our results indicate that O-1602 reduced body fat percentage and epididymal fat pads (epididymal fat weight (g)/ body weight (g) (%)), the data represented in this chapter shows that the mRNA expression of the markers analysed for oxidative capacity, adiponectin signalling and fatty acid oxidation were not altered by treatment with this compound. While mRNA expression alone cannot entirely eliminate the possibility that this compound does not affect the molecular signalling pathways selected in the skeletal muscle, changes in protein expression and phosphorylation of these markers would also need to be quantified to give some further evidence. These findings do indicate the mRNA expression was not altered and therefore based on the evidence provided, O-1602 does not appear to regulate these pathways. On the other hand, considering the metabolically stable C_2C_{12} myotubes treated with O-1602 did not have any alteration in markers of oxidative capacity (NFATc1 and PGC1α), or the positive regulator of adiponectin signalling APPL1, it is therefore not surprising that this compound did not have any effect on the mRNA expression in this DIO model.

O-1918 down regulated the mRNA expression of AdipoR1 in the DIO red gastrocnemius skeletal muscle. AdipoR1 is abundantly expressed in the skeletal muscle (Townsend and Tseng 2012). AdipoR1 knockout mice have reduced PGC1α mRNA and protein expression (Iwabu, Yamauchi et al. 2010). While PGC1α mRNA expression was not down regulated with the treatment of O-1918 in the DIO rat model used in this study, it does not fully discount changes to the function of PGC1α or mitochondrial biogenesis in this tissue. In metabolically stable C_2C_{12} myotubes, silencing AdipoR1 results in inhibition of adiponectin mediated AMPK activation, resulting in reduced fatty acid oxidation and glucose uptake into the skeletal muscle (Townsend and Tseng 2012). Down regulation of AdipoR1 may reduce fatty acid oxidation and glucose uptake in the skeletal muscle in the rats,

which should be investigated in the future. However, given that the administration of O-1918 was for six weeks, it is unclear as to the time course for down regulation of AdipoR1 mRNA expression, but given that there was no change in body fat percentage or weight, (although BAT was decreased in weight compared to body weight %) treatment with this compound did not alter total adiposity. The DIO rats treated with O-1918 in this study (previously described in further detail in *Chapter Five*) did not have altered circulating plasma adiponectin concentrations but did have reduced circulating plasma leptin. McAinch et al. (2007) found that human primary myotubes from lean donors treated with leptin had increased mRNA expression of AdipoR1, indicating a link between leptin and adiponectin signalling, however this effect was not apparent in muscle donors that were obese, T2DM or that had undergone weight loss. While the DIO model described in this thesis focuses on rat and not human, the reduction in both circulating leptin and the AdipoR1 helps to provide some understanding into the effect that this compound is having at a molecular level in skeletal muscle with a higher oxidative capacity. The fact that O-1918 also up regulated APPL2, the negative regulator of adiponectin signalling (Wang, Xin et al. 2009), in the more glycolytic white gastrocnemius skeletal muscle in our study, helps to further support that this compound has an overall role of regulating adiponectin signalling. This data conflicts with other results observed in our laboratory (*Simcocks et al. Unpublished Observations)* in which metabolically stable C_2C_{12} myotubes treated with O-1918 caused an up-regulation in the mRNA expression of NFATc1, PGC1α, PDK4 and a tendency for APPL1 to also be up-regulated, which would be beneficial to obesity and related co-morbidities such as T2DM. However, the results presented in this chapter indicate that the compound would have more detrimental effects for obesity and T2DM if this was translated into humans. The variation in findings could be due to the difference between species, the fact that cell culture is not influenced by circulating hormones and cytokines like *in vivo*, whole tissue versus cell culture and/ or the difference between metabolically stable versus obese skeletal muscle phenotypes.

Taken together, the findings from this study suggest that as GPR18 is expressed in the skeletal muscle obtained from DIO rats, and that modulating this receptor and understanding it's physiological role may be a beneficial future pharmacological target. The effects observed following chronic administration of either O-1602 or O-1918 in this DIO model indicate that O-1602 does not appear to alter oxidative capacity, fatty acid oxidation or adiponectin signalling pathways in the skeletal muscle in obesity. While O-1918 appears to either down or up regulate mRNA expression of key proteins involved in adiponectin signalling (seen to be as detrimental to the obese state) in a fibre type dependant fashion and this may be related to the reduced circulating plasma leptin concentrations apparent in this DIO model.

CHAPTER SEVEN:

7.0 The effect that different dietary fatty acids have on whole body energy homeostasis in a diet induced obesity model.

7.1 Abstract

Background: Not all fatty acids have the same physiological implications based on their chemical structure when grouped as SFA, MUFA and PUFA. An omega-6 PUFA, linoleic acid (LA), is hypothesised to be partially associated with increased obesity rates and associated co-morbidities and the consumption of this fatty acid has increased largely over recent decades, which could partly be attributed to an over active endocannabinoid system. Dietary fatty acids can be synthesised into different endocannabinoids and structurally similar ligands that have an affinity for GPCRs.

LA, which is an omega-6 PUFA is abundant in the Western diet, and is a precursor to arachidonic Acid (AA), as well as the endocannabinoids AEA and 2-AG. The consumption of LA and AA are associated with increased circulating plasma endocannabinoids concentrations, which is associated with weight gain, increased adipose tissue storage, insulin resistance, all of which contribute to the pathogenesis of obesity and T2DM.

Molecules structurally similar to AEA include OEA and PEA which are synthesised by oleic acid, a MUFA, and palmitic acid, a SFA, respectively. OEA induces satiety, concentrations of OEA are reduced in the small intestine due to a HFD and OEA production is reduced in the small intestine of DIO rodents. PEA is reduced in the visceral adipose tissue of ApoE mice and this ligand, down regulates TNFα in human primary adipocytes induced with treatment of LPS.

OEA has been reported to have an affinity to GPR55, in addition to being a potent agonist of GPR119. While one study showed that PEA does activate GPR55, another found that this compound did not elicit a response. It is currently unknown if OEA and/ or PEA are ligands for GPR18. Regardless of

its action on GPR55 or GPR18, the effect that these different dietary fatty acids have on obesity in rats is largely unknown and therefore is the aim of this study.

Materials & Methods: DIO rats were maintained on a 21% (by weight) HFD for nine weeks, then allocated into four groups based on their metabolic characteristics into either: (1) HFD predominately consisting of SFA, (2) HFD predominately consisting of MUFA, (3) HFD predominately consisting of PUFA or (4) a SCD. Additionally a group of rats were maintained on a SCD for fifteen weeks, as a lean control group. Measurements for food intake, body weight, body composition ip. GTT and ip. IST were obtained over the treatment period. Rats were anaesthetised using isoflurane and blood was collected at time of death via cardiac puncture, then adipose fat depots (epididymal, peri-renal and BAT) and the liver were immediately collected and weighed post mortem, then snap frozen for subsequent analysis. Circulating hormones and cytokines were determined using commercially available Bioplex Plates and ELISA assays.

Results/ Discussion/ Conclusion: In DIO rats fed either a HFD predominately composed of SFA, MUFA or PUFA there were no major differences observed between the three groups for body fat percentage, fat pad depots, or circulating hormones or cytokines. However, blood glucose response to insulin in the DIO SFA group compared to the DIO PUFA was increased at 120 and 180 minute time points. This suggests a difference between SFA and PUFA in DIO in terms of whole body insulin sensitivity.

7.2 Background

Dietary fatty acids can be classified according to their chemical structure into either SFA, MUFA or PUFA, but not all fatty acids have the same physiological implications based on these categorisations.

LA, an omega-6 PUFA, is hypothesised to be associated partially to increased obesity rates and associated co-morbidities (Naughton, Mathai et al. 2016). The consumption of LA has increased largely over recent decades and as a result of nutrition transition to a 'Western Diet' plant based vegetable oils that are high in LA are consumed due to convenience of production and cost effectiveness (Naughton, Mathai et al. 2016) and are likely to be some of the factors contributing to the increased consumption of LA.

LA is a precursor to AA, as well as the endocannabinoids AEA and 2-AG, and as would be predicted, dietary LA is associated with increased circulating endocannabinoid concentrations (Alvheim, Malde et al. 2012). An overactive endocannabinoid system results in weight gain (Gatta-Cherifi and Cota 2016), increased lipogenesis (Cota, Marsicano et al. 2003) and insulin resistance (Gatta-Cherifi and Cota 2016), all of which are involved in the pathogenesis of obesity and T2DM.

In addition to endocannbinoids, two other relevant compounds that are structural analogues of AEA include OEA and PEA (Bisogno, Ligresti et al. 2005), these compounds can be synthesised by other fatty acids including oleic acid and palmitic acid, respectively. OEA induces satiety (Gaetani, Fu et al. 2010), short term exposure to a HFD in a rodent model lowers OEA in the small intestine (Diep, Madsen et al. 2011) and the production of OEA is altered in the small intestine of DIO rats and mice (Igarashi, DiPatrizio et al. 2015). Oleic acid consumption causes a post-prandial increase in OEA in humans (Mennella, Savarese et al. 2015). While PEA is proposed to have an anti-inflammatory role in obesity and related co-morbidities, in ApoE knockout mice (a model known to induce atherosclerotic plaque) the VAT concentrations of PEA are decreased (Montecucco, Matias et al. 2009). Furthermore,

PEA down regulates TNFα in human primary adipocytes induced with treatment of LPS and reduced concentrations of leptin (Hoareau, Buyse et al. 2009).

OEA has been reported to have an affinity to GPR55 (Ryberg, Larsson et al. 2007), in addition to being a potent agonist of GPR119 (Overton, Fyfe et al. 2008). While one study shows that PEA does activate GPR55 (Ryberg, Larsson et al. 2007), another found that this compound did not elicit a response (Kapur, Zhao et al. 2009).. It is currently unclear whether these compounds target the other putative cannabinoid receptor GPR18, but is known that GPR55 and GPR18 do have an affinity to a number of other cannabinoid and associated ligands so this could be possible.

Therefore the aim of this study was to determine the role that different dietary fatty acids (SFA, MUFA and omega-6 PUFA) have in obesity with a focus on whole body energy homeostasis.

Hypothesis

It is hypothesised that in DIO rats fed a high fat SFA diet will result in impaired glucose tolerance and insulin sensitivity, increased pro-inflammatory cytokines and decrease anti-inflammatory cytokines. Due to the duration of the diet only being for six weeks it is not hypothesised that there will be any significant change in body fat % or fat pad mass or weight during this time.

It is hypothesised that in DIO rats fed a high fat MUFA diet will cause improvements in glucose tolerance and insulin sensitivity, it is hypothesised that there will be a reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines. Due to the duration of the diet only being for six weeks it is not hypothesised that there will be any significant changes in body fat%, fat pad mass or weight during this time.

It is hypothesised that in DIO rats fed a high fat PUFA diet (LA which is a precursor to endocannabinoids) will cause further impairment in glucose tolerance and insulin sensitivity, it is hypothesised that there would be an increase in pro-inflammatory cytokines and a decrease in anti-

inflammatory cytokines. Due to the duration of the diet only being for six weeks it is not hypothesised that there will be any significant change in body fat pad mass or weight during this time.

It is hypothesised that in DIO rats that recommence a SCD diet (which has a reduced energy conent compared to the HFDs) will cause improvements in glucose tolerance and insulin sensitivity, accompanied by a reduction in body weight, body fat% and fat pad mass, it is also hypothesised that there would be a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines.

7.3 Materials & Methods

7.3.1 Animals, Housing and Dietary Feeding Regime

49 seven week old male Sprague Dawley rats were purchased from the ARC and acclimatised to laboratory conditions for at least one week. The rats were housed singly for the duration of the experiment. The rats were then randomly assigned to either a SCD (4.8% fat in weight 12% fat from digestible lipids) or a HFD SF13-115 (21% fat in weight and 41% fat from digestible lipids) (Speciality Feeds, Glen Forrest, Western Australia, Australia) for nine weeks to induce obesity. Following this, the rats that consumed the HFD were then allocated into four separate groups based on their metabolic characteristics: HFD predominately consisting of SFA (9.76%), HFD predominately consisting of MUFA (16.01%), HFD predominately consisting of PUFA (16.00%) or a SCD. See Table 7.3A.

Table 7.3A: Fatty acid composition of the dietary feeding regimes for SCD, HFD that predominately consisted of SFA, HFD that predominately consisted of MUFA or HFD that predominately consisted of PUFA.

Nutrient Composition	CHOW	HFD SFA SF13-115	HFD MUFA SF13-113	HFD PUFA SF13-114
Saturated Fats C12:0 or less	-	0.07%	TRACE	TRACE
Myristic Acid 14:0	0.03%	0.32%	TRACE	0.03%
Palmitic Acid 16:0	0.50%	5.57%	2.29%	1.31%
Stearic Acid 18:0	0.14%	3.61%	0.53%	0.49%
Other Saturated Fats	-	0.20%	TRACE	0.17%
Palmitoleic Acid 16:1	0.01%	0.36%	0.17%	0.11%
Oleic Acid 18:1	1.90%	7.10%	15.76%	2.72%
Gadoleic Acid 20:1	0.03%	0.15%	0.08%	0.06%
Linoleic Acid 18:2 n6	1.30%	3.02%	1.80%	15.65%
α-Linolenic Acid 18:3 n3	0.30%	0.29%	0.36%	0.35%
Arachadonic Acid 20:4 n6	0.01%	-	-	-
EPA 20:5 n3	0.02%	-	-	-
DHA 22:6 n3	0.05%	-	-	-
Total n3	0.37%	0.33%	0.37%	0.35%
Total n6	1.31%	3.05%	1.82%	15.65%
Total MUFA	2.00%	7.68%	16.01%	2.97%
Total PUFA	1.77%	3.48%	2.18%	16.00%
Total SFA	0.74%	9.76%	2.82%	2.00%

7.3.1.1 Food Intake and Body Weight

Food intake (in grams) and body weight (in grams) were measured and recorded daily throughout the entire study and the weekly average was calculated based off this data and represented. For further detail refer to section 2.3.2.2.

7.3.1.2 Echo MRI

To determine body fat and lean tissue mass the previously validated Echo MRI scan (Taicher, Tinsley et al. 2003) was completed using the Echo MRI^{TM} system (Echo-MRI 900 Houston, United States of America). Measurements were obtained at week nine (baseline of DIO) and week 15 (towards the end of the dietary feeding regime). For further detail refer to section 2.3.2.3.

7.3.1.3 Ip. GTT

To determine the effect a glucose load had on blood glucose concentrations, an ip. GTT was conducted, using previously published methods (Jenkin, O'Keefe et al. 2016). Measurements were obtained at week eight and week fourteen. For further detail refer to section 2.3.2.4.

7.3.1.4 Ip. IST:

To determine the effect a bolus of insulin had on blood glucose concentrations an ip. IST was conducted using previously published methods (Russell, Feilchenfeldt et al. 2003). Measurements were obtained at week eight and week fourteen. For further detail refer to section 2.4.1.2.

7.3.1.5 Anaesthesia, Blood and Tissue Collection

At the end of the different dietary feeding regime (the end of week fifteen) the rats were deeply anesthetised using isoflurane and killed via cardiac puncture, blood was immediately collected, then transferred to a EDTA BD coated vacutainer (Macfarlane Medical, Surry Hills, Victoria) and subsequently placed on ice. Blood tubes were centrifuged at 4°C at 4000 x g for 10 minutes to separate the plasma from the red blood cells. Plasma was transferred to Eppendorf tubes and stored at -80 °C for future analysis. Whole adipose tissue (WAT epididymal and perirenal as well as BAT interscapular

fat pads) and the liver were harvested and the weight (in grams) was recorded, following this a portion of each tissue was snap frozen in liquid nitrogen for subsequent analysis. For further detail refer to section 2.4.1.3, 2.4.1.5 and 2.3.2.10.

7.3.2 Biochemical Analysis

7.3.2.1 Adiponectin Assay

Total circulating plasma concentrations were determined using Adiponectin ELISA Kit (AG-45A-0005TP-KI01) (Adipogen, Liestal, Switzerland) in accordance with the manufacturer's instructions. For further detail refer to section 2.5.2.

7.3.2.2 Bioplex Assay

Bioplex hormone immunoassay (171K1001M) and cytokine assay (YL0000006Q) were used to analyse circulating plasma hormone and cytokine concentrations in accordance with the manufacturer's instructions. For further detail refer to section 2.5.1.

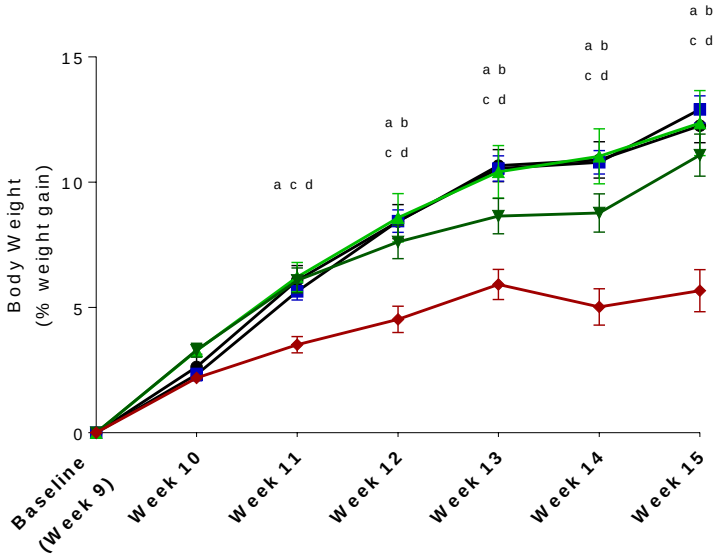
7.3.3 Statistical Analysis

All statistical analyses were completed using Graph Pad Prism Software 7.0. Data sets that compared the different dietary feeding regimes at one time point used a one way ANOVA and a Tukey's Post Hoc multiple comparisons test to determine the statistical differences. The data analysed using this method included body fat (%), organ weights, plasma hormones and cytokines.

Data sets that compared the different dietary feeding regimes for more than one time point used a two-way ANOVA and a Tukey's Post Hoc multiple comparisons test to determine the statistical differences between multiple time points and different feeding regimes in combination. The data analysed using this method included body weight gain (%), energy intake (MJ/ week), food consumption (in grams) and blood glucose concentrations for ip. GTT (mmol/ L) and ip. IST (mmol/ L)

7.4 Results

A



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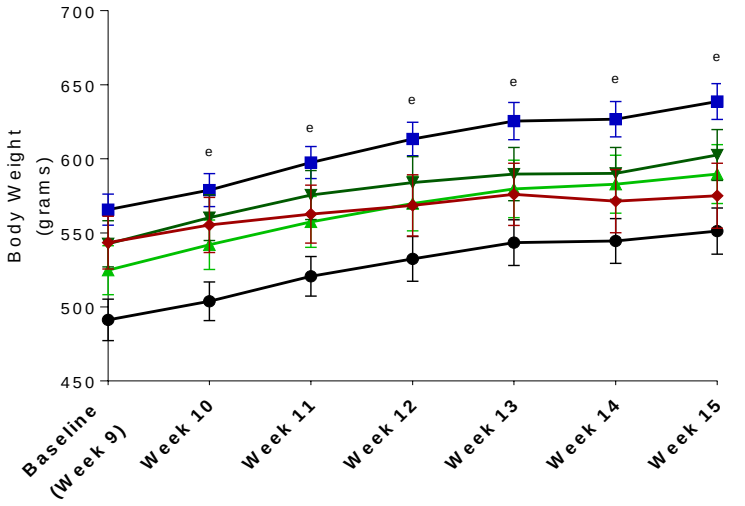

Figure 7.4A: Body Weight

(A) Body weight (% weight gain) and (B) Body weight (in grams) obtained from rats fed either a standard chow diet (SCD) for fifteen weeks (n = 9) or a high fat diet (HFD) for nine weeks to induced obesity (n = 39) then DIO rats were allocated into four dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA), HFD that predominately consisted of monounsaturated fatty acid (MUFA), HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data is reported as ± SEM, data is shown for the six week treatment period. SCD (n = 9) DIO SFA Diet (n = 10) DIO MUFA Diet (n= 10) DIO PUFA (n = 10) DIO SCD (n = 9). Letters indicate significant differences ($p < 0.05$) between groups [a] SCD vs DIO SCD, [b] DIO SFA vs DIO SCD, [c] DIO MUFA vs DIO SCD [d] DIO PUFA vs DIO SCD [e] SCD vs DIO SFA.

Figure 7.4A (A) shows that the DIO SCD fed rats had a significant reduction in weight gain ($p < 0.05$) when compared to the SCD, DIO MUFA and DIO PUFA as a result of the different dietary feeding regimes from week eleven onwards, while the DIO SCD rats compared to the DIO SFA weight gain was reduced ($p < 0.05$) from week twelve onwards. While FigureA (B) shows that the SCD fed rats had a significantly reduced weight gain (in grams) ($p < 0.05$) when compared to the DIO SFA from baseline (this could not be represented on the figure because of the positioning of the axis) through to week 15 of the six week feeding regime.

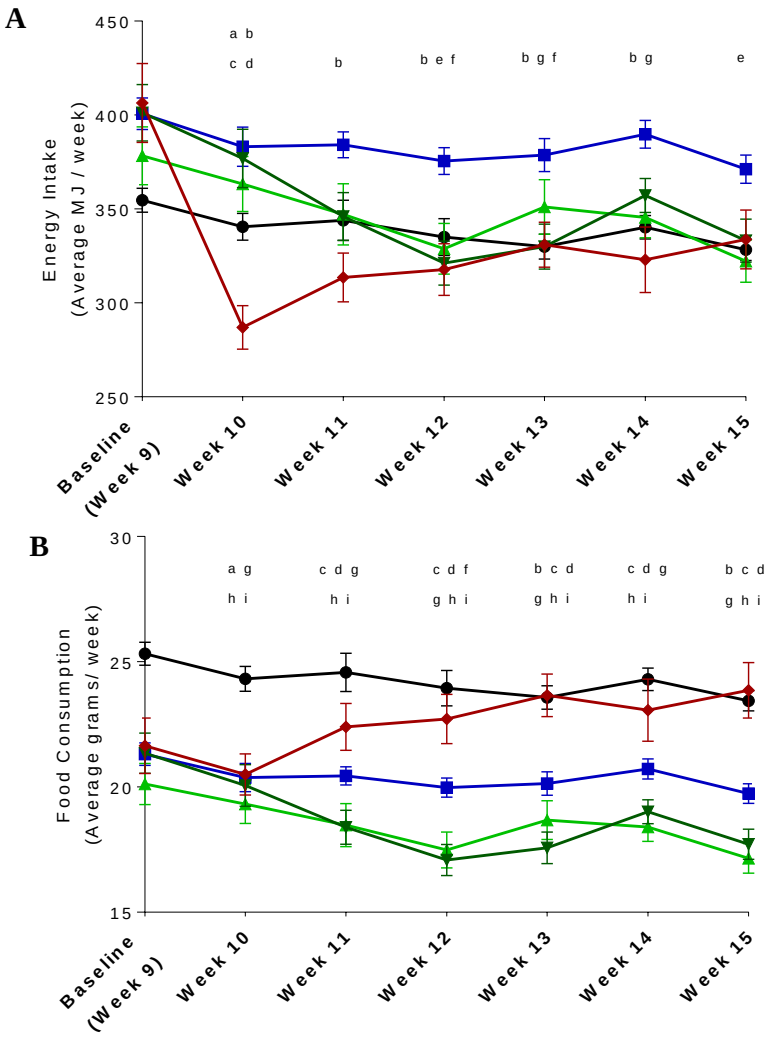

Figure 7.4B : Energy Intake and Food Consumption

(A) Food Consumption (average grams per week) (B) Energy Intake (average MJ per week) obtained from rats fed either a standard chow diet (SCD) for fifteen weeks (n = 9) or a high fat diet (HFD) for nine weeks to induced obesity (n = 39) then DIO rats were allocated into four dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA), HFD that predominately consisted of monounsaturated fatty acid (MUFA), HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data is reported as ± SEM, data is shown for the six week treatment period. SCD (n = 9) DIO SFA Diet (n = 10) DIO MUFA Diet (n= 10) DIO PUFA (n = 10) DIO SCD (n = 9).

Letters indicate a significant difference ($p < 0.05$) between groups [a] SCD vs DIO SCD, [b] DIO SFA vs DIO SCD, [c] DIO MUFA vs DIO SCD [d] DIO PUFA vs DIO SCD [e] DIO SFA vs MUFA [f] DIO SFA vs DIO PUFA [g] SCD vs DIO SFA [h] SCD vs DIO MUFA [i] SCD vs DIO PUFA.

The rats fed the SCD highlighted in Figure 7.4B (A) had a reduced energy intake ($p < 0.05$) compared with the DIO SCD rats at baseline prior to commencing the different feeding regime (this could not be represented on the figure because of the positioning of the axis), however, the energy intake for the SCD was not significantly different to the other rats fed a HFD at baseline. The energy intake data is quite variable in this figure and shows that the DIO SCD rats had a transient reduction in energy intake ($p < 0.05$) at week ten compared to the other four different dietary feeding groups, which increased ($p < 0.05$) at week eleven which was only different from the DIO SFA rats energy intake. At week twelve the DIO SFA group energy intake was increased ($p < 0.05$) compared to the DIO SCD, DIO MUFA and DIO PUFA groups, at week 13 the DIO SFA energy intake was higher ($p < 0.05$) than the SCD, DIO PUFA and DIO SCD rats, at week 14 the DIO SFA energy intake was higher ($p < 0.05$) than the SCD and DIO SCD and then at week 15 the DIO SFA was only increased ($p < 0.05$) compared to the DIO MUFA group.

The rats fed the SCD highlighted in Figure 7.4B (B) had an increased food intake (in grams) ($p < 0.05$) when compared to the other rats fed a HFD for nine weeks at baseline and prior to commencing their dietary feeding regimes (this could not be represented on figure because of the positioning of the axis). The food intake for the DIO SFA, DIO MUFA and DIO PUFA feeding groups remained decreased ($p < 0.05$) when compared to the SCD group for the entire six week feeding regime. From week eleven and onwards the food intake for the DIO SCD group increased ($p < 0.05$) to a similar food intake of the SCD fed rat group, which was increased ($p < 0.05$) compared to the DIO MUFA and DIO PUFA groups at week 11. When the food intake of DIO SCD fed group was compared to the DIO SFA group the data was more variable, there was a trend for the DIO SCD to increase food consumption from week 11 onwards (p 0.29), but was significantly increased ($p <0.05$) at week 12, 13 and 15 but not week 14.

When observing the food intake (in grams) for the three groups of rats consuming the different dietary fatty acid diets and the DIO SCD and SCD was included in the analysis there was a trend for the DIO

PUFA group to be decreased when compared to the DIO SFA group, and the food intake was decrease at the week 12 time point only ($p < 0.05$). Interestingly, however, when the food intake for the three different dietary fatty acid diet groups were analysed excluding the SCD or the DIO SCD rats food intake (this is an statistical analysis observation and is not included on the figure) the DIO SFA group's food intake was increased ($p < 0.05$) from week 11 onwards compared to DIO MUFA and DIO PUFA groups.

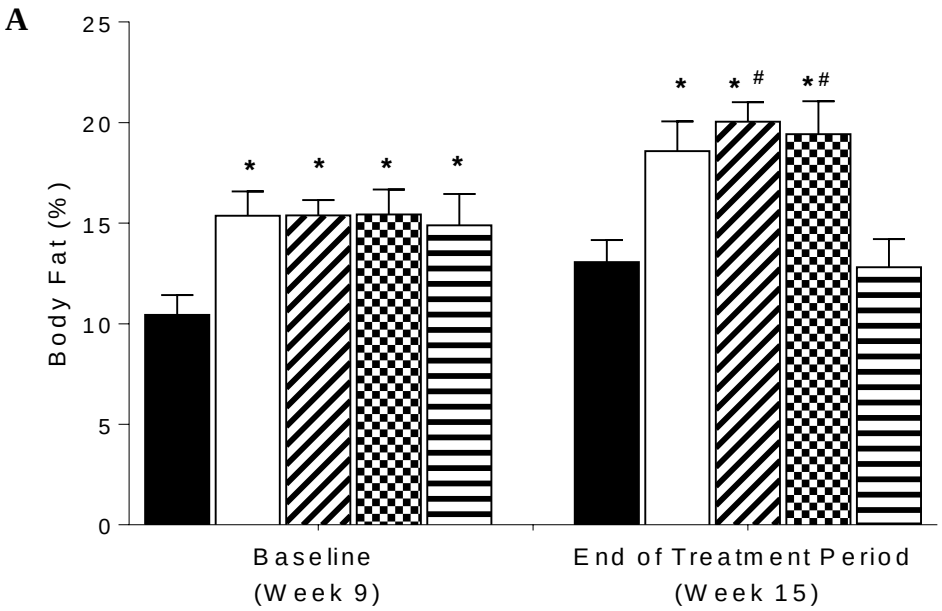

Figure 7.4C: Body Fat Percentage

(A) Body fat (%) obtained from rats fed either a standard chow diet (SCD) for fifteen weeks (n = 9) or a high fat diet (HFD) for nine weeks to induced obesity (n = 39) then DIO rats were allocated into four dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA), a HFD that predominately consisted of monounsaturated fatty acid (MUFA), a HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data is reported as ± SEM, data is shown for baseline and week fifteen measurements only. ■ SCD (n = 9) ▭ DIO SFA Diet (n = 10) ▨ DIO MUFA Diet (n= 10) ▬ DIO PUFA (n = 10) ▯▯▯ DIO SCD (n = 9 - 10). * indicates a significant difference between the SCD group ($p < 0.05$) # indicates a significant difference between the DIO SCD group ($p < 0.05$).

Figure 7.4C (A) shows that at baseline in the DIO rats (that had commenced the HFD for nine weeks) had an increased body fat (%) when compared to the rats fed a SCD ($p < 0.05$). Following the different feeding regimes the body fat (%) of the DIO SFA, DIO MUFA and DIO PUFA groups remained increased when compared to the SCD group. The body fat % of the DIO SCD decreased to a similar group mean value when compared to the SCD fed rats at the end of the dietary feeding regime. The body fat (%) of the DIO SCD group was significantly decreased ($p < 0.05$) when compared to the DIO MUFA and DIO PUFA fed groups but was not statistically significant when compared to the DIO SFA group.

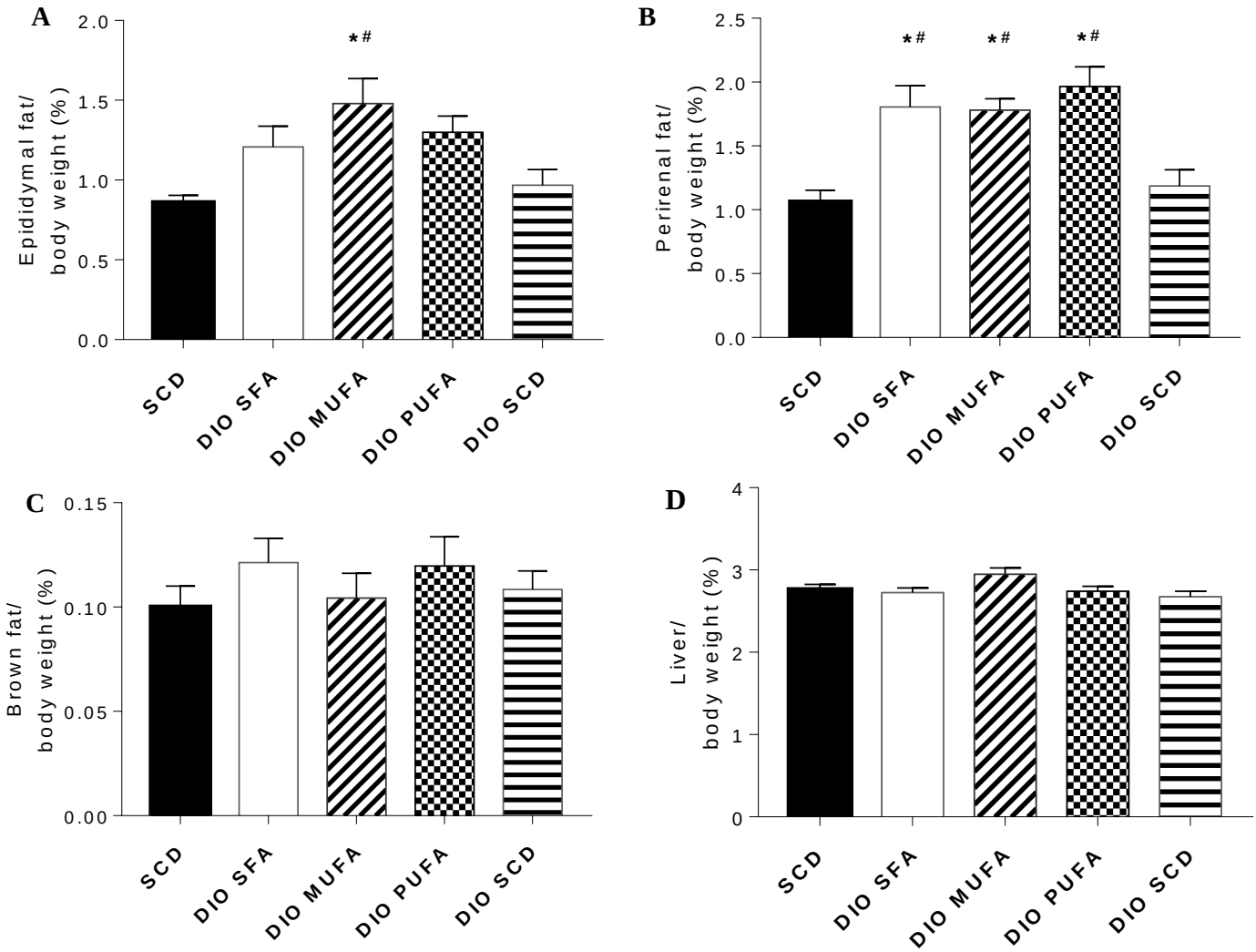

Figure 7.4D: Fat Pad Weights

(A) Epididymal fat weight/ body weight (%) (B) Peri-renal fat weight/ body weight (%) (C) Brown fat weight/ body weight (D) Liver weight/ body weight (%) obtained from rats fed either a standard chow diet (SCD) for fifteen weeks (n = 9) or a high fat diet (HFD) for nine weeks to induced obesity (n = 39) then DIO rats were allocated into four dietary feeding regimes. The different dietary feeding regimes included:a HFD that predominately consisted of saturated fatty acid (SFA), a HFD that predominately consisted of monounsaturated fatty acid (MUFA), a HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data is reported as ± SEM. ■ SCD (n =9) ▭ DIO SFA Diet (n = 10) ▨ DIO MUFA Diet (n= 10) ▬ DIO PUFA (n = 10) ▯▯▯ DIO SCD (n = 8 - 9). * indicates a significant difference between the SCD group ($p < 0.05$) # indicates a significant difference between the DIO SCD group ($p < 0.05$).

Figure 7.4D (A – D) shows that the Epididymal fat depots (Epididymal fat/ body weight %) were increased in the DIO MUFA group when compared to the SCD ($p < 0.05$) and the DIO SCD ($p < 0.05$) fed rats. Perirenal fat depots (Perirenal fat pads/ body weight %) collected from the DIO SFA, DIO MUFA and DIO PUFA were increased ($p < 0.05$) when compared to the SCD and DIO SCD fed rats. The BAT depots (BAT/ body weight %) and liver (liver/ body weight %) were unchanged between all five different feeding regimes.

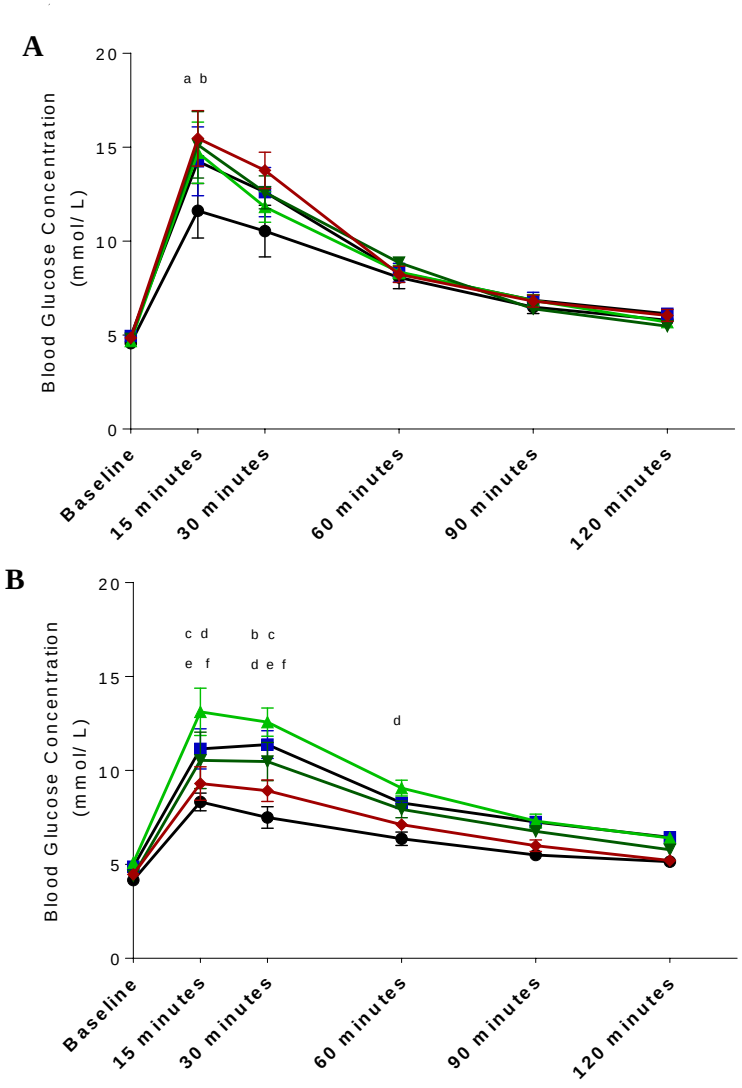

Figure 7.4E: Intraperitoneal Glucose Tolerance Test (ip. GTT)

(A) Pre- dietary feeding regime ip. GTT blood glucose concentration (mmol/ L) (week 8) (B) During the different dietary feeding regime (week fourteen) ip. GTT blood glucose concentrations (mmol/ L) obtained from rats fed either a standard chow (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induce obesity. The DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes include a HFD that predominately consisted of saturated fatty acids (SFA), a HFD that predominately consisted of monounsaturated fatty acids (MUFA), a HFD that predominately consisted of polyunsaturated fatty acids (PUFA) or recommenced a SCD. Grouped data is reported as ± SEM. SCD (n = 8 – 9) DIO SFA Diet (n = 10) DIO MUFA Diet (n = 10) DIO PUFA (n = 10) DIO SCD (n = 9 – 10). Letters indicate a significant difference ($P < 0.05$) between groups [a] SCD vs DIO SCD, [b] SCD vs DIO PUFA, [c] SCD vs DIO SFA [d] SCD vs DIO MUFA [e] DIO MUFA vs DIO PUFA [f] DIO MUFA vs DIO SCD [g] DIO SFA vs DIO SCD.

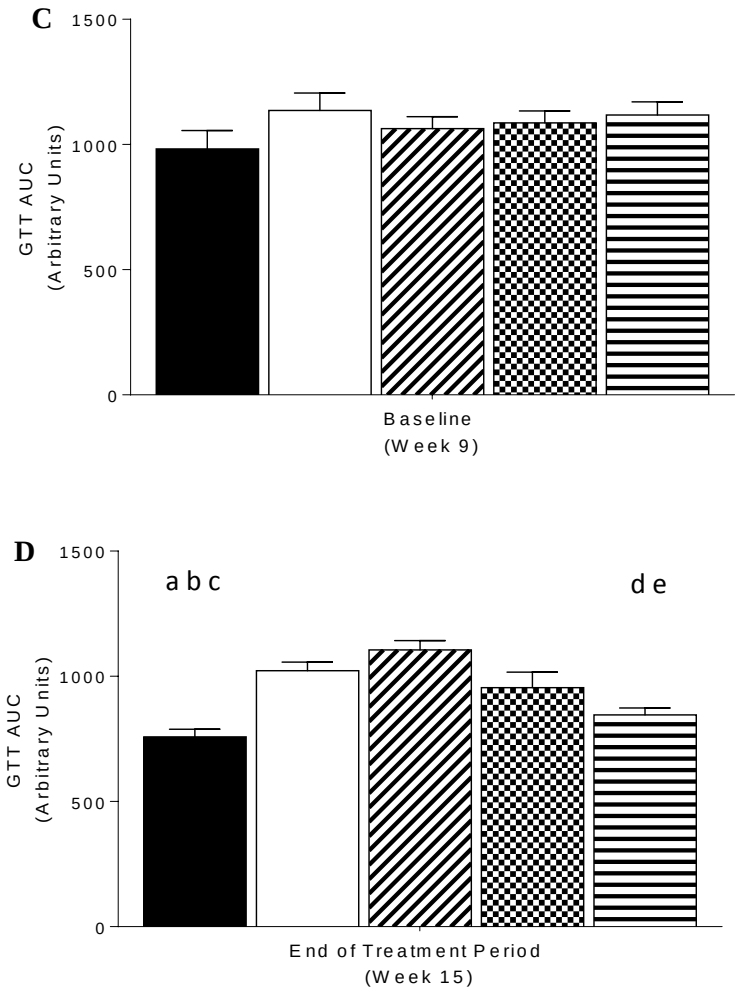

Figure 7.4E: Intraperitoneal Glucose Tolerance Test (ip. GTT)

(C) Pre- dietary feeding regime ip. GTT AUC (arbitrary units) (week 8) (D) During- the different dietary feeding regime ip. GTT AUC (arbitrary units) (week 14) obtained from rats fed either a standard chow (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induce obesity. The DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes include a HFD that predominately consisted of saturated fatty acids (SFA), a HFD that predominately consisted of monounsaturated fatty acids (MUFA), a HFD that predominately consisted of polyunsaturated fatty acids (PUFA) or recommenced a SCD. Grouped data is reported as ± SEM. ■ SCD □ DIO SFA Diet ▨ DIO MUFA Diet ▤ DIO PUFA ▥ DIO SCD. [a] SCD vs DIO SFA, [b] SCD vs DIO MUFA, [c] SCD vs DIO PUFA [d] DIO SFA vs DIO SCD [e] DIO MUFA vs DIO SCD.

Figure 7.4E (A) highlights the ip. GTT results from week eight which is the baseline data prior to to when the rats commenced the different dietary feeding regimes.This figure shows that there were no differences between the rats fed the SCD compared to the rats fed the HFD at baseline prior to

administration of the glucose load. At the 15 minute time point post ip. glucose administration the HFD fed rats that were going to be allocated to the DIO PUFA and DIO SCD had higher ($p < 0.05$) blood glucose concentrations than the SCD fed rats. The following time points of 30, 60, 90 and 120 minutes had no difference in blood glucose concentrations between the five groups. Figure 7.4E (B) highlights the ip. GTT results from week 14 and the data shows that the fasting blood glucose concentrations for all of the different dietary feeding regimes were not different at baseline prior to receiving the ip. glucose administration. 15 minutes post glucose administration DIO SFA and DIO MUFA blood glucose concentrations were increased ($p < 0.05$) when compared to the SCD group, the DIO MUFA was also increased ($p < 0.05$) compared to the DIO PUFA and DIO SCD. At 30 minutes the DIO SFA, DIO MUFA and DIO PUFA blood glucose concentration were all elevated ($p < 0.05$) compared to the SCD group of rats, the DIO SCD was reduced ($p < 0.05$) compared to the DIO SFA and DIO MUFA. At 60 minutes the DIO MUFA blood glucose concentrations remained higher ($p < 0.05$) than the SCD group and from 90 minutes onwards there was no difference between blood glucose concentrations between the different dietary feeding regimes.

Figure 7.4E (C) highlights that the ip. GTT results from week eight (baseline which is prior to when the rats commensed the different dietary feeding regimes) and the results show that there were no differences in blood glucose concentrations when the GTT data was presented as AUC between any of the five dietary groups. Figure 7.4 (D) highlights that the ip.GTT results from week 14 (following five weeks of consuming the different dietary feeding regimes) and the results show that the SCD fed rats had a reduced AUC for blood glucose concentrations (arbitrary units) ($p < 0.05$) when compared with the DIO SFA, MUFA and PUFA groups. The DIO SCD fed rats had a reduced AUC for blood glucose concentrations (arbitrary units) ($p < 0.05$) when compared with the DIO SFA and DIO MUFA groups.

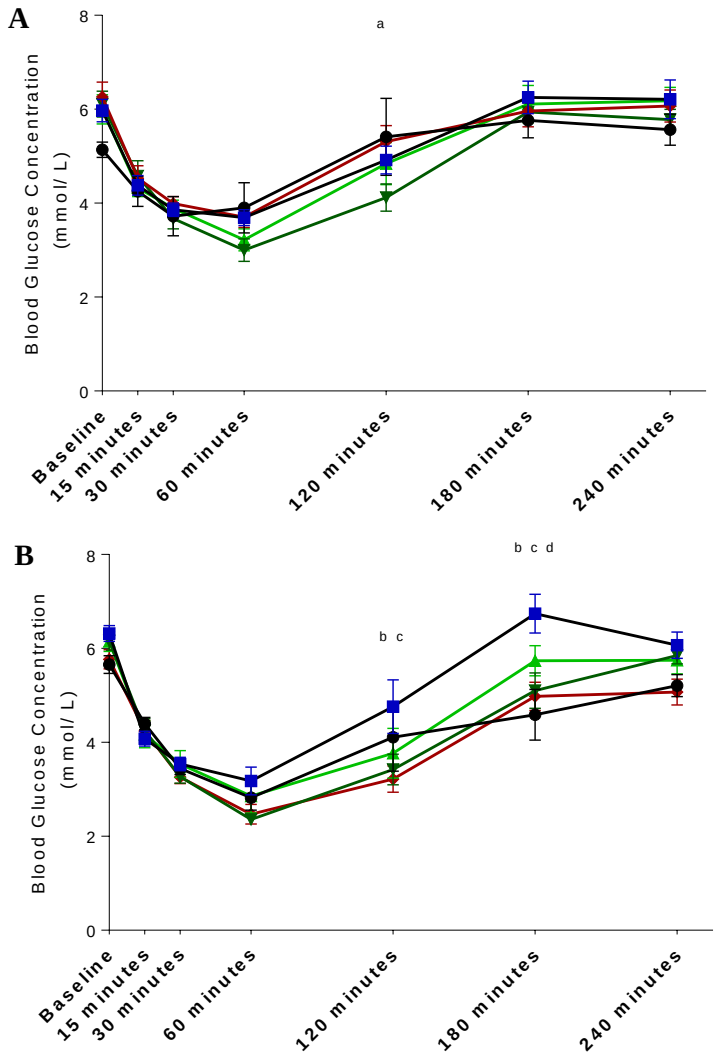

Figure 7.4F: Intraperitoneal Insulin Sensitivity Test (ip. IST)

(A) Pre- dietary feeding regime ip. IST blood glucose concentration (mmol/ L) (week 8) (B) During-dietary feeding regime (week 14) ip. IST blood glucose concentration (mmol/ L) obtained from rats fed either a standard chow (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induce obesity. The DIO rats were then allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acids (SFA), a HFD that predominately consisted of monounsaturated fatty acids (MUFA), a HFD that predominately consisted of polyunsaturated fatty acids (PUFA) or re-commenced a SCD. Grouped data is reported as ± SEM. SCD (n = 9) DIO SFA Diet (n = 10) DIO MUFA Diet (n = 10) DIO PUFA (n = 10) DIO SCD (n = 10). Letters indicate a significant difference ($P < 0.05$) between groups. [a] SCD and DIO PUFA, [b] DIO SFA and DIO SCD, [c] DIO SFA and DIO PUFA, [d] SCD and DIO SFA.

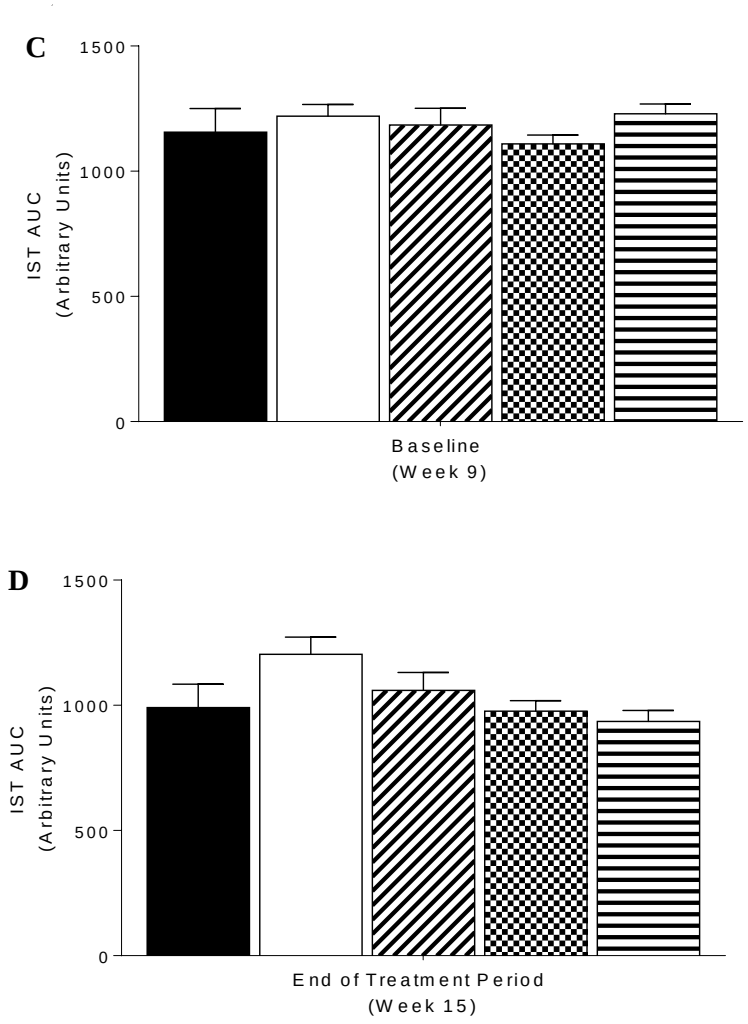

Figure 7.4F: Intraperitoneal Insulin Sensitivity Test (ip. IST)

(C) Pre- dietary feeding regime ip. IST AUC (arbitrary units) (week 8) (D) During- the different dietary feeding regime ip. IST AUC (arbitrary units) (week 14) obtained from rats fed either a standard chow (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induce obesity. The DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes include a HFD that predominately consisted of saturated fatty acids (SFA), a HFD that predominately consisted of monounsaturated fatty acids (MUFA), a HFD that predominately consisted of polyunsaturated fatty acids (PUFA) or recommenced a SCD. Grouped data is reported as ± SEM. ■ SCD □ DIO SFA Diet ▨ DIO MUFA Diet ▭ DIO PUFA ▯ DIO SCD.

Figure 7.4F (A) highlights the ip. IST results from week eight which is the baseline data prior to when the rats commenced the different diet feeding regimes, this figure shows that there were no statistical differences between the rats fed the SCD compared to the rats fed the HFD at baseline prior to the administration of insulin. At the 15, 30 and 60 minute time points there were no differences in blood glucose concentrations between the SCD or HFD rats. At 120 minutes there was a transient increase ($p < 0.05$) in the blood glucose concentrations for the SCD compared to the DIO PUFA rats, then for the 180 and 240 minute time points there were no significant differences between groups. Figure 7.4F (B) highlights the ip. IST results from week 14, the data shows that the fasting blood glucose concentrations that were obtained from the rats on the different dietary feeding regimes were not different at baseline prior to receiving the administration of insulin. The blood glucose concentrations were also not altered at 15, 30 and 60 minutes post insulin administration. At the 120 and 180 minute time points the blood glucose concentrations for the DIO SFA was transiently increased ($p < 0.05$) when compared to the DIO MUFA and DIO PUFA groups, as well as being increased ($p < 0.05$) compared to the SCD group at 180 minutes also.

Figure 7.4F (C) highlights the ip. IST results from week eight (baseline which is prior to when the rats commensed the different dietary feeding regimes) and Figure 7.4 (D) highlights the ip.IST results from week 14 (following five weeks of consuming the different dietary feeding regimes) and the results show that there were no differences in blood glucose concentrations when the IST data was presented as AUC between any of the five dietary groups for either pre or post dietary feeding regime.

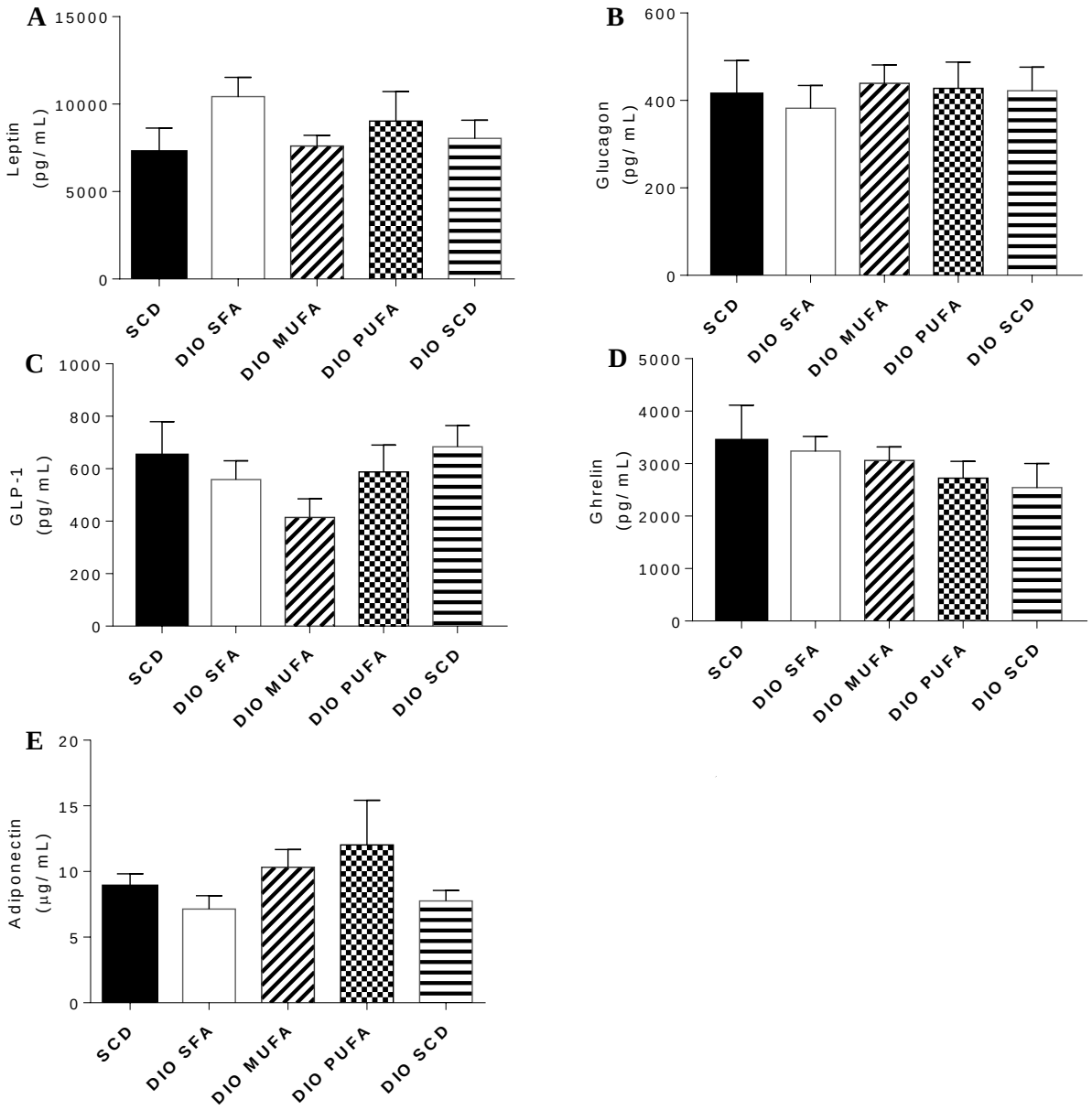

Figure 7.4G: Plasma Hormone Profiles

Plasma concentrations of hormones including; (A) Leptin; (B) Glucagon; (C) Glucagon Like Peptide-1 (GLP-1); (D) Ghrelin; (E) Adiponectin. Plasma was obtained from rats fed either a standard chow diet (SCD) for fifteen weeks (n = 7 - 9) or a high fat diet (HFD) for nine weeks to induced obesity (n = 39) then the DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA) (n = 9 - 10), a HFD that predominately consisted of monounsaturated fatty acid (MUFA) (n = 9 - 10), a HFD that predominately consisted of polyunsaturated fatty acid (PUFA) (n = 4 - 10) or recommenced a SCD (n = 4 - 9). Grouped data for each of the Bioplex analytes µg/ mL was reported as ± SEM. ■ SCD □ DIO SFA ▨ DIO MUFA ▭ DIO PUFA ▯ DIO SCD.

Figure 7.4G shows that the concentrations of circulating hormones involved in appetite, blood glucose regulation and insulin sensitivity (including leptin, glucagon, GLP-1, ghrelin and adiponectin) were not altered in DIO rats allocated different dietary compositions diets that were predominately composed of either SFA, MUFA or PUFA. Further the concentrations of these hormones for the SCD control group compared to any of the DIO different composition fatty acids diets were also not altered between groups.

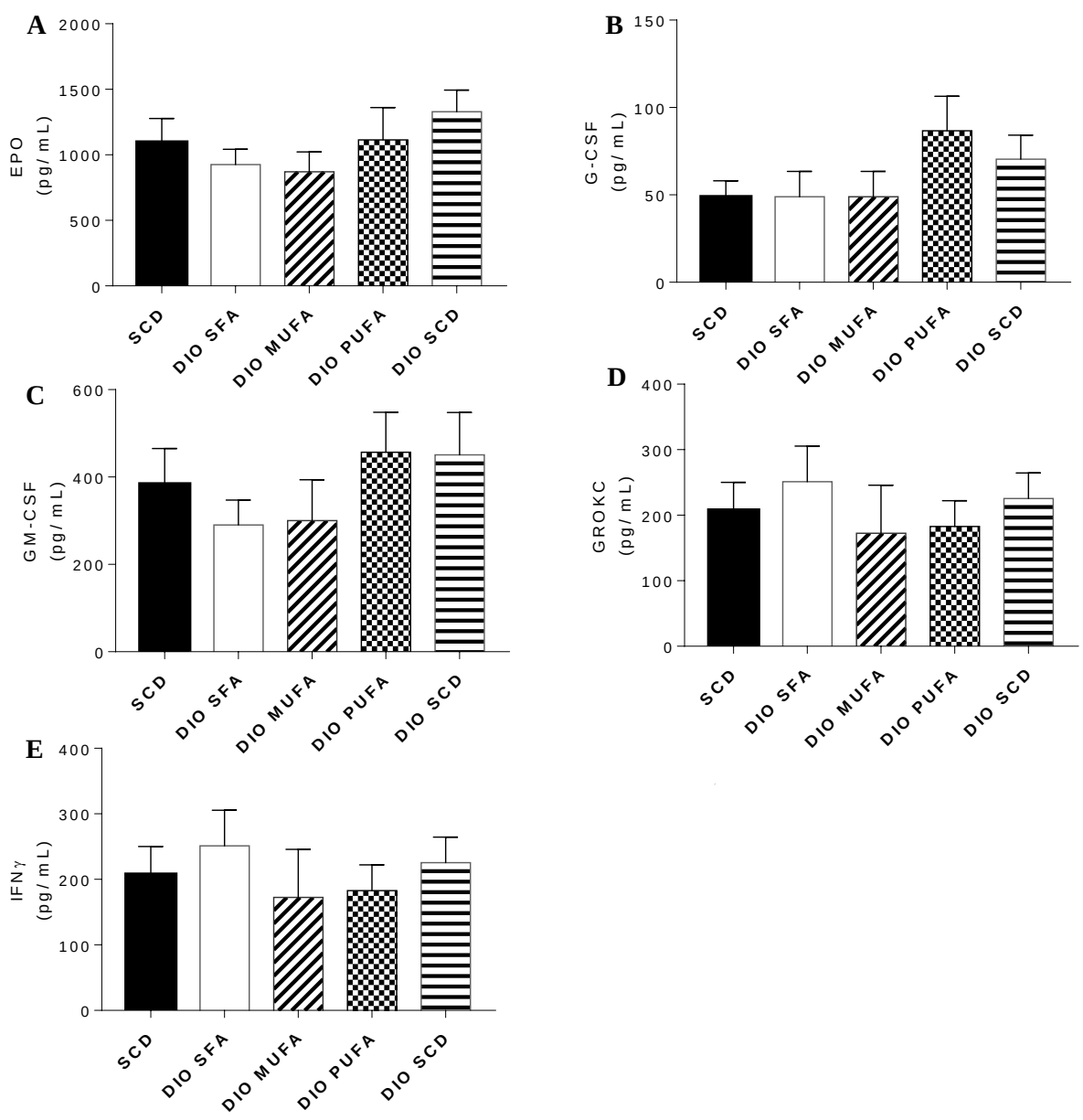

Figure 7.4H: Plasma Cytokine Profiles

Plasma Concentrations of cytokines including: (A) Erythropoietin (EPO); (B) Granulocyte Colony Stimulating Factor (G-CSF); (C) Granulocyte Macrophage Colony Stimulating Factor (GM-CSF); (D) Growth Regulated Oncogene/ Keratinocyte Chemo-attractant (GRO/ KC); (E) Interferon gamma (IFN-γ). Plasma was obtained from rats fed either a standard chow diet (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induced obesity, then the DIO rats were allocated into four dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA), a HFD that predominately consisted of monounsaturated fatty acid (MUFA), a HFD predominately consisting of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data for each Bioplex analyte pg/ mL was reported as ± SEM. ■ SCD (n = 7 - 9) ▭ DIO SFA (n = 8 - 10) ▨ DIO MUFA (n = 8 - 10) ▬ Diet DIO PUFA (n = 7 – 9) ▮▮ DIO SCD (n = 7 – 9).

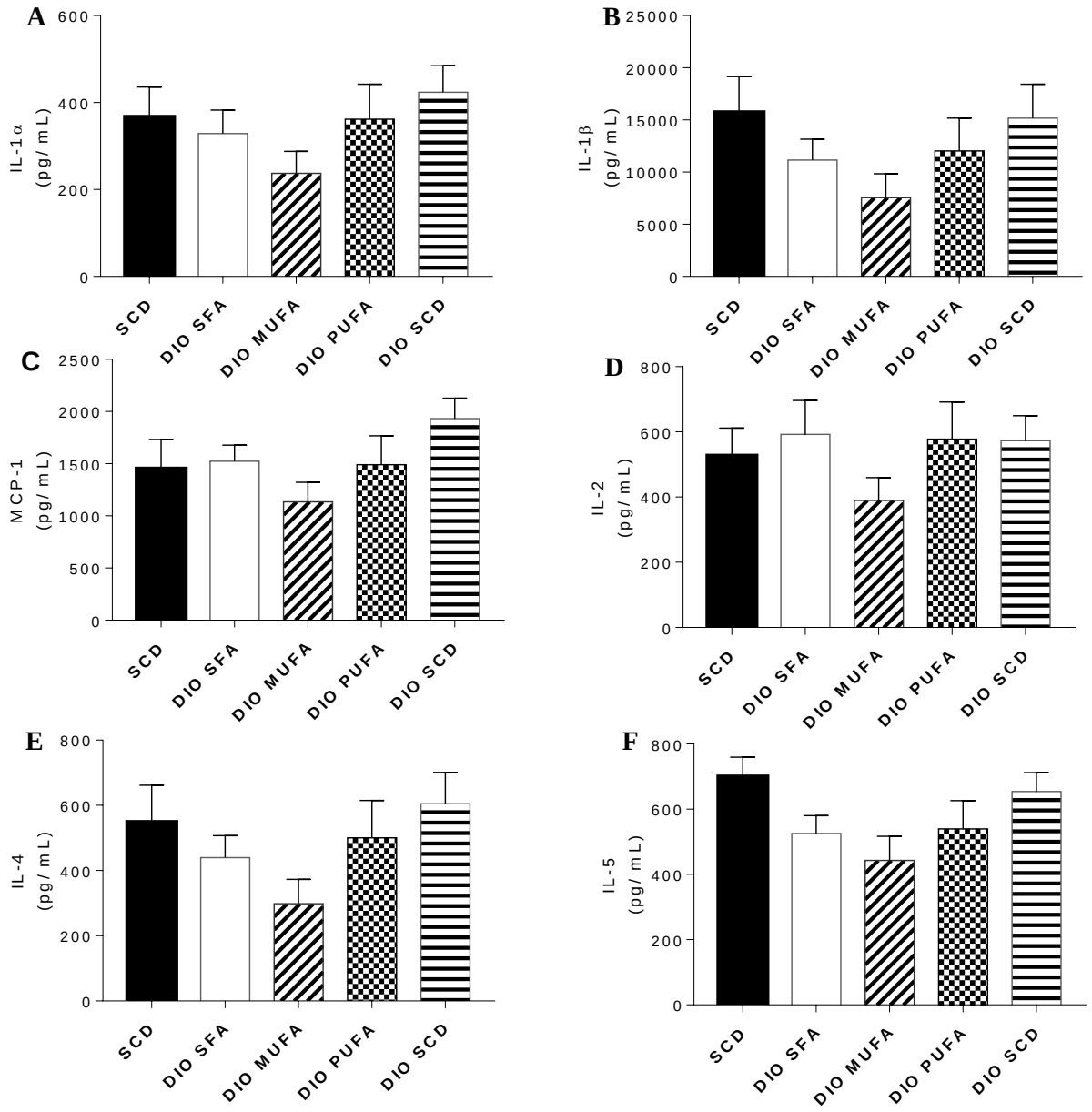

Figure 7.4I Plasma Cytokine Profiles

Plasma concentration of cytokines including: (A) Interleukin 1α (IL-1α); (B) Interleukin 1β (IL-1β); (C) Monocyte Chemo-attractant Protein-1 (MCP-1); (D) Interleukin 2 (IL-2); (E) Interleukin 4 (IL-4); (F) Interleukin 5 (IL-5). Plasma was obtained from rats fed either a standard chow diet (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induced obesity, then the DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acids (SFA), a HFD that predominately consisted of monounsaturated fatty acids (MUFA), a HFD that predominately consisted of polyunsaturated fatty acids (PUFA) or re-commenced a SCD. Grouped data for each Bioplex analyte pg/ mL was reported as ± SEM. ■ SCD (n = 6 - 7) ▭ DIO SFA (n = 10) ▨ DIO MUFA (n = 9 - 10) ▬ DIO PUFA(n = 9) ▮▮ DIO SCD (n = 9).

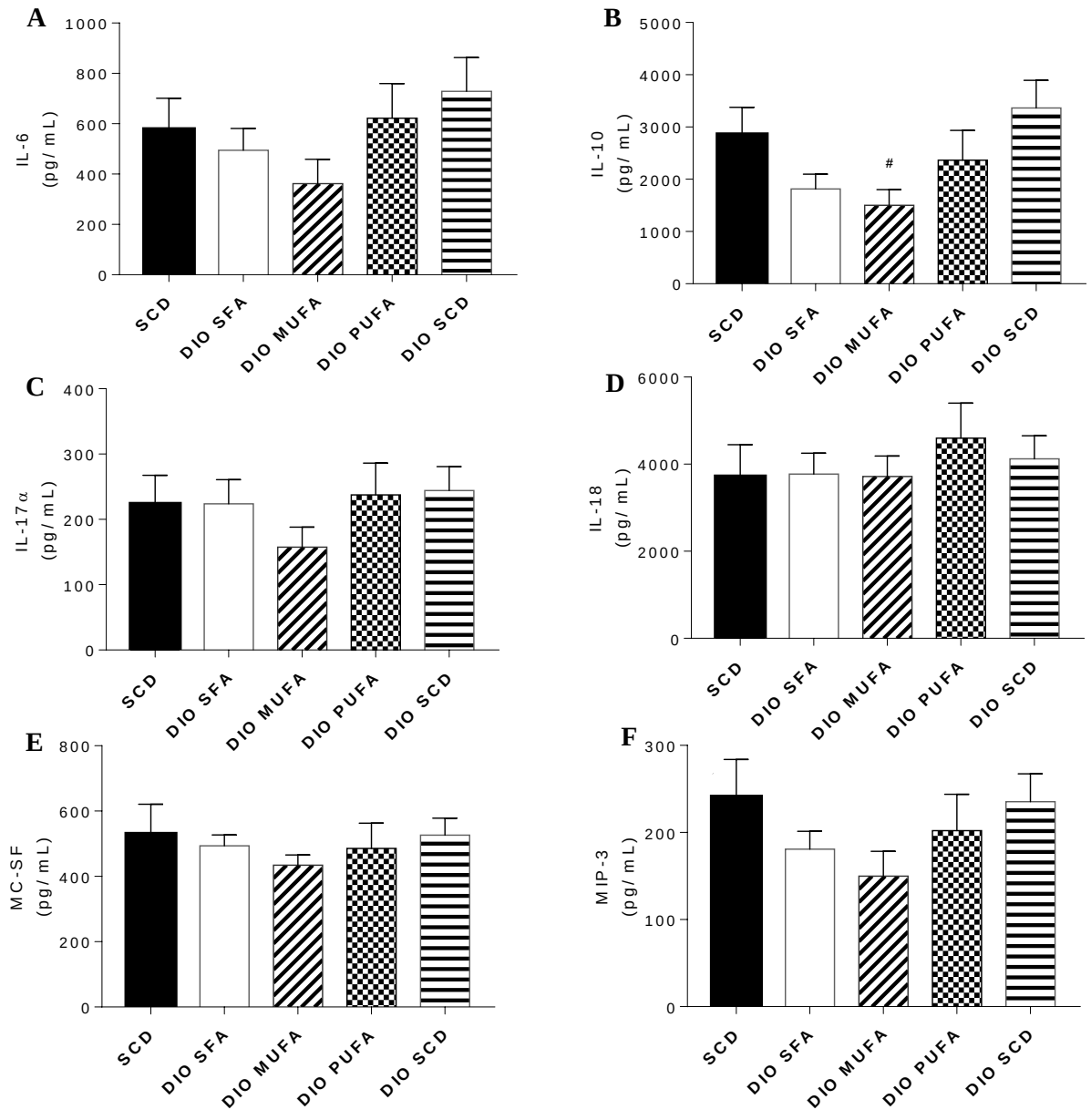

Figure 7.4J: Plasma Cytokine Profiles

Plasma concentration of cytokines including: (A) Interleukin 6 (IL-6); (B) Interleukin 10 (IL-10); (C) Interleukin 17α (IL-17α); (D) Interleukin 18 (IL-18); (E) Macrophage Stimulating Factor (MC-SF); (F) Macrophage Inflammatory Protein (MIP-3). Plasma was obtained from rats fed either a standard chow diet (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induced obesity then the DIO rats were allocated into four different dietary feeding regimes for a further six weeks. The different dietary feeding regimes included: a HFD that predominately consisted of saturated fatty acid (SFA), a HFD that predominately consisted of monounsaturated fatty acid (MUFA), a HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or re-commenced a SCD. Grouped data for each Bioplex analyte μg/ mL was reported as ± SEM. ■ SCD (n = 7) ▭ DIO SFA (n = 10) ▨ DIO MUFA (n = 9 -10) ▬ DIO PUFA (n = 8 – 9) ▯▯▯ DIO SCD (n = 9). # indicates a significant difference to DIO SCD ($P < 0.05$).

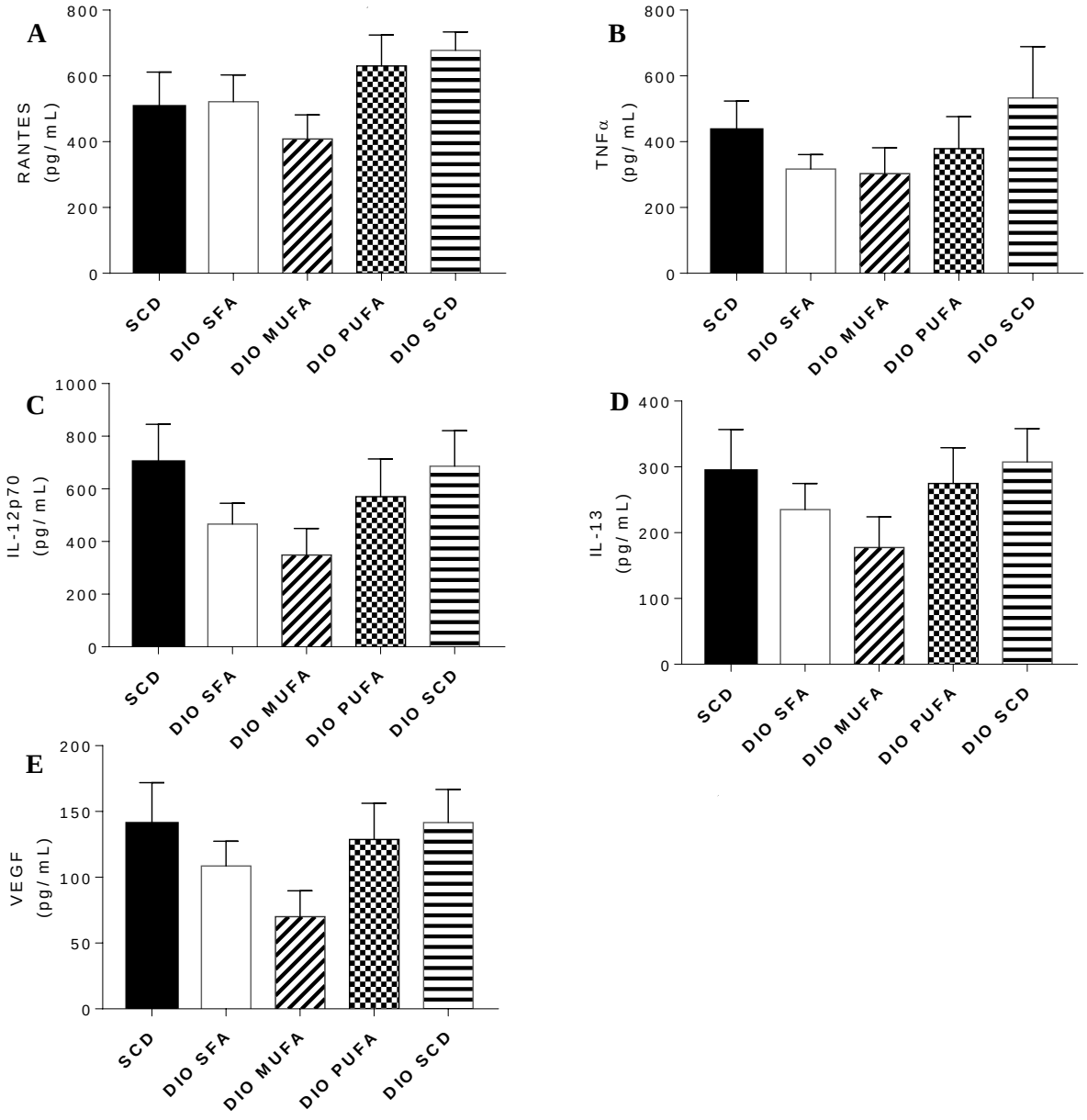

Figure 7.4K: Plasma Cytokine Profiles

Plasma concentration of cytokines including: (A) Regulated on Activation Normal T-Cells Expressed and Secreted (RANTES); (B) Tumour Necrosis Factor Alpha (TNFα); (C) Interleukin 12p70 (IL-10); (D) Interleukin 13 (IL-13); (E) Vascular Endothelial Growth Factor (VEGF). Plasma was obtained from rats fed either a standard chow diet (SCD) for fifteen weeks or a high fat diet (HFD) for nine weeks to induced obesity, then the DIO rats were allocated into four different dietary feeding regimes for a further six week. The different dietary feeding regimes including: a HFD that predominately consisted of saturated fatty acid (SFA), a HFD that predominately consisted of monounsaturated fatty acid (MUFA), a HFD that predominately consisted of polyunsaturated fatty acid (PUFA) or recommenced a SCD. Grouped data for each Bioplex analyte μg/ mLwas reported as ± SEM. ■ SCD (n = 7) ▭ DIO SFA (n = 10) ▨ DIO MUFA (n = 9 -10) ▤ DIO PUFA (n = 8 – 9) ▥ DIO SCD (n = 9) .

Figure 7.4J (B) shows that the circulating plasma IL-10 concentrations were significantly reduced ($p < 0.05$) for the DIO-MUFA fed rats when compared to the DIO-SCD fed rats. Aside from the finding regarding plasma IL-10 concentrations, circulating plasma cytokine concentrations presented in figures 7.4H – 7.4K including (EPO, G-CSF, GM-CSF, GRO/ KC, IFN-γ, IL-1α, IL-1β, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-17α, IL-18, MC-SF, MIP-3, RANTES, TNFα, IL-12p70, IL-13 and VEGF) were not significantly altered between any of the different feeding regimes (SCD, DIO SFA, DIO MUFA, DIO PUFA or DIO SCD).

7.5 Discussion

This study focuses on the effect that HFDs composed of different dietary fatty acids (specifically palmitate and steric acid (SFA), oleic acid (MUFA) or linoleic acid (PUFA)) or re-commencing a SCD (that is reduced in fat compared with the HFD) has on the DIO state while focusing on markers of whole body energy homeostasis.

As would be expected, given that the SCD was less energy dense than the HFDs, the DIO SCD had significantly reduced body weight gain (%) when compared to the other DIO rats continuing on the different dietary fatty acid HFDs. Surprisingly however, statistically the SCD fed rats had the same rate of weight gain as the rats continuing on the HFD but the mean average weight gain (%) was lower. These results conflict with other published findings, Marques et al. (2016) found male Sprague Dawley rats that consumed a HFD for seven weeks caused an increase in weight gain (in grams) when compared to the SCD fed rats (Marques, Meireles et al. 2016). However, in support of the study by Marques et al (2016) the SCD fed rats from the study presented in *Chapter Seven* of this thesis did have reduced body weight (in grams) when compared to the DIO SFA rats (from the baseline of DIO (week 9) which continued to differ for the entire six week feeding regime), the only concern being that the other four HFD groups of rats (DIO MUFA, DIO PUFA and DIO SCD) were statistically considered to be the same weight (in grams) when compared to SCD despite all of the groups of DIO rats consuming the same diet up until baseline of DIO. The rats consuming the different dietary fatty acid HFDs throughout the study presented in this thesis had a lower intake of average daily food consumption (in grams) when compared with the SCD fed diet group. This is consistent with other studies findings in mice and Sprague Dawley rats fed a HFD, as these rodents have a lower daily food intake compared to SCD (Lindqvist, de la Cour et al. 2005; Sugiishi, Kimura et al. 2013). So it is not surprising that once the DIO SCD group commenced the SCD the food intake for this group was increased by the third week when on this feeding regime. Further, the energy intake data presented in this thesis shows that for the first five weeks of the different dietary feeding regimes the DIO SFA

group of rats had a higher energy intake when compared to DIO SCD group, but was not statistically different during the last week of the feeding regime. The DIO SFA group did have a higher energy intake in the last week of the feeding regime when compared to DIO MUFA group.

The body fat (%) of the rats fed a SCD were reduced at baseline compared to the rats fed a HFD (at baseline of DIO prior to commencing the different dietary fatty acid/ SCD feeding regime),which indicates that the rats on the different HFDs were all obese at baseline. Following the six week feeding regime of the different diets the body fat (%) for the DIO SFA, DIO MUFA and DIO PUFA remained increased however, the body fat (%) of the DIO SCD was significantly reduced compared to the DIO MUFA and DIO PUFA groups but not the DIO SFA, even despite the DIO SFA rats having an increased energy intake which could suggest that the DIO SFA group were mainly the obese resistant phenotype of Sprague Dawley rat.

The data in this chapter shows that the two WAT depots (compared to body weight %) were not altered. The epididymal fat pad weights (compared to body weight %) were surprisingly increased in the DIO MUFA group compared to the SCD and DIO SCD fed rats but were not statistically different compared to the DIO SFA or the DIO PUFA group of rats. While the perirenal fat pads were increased for all three different HFD groups compared to the SCD or the DIO SCD groups, this data indicates that there is preferential fat storage dependent of the dietary fatty acid consumed as the MUFA group of rats had storage is both tissue depots. While the study presented in this chapter did not determine the effect that fish oils had on obesity, the study by Hill et al, (1993) found that following three months of consuming the same HFD (that was high in fish oil), only then did these fatty acids have an effect on fat pad mass by reducing both peri-renal and epididymal fat pad mass when compared to diets high in fat derived from beef tallow and corn oil (Hill, Peters et al. 1993). Therefore, the shorter duration of time for the feeding regime that was used as the protocol of the study described in this thesis could play a part in the fact that changes in the peri-renal fat pad mass were not observed between the three different dietary fatty acid feeding groups. The fact that the fat pad weights were similar between the different

HFD groups could be due to the duration of the feeding regime used in this study. Alsaif & Duwaihy (2004) supplemented Wister rats with either a 10% or 20% concentration of different composition dietary fatty acids (either olive oil, corn oil and butter) into their dietsfor a slightly shorter duration of five weeks when compared to the study in this chapter which was for six weeks. In support of the findings in this thesis total VAT was increased in the higher % olive oil supplementation group when compared to the SCD fed rats (Alsaif 2004). However, the corn oil group had lower VAT when compared to the high olive oil and butter groups (Alsaif 2004). While the data in this thesis showed that the peri-renal fat pads between groups was not altered between the different dietary fatty acid feeding regimes, this may be attributed to a different rat strain and the fact that a SCD rather than a HFD was supplemented with these different dietary fatty acids.

The fasting blood glucose concentrations of the HFD or SCD (at DIO baseline), suggests that the rats were not glucose intolerant. However, 15 minutes post administration of glucose for the ip. GTT, the DIO SCD and DIO PUFA had high blood glucose concentrations when compared to the SCD group of rats, however, this was not evident for any of the other time points during the ip. GTT. Following the different dietary feeding regimes for five weeks, the SCD had a lower response to glucose in the ip. GTT at the 15 minute time point when compared to four other DIO rats following the different feeding regimes, but surprisingly the DIO MUFA group continued to have an elevated blood glucose response until the 90 minute time point. Interestingly, the DIO rats that consumed either the SFA, MUFA or PUFA diets did not have any variation in blood glucose responses for the ip. GTT (post dietary feeding regime), suggesting that consuming a HFD composed of different fatty acids diet does not have an impact on glucose tolerance. The response observed in these rats is consistent with observations in mice fed high fat MUFA diet when compared to a high CHO diet, in which there were increases in obesity, hyperglycaemia and triglyceride accumulation in the liver and skeletal muscle in these animals (Tsunoda, Ikemoto et al. 1998).

The Mediterranean diet is high in MUFA, specifically oleic acid, which is derived from olive oil (Visioli, Galli et al. 2000). Epidemiological evidence indicates that the Mediterranean diet which high in MUFA is associated with low prevalence of chronic disease (Keys, Menotti et al. 1986). Research has shown that healthy subjects and individuals predisposed to insulin resistance have improvements in glycaemic response and insulin sensitivity when SFA are replaced with MUFA. Further, insulin resistant subjects consuming a MUFA rich diet had improved insulin sensitivity compared to the SFA rich diet (Paniagua, de la Sacristana et al. 2007). The data in this thesis conflicts with these findings and shows that following the different dietary feeding regimes that the response to insulin in the DIO SFA group rats was less responsive at 120 and 180 minutes compared to the DIO PUFA and DIO SCD following administration of bolus of insulin and 180 minutes only for the SCD but not the DIO MUFA. The DIO MUFA group did follow a similar trend in blood glucose response to the DIO PUFA, DIO SCD and SCD but this was not significant. However, the data in this thesis is supportive of the fact that dietary PUFA may reduce the risk for the development of T2DM while the SFA and MUFA do not appear to be associated with diabetes risk (Salmeron, Hu et al. 2001). Additionally, while palmitic acid was the major component of the high fat SFD used in this study (5.57%), it also comprised of (3.61%) steric acid. Steric acid has been shown to have hypolipidemic effects when compared with other SFAs such as palmitic and lauric acid. Steric acid does not impair glucose tolerance and insulin sensitivity in healthy woman (Louheranta, Turpeinen et al. 1998).

Surprisingly, the circulating hormones quantified in this chapter of the thesis including: leptin, glucagon, GLP-1, ghrelin or adiponectin were not altered as a result of the different dietary feeding regimes. One heavily researched hormone in the field of obesity is the satiety inducing hormone leptin and this hormone has been discussed previously in the results of *Chapters Four and Five*. As mentioned earlier, the circulating concentration of this hormone is reflective of body adipose tissue stores (Considine, Sinha et al. 1996; Rosenbaum, Nicolson et al. 1996), while adiponectin is a hormone that has an anti-inflammatory role and promotes fatty acid oxidation (Ouchi, Kihara et al. 2003) that

is reduced in humans in obesity (Arita, Kihara et al. 1999). The data in this chapter found no difference in the circulating leptin concentrations between the SCD or the DIO SCD in comparison to the DIO different dietary fatty acid groups which would have been expected. However, data from a recently published study found that the concentration of circulating leptin as well as adiponectin obtained from Sprague Dawley rats fed a HFD for a slightly longer duration of time (17 weeks compared with 15 weeks in this thesis) was did not statistically different when compared to the SCD fed Sprague Dawley rats (Marques, Meireles et al. 2016), conversely another group found that 18 weeks of high fat feeding (HFD comprised of 14.4% fat by weight) caused an increase in plasma leptin concentrations when compared to SCD (comprised of 4.6% fat by weight) fed group while there was a trend for the plasma ghrelin concentrations to increase in the HFD group, however this was not statistically significant. Therefore this thesis chapter highlights that the leptin concentration and adiponectin concentrations are not necessarily reflective of DIO in rats fed a HFD despite the changes in these hormones that are observed in obese humans.

Bueno et al. (2008) found that in non-obese mice fed a SCD enriched diet with either: 17.5% soybean oil (high linoleic acid composition), 17.5% lard (high in oleic acid and palmitic acid), 17.5% coconut oil diet (high in lauric acid) or 17.5% fish oil diet (high in palmitic acid, EPA and DHA) both acutely (two days) and chronically (eight weeks) had reduced serum adiponectin concentrations when compared to the control SCD fed mice. The results showed that a HFD, fed to non-obese mice (no matter the composition of dietary fatty acid), had reduced circulating adiponectin concentrations. The data in this thesis conflicts with the findings by Bueno et al. (2008), as there were no differences between groups in the adiponectin plasma concentrations, although in this thesis the data trended towards the DIO MUFA and DIO PUFA groups having an increased circulating adiponectin plasma concentration, the data was not significant and the DIO PUFA group had a large standard error due to a low sample size of n = 4. Further, the animal model used in this thesis was rat whereas the study by Bueno et al. (2008) used mice and the dietary intake of the different diets was for a shorter amount of

time and the mouse model was not obese unlike the rats focused on in this thesis that were DIO (Bueno, Oyama et al. 2008). Further, Bueno et al. (2008) also found that the retroperitoneal mRNA expression of adiponectin was reduced following the acute intake of the different dietary composition SCD enriched higher fat diets, while in the chronically fed mice the epididymal fat depot the adiponectin mRNA expression was decreased in the soy bean and coconut oil diets.

The data in this thesis also conflicts with a study focusing on lean, overweight and obese chimpanzees (Nehete, Magden et al. 2014). However the animals were more so subjectively classified using body conditioning scores rather than BMI or total body composition like in humans and rats, further the weight of these animals were not recorded. Biochemical plasma shows that the hormones leptin, GLP-1 and glucagon plasma concentrations were all elevated in the obese group when compared with the lean group (Nehete, Magden et al. 2014).

The effect that a HFD composed of different dietary fatty acids in DIO has on circulating plasma cytokines is largely unknown. Therefore a number of analytes were quantified in this study. The majority of cytokine analytes were not altered significantly between the different dietary feeding regimes, which is surprising considering that the rats fed a high fat diet and had a greater body fat percentage and peri-renal fat pad mass (peri-renal fat/ body weight %) compared to the lean SCD and DIO SCD groups, or that the type of dietary fatty acids did not have an effect. Obesity is described as a state of chronic low grade inflammation (Weisberg, McCann et al. 2003), however, certain circulating cytokines such as TNFα and IL-6 are up-regulated in obesity and are also implicated in the progression of insulin resistance in humans but were not increased in the DIO rats fed the different composition fatty acids when compared to the SCD fed rats.

Surprisingly the data in this thesis also conflicts with study that was mentioned above that focusing on lean, overweight and obese Chimpanzees (Nehete, Magden et al. 2014). While caution surrounding the subjective nature of classifying these animals into their groups, the biochemical plasma analysis

shows that plasma pro-inflammatory cytokines IFN-γ, IL-6, TNF, IL-1β were up regulated in the obesity, while anti-inflammatory cytokines IL-4, IL-10, and IL-13 were also up-regulated in obesity (Nehete, Magden et al. 2014). MCP-1 circulating concentrations were significantly higher in the obese group. Further a number of analytes are consistent with the findings in this thesis in that IL-12, IL-15, IL-17A, TGF-α, MIP-1α, MIP-1β or RANTES were not significantly altered between obese and non-obese animals (Nehete, Magden et al. 2014).

Surprisingly, the rats fed a MUFA diet had reduced circulating plasma anti-inflammatory hormone IL-10 when compared to the DIO SCD group only. This is inconsistent with the finding by Nehete et al. (2014), however, the chimapanzees were not fed a high fat diet, rather they were fed a high fibre 20% protein diet and had four meals a day. Further, the dietary composition of the diet was not described in the study (Nehete, Magden et al. 2014). Currently there is limited research focusing on the effect oleic acid and obesity have on circulating IL-10 concentrations. IL-10 is positively correlated with whole body insulin sensitivity (Straczkowski, Kowalska et al. 2005). When focusing on 3T3-L1 adipocytes treated with oleic acid there was no effect on the IL-10 mRNA or protein expression in these cells (Bradley, Fisher et al. 2008). In cultured primary rat hepatocytes treated with oleic acid there was an increase in IL-10 production in the liver (Nishitani, Okazaki et al. 2007), however further analysis would be required to understand the mechanism that caused this observation in the liver.

Taken together, while there were some variable observations between the SCD and DIO SCD, the results observed in this chapter of the thesis indicate that the dietary intake of different fatty acids in DIO did not have a major effect on whole body energy homeostasis in this rat model that was used.

CHAPTER EIGHT:

8.0 Overall Discussion & Future Directions

8.1 General Discussion

The endocannabinoid system is a potential therapeutic target for the treatment of obesity and diabetes, as this system is involved in energy homeostasis regulation by in part controlling appetite, hedonic feeding behaviour and glucose and lipid metabolism (Di Marzo, Ligresti et al. 2009). Targeting CB_1 receptors using Rimonobant was a promising therapeutic target for weight control mediated through both central and peripheral pathways (Louheranta, Turpeinen et al. 1998), but unfortunately there was an increased association with increased depressive mood states with this compound caused by central antagonism of CB_1, which resulted in this compound being withdrawn from the pharmaceutical market. Despite the observations surrounding the targeting of the CB_1 receptor, pharmocological modulation of the endocannabinoid system (while still ensuring safety and efficacy of administration of the compounds used) remains a viable target.

Emerging research describes some novel putative cannabinoid receptors including GPR55 and GPR18, these receptors could be potential pharmacological targets for obesity and associated comorbidities and are worth researching. Over recent years GPR55 has been suggested to have a role in obesity (Moreno-Navarrete, Catalan et al. 2012; Simcocks, O'Keefe et al. 2014; Meadows, Lee et al. 2016) and T2DM (Romero-Zerbo, Rafacho et al. 2011; Moreno-Navarrete, Catalan et al. 2012). The role of GPR18 in obesity is unknown, this receptor is highly expressed in a number of tissues involved in immune regulation and therefore as obesity is a state of chronic low grade inflammation GPR18 could be implicated in this state (Rajaraman, Simcocks et al. 2016). Understanding the mechanisms behind modulating either GPR55 or GPR18 in obesity is required to further understand whether these receptors are suitable therapeutic targets to safely be used clinically for the treatment in humans, in combination with reduced energy intake and increased physical activity.

Further, in the literature it is currently unknown about the effect that atypical cannabinoid compounds O-1602 or O-1918 have in the obese state, and these compounds have an affinity for both of the putative cannabinoid receptors GPR55 and GPR18.

Additionally, the role that different dietary fatty acids (which are precursors to endocannabinoid and structurally similar compounds) and their relationship with the putative cannabinoid receptors GPR55 and GPR18 are unknown and understanding this relationship this may be beneficial for dietary advice in obesity and associated co-morbidities in the future.

The results obtained in this thesis has provided novel insights into the role that the atypical cannabinoid compounds either O-1602 or O-1918 (that have an affinity for the putative cannabinoid receptors GPR55 and GPR18) have in the regulation of whole body energy homeostasis and skeletal muscle metabolism (particularly adiponectin signalling, oxidative capacity and fatty acid oxidation) in DIO obesity as well as metabolically stable C_2C_{12} myotubes.

Further, this thesis also provides insight into the role that different dietary fatty acids or recommencing a SCD has on DIO, and proposes a hypothesis concerning how cannabinoid like molecules derived from some of these fatty acids could be regulated by the putative cannabinoid receptor GPR55 and GPR18. The novel findings from this thesis helps to contribute to the knowledge surrounding obesity in relation to O-1602, O-1918, GPR55, GPR18 and different dietary fatty acids, as utilising these compounds or targeting these receptors using pharmacological and / or dietary inventions could be used for future therapeutic targets in the management of obesity.

8.1.1 Expression of GPR18 in skeletal muscle

The results obtained from *Chapter Six* of this thesis show for the first time that GPR18 is expressed in both red and white gastrocnemius skeletal muscle in rat, in which the mRNA expression of GPR18 is up-regulated in red gastrocnemius when compared to white in DIO. Additionally, *Unpublished Observations* from our laboratory show that at the protein level GPR55 is expressed in DIO rat skeletal muscle and C_2C_{12} myotubes (*Simcocks et al.* u*npublished observations*), however this antibody would also need to be used on the muscle of *GPR55 knockout* mice to confirm antibody specificity . The novel finding that GPR18 is expressed in skeletal muscle provides evidence that this receptor has a role in the skeletal muscle homeostasis and therefore understanding it's (patho) physiological function in obesity, insulin resistance and T2DM is warranted.

Given that GPR55 has a role in energy homeostasis (Moreno-Navarrete, Catalan et al. 2012; Simcocks, O'Keefe et al. 2014; Meadows, Lee et al. 2016), it is likely that GPR55 is expressed in the red and white gastrocnemius skeletal muscle, however further work is required to confirm this hypothesis (these are detailed in the future directions section of this thesis).

8.1.2 The role O-1602 has on energy homeostasis

The role that the atypical cannabinoid compound O-1602, has on energy homeostasis both *in vivo* and *in vitro* was determined in *Chapters Three, Four and Six* of this thesis using a metabolically stable non obese skeletal muscle cell culture model derived from mice and a DIO rat model. The compound's effect was assessed in both whole body and skeletal muscle energy homeostasis (while focusing on either red or white gastrocnemius skeletal muscle phenotypes which are either more oxidative or glycolytic in nature respectively). Current knowledge surrounding the role that O-1602 has in energy homeostasis and its suitability as an obesity and T2DM therapeutic target is quite limited in the literature. One study has focused on the compounds effect in non-obese rodents where O-1602 increases adiposity following sub-chronic administration (Diaz-Arteaga, Vazquez et al. 2012). The results provided within this thesis, in *Chapter Four* conflicts with the findings observed by Diaz et al.

(2012) and demonstrates that O-1602 had systemic effects on energy homeostasis and resulted in reduced adiposity (body fat % and epididymal fat pad mass/ body weight %) following chronic administration of this compound in DIO *in vivo*.

Given that skeletal muscle is a whole body regulator of energy homeostasis (Zurlo, Larson et al. 1990), a major site for fatty acid oxidation (Zurlo, Larson et al. 1990) and a suitable pharmaceutical target for the treatment of obesity and T2DM, understanding the role that the atypical cannabinoid compound O-1602 has on whole body skeletal muscle energy homeostasis is therefore important to understand and whether this compound is a suitable target in the obese and T2DM states. 24 hour treatment with O-1602, in a C_2C_{12} myotube model, under homeostatic conditions shows that this compound does not alter markers involved in oxidative capacity, adiponectin signalling or fatty acid metabolism at an mRNA expression level. This is further supported by the findings from *Chapter Six* in the DIO rat model, by which treatment with an ip. injection of O-1602 for a chronic time period did not have any effect on whole red or white gastrocnemius skeletal muscle for markers that regulate oxidative capacity, adiponectin signalling or fatty acid oxidation at an mRNA expression level. Thus, from a mechanistic perspective O-1602 is not contributing to changes in markers that regulate skeletal muscle homeostasis contributing to oxidative capacity, adiponectin signalling or fatty acid oxidation. However to fully discount this, further investigations will need to be undertaken, and will be discussed in the recommendations for future research arising from this thesis.

8.1.3 The role that O-1918 has on energy homeostasis

The role that the atypical cannabinoid compound O-1918, has on energy homeostasis *in vivo* was determined in *Chapters Five* and *Six* of this thesis using a DIO rat model. the compound's effect was assessed in both whole body and skeletal muscle energy homeostasis (while focusing on both red or white gastrocnemius skeletal muscle phenotypes which are either more oxidative or glycolytic in nature respectively).

Current knowledge surrounding the role that O-1918 has in energy homeostasis and determining whether it is a suitable obesity and T2DM therapeutic target has not yet been researched in the literature. *Unpublished observations* from our laboratory show that C_2C_{12} myotubes treated with O-1918 up-regulate the mRNA expression of markers of oxidative capacity, adiponectin signalling and fatty acid oxidation (*Simcocks et al. Unpublished Observations*). The results provided within this thesis, in *Chapter Five* indicate that O-1918 (at a dose of 1 mg/ kg) did not have enough of an effect to cause any alteration in body weight or body fat % following chronic administration of this compound in the DIO rat model utilised. However, O-1918 reduced BAT fat (brown adipose/ body weight %) depots while not altering glucose tolerance or insulin sensitivity. Circulating plasma leptin and ghrelin concentrations were reduced and the pro-inflammatory cytokines including: IL-1α, IL-2, IL-17α, IL-18 and RANTES were reduced in the O-1918 treated group. Circulating plasma AST was also elevated while albumin and ALT were not altered. Further, in the red and white gastrocnemius skeletal muscle, O-1918 down regulated the mRNA expression of AdipoR1 in the red gastrocnemius skeletal muscle of DIO rats, while there was an up-regulation of APPL2 mRNA expression in the white gastrocnemius skeletal muscle. The observations in the rat skeletal muscle conflicts with the previous findings observed in the C_2C_{12} myotubes treated with the same compound (*Simcocks et al. unpublished observations*), in which markers of oxidative capacity, adiponectin signalling and fatty acid oxidation were up-regulated. However, the findings observed are between two different species (mouse versus rats), the fact that cell culture is not influenced by circulating hormones and cytokines but the *in vivo*

model showed a number of changes in circulating cytokines may also have contributed to these observed differences, additionally, a metabolically stable non obese skeletal muscle model was being compared with a DIO model. As O-1918 has an affinity/ putative affinity for GPR55 and GPR18 and both receptors are expressed in tissues involved in energy homeostasis the effects observed in this study suggest that a dose of 1 mg/ kg is not beneficial to obesity but helps to provide some understanding into the effect this compound has in the obese state.

8.1.4 The role that different dietary fatty acids have on whole body energy homeostasis

The last study in this thesis outlined in *Chapter Seven* aimed to determine the role that different dietary fatty acids (based on their chemical structure) had on whole body energy homeostasis in DIO rats. Rats were fed a diet either predominately composed of SFA, MUFA or PUFA and there were no major differences observed between the three groups for body fat percentage, fat pad depots, circulating hormones or cytokines. The blood glucose response to insulin in the DIO SFA group compared to the DIO PUFA was increased at 120 and 180 minutes when blood glucose concentrations were measured. This helps to support findings in the literature in that SFA have a more negative impact on insulin sensitivity than PUFA in DIO (Riserus, Willett et al. 2009) and PUFA are more beneficial for whole body insulin sensitivity. Further recommencing a SCD reduced weight gain, reduced body composition when compared with the DIO MUFA and DIO PUFA groups and decreased peri-renal fat pad mass and increased circulating IL-10 in plasma compared with the DIO MUFA fed rats.

Irrespective of the data presented in this thesis, which found very limited changes in whole body energy homeostasis between the different dietary fatty acids consumed, further research into the role these different dietary fatty acids have on tissues involved in metabolism, nutrient utilisation and storage as well as the circulating concentrations of endocannabinoids and structurally similar molecules is important to understand and is detailed further in Section 8.3 Future Directions.

8.2 Limitations

The two different DIO Sprague Dawley rat models utilised in this thesis (*Chapters Four, Five* and *Seven*) did not have any change in circulating blood glucose concentrations (with the exception of the DIO SCD and the DIO PUFA groups in *Chapter Seven*) when administered either a load of glucose or insulin when compared with the SCD lean control rats. Despite this, the two different DIO models discussed throughout this thesis had increased body fat % and peri-renal adipose tissue depots when compared to the SCD fed control group. To test the effect that the atypical cannabinoid compounds (O-1602 or O-1918) or different dietary fatty acids (in which linoleic acid, oleic acid and palmitic acid are precursors to endocannabinoids and associated compounds inlcuding: AEA, 2-AG, OEA and PEA), had on glucose tolerance and insulin sensitivity it would have been useful to have an animal model displaying characteristics of insulin resistance. A longer duration of the HFD feeding regime may help to assist in creating a phenotype that is glucose intolerant and insulin resistant. Further, grouping the obese prone and obese resistant Sprague Dawley rats may also be beneficial in genetically separating the animals and their susceptibility to obesity and therefore the development of impaired insulin sensitivity and reduced glucose intolerance. Conversely, Stark & Madar (2000) suggested that feeding male Sprague Dawley rats a HFD for three months may not be a suitable model for researching glucose intolerance and insulin resistance. Therefore, Wister rats maybe a more appropriate model to be used although there is not the genetic variability in this model like those observed in humans.

It should also be noted that the administration route of glucose used for both rat studies for the GTT was using an ip. injection. An ip. GTT is more so reflective of hepatic glucose metabolism rather than if an oral GTT was used which may have been more preferable to determine the effects that a glucose load had on skeletal muscle metabolism and would be preferable to use in further studies that focus on skeletal muscle homeostasis.

During the study design phase for this thesis both O-1602 and O-1918 were selected as selective agonists and antagonists for GPR55 to help understand the role that these compounds had in obesity

and associated co-morbidities including T2DM. Since the start of this study however it has since emerged that O-1602 is a biased agonist (Console-Bram, Brailoiu et al. 2014) and O-1918 biased agonist or antagonist (McHugh, Hu et al. 2010; Console-Bram, Brailoiu et al. 2014) for GPR18. The pharmacology for GPCRs can be quite complex and emerging research suggests that GPCR form heteromers, therefore selectivity of compounds are not as "black and white" as once thought. In the endocannabinoid system *in vitro* and *in vivo* work suggests that in tissue where GPR55 and CB_1 are co expressed, these receptors form heteromers and signalling is then influenced by these compounds (Kargl, Balenga et al. 2012; Martinez-Pinilla, Reyes-Resina et al. 2014). Therefore, rather than being able to conclude the effect that these compounds have on specific receptors, this thesis more so determines the effects that these compounds have which may be due to their actions on one or more receptors (which could be counteracting each other's effects).

Another limitation of this study was that the total number of rats included in the O-1602 group reported in this thesis. Initially there was a larger group of animals planned for this group (n=10), however, one of the rats treated with this compound had to be euthanized due to poor health status, in addition to a number of the rats having anatomical abnormalities post treatment (such as abnormalities in the liver as discussed in *Chapter Four*). This resulted in the decision to cease experiments early due to animal welfare. This limitation has then impacted the statistical power for this group due to the low sample size (n=6) which especially has had an influence on the skeletal muscle mRNA expression data which was highly variable. It remains unclear as to the causes of these abnormalities, as hydroxyproline content in the liver and heart was not altered in the DIO O-1602 treated group, but the increase liver mass (liver/ body weight) as well as the increase in circulating plasma RANTES and AST provide some evidence that this compound does cause unwanted side effects as a pharmaceutical compound.

While extensive research has focused on the effect that different dietary fatty acids have in obesity, this study was the first to focus on the effect that different dietary fatty acids, namely linoleic, oleic and palmitic/ steric acid had on a number of different circulating hormones and cytokines. The data in

this thesis demonstrates that the rats were not under a chronic state of low grade inflammation which is often observed in obese humans (Weisberg, McCann et al. 2003) and potentially could also have had some impact surrounding the lack of insulin resistance observed in this model. Circulating hormones and cytokine are often dysregulated in the obese state particularly adiponectin, leptin, IL-6 and TNFα were not in this model. It should however, also be noted that the DIO PUFA and DIO SCD had lower power due to n = 4 for these measurements and could be contributing to a lack of power.

An additional limitation is that the dietary composition of the high fat SFA diet was selected and based around the original cafeteria diet D12451, which is known to induce obesity in rats (Farley, Cook et al. 2003; Marques, Meireles et al. 2016). Minor modifications were made to the diet to meet the Australian manufacturer's limitations. The high fat SFA diet contained 9.76% SFA however this diet still contained a large proportion of MUFA (7.68%). When comparing the high fat SFA diet to the high fat MUFA diet (16% MUFA) and the high fat PUFA (16% PUFA) the SFA was not matched and this could be seen to be a limitation of this study, as a higher composition of MUFA in the high fat SFA diet could provide further beneficial properties compared to if the MUFA content of the high fat SFA diet was lower.

Another limitation in this thesis was that the mRNA expression of GPR55 in the skeletal muscle was unable to be assessed in either red or white gastrocnemius rat skeletal muscle, and this was likely due to a low abundance of the receptor in this tissue. The fact that the primers used for GPR55have been published by Imbernon et al. (2014) suggests that a more concentrated skeletal muscle tissue sample maybe required to determine if this receptor is expressed using 'Real Time' PCR and therefore would help clarify if GPR55 is actually expressed in this tissue.

8.3 Overall Summary

This thesis has helped to identify a role for the atypical cannabinoid compounds either: O-1602 or O-1918 in energy homeostasis and skeletal muscle homeostasis (particularly adiponectin signalling,

oxidative capacity and fatty acid oxidation) in DIO obesity and metabolically stable, non obese C_2C_{12} myotubes. Whereby, the atypical cannabinoid compounds have an affinity for the putative cannabinoid receptors GPR55 and GPR18. Additionally, this thesis has also helped to establish a role that different dietary fatty acids or recommencing a SCD could have on DIO whole body energy homeostatsis.

While the results from this thesis suggest that both O-1602 and O-1918 are not suitable pharmacological targets for obesity especially with the results observed in the DIO *in vivo* model, fully understanding the mechanism behind the changes observed will contribute to the knowledge base as it is not entirely clear which receptor(s) the dosage that was administrated to the rat is actually targeting.

8.4 Recommendations for future research arising from this thesis

The results from this thesis pose a number of further questions that require future investigation especially surrounding the putative cannabinoid receptors either GPR55 or GPR18 with a number of current gaps within the literature at present.

The rats treated and described in the experimental *Chapters Four, Five and Six* had a number of other organs collected for future analysis. Further a SCD fed control group was also utilised in this experiment, where both red and white gastrocnemius skeletal muscle were collected and are currently stored in liquid nitrogen. Therefore, focusing on the mRNA expression of either GPR18 or GPR55 in these tissues would be beneficial to elucidate whether there is a change in the expression of these receptors and the effect that they have in a SCD fed state compared to a DIO state. Given that *Chapter Six* shows that GPR18 is up-regulated in a fibre type specific manner, determining this in SCD group would also be beneficial to provide some understanding about the role of GPR55 or GPR18 in the skeletal muscle.

Given the results observed in the O-1602 or O-1918 treated DIO rats, it would be beneficial to fully understand the effects that these compounds have in other tissues involved in energy homeostasis, particularly when focusing on the DIO O-1918 treated rats, as there was a reduction in BAT mass

(brown adipose/ body weight %). Analysing this tissue and fully understanding the effect that this compound is having systemically with a focus on markers of thermogenesis UCP-1 (Lowell and Spiegelman 2000), PGC1α for thermogenic activation (Bostrom, Wu et al. 2012), PRDM16 a transcription factor that is a co-regulator that controls the development of BAT (Kajimura, Seale et al. 2008) is therefore warranted.

It is also important to understand the tissue specific mechanisms that were observed in the DIO O-1602 treated rats and to investigate the causes of the enlarged liver and increased circulating AST and RANTES, in addition to the observational data obtained from these animals. While the hydroxy proline assay conducted in the liver and heart tissue suggests that this is not fibrosis, this is only one test and the Echo MRI focuses on whole body fat composition no matter the storage of the fat which does suggest (but not definitatively) that there is not an increase in fatty acid storage in the liver of the DIO O-1602 treated rats but this data could be being thrown off by the fact that the epididymal fat pads were reduced contributing to the reduced body fat % in the DIO O-1602 treated rat group. Additional work focusing on the enlarged liver will help to provide some understanding into the effect that O-1602 is having in the liver (comparing DIO Control treated rats to DIO O-1602 treated rats). The proposed analysis includes: (1) Determining the liver lipid content using Oil Red O staining, by sectioning liver portions that have previously been stored in OCT at – 80 °C (2) Determining the liver glycogen content using enzymatic glycogen assay (Keppler 1974; Silva de Oliveira 2007) (3) Further clarification to support that the enlargement was not a result of liver fibrosis by quantifying the mRNA expression of markers involved in fibrosis and sectioning previously stored liver in OCT using Sirus Red staining or (4) In addition to the increased circulating RANTES, determining whether O-1602 was causing chronic inflammation by quantifying plasma C - Reactive Protein (CRP).

To provide a clearer understanding about the role that either O-1602 or O-1918 are having in energy homeostasis and highlighting the differences and similarities in the cellular signaling that these compounds have between species (for example mouse, rat and human) fully investigating these

compounds in cell culture, rat models and human primary cell culture will help to highlight the similarities and differences that occur between species as well as the different effects observed in the lean, obese, insulin resistant and T2DM states.

Previously preliminary data from my honours work indicates that a dose response range of O-1918 does not appear to alter a number of markers involved in oxidative capacity, adiponectin signalling or fatty acid metabolism in human primary myotubes obtained from individuals that are classified as obese or have T2DM (*Simcocks et al, Unpublished Observations*). Therefore treating lean and insulin resistant human primary myotubes with either O-1602 or O-1918 would also help to provide some understanding about the role that these compounds have in human skeletal muscle homeostasis in addition to the observations and the data we already have in C_2C_{12} myotubes obtained from mice, to determine the effect between different species as well as comparing physiological to pathophysiological states. To determine the specific action for either GPR55 or GPR18 in these cells a GTPγS binding assay could be used in combination with silencing each of the receptors. Glucose uptake and fatty acid oxidation in these cells could also be assessed using a Seashorse.

Further to fully understand both of the putative cannabinoid receptor's role in human skeletal muscle, analysing and quantifying the mRNA expression of putative cannabinoid receptors GPR55 or GPR18, and determining the difference in expression between lean, obese, insulin resistant and T2DM skeletal muscle would help to provide some novel insight into whether there is a difference in expression of these receptors are occuring within the certain tissue types. While this does not neccearily highlight the function of the receptor in these conditions, it helps to clarify whether the expression is altered in these states.

Following on from this, given that a number of new generation of GPR55 agonists and antagonists have been developed over recent years (although they are still relatively new in the literature) (Kotsikorou, Madrigal et al. 2011; Kargl, Balenga et al. 2012; Kargl, Brown et al. 2013; Kotsikorou,

Sharir et al. 2013). The compounds are structurally similar to the potent endogenous non endocannabinoid GPR55 ligand LPI. One of the agonists for GPR55 GSK319197A has been shown to only activate human GPR55 but not rat (Brown, Daniels et al. 2011; Kargl, Balenga et al. 2012) so would not be a useful candidate to use in a DIO rat model and the other agonists for GPR55 have not been used *in vivo* as yet.

One antagonist for GPR55, CID16020046, has been tested in mice, this antagonist reduces leukocyte migration and activates intestinal inflammation (Stancic, Jandl et al. 2015). Further, this compound decreases neutrophil activation and atherogenesis (Montecucco, Bondarenko et al. 2016), with researchers describing this compound as well being tolerated by the transgenic ApoE knockout mice (a model that is used for atherosclerosis) which were fed either a SCD or a high cholesterol diet. The compound CID16020046 has also been tested *in vivo* in mice and *ex vivo* in isolated rat arteries, and therefore may be a suitable antagonist to use for targeting GPR55. The effect this compound has in obesity or T2DM has not yet been determined in the literature. Therefore, it would be beneficial to use an antagonist that is more selective to GPR55 (CID16020046) and determine its effect on skeletal muscle homeostasis and obesity and related co-morbidities including T2DM. The findings could be further confirmed in *GPR55 knockout* mice to help ensure the antagonists specificity.

In addition to this either (1) silencing GPR55 or and GPR18 or transfecting cells the receptors into C_2C_{12} myotubes or human primary myotubes and/ or (2) using a process called electroporation to increase one or both of the receptors (GPR55 and GPR18) in rodent muscle (Potter and Heller 2010) would help to provide some understanding surrounding the cellular signalling that these receptor have in the skeletal muscle. Performing glucose uptake and fatty acid oxidation experiments in afore mentioned models would also help to provide some understanding in relation to the roles for each receptor either in isolation or combination in the skeletal muscle and therefore subsequently energy homeostasis in this tissue. Depending on the results obtained from the fatty acid oxidation and glucose uptake experiments, could then lead onto determining the changes in mRNA expression and protein

expression in cellular signalling pathways forfatty acid oxidation and glucose uptake. Additional analysis could also be undertaken to assess mitochondrial function in these tissues using an Oroborous which measures mitochondrial oxygen consumption.

Future directions following on from the study detailed in *Chapter Seven*, include measuring the mRNA content of adiponectin and leptin in adipose tissue fat pad depots to determine the effect that different dietary fatty acids have on the expression of these proteins *in vivo.* This work will help to provide some insight into the role that different dietary fatty acids have on these hormones in this DIO rat model, as well as understanding the molecular signalling that is occurring in organs that are involved in energy homeostasis such as in the hypothalamus, gastrointestinal tract, pancreas, liver, WAT, BAT and the skeletal muscle. Further, the tissue distribution and expression of the putative cannabinoid receptors GPR55 and GPR18 is currently unknown and understanding the changes in circulating endocannabinoid and associated molecules that target both of these putative cannabinoid receptors would be of benefit to research.

The chapters in this thesis helps to provide some understanding into the effects that either compounds O-1602 or O-1918 have on whole body energy homeostasis and skeletal muscle metabolism, as well as the effect that different dietary fatty acids have on whole body energy homeostasis. The novel finding that GPR18 is expressed in the skeletal muscle indicates that this receptor is likely to have a function in this tissue. As the skeletal muscle is a regulator of whole body energy metabolism, understanding the role of GPR18 in this tissue maybe a useful future target for obesity and related co-morbidities such as insulin resistance and T2DM. However, specific ligands that target GPR18 are required to focus on the role that this receptor has or using models such as *GPR18 knockout* mice to fully understand the effect of the receptor. Further the development of muscle specific compounds that target GPR55 and/ or GPR18 maybe more beneficial to help reduce the undesired central side-effects that have preiovuly been observed with the use of Rimonabant.. The effects that different dietary fatty acids have on GPR55 and/ or GPR18 in obesity and associated comorbidities such as T2DM can also

be determined. Specifically, focusing on changes in mRNA expression for the putative cannabinoid receptors GPR55 or GPR18 in adipose tissue and the skeletal muscle, as well as quantifiying the circulating concentrations of endocannabinoid ligands/ putative endocannbinoid ligands (OEA and PEA) for these recepotrs in the plasma using High Performance Liquid Chromatography (HPLC) which will also help to provide some understanding regarding dietary advice and the consumption of different dietary fatty acids.

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GPR55

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Synonyms

G protein-coupled receptor 55; Gm218; GPR55; LPIR1

Historical Background

G protein-coupled receptor 55 (GPR55) was first cloned in 1999 and is a 319 amino acid seven transmembrane G protein-coupled receptor (GPCR) that is mapped to chromosome 2q37 (human) (Sawzdargo et al. 1999). It displays features common with other Family A GPCRs including a short extracellular N-terminal and C-terminal tail and contains two highly conserved extracellular cysteine residues that form disulfide bonds to help stabilize the receptor structure. Glycosylation sites in its N-terminus are present, while its intracellular loops and C-terminal tail have a number of potential phosphorylation sites. Orthologues for GPR55 have been found in a number of mammalian species including rat, mouse, dog, cow, chimpanzee, and human (Baker et al. 2006) (Figs. 1 and 2).

Following the characterization of the cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2) ~~receptors~~, it was then postulated that there was a third or "atypical" cannabinoid receptor, "CBx." This receptor is sensitive to both anandamide (AEA) and the "atypical" cannabinoid abnormal cannabidiol (Abn-CBD)~~,~~ in endothelial cells (Jarai et al. 1999; Wagner et al. 1999). This unknown receptor was found to mediate mesenteric vasodilation distinct from activation of CB_1 and CB_2 (Jarai et al. 1999; Wagner et al. 1999). It was then hypothesized that GPR55 may be the unknown cannabinoid receptor, as GPR55 is sensitive to the "atypical" cannabinoids, and an in silico screen later indicated that GPR55 was a cannabinoid receptor (Baker et al. 2006). However, Johns et al. (2007) showed that the vasodilatory effects of Abn-CBD in the presence and absence of O-1918 (a nonspecific putative GPR55 antagonist) were similar in mesenteric vessels obtained from GPR55 knockout mice and wild-type mice, in addition to no difference

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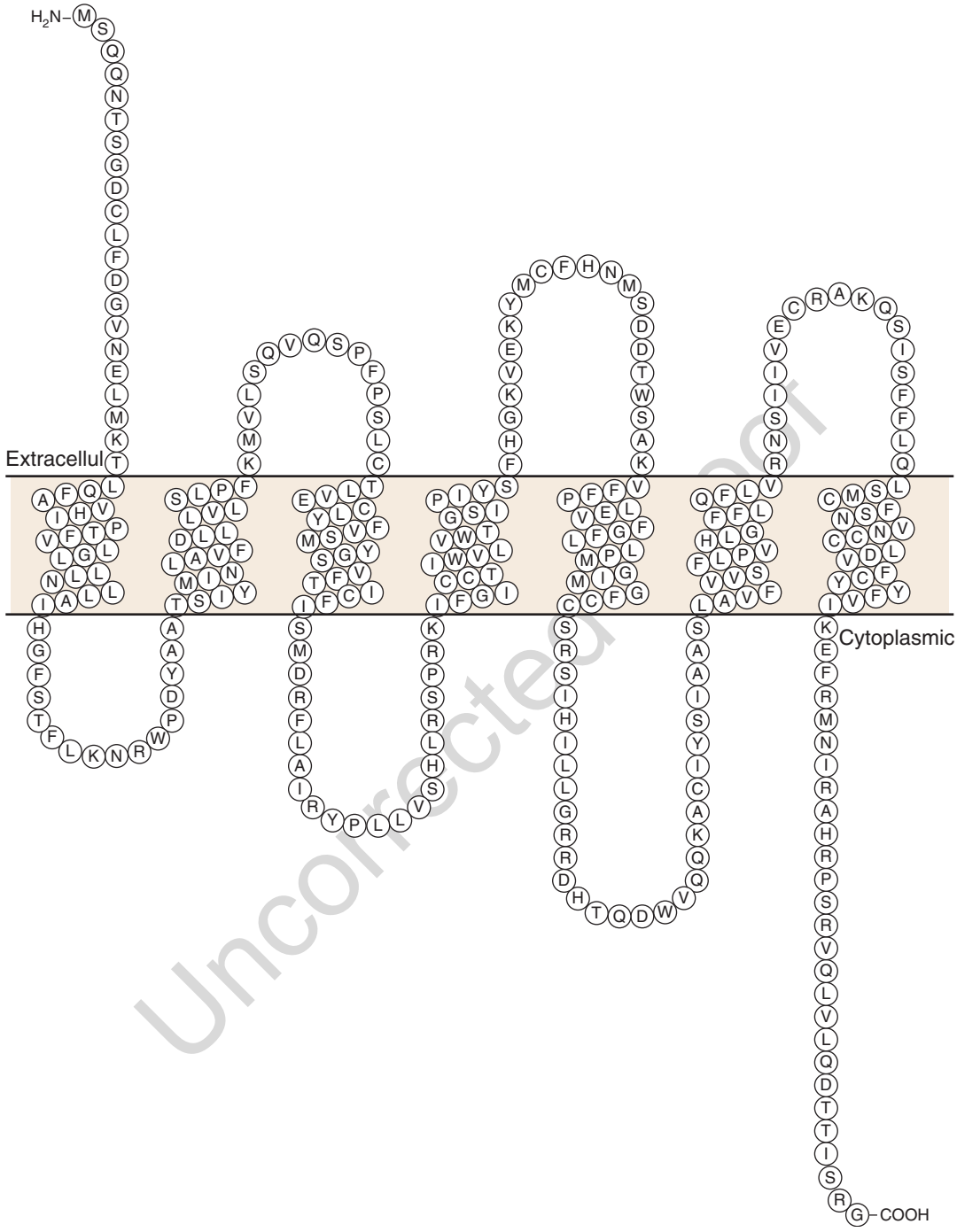
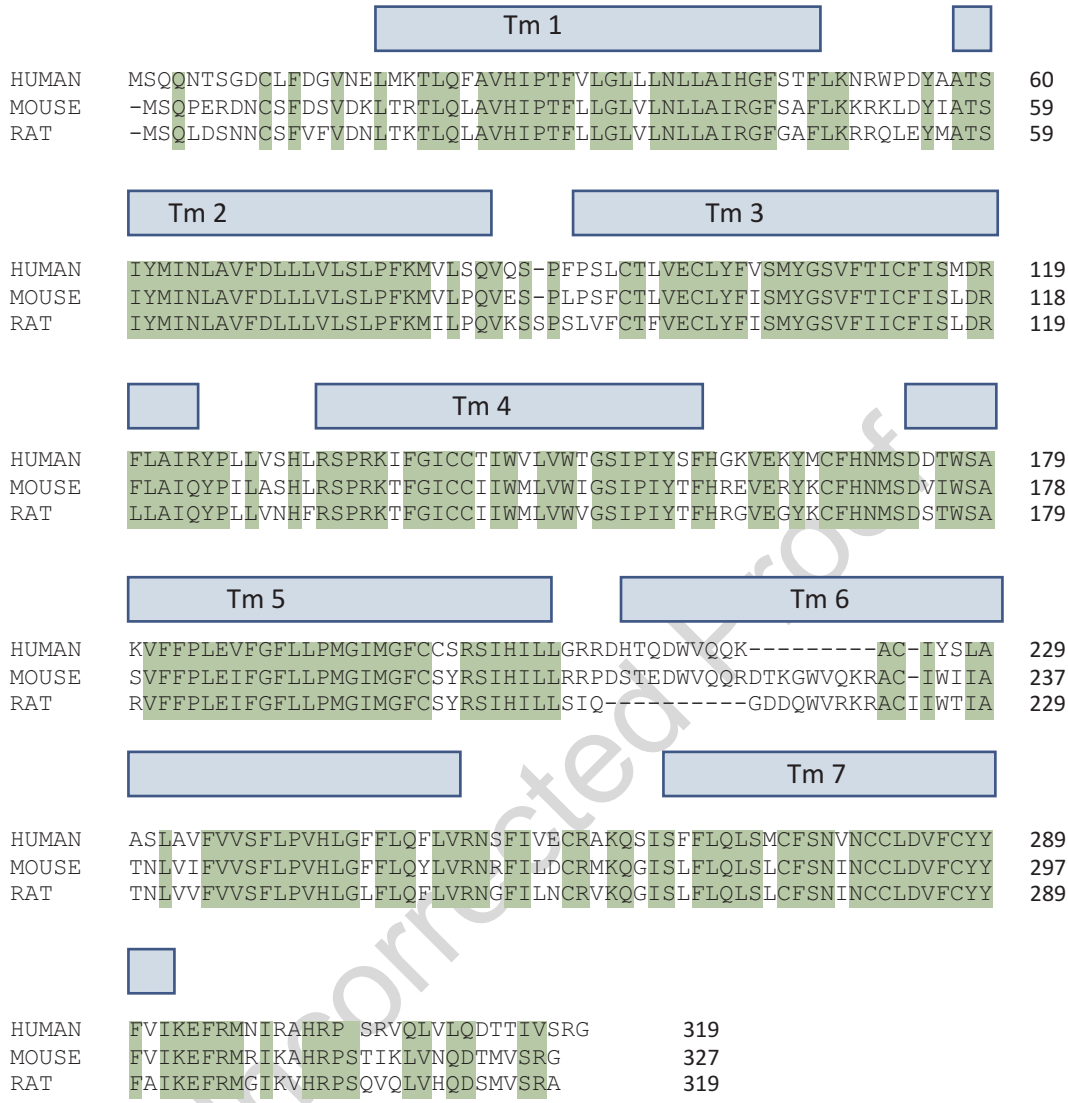


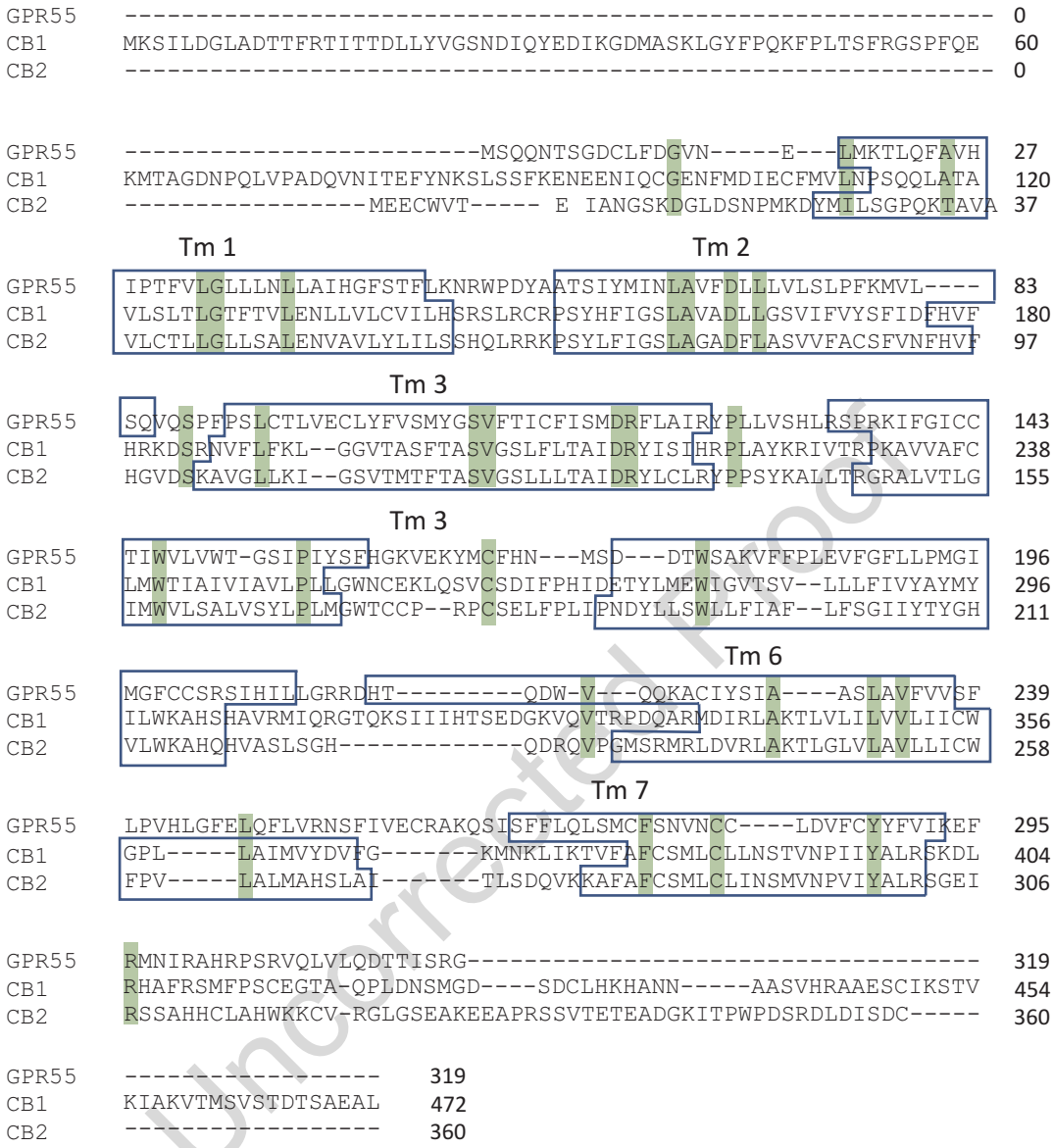
GPR55, Fig. 1 Snake plot diagram of the human GPR55 receptor. GPR55 is a seven transmembrane spanning receptor with an extracellular N-terminal domain and an intracellular C-terminal domain (Diagram generated by Protter (Omasits et al. 2013))



GPR55, Fig. 2 Amino acid sequence alignment of mouse (NP_001028462.2), rat (XP_006245556.1), and human (NP_005674.2) GPR55 genes. *Blue boxes* are transmembrane regions, and *green shaded regions* are amino acids conserved between all three receptors. Alignment performed by CLUSTAL Omega (1.2.2)

in resting heart rate or blood pressure between these two mice strains. The authors also stated that a limitation of the study is that they did not determine the antagonistic effect of O-1918 on Abn-CBD ~~induced~~ GTPγS activation in GPR55 transfected cells, which would have helped to further support the observations in the mice (Johns et al. 2007).

It has since been established that both GPR55 and G protein-coupled receptor 18 (GPR18) are sensitive to Abn-CBD (Johns et al. 2007; McHugh et al. 2010; Ryberg et al. 2007), as well as other cannabinoid compounds, and as such are both cannabinoid receptor candidates (McHugh et al. 2012; Ryberg et al. 2007), although GPR55 has a low homology (10–15%) when compared to the cannabinoid receptors CB_1 and CB_2 (Baker et al. 2006). Figure 3~~: Amino acid alignment CB_1, CB_2, and GPR55~~. Additional research is required before a decision surrounding classification of



GPR55, Fig. 3 Amino acid sequence alignment of human CB1 (NP_001153698.1), CB2 (NP_001832.1), and GPR55 (NP_005674.2) genes. *Blue boxes* are transmembrane regions, and *green shaded regions* are amino acids conserved between all three receptors. Alignment performed by CLUSTAL Omega (1.2.2)

GPR55 as a cannabinoid receptor can be made by the International Union of Basic and Clinical Pharmacology (IUPHAR) (Pertwee et al. 2010). One area of concern is that the pharmacological profile of this receptor remains controversial, with the receptor's affinity to several cannabinoid ligands providing inconsistent results (Henstridge et al. 2010; Kapur et al. 2009; Pertwee et al. 2010).

Regardless of whether GPR55 is classified as a cannabinoid receptor, emerging research over the past two decades has been undertaken to help understand this receptor's physiological/pathophysiological role for pharmacological purposes.

GPR55 Tissue Expression

GPR55 receptor expression has been demonstrated both peripherally and centrally in human, mouse, and rat with some similarities and differences in the distribution between species. Receptor expression has primarily been assessed through the measurement of mRNA levels (Table 1).

Pharmacology

The pharmacology of GPR55 is quite complex as there have been conflicting findings surrounding this receptor and some of its cannabinoid ligands (Kapur et al. 2009; Lauckner et al. 2008; Oka et al. 2007; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008). This is likely due to a number of reasons including a number of fatty acid- and plant-derived compounds which are not specifically selective to GPR55 and thus have off-target effects. For example, oleoylethanolamide (OEA) is an agonist for both GPR55 and G protein-coupled receptor 119 (Overton et al. 2008). Additionally, research has focused on a number of different cell lines/types and different assays which have been utilized to determine agonist and antagonist binding and signaling properties (Kapur et al. 2009; Lauckner et al. 2008; Oka et al. 2007; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008). Therefore, variability in the experimental design could also add to these conflicting results (Pertwee et al. 2010), as well as the possibility of biased signaling (Henstridge et al. 2010). To add to the complexity surrounding GPR55 pharmacology, GPR55 signaling can also be influenced by the two cannabinoid receptors CB_1 and CB_2, as GPR55 forms heteromers with these GPCRs (Balenga et al. 2014; Kargl et al. 2012; Martinez-Pinilla et al. 2014), which will be discussed in more detail in the GPR55 signaling pathways section.

A number of different ligands act as agonists and antagonists for GPR55, these include plant-derived, fatty acid-derived, and chemically synthesized compounds (Ryberg et al. 2007). A summary of these compounds is included in Table 2. Synthetic cannabinoid derivatives of plant-derived cannabidiol (CBD), including Abn-CBD and O-1602, have been shown to act as agonists for GPR55 (Ryberg et al. 2007; Whyte et al. 2009), whereas CBD acts as an antagonist (Ryberg et al. 2007; Whyte et al. 2009). The CBD analog, O-1918, is thought to be an antagonist for the CBx receptor (Pertwee et al. 2010) as well as a putative GPR55 antagonist (Henstridge et al. 2011; Kremshofer et al. 2015), although there is no evidence for binding of O-1918 to GPR55 and further research into this is required. These atypical cannabinoid compounds have previously been used to help elucidate the role of GPR55 in human (patho)physiology (Li et al. 2013; Whyte et al. 2009). However, some conflicting findings have been observed in GPR55 knockout mice (Johns et al. 2007; Schicho et al. 2011). It was established that Abn-CBD, O-1602, and CBD also have an affinity toward GPR18 (McHugh et al. 2012). Further research using the new-generation agonists and antagonists (Table 2), with the support of GPR55 knockout models, is required to provide further understanding surrounding the (patho)physiological role of GPR55.

GPR55 Signaling Pathways

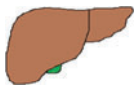
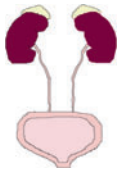
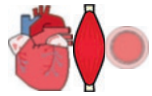
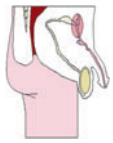
A number of studies investigating the signaling pathways activated by GPR55 have been conducted in HEK293 (human embryonic kidney 293) cells transfected with the hGPR55, as well as other cells such as human bone osteosarcoma (U2OS) cells expressing GPR55, mouse dorsal root ganglion primary cells, human and mouse osteoclast primary cells, and placental venous endothelial cells (EA.hy926 cells) (Henstridge et al. 2010; Kapur et al. 2009; Lauckner et al. 2008; Oka et al. 2010; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008). GPR55 is coupled to $G_{\alpha 12/13}$ and/or $G_{\alpha q}$ (Pertwee et al. 2010). This subsequently leads to activation of a number of intracellular signaling pathways depending on the ligand utilized, including the Ras homolog gene family member RhoA-associated protein kinase (RhoA-ROCK)

GPR55, Table 1 A comparison of GPR55 expression between mouse, rat, and human

Organ/cell	Mouse	Rat	Human	
 Central nervous system	+ Cortex	+ Hippocampus	– Hippocampus	
	+ Striatum	+ Thalamic nuclei	– Thalamus	
	+ Hypothalamus	+ Midbrain	– Cerebellum	
	+ Hippocampus		– Frontal cortex	
	+ Brain stem	Sawzdargo et al. (1999)	– Pons	
	+ Spinal cord		+ Amygdala	++ Nucleus accumbens
			++ Caudate nucleus	+ Parahippocampal gyrus
			+ Cerebellum	+ Pituitary gland
			+ Cingulate gyrus	++ Putamen
			+ Globus pallidus	+ Spinal cord
			+ Hippocampus	++ Striatum
			+ Hypothalamus	+ Substantia nigra
			+ Locus coeruleus	+ Superior frontal gyrus
			+ Medial frontal gyrus	+ Thalamus
			+ Medulla oblongata (– using Northern Blot; + using quantitative "real-time" PCR)	
			Henstridge et al. (2011), Kremshofer et al. (2015), Oka et al. (2010), and Sawzdargo et al. (1999)	
 Respiratory system	+ Lung		+ Trachea	
	Ryberg et al. (2007)		+ Lung	
			+ Cartilage	
			Henstridge et al. (2011, Kremshofer et al. (2015), and Oka et al. (2010)	
 Gastrointestinal tract	+ Esophagus	+ Small intestine	+ Salivary glands	
	+ Stomach	+ Colon	+ Esophagus	
	+ Jejunum	Sawzdargo et al. (1999) and Lin et al. (2011)	+ Stomach	
	+ Ileum		++ Small intestine	
	+ Colon		+ Colon	
	+ Mucosa		Henstridge et al. (2011), Kremshofer et al. (2015), and Oka et al. (2010)	
	+ Muscle layer			
	Ryberg et al. (2007 and Schicho et al. (2011)			
 Pancreas	+ Pancreas	+ Pancreatic islets	+ Pancreas	
	McKillop et al. (2013)	+ β islets (protein)	Henstridge et al. (2011)	
		– α and δ islets (protein)		
		+ BRIN-BD11 cells		
		McKillop et al. (2013 and Romero-Zerbo et al. (2011)		

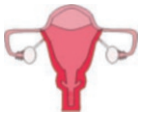
(*continued*)

GPR55, Table 1 (continued)

Organ/cell	Mouse	Rat	Human
 Liver	+ Liver	+ Liver	− /+ Liver (− Northern Blot; + quantitative "real-time" PCR)
	Ryberg et al. (2007)	Romero-Zerbo et al. (2011)	Henstridge et al. (2011) and Sawzdargo et al. (1999)
 White adipose tissue (WAT)	+ WAT	+ WAT	+ WAT
	(Ryberg et al. 2007)	(Romero-Zerbo et al. 2011)	+ Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT)
			↑ GPR55 VAT and SAT in obesity
			↑ GPR55 obese T2D VAT
			Henstridge et al. (2011) and Moreno-Navarrete et al. (2012)
 Renal system	+ Kidney	+ Kidney	+ HK2 proximal tubule cell line
		Jenkin et al. (2010)	+ Adrenal glands
	+ Adrenal glands		+ Kidney
	++ Bladder		+ Bladder
	Ryberg et al. (2007)		Jenkin et al. (2010), Kremshofer et al. (2015), Oka et al. (2010), and Henstridge et al. (2011)
 Heart, skeletal muscle, and endothelial cells	− Heart		+ Heart
	Ryberg et al. (2007)		+ Skeletal muscle
			+ Placental venous endothelial cell line EA.hy926
			Henstridge et al. (2011), Kremshofer et al. (2015), and Waldeck-Weiermair et al. (2008)
 Bone	+ Osteoblasts (protein also)		+ Osteoblasts (protein also)
	+ Osteoclasts (proteins also)		+ Osteoclasts (protein also)
	Whyte et al. (2009)		+ Bone
			+ Bone marrow
			Henstridge et al. (2011) and Whyte et al. (2009)
 White blood cells and platelets			+ Peripheral blood Mononuclear cells
			+ Macrophages
			+ CD+T cells
			+ B cells
			++ Natural killer cells
			++ Monocytes
			+ Platelets
			Chiurchiu et al. (2015) and Henstridge et al. (2011)
 Male reproductive system			++ Testes
			+ Prostate
			Henstridge et al. (2011), Kremshofer et al. (2015), and Oka et al. 2010)

(*continued*)

GPR55, Table 1 (continued)

Organ/cell	Mouse	Rat	Human
 Female reproductive system	++ Uterus	+ Fetal tissues	+ Cervix
	Ryberg et al. (2007)	Sawzdargo et al. (1999)	+ Uterus
			+ Human placenta
			↑ mRNA full-term compared to first trimester placenta
			Henstridge et al. (2011), Kremshofer et al. (2015, and Oka et al. (2010)
 Thymus, spleen, and thyroid gland	+ Spleen	+ Spleen	++ Spleen
	Ryberg et al. (2007)	Romero-Zerbo et al. (2011) and Sawzdargo et al. (1999)	++ Thymus
			+ Thyroid gland
			Henstridge et al. (2011), Kremshofer et al. (2015), and Oka et al. (2010)

This table highlights the expression of GPR55 in tissues or cells across three species including mouse, rat, and human
+ Indicates mRNA expression for GPR55 in tissue or cell (protein is specified if analyzed). Caution is advised when interpreting protein results, as GPCR antibodies tend to lack selectivity
++ Indicates abundant mRNA expression for GPR55 in tissue or cell
– Indicates no evidence of receptor expression for GPR55 in tissue

pathway, resulting in downstream signaling to p38 mitogen-activated protein kinase (p38MAPK) and activating transcription factor-2 (ATF-2) phosphorylation (Oka et al. 2010) and/or phosphoinositide phospholipase C (PLC), Ca^{2+} mobilization, and subsequently nuclear factor of activated T cells (NFAT) nuclear translocation (Henstridge et al. 2009). Additionally, activation of PLC causes increase in Ca^{2+} release from the endoplasmic reticulum, leading to initiation of protein kinase C (PKC) and extracellular signal-regulated kinase (ERK1/2) phosphorylation. ERK1/2 activates cAMP response element-binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) (Henstridge et al. 2010). Stimulation of GPR55 can also result in β-arrestin activation and internalization of the receptor (Kapur et al. 2009) (Refer to Fig. 4 – GPR55 cellular signaling – for a detailed diagram).

GPCRs can act not only as monomers but also as heteroreceptor complexes, which can impact on activation of receptor signaling pathways. GPR55 forms heteromers with both CB_1 and CB_2 (Balenga et al. 2011; Kargl et al. 2012; Martinez-Pinilla et al. 2014). In vitro experiments indicate that both GPR55 and CB_1 form heteromers in HEK293 cell lines when both receptors are expressed (Kargl et al. 2012). Co-immunoprecipitation experiments show that HEK-CB_1-GPR55 cells interact when compared with HEK293 cells singly expressing either CB_1 or GPR55. Although, unstimulated CB_1 and GPR55 do not appear to co-internalize (Kargl et al. 2012). Using a range of different agonists, it has been determined that in the presence of CB_1, GPR55-mediated signaling is reduced or inhibited, and furthermore, CB_1-mediated ERK1/2 and NFAT activation are enhanced in the presence of GPR55, and blocking CB_1 inhibits GPR55 signaling (Kargl et al. 2012). Supporting these findings, another study used bioluminescence resonance energy transfer (BRET) and proximity ligation assay (PLA) which showed a direct interaction between CB_1 and GPR55 and formation of heteromers in HEK293 cells transiently co-transfected with human CB_1 and GPR55 (Martinez-Pinilla et al. 2014). The same study also found that ~~in vivo~~ GPR55 and CB_1 are co-expressed in rat and monkey striatum and these receptors also form heteromers in these tissues (Martinez-Pinilla et al. 2014).

~~In vitro~~ experiments also indicate that both GPR55 and CB_2 form heteromers (Balenga

GPR55, Table 2 Ligands for GPR55

Compound	Compound type	Some of the observed actions at GPR55
L-α-lysophosphatidylinositol (LPI)	Endogenous fatty acid	**Agonist** (Henstridge et al. 2009, Henstridge et al. 2010, Oka et al. 2007)
		Induces GTPγS binding (Oka et al. 2007), ERK1/2 phosphorylation (Oka et al. 2007), and Ca^{2+} mobilization (Henstridge et al. 2009; Lauckner et al. 2008; Oka et al. 2007) in HEK293 cells transfected with human GPR55 (hGPR55)
		Induces RhoA activation in both human and mouse osteoclast primary cells (Whyte et al. 2009)
		Mediates ERK1/2 phosphorylation, β-arrestin activation, and GPR55 internalization in U2OS cells expressing GPR55 (Kapur et al. 2009)
Anandamide (AEA)	Endogenous fatty acid – endocannabinoid	**Agonist** (Lauckner et al. 2008; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008)
		Inconsistent findings depending on the functional assay and cells used
		Induces [^{35}S] GTPγS binding, RhoA activation (Ryberg et al. 2007), and Ca^{2+} mobilization (Lauckner et al. 2008) in HEK293 cells transfected with (hGPR55)
		Induces ERK1/2 phosphorylation in EA.hy926 cells (Waldeck-Weiermair et al. 2008)
		No effect for ERK1/2 phosphorylation, β-arrestin activation, or GPR55 internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)
2-Arachidonoylglycerol (2-AG)	Endogenous fatty acid – endocannabinoid	**Agonist** (Ryberg et al. 2007)
		Inconsistent findings depending on functional assay and cells type used
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		No effect in HEK293 cells transfected with hGPR55 for Ca^{2+} mobilization and ERK1/2 phosphorylation (Oka et al. 2007)
		No effect for Ca^{2+} mobilization in dorsal root ganglion derived from mice (Lauckner et al. 2008)
		No effect for ERK1/2 phosphorylation, β-arrestin activation, or GPR55 internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)
Noladin ether	Endogenous fatty acid – endocannabinoid	**Agonist** (Ryberg et al. 2007)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
Oleoylethanolamide (OEA)	Endogenous fatty acid	**Agonist** (Ryberg et al. 2007)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
Palmitoylethanolamide (PEA)	Endogenous fatty acid	**Agonist** (Ryberg et al. 2007)
		Inconsistent findings depending on functional assay and cells type used
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)

(*continued*)

GPR55, Table 2 (continued)

Compound	Compound type	Some of the observed actions at GPR55
Δ^9Tetrahydrocannabinol (Δ^9THC)	*Cannabis sativa* plant derivative	**Agonist** (Ryberg et al. 2007)
		Inconsistent findings depending on functional assay and cells type used.
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		Stimulates Ca^{2+} mobilization and RhoA activation in hGPR55-transfected HEK293 cells (Lauckner et al. 2008)
		Stimulates Ca^{2+} hGPR55-transfected HEK293 cells (Lauckner et al. 2008)
		No effect for β-arrestin activation or GPR55 internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)
Cannabidiol (CBD)	*Cannabis sativa* plant derivative	**Antagonist** (Ryberg et al. 2007; Whyte et al. 2009)
		Inhibits agonist for [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		Antagonizes the effect that LPI had on ERK1/2 phosphorylation in human osteoclasts cells (Whyte et al. 2009)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
Abnormal cannabidiol (Abn-CBD)	Synthetic regioisomer of CBD	**Agonist** (Ryberg et al. 2007)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		No effect on Ca^{2+} mobilization in HEK293 cells transfected with hGPR55 (Oka et al. 2007)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
O-1602	Synthetic derivative of Abn-CBD	**Agonist** (Johns et al. 2007; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		Induces ERK1/2 phosphorylation and RhoA activation in human osteoclasts cells (Whyte et al. 2009)
		Initiates RhoA activation in mouse osteoclast cells (Whyte et al. 2009)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
O-1918	Synthetic derivative of CBD	**Putative GPR55 antagonist** (Henstridge et al. 2011; Kremshofer et al. 2015)
		Structurally similar to CBD; however, no studies show that this compound actually binds to GPR55
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
SR141716A (rimonabant)	Synthetic cannabinoid – diarylpyrazole	**Agonist at higher μM concentrations and antagonist at lower μM concentrations**
		Antagonizes a number of GPR55 agonists in HEK293 cells transfected with hGPR55 and dorsal root ganglion from mice (Lauckner et al. 2008)
		Induces β-arrestin activation and receptor internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)

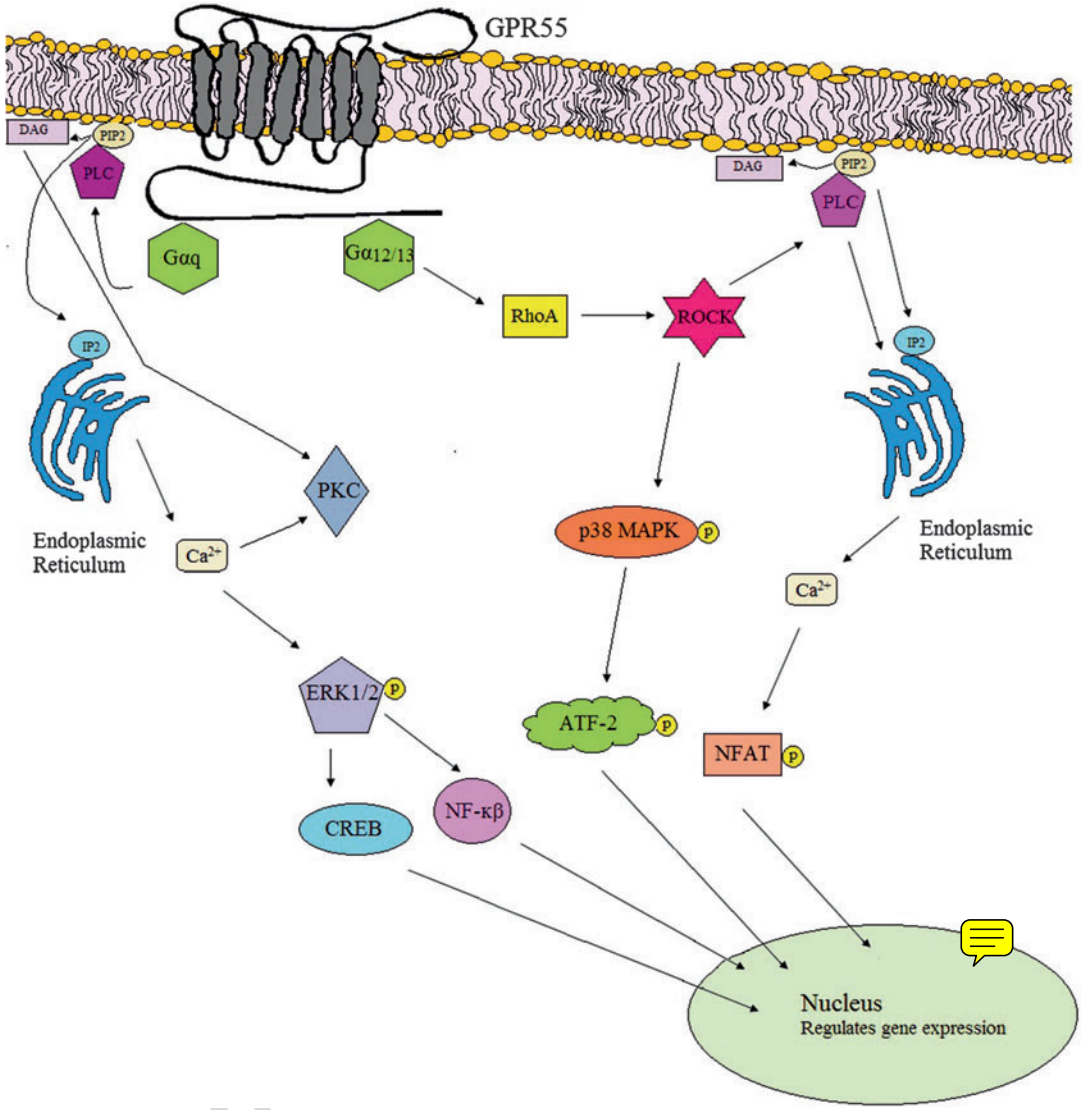
(*continued*)

GPR55, Table 2 (continued)

Compound	Compound type	Some of the observed actions at GPR55
AM251	Synthetic cannabinoid – diarylpyrazole	**Agonist** (Henstridge et al. 2010; Ryberg et al. 2007)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		Induces β-arrestin activation and receptor internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)
AM281	Synthetic cannabinoid	**Weak agonist** (Henstridge et al. 2010)
		No effect for [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		No effect β-arrestin activation and receptor internalization in U2OS cells expressing GPR55E (Kapur et al. 2009)
JWH-015	Synthetic cannabinoid	**Agonist** (Ryberg et al. 2007)
		Stimulates Ca^{2+} mobilization in hGPR55-transfected HEK293 cells and dorsal root ganglion derived from mice (Lauckner et al. 2008)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
HU-210	Synthetic cannabinoid	**Agonist** (Lauckner et al. 2008; Ryberg et al. 2007)
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		No effect for β-arrestin activation in U2OS cells expressing GPR55E (Kapur et al. 2009)
Virodhamine	Synthetic cannabinoid	**Agonist**
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
CP55940	Synthetic cannabinoid	**Agonist at lower concentrations and antagonist at high concentrations**
		Induces [^{35}S] GTPγS binding in HEK293 cells transfected with hGPR55 (Ryberg et al. 2007)
		Blocks formation of β-arrestin, receptor internalization, and phosphorylation of ERK1/2 in U2OS cells expressing GPR55E (Kapur et al. 2009)
GSK494581A	Synthetic	Selective agonist (Kargl et al. 2012)
GSK319197A	Synthetic	Selective agonist (Kargl et al. 2012)
CID1792197	Synthetic	Selective agonist (Kotsikorou et al. 2011)
CID1172084	Synthetic	Selective agonist (Kotsikorou et al. 2011)
CID2440433	Synthetic	Selective agonist (Kotsikorou et al. 2011)
CID23612552 (ML191)	Synthetic	Selective antagonist (Kotsikorou et al. 2013)
CID1434953 (ML192)	Synthetic	Selective antagonist (Kotsikorou et al. 2013)
CID1261822 (ML193)	Synthetic	Selective antagonist (Kotsikorou et al. 2013)
CID16020046	Synthetic	Selective antagonist (Kargl et al. 2013)

This table highlights the endogenous fatty acids, plant derivatives and synthetic compounds which act as agonist or antagonists for GPR55

et al. 2014). GPR55-mediated signaling is diminished in a number of downstream signaling pathways including the activation of NFAT, NF-κβ, and CREB, yet in contrast ERK1/2 MAPK activation was improved with the formation of a heteromers between GPR55 and CB_2 (Balenga et al. 2014). CB_2-mediated signaling was also altered in the presence of GPR55 (Balenga



GPR55, Fig. 4 GPR55 cellular signaling. Activation of GPR55 can initiate $G_{\alpha 12/13}$ (Henstridge et al. 2009; Lauckner et al. 2008; Ryberg et al. 2007) and $G_{\alpha q}$ subunits (Lauckner et al. 2008; Pertwee et al. 2010). The $G_{\alpha 12/13}$ subunit stimulates the RhoA (Henstridge et al. 2009; Lauckner et al. 2008; Ryberg et al. 2007). Activation of the RhoA-ROCK pathway induces the phosphorylation of p38 MAPK and then subsequently phosphorylation of ATF-2 (Oka et al. 2010). The initiation of the RhoA-ROCK pathway also results in activation of PLC, resulting in Ca^{2+} release from the endoplasmic reticulum triggering activation of transcription factor NFAT, resulting in nuclear translocation (Henstridge et al. 2009). Whereas the $G_{\alpha q}$-mediated PLC activation results in the release of DAG and Ca^{2+} (Lauckner et al. 2008), which activates PKC and ERK1/2 phosphorylation, triggering CREB and NF-κβ (Henstridge et al. 2010). As a result, activation of the transcription factors ATF-2, NFAT, CREB, and NF-κβ may regulate gene expression within the nucleus of the cell (Henstridge et al. 2009, 2010; Lauckner et al. 2008; Oka et al. 2010; Ryberg et al. 2007; Whyte et al. 2009).

et al. 2014). CB_2 and GPR55 are both expressed in human neutrophils, and when both receptors are activated, the signaling pathways RhoA and cdc42 are enhanced, while Rac2 signaling is diminished (Balenga et al. 2011). In cancer cells, GPR55 and CB_2 also have been found to form heteromers resulting in unique signaling properties (Moreno et al. 2014).

Taken together, as GPR55 is expressed in a number of tissues where CB_1 and CB_2 are also expressed, these studies suggest there could be pharmacological implications for the GPR55 and CB_1 and CB_2 heteromers, as traditional signaling of each receptor is altered when heteromers are formed.

(Patho)physiological Role of GPR55

GPR55 is distributed throughout the body in humans, which suggests that this receptor may have a number of (patho)physiological roles. As described below, emerging research has shown this receptor to have a role physiologically in the pancreas, gastrointestinal tract, vasculature, and bone. GPR55 has also been implicated in a number of pathophysiological conditions including cancer, neuropathic pain, inflammatory conditions, obesity, and diabetes (Fig. 5).

Bone

GPR55 has been demonstrated to be involved in regulating osteoclast formation and function in vitro. Activation of GPR55, using either LPI or O-1602, stimulates osteoclast polarization and reabsorption (Whyte et al. 2009). Male GPR55 knockout mice but not the female knockout mice have proportionally higher osteoclast numbers in long bones and impaired osteoclast function when compared to male and female wild-type mice, respectively (Whyte et al. 2009). The same study suggests that blocking GPR55 using CBD can inhibit bone resorption in vivo. Taken together these findings add to the hypothesis that blocking GPR55 may be beneficial for bone turnover and arthritic diseases (Whyte et al. 2009).

Gastrointestinal Tract

GPR55 is expressed throughout the gastrointestinal tract (Henstridge et al. 2011) and is abundantly expressed in the small intestine (Henstridge et al. 2011). This receptor has been located in mucosal scrapings (Schicho et al. 2011) and myenteric plexus (Schicho et al. 2011) in the rat colon, as well as in enteric neurons (Lin et al. 2011; Schicho et al. 2011). Activating GPR55 has been shown to slow gastrointestinal motility (Li et al. 2013). GPR55 therefore may play a role in gastrointestinal function, specifically, in secretion and motility.

GPR55 has also been shown to be involved in gastroparesis in a type 1 diabetes model using STZ mice (Lin et al. 2014). The expression of GPR55 is upregulated in the stomach in this condition, and treatment with the potent agonist LPI helps to protect against gastroparesis in these mice.

Inflammation

GPR55 may have a pro-inflammatory role in colitis. One study used two different experimentally induced models of colitis, either by administrating dextran sulfate sodium into the drinking water or by intrarectally applying trinitrobenzene sulfonic acid (Stancic et al. 2015). Antagonizing GPR55 using highly selective antagonist CID16020046 in both models had an anti-inflammatory affect by reducing pro-inflammatory cytokines (Stancic et al. 2015). When GPR55 was antagonized using CID16020046, this compound also interfered with macrophage and lymphocyte recruitment in the colon, thereby protecting against inflammation in the colon (Stancic et al. 2015). In addition to the pharmacological modulation, GPR55 knockout mice have a reduction in inflammatory scores when compared to wild-type mice (Stancic et al. 2015). In contrast, another study found that administrating O-1602, which acts as an agonist for GPR55, had anti-inflammatory properties and ameliorated experimentally induced colitis (Schicho et al. 2011). However, this anti-inflammatory effect was still apparent in GPR55 knockout mice, suggesting that this compound was targeting a putative cannabinoid receptor other than GPR55 (Schicho et al. 2011). Therefore the current evidence suggests that blocking GPR55 may be beneficial in the treatment of inflammatory bowel disease; however additional supporting studies are required before any conclusive decisions can be made.

GPR55 is highly expressed in monocytes and natural killer cells; activation of these cells by LPI results in secretion of pro-inflammatory cytokines (Chiurchiu et al. 2015). Conversely, in a cerulein-

GPR55, Fig. 5 Summary of (patho)physiological roles for GPR55. This figure summarizes the physiological roles GPR55 plays in the bone, gastrointestinal tract, vasculature, and pancreas. This figure also highlights the pathophysiological role GPR55 plays in inflammation, cancer, inflammatory, and neuropathic pain, as well as obesity and T2D. ↑ increased, *AEA* anandamide, *GPR55* G protein-coupled receptor 55, *GI* gastrointestinal, *LPI* L-α-lysophosphatidylinositol, *SAT* subcutaneous adipose tissue, *T1D* type 1 diabetes mellitus, *T2D* type 2 diabetes mellitus, *VAT* visceral adipose tissue

induced acute pancreatitis model, GPR55 expression is reduced with treatment of either O-1602 or CBD (an agonist and antagonist for GPR55), ~~with~~ O-1602 treatment ~~improving~~ pathological changes (Li et al. 2013). Taken together, these studies indicate GPR55 may be a potential target for inflammatory-related conditions in the future, which may vary depending on the associated condition.

Cancer

A large body of evidence demonstrates that GPR55 and the GPR55 potent agonist LPI have a role in cancer progression. Circulating LPI levels are increased in individuals with colon cancer when compared with healthy individuals (Kargl et al. 2016). Furthermore, GPR55 expression has been correlated with cancer aggressiveness. GPR55 expression and LPI have been

associated with proliferation in a number of cancers including ovarian, prostate, breast, and glioblastoma while being involved in migration of breast cancer (Leyva-Illades and Demorrow 2013) and colon cancer (Kargl et al. 2016). Given that GPR55 has a role in cancer progression, it is not surprising that the receptor is expressed in a number of cancers and cancer cell lines including cholangiocarcinoma, breast cancer, prostate cancer cell lines, ovarian cancer cell lines, glioblastoma, human pancreatic ductal adenocarcinoma, human skin tumors and other squamous cell carcinomas, lymphoblastoid cell lines, human astrocytoma, melanoma, B lymphoblastoma, and lung cancer (for an in-depth review on GPR55 as an emerging target for cancer therapy, refer to Leyva-Illades and Demorrow 2013).

GPR55 has a role in migration and metastasis in colon cancer using human colorectal carcinoma 116 (HCT116) cells as a colon cancer model (Kargl et al. 2016). One study found that migration of cancer cells was induced when the potent GPR55 agonist, LPI, was added to HCT116 cells overexpressing GPR55 and that this effect was blocked by GPR55 antagonists (Kargl et al. 2016). Furthermore, chemotactic assays showed that invasion and migration of cancer cells were both inhibited by the GPR55 antagonists CID16020046 and CBD (Kargl et al. 2016).

Inflammatory and Neuropathic Pain

GPR55 appears to have a role in nociception. GPR55 knockout mice ~~have been shown to be~~ resistant to neuropathic and inflammatory pain (Staton et al. 2008), while the GPR55 agonist O-1602 has ~~been shown to have~~ pronociceptive effects (Staton et al. 2008). Therefore it ~~can~~ be hypothesized that activating GPR55 has pronociceptive properties for neuropathic pain while blocking the receptor may have antinociceptive results.

Obesity

Moreno-Navarrete et al. (2012) found that GPR55 expression is increased in obesity, specifically in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT), with circulating levels of LPI also increased in human obesity (Moreno-Navarrete et al. 2012). LPI has also been shown to increase lipogenic genes in a human adipose tissue cell culture model (Moreno-Navarrete et al. 2012). In contrast, deletion of GPR55 in mice was found to promote obesity as GPR55 knockout mice had significantly reduced voluntary physical activity which was associated with the mice also having increased adiposity and increased insulin resistance (Meadows et al. 2016). Interestingly, the food intake of the GPR55 knockout mice was not altered (Meadows et al. 2016).

Diabetes Mellitus

As GPR55 is expressed in the insulin-secreting β cells in the islets of Langerhans, this suggests that this receptor has a role in insulin secretion (Romero-Zerbo et al. 2011). Romero-Zerbo et al. (2011) demonstrated that activating GPR55 using O-1602, in lean rats under hyperglycemic conditions, causes an improvement in glucose-stimulated insulin secretion. This effect was not evident in GPR55 knockout mice, supporting this receptor's role in blood glucose regulation (Romero-Zerbo et al. 2011). Further, this study also demonstrated that acute administration of O-1602, in Wistar rats, caused an increase in glucose tolerance accompanied by an increase in plasma insulin levels (Romero-Zerbo et al. 2011). These findings are further supported by more recent work using a number of cannabinoid agonists known to activate GPR55, which increased insulin secretion in BRIN-BD11 cells (a glucose-sensing and insulin-secreting line derived from isolated rat pancreatic ~~beta~~ cells) (McKillop et al. 2013).

Co-localization experiments from the same study also showed that GPR55 is co-localized with insulin in both BRIN-BD11 and pancreatic islets from m[illegible] while there was no evidence of GPR55 in the ~~alpha~~ islets that secrete glucagon (McKillop et al. 2013). These findings further support the study by Romero-Zerbo et al. (2011) which found that mRNA and protein expression of GPR55 are expressed in β islets but not in the α or δ islets in rats. Taken together, these two studies support the hypothesis that activating GPR55 in

beta islets of the pancreas may enhance beta cell function and could therefore be a beneficial therapeutic target in the treatment of diabetes mellitus.

Summary

Since the discovery of GPR55 in 1999, almost two decades of research has found that this receptor is diversely expressed throughout the human body. GPR55 has a number of different ligands, some of which are cannabinoid compounds and derivatives, with the non-cannabinoid endogenous fatty acid LPI being the most potent agonist for this receptor. GPR55 is a putative cannabinoid receptor which has a number of physiological roles in the bone, gastrointestinal tract, pancreas, and vasculature. Targeting this receptor may also be of benefit in inflammatory conditions, diabetes, inflammatory and neuropathic pain, cancer, and obesity. Signaling properties of GPR55 vary depending on the agonist/antagonist utilized. This receptor has also been found to form heteromers with both cannabinoid receptors CB_1 and CB_2. The receptors' ability to form heteromers alters signaling properties of the receptors involved and thus may be leading to some variation in the literature regarding the effects of GPR55 in various tissues and pathophysiological conditions. Future directions should focus on the effect that the second-generation GPR55 agonists and antagonists have in different disease states, as well as these compounds' effects on signaling using both in vitro and in vivo models. Further investigation into this receptors role is required to elucidate the therapeutic potential of GPR55 in current known and newly identified pathophysiological conditions.

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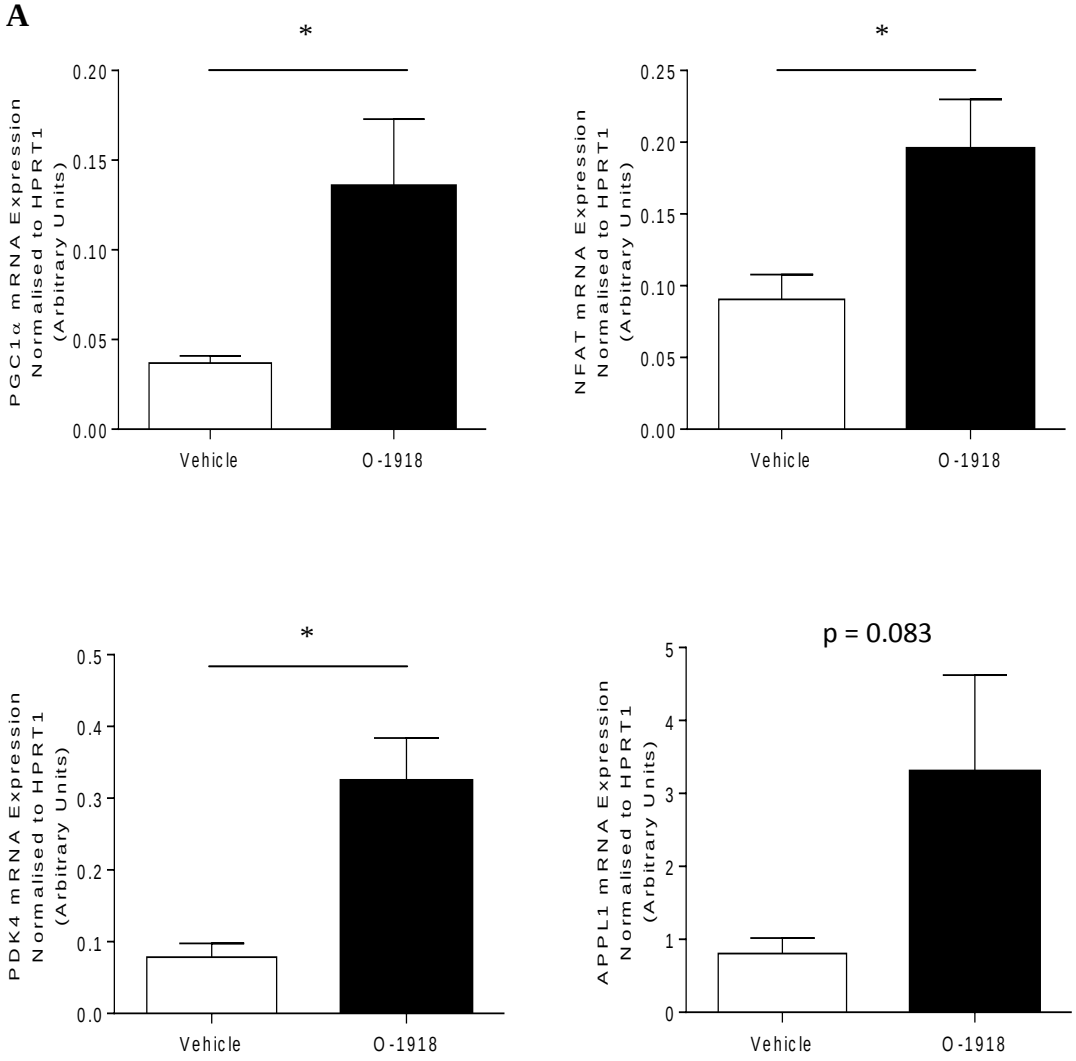
APPENDIX TWO

A



The effect 24 hours of treatment with 100 nM of O-1918 has on C_2C_{12} myotubes, on markers involved in oxidative capacity and adiponectin signalling regulation. The abundance in mRNA expression of these markers were normalised to the housekeeping gene Hypoxanthine Phosphoribosyltransferase (HPRT1) and expressed as mean ± SEM (n = 7 – 8). (A) Nuclear Factor of Activated T- Cells (NFAT), (B) Peroxisome proliferator-activated receptor gamma co activator 1-alpha (PGC1α), (C) Pyruvate Dehydrogenase Kinase 4 (PDK4) and (D) Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1 (APPL1). Open Bars – Control, C_2C_{12} myotubes treated with vehicle 0.1% EtOH, Closed Bar – C_2C_{12} myotubes treated with 0.1% EtOH and 100 nM O-1918. * indicates significance ($p < 0.05$).