# Renal Cannabinoid Receptor Expression and Function: Their Role in Obesity and Diabetes

A thesis submitted by

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# Abstract

Obesity and diabetes are clearly established independent risk factors for renal disease. Therapeutic targets have been investigated for their role in treating obesity and diabetic associated renal damage. The endocannabinoid system is an important endogenous lipid signalling system known to mediate glucose and lipid metabolism, inflammation and energy storage. Specifically, diabetes mellitus and obesity induces alterations in the expression of cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2) and putative cannabinoid receptor G-protein coupled receptor 55 (GPR55) in a tissue specific manner. Renal expression and function of these receptors, particularly within the pathophysiological context of obesity and diabetes related renal damage is poorly understood. The research presented in this thesis examines the renal expression and function of CB1, CB2 and GPR55. The significant aim of this PhD candidature was to examine the expression of cannabinoid receptors in the kidney in obese and diabetic conditions. Subsequent studies sought to evaluate the actions of selective manipulation of the receptors by synthetic compounds on markers of renal damage and structure in an animal model of diet induced obesity (DIO).

The first study demonstrated that *in vivo,* expression CB1 is upregulated in whole kidney of diabetic animals. An *in vitro* model of diabetic nephropathy leads to increases to both CB1 and GPR55 expression specifically within proximal tubule cells. The second study identified that expression of CB2 was downregulated in an *in vivo* and *in vitro* model of diabetes, and that expression of CB2 is modulated by

internalisation of albumin in proximal tubule cells. Activity of albumin uptake by proximal tubule cells may be mediated by CB2 independent of ERK1/2 signalling. The third study examined renal cannabinoid receptor expression in a DIO model in rats. Study 3 found that the receptors show a similar pattern of renal expression to what was demonstrated in Study 1 and 2 with diabetic conditions, where CB1 and GPR55 are upregulated and CB2 is downregulated in renal tissue of obese rats. Studies 4, 5 and 6 identified the renal effects of chronic administration of cannabinoid receptor specific compounds which are used to block or stimulate the activity of CB1, CB2 and GPR55 respectively in obese rats.

This research has established that renal cannabinoid receptor expression of CB1, CB2 and GPR55 are altered under diabetic and obese conditions. Further, pharmacologically targeting these receptors *in vivo* in a DIO model resulted in changes to measures of renal damage. In conclusion, the cannabinoid receptors play a significant role in a number of important signalling pathways in the diabetic and obese milieu. This indicates that these receptors could be potential therapeutic targets for the treatment of kidney disease caused by obesity and diabetes.

# Table of Contents

# Declaration

I, Kayte Jenkin, declare that the PhD thesis entitled '*Renal Cannabinoid Receptor Expression and Function: Their Role in Obesity and Diabetes'* is no more than 100,000 words in length including quotes and exclusive of 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

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# Publications

## Published Manuscripts Arising From This Thesis

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**Jenkin, KA**, Verty, AN, McAinch, AJ, Hryciw, DH 2012, 'Endocannabinoids and the Renal Proximal Tubule: An emerging role for diabetic nephropathy' *The International Journal of Biochemistry and Cell Biology* 44(11), 2028- 2031.

## Manuscripts in Preparartion

**Jenkin, KA**, O'Keefe, L, Simcocks, AC, Mathai, ML, McAinch, AJ and Hryciw, DH, 'Chronic Administration with AM251 Improves Cardio-Renal Outcomes in Obese Rats'.

**Jenkin, KA**, O'Keefe, L, Simcocks, AC, Mathai, ML, McAinch, AJ and Hryciw, DH, 'Renal Effects of Chronic Pharmacological Manipulation of CB2 in Rats with Diet Induced Obesity'.

**Jenkin, KA**, Simcocks, AC, O'Keefe, L, Mathai, ML, McAinch, AJ and Hryciw, DH, 'The Effects of Chronic Administration of O-1602 and O-1918 in Diet Induced Obese Rats on Metabolic and Cardio-Renal Parameters'.

**Jenkin, KA**, McAinch, AJ, Andrew J McAinch, Zhang, Y, Kelly, DJ and Hryciw, DH, 'Elevated CB1 and GPR55 receptor expression in proximal tubule cells and whole kidney exposed to diabetic conditions'.

**Other Publications**

Simcocks, AC, O'Keefe, L, **Jenkin, KA,** Mathai, ML, Hryciw, DH and McAinch, AJ, 2014 'A potential role for GPR55 in the regulation of energy homeostasis' *Drug Discovery Today,* 19(8), 1145-1151.

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' *Cellular Physiology and Biochemistry,* 5(44), 815-823

Slattery, C, **Jenkin, K**, Lee, A, Simcocks, A, McAinch, AJ, Poronnik, P & Hryciw, D 2011 'Na+/H+ exchanger regulatory factor 1 (NHERF1) binds an internal binding site in the scavenger receptor megalin' *Cellular Physiology and Biochemistry,* 27(2), 171-178

**Jenkin, KA**, McAinch, AJ, Grinfeld, E & Hryciw, DH 2010, 'Role for cannabinoid receptors in human proximal tubule cell hypertrophy' *The International Journal of Biochemistry & Cell Biology,* 26(6), 879 – 886

# Presentations

## Oral Presentations

**Jenkin, KA,** McAinch, AJ, Zhang, Y, Kelly, DJ and Hryciw, DH, 2013 'Elevated CB1 and GPR55 cannabinoid receptor expression in proximal tubule cells and whole kidney exposed to diabetic conditions' *Australian Physiological Society (AuPS).* (Geelong, Australia, December 2013)

**Jenkin, KA,** 2013 'Elevated CB1 and GPR55 cannabinoid receptor expression in proximal tubule cells and whole kidney exposed to diabetic conditions' *College of Health and Biomedicine, Research Student Conference.* Victoria University. (Melbourne, Australia, November 2013)

**Jenkin, KA,** 2013 'The influence of endocannabinoids on renal function in diet induced obese rats' *Victorian Obesity Consortium Symposium (VOC).* (Melbourne, Australia, August 2013)

**Jenkin, KA,** Simcocks, A, O'Keefe, L, Mathai, M, McAinch, AJ and Hryciw, DH 2013 'Cardio-Renal effects of chronic administration with CB2 agonist AM1241 and CB2 antagonist AM630 in rats with diet induced obesity' *International Cannabinoid Research Society (ICRS).* (Vancouver, Canada, June 2013)

**Jenkin, KA,** 2013 'The cardio-renal effects of targeting cannabinoid receptor 2 in a rat model of diet induced obesity' *Health Research Symposium* University of Texas El Paso and Victoria University. (Melbourne, Australia, May 2013)

**Jenkin, KA,** 2013 'The cardio-renal effects of targeting cannabinoid receptor 2 in a rat model of diet induced obesity' *Biomedical and Lifestyle Disease Unit (BioLED) Scientific Meeting,* Victoria University. (Melbourne, Australia, May 2013)

**Jenkin, KA,** McAinch, AJ and Hryciw, DH 2011 'Effect of elevated glucose and albumin on endocannabinoid receptor expression in PTC' *Australian and New Zealand Nephrology Society (ANZOS).* (Adelaide, Australia, September 2011).

**Jenkin, KA,** McAinch, AJ and Hryciw, DH 2011 'Effect of Elevated Glucose and Albumin Levels on Endocannabinoid Receptor Expression in Proximal Tubule Cells' Victoria University Faculty of Health, Engineering and Science 2011 Postgraduate Research Conference, Victoria University (Melbourne, Australia, July 2011).

**Poster Presentations**

**Jenkin, KA,** McAinch, AJ and Hryciw, DH 2012 'Cannabinoid receptor 2 expression in proximal tubule cells exposed to elevated glucose and albumin' *Australian Physiological Society (AuPS).* (Sydney, Australia, December 2012).

McAinch, AJ and Hryciw, DH, Grinfeld, E and **Jenkin KA** 2010 'Role and Expression of Cannabinoid Receptors in Kidney Tissue and Proximal Tubule Cells' *Victoria University Faculty of Health, Engineering and Science 2010 Postgraduate Research Conference*, Victoria University (Melbourne, Australia, July 2010).

# List of Figures

# List of Tables

# List of Abbreviations

| | |
|---|---|
| 2-AG | 2-Arachidonoylglycerol |
| AEA | Anandamide |
| AEC | Animal Ethics Committee |
| BCA | Bicinchoninic Acid |
| BLAST | Basic Local Alignment Search Tool |
| BMI | Body Mass Index |
| BSA | Bovine Serum Albumin |
| BUN | Blood Urea Nitrogen |
| CB1 | Cannabinoid Receptor 1 |
| CB1 -/- | Cannabinoid Receptor 1 Knockout Mouse |
| CB2 | Cannabinoid Receptor 2 |
| CBD | Cannabidiol |
| CCR2 | C-C Motive Receptor 2 |
| cDNA | Complementary Deoxyribonucleic Acid |
| CHOP | C/EBP homologous protein |
| CKD | Chronic Kidney Disease |

| | |
|---|---|
| CRIP | Cannabinoid Receptor Interacting Protein |
| CTGF | Connective Tissue Growth Factor |
| DEPC | Diethylpyrocarbonate Treated Water |
| DMEM/F12 | Dulbecco's Modified Eagle Media/ Nutrient Mixture F12 |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGF | Epidermal Growth Factor |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ERK1/2 | Extracellular Signal-Regulated Kinase 1/2 |
| ESRD | End Stage Renal Disease |
| FBS | Foetal Bovine Serum |
| *fa/+* | Obese Zucker rat strain containing a heterogeneous mutation to the *fa* gene |
| *fa/fa* | Obese Zucker rat strain containing a homozygous mutation to the *fa* gene |
| FAAH | Fatty Acid Amide Hydrolase |
| FCA | Fruend's Complete Adjunct |

| | |
|---|---|
| GAPDH | Glyceraldehydes-3-Phosphate Dehydrogenase |
| GFR | Glomerular Filtration Rate |
| GPCRs | G-Protein Coupled Receptors |
| GPR55 | G-Protein Coupled Receptor 55 |
| GPR55 -/- | GPR55 Knockout Mouse |
| GPR55+/+ | GPR55 Wild-type Mouse |
| H&E | Haematoxylin and Eosin |
| HEK293 | Human Embryonic Kidney 293 Cell Line |
| HEPES | 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid |
| HFD | High Fat Diet |
| HK2 | Human Kidney Proximal Tubule Cell Line |
| HRP | Horseradish Peroxidase |
| IFNγ | Interferon γ |
| IGF-1 | Insulin Like Growth Factor 1 |
| IL | Interleukins |
| IP | Intraperitoneal |
| ITS | Human Insulin, Transferrin and Sodium Selenite Liquid Media Supplement |

| | |
|---|---|
| kD | Kilo Daltons |
| LPI | L-α-lysophosphatidylinositol |
| MAPK | Mitogen-Activated Protein Kinase |
| MCP-1 | Monocyte Chemoattractant Protein |
| mg/dl | Milligram per Decilitre |
| mg/g | Milligram per Gram |
| mg/kg/day | Milligram per Kilogram per Day |
| mg/ml | Milligram per Millilitre |
| MJ/kg | Megajoules per Kilogram |
| ml/min | Millilitres per Minute |
| ml/min/kg | Milliltres per Minute per Kilogram |
| mmol/l | Millimole per Litre |
| MOPS | 3-(N-morpholino) Propanesulfonic Acid |
| mRNA | Messenger Ribonucleic Acid |
| ng/ml | Nanogram per Millilitre |
| NHE-3 | Sodium-Hydrogen Exchanger-3 |
| NOS | Nitric Oxide Synthase |
| ob/ob | Obese mouse strain demonstrating leptin deficiency |

| | |
|---|---|
| OCT | Optimal Cutting Temperature Compound |
| OK | Opossum Kidney Proximal Tubule Cell Line |
| PAS | Periodic Acid Schiffe |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| pg/ml | Picogram per Millilitre |
| PPAR | Peroxisome Proliferator Activated Receptors |
| RAAS | Renin-Angiotensin-Aldosterone System |
| RNA | Ribonucleic Acid |
| SGLT | Sodium-Glucose Transporters |
| STZ | Streptozotocin |
| T2DM | Type 2 Diabetes Mellitus |
| TBS | Tris Based Saline |
| TBST | Tris Based Saline Tween 20 |
| TGF-β1 | Transforming Growth Factor β1 |
| THC | $\Delta^9$ Tetrahydrocannabinol |
| TNF-α | Tumour Necrosis Factor-α |
| TR-albumin | Texas Red Albumin |

| | |
|---|---|
| TRPV1 | Transient Receptor Vanilliod Type I Receptor |
| µg/ml | Microgram per milliltre |
| VEGF | Vascular Endothelial Growth Factor |
| vol/vol | Volume for Volume |
| x *g* | Times gravity |

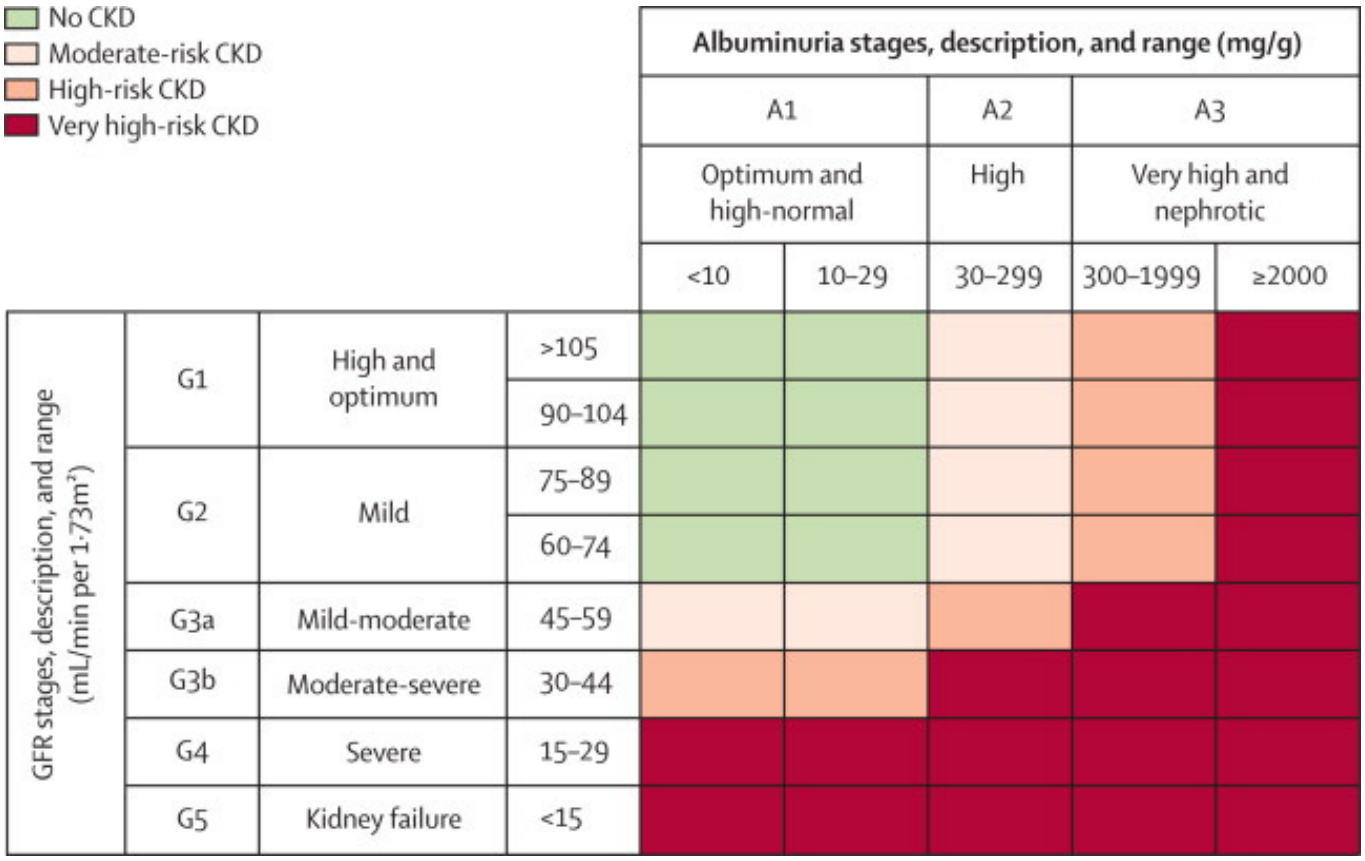
# Chapter 1 - Literature Review

## *1.1 Renal Disease: Chronic Kidney Disease*

The renal system plays a crucial role in the homeostatic balance of fluids, essential ions, proteins and nutrients (MacIsaac and Watts, 2005). In Australia, 16% of adults over the age of 65 have at least one indicator of renal disease (Chadban et al., 2003, Boudville et al., 2012). Damage to the renal system resulting in the development of chronic kidney disease (CKD) is defined as any state where loss of renal function or kidney damage occurs for 3 months or longer (Cass et al., 2006). CKD is the result of structural or functional changes that affects the kidney, and can be varied in severity (Levey et al., 2003). CKD is categorised as one of five stages, with the fifth and final stage being end stage renal failure (AIHW, 2009, AIHW, 2011, Tong and Stevenson, 2007). The classification of these stages have been modified over the past decade to ensure that marker of renal damage, such as albuminuria can be incorporated with the more traditionally established markers of renal disease, namely glomerular filtration rate (GFR), see Table 1.1 below (Levey and Coresh, 2012).

The most basic measurement of kidney function is GFR, which is the quantity of glomerular filtrate produced by the kidneys per minute. GFR has been used for many years as a foundation to detect and evaluate the severity of CKD. Normal GFR levels are considered to be 90 ml/min or above while moderate to severe renal impairment are considered at a GFR of below 40 ml/min (Levey and Coresh, 2012, Levey et al., 2003, Regeniter et al., 2009).

**Table 1.1: Prognosis of Chronic Kidney Disease by GFR and Albuminuria .** GFR and albuminuria are established indicators for assessment of renal health outcomes. As GFR declines and albuminuria progresses, worse patient prognostic outcomes are expected. Taken from Levy and Coresh (2012).



In order to maintain osmotic homeostasis, the proximal tubule is required to reabsorb several grams of protein each day (Christensen et al., 2007), making urinary protein excretion an important factor in the diagnosis and evaluation of CKD. When the cellular structure of the kidneys are damaged, the filtration process becomes impaired (causing a drop in GFR), and the reabsorptive pathways also diminishes resulting in an increase in total proteins present in the urine. Albumin makes up the major component of plasma, and is essential for the maintenance of oncotic pressure. Albumin present in the urine is often the first indicator of renal damage (Thrailkill et al., 2009).

An important feature of nephropathy is that up to 90% of the kidney can be damaged before symptoms start to appear (Go et al., 2004), which underlies important recognition by worldwide health authorities that CKD needs to be identified and properly managed during its early stages (Levey and Coresh, 2012). In Australia, CKD affects 1 in 7 adults and treatment of CKD, particularly dialysis, is one of the leading causes for outpatient hospitalisations (AIHW, 2012). CKD is both a national and international concern as available treatments (including dialysis and transplantation) are expensive and the prevalence of CKD is increasing globally (AIHW, 2011). CKD has multiple adverse outcomes including premature death and cardiovascular disease. The most severe form of CKD is end stage renal disease (ESRD), which is projected to escalate 80% in Australia from rates reported in 2009 (AIHW, 2011). In 2009, 11 people per 100,000 population had ESRD, and in 2020, the projected rates increase to 19 per 100,000 population (Levey et al., 2005, AIHW, 2011), see Figure 1.1. Further, in Australia, males develop ESRD at 1.5 times the

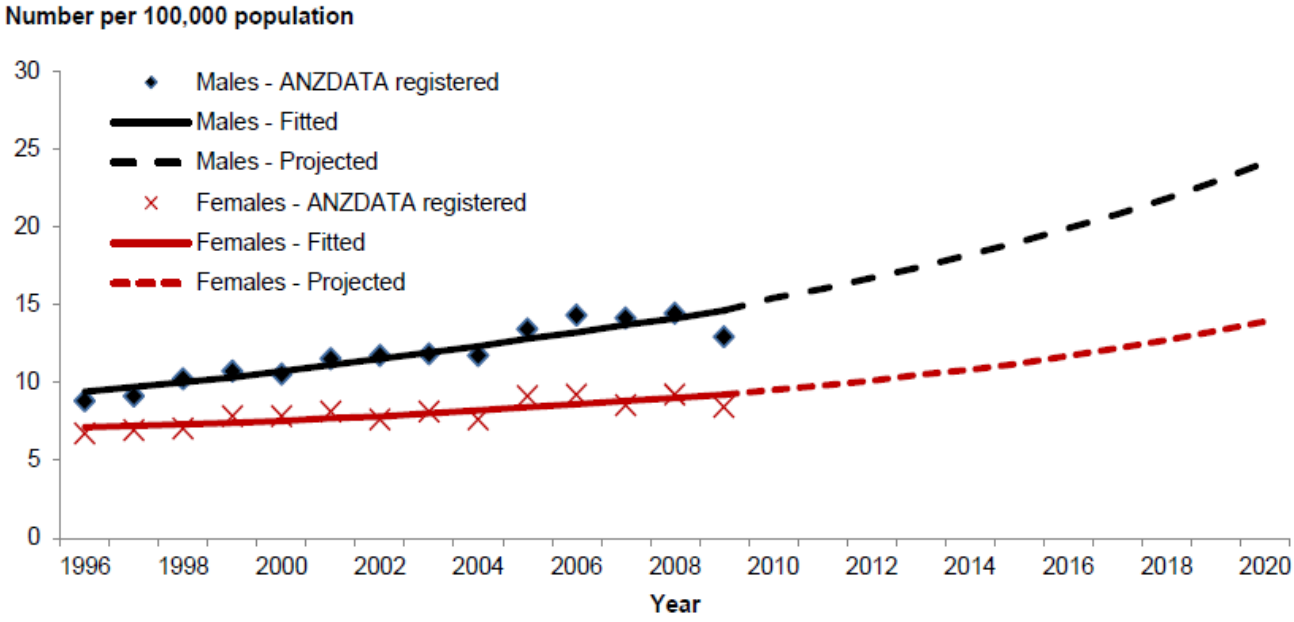



**Figure 1.1: Incidence rates of current and projected end stage renal disease (ESRD) by sex in Australia 1996-2020**. From Australian Institute of Health and Welfare National Health Survey (2011).

rates observed in the female population (Figure 1.1) and also have higher rates of hospitalisation for CKD than females (AIHW, 2011). This pattern is consistent with worldwide trends of ESRD (Iseki et al., 1996), and it is postulated that this discrepancy between the sexes may due to higher rates of associated risk factors for CKD, including hypertension, which is present at a higher rate in males (Klag et al., 1996)

### *1.1.1 Risk Factors for CKD*

CKD is frequently the consequence of a complex interaction of a number of lifestyle and clinical factors (Ekundayo et al., 2009, Foster et al., 2008, Kramer et al., 2005). Risk factors for CKD include age, sex (male), family history of CKD, smoking status, alcohol consumption, hypertension, diabetes mellitus and obesity (AIHW, 2012). High caloric intake and a sedentary lifestyle are major factors which have contributed to the rise in CKD globally, and an overall increase in the prevalence of "lifestyle diseases", including obesity and type 2 Diabetes Mellitus (T2DM) (Chadban et al., 2003). Identifying risk factors is important for understanding any disease, it is paramount in ensuring those patients with multiple risk factors have renal function evaluated. Interventions to prevent CKD and its progression to ESRD have the potential to save many lives as well as decrease health-care costs (Wang et al., 2008b).

*1.1.2 Diabetes as a Risk Factor for CKD*

T2DM is a multifactorial metabolic disorder characterised by hyperglycaemia and associated with a relative deficiency of insulin secretion, along with insulin resistance (a reduced response of target tissues to insulin) (Surampudi et al., 2009). The link between diabetes and kidney disease has been long established, with observations of renal damage from diabetes being observed as early as the 19$^{th}$ Century (Wolf and Ziyadeh, 1999). AusDIAB, the largest longitudinal study undertaken in Australia which examined diabetes, pre-diabetes, heart disease and kidney disease in the adult population, found that several markers of renal damage were higher amongst the diabetic population than non-diabetic population (Chadban et al., 2003). In addition, proteinuria was four times more likely to be observed in diabetic individuals and renal impairment, as indicated by a reduced GFR of ≤ 60 ml/min, was three times more prevalent in the diabetic cohort (Chadban et al., 2003). Worldwide, diabetic nephropathy will develop in up to 40% of people with type 2 diabetes mellitus, and is the leading cause of ESRD (Rossing et al., 2004). In Australia, a third of Australian patients with ESRD result from renal damage from diabetic nephropathy (Colagiuri et al., 2003). Recently, a longitudinal study highlighted the significance of CKD as a co-morbidity of diabetes by examining diabetic and healthy people with and without kidney disease (Afkarian et al., 2013). It was found that mortality rates of people with both diabetes and kidney disease was up to 31.1% over a ten year period compared to rates of 11.5% mortality for diabetic people without kidney disease and 7.7% for non-diabetic people without kidney disease (Afkarian et al., 2013). Optimizing glycaemic control and blood pressure can slow the

progression of diabetic nephropathy. Importantly, no treatment as yet for CKD prevents progression to ESRD (Detournay et al., 2012, Wang et al., 2012).

### *1.1.3 Obesity as a Risk Factor for CKD*

Obesity is a disease strongly associated with many risk factors linked to CKD, namely hypertension, hyperglycaemia and dyslipidaemia (Abrass, 2004, De Jong et al., 2002). However, studies have demonstrated that even accounting for confounding factors such as diabetes and hypertension, obesity is considered an independent risk factor of CKD and its progression to ESRD (Kramer et al., 2005, Mathew et al., 2011, Foster et al., 2008, Hsu et al., 2006). Conclusive evidence shows that obesity is a significant risk factor for the incidence and progression of CKD (Kramer et al., 2005, Mathew et al., 2011, Hsu et al., 2006). Recent research into obesity associated kidney disease has found that overweight individuals have a 40% increase in risk of kidney disease than individuals of normal weight (Abrass, 2004, Wang et al., 2008b). Further, overweight or obese individuals have worse outcomes regarding treatment options such as successful kidney transplants and increased tendency of progression to end stage renal failure (Wang et al., 2008b). In turn, weight loss, particularly through interventions such as bariatric surgery which can achieve significant weight loss, is positively associated with improvements to markers of renal damage (decrease in proteinuria, improved GFR and creatinine clearance) as well as minimising confounding risk factors such as hypertension and insulin sensitivity (Mathew et al., 2011).

### *1.1.4 Pathophysiology of Renal Disease*

In a healthy kidney, the blood is filtered by the glomerulus, and initially the proximal tubule cells are responsible for the majority of reabsorption of fluid and solutes from the filtrate. Filtration of blood plasma from the capillary bed to the glomerular capsule is primarily driven by the blood hydrostatic pressure, which maintains vasomotor tone of the afferent arteriole entering the glomerulus, and the efferent arteriole exiting the glomerulus (Zatz et al., 1986). Water and solutes pass through fenestrations of the endothelium and this 'slit diaphragm' is maintained by a population of podocyte and mesangial cells (Schlöndorff and Banas, 2009). The filtrate then flows along the nephron past the proximal tubule, loop of Henle and distal tubule where solutes and water are reabsorbed from the filtrate and taken up by the surrounding peritubular capillaries and *vasa recta* (Hijikata and Sakai, 1991). Substances including metabolic waste products, too large to be filtered can be secreted from the surrounding capillaries and are transported through the tubules into the filtrate. What remains in the filtrate following its passage through the nephron forms the urine. While the glomerulus is responsible for filtration, the proximal tubule accounts for the majority of reabsorption and secretion which occurs. Proximal tubule cells are enriched with transporters at both the apical and basolateral membranes, to facilitate the passage of solutes and nutrients out of the filtrate, to the blood (Christensen et al., 1998, Walmsley et al., 2010). In CKD damage to both the glomerulus and tubules contribute to the decline in renal function.

The molecular mechanisms behind glomerular and tubular damage in obesity and diabetes include extensive changes to microvascular structures, modification of the

Renin-Angiotensin-Aldosterone (RAAS) System, signalling molecules, inflammatory and pro-fibrotic cytokines, adipokines and oxidative stress pathways (Declèves et al., 2011, Boner and Cooper, 2006, Carey and Siragy, 2003, Mezzano et al., 2003) . These mechanisms give way to structural and functional changes within the kidney and allow the progression of CKD to occur. The following sections will outline how these mechanisms mediate CKD pathogenesis.

### *1.1.5 The Renin-Angiotensin-Aldosterone System in CKD*

In both diabetic nephropathy and obesity, RAAS becomes increasingly activated (Jayasooriya et al., 2008, Linden et al., 2006, Schiffrin et al., 2007). The classical RAAS pathway is a cascade of hormones which is initiated by the release of renin from specialised juxtaglomerular cells of the afferent arteriole (Carey and Siragy, 2003). The resulting effect is an increase in blood pressure and alterations in water and sodium retention by the kidneys. Compounding the effects of changes to renal haemodynamics, when renin binds to its receptor a number of signalling pathways are initiated including mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK1/2). This signal cascade induces increased DNA synthesis, as well as enhanced levels of collagen, transforming growth factor β (TGF-β1) and fibronectin which are responsible for aspects of the microstructural remodelling which occurs in CKD (Abassi et al., 2009). A number of clinical trials have shown that the prevention of hypertension through the use of synthetic RAAS inhibitors can slow the progressive decline of renal function in patients with diabetic nephropathy as well as non-diabetic patients with nephropathy (Lewis et al., 1993, Brenner et al., 2001). The RAAS inhibitor, Losartan, which acts as an angiotensin II

receptor agonist, has been shown in clinical trials to significantly reduce proteinuria, serum creatinine content and progression to end stage renal disease (Brenner et al., 2001). These outcomes were primarily attributed to reduction in blood pressure, and highlight the importance of regulating blood pressure in ameliorating the progression of CKD.

### *1.1.6 Hemodynamic Changes in CKD*

In the early stages of nephropathy, specific haemodynamic changes such as hyperfiltration and increased filtration fraction is evident often long before renal function begins to decline. Underpinning the local haemodynamic changes to the kidney is disruption to the RAAS, although glomerular pressure can be modulated independently of system blood pressure (Cooper, 2001, Zatz et al., 1986). Through micropuncture studies, it has been demonstrated that hyperfiltration is the result of an increased glomerular plasma flow rate and increased intracapsular pressure within the glomerulus (Hostetter et al., 1981). Increased intracapsular pressure, while associated with systemic hypertension can occur even in a normotensive setting (Cooper, 2001), illustrating that vasomotor tone of the afferent and efferent arteriole are sensitive to alterations in hormone, cytokine and growth factor signalling. Increased intracapsular pressure leads to changes in glomerular permeability to proteins and other solutes which then contributes to an increase reabsorptive load placed upon the tubules (Sánchez-Lozada et al., 2003). Beyond the role of the RAAS, it has also been identified that other hormones such as atrial natriuretic peptide (Niehaus et al.), bradykinins and nitric oxide synthase (NOS) are also

mediators of vasomotor tone of glomerular afferent and efferent arterioles, thus contribute to overall intracapsular pressure (Cooper, 2001).

*1.1.7 Signalling Pathways Involved in CKD*

There are a number of key signalling pathways which have been identified in playing a crucial role in mediating structural changes in CKD. Hyperglycaemia, hyperlipidemia and hypertension activate a number of key cytokines and growth factors which are produced by cells of the kidney during renal damage. Figure 1.2 (below) highlights the complex signalling changes which occur within renal tubules sequelae to metabolic syndrome (Vallon, 2011b).

TGF-β1 is a cytokine known to be an important mediator of pathogenesis in the renal proximal tubule (Vallon et al. 2011). It has been well established that increased renal expression of TGF-β1 occurs with hyperglycaemia, and contributes to a number of pathophysiological processes such as mediating fibrosis, promoting the production of extracellular proteins, and interfering with albumin handling in the proximal tubules (Hryciw et al. , 2004, Qi et al., 2008a). TGF-β1 regulates the production of many matrix and basement proteins, including collagen type I and IV. Thus, elevation in TGF-β1 productions leads to an accumulation of extracellular matrix, renal fibrosis and thickening of the basement membrane (Branton and Kopp, 1999). In addition to its key regulatory role in protein production, TGF-β1 also has potent chemotactic properties which lead to the accumulation of macrophages in renal structures (Qi et al., 2008b).

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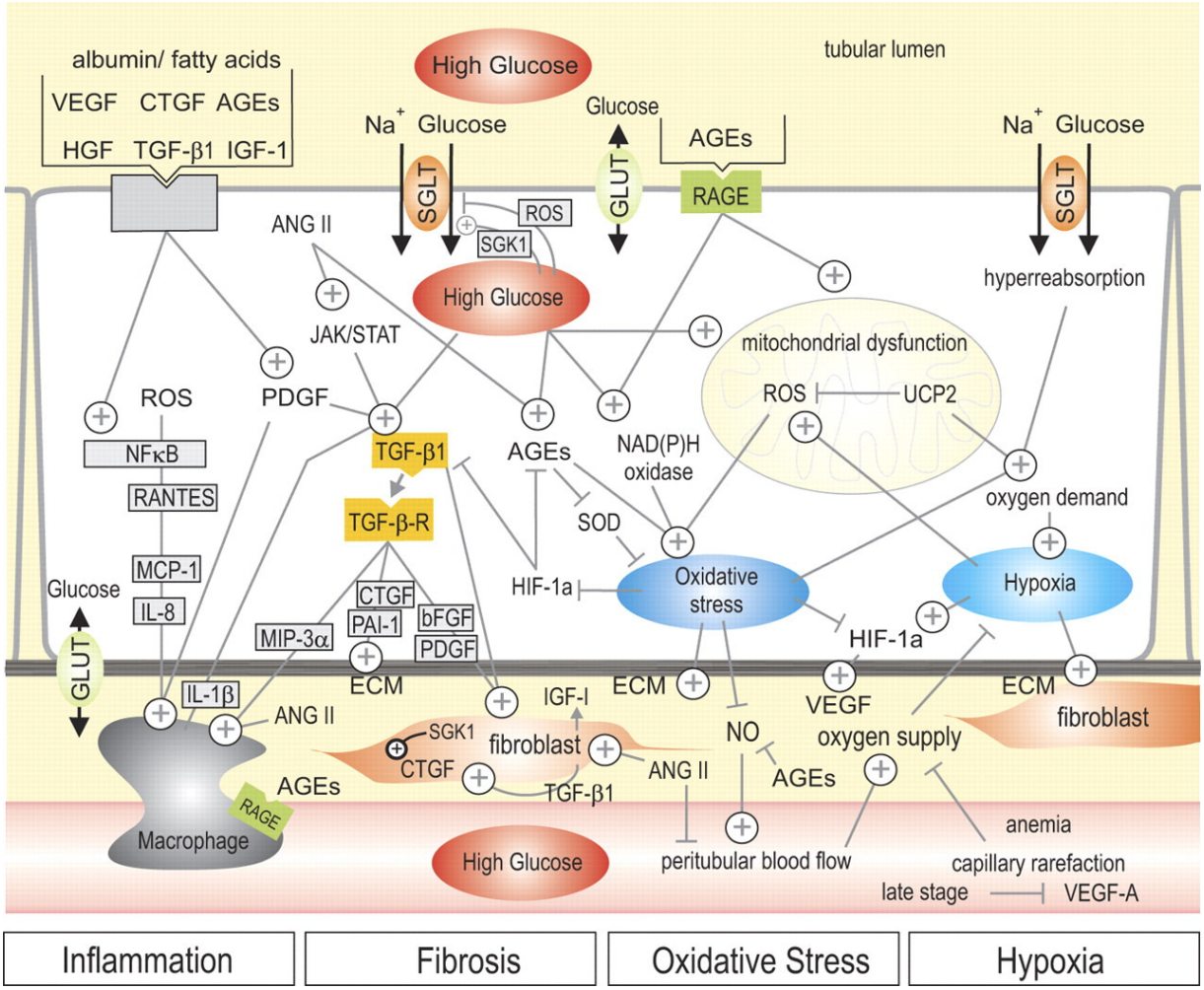



**Figure 1.2: Signalling events leading to tubular injury in response to diabetes mellitus.** Illustrated is the influence of hyperglycaemia, luminal factors (derived from glomerular filtration and tubular release), tubular transport work, and peritubular blood flow on the interaction of proximal tubular cells with fibroblasts and inflammatory cells. Taken from Vallon (2011b).

Vascular endothelial growth factor (VEGF) is a growth factor derived from mesangial and podocyte cells in the glomerulus and to a lesser extent, tubular cells. VEGF acts to increase the permeability of endothelial cells (Boner and Cooper, 2006, Khamaisi et al., 2003). VEGF production can be stimulated by angiotensin, high glucose and low oxygen environments which it induces vasodilation and increased endothelial permeability via NOS dependant mechanisms (Khamaisi et al., 2003). It is hypothesised that VEGF has an important role in CKD by mediating increased glomerular filtration. Evidence to support the role of VEGF in microvascular complications of CKD has been shown in humans where a type 2 diabetic cohort of patients with and without renal damage (measured by the level of proteinuria) showed that individuals with overt proteinuria has significantly higher levels of plasma and urinary VEGF than those with normo- or micro-proteinuria (Cha et al., 2000).

### *1.1.8 Structural Changes to the Nephron in CKD*

Initial structural changes to the kidney include hypertrophy (increase in cell size) and hyperplasia (increase in cell number) (Magri and Fava, 2009). Renal hypertrophy and hyperplasia are structural changes induced by both haemodynamic and metabolic vicissitudes. In rats, spontaneously hypertensive rats demonstrate an increased glomerular and tubular basement membrane thickening compared to normotensive Wistar Kyoto rats and further, induction with diabetes compounded these differences (Amazonas and Lopes de Faria, 2006, Cooper, 2001, Fogo, 2000). It is hypothesised that increased intracapsular pressure leads to altered gene expression in glomerular cells of several key growth factors including TGF-β1, insulin

like growth factor 1 (IGF-1), interleukins 1 and 6 (IL-1, IL-6), and epidermal growth factor (EGF) which induce hypertrophy of the glomerulus, leading to downstream effects in the nephron (Fogo, 2000). A competing hypothesis is one which is focused on the role of tubular hypertrophy being the primary driving force behind further structural and functional changes in CKD (Vallon, 2011b). Here, it is argued that increased tubular reabsorption comes before hyperfiltration and leads to not only structural changes including tubular hypertrophy, but it also impairs the tubular glomerular feedback system which *then* results in hyperfiltration (Magri and Fava, 2009). Renal tubule cells account for most of the renal growth associated with diabetic nephropathy and it is the degree of tubular-interstitial damage rather than glomerular injury which most closely correlates with decline in renal function (Nath, 1992). Therefore renal hypertrophy may largely be due to the increased load for tubular reabsorption. Whether hypertrophy is due to primarily glomerular or tubular functional alterations is yet to be fully elucidated (See Figure 1.3).

A number of metabolic factors associated with obesity and diabetes have been linked to renal growth including high protein or salt diets and hyperglycaemia (Fogo, 2000, Ziyadeh et al., 1990). These factors then lead to alterations in hormonal (angiotensin, glucocorticoids, endothelin) and growth factors (IGF, TGF-β1) production stimulating an increase in renal cell growth and number (Wolf and Ziyadeh, 1999).

As CKD progresses, the characteristic changes which are observed are glomerulosclerosis, podocyte loss, tubular fibrosis, thickening of glomerular and tubular basement membranes and accumulation of extracellular matrix (Boner and

Cooper, 2006, Magri and Fava, 2009, Ritz et al., 1999, Thomas et al., 2005, Wang et al., 2001). The mechanisms underlying these further structural changes are closely linked to the dysregulation of signalling pathways outlined in Section 1.1.8. Many of these signalling pathways not only disrupt the balance of matrix turnover and endothelial permeability, but they also are important regulators of inflammation. Macrophage infiltration and an overproduction of leukocyte adhesion molecules has been identified in both human and experimental models of nephropathy (Elmarakby and Sullivan, 2012). TGF-β1 and NOS are potent chemo attractants and infiltration of T cells and monocytes can initiate structural renal damage. In ESRD, elevated inflammatory states is a risk factor for cardiovascular morbidity and mortality (Oberg et al., 2004). One study which examined inflammatory and oxidative stress markers in a cohort of patients with CKD found that while the markers were significantly elevated in the CKD group compared to the healthy group, the link between inflammation and renal function is weak, which indicates that markers for inflammation may not provide the optimal prognostic diagnostic tools for CKD (Oberg et al., 2004). The fibrotic processes which occur within the kidneys in response to diabetes are complex and involve a number of both fibrotic and inflammatory signalling molecules (Figure 1.3). These pathways can result in structural adaptations to all structures of the nephron, including the glomerulus and tubules. Figure 1.3 below outlines the complex pathways involved in the glomerular and tubular adaptions which occur in diabetic nephropathy.

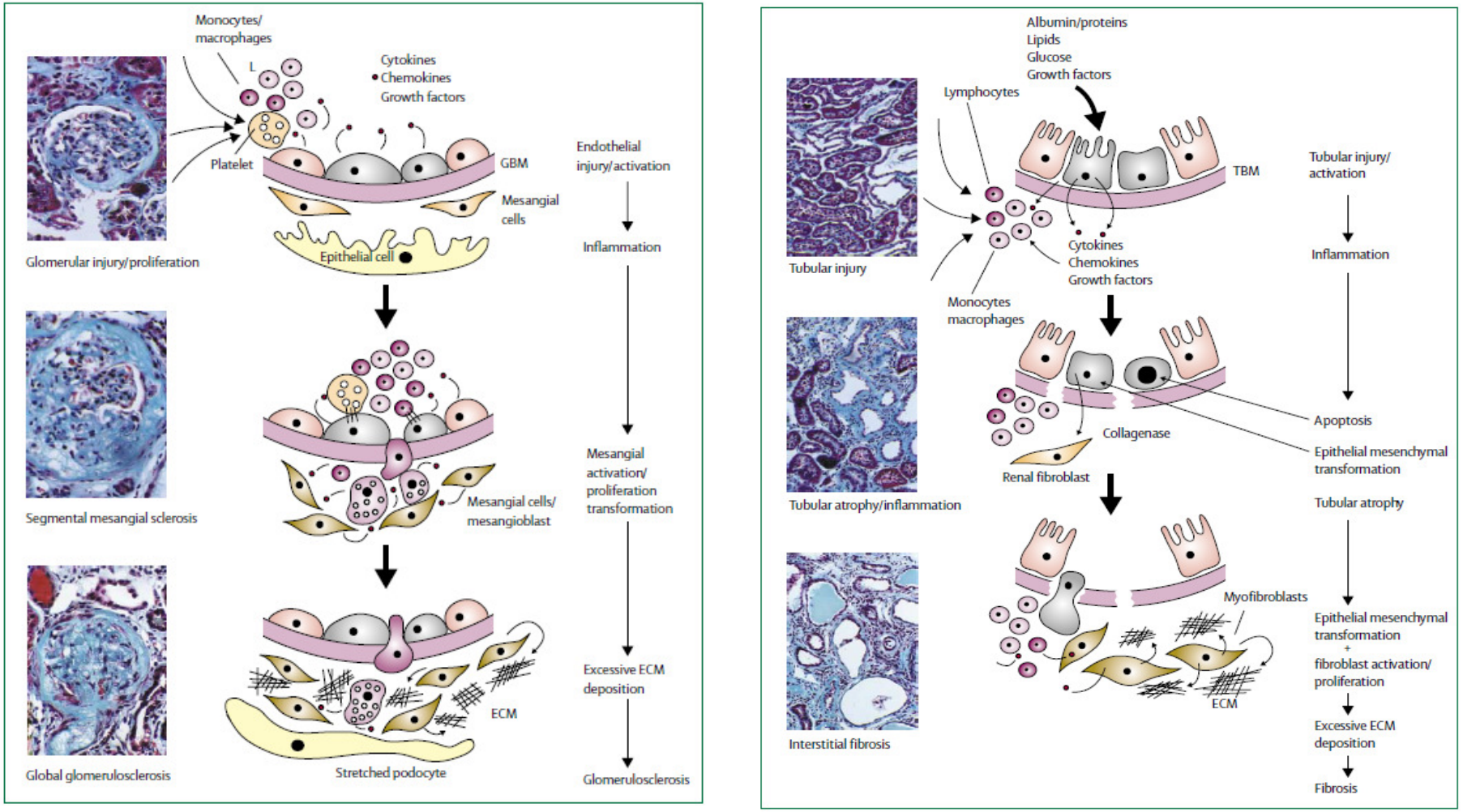



**Figure 1.3: Stages of glomerulosclerosis and tubulo-interstitial fibrosis and role of signalling and inflammatory molecules.**

Representative figure for the stages of glomerular and tubular damage in nephropathy adapted from El Nahas and Bello (2005).

### *1.1.9 Functional Changes in CKD*

Functional changes such as GFR and albuminuria are important for the diagnosis and evaluation of CKD for patient care and prognosis. The structural characteristics and changes outlined in Section 1.1.8 ultimately contribute to the reduction of markers in renal damage, including decline in GFR, increased protein and albumin excretion, reduced creatinine clearance and increase sodium excretion. This section details what functional changes are expected as a result of CKD.

#### *1.1.9.1 Glomerular Filtration Rate*

GFR is the most basic measurement of renal function and evaluation of GFR has been used for decades to monitor renal function. It represents the quantity of glomerular filtrate produced by the kidneys in one minute. Generally, a GFR for a healthy adult is 90 ml/min or more, while chronic kidney disease is indicated by a reduction in GFR to below 60 ml/min for a period of 3 months or longer (Levey et al., 2003). Obesity and diabetes are major contributing factors which can damage structures within the nephron and initially lead to an increase in filtration rate in response to hyperglycaemia and hyperlipidaemia, resulting in hyperfiltration (GFR 125 ml/min or above) as a compensatory mechanism (Declèves et al., 2011, Vallon, 2011b). However, as structural changes to the nephron progress, a gradual decline in GFR continues and may drop as low as 15 ml/min in the case of ESRD (Levey and Coresh, 2012, Metcalfe, 2007). In experimental models, estimating GFR using small animals such as rats, it has been reported that normal ranges for adult animals range between 1 ml/min – 3 ml/min (Nehiri et al., 2008). It is difficult to directly measure GFR; however substances which are cleared by the kidneys, such as

creatinine, cystatin C or urea can be used as estimates of GFR. Creatinine clearance is a method commonly used in both a clinical and experimental setting to evaluate GFR (Regeniter et al., 2009). Creatinine is a by-product of muscle metabolism and is primarily removed from the blood via glomerular filtration, making it an ideal marker for GFR (Toffaletti and McDonnell, 2008). When GFR declines, creatinine urinary excretion also declines, and higher levels of creatinine remain in the blood. As creatinine is a by-product of muscle metabolism, serum levels and urinary excretion are dependent on an individuals' muscle mass, and so sex, age and race are factors which may be included in calculating GFR based off creatinine clearance (Levey et al., 2003, Regeniter et al., 2009, Toffaletti and McDonnell, 2008).

*1.1.9.2 Proteinuria and Albuminuria*

In a clinical setting, proteinuria is one of the most frequently assessed marker of CKD (Levey et al., 2003). The AusDIAB study found that not all patients with CKD exhibit reduced GFR, with approximately 57% of CKD participants showing albuminuria and proteinuria with a GFR within the normal range of 60 ml/min or higher (Chadban et al., 2003). This demonstrates that it is imperative to consider more than just GFR when assessing renal function. Protein reabsorption in the proximal tubule takes place via a complex system containing a number of transmembrane and scaffold proteins within the microvilli (Hryciw et al., 2012a, Slattery et al., 2011). In an adult human, the serum protein albumin has a fractional clearance of 0.00062, and approximately 3.3 g of albumin is filtered by glomerulus daily (Tojo and Kinugasa, 2012). However, all filtered albumin is completely reabsorbed from the filtrate in healthy individuals by the proximal tubule via

endocytosis. Due to its size, albumin is the most commonly increased urinary protein across all forms of nephropathy (Johnson et al., 2012). The gold standard of evaluating levels of albumin is by using urinary albumin to creatinine ratio (mg/g) from either a first void urinary sample or a timed 24 hour urinary sample. Currently in Australia, set points for normal reference ranges for urinary albumin vary depending on the pathology laboratory in which the sample is tested (Johnson et al., 2012), although albumin excretion ranges are usually considered optimal at 29 mg/g or below, microalbuminuria is considered at ranges of 30-299 mg/g and macroalbuminuria, 300 mg/g or above (Levey et al., 2003). Recently an expert panel, the Australasian Proteinuria Consensus Working Group, recommended all pathology laboratories adhere to set threshold ranges for albumin to creatinine ratio and suggested that due to the correlation with urinary creatinine excretion and muscle mass, sex specific thresholds for albuminuria could be developed (Johnson et al., 2012).

Interestingly, total urinary protein excretion (proteinuria) has also been found to be a strong predictor for the presence of renal disease. Further, proteinuria may be a robust biomarker for specifically the progression of renal disease within the general population (De Jong et al., 2007). The potential for proteinuria being used as a biomarker for renal damage was highlighted in a population study conducted in the Netherlands. This study found that just 0.6% of 8592 of adult participants studied exhibited proteinuria, however these participants exhibited a significantly greater loss of renal function (measured by creatinine clearance) at the one year follow up period than individuals with lower renal function at baseline testing (Halbesma et al., 2006).

There appears to be nuances in how microalbuminuria and total urinary protein concentrations may be used to predict other co-founding risk factors of CKD, such as cardiovascular events (De Jong et al., 2007). It is generally accepted that microalbuminuria signals the initial reversible phase of kidney damage, while proteinuria heralds the progression of irreversible CKD and is a much stronger indicator of risk of ESRD (Snyder and Pendergraph, 2005, De Jong et al., 2007). This has led to calls for discrete testing of microalbuminuria and proteinuria in the clinical setting for best prognosis and patient outcomes (De Jong et al., 2007).

*1.1.9.3 Urinary Sodium Excretion*

The kidneys play a major role in regulation of ion levels, and control ion excretion and reabsorption which is essential for maintenance of normal homeostasis. Sodium constitutes one of the major cations found in the body and is responsible for influencing fluid shifts between intracellular and extracellular compartments, maintenance of blood pressure and nerve excitation (Paradiso, 1999). Sodium has a complex relationship with factors affecting renal disease. Increased sodium intake is linked to increased blood pressure and thus implicated with the haemodynamic and structural changes which progress renal damage (Wright and Cavanaugh, 2010). It has been demonstrated that in obese patients, elevated plasma levels of all constituents of the RAAS system occurs while simultaneously, increased tone of the sympathetic nervous system leads to increased renal sodium reabsorption and ultimately elevated blood pressure (Cignarelli and Lamacchia, 2007a, Wickman and Kramer, 2013). Leptin, a hormone released by adipose tissue which is significantly higher in obese individuals, may play a significant role in mediating changes to renal

handing of sodium via alterations to sympathetic tone and expression of renal sodium transporters, sodium–hydrogen exchanger-3 (NHE3) (Iwashita et al., 2002). In experimental models of obesity, diet induced obese rats have been shown to have significantly reduced levels of urinary sodium excretion, whilst exhibiting increased sympathetic and RAAS activation (Iwashita et al., 2002). In contrast in a genetic model of obesity caused by deficiency in the leptin receptor, obese Zucker rats exhibit up to 3 times greater urinary sodium excretion compared to lean animals and reduced expression of renal sodium transporters NHE3, highlighting the importance of this hormone in regulating sodium urinary excretion (Bickel et al., 2002). In diabetic nephropathy, a reduced ability to excrete sodium also occurs, which in addition to alterations in RAAS activation and sympathetic tone, is hypothesised to be a consequence of increased tubular glucose reabsorption through sodium-glucose transporters (SGLT) (Pietinen et al., 1988, Thomas et al., 2011, Wright and Cavanaugh, 2010, Bickel et al., 2002). SGLT co-transports both sodium and glucose across the membrane in the same direction, under hyperglycaemic conditions typical of diabetes, kidneys increase glucose reabsorption from the tubular lumen into the blood, coinciding with increased sodium reabsorption. Urinary sodium excretion in humans may range from 40 - 220 mmol/24 hours, depending on dietary salt intake, hydration status and hormonal regulatory input (Pietinen et al., 1988). Due to the extensive factors mediating urinary sodium excretion, it has been established that this marker alone does not accurately predict progression or prognosis of CKD (Thomas et al., 2011).

### *1.1.10 Future Therapeutic Targets for CKD*

The renal system plays a crucial role in the homeostatic balance of fluids, essential ions, proteins and nutrients (MacIsaac and Watts, 2005). Section 1.1 has given an overview as to how both obesity and diabetes are implicitly linked with CKD. Given the impact both diabetes and obesity has on renal structure and function, it is imperative that viable treatment options are identified for CKD. Currently, emerging research has shown that the endocannabinoid system may be involved in mediating processes in both diabetes and obesity; however the potential clinical relevance of targeting this system to reduce the impact of renal disease has yet to be fully examined.

## ***1.2 The Endocannabinoid System***

### *1.2.1 A Brief History of Endocannabinoid Research*

The endocannabinoid system derives its name from the *cannabis sativa* plant, which for centuries has been used for a range of medicinal and recreational purposes, however identification of the plant's active component $\Delta^9$ tetrahydrocannabinol (THC) was only first described in 1964, along with several other plant derived cannabinoids known as "phytocannabinoids" (Nagy et al., 2008). Ultimately it took three more decades of research into the psychotropic effects of phytocannabinoids before the notion that THC acts via a through a specific binding site rather than the mechanism of action being due to plasma membrane perturbation or via the lipid solubility properties of cannabinoids (Devane et al., 1988, De Petrocellis and Di Marzo, 2009). The formation of a new pedagogy in cannabinoid research

subsequently led to the discovery of the gene encoding the receptor cannabinoid receptor 1 (CB1), which was identified in 1990 (Matsuda et al., 1990). A second cannabinoid receptor designated “CB2” was described in 1993 and compared to the CB1 receptor, it was found to have a different pattern of distribution and amino acid sequence (De Petrocellis and Di Marzo, 2009, Munro et al., 1993, Nagy et al., 2008). The identification of cannabinoid receptors in the early 1990’s led to an increase in cannabinoid research; endogenous ligands known as “endocannabinoids” were classified, degradation enzymes were described and there was an increased awareness of how the endocannabinoid system may play a role in both health and disease (Onaivi et al., 2005). Section 1.2 of this literature review will outline the structure of the cannabinoid receptors CB1 and CB2, along with the putative cannabinoid receptor GPR55 and their endogenous ligands and degradation pathways.

### *1.2.2 An Overview of the Endocannabinoid System*

The endocannabinoid system is an important endogenous lipid signalling system. It is a system comprised of cannabinoid receptors, their endogenous ligands (endocannabinoids) and the enzymes responsible for the formation and degradation of those ligands (Muccioli, 2010, Wang and Ueda, 2008). In addition to endocannabinoids, these receptors can also be activated by plant derived ‘phytocannabinoids’ and synthetic constructs, with each compound having a varying affinity and selectivity for cannabinoid receptors (Wang and Ueda, 2008). Recent evidence highlights the promiscuity of the endocannabinoid system whose actions are not limited to the two receptors CB1 and CB2. All classes of cannabinoid ligands

are also able to bind to and activate orphan G-protein coupled receptor GPR55, the ion gated channel transient receptor vanilliod type I (TRPV1) as well as a two classes of peroxisome proliferator activated receptors (PPAR) known as PPARα and PPARγ (De Petrocellis and Di Marzo, 2009).

Cannabinoid receptors are members belonging to a class of G-Protein Coupled Receptors (GPCR). These types of receptors are usually tightly regulated and normally exhibit a specific pattern of events which originate with activation by ligand binding, signalling of the receptor is then abated by a desensitisation process and internalisation into the cell. Once internalised, the receptor either gets resensitized via a process of dephosphorylation and recycled back to the plasma membrane, or alternatively gets degraded by lysosomes leading to the down regulation of receptor expression (Atwood et al., 2012). GPCR's are capable of exhibiting multiple conformations, leading to different signalling events (Turu and Hunyady, 2010). Thus, the endocannabinoid system is a network which has a broad range of physiological functions including control of energy homeostasis, glucose and lipid metabolism, energy intake and expenditure, mood, cardiovascular tone, reproduction, bone remodeling as well being an intermediate for a number of pathological conditions including nephropathy (Di Marzo, 2008, Maccarrone and Finazzi-Agro, 2003).

### *1.2.3 Ligands and Degradation Pathways of the Endocannabinoid System*

Cannabinoid receptors can be activated by a range of endogenous, plant derived and synthetic ligands. The actions of the phytocannabinoid THC has been known and used for medicinal and recreational purposes for centuries (Di Marzo, 2008,

Horváth et al., 2012), although over 70 additional phytocannabinoids have been identified including cannabidiol (CBD), which can also be derived from the *cannabis sativa* plant (Yamaori et al., 2010). The lipophilic properties of phytocannabinoids allow them to be readily metabolised by the body and although structurally very similar, THC and CBD (Figure 1.4) are physiologically quite disparate in their effects. Figure 1.4 highlights the structural similarity between the two compounds.

THC binds readily to centrally distributed CB1 receptors and is the main psychoactive ingredient of cannabis, inducing changes to cognitive and psychomotive functioning. However the psychotropic side effects of THC severely limit its use therapeutically (Horváth et al., 2012). CBD is the main constituent of the cannabis plant which doesn't induce psychotropic effects and has been shown to potentially play a protective role in a number of disease models including diabetes, however its binding capacity with cannabinoid receptors is limited (Di Marzo, 2008).Following the identification of the CB1 and CB2 receptors in the early 1990's, research turned to discovering endogenous substances capable of binding to these receptors. Endocannabinoids are substances which are synthesised on demand and are capable of binding to and activating cannabinoid receptors (McPartland et al., 2006). The two most well research endocannabinoids are *N*-arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG) (Di Marzo, 2008), however a number of other putative endogenous cannabinoid receptor ligands have also been established (Figure 1.5). The structures of these compounds are quite similar as is highlighted by Figure 1.5, but can mediate a diverse range of physiological effects.Anandamide (AEA) derives its name from the Sanskrit word *ananda*, which translates to "bliss", and is an important mediator in a

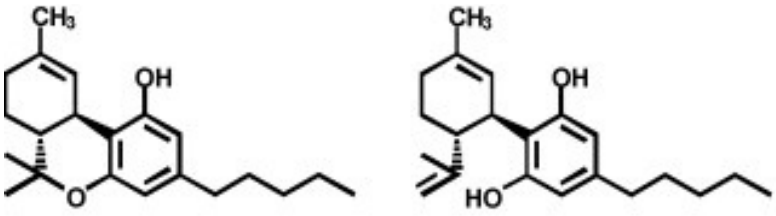
number of physiological processes including analgesia, appetite and hypothermia (Fonseca et al., 2013). It acts as a partial agonist for both the CB1 and CB2 receptor but can activate other receptors including GPR55. AEA preferentially binds to CB1 with a relatively higher efficacy and affinity than what it displays for the CB2 receptor (Pertwee et al., 2010).

### *1.2.4 The Cannabinoid Receptor 1 (CB1)*

The CB1 receptor was the first cannabinoid receptor to be identified and cloned (Matsuda et al., 1990). It belongs to a class of GPCR which typically exhibit seven transmembrane domain regions and the CB1 receptor maintains a high degree of homology across both vertebrate and invertebrate species whilst only sharing about 40% homology with the CB2 receptor (see Figure 1.6) (Abood, 2005, Yao and Mackie, 2009). The extracellular loops have been shown to play a critical role in receptor signalling and ligand binding, while the function of the intracellular loops are critical for internalisation and sensitisation of the receptor (Marcu et al., 2013, Hsieh et al., 1999). The CB1 receptor, like many GPCR's provide a range of signalling capabilities and so, expression and sensitisation is regulated by cells *in situ* via the C terminus and is achieved either through general GPCR regulatory pathways such as via β arrestin, or through CB1 specific interacting proteins like the recently identified Cannabinoid Receptor Interacting Proteins (CRIP) CRIP 1a and CRIP 1b (Niehaus et al., 2007, Howlett et al., 2010).

1.2.4.1 The CB1 Receptor in Health and Disease

The CB1 receptor is predominantly expressed in the central nervous system and in almost all peripheral tissues to varying but lesser degrees (Gomez et al., 2002, Svíženská et al., 2008). Due to its extensive distribution in the nervous system particularly in the hippocampus region it is known to be involved in a number of cognitive processes including memory and learning (Martin et al., 2002). The CB1 receptor is also implicated in a number of neurodegenerative disease states such as Parkinson's and Huntington's disease which involve the basal ganglia and cerebellum where the receptor is expressed in high density (Svíženská et al., 2008).,




**THC** **CBD**

**Figure 1.4: THC and CBD: The two major phytocannabinoids derived from the *cannabis sativa* plant.** Shown are the chemical structures of $\Delta^9$ tetrahydrocannabinol (THC), Cannabidiol (CBD). Adapted from Yamaori et al. (2010).

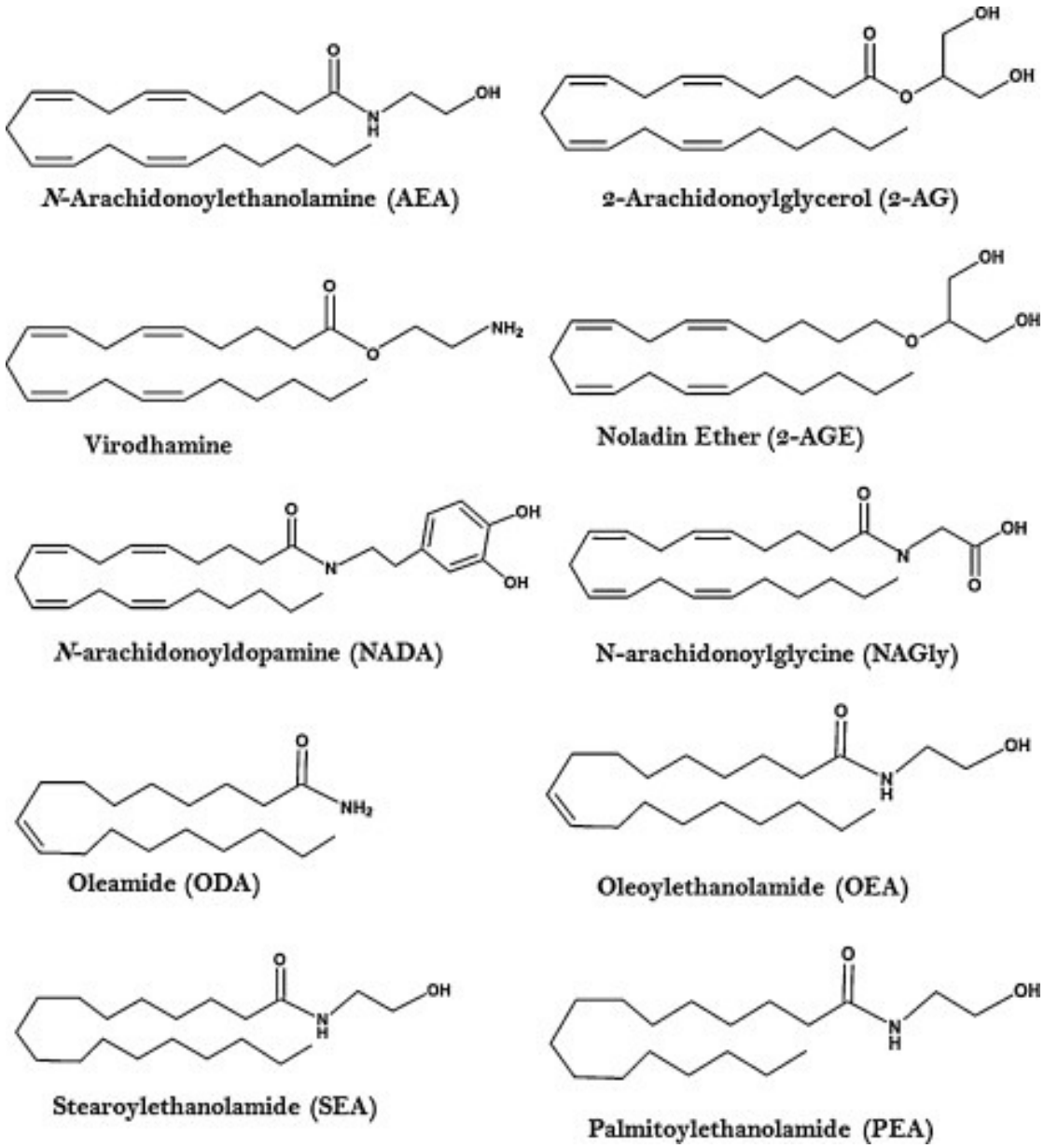



**Figure 1.5: Chemical structure of endogenous endocannabinoids and cannabimimetic molecules**.Taken from Fonseca et al. (2013).

Food intake is also regulated by CB1, where the receptor plays a twofold role, firstly through the limbic system where it mediates the reward pathway when food is consumed and secondly, via the hypothalamus where it induces the release of orexogenic mediators following fasting periods (Di Marzo and Matias, 2005). When observations were made showing the synthetic CB1 specific antagonist Rimonabant (SR141716) decreased palatable food intake by rodents, it was initially thought that the receptor exerted its effects via the central nervous system alone (Arnone et al., 1997). However, it has since been identified that the CB1 receptor also mediates food intake via peripheral pathways too (Gomez et al., 2002). In a CB1 knockout mouse model, rodents without the CB1 receptor displayed a lean phenotype, were resistant to diet induced obesity and exhibited diminished insulin sensitivity typical of a high fat diet (HFD) (Kunos and Btákai, 2001). The notion that the CB1 receptor could be harnessed to reduce food intake and weight gain led to a boom in both experimental and clinical trials examining the effects of CB1 antagonism on food intake and weight. The RIO (Rimonabant in Obesity) study was the largest clinical trial utilising the CB1 specific antagonist Rimonabant. The study was conducted in both Europe and North America with over 6000 participants (Van Gaal et al., 2005). Obese individuals on 20 mg Rimonabant showed significant reduction in weight and waist circumference measurements, as well as significant improvements in several measures of cardiovascular and metabolic risk factors (Van Gaal et al., 2005).

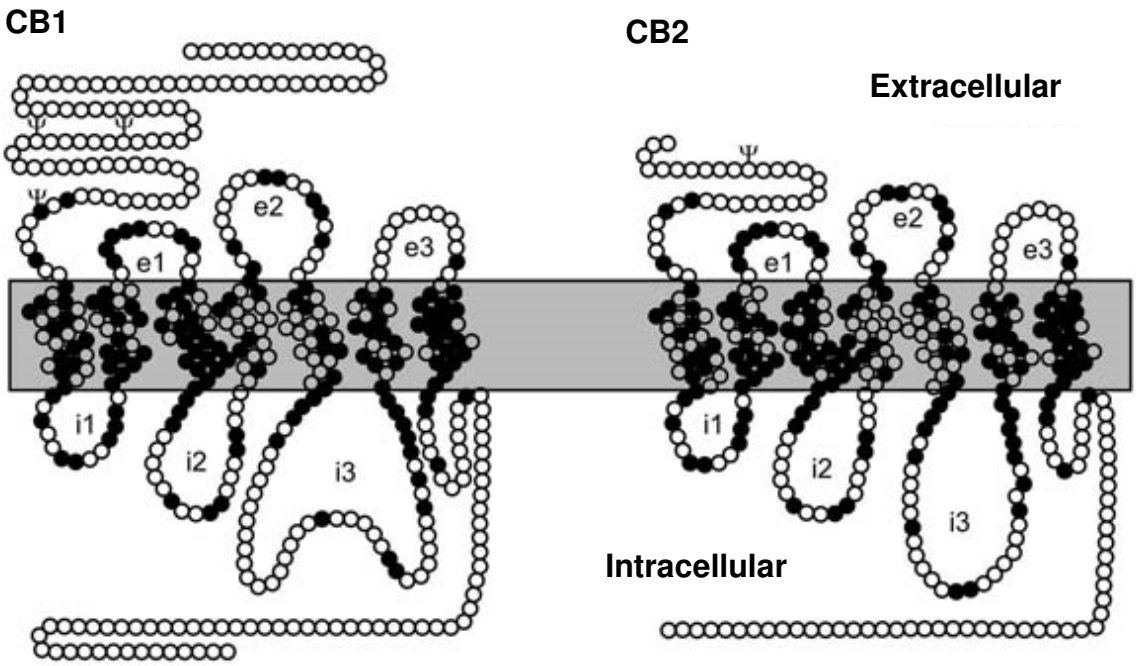



**Figure 1.6: Schematic representation of the CB1 and CB2 receptors.** . Receptors have seven transmembrane segments with homologous amino acid residues between the CB1 and CB2 receptors depicted as closed circles and differences in amino acid residues are depicted open circles. The symbol Ψ indicates glycosylation sites. Extracellular loops are labelled e1, e2 and e3. Intracellular loops are labelled i1, i2 and i3. Taken from Rodríguez et al. (2005).

However, due to the number of psychological adverse side effects, including depressive symptoms, Rimonabant is no longer a clinical option for the treatment of obesity. A new generation of CB1 antagonist compounds that do not cross the blood brain barrier are currently being explored (Isoldi and Aronne, 2008, Rosenstock et al., 2008, O'Keefe et al., 2013). These compounds are primarily peripherally acting and emerging research indicates that these new generation CB1 antagonists are able to reduce weight without adverse neurological effects (O'Keefe et al., 2013).

The CB1 receptor is expressed in a number of metabolically active organs including skeletal muscle, liver and pancreas (Cavuoto et al., 2007, Di Marzo, 2008, Linari et al., 2009). It has been shown that in obese and diabetic states the CB1 receptor can become over stimulated and thus has been a pivotal element in understanding the role of endocannabinoid control of glucose and lipid metabolism in the diabetic milieu (Di Marzo, 2008, Matias et al., 2008). Figure 1.7 highlights the potential role the endocannabinoid system has in tissues under different metabolic disturbances including diabetes and obesity. Three key changes mediate the move from pre-diabetic to type 2 diabetic states; this includes a decrease in functional mass of insulin secreting beta cells within the pancreas, increased glucose production by the liver and a decrease in insulin stimulated glucose uptake by skeletal muscle (Lafontan et al., 2007). To date, there has been extensive research into how the CB1 receptor is involved in the control of energy balance and glucose metabolism and, it appears that the receptor can mediate all three changes important in the transition to a diabetic state (Lafontan et al., 2007).

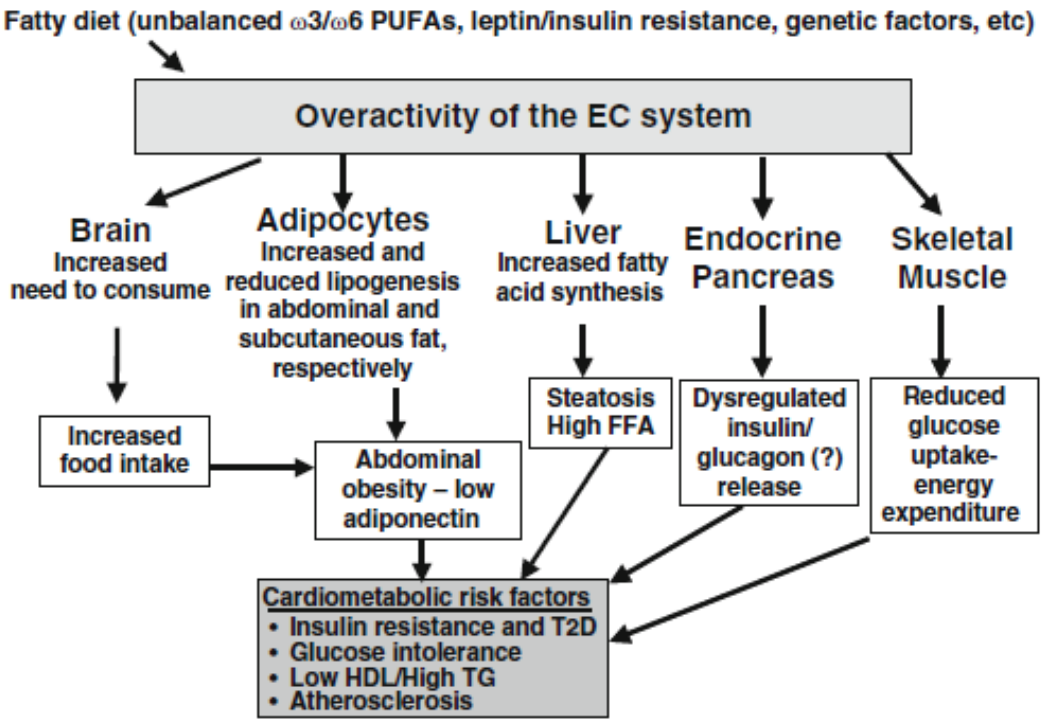



**Figure 1.7: Central and peripheral mechanisms of CB1 receptor signalling in obese and diabetic disease states** This figure illustrates the potential metabolic consequences of endocannabinoid dysregulation. Abbreviations include FFA: Free Fatty Acids, HDL: High Density Lipoproteins, T2D: Type 2 Diabetes, TG: Triglycerides. Taken fromDi Marzo (2008).

### *1.2.5 The Cannabinoid Receptor 2 (CB2)*

The CB2 receptor was first identified in the marginal zone of the spleen two decades ago by Munro et al. (1993). Like CB1, CB2 is a receptor which belongs to the class of GPCR and exhibits seven transmembrane segments with three intracellular and extracellular loops (Figure 1.8). In contrast to CB1, the CB2 receptor does not maintain a high degree of homology across species, with the overall corresponding sequence between mammals being about 70%, although between some species receptor homology is much higher (Yao and Mackie, 2009). The mechanisms behind the internalisation and resensitisation of the CB2 receptor is not clearly understood, however it is suggested that the internalisation of the receptor is transient and can be mediated by certain agonists, whilst other agonists activate signalling pathways (Atwood et al., 2012).

Initially considered the 'peripheral cannabinoid receptor', the CB2 receptor is widely distributed throughout peripheral tissues and is highly abundant within cells of the immune system. Originally it was thought that the CB2 receptor expression was completely devoid within the central nervous system (Pacher and Mechoulam, 2011, Griffin et al., 1999). A number of studies have now established that CB2 is found throughout the nervous system, although with varying densities of expression within brain specific structures, for review see (Svíženská et al., 2008).

#### *1.2.5.1 The CB2 Receptor in Health and Disease*

Considering the relatively high expression of the CB2 receptor in immune cells, the role of the receptor in modulating inflammation and immune pathways is evident. Indeed, activation of immune cells often results in an increase in CB2 receptor

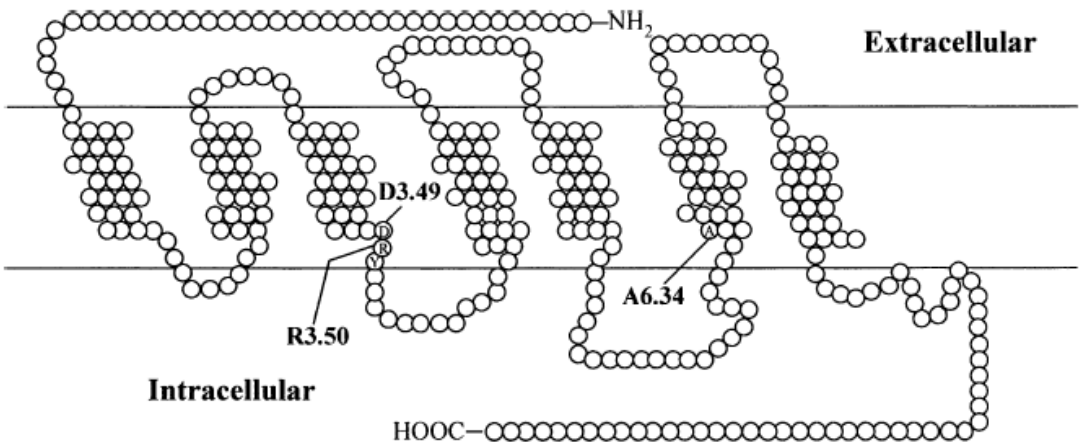



**Figure 1.8: Schematic model of the CB2 receptor.** In the third and sixth transmembrane domains, the three highly conserved amino acid sequences critical for ligand binding and conformation of the CB2 receptor are indicated. Taken from Feng and Song (2003).

expression in a number of immune subpopulations, including B cells and macrophages (Lee et al., 2001, Pacher and Mechoulam, 2011). An *in vitro* study demonstrated that macrophage expression of CB2 increased in response to a step-wise activation of the murine macrophage cell line, indicating that in a primed state macrophages are most sensitive to cannabinoids (Carlisle et al., 2002). However CB2 expression can be influenced by various experimental triggers used for immune cell activation as well as the subpopulation of immune cells. Specifically in B lymphocytes, activation of rodent B lymphocytes with CD-40 caused a transient increase of the receptor, but after 24 hours, CB2 expression had returned to baseline (Carlisle et al., 2002). Conversely, in mouse B lymphocytes treated with bacterial lipopolysaccharide caused a decrease in CB2 receptor expression (Lee et al., 2001). These studies, taken together with other works examining immune regulation of CB2 (Galiègue et al., 1995, Lombard et al., 2007, Pacher and Gao, 2008, Roth et al., 2002), highlight the complex role of various immune activators in regulating CB2 expression.

While expression of the CB2 receptor is influenced by a range of immuno-related signalling proteins, the receptor itself can mediate production of the very same signalling molecules. Peripheral blood lymphocytes treated with THC induced a significant increase in TGF-β1, however a CB2 specific antagonist, SR14452 mitigated this response while a CB1 specific antagonist did not (Gardner et al., 2002). Further, treatment of TGF-β1 to peripheral blood lymphocytes resulted in a decrease in the CB2 receptor thus demonstrating that TGF-β forms an autocrine regulatory loop with CB2, see Figure 1.9 below (Gardner et al., 2002). However, while the CB2 receptor does have a complex and intricate relationship with

inflammatory signalling proteins, a substantial amount of evidence indicates that activation of the CB2 receptor through endogenous and synthetic ligands have primarily immunosuppressive effects (Pacher and Mechoulam, 2011). In a microglial cell model treatment with cannabinoid agonist AEA induced an down regulation in cytokine production of interleukins IL-23 and IL-12 which was mediated specifically by the CB2 receptor (Correa et al., 2009). The observations of the immunosuppressive actions of the CB2 receptor have also been demonstrated in pathological conditions ranging from cardiomyopathy, pancreatitis, and neuroinflammatory disorders to diabetic nephropathy. In these conditions, treatment with CB2 agonists can attenuate inflammation via modulation of cytokines, prevention of immune cell infiltration and abrogation of fibrotic factors (Pacher and Mechoulam, 2011).

### *1.2.6 The G-Protein Coupled Receptor 55 (GPR55)*

GPR55 was first identified and cloned in 1999 using expressed sequence tags highly conserved within GPCR (Sawzdargo et al., 1999). Immuno-localisation analysis determined that it was expressed within some brain structures including the putamen and caudate nucleus (Sawzdargo et al., 1999). The receptor shares less than a 15% sequence homology with either the CB1 or CB2 receptors, and in many tissues its expression is much lower than the two established cannabinoid receptors (Ross, 2009). GPR55 has garnered significant debate over its classification as an endocannabinoid receptor (Williams, 2000, Ross, 2009). Early research on GPR55 conducted by GlaxoSmithKline had showed it to be activated by a limited range cannabinoids (Brown, 2007). It has since been shown to be activated

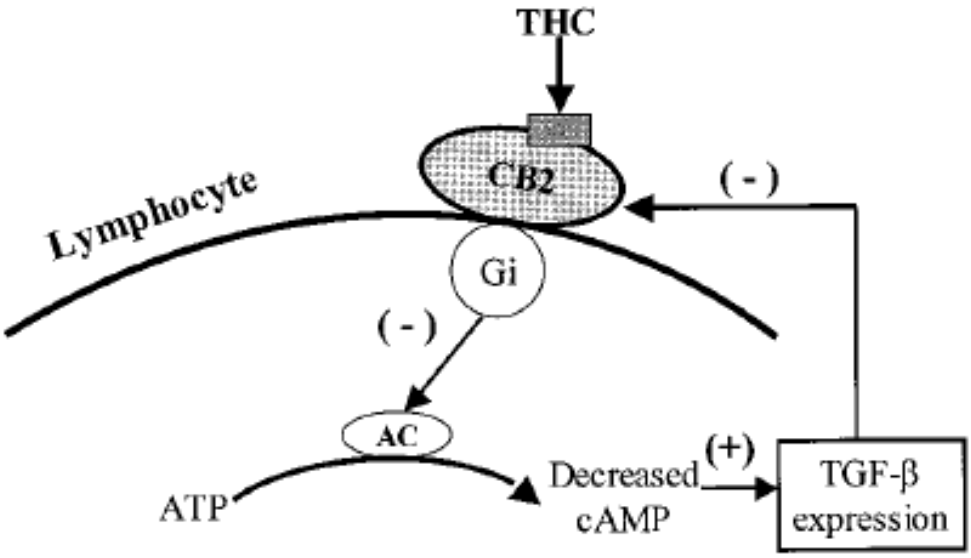



**Figure 1.9: Depiction of the TGF-β mediated regulatory loop of CB2 receptor expression in lymphocytes**. Activation of the CB2 receptor with THC stimulates TGF-β production via a decrease in cyclic AMP. An increase in TGF-β results in reduced expression of CB2. Taken from Gardner et al. (2002).

by THC, AEA, 2-AG, HU-210 and CP55940, which are all established agonists for both CB1 and CB2 (Ryberg et al., 2007). The factor that gives the most support to the opposition of GPR55 being classified as a cannabinoid receptor is that the most potent endogenous ligand for GPR55 is the non-cannabinoid lipid L-α-lysophosphatidylinositol (LPI). However, while the receptor's affinity to certain cannabinoid ligands is significantly less when compared to CB1 and CB2, there is also a number of cannabinoids to which GPR55's binding capacity is quite similar (Hiley and Kaup, 2007, Ryberg et al., 2007, Henstridge, 2012). Computational modelling of the receptor has identified a few key structural features involved in GPR55 activation, docking and distinct ligand binding sites, see Figure 1.10 (Sharir and Abood, 2010). However, the pharmacology of GPR55 is still not well understood (Elbegdorj et al., 2012).

#### *1.2.6.1 GPR55 in Health and Disease*

GPR55 is widely distributed through a number of tissues within the body, and is predominately expressed in the adrenal glands, spleen, gut and certain brain structures (Henstridge et al., 2009, Ryberg et al., 2007), indicating that the receptor may have a wide range of physiological functions (Ross, 2009). Recently it has been established that the primary endogenous ligand for GPR55, LPI, positively correlates with body fat percentage and BMI in humans (Moreno-Navarrete et al., 2012). Further, expression of GPR55 is significantly higher in liver and adipose tissue of obese compared to lean individuals and expression of this receptor is also tissue specific (Moreno-Navarrete et al., 2012).

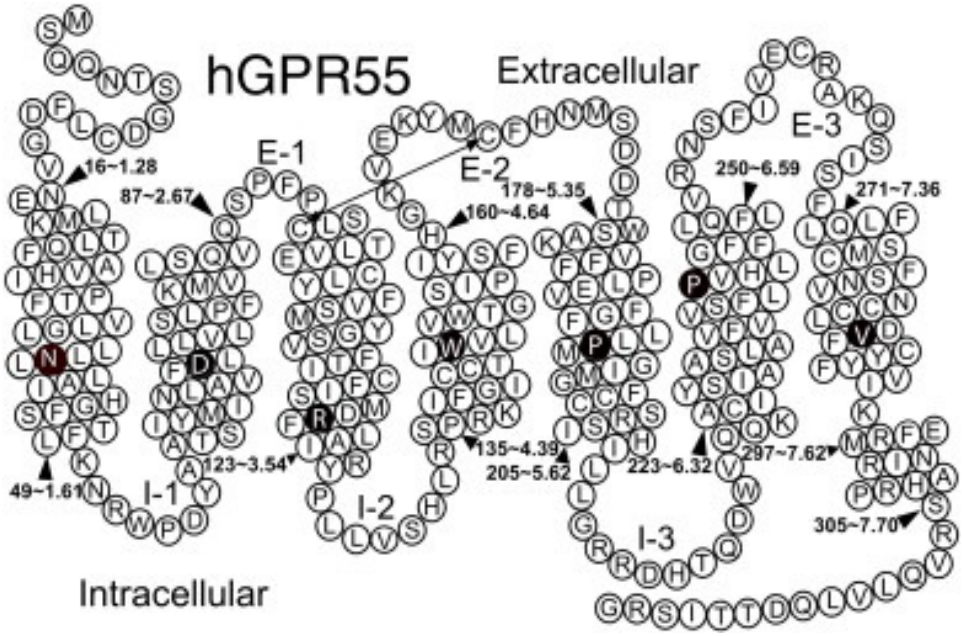



**Figure 1.10: Schematic representation of GPR55.** Amino acid residues depicted as closed circles shows those residues highly conserved among the rhodopsin group of G-Protein Could Receptors. Extracellular loops are labelled E-1, E-2 and E-3. Intracellular loops are labelled I-1, I-2 and I-3. Residues which may be important for GPR55 activation, docking and ligand binding are indicated by closed arrows. Taken from Sharir and Abood (2010).

GPR55 within endothelial cells has been found to be activated by the global endocannabinoid AEA (Waldeck-Weiermair et al., 2008). Whilst it is acknowledged that GPR55 and CB1 activate separate signalling pathways, it has also been suggested that interaction of the two receptors occur when activated by AEA, with CB1 activation leading to inhibition of GPR55 signalling (Waldeck-Weiermair et al., 2008). In contrast to these findings, a study by Kapur et al. (2009) showed that GPR55 in human embryonic kidney 293 cell lines (HEK293) was not in fact activated by AEA, although activation of GPR55 was found trigged by a number of other known cannabinoids like 2-AG, virodhamine, THC and cannabidiol, which contradicts the finding of both Ryberg et al. (2007) and Waldeck-Weiermair et al. (2008). These studies are representative of the overall contradictive reports present in the current literature, highlighting the complex nature of GPR55 pharmacology. A possible explanation may be due to the cross talk between GPR55 and the CB1 receptor. It has been demonstrated that CB1 and GPR55 are co-expressed in many tissues at similar levels particularly within the nervous system. A recent study reports that in HEK293 cells, GPR55 can be activated by synthetic compounds, previously thought to be specific for the CB1 receptor including AM251 and Rimonabant (Kargl et al., 2012). Further, these two receptors are able to form heteromers and can influence individual receptor signalling properties (Kargl et al., 2012). Currently, there are limited synthetic compounds available which are highly specific for GPR55, and compounds such as agonists HU-210, SR141716 and O-1602 and antagonists O-1918 have been found to act upon the receptor has also been shown to interact at different concentrations with cannabinoid receptors CB1 and CB2 as well as other G-protein coupled receptors GPR18 (Simcocks et al., 2014).

Whilst a substantial proportion of GPR55 research has been accomplished using *in vitro* models using various compounds with conflicting results, studies that have looked at the receptor using *in vivo* methods may give a more congruent overview of the role of the receptor physiologically. A GPR55 knockout (GPR55 -/-) mouse model was used to evaluate hyperalgesia associated with both inflammatory and neuropathic pain models (Staton et al., 2008). It was found that mice lacking GPR55 abolished the hyperalgesia elicited by Fruend's Complete Adjunct (FCA) induced inflammation model of mechanical heat sensation and nerve ligation compared to their wild type (GPR55+/+) litter mates. Previous to the induction of inflammation, no differences were seen between the groups with pain associated with heat sensation alone. Of the mice where inflammation had been induced, GPR55 -/- showed a different cytokine profile compared to wild type mice, with higher levels of interleukin 4 and 10 (IL-4 and IL-10), and interferon γ (IFNγ). As there were no changes in CB1 or CB2 expression in the groups, this indicates that GPR55 may have a role in regulating cytokine production during inflammatory responses (Staton et al., 2008). However a major limitation to this study is that expression of GPR55 and the CB1 receptor was evaluated using brain tissue and the CB2 receptor with splenic tissue, whilst the model of inflammation was somewhat localised to the plantar region of the mice and so CB2 expression may have been altered in this region and could be responsible for mediating the effects of altered cytokine profile.

GPR55 has recently been highlighted for its possible role in cancer (Perez-Gomez et al., 2012, Pisanti et al., 2013, Ross, 2011). Its most potent endogenous ligand, LPI has been shown to be upregulated in proliferating cancer cell lines as well as high LPI plasma levels being identified as a risk factor for poor prognosis in ovarian

cancer (Ross, 2011). Control of cell proliferation has been shown to be regulated by GPR55 in several models of cancer (Henstridge, 2012). GPR55 antagonists may provide an attractive therapeutic target for a number of different diseases, including cancer and inflammation, although development of highly specific GPR55 compounds are only just beginning to be developed (Kotsikorou et al., 2011), and elucidating the physiological role of GPR55 in both healthy and disease states is required.

## ***1.3 Endocannabinoids in Renal Disease***

Currently, limited investigations have been performed concerning the renal expression and function of the cannabinoid receptors CB1 and CB2 particularly within the pathophysiological context of obesity and diabetes related renal damage. The clinical implications of targeting GPR55 in renal disease have yet to be explored. In 1997 expression of the CB1 and CB2 receptor and their endogenous ligand AEA was first described in rat kidney tissue and *in vitro* in human renal mesangial cells (Deutsch et al., 1997), however it was not until a decade later that a possible functional role for these receptors in nephropathy was identified (Janiak et al., 2007). Currently, few studies have focused on the role of the endocannabinoid system specifically in the kidneys.

### *1.3.1 Expression of Cannabinoid Receptors in Renal Tissue*

Initially, after cannabinoid receptors CB1 and CB2 were first identified, qualitative polymerase chain reaction (PCR) analysis failed to detect mRNA expression of these receptors in a number of human peripheral tissues, including the kidneys (Galiègue et al., 1995, Nieri et al., 2006). Indeed, compared to the relatively high expression of

these receptors in the central nervous system and immune cell populations, many tissues in which the endocannabinoid system is now thought to have significant physiological roles such as skeletal muscle, liver, placental and retinal tissues were initially reported as not expressing either the CB1 or CB2 receptor (Galiègue et al., 1995). Since then, multiple studies have confirmed the presence of CB1 and CB2 protein and mRNA transcripts in human and animal kidney tissue as well as renal cell lines (Barutta et al., 2010, Barutta et al., 2011, Deutsch et al., 1997, Janiak et al., 2007, Jenkin et al., 2010, Matias et al., 2008, Mukhopadhyay et al., 2010a, Mukhopadhyay et al., 2010b, Lim et al., 2010, Jenkin et al., 2013). Further, studies indicate that renal expression of the receptors vary in physiologically stressful context such as diabetes, ischemia or inflammation (Barutta et al., 2010, Barutta et al., 2011, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Mukhopadhyay et al., 2010b). GPR55 was first identified in 1999 (Sawzdargo et al., 1999), and initial investigations into tissue distribution showed a similar pattern of expression to that of the CB1 receptor. It is highly expressed in the central nervous system, adrenal glands and parts of the digestive tract, however it was later found to be also expressed in a number of peripheral tissues including the kidney (Ryberg et al., 2007). Our group was the first to identify GPR55 protein and mRNA transcripts specifically within the renal HK2 proximal tubule cell line (Jenkin et al., 2010).

### *1.3.2 Functional Role of Cannabinoid Receptors in Renal Physiology*

Given the kidneys are a highly vascular organ, receiving 20-25% of cardiac output, it is apt that the initial study indicating the endocannabinoid system may play a functional role in the renal system identified a vascular physiological role by

demonstrating the endogenous endocannabinoid AEA exerts vasodilatory effects in rat kidney via NOS signalling (Deutsch et al., 1997). Further, the authors showed that renal endothelial and mesangial cells can produce and metabolise AEA, as well as express both CB1 and CB2 receptors (Deutsch et al., 1997). Consequently, much of the research has since focused on the role of the system within the context of nephropathy. It is likely that due to its relatively low expression in a number of tissues, including the kidneys, under normal physiological conditions the cannabinoid system may not exert a large functional role. However, under chronic or acute diseases or cellular stress, it is becoming increasingly clear that cannabinoid receptors mediate a variety of important signalling pathways which lead to both structural and functional changes (Di Marzo, 2009).

Following the initial success of CB1 antagonist Rimonabant as a weight loss compound, intense expansion into clinical and experimental trials lead to the initial observation that pharmacologically blocking CB1 activity induced improvements in renal function in an animal model of T2DM (Janiak et al., 2007). This study used obese *fa/fa* Zucker rats, a breed which spontaneously exhibits characteristics of obesity, hypertension, proteinuria, and glucose intolerance compared to lean *fa/+* animals (Takaya et al., 1996). Here, lean and obese rats were fed a diet containing either a vehicle (placebo) or Rimonabant at low and high concentrations (3 mg/kg/day or 10 mg/kg/day). Obese *fa/fa* rats on Rimonabant treatment exhibited higher rates of survival and weight loss as well as improved metabolic and renal function (Janiak et al., 2007). Improved renal parameters was demonstrated by significantly delayed increased proteinuria, lower urinary N-acetyl-beta-D-glucosaminidase (a marker for tubular damage), improved creatinine clearance and

significant reductions to glomerular and tubular lesions and fibrosis compared to obese pair fed animals (Janiak et al., 2007). Further, the lower 3 mg/kg/day dosage treated group didn't exhibit reduced weight or improved fasting plasma glucose and insulin levels compared to pair fed obese animals, but a number of renal measurements were still ameliorated by the CB1 blockade (Janiak et al., 2007), indicating that improvements may not wholly be the result of changes to metabolic parameters. One limitation to the study was the model of obesity used by the authors. Obesity in *fa/fa* Zucker rats is induced by a mutation to the leptin receptor, which impairs feeding behaviour, thus obese *fa/fa* Zucker rats exhibit significant hyperphagia compared to lean animals (Takaya et al., 1996). Leptin however, is an important adipokine which has been shown to be involved in metabolism of proximal tubule cells as well as mediate the renal handling of protein (Briffa et al., 2013, Kaseda et al., 2007). The limitation of using the Zucker model of obesity is that it does not reflect the true mechanisms which underlie obesity related nephropathy. Obesity in the Zucker strain is due to the inhibition of a functioning leptin signalling pathway, and consequently, limited conclusions can be drawn from the role of the receptor in obesity related renal damage in humans. Further, the authors contribute improved renal function to changes within the glomerulus, citing that obese patients (as represented by the Zucker rats) are prone to glomerulosclerosis and glomerular hypertrophy, however despite the fact that proteinuria can be the result of glomerular or proximal tubule damage; no investigations into the tubular changes were attempted.

A subsequent study which investigating the endocannabinoid system in obesity also indicated that the endocannabinoid system becomes deregulated in a number of

tissues, including the kidneys after just a few weeks on a HFD (Matias et al., 2008). This study used a mouse model of diet induced obesity (DIO), using two different HFDs containing 20- 34% fat, with different fatty acid composition and showed that both AEA and 2-AG become elevated after just three weeks on a HFD in metabolically active tissues such as skeletal muscle and brown fat, even before significant changes in fasting glucose levels was detected (Matias et al., 2008). In the kidneys, it was observed that after 8 weeks on a HFD, AEA was significantly elevated in renal tissue of animals fed both HFDs compared to standard chow fed control, and this effect was sustained in the animals which were maintained on the diets for 14 weeks (Matias et al., 2008). Renal concentrations of 2-AG were also significantly altered in DIO, however, this was only demonstrated in one group feeding on the HFD, and only 14 weeks of DIO, indicating that fatty acid composition of the diet may be an important factor mediating renal changes to endocannabinoid signalling. While this study added to evidence which supports the endocannabinoid system becomes disrupted in the kidneys in response to a HFD, it did not examine receptor expression within the tissues investigated, or whether changes to AEA and 2-AG levels corresponded with functional changes to the tissues.

One group has recently published two papers on pharmacological targeting the CB1 and CB2 receptor in a type 1 model of diabetes, using streptozotocin (STZ) to induce diabetes in C57BL6/J mice (Barutta et al., 2010, Barutta et al., 2011). In these studies, lean diabetic mice were treated for 14 weeks with CB1 antagonist AM251 (1 mg/kg) and CB2 agonist AM1241 (3 mg/kg), and both treatments in comparison to sham injected diabetic animals lead to reduced proteinuria and podocyte loss, a cell type important for maintaining the structural integrity of the slit diaphragm within the

glomerulus (Barutta et al., 2010, Barutta et al., 2011). A large body of evidence shows that in particular, CB1 antagonism leads to improvements in a number of metabolic parameters including body weight, blood glucose and insulin sensitivity (Di Marzo, 2009, Irwin et al., 2008, Janiak et al., 2007, Rosenstock et al., 2008, Van Gaal et al., 2005). However, as previous research into the renal effects of CB1 antagonism have only focused on streptozotocin (STZ) induced diabetes in mice (Barutta et al., 2011, Nam et al., 2012) or obese animals without functional leptin receptors (Janiak et al., 2007), the functional renal outcomes of CB1 antagonism in a model of obesity, which more closely resembles the physiological and inflammatory state in obese humans has not yet been explored.

Renal hypertrophy is an important structural change which takes place in CKD, and is an early indicator of nephropathy (outlined in Section 1.1.8). Our group has previously identified expression of CB1, CB2 and GPR55 mRNA transcripts and protein in human proximal tubule HK2 cells (Jenkin et al., 2010). We also identified a functional role of the receptors *in vivo*, showing that cannabinoid receptors mediate tubular hypertrophy (Jenkin et al., 2010). The non-specific agonist AEA significantly increases proximal tubule hypertrophy, while receptor specific antagonists were also shown to mediated this process (Jenkin et al., 2010). Briefly, we found that treatment with CB1 antagonist AM251 reduces hypertrophy while treatment with CB2 (AM630) antagonists increases hypertrophy. GPR55 antagonist treatment with O-1918 in HK2 cells did not alter proximal tubule cell hypertrophy (Jenkin et al., 2010). The results indicated that the receptors are likely to activate different cell signalling pathways, however identifying these were beyond the scope of the study. One limitation of the study is that even though GPR55 was shown not be involved in mediating proximal

tubule hypertrophy through O-1918, the CB1 antagonist AM251 may also have agonistic effects on GPR55 at the concentrations used in our study (0.5 – 5 μM), making it difficult to rule out actions of GPR55 mediating hypertrophy in this cell line (Henstridge et al., 2011). Further evaluation of pharmacological activation of GPR55 in tubule cells is required. Beyond our *in vitro* study, to date the functional effects of GPR55 in renal physiology has not been examined.

### *1.3.3 Signalling Pathways Mediated by Cannabinoid Receptors in Renal Physiology*

Elevated cytokine production (particularly TGF-β1) mediates renal fibrosis and thickening of the basal membrane of proximal tubule cells during the progression of diabetic nephropathy. The studies by Barutta et al. (2010, 2011) also examined the role of cytokines which may mediate structural and functional consequences of cannabinoid pharmacological treatments. Here, they found that in isolated glomeruli of diabetic compared to non-diabetic animals TGF-β1, CTGF and fibronectin mRNA transcripts were all upregulated, and treatment with CB1 antagonist AM251 or CB2 agonist AM1241 did not alter these levels (Barutta et al., 2010, Barutta et al., 2011). Our group has previously shown that *in vitro* HK2 cells treated with global agonist AEA significantly increases tubule hypertrophy in proximal tubule cells, while treatment with CB1 antagonist AM251 significantly reduces hypertrophy, and CB2 antagonist AM630 significantly increased hypertrophy even beyond levels caused by AEA treatment (Jenkin et al., 2010). CB2 activation by various methods has been shown to mediate reductions in a number of pro-fibrotic cytokines including TGF-β1 (Correa et al., 2009, Julien et al., 2005, Rajesh et al., 2007, Gardner et al., 2002). Further, animal models of liver fibrosis has shown that CB1 blockade significantly

reduces TGF- β1 expression (Teixeira-Clerc et al., 2006) as well as other fibrotic markers (Chen et al., 2012). Many cell types, including proximal tubule cells can contribute to cytokine production. Ultimately, the renal structural remodelling which occurs in diabetic nephropathy is a contribution of many cell types, and the failure to investigate beyond glomerular cells by the authors Barutta et al. (2010, 2011) highlights that further investigation into pharmacological cannabinoid treatment affects kidney expression of these factors is needed.

To date, studies have not characterised the effects of pharmacological cannabinoid interventions on mediating markers of renal fibrosis. In animal and cell culture models of nephropathy, pharmacological modulation of cannabinoid receptor activity has been linked to mediating inflammatory effects (Pacher and Mechoulam, 2011, Barutta et al., 2011, Lim and Tesch, 2012); however the role of these treatments in renal fibrosis has yet to be elucidated. Currently the role of GPR55 in nephropathic related inflammation has not been investigated. Inflammation in nephropathy induced by the chemotherapeutic drug cisplatin, use of CB1 receptor antagonist AM281 and SR141716 attenuated markers of renal damage in general - lowering serum creatinine and blood urea nitrogen (BUN) levels, but also specifically reduced markers of inflammation such as tumour necrosis factor α (TNF-α), interleukin 1 β (IL-1β) and C-reactive protein (Mukhopadhyay et al., 2010a). It has also been demonstrated that both inflammation and cell death was significantly reduced in retinal cells from STZ induced diabetic CB1 -/- knockout mice, compared to wild type littermates, with this effect remaining in wild type diabetic mice treated with CB1 receptor specific antagonist Rimonabant (El-Remessy et al., 2011).

While CB1 antagonist treatment can reduce inflammation in certain models of cell damage, it is the CB2 receptor which is highly expressed in inflammatory cells that has increasingly garnered attention on mediating inflammatory effects both in CKD and other models of diseases (Barutta et al., 2011, Bermudez-Silva et al., 2007, Cabral and Griffin-Thomas, 2009, Gardner et al., 2002, Jenkin et al., 2010, Jenkin et al., 2012, Mukhopadhyay et al., 2010b, Pacher and Mechoulam, 2011). Overwhelmingly, it seems that the presence or activation of the CB2 receptor acts as an anti-inflammatory mediator (Pacher and Mechoulam, 2011), even in states where receptor expression in the tissue examined remains unchanged (for review, see Pacher and Mechoulam 2011). Specifically in nephropathy, CB2 selective agonists have been shown to limit inflammation, inflammatory markers chemokine c–c motive receptor 2 (CCR2) and monocyte chemo attractant protein (MCP-1), recruitment of lymphocytes and expression of adhesion molecules (Barutta et al., 2011, Mukhopadhyay et al., 2010b).

In summary, as outlined in this review of the literature, rising levels of obesity and diabetes are principal factors leading to increased incidence of CKD globally. Viable therapeutic targets need to be established to safely combat the progression of renal damage associated with these chronic diseases. Cannabinoid receptors have been shown clinically mediate a number of physiological effects important in the pathophysiology of both obesity and diabetes. Emerging research also highlights that the endocannabinoid system has potentially a significant role in renal function. It is postulated that cannabinoid receptors may mediate CKD via a number of mechanisms including energy storage and expenditure, cardiovascular tone and the modulation of inflammatory and hypertrophic pathways. However, mechanistic

pathways of the endocannabinoid system in obesity and diabetic related renal damage is still poorly understood. Pharmacologically targeting cannabinoid receptors may provide useful targets for the clinical treatment of CKD. This thesis intends to examine renal expression and function of CB1, CB2 and GPR55 receptors, particularly within the pathophysiological context of obesity and diabetes related renal damage. Further, to elucidate whether pharmacologically targeting cannabinoid receptors may abrogate obesity related renal damage.

## ***1.4 Aims and Hypothesis***

This thesis aims to examine the expression of cannabinoid receptors in the kidney in obese and diabetic conditions, and how pharmacological targeting of the receptors may affect markers of renal damage and expression of cannabinoid receptors and key signalling proteins in the kidneys.

The studies described within this thesis tested the following hypotheses that:

i) Exposure to diabetic conditions (elevated glucose and albumin) significantly alters CB1, CB2, and GPR55 expression within proximal tubule cells as well as the whole kidney.

ii) Expression of the CB2 receptor in proximal tubule cells is dependent on albumin internalisation.

iii) DIO in male Sprague Dawley rats will significantly alter renal structure and function.

iv) DIO will lead to significant changes in CB1, CB2 and GPR55 receptor expression in kidney tissue of Sprague Dawley rats.

v) Pharmacological blockade of the CB1 receptor will improve renal structure and function in a model of DIO, and also lead to alterations in key pro-fibrotic cytokines within the kidney.

vi) Pharmacological activation, but not blockade of the CB2 receptor will improve renal structure and function in a model of DIO, and lead to alterations in key pro-fibrotic cytokines in the kidney.

vii) Pharmacological blockade, but not activation of GPR55 will improve renal structure and function in a model of DIO, and lead to alterations in key pro-fibrotic cytokines in the kidney.

# Chapter 2 - Materials and Methods

## *2.1 Cell Culture*

### *2.1.1 Immortalised Human Proximal Tubule Cell Line*

All cell culture reagents used in these studies were purchased from Gibco (distributed by Invitrogen, Carlsbad, CA) unless otherwise stated. Cell lines were a kind gift from Professor Daniel Markovich (University of Queensland). Human proximal tubule cell line (HK2) cells were maintained in Dulbecco's modified Eagle media: nutrient mixture F12 (DMEM/F12) media supplemented with 10% foetal bovine serum (FBS; vol/vol), 1% penicillin streptomycin (vol/vol), 1% 1 X ITS (Human Insulin, Transferrin and Sodium Selenite) Liquid Media Supplement (Sigma, St Louis, MO vol/vol), 10 ng/ml epidermal growth factor (EGF; Sigma, St Louis, MO) and 2 mM l-glutamine (Sigma, St Louis, MO). Opossum Kidney (OK) cells were maintained in the same media as described for HK2 cells, excluding ITS and EGF supplementation. Cells were maintained at 37℃, in 5% $CO_2$, with media being changed every second day.

### *2.1.2 Cannabinoid Receptor Expression Treatment*

Prior to treatment HK2 cells were grown to 80% confluence, followed by 24 hours incubation in DMEM/F12 media supplemented with 1% penicillin streptomycin (vol/vol) without FBS. Cells were then briefly rinsed in sterile 1 x phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) and treated for a period of 4, 6, 18 and 24 hours with either a control media

containing physiological levels of glucose (5 mM) and no albumin, a high glucose media (25 mM) containing no albumin, a high albumin media (1 mg/ml) containing 5 mM glucose, or treated with a media incorporating both high glucose (25 mM) and high albumin (1 mg/ml) (Zafiriou et al., 2004). Glucose and albumin were purchased from Sigma Aldrich (St Louis, MO). Stock solutions of 1 M glucose and 100 mg/ml albumin were made using sterile PBS.

### *2.1.3 Albumin Uptake*

OK cells were seeded to confluence and grown for 5 days to allow the formation of a polarised monolayer. Cells were then exposed to albumin conjugated to Texas Red (TR-albumin) (50 µg/ml) in the presence of specific inhibitors for two hours at 37℃ as previously described (Hryciw et al., 2005). At the end of the albumin uptake period, cells were washed in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and lysed in MOPS (3-(N-morpholino) propanesulfonic acid) buffer (20 mM MOPS, 0.1% Triton X-100, pH 7.4). TR-albumin florescence was determined using an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA) at 580 nm excitation and 630 nm emission wavelengths. TR-albumin uptake was adjusted for background and standardized to treatment without the presence of inhibitors (control).

## ***2.2 mRNA Analysis***

### *2.2.1 RNA Extraction*

Following treatment in 6 well plates, HK2 cells were lysed for 5 minutes at room temperature in 800 µl of TRIzol (Invitrogen, Carlsbad, CA) and transferred to 1.5 ml

Eppendorf tubes. To each sample 200 µl of chloroform (Sigma Aldrich, St Louis, MO) was added and briefly vortexed for approximately 15 seconds and then centrifuged at 12000 times gravity (x *g*) for 15 minutes at 4℃. The clear upper layer was removed and added to fresh 1.5 ml Eppendorf tubes containing an equal volume (approximately 600 µl) of 2-isopropanol (Sigma Aldrich, St Louis, MO) and incubated on ice for 10 minutes. Samples were centrifuged at 12000 x *g* for 10 minutes at 4℃, forming a small white RNA pellet. Supernatant was then removed and the RNA pellet was carefully washed with 500 µl freshly prepared 75% (vol/vol) ethanol made with diethylpyrocarbonate (DEPC) water (Invitrogen Life Sciences). Samples were then centrifuged at 9000 x *g* for 8 minutes at 4℃. Ethanol was aspirated off and the RNA pellet was left to briefly air dry for 10 -15 minutes at room temperature. The pellet was then dissolved in 10 µl of DEPC water. Total RNA was quantified using nanodrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 nm. Samples were stored at -80℃ until analysis.

### *2.2.2 DNAse Treatment of RNA Samples*

Prior to reverse transcription, 1 µg total RNA was digested for 30 minutes at 37℃ using 1 unit of RQ1 RNase-free DNase (Promega, Corporations, Madison, WI), followed by 10 minutes at 65℃ in a buffer containing 20 mM ethylene glycol tetraacetic acid (EGTA, pH 8), according to manufacturer's instructions.

### *2.2.3 Reverse Transcription of RNA samples*

The DNAse treated RNA samples were measured for total RNA using NanoDrop 2000 spectrophotometer and 0.5 µg of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Melbourne, Australia). The PCR

amplification was performed using a MyCycler™ Thermo Cycler (Bio-rad Laboratories, Hurcules, CA), set to the following program: 5 minutes at 25℃, 30 minutes at 42℃ and 5 minutes at 85℃.

*2.2.4 'Real-Time' Polymerase Chain Reaction (PCR)*

'Real-time' PCR was conducted using MyiQ™ multiplex 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Forward and reverse oligonucleotide primers for the gene was designed using OligoPerfect™ Suite (Invitrogen, Carlsbad, CA) and can be found outlined in Table 2.1 below. Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was also measured. 'Real-time' PCR reactions were run for 40 - 50 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds. Relative changes in mRNA abundance was normalised to housekeeping GAPDH gene and then quantified using the $2^{-\Delta\Delta CT}$ method. Treatment groups were expressed relative to control treatment. $C_T$ values for GAPDH were not altered by treatments.

**Table 2.1: Human Primer Sequences Used For 'Real-time' PCR Analysis**

| Gene | Forward Primer (5' - 3') | Reverse Primer (5' – 3') | Accession Number |
|---|---|---|---|
| CB1 | GCCTTGCAGATACCACCTTC | GTTCTCCCCACACTGGATGT | NM_016083.4 |
| CB2 | ACTCCATGGTCAACCCTGTC | GATCTCGGGGCTTCTTCTTT | NM_001841.2 |
| GPR55 | CCCATTCAAGATGGTCCTGT | TTCCGTACATGCTGACGAAG | NM_005683.3 |
| GAPDH | CAACGACCACTTTGTCAAGC | TTACTCCTTGGAGGCCATGT | NM_002046.3 |

## ***2.3 Protein Analysis***

### *2.3.1 Protein Extraction*

HK2 cells were cultured in 75cm² flasks, and prepared for experimental use as described in Section 2.1.2. Following experimental treatment, HK2 cells were rinsed briefly three times with PBS and lysed with 400 µl of lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% (vol:vol) NP- 40; with the pH adjusted to 7.5, supplemented with a Complete Mini Protease Inhibitor Cocktail tablet (Roche Products Pty Limited, Dee Why, NSW). When extracting protein for analysis of phosphorylated proteins, a Halt Phosphatase Inhibitor Cocktail (Pierce Chemical Company, Rockford, IL) was added to the buffer at a 1:1000 dilution. Cells were lysed for 30 minutes at 4℃ with agitation. Following the incubation period, cells were thoroughly scraped using a cell scraper and lysates added to clean 1.5 ml Eppendorf tubes and centrifuged at 12000 x *g* for 10 minutes at 4℃. Samples were stored at -20 °C until analysis.

### *2.3.2 Total Protein Quantification*

Total protein content of each cell culture and kidney tissue samples were quantified with a bicinchoninic acid (BCA) Protein Assay Reagent Kit (Thermo Scientific, Rockford IL), with bovine serum albumin (BSA) used as standards. Cell culture lysate was diluted 1:5 with MilliQ water and a series of nine standards (0, 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml) were prepared via serial dilution of stock BCA using MilliQ water as diluent. To 200 µl of Pierce BCA protein assay reagents B and A (prepared with 1:50 vol:vol ratio), 25 µl of diluted sample or standards were added and incubated at 37℃ for 30 minutes. Absorbance was read at 562 nm with

an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). A standard curve was prepared and protein content for each sample was calculated.

*2.3.3 Western Blot Analysis*

To quantify levels of specific protein expression of cannabinoid receptors and signalling molecules in cell lysate and kidney samples, Western blot analysis was performed. All reagents used for Western blot analysis were purchased from Bio-Rad Laboratories (Hurcules, CA), unless otherwise stated. 40 µg of sample protein were denatured in Laemmli loading buffer containing 2.5% beta-mercaptoethanol and boiled for 5 minutes at 70 -100℃ followed by a brief spin down. Samples were then loaded onto pre-cast 7.5 - 20% or 10% mini TGX polyacrylamide gels. Following electrophoresis (200 V for 40 minutes), proteins were transferred on to Trans-blot Turbo ™ nitrocellulose membrane and blocked for 1 hour with Tris Based Saline Tween 20 (TBST) blocking buffer containing 140 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20 and 5% skim milk powder. Following this, membranes were incubated overnight at 4℃ with fresh TBST blocking buffer containing skim milk powder with primary antibody, for antibody dilution and company supplier of primary antibody, see Table 2.2 below. The membrane was then washed three times with TBST buffer and incubated for 1 hour at room temperature with the secondary antibody diluted in TBST buffer containing either skim milk powder of BSA (Sigma Aldrich, St Louis, MO). Membranes were washed and incubated with chemiluminiscent detection solution (Super Signal West-Femto Detection Kit, Pierce, Rockford, IL) for 5 minutes at room temperature. Blot images were captured and

protein content of samples quantified using Image Lab software (Bio-rad Laboratories, Hercules, CA). Protein concentration of treatment groups were normalised to control treatments. To ensure equal loading of samples, β-Actin (Sigma Aldrich, St Louis, MO) was chosen as loading control.

**Table 2.2: Supplier Information and Dilution Factor of Antibodies Used in Western Blot Analysis**

| Antibody | Supplier (catalogue number) | Dilution |
|---|---|---|
| CB1 | Cayman Chemicals (10006590) | 1:1000 |
| CB2 | Cayman Chemicals (101550) | 1:1000 |
| GPR55 | Novus Biologicals (NLS6817) | 1:500 |
| phosphorylated AKT (Ser 473) | Cell Signaling (4060) | 1:1000 |
| AKT | Cell Signaling (4691) | 1:1000 |
| phosphorylated p38 (Thr180/Tyr182) | Cell Signaling (4511) | 1:1000 |
| p38 | Cell Signaling (8690) | 1:1000 |
| phosphorylated ERK1/2 (Thr202/Tyr204) | Cell Signaling (4370) | 1:1000 |
| ERK1/2 | Cell Signaling (4372) | 1:1000 |
| VEGF | Abcam (ab46154) | 1:1000 |
| Collagen IV | Abcam (ab6586) | 1:5000 |
| TGF-β1 | Abcam (ab66043) | 1:10000 |
| β-Actin | Sigma (A-2228) | 1:2000 |

## ***2.4 Experimental Outline of in vivo Studies***

### *2.4.1 Diabetic Rat Study*

Kidney tissue analysed in Chapter 3 and Chapter 4 was kindly provided by Dr Yuan Zhang and Prof Darren Kelly from Department of Medicine, St Vincent's Hospital, Fitzroy, Australia. Six-week old male Sprague–Dawley rats were randomised to receive either 55 mg/kg of STZ (Sigma, USA) diluted in 0.1 M citrate buffer pH 4.5 or citrate buffer (control) by tail vein injection following an overnight fast (n = 8 per group). Blood glucose was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, Australia) and only STZ-treated animals with blood glucose >20 mmol/L were considered diabetic. All animals were housed in a stable environment maintained at 22 ± 1℃ with a 12 hour light/dark cycle (7:00 – 19:00). 16 weeks after STZ treatment animals were killed and kidney tissues were stored at -80℃. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Research Ethics Committee of St. Vincent's Hospital.

### *2.4.2 High Fat Obese and Lean Rat Study*

To determine the effects of a HFD on markers of kidney damage and renal cannabinoid receptor expression this study was completed in collaboration with Ms Lannie O'Keefe and Mrs Anna Simcocks. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036). Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour light/dark cycle (7:00 -

19:00). Rats were randomly assigned to receive either a HFD (21% fat; Specialty Feeds, Glen Forrest, Australia) or a control diet (standard rodent chow; 5% fat; Barastoc Ltd, Melbourne, Australia) for a period of three or 10 weeks (n = 4 per group), see Table 2.3 for nutritional outline of standard chow and HFDs. *Ad libitum* access to food and water was maintained throughout the duration of the study, body weight and food consumption was recorded daily. In the final week of the experimental period, a number of metabolic parameters were measured including blood pressure, body composition, insulin and glucose tolerance and 24 hour urine sample collection. Protocols for these measurements will be outlined in more detail in the following sections. Rats were then deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanised via cardiac puncture. Kidneys were removed, weighed and stored for further analysis.

**Table 2.3: Calculated Nutritional Parameters of Standard Chow and High Fat Diets (HFD)**

| Calculated Nutritional Parameters | Standard Chow Diet | HFD |
|---|---|---|
| Protein (%) | 20.0 | 19.0 |
| Total Fat (%) | 5.0 | 21.0 |
| Crude Fibre (%) | 5.0 | 4.7 |
| Digestible Energy (MJ/kg) | 17.25 | 19.4 |
| Total calculated digestible energy from lipids (%) | 10.7 | 40.0 |
| Total calculated digestible energy from Protein (%) | 19.7 | 17.0 |

*2.4.3 Chronic Administration of Cannabinoid Receptor Compounds in Obese Rats*

This study determined the renal effects of chronic administration of cannabinoid receptor specific compounds for CB1, CB2 and GPR55 in a model of DIO. This study was completed in collaboration with Ms Lannie O'Keefe and Mrs Anna Simcocks. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036). 53 seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour light/ dark cycle (7:00 - 19:00). Nine weeks of a HFD was sufficient to induce obesity, with rats exhibiting significant increases in body weight, body fat composition and hypertension and reduced markers of renal damage as measured by higher urinary protein and albumin excretion. The measurements taken at nine weeks were similar to the obese changes observed in our 10 week HFD model, and therefore it was deemed as unnecessary for the animals to be subjected to a longer period of treatment than what was required. Rats were fed a HFD (detailed in Table 2.3, containing 21% fat, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Rats were then matched using a number of metabolic parameters and put into one of six treatment groups, outlined in Table 2.4 below. For six weeks, rats were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution (McFarlene Medical) containing 0.75% Tween 80 (Science Supply, Labchem) or experimental compound dissolved in vehicle solution via intraperitoneal (IP) injection (n = 6 – 10 per group). Compounds for CB1 and CB2 receptors AM251, AM1241 and AM630 were purchased from Cayman Chemicals (Ann Arbour, MI) and GPR55 specific compounds (O-1602 and O-1918) were purchased from Tocris Bioscience (Bristol, UK). Stock solutions were prepared,

and stored at -80℃, working stock was made as required by diluting stock solutions with sterile 0.9% isotonic saline solution to achieve the desired concentration (see Table 2.4). The concentration of compounds utilised for the CB1, CB2 and GPR55 treatments were based off previously published experimental studies using mice and rats, as indicated in Table 2.4 below (Curto-Reyes et al., 2010, Janiak et al., 2007, Offertaler et al., 2003, Shoemaker et al., 2007).

**Table 2.4: Outline of Treatment Concentrations for Experimental Groups**

| Treatment Group | Compound | Concentration (mg/kg) | References for concentration of compounds |
|---|---|---|---|
| Control | Saline | | |
| CB1 Antagonist | AM251 | 3 | (Janiak et al., 2007) |
| CB2 Agonist | AM1241 | 3 | (Curto-Reyes et al., 2010, Shoemaker et al., 2007) |
| CB2 Antagonist | AM630 | 0.3 | |
| GPR55 Agonist | O-1602 | 5 | (Offertaler et al., 2003) |
| GPR55 Antagonist | O-1918 | 1 | |

Before administration of cannabinoid receptor compounds and in the final week of the experimental period, a number of metabolic parameters were measured including blood pressure, body composition, insulin and glucose tolerance and 24 hour urine sample collection. Protocols for these measurements will be outlined in more detail in the following sections. At the conclusion of the experimental period, rats were deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanised via cardiac puncture. Kidneys were removed, weighed and stored for further analysis.

## ***2.5 Metabolic Measurements for Animal Studies***

### *2.5.1 Weight and Food Consumption*

Rat weight and food consumption was monitored and recorded daily, typically during the morning hours between 7:00 -11:00 am, at the beginning of the light cycle.

### *2.5.2 Body Composition*

Body composition of rats were analysed using EchoMRI ™ Whole Body Composition Analyzer (EchoMRI, Houston TX). The machine was calibrated according to manufacturer's instructions using canola oil as calibration medium. Live, conscious rats were restrained within a clear plastic cylinder which limited their ability to turn around, but did not restrict breathing. The restrained rats were then placed inside the EchoMRI™ Analyzer for a two minute scan, which determined fat and lean tissue as well as free and total body water content of the animals. Each animal was scanned in duplicate, to ensure accuracy of measurements. Animals were restrained for no longer than 10 minutes and were returned to cages following the scans.

*2.5.3 Blood Pressure*

Blood pressure was analysed using a non-invasive tail-cuff method with a volume pressure recording software CODA 2 (Kent Scientific, Torrington, CT). Measurements for systolic and diastolic blood pressure were obtained from conscious rats restrained in a clear plastic cylinder. Rats were placed in a heating chamber set to 30℃ for 10 minutes to warm the tail vein for increased circulation. Rats then had the tail cuff and volume pressure sensor placed on the tail and went through 5 acclimatisation cycles followed by 10 - 20 cycles of cuff inflation with a 5 second interval time between cycle sets. Data was analysed using CODA software and only accepted blood pressure readings were used for data analysis.

*2.5.4 Urine Collection*

To evaluate markers of kidney damage, throughout the experimental period, 24 hour urine collection was taken. Rats were placed into metabolic cages with access to food and water *ad libitum*. Urine collection containers were weighed before and after the collection period to determine total volume of urine voided. Food and water consumed during the collection period was also recorded. Urine samples were transferred to 15 ml falcon tubes and centrifuged at 4000 x *g* for 10 minutes at 4℃ to remove any food or faecal contaminates. Samples were then aliquoted into 2 ml Eppendorf tubes and stored at -80℃ until further analysis.

*2.5.5 Plasma Collection*

At the end of the experimental period, rats were deeply anesthetised and killed via cardiac puncture using a 10 ml syringe with 18 gauge needle. Blood was transferred into 10 ml Ethylenediaminetetraacetic acid (EDTA) BD Vacutainer® tubes

(McFarlene Medical). Tubes containing blood were gently mixed to ensure even distribution of EDTA within samples to prevent clotting and samples were stored on ice until samples were centrifuged at 4000 x *g* for 10 minutes at 4℃. The plasma layer was carefully aspirated off and aliquoted into 2 ml Eppendorf tubes and stored at -80℃ until further analysis. An aliquot of the haematocrit layer containing red blood cells was also kept for further analysis.

## ***2.6 Tissue Collection and Analysis***

### *2.6.1 Kidney Dissection*

At the end of the experimental period, rats were deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanised via cardiac puncture. Kidneys were dissected out, and the surrounding peri-renal fat and capsule were carefully removed and kidney weight was recorded. The kidneys were then dissected transversely down the axis. One half of each kidney was put into cryotubes (Thermo Fisher Scientific, Wilmington, DE) and immediately frozen in liquid nitrogen for RNA and protein analysis.

The other half of the kidney was dissected and preserved for fixed or fresh frozen histological analysis. Here the poles of the kidney were removed and the remaining kidney section was dissected in half at the renal sinus.

The first portion was placed into cryomolds (Sakura Finetek, Leiden) containing sufficient volume of optimal cutting temperature compound (OCT Tissue-Tek, Torrance, CA) so that the kidney sections were completely immersed. The cryomolds were then placed into 2-methylbutane (Sigma Aldrich St Louis, MO) pre-

cooled to -150℃ set in liquid nitrogen “bath” until the OCT media completely set. Samples were then transferred to dry ice until they could be stored at -80℃ until further analysis on “fresh frozen” kidney tissue.

The second portion was used for fixed histological analysis. Here, the section was placed into McCarthy bottles containing 4% paraformaldehyde in 0.1 M PBS solution at 4℃ overnight followed by a sequential incubation in a 30% sucrose solution overnight at 4℃. Embedding was performed in OCT solution and embedded samples were stored at -80℃ until further analysis.

*2.6.2 Kidney Lysate Preparation*

Kidney tissue was sectioned, weighed and finely diced using a sharp scalpel blade. Samples were then homogenised in HNT buffer (containing 20 mM HEPES, 120 mM NaCl, 0.6% Triton-X, 5 mM EDTA and Roche Complete protease inhibitor cocktail) in a 2:1 buffer volume to kidney weight ratio using Miccra D1 homogeniser (Milan SA, Geneva) for 30 seconds. Homogenates were gently agitated for 3 hours at 4℃ and then centrifuged at 45000 x *g* for 10 minutes and the supernatant was collected. An aliquot of each sample was diluted 1:50 using MilliQ water and total protein was quantified using BCA protein assay kit as outlined Section 2.3.2 above. Samples were stored at -80℃ until analysis.

*2.6.3 Histological Analysis*

Fixed or fresh frozen kidney samples were cut into 5 µm-thick sections using HM 550 Cryostat (Thermo Fisher Scientific, Australia) and stored at -80℃ until time of analysis.

Haematoxylin and Eosin (H&E) stain was performed using a modified protocol described previously (Hughes and Gobe, 2007). Kidney sections were air dried for 1 hour and then submerged in PBS solution for 2 minutes, sections were then stained with Mayers haematoxylin (Sigma Aldrich) for 10 seconds, washed in tap water for 6 minutes and immersed in Scotts tap water 'blueing solution' (20 g $NaHCO_3$, 3.5 g $MgSO_4$ per 1 L $dH_2O$) for 3 minutes, then returned to tap water for 30 seconds. Kidney sections were then dehydrated in 70% ethanol (1 minute), 95% ethanol (2 x 2 minutes) and 100% ethanol (1 minute). Sections were then stained with Eosin (1%, Sigma Aldrich) for 2 minutes followed by a brief wash in tap water (30 seconds) then immersed in 100% ethanol (3 x 2 minutes), cleared in xylene (3 x 2 minutes) and mounted with Cytoseal mounting medium (Thomas Scientific).

Periodic Acid Schiff (PAS) stain was performed on kidney sections air dried for 30 minutes at room temperature and then submerged in PBS for 2 minutes. Sections were oxidised in 0.75% periodic acid solution (Thermo Fisher Scientific, Australia), rinsed briefly in distilled water and then stained with Schiffe Reagent (Thermo Fisher Scientific, Australia), for 15 minutes, then washed in lukewarm tap water for 5 minutes. Sections were then counterstained in Mayers haematoxylin, dehydrated and mounted according to the same protocol as described for H&E staining.

Renal sections from the cortex were imaged at 200 X magnification (Carl Zeiss microscope) and at least 6 random glomeruli and renal proximal tubule sections from four individual rats in each group were analyzed at the widest point for cross sectional diameter using AxioVision 4.8 software.

## ***2.7 Urine Analysis***

### *2.7.1 Total Urinary Protein Content*

Urine samples were diluted 1:100 with MilliQ water and total protein content was quantified using BCA Assay analysis as outlined in Section 2.3.2.

### *2.7.2 Urinary Albumin Content*

Total albumin content of urine samples was quantified with a Rat Albumin ELISA kit (ALPCO Diagnostics, Salem, NH). Urine samples were diluted 1:500 using 1 x diluent, provided by the kit. A series of seven rat albumin standards (0, 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml) were prepared via serial dilution of stock rat albumin calibrator with the 1 x diluent. Reagents were brought to room temperature before use and 100 µl of diluted sample or standard were added to 96 well plates pre-coated with affinity purified anti-rat albumin and incubated for 30 minutes at room temperature. Following the incubation period, samples and standards were aspirated off and the plates were washed four times with wash solution (ALPCO Diagnostics, Salem, NH). 100 µl of enzyme antibody conjugate was then added to each well and incubated for a further 30 minutes at room temperature. Subsequent to the incubation period, the enzyme antibody conjugate was removed and the plate washed as described above. 100 µl light sensitive 3,3'5,5'-tetramethylbenzidine (TMB) containing hydrogen peroxide and citric acid buffer (pH 3.3, ALPCO Diagnostics, Salem, NH) was then added to each well and the plate was incubated in the dark for 10 minutes, followed by 100 µl of 'stop solution' containing 0.3 M sulphuric acid added to each well. Absorbance was read at 450 nm with an xMark

Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). A standard curve was prepared and protein content for each sample was calculated.

*2.7.3 Urinary Creatinine Excretion*

Total creatinine urine content was quantified using Creatinine Urinary Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Urine samples were diluted 1:50 using MilliQ water as diluent. A series of eight standards (0, 2, 4, 6, 8, 10, 12, 15 mg/dl) were prepared using a serial dilution of creatinine standard with MilliQ water as diluent. Alkaline Picrate Solution was prepared using reagents supplied by the kit including Sodium Borate, Surfactant, Colour reagent and Sodium Hydroxide. 15 µl of standard or sample were added to a 96 well microplate (Greiner-Bio One, Germany) followed by 150 µl of the Alkaline Picrate Solution. The plate was then incubated for 10 minutes with gentle orbital agitation, and 'initial' absorbance was read at 490 nm with an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). 5 µl of acid solution containing sulphuric and acetic acid (supplied with kit) was then added to each well, incubated at room temperature for 20 minutes with gentle orbital agitation. 'Final' absorbance was read at 490 nm with an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). To determine urinary creatinine content, 'final absorbance' was subtracted from 'initial absorbance' for absorbance readings of both standards and samples. A standard curve was generated and sample urinary creatinine content was calculated.

*2.7.4 Urinary Sodium Excretion*

Urinary sodium excretion was quantified using COBAS Integra® 400 plus (Roche Diagnostics, Switzerland). The system was calibrated according to manufacturer's

instructions and undiluted samples were analyzed for sodium content. Sodium excretion for each sample was expressed as mmol/L.

## ***2.8 Plasma Analysis***

### *2.8.1 Plasma Creatinine Analysis*

Total creatinine plasma content was quantified using Creatinine Plasma Assay Kit (Cayman Chemical Company, Ann Arbor, MI). A series of eight standards (0, 0.5, 1, 1.5, 2, 3, 4 and 5 mg/dl) were prepared using a serial dilution of creatinine standard with MilliQ water as diluent. Creatinine Reaction Buffer was prepared according to manufacturer's instructions using reagents supplied by the kit including Sodium Borate, Surfactant, Colour reagent and Sodium Hydroxide. 15 µl of standard or undiluted plasma samples were added to a 96 well microplate (Greiner-Bio One, Germany) followed by 100 µl of the Creatinine Reaction Buffer and 100 µl of Creatinine Colour Reagent. The plate was then incubated for 1 minute, and 'initial' absorbance was read at 490 nm with an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The plate was then incubated for seven minutes at room temperature and 'final' absorbance was read at 490 nm. To determine urinary creatinine content, 'final absorbance' was subtracted from 'initial absorbance' for absorbance readings of both standards and samples. A standard curve was generated and sample plasma creatinine content was calculated.

*2.8.2 TGF-β1 Plasma Analysis*

TGF-β1 of plasma samples were quantified using the TGF-β1 $E_{max}$® Immunoassay System (Promega, Madison, WI). In order to quantify the bioactive form of TGF-β1, samples underwent 'acid treatment', according to manufacturer's instructions. Plasma samples were diluted 1:5 using Dulbecco's PBS (containing 2.7 mM KCl, 137 mM NaCl, 1.5 mM $KH_2HPO_4$, 8 mM $Na_2HPO_4$, 0.004 mM $MgCl_2 \bullet 6H_20$, 0.009 mM $CaCl_2 \bullet 2H_20$, pH 7.35) and samples were acidified to pH 3.0 using 1 M HCl and incubated for 15 minutes at room temperature. Following the incubation period, samples were neutralised using 1 M NaOH and brought to pH 7.6 Acid treated samples were stored at -20℃ until analysis. To perform the TGF-β1 assay, 96 well microplates (Greiner-Bio One, Germany) were pre-coated with TGF-β1 Coat antibody diluted 1:2 in carbonate coating buffer (containing 25 mM sodium bicarbonate and 25 mM sodium carbonate, pH 9.7) and incubated at 4℃ overnight. Following the incubation period, the plate was brought to room temperature, TGF-β1 coating antibody was removed and the plate was blocked for 35 minutes at 37℃ with 1 x TGF-β1 block buffer, prepared with MilliQ water. During the blocking incubation period, eight TGF-β1 standards (15.6, 31, 62, 125, 250, 500 and 1000 pg/ml) were prepared via serial dilution with 1 x sample buffer. Acidified samples were diluted with 1 x sample buffer to achieve a final dilution of 1:150. Following blocking incubation of the plate, the blocking buffer was removed and the plate washed five times with 1 x TBST wash buffer (containing 20 mM Tris-HCl, 150 mM NaCl and 0.05% vol/vol Tween20). 100 µl of standard or diluted sample were added in triplicate to each well and was then incubated for 90 minutes at room temperature with orbital agitation. Following incubation, samples and standards were aspirated

from each well, the plate was washed five times as described above, and 100 µl of Anti-TGF-β1 antibody was added to each well. The plate was incubated for 2 hours at room temperature on an orbital shaker. The plate was then washed five times, and 100 µl of 1 x TGF-β1 HRP conjugate, prepared with 1 x sample buffer was added to each well and incubated for two hours at room temperature with gentle agitation. The plate was washed five times and 100 µl of TMB One Solution was added for colour development to each well and incubated at room temperature for 15 minutes. A 'stop' solution of 1 M HCl, prepared with MilliQ water was then added to a volume of 100 µl to each well and absorbance was read at 450 nm with an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). A standard curve was prepared and TGF-β1 content of plasma samples was calculated.

## ***2.9 Creatinine Clearance***

As a measure of renal function, creatinine clearance was calculated using urinary and plasma creatinine measurements outlined in Sections 2.5.6, 2.7.3 and 2.8.1. Creatinine clearance (ml/min) was calculated using a previously published formula for creatinine clearance in Sprague Dawley rats (Keenan et al., 2000):

*[Urinary vol (ml/min) x Urinary Creatinine Concentration (mg/dl)] / Plasma Creatinine (mg/dl)*

Creatinine clearance was then adjusted for body weight of each animal to determine relative creatinine clearance for each animal (ml/min/kg body weight) (Keenan et al., 2000).

## ***2.10 Quantification of Results and Statistical Analysis***

All statistical analysis was conducted using PASW statistics version 18 (SPSS Inc., Chicago, USA). All data is reported as mean ± SEM. Independent two tailed t-tests or analysis of variance (ANOVA) with Tukey's post-hoc test were performed on the samples to determine between group differences. Mixed model ANOVA analysis was used to determine differences between pre- and post-treatment measurements in the *in vivo* studies. Statistical significance was accepted at $p < 0.05$.

# Chapter 3 – Elevated CB1 and GPR55 cannabinoid receptor expression in proximal tubule cells and whole kidney exposed to diabetic conditions.

## *3.1 Summary*

Hyperglycaemia has been implicated in the etiology of diabetic nephropathy, which typically leads to elevated albumin levels in the filtrate. Diabetes mellitus has been shown to induce up regulation of the CB1 receptor and GPR55 in a tissue specific manner. To date, there has been little investigation into changes in the expression of the CB1 and GPR55 receptors in the proximal tubule under diabetic conditions. We investigated if elevated albumin and glucose alter tubular levels of CB1 and GPR55 across multiple time points in HK2 cells. Further, we characterised whole kidney protein expression of these receptors in diabetic animals. We found that CB1 protein expression was significantly higher in whole kidney tissue of diabetic rats compared to non-diabetic animals. Specifically in proximal tubule cells, exposure to high albumin levels alone and in combination with high glucose media increased CB1 expression *in vitro.* GPR55 expression was not altered in whole kidney of diabetic animals, but was significantly elevated in proximal tubule cells *in vitro* in response to elevated glucose alone and in combination with albumin. We have demonstrated that *in vivo* the CB1 receptor is upregulated in whole kidney of diabetic animals. Further an *in vitro* model of diabetes lead to alterations in the level of CB1 and GPR55 expression specifically within proximal tubule cells.

## ***3.2 Background***

The link between diabetes and kidney disease has been long established (Rossing et al., 2004, Wolf and Ziyadeh, 1999). It has become increasingly important to investigate physiological targets which may be useful in safely reducing the impact diabetes has on the pathophysiology of the kidneys. One target currently being investigated is the endocannabinoid system. It has been demonstrated that regulation of cannabinoid receptor expression and their endogenous ligands becomes disrupted in response to metabolic perturbations associated with T2DM (including hyperglycaemia and hyperlipidaemia) (Jagerovic et al., 2008, Janiak et al., 2007, Di Marzo, 2009).

Prolonged activation of CB1 receptor elicits detrimental effects on systemic metabolic homeostasis derived from both central and peripheral signalling consequences of the receptor (Di Marzo, 2008, O'Keefe et al., 2013). Using diabetic animal models, and *in vitro* techniques it has been shown that the receptor is upregulated in kidney tissue exposed to elevated glucose levels (Barutta et al., 2010, Nam et al., 2012). Physiologically, CB1 receptor blockade in diabetic models ameliorates the progression of diabetic nephropathy along with improvements in other metabolic parameters such as weight and survival rates (Barutta et al., 2010, Giuffrida et al., 2001, Janiak et al., 2007, Nam et al., 2012, Silvestri and Di Marzo, 2012). Although the receptor has been identified in both glomerular and tubular cells, the protective renal effects of CB1 blockade in diabetes has largely been attributed to CB1 localisation within podocyte cells of the glomerulus (Barutta et al., 2010, Nam et al., 2012). Structural modifications to the tubules are also mechanistically

important to the pathophysiological changes that occur during diabetic nephropathy however, we still don't fully understand how CB1 expression may be modified in a diabetic environment in this cell type.

GPR55, a putative cannabinoid receptor, shows a similar pattern of expression throughout central and peripheral tissues comparable to what is observed with the CB1 receptor and can be activated by some of the same compounds as CB1 (Kargl et al., 2012). While our current understanding of GPR55 in diabetes is quite limited, a handful of studies indicate that expression of this receptor may be mediated by diabetic conditions. In humans, GPR55 expression was seen to be significantly elevated in the adipose tissue of obese compared to lean individuals, with further elevations in expression observed in obese patients with diabetes and impaired glucose tolerance compared to obese normo-glycaemic individuals (Moreno-Navarrete et al., 2012). It was also demonstrated that modification to GPR55 expression was found to be tissue specific, with GPR55 expression in liver tissue not affected by an individual's diabetic status (Moreno-Navarrete et al., 2012). Our group has previously established that GPR55 is expressed in renal proximal tubule cells (Jenkin et al., 2010), however expression of this receptor in renal tissue exposed to the diabetic milieu is yet to be elucidated. The aim of this study was to therefore examine how expression of CB1 and GPR55 are affected in an *in vivo* and *in vitro* model of diabetes in proximal tubule cells and whole kidney.

## ***3.3 Materials and Methods***

### *3.3.1 Diabetic Kidney*

Six-week old male Sprague–Dawley rats were randomised to receive either 55 mg/kg of STZ (Sigma, USA) diluted in 0.1 M citrate buffer pH 4.5 or citrate buffer (control) by tail vein injection following an overnight fast (n = 8 per group). 16 weeks after STZ treatment animals were killed and kidney tissues harvested. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Research Ethics Committee of St. Vincent's Hospital, as outlined in Section 2.4.1.

### *3.3.2 Cell Culture*

HK2 cells were maintained as outlined in Section 2.1. Cells were grown to 80% confluence, followed by 24 hour incubation in 5 mM glucose DMEM/F12 media without FBS (control). Cells were then treated for 4, 6, 18 and 24 hours with control media or high glucose (25 mM), high albumin media (1 mg/ml) or both high glucose (25 mM) and high albumin (1 mg/ml).

### *3.3.3 'Real-time' PCR*

Following treatment, mRNA was extracted from HK2 cells and 'real-time' PCR analysis was performed. CB1 (Accession number NM_016083.4) was amplified with forward (5'-3' *GCCTTGCAGATACCACCTTC*) and reverse (5'-3' *GTTCTCCCCACACTGGATGT*) primers. GPR55 (Accession number NM_005683.3) was amplified using forward (5'-3' *CCCATTCAAGATGGTCCTGT*) and reverse (5'-3'

*TTCCGTACATGCTGACGAAG*) primers. Selective gene homology for genes of interest was confirmed by BLAST. To compensate for variations in RNA input amounts and reverse transcriptase efficiency, GAPDH (Accession number NM_002046.3) was used as a housekeeping gene, with forward (5'-3' *CAACGACCACTTTGTCAAGC*) and reverse (5'-3' *TTACTCCTTGGAGGCCATGT*). Relative changes in mRNA abundance was normalised to housekeeping GAPDH gene and then quantified using the $2^{-\Delta\Delta CT}$ method. Treatment groups were expressed relative to control treatment. $C_T$ values for GAPDH were not altered by treatments.

*3.3.4 Western Blot Analysis*

HK2 cells were lysed and analyzed via Western blot using previously described methods (Jenkin et al., 2010) and outlined in Section 2.3.1 and 2.3.3. In addition, kidney lysate from STZ rats was homogenised in HNT buffer (containing 20 mM HEPES, 120 mM NaCl, 0.6% Triton-X, 5 mM EDTA and protease inhibitor cocktail). 40 µg of protein from HK2 lysate and 100 µg of whole kidney lysate were analyzed via Western blot. A polyclonal antibody for CB1 was purchased from Cayman Chemicals (Ann Arbour, MI) and polyclonal antibody for GPR55 was purchased from Novus Biologicals (Littleton, CO). Secondary antibodies anti-mouse and anti-rabbit were purchased from Sigma Aldrich (St Louis, MO). β-Actin was used as loading control (Sigma Aldrich, St Louis, MO) control.

## ***3.4 Results***

### *3.4.1 Protein Expression of CB1 Receptor in Whole Kidney Lysate from Diabetic Rats*

Here we have demonstrated that in a similar diabetic animal model using Sprague Dawley rats, a similar pattern for CB1 expression is seen (Figure 3.1). Whole kidney lysate analyzed for CB1 expression using immunoblotting techniques showed that diabetic STZ animals had a 2.3 ± 0.37 (n = 8) times higher expression of the receptor relative to non-diabetic control animals (1 ± 0.29, n = 8).

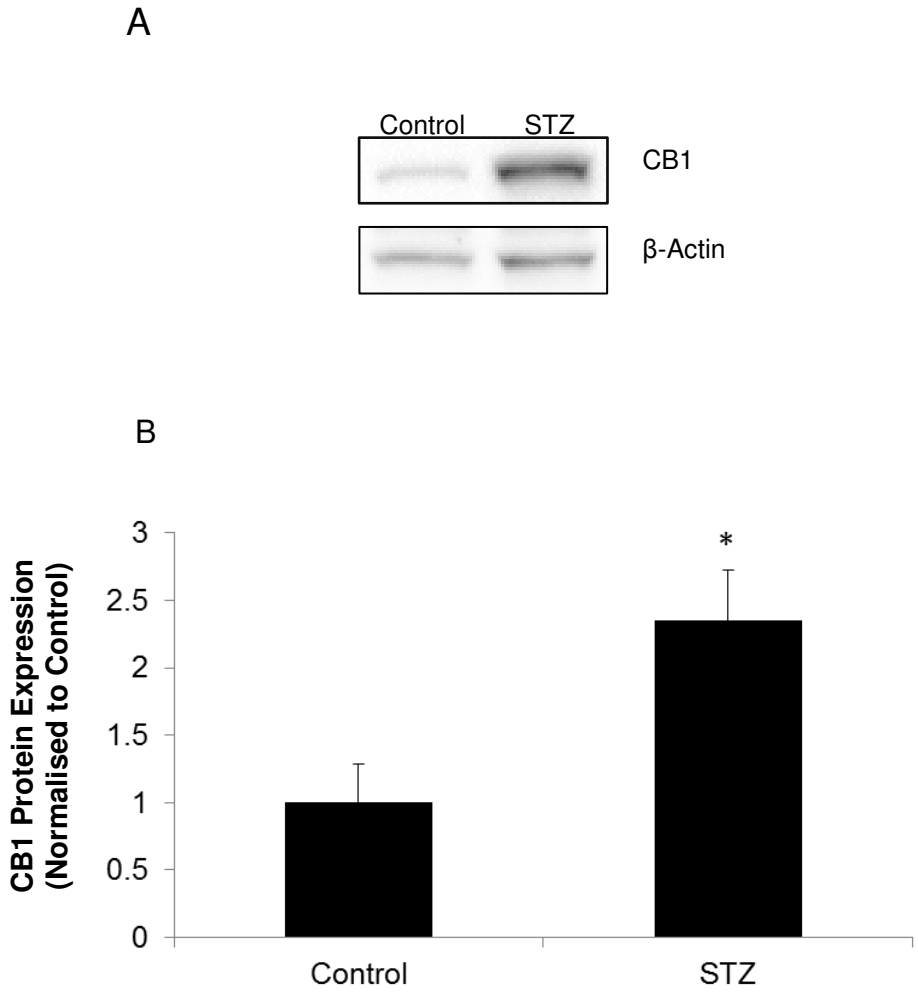



**Figure 3.1: Expression of CB1 in whole kidney lysate of diabetic and non-diabetic animals. A.** Representative Western Blot of CB1 receptor expression in kidney tissue of control animals and diabetic STZ animals. β-Actin was used as loading control. **B.** CB1 receptor expression in whole kidney lysate was significantly increased in diabetic STZ treated animals compared to control. Data expressed as normalised to control animals, mean ± SEM. Significance is indicated by * ($p < 0.05$, $n = 8$).

*3.4.2 mRNA Expression of CB1 Receptor in Proximal Tubule Cells in vitro*

'Real-time' PCR analysis was used to determine if mRNA of the CB1 receptor is altered in proximal tubule cells exposed to the diabetic milieu. Across 4, 18 and 24 hour time points, it was shown that treatment with high glucose and albumin alone or in combination did not have a significant effect on CB1 mRNA compared to control treatment (see Figure 3.2, n = 9). However, treatment for 6 hours with high glucose and albumin together exhibited significantly higher (1.88 ± 0.31) CB1 receptor expression relative to control treatment (1 ± 0.15, $p > 0.05$, n = 9, Figure 3.2).

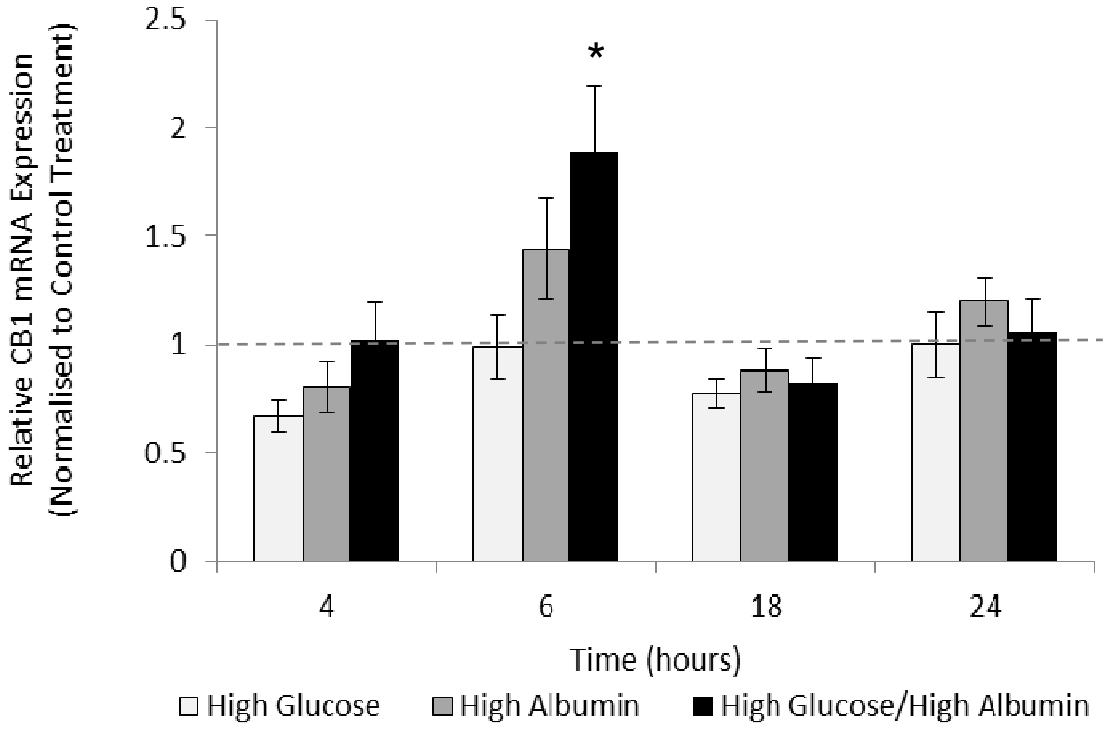



**Figure 3.2: 'Real-time' PCR analysis of proximal tubule expression of CB1 receptor in HK2 cells**. Cells treated with high glucose (25 mM), high albumin (1 mg/ml) or combination high glucose/ high albumin for 4, 6, 18 or 24 hours. Data expressed as normalised to control treatment (5 mM glucose, no albumin), mean ± SEM. Significance is indicated by * $p < 0.05$, $n = 9$.

### *3.4.3 Protein Expression of CB1 Receptor in Proximal Tubule Cells in vitro*

Western blot analysis was used to confirm that an alteration in protein expression of CB1 in proximal tubule cells is induced by high glucose and albumin environments. At 4 hours (Figure 3.3 A) there was a significant increase in CB1 protein expression for high albumin treatment (2.3 ± 0.06) and treatment with high glucose with albumin (1.5 ± 0.06), when normalised to control media treatment. At the 4 hour time period, there was no significant alteration in CB1 receptor expression in response to high glucose alone (1.0 ± 0.23). All other time intervals (6, 18 and 24 hours) glucose and albumin treatment alone or together showed no significant changes to the CB1 receptor compared to control media (Figure 3.3 B-D).

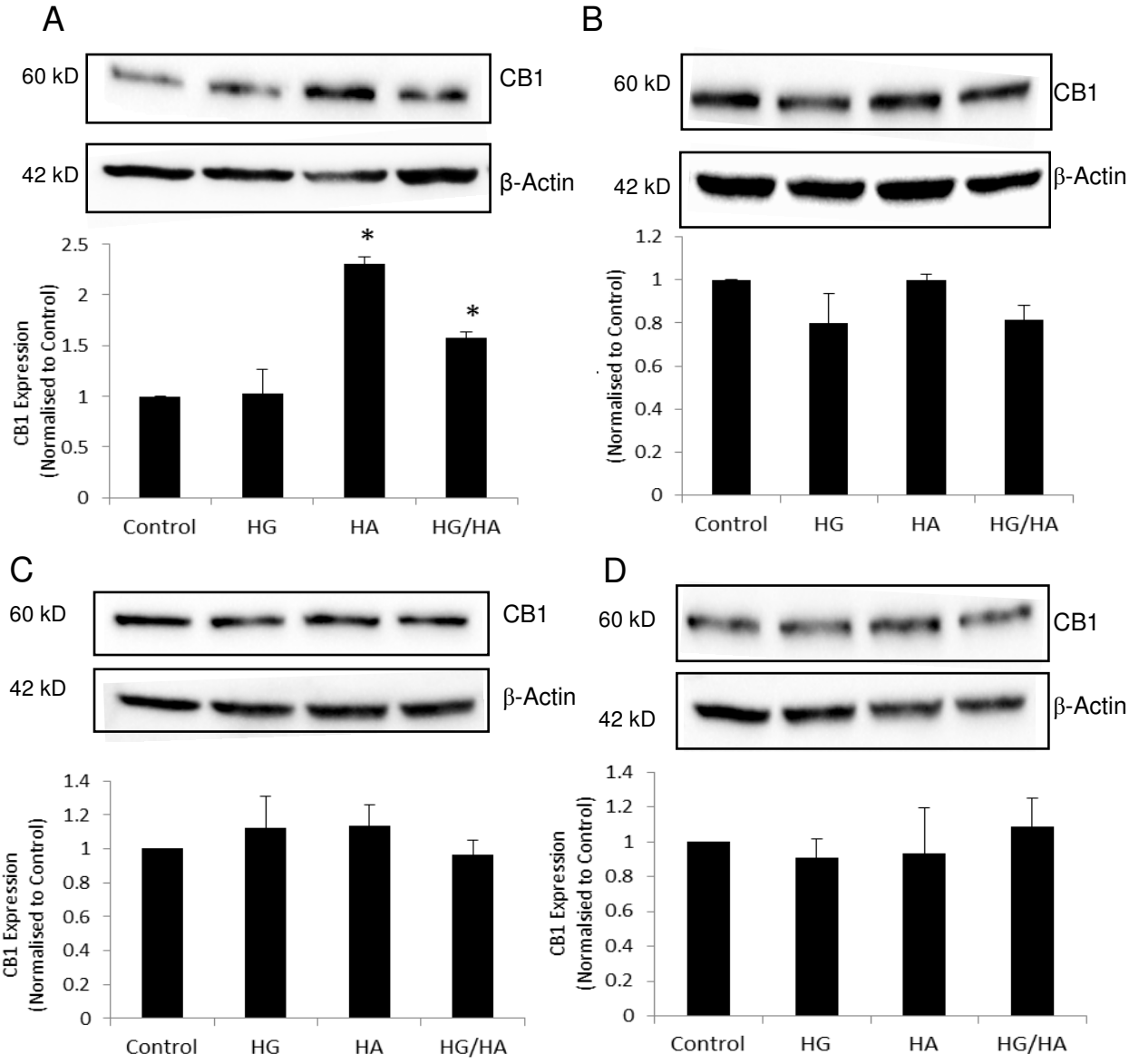



**Figure 3.3: Representative Western blot and quantification of CB1 receptor expression in HK2 cells**. Cells treated with high glucose (HG; 25 mM), high albumin (HA; 1 mg/ml) and a combination of high glucose/ high albumin (HG/HA). Protein abundance was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM. Significance is indicated by * $p < 0.05$ compared to control treatment (n = 3 – 4). β-Actin loading control was not altered by treatment. **A.** Treatment for 4 hours **B.** Treatment for 6 hours. **C.** Treatment for 18 hours. **D.** Treatment for 24 hours.

### *3.4.4 Protein Expression of GPR55 in Whole Kidney Lysate from Diabetic Rats*

Here we have demonstrated (Figure 3.4) that in an animal model of diabetes (n = 8), there were no significant differences in whole kidney tissue for GPR55 protein expression in diabetic rats (1.04 ± 0.09) relative to non-diabetic control animals (1.0 ± 0.07).

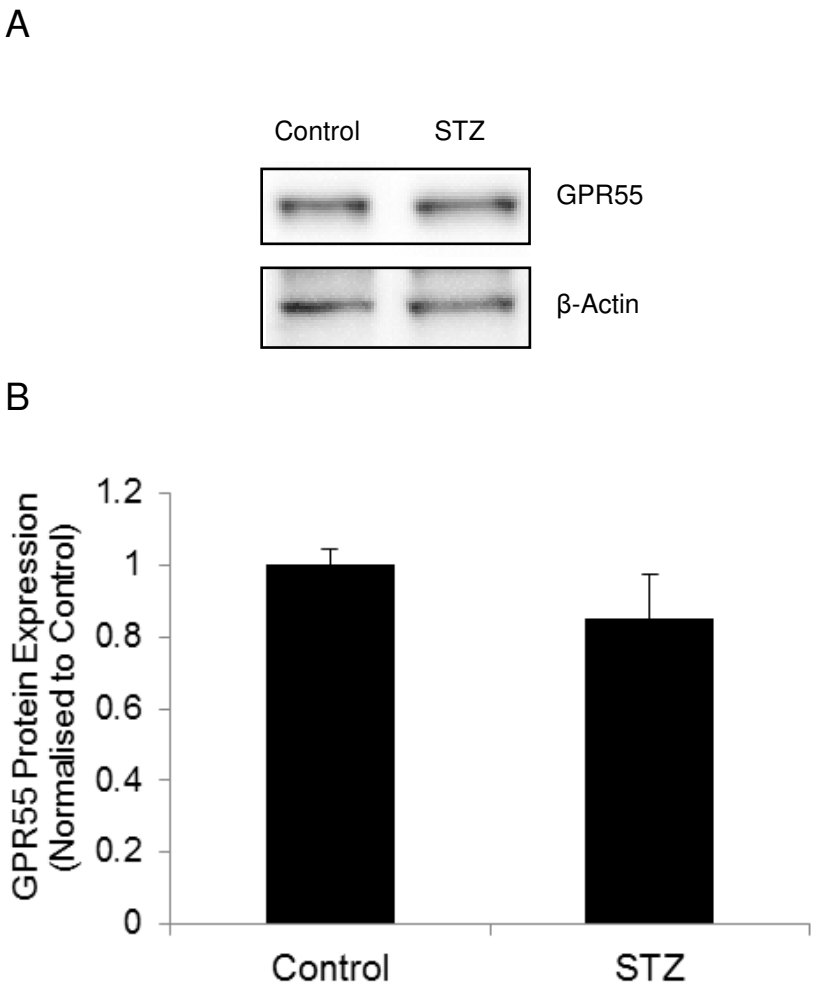



**Figure 3.4: Expression of GPR55 in whole kidney lysate of diabetic and non-diabetic animals.A.** Representative Western Blot of GPR55 expression in kidney tissue of control animals and diabetic STZ animals. β-Actin was used as loading control. **B.** GPR55 expression in whole kidney lysate from control and diabetic STZ treated animals, data expressed as mean ± SEM.

*3.4.5 mRNA Expression of GPR55 in Proximal Tubule Cells in vitro*

To further investigate whether renal tissue may contain specific cells in which GPR55 expression may be responsive to elevated glucose or albumin environments mimicking the diabetic milieu, 'real-time' PCR analysis was used to examine GPR55 mRNA expression in proximal tubule HK2 cells. Following 4 hours of treatment with high glucose and albumin, no significant changes to GPR55 expression was observed compared to control media (Figure 3.5, n = 9). Indeed, at any time interval, high albumin alone does not appear to have any impact on GPR55 expression in proximal tubule cells. The later time points of 6, 18 and 24 hours showed that expression of GPR55 is continually elevated in response in high glucose alone and in combination with high albumin compared to control treatment. Significantly higher mRNA expression of GPR55 at the 24 hour time point was seen with high glucose conditions (1.56 ± 0.08) compared to control media treatment (1.0 ± 0.12). Significant increases in GPR55 mRNA expression were also observed with treatment of high glucose with high albumin for 6 hours (1.76 ± 0.28) and 24 hours (1.58 ± 0.09) compared to control. There was a trend for increased expression of GPR55 with 18 hours treatment with high glucose and high albumin (1.51 ± 0.22), however differences between groups were not statistically significant when compared to control treatment (1.0 ± 0.12, p = 0.052).

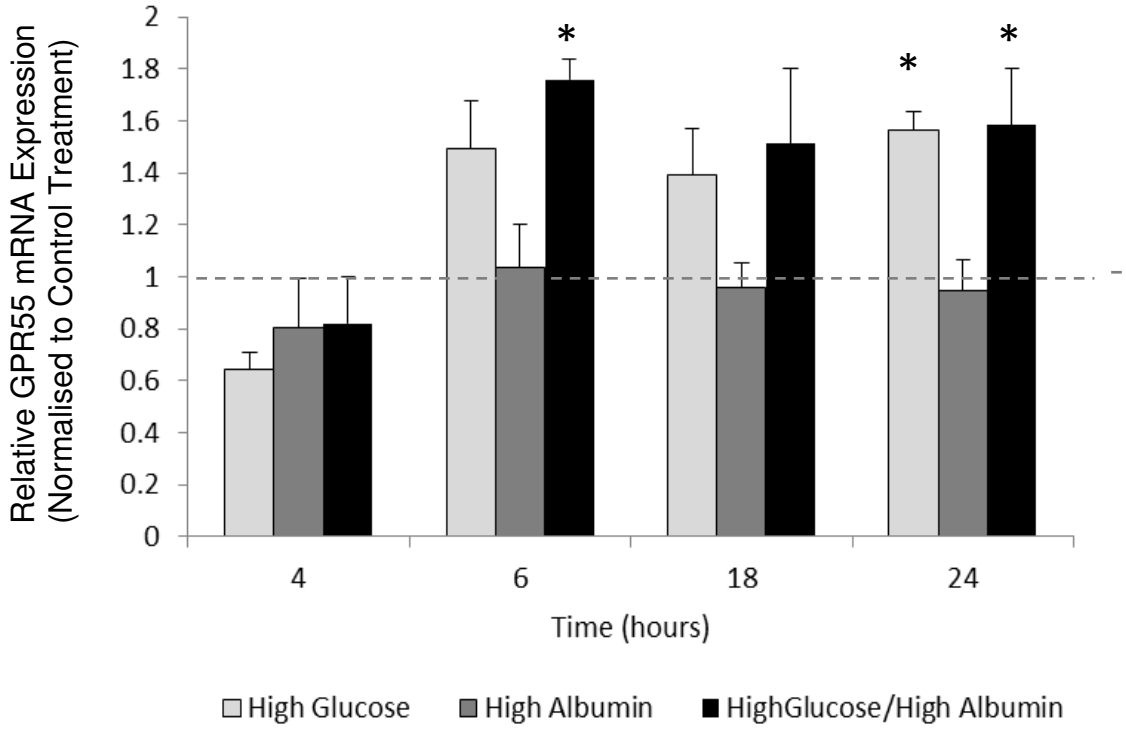



**Figure 3.5: 'Real-time' PCR analysis of proximal tubule expression of GPR55 in HK2 cells**. Cells treated with high glucose (25 mM), high albumin (1 mg/ml) and combination of high glucose/ high albumin for 4, 6, 18 or 24 hours. GAPDH was used as housekeeping gene. Data expressed as normalised to control treatment (5 mM glucose, no albumin), mean ± SEM. Significance is indicated by * $p < 0.05$ compared to control treatment, n = 9.

### *3.4.6 Protein Expression of GPR55 in Proximal Tubule Cells in vitro*

In addition to 'real-time' PCR quantification of GPR55 mRNA, Western blot analysis was used to confirm that protein for GPR55 is upregulated in proximal tubule cells exposed to a high glucose environment. Figure 3.6 demonstrates that the pattern of increased GPR55 expression in high glucose treatments (either alone or with albumin) compared to control, were similar to the mRNA findings seen in Figure 3.5. However only at the 6 hour time period high glucose treatment showed a significant increase (1.78 ± 0.31) in GPR55 protein expression relative to control.

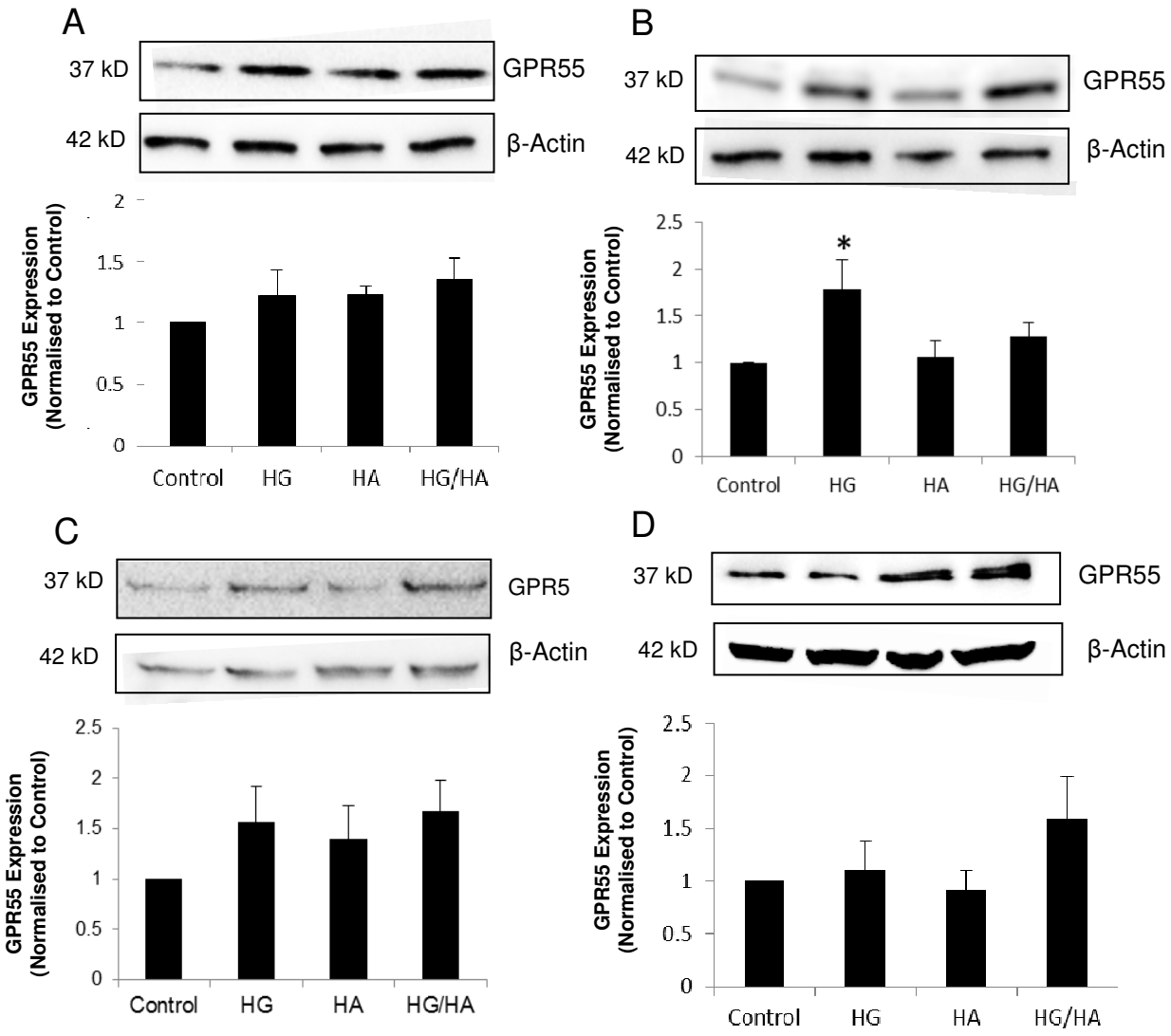



**Figure 3.6: Representative Western blot and quantification of GPR55 expression in HK2 cells**. Cells treated with high glucose (HG; 25 mM), high albumin (HA; 1 mg/ml) and a combination of high glucose/ high albumin (HG/HA). Protein abundance was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM. Significance is indicated by * $p < 0.05$, n=3 compared to control treatment. β-Actin loading control was not altered by treatment **A.** Treatment for 4 hours **B.** Treatment for 6 hours. **C.** Treatment for 18 hours. **D.** Treatment for 24 hours.

## ***3.5 Discussion***

Increases in CB1 and GPR55 expression in response to T2DM have previously been noted in a range of tissues (Lafontan et al., 2007, Moreno-Navarrete et al., 2012, Zhang et al., 2007). The positive effects of CB1 blockade on peripheral metabolism has been extensively reported in both human and animal models of obesity and diabetes (Isoldi and Aronne, 2008, Janiak et al., 2007, Silvestri and Di Marzo, 2012, Croci et al., 2003, Dol-Gleizes et al., 2009, Gary-Bobo et al., 2006). Rimonabant was the first selective compound made available to treat obesity related metabolic disruptions by blocking the actions of the CB1 receptor; however adverse centrally mediated side effects resulted in the compound being withdrawn from the market (O'Keefe et al., 2013). Currently, research is looking to find 'second generation' CB1 selective compounds which act peripherally, in order to attain the beneficial effects of CB1 antagonism without the adverse effects on mood (O'Keefe et al., 2013, Silvestri and Di Marzo, 2012). It is important to consider that given the varied tissue specific expression of CB1, signalling of the receptor in peripheral tissues is likely to activate organ specific signalling pathways.

If further research is conducted regarding whether the CB1 receptor may be a viable physiological target for the treatment of diabetic nephropathy, it is crucial to first understand how CB1 expression is modified in renal cells exposed to the diabetic milieu. One of the first studies to examine the role of the cannabinoid receptors in the kidneys demonstrated that the CB1 receptor is expressed in renal cells, particularly in the glomeruli and to a lesser extent, tubular cells (Janiak et al., 2007). Histologically, it has been shown that there is a 1.5 fold increase in staining for the

CB1 receptor in C57BL6/J mice made diabetic with STZ compared to control treated animals (Barutta et al., 2010). Specifically within the realm of renal physiology, the action of blocking the activity of the CB1 receptor seems to have protective effects in combating obesity and diabetic related renal damage, through the amelioration of proteinuria, albuminuria, improved creatinine clearance and reduced loss of podocyte cells (Barutta et al., 2010, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Nam et al., 2012). Currently, it has been postulated that the beneficial effects of blocking CB1 actions in the kidneys arise from actions of the receptor solely localised within cells of the glomerulus (Barutta et al., 2010, Janiak et al., 2007). However, it is the degree of tubular and interstitial damage which most closely correlates with the progression of diabetic nephropathy, and yet expression and function of the CB1 receptor within these cells have largely been overlooked. We have previously established a functional role for CB1 in proximal tubular cells, where activation with the endogenous agonist AEA lead to tubular hypertrophy and blockade of CB1 with antagonist AM251 saw a significant reduction in hypertrophy (Jenkin et al., 2010). Tubular hypertrophy is one of the very first structural changes proximal tubule cells undergo in nephropathy, as a result of hyperfiltration (Vallon, 2011b), and yet CB1 expression and function in these cells are continually overlooked in studies examining the role of the endocannabinoid system in the kidneys. Here we have clearly shown that *in vitro,* when proximal tubule cells are exposed to pathophysiological levels of albumin alone, or with elevated glucose, CB1 receptor expression is significantly elevated in these cells. We have also established that CB1 has over a two fold increase in whole kidney tissue of diabetic

compared to non-diabetic animals, congruent with previous findings (Barutta et al., 2010, Janiak et al., 2007).

Currently there is very limited research examining the role of GPR55 in T2DM, and to date, the role of this receptor has not been examined at all in relation to diabetic nephropathy. In humans, GPR55 expression was recently reported to be significantly elevated in the adipose tissue of obese compared to lean individuals, with further elevations in expression observed in obese patients with diabetes and impaired glucose tolerance compared to obese normo-glycaemic individuals (Moreno-Navarrete et al., 2012). Interestingly, it has been demonstrated that GPR55 is also expressed in pancreatic islet cells, particularly in insulin producing beta cells (McKillop et al., 2013, Romero-Zerbo et al., 2011) Activation of this receptor induces a strong response towards insulin release in mice, and has been shown to increase both glucose tolerance and plasma insulin levels (McKillop et al., 2013, Romero-Zerbo et al., 2011). These data taken together make it difficult to determine whether this receptor may be a beneficial target in the treatment of diabetic nephropathy. We have previously demonstrated that the receptor is expressed in human proximal tubule cells, but it does not mediate hypertrophic processes that CB1 and CB2 both appear to regulate (Jenkin et al., 2010). Here, we have demonstrated that although GPR55 is significantly upregulated in proximal tubule cells exposed to high glucose conditions alone and in combination with elevated albumin, in whole kidney tissue of diabetic animals there is no change in expression.

There are potentially a few reasons which may be contributing to these disparate *in vivo* and *in vitro* results. Firstly, it is clear from past studies that GPR55 is expressed

differentially in both central and peripheral tissues (Johns et al., 2007, McKillop et al., 2013, Moreno-Navarrete et al., 2012, Romero-Zerbo et al., 2011, Schicho and Storr, 2012, Staton et al., 2008). Whole kidney tissue contains within it a variety of glomerular, tubular, neuronal and endothelial cells. In diabetic nephropathy, advancement of renal damage can lead to an increase in infiltrating macrophages, T-lymphocytes and the subpopulation of regulatory T cells which leads to inflammation of renal tissue (Lim and Tesch, 2012). The variety of cell types in whole kidney lysate and various expression of GPR55 within these cells may impact the overall expression of GPR55 within whole kidney tissue. Secondly, GPR55 has recently been demonstrated to form heteromers with other GPCR, including CB1, which can ultimately affect function and regulation of the expression of these receptors (Kargl et al., 2012). Further investigation is required to elucidate the factors governing GPR55 expression in whole kidney tissue however; we have established that specifically within proximal tubule cells exposed to high glucose conditions alone or in combination with elevated levels of albumin, GPR55 expression is significantly increased.

## ***3.6 Conclusion***

We have demonstrated that *in vivo* the CB1 receptor is upregulated in whole kidney of diabetic male Sprague Dawley rats. Further, an *in vitro* model of diabetic nephropathy leads to increases to both CB1 and GPR55 receptor expression specifically within proximal tubule cells. If the endocannabinoid system is a viable physiological target for the treatment of diabetic nephropathy, it is crucial to first understand how CB1 and GPR55 receptor expression in the kidneys are modulated

in response to diabetes both on a whole organ system basis and at a cellular level. Here we have demonstrated that both CB1 and GPR55 are significantly increased in the renal system under diabetic conditions. Additional investigation may indicate that these receptors may provide useful physiological targets for the treatment and prevention of diabetic nephropathy

# Chapter 4 - Cannabinoid receptor 2 expression in human proximal tubule cells is regulated by albumin

## *4.1 Summary*

Expression of CB2 has been shown to be reduced in podocytes of animals and humans with T2DM, with activation of CB2 ameliorating albuminuria. We investigated if elevated albumin and glucose alter tubular levels of CB2. Further, we characterised total CB2 protein in diabetic animals. There were no changes to CB2 expression in total kidney lysate from diabetic rats. In proximal tubule HK2 cells, expression of CB2 was unaltered in cells exposed to high glucose. Conversely, high albumin treatment alone and in combination with high glucose, resulted in a significant reduction in CB2 receptor mRNA expression at 6 and 18 hours. CB2 protein expression was also reduced at 6 and 24 hours, in high albumin and in combination with high glucose. We have demonstrated that elevated levels of albumin in the presence and absence of glucose significantly reduces CB2 mRNA and protein expression in proximal tubules *in vitro*. Consequently, altered expression of CB2 in both the podocytes and tubules may contribute to the albuminuria observed in T2DM.

This chapter has been published largely in the manuscript:

JENKIN, K.A, MCAINCH, A.J, BRIFFA, J.F, ZHANG, Y., KELLY, D.J, POLLOCK, C.A, PORONNIK, P. & HRYCIW, D.H 2013. Cannabinoid Receptor 2 Expression in Human Proximal Tubule Cells is Regulated by Albumin Independent of ERK1/2 Signaling. *Cellular Physiology and Biochemistry,* 32**,** 1309-1319.

## ***4.2 Background***

Diabetic nephropathy increases the risk of developing end stage renal failure (Rossing et al., 2004), with a number of molecular targets identified as playing a role in diabetic pathophysiology. Recent studies focused on the CB2 receptor, which is expressed in kidneys tissue (Pacher and Mechoulam, 2011). In humans with diabetic nephropathy, CB2 expression is reduced in glomerular podocytes and the endocannabinoid agonist 2-AG is reduced in the kidney (Barutta et al., 2011). Further, in a diabetic animal model, CB2 protein expression is reduced in podocyte cells of the glomerulus (Barutta et al., 2011). However interestingly, the level of CB2 mRNA in diabetic whole kidneys is unaltered compared to control (Barutta et al., 2011), perhaps due to recruitment of inflammatory cells in renal tissue. However, expression of CB2 specifically in proximal tubule cells exposed to hyperglycemic conditions is unknown.

Altered expression of CB2 mRNA and protein in podocytes is important in the regulation of the normal physiological processes in the glomerulus. It is important to note that albuminuria is the result of both glomerular and proximal tubule dysfunction *in vivo* and proximal tubule cells exposed to higher levels of albumin in diabetic nephropathy and are responsible for its reabsorption from the filtrate via endocytosis (Christensen and Verroust, 2008). The link between activation of the receptor and altered albumin handling by the glomerulus has clearly been established; however the contribution of CB2 to tubular processing of albumin has not received much attention.

The serine/threonine kinase AKT protein has been identified as an important regulator of renal hypertrophy and apoptosis induced by hyperglycaemia (Rane et al., 2010). Downstream, phosphorylation of AKT leads to the activation of a signalling cascade which regulates cell proliferation, migration, differentiation and cell survival, known as the mitogen activated protein kinase (MAPK) pathway. The MAPK cascade subsumes several proteins, including p38 and ERK1/2 which have been demonstrated to be involved in the pathogenesis of diabetic nephropathy (Nishimoto and Nishida, 2006, Feliers and Kasinath, 2011, Rane et al., 2010). Activity of the CB2 receptor has been linked to the AKT/MAPK signalling pathway (Correa et al., 2009, Romero-Sandoval et al., 2009). *In vitro* CB2 activation results in reduced phosphorylated ERK1/2 protein expression (Romero-Sandoval et al., 2009) and conversely, CB2 antagonists can lead to greater and sustained activation of MAPK proteins (Correa et al., 2009), indicating that the mechanism of the CB2 receptor in diabetic nephropathy may mediate changes via the AKT/MAPK signalling pathway. The association between CB2 expression and AKT/MAPK has not been examined in the role of renal tubular damage in diabetic nephropathy.

In the early stages of diabetic nephropathy, downstream renal proximal tubule cells are exposed to high levels of glucose, with increases in hyperfiltration leading to elevated albumin and glucose in the filtrate and altered AKT/MAPK signalling (Phillips and Steadman, 2002, Feliers and Kasinath, 2011). Therefore, the objectives of this study were to determine: 1. expression of the CB2 receptor and its relationship with AKT/MAPK signalling in proximal tubule cells mimicking the diabetic phenotype, 2. determine if CB2 expression alters tubular processing of albumin and

if so, 3. characterise whether CB2 receptor expression in proximal tubule cells is dependent on albumin internalisation.

## ***4.3 Materials and Methods***

### *4.3.1 Diabetic Kidney*

Six-week old male Sprague Dawley rats were randomised to receive either 55 mg/kg of STZ (Sigma, USA) diluted in 0.1 M citrate buffer pH 4.5 or citrate buffer (control) by tail vein injection following an overnight fast. 16 weeks after STZ treatment animals were killed and kidney tissues harvested. Experimental procedures have been outlined in Section 2.4.1.

### *4.3.2 Cell Culture*

HK2 and OK cells were maintained as previously described in Section 2.1 (Jenkin et al., 2010). Cells were grown to 80% confluence and then incubated in 5 mM glucose DMEM/F12 media without FBS (control) for 24 hours. Cells were then treated for 4, 6, 18 and 24 hours with control media or high glucose (25 mM), high albumin media (1 mg/ml) or both high glucose (25 mM) and high albumin (1 mg/ml).

To determine if ERK1/2 mediates CB2 receptor expression, HK2 cells were treated for 6 hours with control, high glucose, high albumin or the combination high glucose and albumin media as described above with 10 µM ERK1/2 inhibitor U0126 (Cell Signaling Technology, Beverly, MA). Before experimental use, U0126 was dissolved in Dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) to make a 10 mM stock solution.

To determine whether the expression of CB2 is dependent on albumin internalisation, HK2 cells were exposed to latrunculin A to fragment the cytoskeleton and thus inhibit albumin endocytosis, according to previously described published methods with minor modifications (Hryciw et al., 2005). Briefly, HK2 cells were treated for six hours with 1.5 μM latrunculin A (Sigma Aldrich, St Louis, MO) with control, high glucose, high albumin or both high glucose and albumin media. Cells were then lysed and analyzed for CB2 receptor expression with Western blot analysis.

*4.3.3 'Real-time' PCR*

In HK2 cells, following treatment, mRNA was extracted and 'real-time' PCR performed as outlined in Section 2.2, using previously described methods (Jenkin et al., 2010). CB2 (Accession number NM_001841.2) was amplified with forward (5'-3' *ACTCCATGGTCAACCCTGTC*) and reverse (5'-3' *GATCTCGGGGCTTCTTCTTT*) primers. To compensate for variations in RNA input amounts and reverse transcriptase efficiency, GAPDH (Accession number NM_002046.3) was used as a housekeeping gene, with forward (5'-3' *CAACGACCACTTTGTCAAGC*) and reverse (5'-3' *TTACTCCTTGGAGGCCATGT*). Selective gene homology for genes of interest was confirmed by BLAST. Relative changes in mRNA abundance was normalised to housekeeping GAPDH gene and then quantified using the $2^{-\Delta\Delta CT}$ method. Treatment groups were expressed relative to control treatment. $C_T$ values for GAPDH were not altered by treatments.

*4.3.4 Western Blot Analysis*

HK2 cells were lysed and analyzed via Western blot as outlined in Section 2.3. In addition, kidney lysate from STZ rats was homogenised in HNT buffer (containing 20 mM HEPES, 120 mM NaCl, 0.6% Triton-X, 5 mM EDTA and protease inhibitor cocktail). 40 µg of protein from HK2 lysate and 100 µg of whole kidney lysate was analyzed via Western blot as described previously (Jenkin et al., 2010). A polyclonal antibody for CB2 was purchased from Cayman Chemicals (Ann Arbour, MI), antibodies for phospho AKT (Ser473), AKT, phospho p38 (Thr180/Tyr182), p38, phospho ERK1/2 (Thr202/Tyr204) and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Secondary antibodies anti-mouse and anti-rabbit were purchased from Sigma Aldrich (St Louis, MO). Protein concentration were normalised to β-Actin (Sigma Aldrich, St Louis, MO) control.

*4.3.5 Albumin Uptake*

Albumin uptake was performed in OK cells, a cell line shown to have express all constituents of the macromolecular complex required for tubular absorption of albumin via endocytosis (Amsellem et al., 2010, Birn and Christensen, 2006, Hryciw et al., 2012b). HK2 cells express a relatively low level of the transmembrane protein megalin, an important component of the complex, which reduces the capacity of this cell line to absorb albumin via receptor mediated processes (Sawada et al., 2012). To measure the effect of CB2 and MAPK regulation of albumin uptake, OK cells were exposed to albumin conjugated to Texas Red (50 µg/ml) in the presence of specific inhibitors for two hours at 37℃ as previously described (Hryciw et al., 2005) CB2 inhibitor AM630 (Cayman Chemical, Ann Arnour, MI) was used at 10 µM

concentrations. ERK1/2 inhibitor U0126 (10 mM) and p38 inhibitor SB203580 was purchased from Cell Signaling Technology (Beverly, MA). At the end of the albumin uptake period, cells were washed in HEPES buffer and lysed in MOPS buffer (20 mM MOPS, 0.1% Triton X-100, pH 7.4). TR-albumin florescence was determined using an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). at 580 nm excitation and 630 nm emission wavelengths. TR-albumin uptake was adjusted for background and standardised to treatment without the presence of inhibitors (control).

### *4.3.6 Statistical Analysis*

The SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group analysis or One-Way ANOVA with Tukey's post-hoc tests for analysis of groups greater than two. Significance was accepted when $p < 0.05$.

## ***4.4 Results***

### *4.4.1 Protein Expression of CB2 Receptor in Whole Kidney Lysate from Diabetic Rats*

Despite, a previous study demonstrating that podocyte levels of CB2 are reduced in animal and humans with T2DM (Barutta et al., 2011), in whole kidney lysate, there was no difference in CB2 receptor protein levels between STZ and control animals relative to the β-actin loading control (n = 8, Figure 4.1).

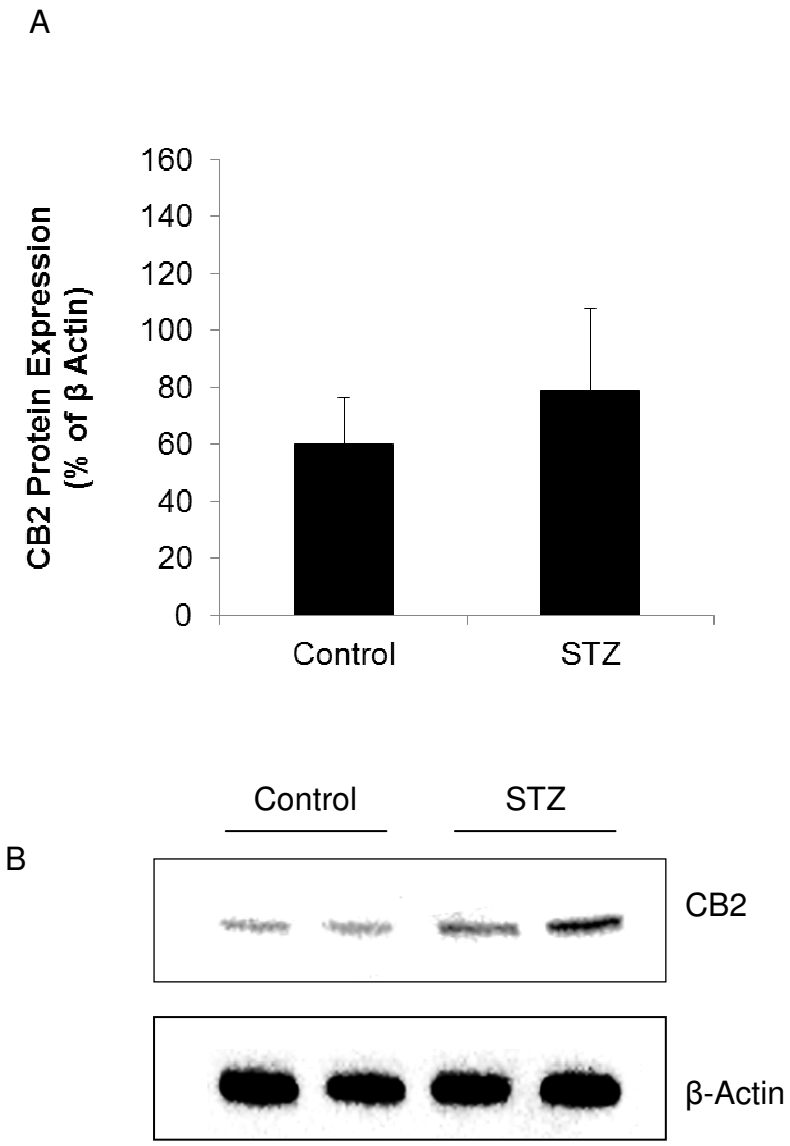



**Figure 4.1: Expression of CB2 in whole kidney lysate of diabetic and non-diabetic animals.** Quantification of the CB2 receptor expression in whole kidney lysate from control and diabetic STZ treated animals. Data was normalised to control animals and expressed as mean ± SEM (n = 8). **B.** Representative Western Blot of CB2 receptor expression in Control and STZ kidney lysate. β-Actin was used as loading control.

*4.4.2 mRNA Expression of CB2 Receptor in Proximal Tubule Cells in vitro*'Real-time' PCR analysis was used to determine if mRNA of the CB2 receptor is altered in proximal tubule cells exposed to the diabetic milieu. Following 4 and 24 hours exposure to the different treatments, CB2 mRNA expression was unaltered across treatment groups (n = 9). 'Real-time' PCR analysis showed that proximal tubule cells exposed to high albumin exhibited significantly lower CB2 receptor expression ($p < 0.05$) following 6 hours (0.38 ± 0.12) and 18 hours (0.45 ± 0.05) treatment periods (Figure 4.2A) compared to control treatment (1.00 ± 0.19) conditions (n = 9). Further, CB2 receptor expression was also significantly reduced ($p < 0.05$) when high albumin combined with high glucose treatment (0.40 ± 0.04) was applied for 18 hours (n = 9, Figure 4.2). High glucose treatment alone did not significantly alter CB2 receptor expression when compared to control treatment at any time interval.

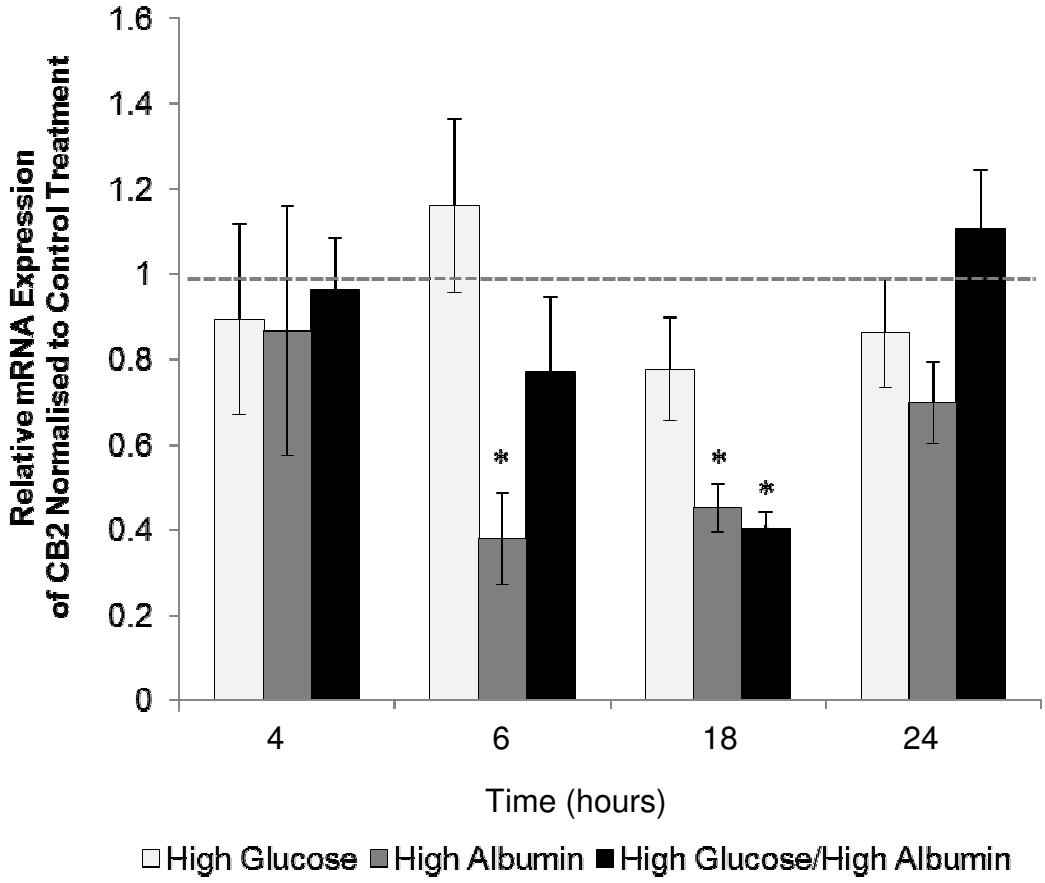



**Figure 4.2: 'Real-time' PCR analysis of proximal tubule expression of CB2 receptor in HK2 cells**. Cells treated with high glucose (25 mM), high albumin (1 mg/ml) and combination of high glucose/ high albumin for 4, 6, 18 or 24 hours. GAPDH was used as housekeeping gene. Data expressed as normalised to control treatment (5 mM glucose, no albumin), mean ± SEM. Significance is indicated by * $p < 0.05$ compared to control treatment (n = 9).

*4.4.3 Protein Expression of CB2 Receptor in Proximal Tubule Cells in vitro*

In addition to 'real-time' PCR quantification of CB2 mRNA, Western blot analysis was used to confirm alterations in protein expression of CB2. At 4 hours (Figure 4.3A) and 18 hours (Figure 4.3C) there was no significant change in CB2 receptor protein expression. Six hours high albumin treatment alone caused a significant reduction in CB2 receptor protein expression ($0.50 \pm 0.09$, $p < 0.05$, $n = 3$ Figure 4.3B) when compared to control treatment. The combination of high glucose and high albumin caused a significant reduction in CB2 protein expression following 6 and 24 hours treatment ($0.43 \pm 0.08$ and $0.42 \pm 0.05$, $p < 0.05$, $n = 3$ Figure 4.3B and 4.3D, respectively). High glucose conditions did not significantly alter CB2 expression in HK2 proximal tubule cells at any time interval.

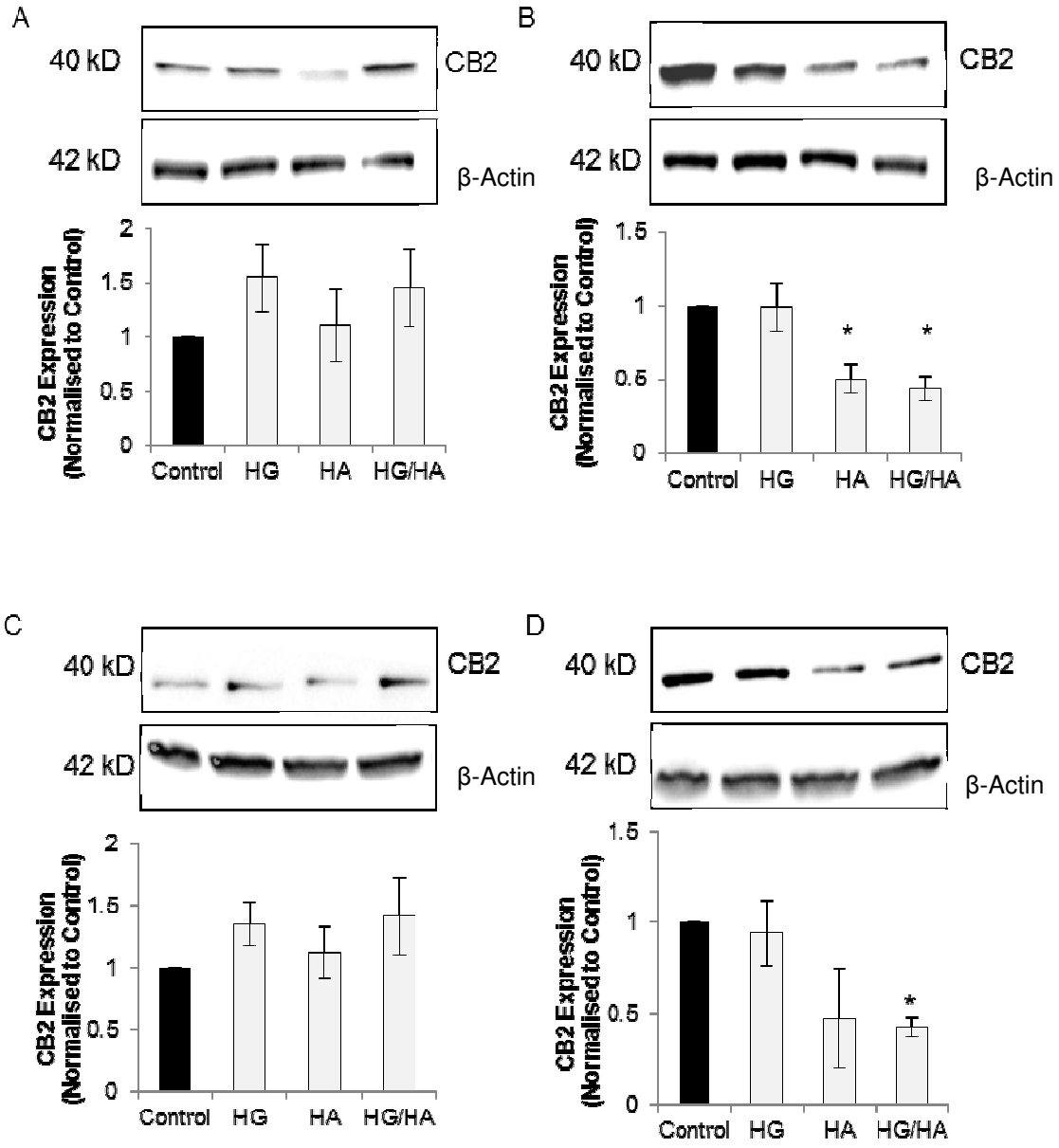
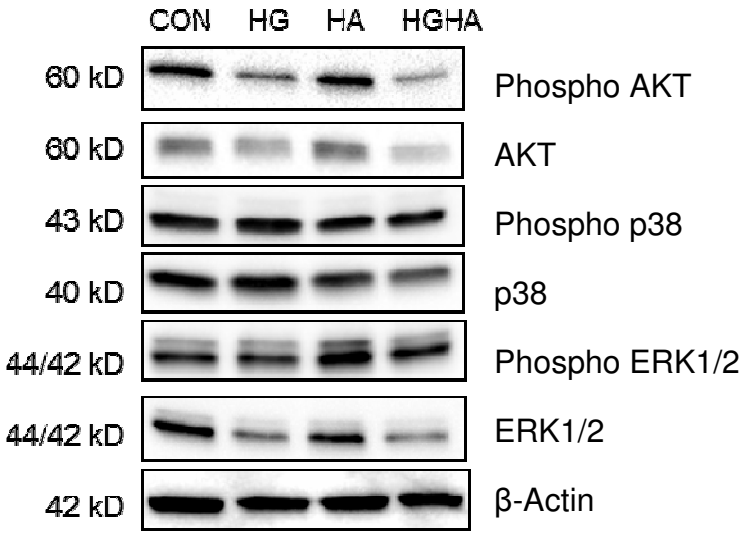



**Figure 4.3: Representative Western blot and quantification of CB2 receptor expression in HK2 cells.** Cells treated with high glucose (HG; 25 mM), high albumin (HA; 1 mg/ml) and a combination of high glucose/ high albumin (HG/HA). CB2 protein expression was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM, n = 3. β-Actin loading control was not altered by treatment Significance is indicated by * $p < 0.05$ compared to control treatment. **A.** Treatment for 4 hours **B.** Treatment for 6 hours. **C.** Treatment for 18 hours. **D.** Treatment for 24 hours.

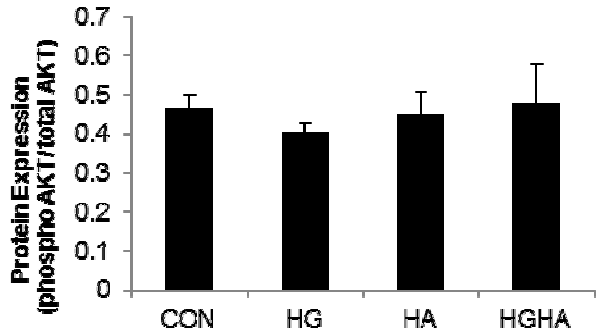
*4.4.4 Alterations in AKT/MAPK Signalling Proteins in Proximal Tubule Cells*

To investigate whether changes in CB2 receptor expression corresponds with changes to AKT/MAPK signalling alterations, HK2 cells were treated for six hours with high glucose or high albumin media. It was found that treatments did not alter AKT phosphorylation profile for any treatment group, while high glucose combined with high albumin led to a significant increase in phosphorylation of p38 ($p < 0.05$, $n = 3$, Figure 4.4). Alterations in phosphorylation of ERK1/2 was significantly increased in HK2 cells treated with both high albumin alone and in combination with high glucose ($p < 0.05$, $n = 3$, Figure 4.4). This data highlights that changes to ERK1/2 proteins reflected alterations seen in CB2 receptor expression, which had reduced expression in these treatment groups with 6 hour time point.

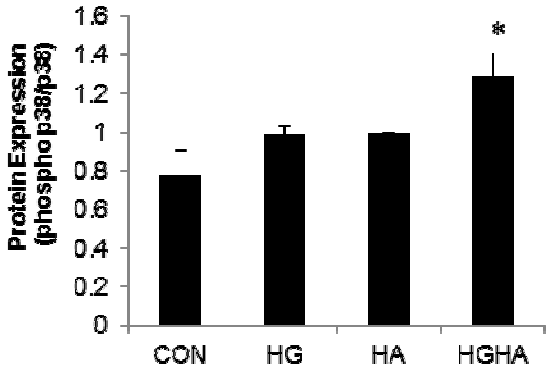
A


CON
HG
HA
HGHA
60 kD
Phospho AKT
60 kD
AKT
43 kD
Phospho p38
40 kD
p38
44/42 kD
Phospho ERK1/2
44/42 kD
ERK1/2
42 kD
β-Actin

B


Protein Expression (phospho AKT/total AKT)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CON
HG
HA
HGHA

C


Protein Expression (phospho p38/p38)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
CON
HG
HA
HGHA

D

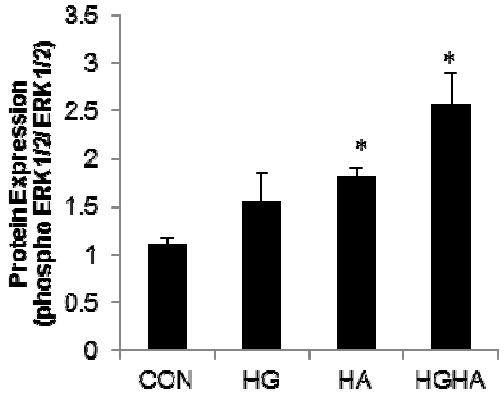

Protein Expression (phospho ERK1/2/ERK1/2)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
CON
HG
HA
HGHA

**Figure 4.4: Characterisation of HK2 levels of active and total AKT, ERK1/2 and p38 in response to 6 hour treatment with glucose and albumin** Protein abundance was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM. β-Actin loading control was not altered by treatment **A.** Representative Western blot of phosphor AKT, AKT, phosphor p38, p38, phosphor ERK1/2 and ERK1/2 **B.** Quantification of phosphor AKT/AKT normalised to control **C.** Quantification of phosphor p38/p38 normalised to control **D.** Quantification of phosphor ERK1/2/ ERK1/2 normalised to control. Data expressed as mean ± SEM. Significance is indicated by * $p < 0.05$ compared to control treatment ($n = 3$).

*4.4.5 CB2 Receptor Expression is Regulated by Albumin Internalisation*

To determine whether CB2 receptor expression is mediated by exposure to increased albumin alone, or dependent on tubular internalisation of albumin, latrunculin A (1.5 μM) was used to disrupt the actin microfilament network and abolish albumin uptake. Here, we demonstrated that when HK2 cells exposed to high glucose and albumin for six hours in conjunction with latrunculin A, there were no significant differences in CB2 receptor expression between treatment groups (n = 3, Figure 4.5), indicating that the process of albumin endocytosis by proximal tubule cells is required for CB2 down regulation.

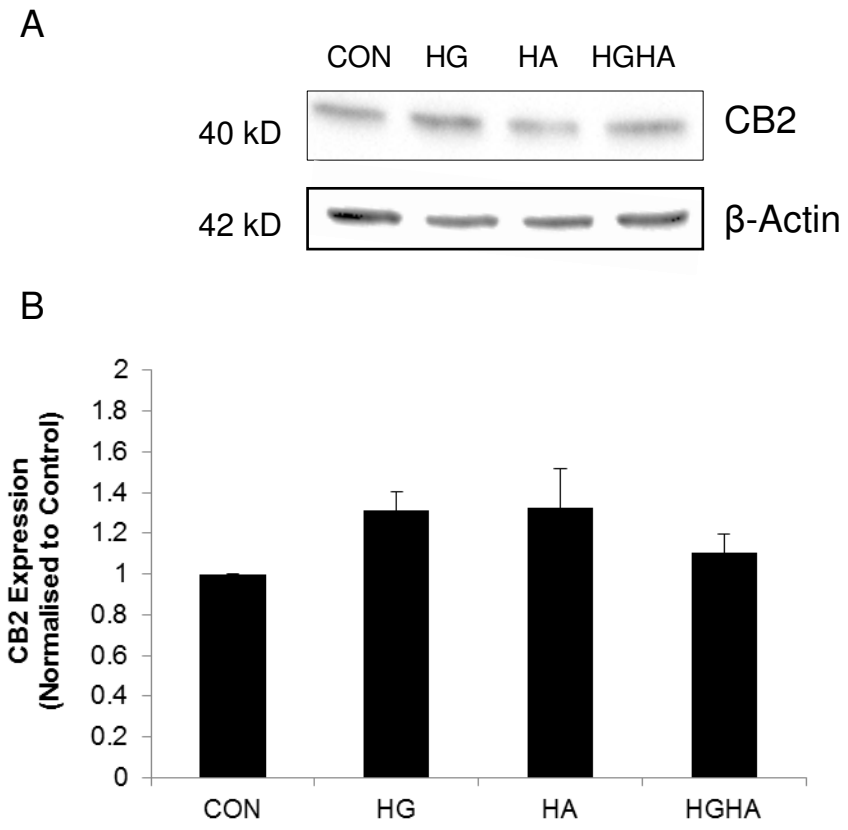



**Figure 4.5: CB2 expression in HK2 cells treated for 6 hours with latrunculin inhibited albumin uptake. A.** Representative Western blot and quantification of CB2 receptor expression in HK2 cells treated with latrunculin A in conjunction with high glucose (HG; 25 mM), high albumin (HA; 1 mg/ml) and a combination of high glucose/ high albumin (HG/HA). β-Actin loading control was not altered by treatment. **B.** CB2 protein expression was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM, n = 3. No significant differences between treatment groups was observed.

*4.4.6 Regulation of Albumin Uptake in Proximal Tubule Cells by CB2 and ERK1/2*

We have demonstrated that high albumin leads to increase phosphorylation of ERK1/2 (Figure 4.5A) and that endocytosis of albumin in proximal tubule cells is essential for regulating CB2 receptor expression (Figure 4.4). To determine whether these cellular alterations lead to changes in proximal tubular handling of albumin, OK cells were treated for 2 hours with TR-albumin in the presence of specific CB2 and ERK1/2 inhibitors. Here, we showed that inhibition of OK cells with CB2 specific antagonist AM630 (10 μM) led to a significant increase in albumin uptake of OK cells ($n = 3$, $p < 0.05$, Figure 4.6). Inhibition of ERK1/2 with antagonist U0126 (10 mM) and MAPK inhibitor SB203580 had no significant effect on albumin uptake in proximal tubular cells. This indicates that while CB2 receptor expression can mediate albumin endocytosis in proximal tubule cells, this process is not affected by ERK1/2 signalling pathways.

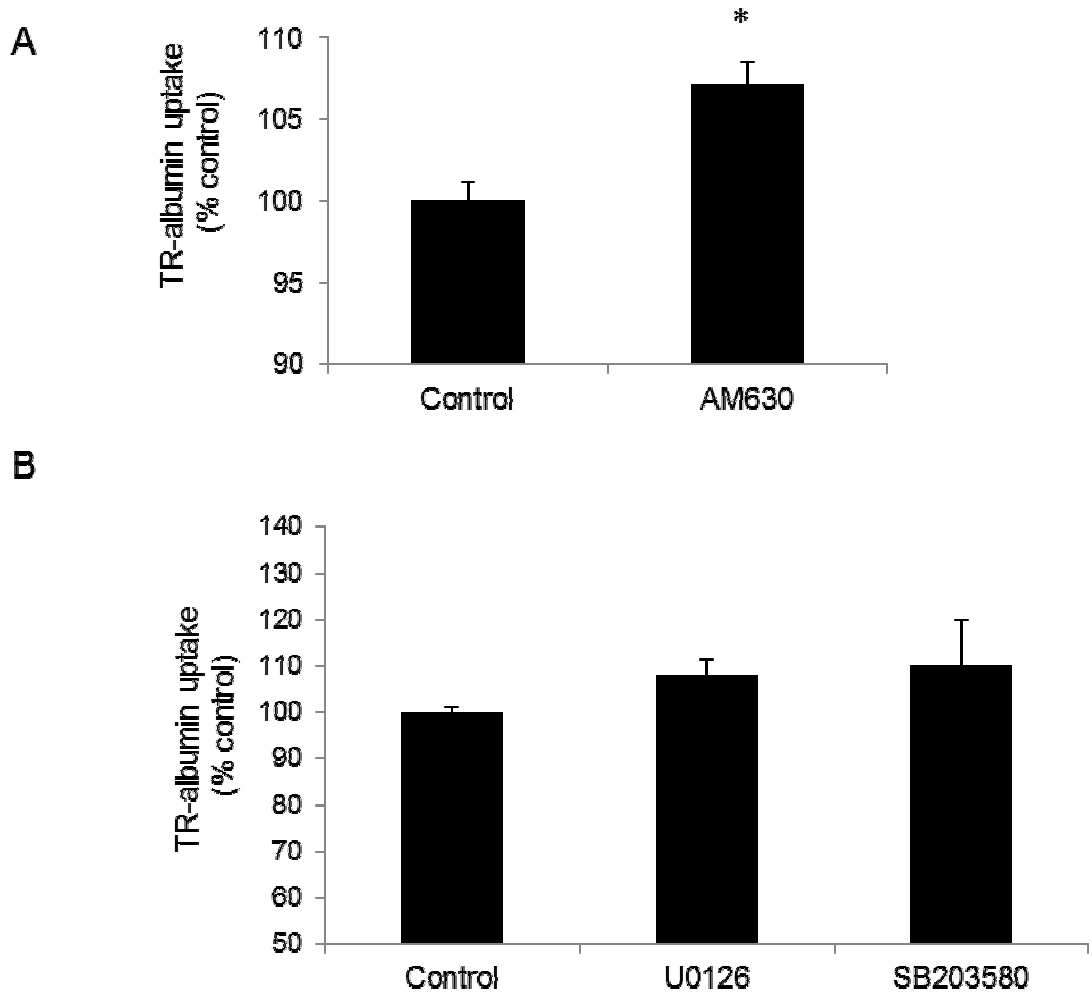



**Figure 4.6: Albumin uptake of proximal tubule OK cells.** Albumin uptake was adjusted for background and normalised to control treatment (no inhibitor), data expressed as mean ± SEM. **A.** OK cells treated with CB2 inhibitor AM630 had significantly higher albumin uptake compared to control treatment ($p < 0.05$, $n = 3$) **B.** OK cells treated with ERK1/2 inhibitor U0126 or MAPK inhibitor SB203580 showed no significant difference in albumin uptake compared to control treatment ($n = 3$).

*4.4.7 CB2 Receptor Expression is not Mediated by ERK1/2*

To determine whether CB2 receptor expression is mediated by increased ERK1/2 phosphorylation, HK2 cells were treated for six hours with high glucose and high albumin media in the presence of ERK1/2 inhibitor U0126 (10 mM). Treatment with the inhibitor resulted in a significant reduction of CB2 receptor expression across all treatment groups compared to control ($p < 0.05$, $n = 3$, Figure 4.7A). These data, along with the results seen in Figure 4.2 suggests that CB2 receptor expression is not mediated by ERK1/2 in high albumin conditions, but may play a role in maintaining receptor expression in hyperglycemic conditions.

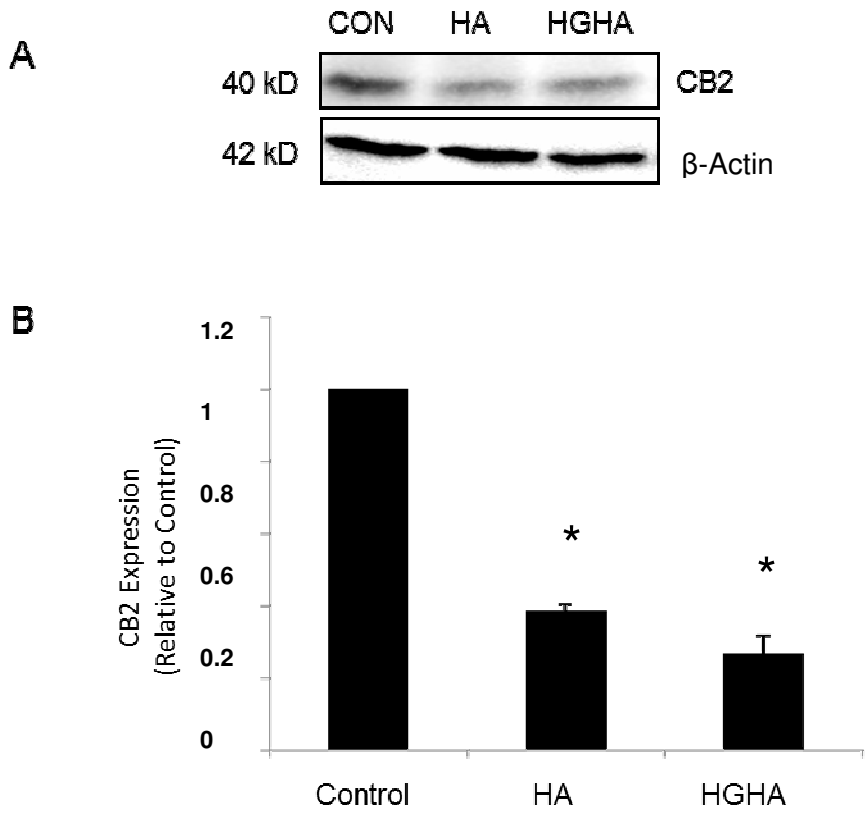



**Figure 4.7: CB2 protein expression of HK2 cells treated with ERK1/2 inhibitor U0126 in conjunction high albumin and a combination of high glucose/ high albumin.** Protein abundance was normalised to control treatment (5 mM glucose, no albumin), data expressed as mean ± SEM. β-Actin loading control was not altered by treatment. **A.** Representative Western blot **B.** Quantification of CB2 receptor expression. Significant reductions in CB2 expression was seen in all treatment groups compared to control ($p < 0.05$, $n = 3$).

## ***4.5 Discussion***

A reduced expression of CB2 in the diabetic kidney has been targeted in the development of therapeutics for diabetic nephropathy (Barutta et al., 2011). Down-regulation of CB2 protein in the glomerulus of animals and humans with diabetes may lead to albuminuria, with activation of this receptor reversing this phenotype (Barutta et al., 2011). Importantly, both glomerular and tubular dysfunction leads to defects in the albumin handling pathway in the kidney (El Nahas and Bello, 2005). To add to our understanding of the role of CB2 in diabetic nephropathy, our study is the first to show that CB2 expression in proximal tubule cells *in vitro* can be modulated by high albumin (1 mg/ml) in the presence and absence of high glucose (25 mM). The reduction in CB2 levels was observed at both the mRNA and protein levels, at specific time points. Interestingly, high glucose alone did not significantly alter CB2 receptor mRNA or protein expression at any time point. Potentially, while transient fluctuations in glucose levels does not contribute to alterations in receptor expression at a cellular level in the kidney, it can alter cannabinoid signalling pathways which may contribute to the eventual decline in CB2 receptor expression. *In vitro,* hyperglycemic conditions sustained for 24 hours in pancreatic islet cells, led to changes to calcium signalling but the effect is not mediated by CB2, but by CB1 (Juan-Picó et al., 2006). Further, it has been demonstrated that in early diabetic models of diabetic nephropathy in mice isolated glomeruli showed no significant differences in CB2 expression, while in human patients with advance diabetic nephropathy have significantly reduced CB2 receptor expression in the renal cortex (Barutta et al., 2011). We have added to this knowledge, demonstrating that despite

a reduction in CB2 expression in specific cells within kidney, in the whole kidney of STZ animals, expression of the CB2 receptor is the same as control animals.

CB2 expression in podocytes (Barutta et al., 2011) and tubular cells is reduced (data presented in this chapter). Why then in an *in vivo* setting, is the expression of CB2 protein in diabetic (STZ) rats unaltered? It is well established that the CB2 receptor is highly expressed in cells of the immune system and assume a diverse range of functions mediating immunological processes in health and disease states (Cabral et al., 2008, Pacher and Mechoulam, 2011, Tanasescu and Constantinescu, 2010). Specifically in diabetic nephropathy, advancement of the renal damage leads to an increase in infiltrating macrophages, T-lymphocytes and the subpopulation of regulatory T cells which leads to inflammation (Lim and Tesch, 2012). In diabetic STZ animals, hallmark indicators of renal damage are elevated such as TGF-β and fibronectin, in addition to the development of albuminuria (Diwakar et al., 2007). Significantly, there is also an infiltration of macrophages, which abundantly express CB2 (Carlisle et al., 2002). Thus, it is likely that the CB2 receptor expression in whole kidney samples is influenced by infiltrating macrophages offsetting the down regulation of CB2 in podocytes and proximal tubule cells. In support of this, in the glomeruli from STZ treated animals, there is an infiltration of CB2-expressing macrophages (Barutta et al., 2011). Diabetic nephropathy is associated with tubular damage primarily due to the exposure to elevated levels of albumin and glucose (Vallon, 2011b, El Nahas and Bello, 2005). Studies have shown that exposure to elevated albumin specifically, results in proximal tubular cells secreting cytokines such as TGF-β1, endothelin-1, MCP-1, regulated upon activation, normal T cell expressed and secreted (RANTES), interleukin-8 and fractalkine. These secreted

cytokines recruit and stimulate interstitial macrophages, which are likely to transform interstitial cells into myofibroblasts, resulting in inflammation and fibrosis *in vivo* (Pacher and Mechoulam, 2011). Our research demonstrates that in tubular cells, exposure to elevated albumin in the presence and absence of high glucose alters CB2 expression. A role for TGF-β1 in the modulation of CB2 renal expression levels has been suggested by Barutta et al. (2011). Importantly, exposure to 1 mg/ml of albumin, for a similar exposure time to our study, resulted in a significant up regulation of TGF-β1 in a proximal tubule cell line (Diwakar et al., 2007). This suggests that CB2 expression in proximal tubule cells may be modulated by cytokines.

The AKT/MAPK signalling cascade is known to mediate to some degree almost every structural change characteristic of diabetic nephropathy, including hypertrophy, cellular stress pathways and apoptosis (Lakshmanan et al., 2012). Here we have demonstrated that changes in CB2 receptor expression corresponds to changes in ERK1/2 profile, but not AKT or p38. While high albumin alone and in combination of high glucose significantly reduced CB2 levels in proximal tubule cells, treatment of HK2 cells with ERK1/2 antagonist does not affect CB2 receptor expression, indicating that ERK1/2 does not mediate CB2 expression in proximal tubule cells. However, a link between the activity of the CB2 receptor and ERK1/2 has been established (Correa et al., 2009, Romero-Sandoval et al., 2009). *In vitro* CB2 activation results in reduced phosphorylated ERK1/2 protein expression (Romero-Sandoval et al., 2009) and conversely, CB2 antagonists can lead to greater and sustained activation of MAPK proteins (Correa et al., 2009), indicating that the mechanism of the CB2 receptor in diabetic nephropathy may mediate changes via

the ERK1/2 proteins, while the AKT/MAPK pathway does not directly mediate changes to CB2 receptor expression.

Diabetic nephropathy is associated with glomerular and proximal tubule damage that leads to albuminuria. A recent study demonstrated that activation of CB2 ameliorated diabetes induced albuminuria *in vivo*. In addition, others have shown that activation of CB2 attenuates renal apoptosis (Mukhopadhyay et al., 2010b), inflammatory mediators (Gardner et al., 2002, Mukhopadhyay et al., 2010b), while blocking CB2 initiates hypertrophy in proximal tubule cells (Jenkin et al., 2010). Collectively, these data suggest that CB2 plays a protective role in renal cell physiology, presumably by abating cellular damage caused by inflammatory mediators. Down regulation of CB2 in podocytes in the diabetic model has been described, but the specific mechanism for this has not been investigated. Upstream damage to the glomerulus results in proximal tubule cells being exposed to higher levels of albumin, but these cells are also responsible for endocytosis of albumin for its reabsorption. Diabetic nephropathy leads to an increased workload for tubular reabsorption of albumin. We have showed that by blocking the CB2 receptor with antagonist AM630 leads to a significant increase in albumin uptake by proximal tubule cells. Inhibition of ERK1/2 with antagonists showed no changes to albumin uptake under physiological conditions. Further, here we demonstrate that internalisation of albumin by proximal tubule cells, and not increased exposure to albumin alone regulates CB2 levels in proximal tubule cells. This may indicate that a possible cross regulatory loop between albumin endocytosis and CB2 receptor expression occurs in proximal tubule cells. Complex cross regulation pathways of CB2 have been identified in other cell types (Correa et al., 2009, Lakshmanan et al., 2012).

## ***4.6 Conclusion***

In summary, these data demonstrated that CB2 receptor expression in proximal tubule cells is modulated by internalisation of albumin. Activity of albumin uptake by proximal tubule cells may be mediated by CB2. Alterations in CB2 expressions may lead to changes in ERK1/2 signalling, however this pathway does not mediate changes to albumin handling by tubular cells. In addition to this, our study has demonstrated that CB2 protein expression is unaltered in the whole kidney from diabetic animals, suggesting that infiltration of inflammatory cells is influencing the whole renal expression levels of CB2. Future studies should characterise signalling targets that are altered in response to altered CB2 expression in diabetic models *in vivo*, to ensure that therapeutic modulation of CB2 is an effective therapy for diabetic nephropathy.

# Chapter 5 – Cannabinoid Receptor Expression in a Model of Diet Induced Obesity

## *5.1 Summary*

Obesity is fast becoming a worldwide epidemic, partially due to increased high caloric intake and sedentary lifestyle. Obesity is strongly associated with many risk factors linked to CKD. It is clear that alterations to the endocannabinoid system occur in a number of disease states such as diabetes, however only a handful of studies have examined this system specifically within the kidneys under obesogenic conditions. The aim of this study was to determine metabolic and renal adaptations which occur in an animal model of DIO. Further, to characterise expression of CB1, CB2 and GPR55 in renal tissue of lean and obese animals. We have demonstrated that DIO can lead to significant renal and metabolic adaptations, including increased proteinuria and body fat composition within three weeks, with effects being exacerbated further when the diet is sustained for a period of up to 10 weeks. We have demonstrated that DIO leads to significant alterations to renal cannabinoid receptor expression including increases in CB1 and GPR55, and a reduction in the CB2 receptor in kidney tissue of obese animals compared to lean standard chow fed controls. Understanding cannabinoid receptor expression within the context of renal pathology is an important first step in comprehending how this endogenous lipid signalling system may mediate the progression of obesity related nephropathy.

## ***5.2 Background***

We have shown in Chapters 3 and 4 that CB1, CB2 and GPR55 renal expression can be significantly when exposed to diabetic conditions. Research has highlighted that the endocannabinoid system has a broad range of physiological functions which mediate the imbalance between caloric intake and energy expenditure, influencing the progression of obesity including control of energy homeostasis, glucose and lipid metabolism, satiety signals and hedonistic eating behavior (Di Marzo, 2008). In animal models DIO, changes in the concentration of the endogenous cannabinoid ligands AEA and 2-AG are dependent on tissue type, and the duration of HFD consumption and the fatty acid composition of the diet (Matias et al., 2008). It is clear that alterations to the endocannabinoid system occur in a tissue specific manner, yet only a handful of studies have examined this system specifically within the kidneys under obesogenic conditions.

*In vivo,* renal proximal tubule cells treated with palmitic acid as a model of hyperlipidaemia, significantly up regulates CB1 receptor protein expression in HK2 cells (Lim et al., 2010). While in db/db diabetic obese mice, CB1 expression has been shown to be significantly upregulated in podocyte glomerular cells (Nam et al., 2012). These studies link altered CB1 expression with an *in vitro* model of obesity linked nephropathy.

In addition, considering the relatively high expression of the CB2 receptor in immune cells, the role of the receptor in modulating inflammation and immune pathways is evident. Obesity has categorically been shown to be associated with a state of chronic, low grade inflammation, which is thought to mediate the numerous systemic

pathologies in which obesity is a risk factor (Gregor and Hotamisligil, 2011). Further, it has been demonstrated in a range of both chronic and acute nephropathies not associated with obesity, activation of the CB2 receptor can ameliorate the progression of renal damage (Barutta et al., 2011, Mukhopadhyay et al., 2010b, Bátkai et al., 2007). Given the increasing evidence of the CB2 receptor in renal pathology, in addition with the established role of the receptor in mediating inflammatory effects, it is surprising that the expression of the CB2 in obesity related renal damage has yet to be explored.

GPR55 has previously been identified in renal proximal tubule cells by our research group (Jenkin et al., 2010). Recently it has been established that the primary endogenous ligand for GPR55, LPI, positively correlates with body fat percentage and BMI in humans (Moreno-Navarrete et al., 2012). Further, expression of GPR55 is significantly higher in liver and adipose tissue of obese compared to lean individuals (Moreno-Navarrete et al., 2012), and expression of this receptor is also tissue specific. The physiological role of GPR55 within the context of obesity and nephropathy has yet to be elucidated. Currently, while obesity has been shown to induce changes to GPR55 expression in adipose tissue, it has yet to be examined whether obesity also induces changes to GPR55 expression within the kidneys.

The aim of this study was to determine metabolic and renal adaptations, including structural remodelling which occur in an animal model of DIO. Further, to characterise expression of cannabinoid receptors CB1, CB2 and GPR55 in renal tissue of lean and obese animals. It is hypothesised that a HFD will result in detrimental metabolic and renal outcomes compared to lean standard chow fed

animals, and that DIO will lead to significant alterations in receptor expression in kidney tissue.

## ***5.3 Materials and Methods***

### *5.3.1 Animal Model and Diet Induced Obesity*

Experimental procedures were approved by the Howard Florey Institute Animal Ethics Committee (AEC 09-050). Sixteen, six-week old male Sprague Dawley rats (mean initial body weight approximately 178 g) were housed within individual cages in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour light/ dark cycle (7:00 - 19:00). Rats were randomly assigned to receive either a HFD (21% fat; Specialty Feeds, Glen Forrest, Australia) or a control diet (standard rodent chow; 5% fat; Barastoc Ltd, Melbourne, Australia) (n = 4 per group) for a period of three or ten weeks. *Ad libitum* access to food and water was maintained throughout the duration of the study. Rats were then deeply anaesthetised with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanised via cardiac puncture. Kidneys were dissected out, and surrounding peri-renal fat and capsule were removed and kidney weight was recorded. Kidneys were dissected transversely down the axis. One half of the tissue sample was put into cryotubes (Thermo Fisher Scientific, Wilmington, DE) and immediately frozen in liquid nitrogen for protein analysis, while the remaining half was prepared for histological analysis, as outlined in Section 2.6.1.

*5.3.2 Metabolic and Renal Measurements*

Rat weight and food was recorded daily throughout the experimental period. During the final week of the three or ten week experimental period, body composition and blood pressure was analyzed (as outlined in Section 2.5) and renal measurements including creatinine clearance, urinary protein, albumin and sodium excretion were evaluated using 24 hour urine sample and plasma collected at time of death, as outline in Section 2.7.

*5.3.3 Histological Analysis*

To determine what structural changes occurred in response to this model of DIO in Sprague Dawley rats, histological analysis examining glomerular and tubular cross sectional area was performed. Kidneys were extracted and fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution at 4℃ overnight followed by a sequential incubation in a 30% sucrose solution overnight at 4℃. Embedding was performed in OCT (Tissue-Tek, Torrance, CA), and embedded samples were stored at -80℃. Samples were cut into 5 µm-thick sections. H&E stain was performed as outlined in Section 2.6 (Hughes and Gobe, 2007). Stained kidney sections were imaged at 200 X magnification (Carl Ziess microscope) and glomerular and tubular cross sectional area was analyzed using AxioVision 4.8 software.

*5.3.4 Protein Extraction and Western Blot Analysis*

Kidney tissue was homogenised and 40-100 µg of protein was used for Western blot analysis as outlined in Section 2.3. The following primary antibodies used include CB1, CB2, GPR55 and β-Actin (supplier information and dilution of antibodies are outlined in Table 2.2). Secondary antibodies anti-mouse and anti-rabbit were

purchased from Sigma Aldrich (St Louis, MO). Band densitometry was analyzed using Image Lab software.

### *5.3.5 Statistical Analysis*

Differences between groups were determined using independent samples T-test, where significance was accepted if $p < 0.05$.

## ***5.4 Results***

### *5.4.1 Metabolic Parameters of Animals with Diet Induced Obesity*

In rats fed a HFD (21% fat) for 3 and 10 weeks there was a significant increase in body fat composition compared to animals fed a control chow diet (5% fat), however no significant differences in overall body weight were detected, likely due to large within group variation (Table 5.1). Further, at 10 weeks there was a significant increase in the ratio of kidney to body weight, and increase in both diastolic and systolic blood pressure (Table 5.1).

**Table 5.1: Metabolic Parameters of Lean and High Fat Fed (HFD) Animals.** Rodents fed either a standard chow diet (Lean), or high fat diet (HFD) for a period of 3 or 10 weeks. Significance is indicated by *, where $p < 0.05$ compared to lean animals of the same feeding period (n = 4 per group).

| Measure | 3 Week Lean | 3 Week HFD | 10 Week Lean | 10 Week HFD |
|---|---|---|---|---|
| | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* |
| Weight (g) | 470.00 ± 11.05 | 493.10 ± 21.16 | 570.60 ± 11.97 | 624.63 ± 43.75 |
| Body Composition (% Fat) | 7.69 ± 0.37 | 12.99 ± 0.70 * | 7.29 ± 0.31 | 10.75 ± 0.46 * |
| Gross Kidney Weight (g) | 1.47 ± 0.11 | 1.52 ± 0.03 | 1.80 ± 0.08 | 1.66 ± 0.05 |
| Kidney Weight/Body Weight (%) | 0.31 ± 0.02 | 0.30 ± 0.01 | 0.32 ± 0.01 | 0.27 ± 0.01 * |
| Diastolic BP (mmHg) | 101.04 ± 0.81 | 96.69 ± 5.35 | 85.36 ± 6.88 | 131.08 ± 5.33 * |
| Systolic BP (mmHg) | 148.26 ± 1.89 | 143.69 ± 8.10 | 132.44 ± 8.13 | 166.19 ± 9.60 * |

### *5.4.2 Functional Renal Outcomes of Diet Induced Obesity*

Rats fed for a period of 3 weeks had exhibited increased total urinary protein excretion, in rats fed a HFD (Table 5.2). Three weeks of high fat feeding did not alter urinary albumin, creatinine or sodium excretion or plasma creatinine and creatinine clearance compared to lean standard chow fed animals (Table 5.2). In animals undergoing 10 week dietary treatment, a number or renal parameters were significantly altered in rats fed a HFD compared to rats on the standard chow diet. In these animals, obese rats showed significantly elevated urinary protein, and albumin, and significantly reduced urinary sodium and creatinine clearance compared to lean animals (Table 5.2).

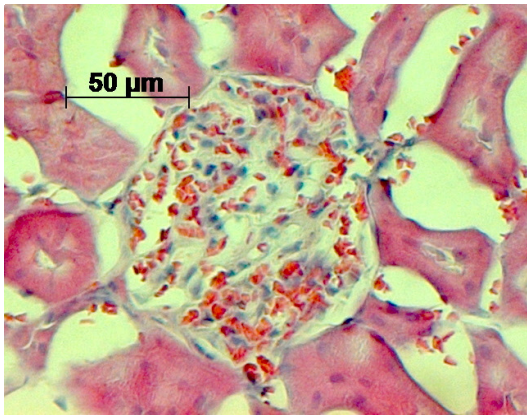
**Table 5.2: Functional Renal Parameters Parameters of Lean and High Fat Fed DIO animals.** Rodents fed either a standard chow diet (Lean) or high fat diet (HFD) for a period of 3 or 10 weeks. Significance is indicated by *, where $p < 0.05$ compared to lean animals of the same feeding period (n = 4 per group)

| **Measure** | **3 Week Lean** | **3 Week HFD** | **10 Week Lean** | **10 Week HFD** |
|---|---|---|---|---|
| | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* |
| Total Urinary Protein (µg/ml) | 10.75 ± 1.96 | 19.67 ± 2.46 * | 10.38 ± 0.76 | 16.04 ± 0.98 * |
| Urinary Albumin (µg/ml) | 53.9 ± 20.91 | 107.8 ± 50.59 | 48.7 ± 11.26 | 202.5 ± 52.72 * |
| Urinary Creatinine (mg/dl) | 97.94 ± 12.72 | 183.02 ± 33.33 | 212.34 ± 28.26 | 266.48 ± 53.45 |
| Plasma Creatinine (mg/dl) | 0.61 ± 0.17 | 0.68 ± 0.17 | 1.06 ± 0.05 | 1.16 ± 0.11 |
| Creatinine Clearance (ml/min/kg body weight) | 2.40 ± 0.59 | 2.10 ± 0.38 | 2.16 ± 0.27 | 1.37 ± 0.06 * |
| Urinary Sodium (mmol/L) | 126.75 ± 21.85 | 173.25 ± 24.06 | 199.25 ± 19.93 | 128.25 ± 14.92 * |

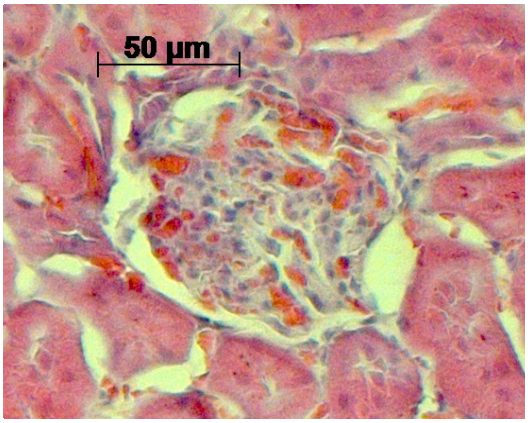
### *5.4.3 Histological Analysis of Kidney Sections from Animals with Diet Induced Obesity*

H&E stained kidney sections from rats fed a HFD for a period of 3 or 10 weeks showed no significant differences in glomerular size from lean rats fed a standard chow diet (Figure 5.1). Staining of the kidney sections showed no significant differences in tubular cross section between high fat and standard chow fed rats at 3 weeks, however after 10 weeks on a HFD, animals showed significant increase in tubular cross section (10.15 ± 0.23 μm) compared to lean standard fed animals (7.15 ± 0.19 μm, $p < 0.05$, $n = 4$), see Figure 5.1.

A




B

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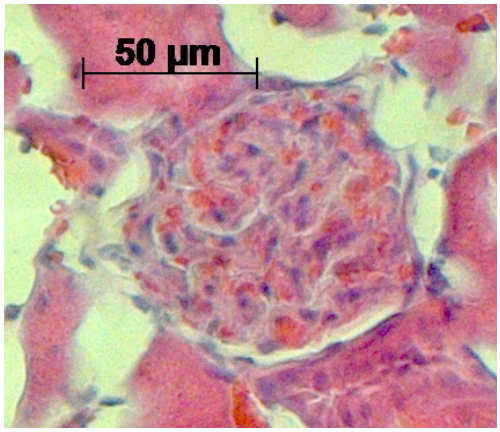
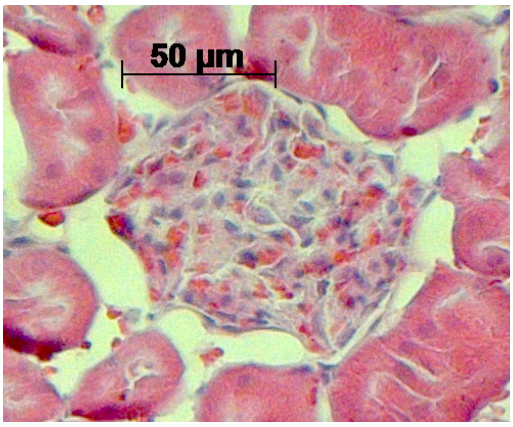



D




E

| Measure | 3 Week Lean | 3 Week HFD | 10 Week Lean | 10 Week HFD |
|---|---|---|---|---|
| | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* | *Average ± SEM* |
| Glomerular Cross Section (µm) | 110.97 ± 5.45 | 120.50 ± 3.35 | 113.00 ± 3.24 | 122.21 ± 5.29 |
| Tubular Cross Section (µm) | 8.22 ± 0.39 | 8.47 ± 0.45 | 7.15 ± 0.19 | 10.15 ± 0.24 * |

**Figure 5.1: Renal structural analysis of rats fed a standard chow (Lean) or high fat diet (HFD).** H&E stain of renal glomerulus and tubules, 200 x Magnification **A:** 3 week standard chow fed lean rat, **B:** 3 week HFD fed obese rat, **C:** 10 week standard chow lean rat, **D:** 10 week HFD fed obese rat. **E:** Average glomerular and tubular cross sectional area for lean and obese rats. Significance is indicated by * where $p < 0.05$ compared to lean animals of the same feeding period ($n = 4$ per group).

*5.4.4 Cannabinoid Receptor Expression in Diet Induced Obesity*

In whole kidney extract, the CB1 receptor was significantly increased in rats fed a HFD for a period of both 3 (1.55 ± 0.14) and 10 weeks (1.54 ± 0.24) compared to lean standard chow fed animals (1.0 ± 0.22 and 1.0 ± 0.11, respectively), see Figure 5.2. Conversely, it was demonstrated that the protein expression of the CB2 receptor was significantly reduced in rats fed a HFD for a period of 10 weeks (0.42 ± 0.05) compared to standard chow fed animals (1.0 ± 0.17), Figure 5.3. No significant differences for CB2 expression was found in animals fed a high fat or standard diet for 3 weeks (0.56 ± 0.10 and 1 ± 0.23, respectively), Figure 5.3. GPR55 also showed no significant difference in renal expression after 3 weeks of high fat compared to standard chow feeding (in order, 1.13 ± 0.12 and 1 ± 0.25). However, GPR55 protein expression was significantly increased in whole kidney lysate succeeding 10 weeks of DIO (1.44 ± 0.18), compared to lean animals fed a standard chow diet (1 ± 0.08), see Figure 5.4.

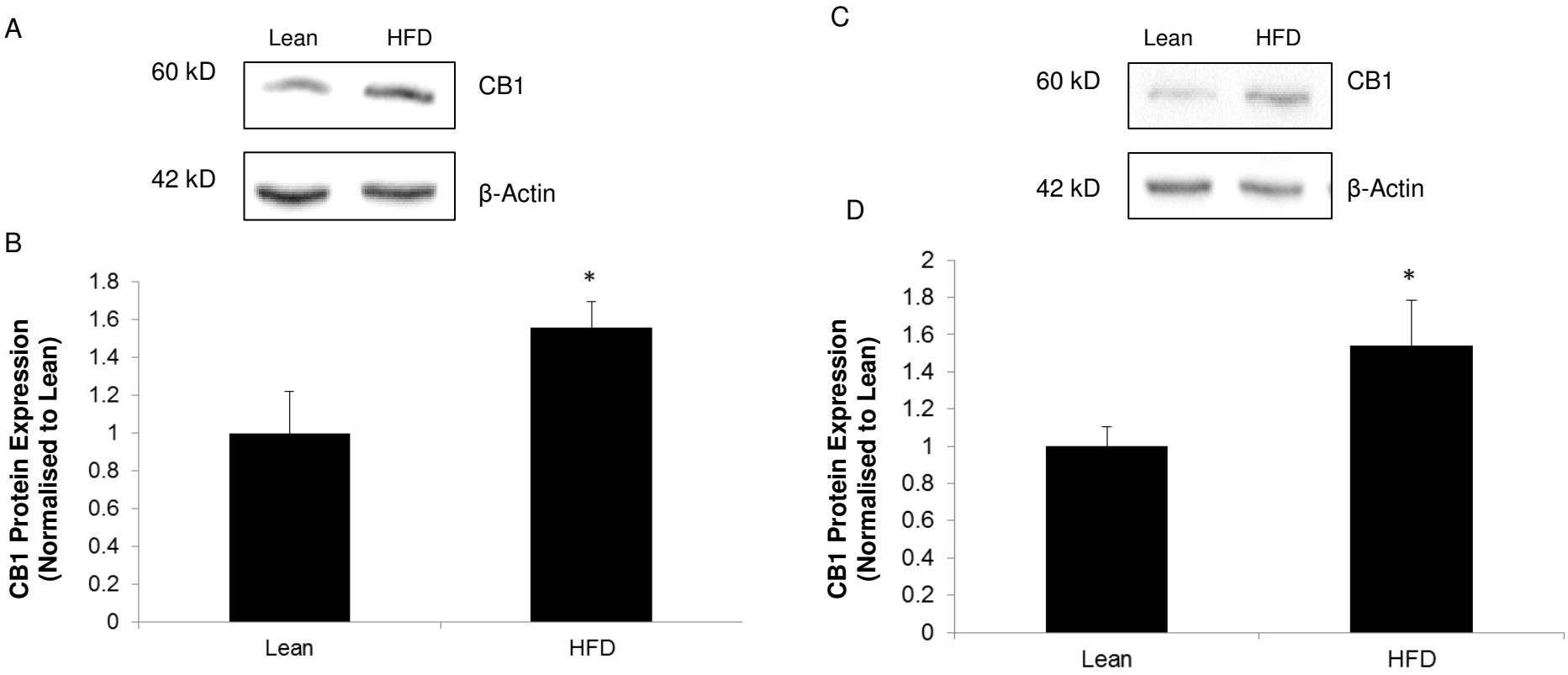



**Figure 5.2: CB1 protein expression in whole kidney lysate of rats fed a standard chow (Lean) or high fat diet (HFD)**. **A.** Representative Western blot of 3 week fed rats **B.** Quantification of CB1 receptor expression of 3 week fed rats. **C.** Representative Western blot 10 week fed rats **D.** Quantification of CB1 receptor expression of 10 week fed rats. Significance is indicated by * compared to lean standard chow fed animals ($p < 0.05$, $n = 4$).

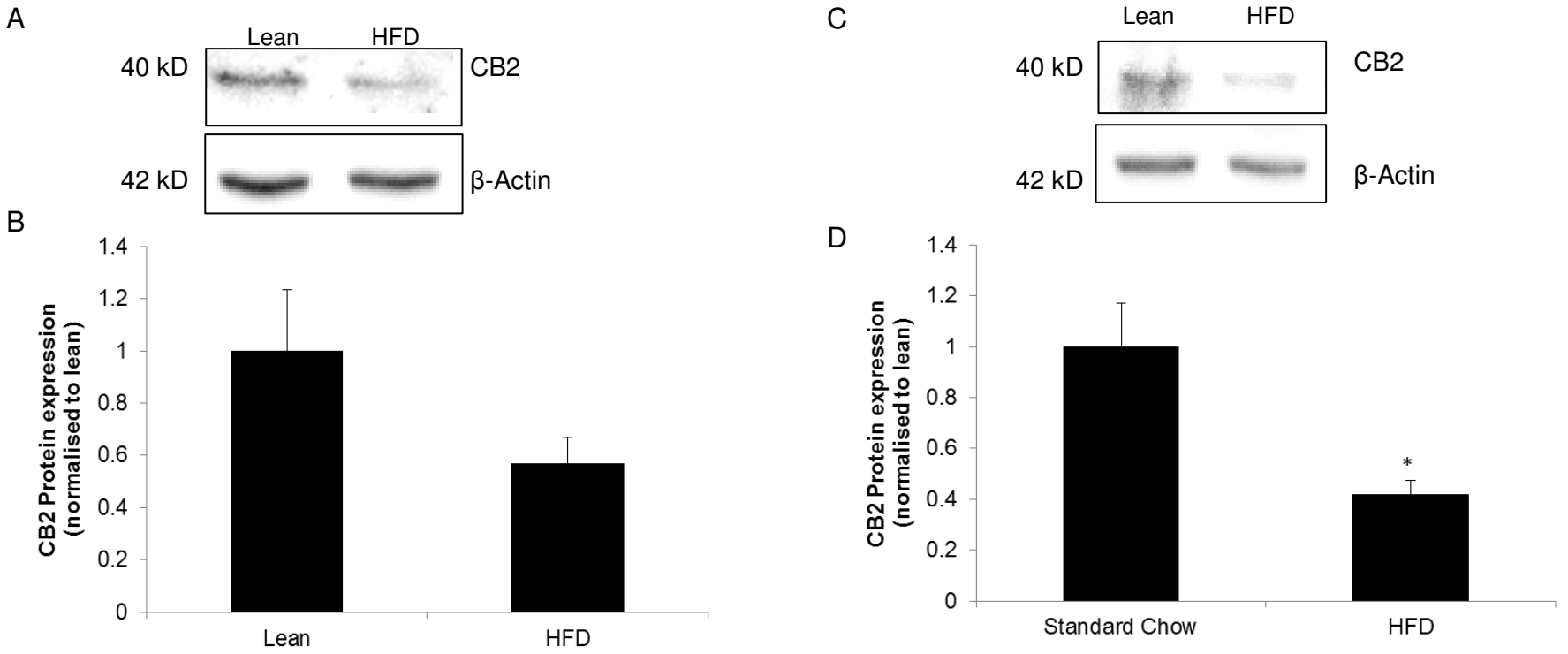



**Figure 5.3: CB2 protein expression in whole kidney lysate of rats fed a standard chow (Lean) or high fat diet (HFD)**. **A.** Representative Western blot of 3 week fed rats **B.** Quantification of CB2 receptor expression of 3 week fed rats. **C.** Representative Western blot 10 week fed rats **D.** Quantification of CB2 receptor expression of 10 week fed rats. Significance is indicated by * compared to lean standard chow fed animals ($p < 0.05$, $n = 4$).

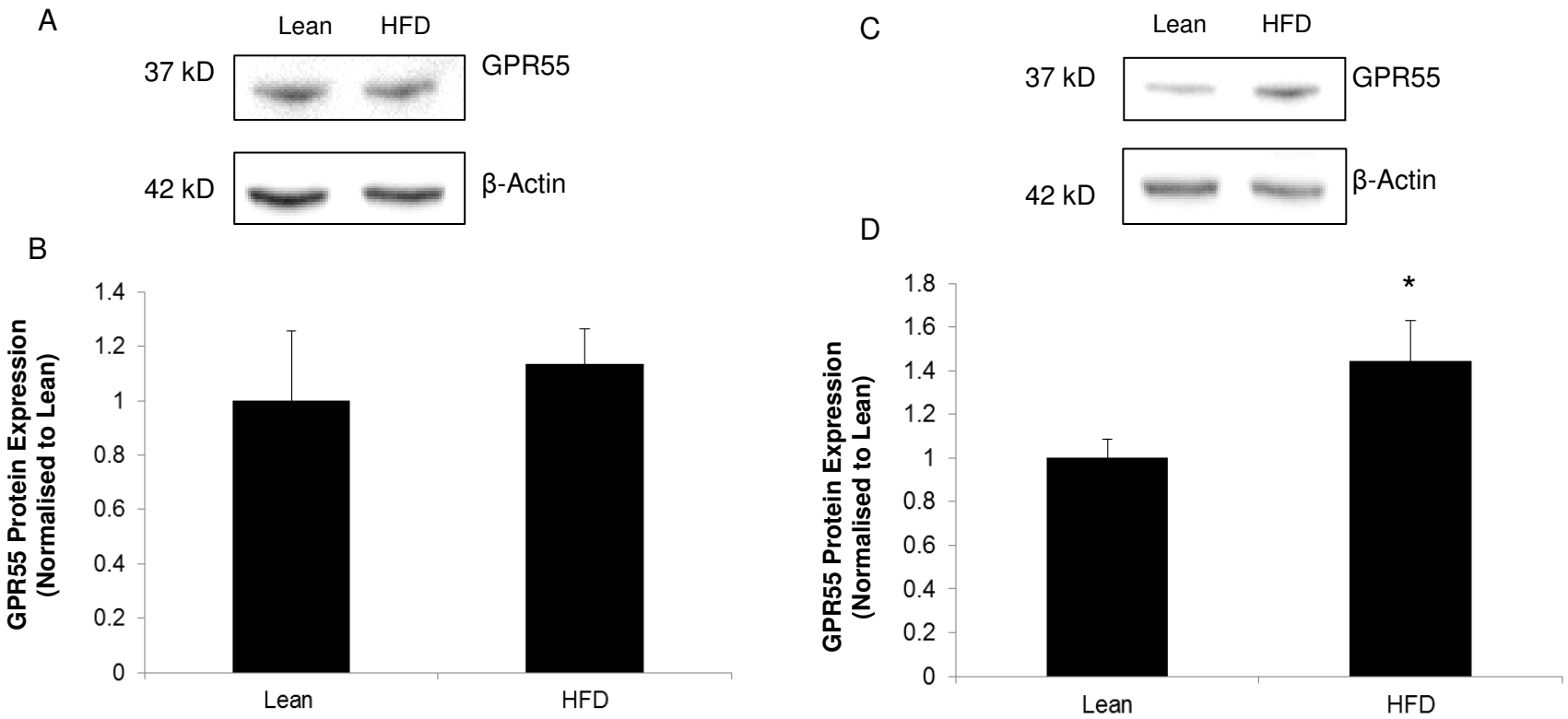



**Figure 5.4: GPR55 protein expression in whole kidney lysate of rats fed a standard chow (Lean) or high fat diet (HFD)**. **A.** Representative Western blot of 3 week fed rats **B.** Quantification of GPR55 expression of 3 week fed rats. **C.** Representative Western blot 10 week fed rats **D.** Quantification of GPR55 expression of 10 week fed rats. Significance is indicated by * compared to lean standard chow fed animals ($p < 0.05$, $n = 4$).

## ***5.5 Discussion***

It has been well documented that high caloric intake typical of a 'Western style' diet contributes to obesity (Booth et al., 2005, Stanton, 2009). Here, it has been demonstrated in an animal model of DIO that a HFD for just three weeks can lead to increased body fat composition and urinary protein excretion compared to lean standard chow fed animals. Sustained consumption of a HFD for a period of 10 weeks also lead to other physiological perturbations including increased kidney weight (adjusted for body weight), and significantly higher systolic and diastolic blood pressure, similar to what has been reported in models of animal obesity elsewhere (Dobrian et al., 2000).

The Framingham Heart study, a longitudinal epidemiological study identifying risk factors for cardiovascular diseases established that obesity is the primary controllable contributor to hypertension, estimating that 65 to 75% of hypertension cases identified could be attributed to excess weight (Garrison et al., 1987, Wickman and Kramer, 2013). Indeed, it has been shown that adipose tissue has many endocrine functions and produces a variety of hormones important for regulating blood pressure either directly or indirectly, including intermediates of the RAAS system and glucocorticoids (Ahima and Flier, 2000b). The mechanisms underlying obesity related hypertension is thought to be a multimodal, with a number of contributing factors being identified in its progression, including activation of the RAAS and sympathetic nervous system, increased circulating pro-inflammatory and fibrotic mediators and altered renal function (Abassi et al., 2009, Cignarelli and Lamacchia, 2007a, Kramer et al., 2005). Significant increases in diastolic and

systolic blood pressure in obese animals fed a HFD for ten weeks, is expected to be primarily mediated through these established mechanisms.

In relation to renal damage, three weeks on a HFD induced significantly higher levels of proteinuria compared to lean standard chow fed animals. At this time point, total urinary protein was the only renal parameter that was significantly altered by the HFD. Clinically, detection of elevated protein excretion preceding changes to glomerular filtration is a characteristic of obesity related nephropathy (Cignarelli and Lamacchia, 2007a). We have demonstrated here that in an animal model of DIO, ten weeks on a HFD leads to substantial increases in measures of renal damage, including significantly increased proteinuria, albuminuria and significant decline in creatinine clearance and reduced urinary sodium excretion. These observations are similar to what has been reported by Keenan et al. (2000), where Sprague Dawley rats fed *ad libitum* showed a marked increase in the measures of kidney damage than animals on a restricted caloric diet. Elevated proteinuria and albuminuria observed in obesity related renal damage is thought to be due to excess metabolic excretory load, imbalance of nephron number relative to total body weight and structural renal remodeling including glomerulosclerosis (Afshinnia et al., 2010). Histologically, no evidence for glomerulosclerosis was evident, however significant increases in renal tubular size found in 10 week DIO animals compared to lean animals of the same feeding period. An increase in cellular size without changes to cell number is classified physiologically as hypertrophy (Vallon, 2011). Here we have used cross sectional tubular and glomerular size as a measure of renal hypertrophy. Hypertrophy is one of the initial adaptations which occurs within the kidneys in response to increased reabsorptive pressures derived from metabolic disturbances

like hyperlipidemia, and is associated with alterations in protein handling by the kidneys (Vallon, 2011b).

Creatinine clearance is often used to estimate GFR, which is the most basic measurement of renal function and evaluation. GFR has been used for decades to monitor and assess (in both clinical and in experimental models) renal function. Typically it is observed in obesity that structural adaptations to the nephron occur in response to hyperlipidaemia and altered metabolic load, and as functional consequences follow these structural adaptations, a gradual decline in GFR occurs. This study has established that rats fed a HFD for 10 weeks have significantly reduced GFR, as measured by creatinine clearance compared to lean chow fed animals. This is consistent with previous findings using a similar model DIO in the Sprague Dawley rat, which showed rats fed *ad libitum* HFDs have reduced creatinine clearance after 39 weeks, compared to animals whose food intake was calorie restricted (Keenan et al., 2000). Of particular note, is that even within a relatively short amount of time (10 weeks), basic markers of renal damage are increased by high fat feeding. The model used in this study is a robust model of obesity, as HFD interventions leading to renal disease are much higher in male Sprague Dawley compared to female rats (Hard and Khan, 2004), and it does not involve manipulation of the leptin receptor, which may limit translation of results in human renal pathology. However, it should be considered that when using Sprague Dawley rats for a DIO model, a range of physiological adaptations within the population can occur, as it has been reported that up to 50% of outbred Sprague Dawley rats are resistant to obesity induced by high fat or caloric intake (Levin et al., 1997). Providing further support that this model is a robust model of DIO, due to the diverse

physiological outcomes which are exhibited by Sprague Dawley rats in response to a HFD are comparable to what is observed in humans.

Sodium urinary excretion was found to be significantly reduced in obese animals, and this could be a factor driving the hypertensive state also observed in the high fat fed animals. Mechanistically, reduced urinary sodium excretion (and thus increased sodium retention) is associated with hypertension, as increased renal sodium reabsorption corresponds with increased fluid retention and plasma volume leading to increases in systemic blood pressure. Adipose tissue has been shown to be capable of producing angiotensin, the primary constituent in the cascade of RAAS signalling, which has been shown to increase sodium reabsorption via increased sodium transporter expression in renal tubules (Ahima and Flier, 2000b). The tubular-glomerular feedback mechanism which usually regulates GFR and tubular reabsorption is also impaired via structural compression of the vasa recta and loop of Henle which slows the rate of filtrate flow through the nephron and increases sodium reabsorption back into surrounding capillaries, ultimately leading to activation of the RAAS (Vallon, 2011a). Increased adipose stores also leads to activation of RAAS and sympathetic tone which can induce systemic vasoconstriction, compounding hypertensive effects or increased sodium retention as well as contributing to the progression of renal function decline (Kramer et al., 2005).

In addition to demonstrating that a HFD can have significant impact on both metabolic and measurements of kidney damage, this study has also been the first to characterise timelines for altered expression of cannabinoid receptors CB1, CB2 and GPR55 in renal tissue of animals with DIO. It is clear that obesity can affect

expression of constituents of the endocannabinoid system in a tissue specific manner (Atwood et al., 2012, Cavuoto et al., 2007, Crespillo et al., 2010, Mendez-Sanchez et al., 2007, Moreno-Navarrete et al., 2012). We have demonstrated that obese conditions lead to significant alterations to cannabinoid receptor expression, including increases in CB1 and GPR55, and a reduction in the CB2 receptor in renal tissue of obese animals compared to lean counterparts. It is important to consider that given the varied tissue specific expression of these receptors, signalling of the receptor in peripheral tissues is likely to activate organ specific signalling pathways.

Here we have shown that after just three weeks, increased expression of CB1 in kidney tissue in animals with DIO compared to lean animals, and this is sustained for extended high fat feeding of 10 weeks. Obesity induces significant up regulation of CB1 within many tissues and the receptor has been shown to not only regulate food intake through central and peripheral mechanisms, (Arnone et al., 1997, Gomez et al., 2002) but also promotes adipogenesis, lipogenesis and fatty acid synthesis (Di Marzo, 2008). CB1 antagonism has been shown to have beneficial effects in ameliorating the progression of diabetic nephropathy (Barutta et al., 2010, Janiak et al., 2007), blunting the effects of AEA induced hypertrophy in renal proximal tubule cells (Jenkin et al., 2010), and reducing oxidative stress in an acute experimental model of nephropathy (Mukhopadhyay et al., 2010a).

Prior to this study, expression of the CB2 receptor in kidney tissue of obese animals has not been examined. Our data demonstrates that after 10 weeks on a HFD, CB2 protein expression in kidney tissue is significantly reduced compared to lean standard chow fed animals. The CB2 receptor has been identified in kidney tissue

previously (Deutsch et al., 1997), and renal expression has been found to be markedly reduced in the glomeruli of patients with advanced diabetic nephropathy compared to non-diabetic patients (Barutta et al., 2011) as well as in HK2 cells exposed to high albumin media (Chapter 4). In contrast to research showing pharmacological blockade of CB1 ameliorates progression of renal disease (Barutta et al., 2010, Janiak et al., 2007), pharmacological activation of CB2 receptor has been shown to have beneficial outcomes in a range of nephropathy models (Barutta et al., 2011, Mukhopadhyay et al., 2010b, Horvath et al., 2012). These studies indicate that activation of CB2 is likely to protect renal cells from cytokine mediated cellular damage. Regarding CB2 modulating energy intake, in models of CB2 knockout mice, deletion of the receptor appears to promote obesity and food intake, whilst also leading to improvements in insulin sensitivity and alterations to inflammatory markers (Agudo et al., 2010, Deveaux et al., 2009). Conversely, it has also been shown that CB2 agonist, CBD can reduce weight gain in rodents (Ignatowska-Jankowska et al., 2011) and overexpression of the CB2 receptor in hypothalamic regions of the brain leads to a lean phenotype with reduced glucose tolerance (Romero-Zerbo et al., 2012). This highlights that specifically within the context of obesity, the physiological role of the CB2 receptor is unclear.

Here, we have demonstrated in a model of DIO that after 10 weeks, animals have significantly elevated GPR55 expression in renal tissue compared to lean standard chow fed animals. This is the first study to examine expression of this receptor in relation to nephropathy of any kind. Despite GPR55 and CB1 being co-expressed in a number of tissues at similar levels and in HEK293 cells, it has been demonstrated that these two receptors are able to form heteromers, mediating changes to

signalling events *in vivo (*Kargl et al., 2012). Emerging research shows a potential role for GPR55 in obesity (Imbernon et al., 2013, Moreno-Navarrete et al., 2012). In humans, obese patients have significantly elevated plasma levels of endogenous GPR55 ligand LPI and GPR55 expression is upregulated in adipose tissue in obese patients (Moreno-Navarrete et al., 2012). Further, *in vivo,* activation of GPR55 with synthetic agonist O-1602 administered centrally and peripherally leads to stimulation of food intake and increases in fat mass in rodents, whilst *in vitro,* O-1602 treatment increased calcium signalling and lipogenesis in adipocytes (Díaz-Arteaga et al., 2012). Our group has previously demonstrated that GPR55 is expressed in renal proximal tubule cells (Jenkin et al., 2010), however it has yet to be examined what effect obesity has on renal expression of the receptor. Given only a handful of studies have examined GPR55 in relation to the pathogenesis of obesity and related complications, it is clear further investigation needs to be directed towards the possible physiological role the receptor may be responsible for in normal and pathological renal physiology.

## ***5.6 Conclusion***

This study has shown that DIO can lead to significant renal and metabolic adaptations within just three weeks, with effects being exacerbated further when the diet is sustained for a period of up to 10 weeks. A diet high in fat also leads to significant alterations in renal expression of CB1 and CB2, and GPR55. Understanding cannabinoid receptor expression within the context of pathology is an important first step in comprehending how this endogenous lipid signalling system may mediate the progression of obesity related nephropathy.

# Chapter 6 – Chronic Administration with CB1 Antagonist AM251 Reduces Proteinuria, Albuminuria and Renal Tubule Size in Rats with DIO

## *6.1 Summary*

Pharmacological blockade of the CB1 receptor in Type 1 and Type 2 models of diabetes has previously been shown to ameliorate progression of renal disease (Barutta et al., 2010, Janiak et al., 2007, Nam et al., 2012). It has yet to be investigated whether this therapeutic approach also confers renal improvements in a DIO model. The aim of this study was to determine metabolic and renal adaptations which occur in response to treatment with the CB1 antagonist AM251 in an animal model of DIO. Male Sprague Dawley rats were fed a HFD for nine weeks to induce obesity. Following this, animals were maintained on the HFD for a further six weeks and treated daily with 3 mg/kg AM251 or saline via IP injection (n = 9 per group). Obese AM251 treated animals exhibited significant improvements in both metabolic and renal parameters including reduction in weight gain, systolic blood pressure, proteinuria, albuminuria, plasma creatinine levels and renal tubule cross sectional area compared obese controls. Treatment with AM251 in obese animals did not significantly affect diastolic blood pressure, sodium excretion, estimated creatinine clearance or renal cytokine profile. Further investigation is required to determine the signalling pathways by which AM251 treatment in an obese model is able to improve markers of kidney damage. Nonetheless, this study identifies a potential role of CB1 blockade for the treatment of obesity related renal damage.

## ***6.2 Background***

In recent years, there has been building evidence for the CB1 receptor and its role in renal physiology. Overexpression of the receptor has been identified in glomerular cells of animals with diabetic nephropathy as well as human proximal tubular cells (Barutta et al., 2010, Lim et al., 2010, Nam et al., 2012, Jenkin et al., see Chapter 3 and 5). In Chapter 5, we have demonstrated that the CB1 receptor is significantly increased in kidney tissue of rats with DIO after just three weeks. It has been identified that antagonism of the CB1 receptor may be a potential therapeutic target for the treatment of diabetic nephropathy via improved metabolic parameters, reduced podocyte loss and tubule apoptosis (Barutta et al., 2010, Janiak et al., 2007, Lim et al., 2010). In obese humans it has been shown that selective CB1 antagonism is effective in reducing weight and prevalence of metabolic syndrome, parameters known to contribute to renal damage (Després et al., 2005, Van Gaal et al., 2005).

Treatments targeting CB1 may also influence the leptin signalling pathway (Tam et al., 2012). Leptin is a hormone primarily produced by adipose tissue and hyperleptinaemia is a characteristic of the obese state (Ahima and Flier, 2000a). Leptin is principally cleared by the kidneys (Hama et al., 2004) and elevated leptin levels have been associated with increased collagen deposition and transforming growth factor 1 (TGF-β1) secretion by renal glomerular cells, leading to increased renal fibrosis (Wolf et al., 1999). Kidney damage associated with obesity also leads to increased levels of protein and albumin in the urine, and obesity has been shown to alter renal expression of megalin (Tam et al., 2012), a transmembrane protein which regulates both albumin and leptin absorption in the tubules (Birn and

Christensen, 2006, Hama et al., 2004). CB1 antagonism has been shown to reverse hyperleptinaemia and improve leptin sensitivity in obese animals (Tam et al., 2012).

It is likely that improvements in markers of kidney damage and structure demonstrated in these studies partially can be attributed to systemic metabolic changes, but potentially CB1 antagonism may affect signalling pathways specifically within renal tissue, as has been demonstrated *in vitro* (Mukhopadhyay et al., 2010a, Lim et al., 2010). A number of cytokines are known to mediate structural changes in obesity related renal damage, including TGF-β1. TGF-β1 regulates the production of many matrix and basement proteins, including collagen type IV and elevation in TGF-β1 production leads to an accumulation of extracellular matrix, renal fibrosis and thickening of the basement membrane (Qi et al., 2008b). We have recently established that CB1 antagonist AM251 *in vitro,* significantly reduces proximal tubule hypertrophy under normal physiological conditions (Jenkin et al., 2010), however the signalling pathways mediating this event was not explored. The first aim of this study was to determine metabolic and renal adaptations which occur in response to the CB1 antagonist AM251 in an animal model of DIO. The second aim was to identify whether cytokines which are known to mediate structural renal changes are altered in response to AM251 treatment.

## ***6.3 Materials and Methods***

### *6.3.1 Animals and Experimental Protocol*

Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour

light/ dark cycle (7:00 - 19:00). Rats were fed *ad libitum* a HFD (containing 21% fat, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Rats were then matched using a number of metabolic parameters and put into either obese control or CB1 antagonist treatment groups (n = 9 per group). For six weeks, rats were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80 or 3 mg/kg of AM251 dissolved in vehicle solution via IP injection. Following the conclusion of the experimental protocol, rats were deeply anesthetised and killed via cardiac puncture. Organs were removed, weighed and stored for further analysis, as outlined in Section 2.6. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036).

### *6.3.2 Metabolic Measurements*

Rat weight and food was recorded daily throughout the experimental period. Body composition and blood pressure was analyzed (as outlined in Section 2.5) for pre- and post-treatment time points. 'Pre-treatment' measurements were conducted the week before saline or AM251 treatments commenced and 'post-treatment' measurements in the final week of treatment.

### *6.3.3 Functional Measurements of Renal Outcomes*

Renal measurements including creatinine clearance, urinary protein, albumin and sodium excretion were evaluated using 24 hour urine samples collected pre- and post-treatment periods and plasma collected at time of death, as outline in Section 2.7.

*6.3.4 Histological Analysis*

To determine what structural changes occurred in response to AM251 treatment in this model of DIO in Sprague Dawley rats, histological analysis examining glomerular and tubular cross sectional area was performed. Following kidney dissection, a portion of kidney tissue was fresh frozen, as outlined in Section 2.6.1 and sections were stained. H&E and PAS staining was performed as outlined in Section 2.6. Sections were imaged at 200 X magnification (Carl Ziess microscope) and glomerular and tubular cross sectional area was analyzed using AxioVision 4.8 software.

*6.3.5 Cytokine Profile*

Cannabinoid receptors have been known to mediate cytokine signalling. To determine whether AM251 treatment affects renal cytokine profile, a number of key proteins known to mediate renal structural changes in DIO were examined. TGF-β1 plasma expression was analyzed using immunoassay techniques outlined in Section 2.8.2. Kidney tissue was analyzed for protein expression of TGF-β1, Collagen IV and VEGF via Western blot analysis. 40 - 100 µg of protein was used for Western blot analysis as outlined in Section 2.3. The following antibodies were used: TGF-β1, Collagen IV, VEGF and β-Actin. Concentrations and supplier information are outlined in Section 2.2.3 in Table 2.2. Secondary antibodies anti-mouse and anti-rabbit were purchased from Sigma Aldrich (St Louis, MO). Band densitometry was analyzed using Image Lab software.

*6.3.6 Statistical Analysis*

The SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests for analysis of pre- and post-treatment measurements between obese control and AM251 treated groups. Significance was accepted when $p < 0.05$.

## ***6.4 Results***

*6.4.1 Metabolic Parameters in Control and AM251 Treated Obese Animals*

Following treatment with AM251, CB1 antagonism induced improvements in percentage weight gain and systolic blood pressure of animals with DIO. Obese control and obese AM251 animals were at similar weights pre-treatment (582.9 ± 14.35 g and 576.0 ± 11.51 g, respectively), but obese AM251 animals were significantly lighter (607.2 ± 12.43 g), at the conclusion of the 6 week treatment period compared to control obese animals (655.9 ± 19.23 g). This was due to significantly reduced weight gain (expressed as a percentage of pre-treatment weight) across the six week treatment period, compared to obese control animals (Figure 6.1). Obese animals treated with AM251 also exhibited significant reductions in systolic blood pressure (151 ± 3.38 mmHg pre-treatment, 135 ± 7.43 mmHg post-treatment) compared to obese control animals (Figure 6A;137 ± 4.89 mmHg pre-treatment, 144 ± 7.07 mmHg post-treatment). Diastolic blood pressure in AM251 treated obese animals (105 ± 3.38 mmHg pre-treatment, 91± 5.59 mmHg post-

treatment) was not significant compared to obese control animals (Figure 6B; 100 ± 5.86 mmHg pre-treatment, 105 ± 5.83 mmHg post-treatment).

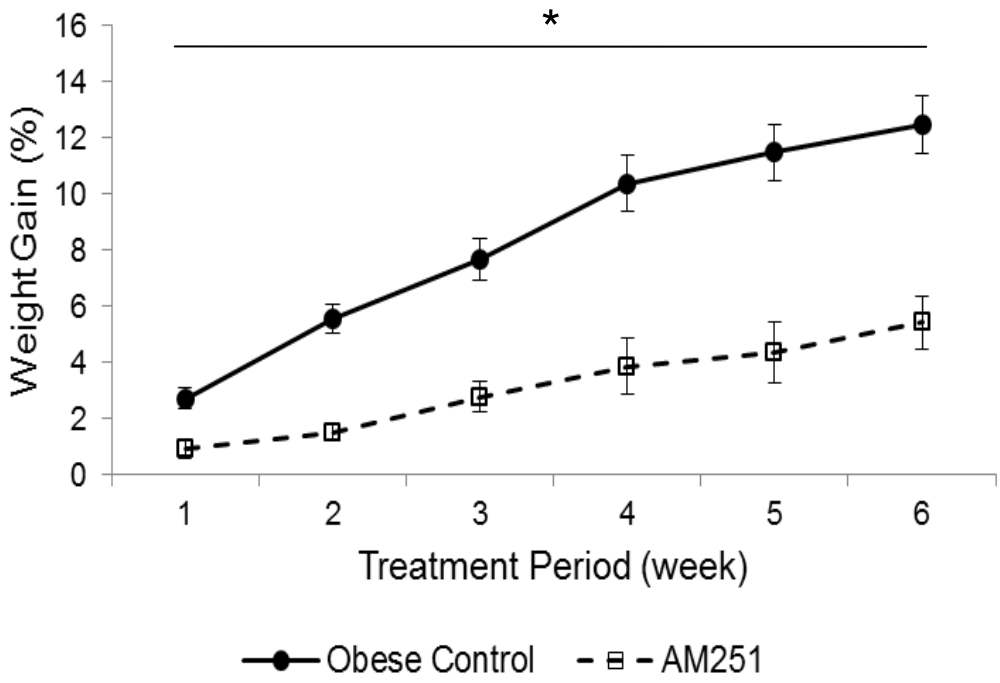



**Figure 6.1: Percentage weight gain in obese AM251 treated animals, following nine weeks of DIO compared to obese controls.** Obese AM251 treated animals exhibited significantly reduced weight gain (% of pre-treatment weight) from Week 1 to Week 6 of treatment compared to obese control animals receiving saline. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).

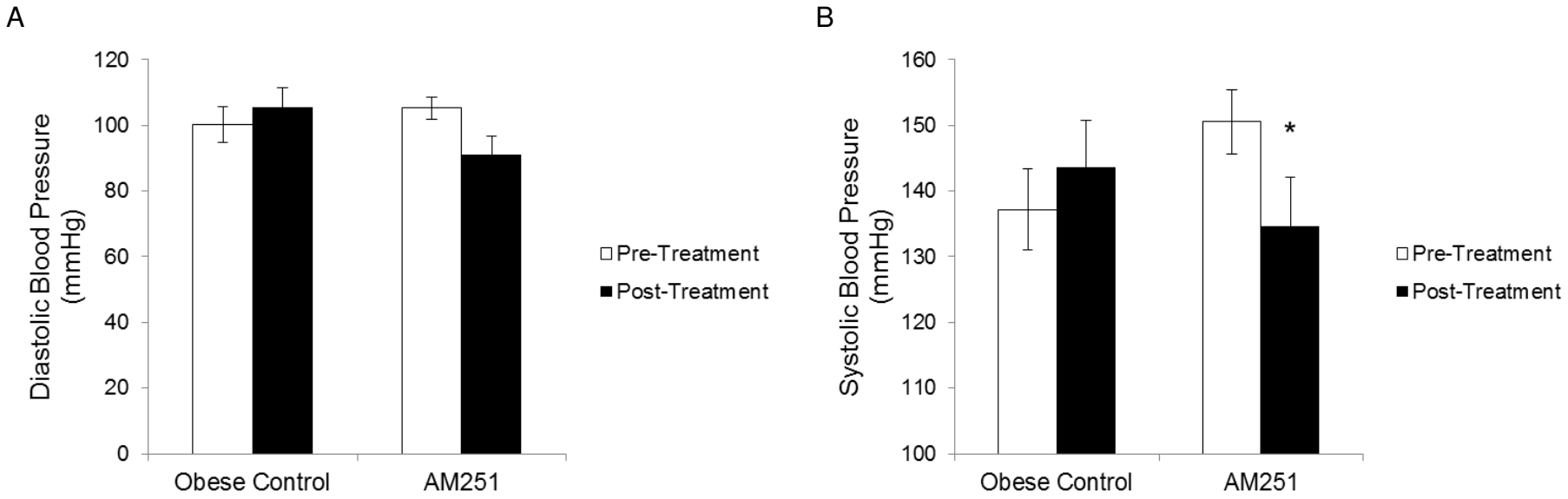



**Figure 6.2: Blood pressure of obese AM251 treated obese animals compared to obese controls. A.** Diastolic blood pressure was not significantly altered in obese animals by AM251 treatment compared to control ($p= 0.059$, $n = 9$) . **B.** In obese animals, systolic blood pressure was significantly reduced by AM251 treatment compared to obese control animals recieving saline. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).

*6.4.2 Functional Renal Outcomes in Control and AM251 Treated Obese Animals*

As outlined in Chapter 5, our model of DIO leads to a significant increase in markers of kidney damage compared to lean animals. In this study, treatment with AM251 did not significantly alter urinary sodium or GFR as estimated by creatinine clearance compared to obese animals (Figure 6.3 C and 6.3 E). However, renal markers of damage including proteinuria, albuminuria and plasma creatinine were significantly reduced by AM251 treatment in obese animals. Total urinary protein excretion was higher in obese AM251 treated animals pre-treatment (22.5 ± 4.37 µg/ml) compared to obese control (15.3 ± 1.14 µg/ml). However, obese AM251 animals exhibited significant reductions in urinary protein (16.74 ± 2.32 µg/ml, post-treatment) across the treatment period, while obese control animals had higher levels of protein present in the urine post-treatment (19.1 ± 2.96 µg/ml, post-treatment). Pre-treatment, urinary albumin concentration was similar between obese AM251 treated (293.28 ± 43.12 ng/ml) and obese control (218.7 ± 36.13 ng/ml) groups. However, across the treatment period, mixed model ANOVA analysis showed that obese control animals exhibited increased urinary albumin excretion (247.8 ± 49.50 ng/ml, post-treatment) while obese AM251 treated animals had significantly reduced levels of urinary albumin excretion (Figure 6.3C; 274.1 ± 31.01 ng/ml, post-treatment). Plasma creatinine was also significantly reduced in animals treated with AM251 (0.47 ± 0.06 mg/dl) compared to obese control (0.98 ± 0.16 mg/dl), Figure 6.3D.

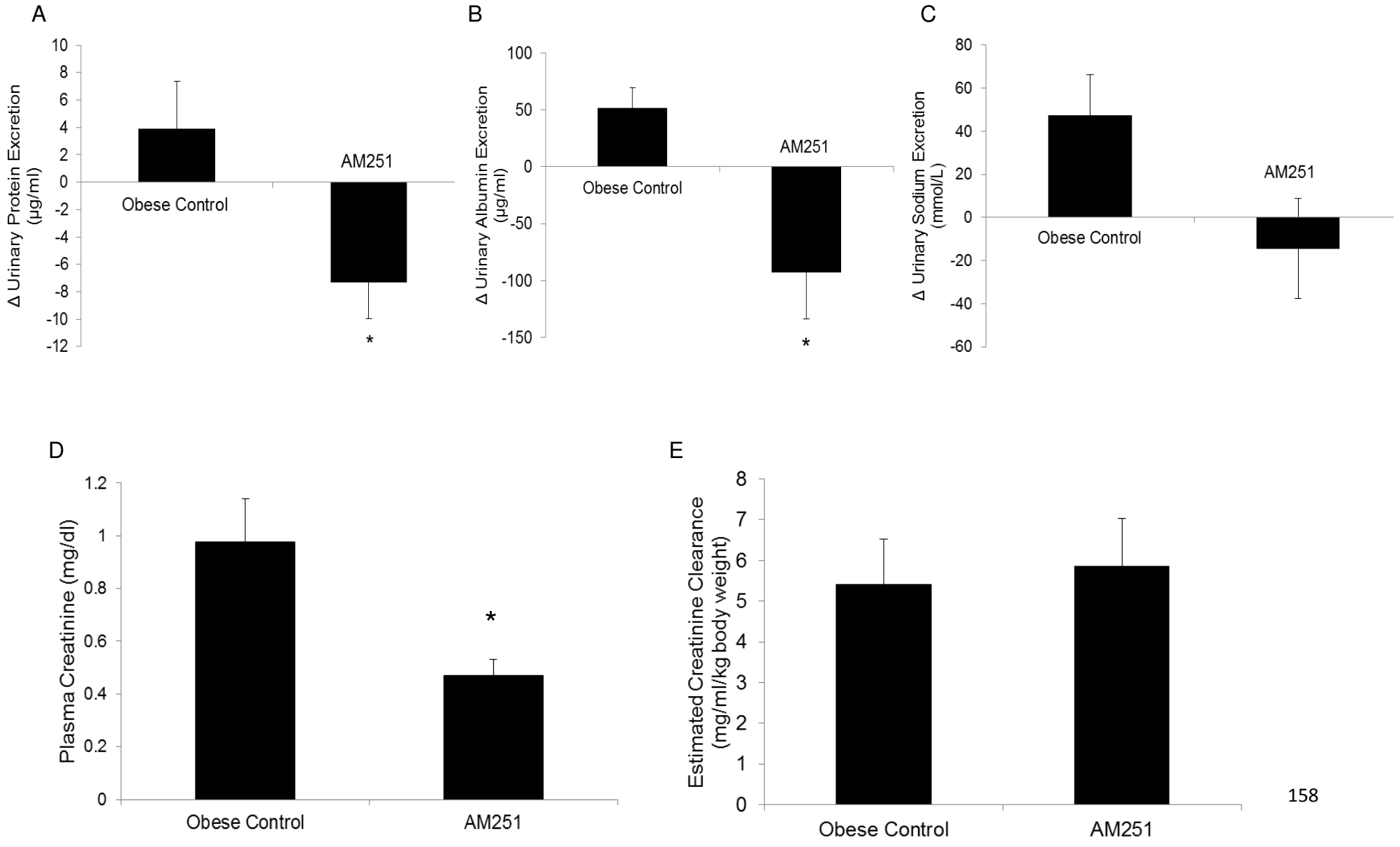
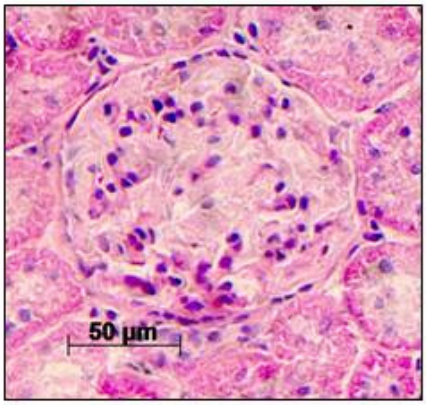

A
Δ Urinary Protein Excretion (µg/ml)
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
Obese Control
AM251
*
B
Δ Urinary Albumin Excretion (µg/ml)
100
50
0
-50
-100
-150
Obese Control
AM251
*
C
Δ Urinary Sodium Excretion (mmol/L)
80
60
40
20
0
-20
-40
-60
Obese Control
AM251
D
Plasma Creatinine (mg/dl)
1.2
1
0.8
0.6
0.4
0.2
0
*
Obese Control
AM251
E
Estimated Creatinine Clearance (mg/ml/kg body weight)
8
7
6
5
4
3
2
1
0
Obese Control
AM251

**Figure 6.3: Functional renal outcomes obese animals treated with AM251 compared to obese control. A.** Urinary Protein was significantly reduced in obese AM251 treated compared to obese control animals. **B.** Urinary albumin was significantly reduced in obese animals treated with AM251 compared to obese control across the treatment period. **C.** Urinary sodium excretion was not significantly altered by AM251 treatment in obese animals compared to obese control animals **D.** Plasma creatinine levels were significantly lower in obese AM251 treated animals compared to obese control. **E.** Estimated creatinine clearance was not significantly different between obese treated AM251 and obese control groups. Data represented average ± SEM, significance is indicated with * ($p < 0.05$ $n = 9$).

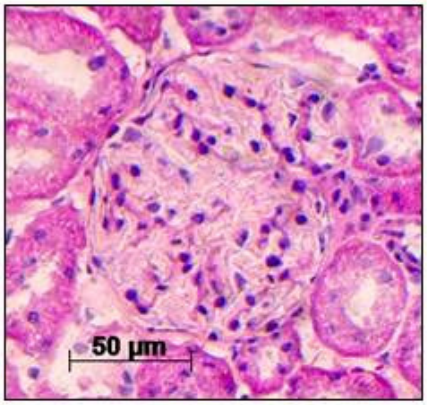
### *6.4.3 Effect of AM251 Treatment in Obese Animals on Renal Histology*

In obese rats, AM251 treatment did not have any significant effects on gross kidney weight, kidney weight standardised for body weight or glomerular cross sectional area compared to obese controls (Figure 6.4E). Histological analysis with PAS staining showed that tubular cross sectional area was significantly smaller in obese AM251 treated animals (10.6 ± 0.32 μm) compared to obese control (12.5 ± 0.23 μm), Figure 6.4C-E.

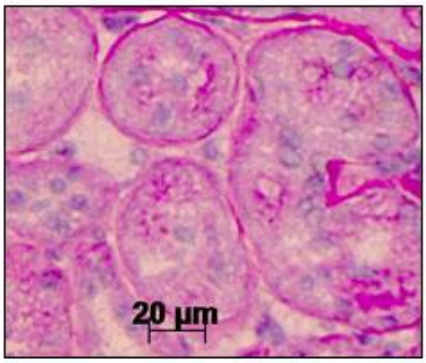
A



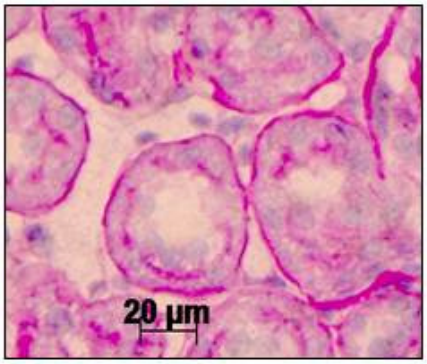

B




C




D




E

| Measure | Obese Control | AM251 |
|---|---|---|
| Kidney weight (g) | 1.67 ± 0.05 | 1.68 ± 0.05 |
| Kidney weight/ Body weight (%) | 0.25 ± 0.01 | 0.27 ± 0.01 |
| Glomerular Cross Section (µm) | 126.2 ± 4.49 | 120.7 ± 3.22 |
| Tubular Cross Section (µm) | 12.5 ± 0.23 | 10.6 ± 0.32 * |

**Figure 6.4: Effect of AM251 treatment in obese rats on renal histology**. Glomerular H&E stained sections and PAS stained tubular sections were from 5 animals in each group; imaged at 200X magnification and analyzed using AxioVision software for cross sectional area. **A.** Glomerulus of obese control animal **B.** Glomerulus of AM251 treated obese animal **C.** Tubules of obese control animal **D.** Tubules of AM251 treated obese animal **E.** Gross and histological measurements of renal tissue, data represented group average ± SEM, significance is indicated by * ($p < 0.05$, $n = 9$).

### *6.4.4 Cytokine Profile of AM251 Treated Animals*

A number of cytokines, including TGF-β1 and VEGF are known to mediate structural changes including increased collagen deposition in obesity related renal damage. To identify if AM251 treatment mediate structural and functional improvements in DIO via these cytokines, immunoassays of plasma and Western blot analysis of kidney tissue was performed. In obese rats, plasma concentrations of TGF-β1 were not altered by treatment with AM251 compared to obese controls (Figure 6.5). Western blot analysis of Collagen IV, TGF-β1 and VEGF showed that treatment with AM251 in obese rats did not significantly alter levels of these proteins in kidney tissue (Figure 6.6).

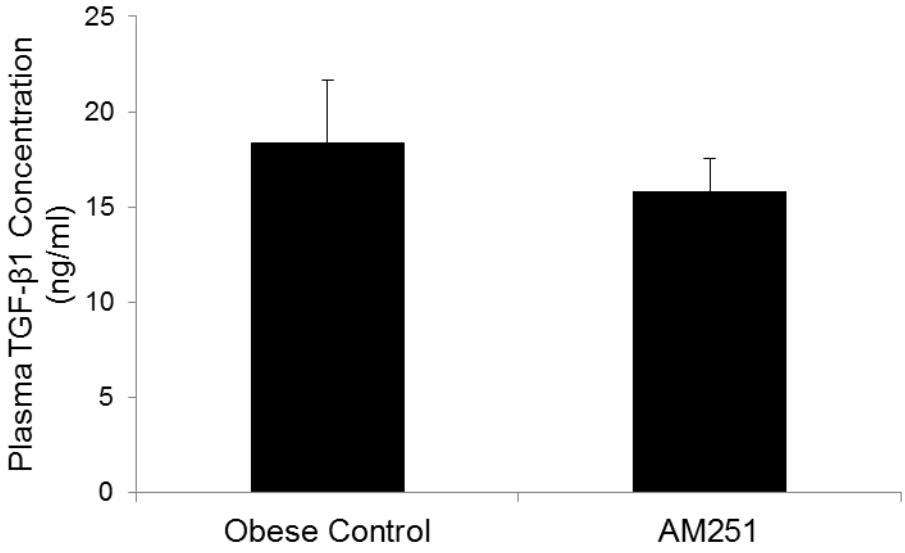
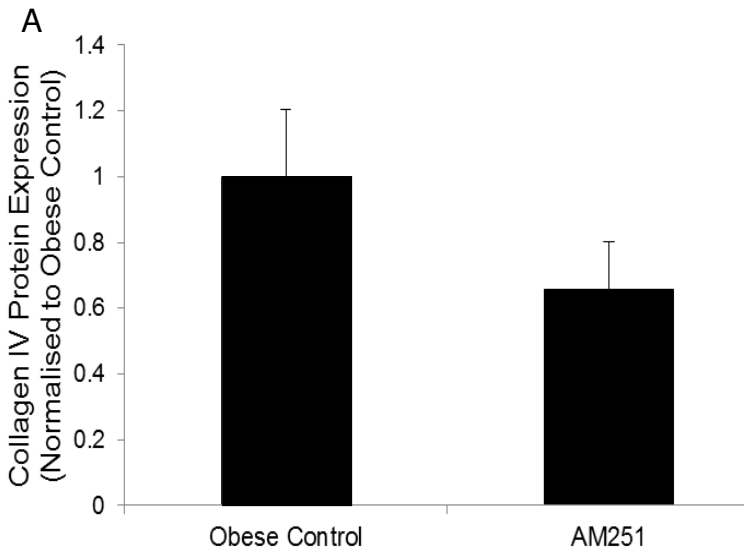
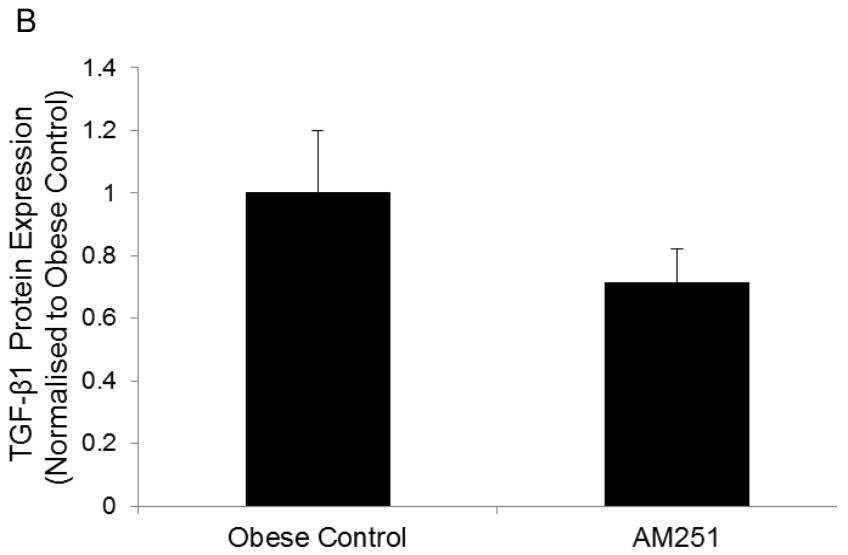
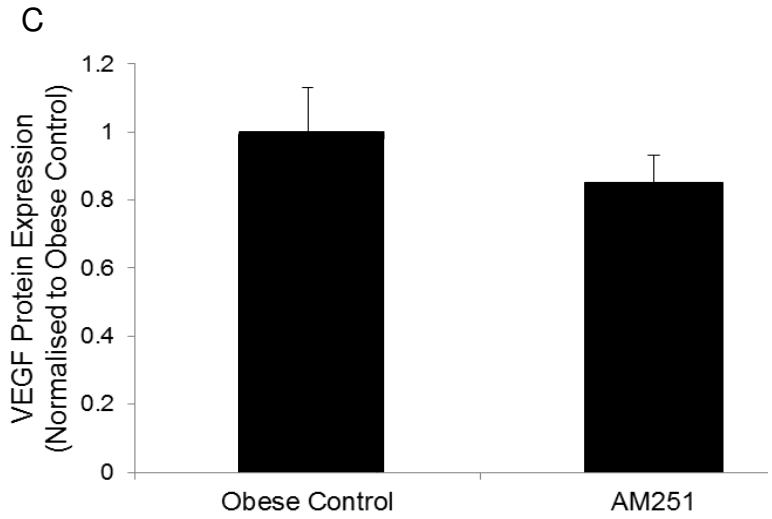



**Figure 6.5: Plasma concentrations of TGF-β1 in AM251 treated obese animals compared to obese controls.** No significant differences in plasma TGF-β1 concentreations was detected between groups (n = 9).


A
Collagen IV Protein Expression (Normalised to Obese Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
AM251


B
TGF-β1 Protein Expression (Normalised to Obese Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
AM251


C
VEGF Protein Expression (Normalised to Obese Control)
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
AM251

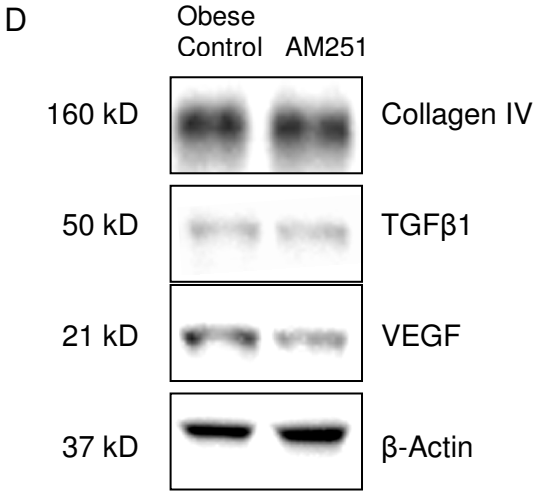

D
Obese Control
AM251
160 kD
Collagen IV
50 kD
TGFβ1
21 kD
VEGF
37 kD
β-Actin

**Figure 6.6: Protein expression of cytokines in kidney tissue of obese rats following AM251 treatment compared to obese control.** . **A.** Densitometry of Collagen IV protein expression demonstrated no significant differences between obese control and AM251 treated obese animals. **B.** Densitometry of TGF-β1 protein expression demonstrated no significant differences between obese control and AM251 treated obese animals. **C.** Densitometry of VEGF protein expression demonstrated no significant differences between obese control and AM251 treated obese animals **E.** Representative Western blots of proteins in kidney tissue of obese control and AM251 treated obese animals.

## *6.5 Discussion*

Recent research into obesity associated kidney disease has found that overweight individuals have a 40% increase in risk of kidney disease than individuals of normal weight (Abrass, 2004, Wang et al., 2008b). Further, overweight or obese individuals have worse outcomes regarding progression to end stage renal failure and treatment options such as successful kidney transplantation (Wang et al., 2008b), highlighting that obesity is an important risk factor for the development and progression of CKD (Abrass, 2004, De Jong et al., 2002). The positive effects of CB1 blockade on peripheral metabolism has been extensively reported in both human and animal models of obesity and diabetes (Isoldi and Aronne, 2008, Janiak et al., 2007, Silvestri and Di Marzo, 2012, Croci et al., 2003, Dol-Gleizes et al., 2009, Gary-Bobo et al., 2006). Recently, it has been proposed that CB1 antagonists contribute to not only systemic metabolic improvements, but also ameliorates pathophysiological changes which occur within the kidneys under diabetic conditions (Barutta et al., 2011, Janiak et al., 2007, Lim et al., 2010, Nam et al., 2012). Here we have demonstrated CB1 antagonism with AM251 in a model of DIO results in both metabolic and renal improvements in obese rats.

In agreement with previously published data (Després et al., 2005, Rosenstock et al., 2008, Van Gaal et al., 2005), we have shown that CB1 blockade limits weight gain in DIO. The effect was found to be significant compared to obese control animals after just one week of treatment of 3mg/kg/day AM251 and was sustained throughout the six week experimental period. Hyperleptinaemia positively correlates with the degree of obesity (Tam et al., 2012, Wolf et al., 2002) and is also associated with increased

TGF-β1 production and collagen deposition leading to increased fibrosis in CKD (Briffa et al., 2013, Wolf, 1999). CB1 antagonism has been shown to reverse hyperleptinaemia in obesity (Tam et al., 2012). Leptin is primarily reabsorbed from the filtrate by the transmembrane protein megalin in the renal proximal tubule (Hama et al., 2004). Even in the presence of hyperleptinaemia, the amount of leptin present in the urine is negligible, thus indicating that in the obese state, renal tubular absorption of leptin is significantly increased (Cumin et al., 1997). We have found that AM251 treatment in obese rats treated with AM251 had significantly reduced weight gain and body fat composition, indicating that reduced circulating leptin levels may also be contributing to improved renal function.

Further, AM251 also significantly reduced systolic blood pressure of DIO animals compared to control animals. The role of CB1 in the cardiovascular system is complex; the effect of the receptor on mediating haemodynamics depends on the experimental context, species of animal and in clinical trials, patient background (Sarzani, 2008). For instance, activation of CB1 with AEA and THC (endogenous and plant derived cannabinoid receptor ligands) in both humans and rodents have been shown to exert potent reductions in blood pressure, however this effect was mainly attributed to reduced cardiac contractility rather than reductions to peripheral resistance (Pacher et al., 2005). In contrast, CB1 blockade with Rimonabant in an animal model of liver cirrhosis, treatment was observed to significantly reduced systolic blood pressure in rats (Bátkai et al., 2001). It has been suggested that obesity may be a key factor mediating the role of CB1 in cardiovascular function, which in some part may explain our finding that systolic blood pressure was significantly reduced with treatment of AM251 in our model of DIO, while in other

animal models of nephropathy, this effect was not observed (Barutta et al., 2010, Janiak et al., 2007). It is possible that antagonism of CB1 with AM251 may be affecting systolic blood pressure via either sympathetic nervous system activity or RAAS activation; both systems are implicated in changes to systemic haemodynamics. While CB1 is known to be distributed extensively throughout the nervous system (Svíženská et al., 2008), traditionally treatment with CB1 antagonists increases activation of the peripheral sympathetic nervous system and norepinephrine production (Bellocchio et al., 2013, Ishac et al., 1996, Quarta et al., 2011). This suggests that it is unlikely that AM251 maintains renal structure and reduces systolic blood pressure via decreased sympathetic tone. While we have not investigated the mechanism behind AM251 treatment leading to reduction of systolic blood pressure in obese rats, importantly, systolic blood pressure is correlated with degree of nephropathy in Zucker rats (González-Albarrán et al., 2003).

This study found that in obese animals treated with AM251, significant improvements in some functional renal markers was observed, including reductions in total urinary protein and albumin levels. However, AM251 treatment in obese animals did not significantly alter sodium excretion and creatinine clearance compared to obese control animals. These findings are congruent with past studies of CB1 blockade ameliorating the progression of albuminuria in diabetic nephropathy (Barutta et al., 2010, Janiak et al., 2007, Nam et al., 2012). While these authors contributed improvements in renal function to effects which can be categorised as either systemic metabolic outcomes (weight loss, reduction in sympathetic nervous system activity) or glomerular outcomes (reduction in podocyte loss), they failed to examine the possible role of targeting CB1 within the tubules of the nephron. Here, we found

that obese control animals had significantly greater tubular cross sectional area than AM251 treated obese animals. In nephropathy, often the first structural modification to occur is hypertrophy (Wolf and Ziyadeh, 1999, Zerbini et al., 2006). We have previously established that in proximal tubule cells, CB1 can mediate hypertrophy under normal conditions, with AM251 causing significant reductions in HK2 cell hypertrophy *in vitro* (Jenkin et al., 2010). Albumin is a plasma protein which is filtered by the glomerulus, and is largely reabsorbed by proximal tubule cells; damage to either of these structures can result in albuminuria (Birn and Christensen, 2006). In this study no significant changes to glomerular histology or size was observed, however significant differences in tubular size in AM251 treated animals was detected, indicating that CB1 blockade may exert changes to urinary albumin and total urinary protein via improvements in tubular structure. It is unlikely however that reduced tubular size is the only contributing factor to improved albuminuria levels in AM251 treated animals with DIO. Reductions in systolic blood pressure and weight gain were also observed in these animals, which may affect the progression of proteinuria and albuminuria, along with a number of other parameters (including podocyte loss, RAAS system involvement, renal ROS production and sympathetic nervous system activity) were not examined.

This study also showed that AM251 treatment in obese animals significantly altered plasma creatinine levels, which was significantly reduced compared to obese control animals. Creatinine clearance is a method commonly used in both a clinical and experimental setting to evaluate GFR (Regeniter et al., 2009). Creatinine is a by-product of muscle metabolism and is primarily removed from the blood via glomerular filtration, making it an ideal marker for GFR (Toffaletti and McDonnell,

2008). When GFR declines, creatinine urinary excretion also declines, and higher levels of creatinine remain in the blood. Interestingly, although we observed a significant reduction in plasma creatinine of obese animals treated with AM251, no differences were found between groups for estimated creatinine clearance standardised to body weight. It is likely that due to the diverse response Sprague Dawley rats exhibit when consuming a high fat diet, as noted by Levin et al., (1997), a measurement reliant on body weight may have been too underpowered (n = 9) to pick up significant differences in creatinine clearance standardised for body weight.

Finally, it was found that treatment with AM251 in obese animals did not significantly alter TGF-β1, VEGF or collagen IV protein expression in the kidney. While these proteins are known to be important in the structural modifications which take place in nephropathy, other studies have previously identified that CB1 blockade in diabetes does not significantly affect these markers (Barutta et al., 2010). To fully elucidate the role of CB1 within the renal system, it is important to identify key signalling pathways through which CB1 may be exerting physiological effects. *In vitro*, CB1 antagonism with AM251 in proximal tubule cells exposed to hyperlipidemia conditions reduced apoptosis via signalling the CHOP (C/EBP homologous protein) pathway which evokes endoplasmic reticulum stress (Lim et al., 2010). In a model of nephropathy induced by the chemotherapeutic drug cisplatin, use of CB1 receptor antagonist AM281 and SR141716 attenuated markers of oxidative/nitrosative stress and cell death (Mukhopadhyay et al., 2010a). Further investigation is required to determine which downstream targets the CB1 receptor may be mediating in a DIO of nephropathy.

## ***6.6 Conclusion***

This study has demonstrated that in a model of DIO, AM251 treatment can produce significant improvements in both metabolic and renal parameters. CB1 antagonism resulted in significant reductions in weight gain, systolic blood pressure, proteinuria, albuminuria, plasma creatinine levels and renal tubule cross sectional area compared obese controls. This study identifies a potential role of CB1 blockade for the treatment of obesity related renal damage. Further investigation is required to identify which signalling pathways the CB1 receptor may be acting on to fully elucidate the role of CB1 within the renal system under obese conditions.

# Chapter 7 – Renal Effects of Chronic Pharmacological Manipulation of CB2 in Rats with Diet Induced Obesity

## *7.1 Summary*

Activation of CB2 may be beneficial in ameliorating the effects of diabetic nephropathy through the reduction of albuminuria, podocyte loss and macrophage infiltration (Barutta et al., 2011), however the role of CB2 in modulating the progression of obesity related renal damage has yet to be elucidated. The aim of this study was to determine metabolic and renal adaptations which occur in response to the CB2 agonist AM1241 and CB2 antagonist AM630 in an animal model of DIO. Following nine weeks of a HFD to induce obesity, male Sprague Dawley rats were injected with either 3 mg/kg body weight of AM1241 (n = 9) , 0.3 mg/kg body weight of AM630 (n = 10), or saline (n = 9) for six weeks, while being maintained on the HFD. Treatment with AM1241 in obese animals significantly reduced systolic blood pressure, urinary protein, albumin and sodium excretion and significantly lowered renal protein expression of the fibrotic markers collagen IV, TGF-β1 and VEGF compared to obese control animals. CB2 antagonist treatment with AM630 lead to significant increases in urinary albumin, kidney to body weight ratio and a reduction in TGF-β1 protein expression in renal tissue of obese animals compared to obese controls. Treatment with both CB2 compounds independently, was shown to have no effect on body weight, diastolic blood pressure, creatinine clearance or plasma creatinine or plasma TGF-β1 concentrations in animals with DIO. This study demonstrates that independent of body weight changes, activation of CB2 leads to

improvements in a number of key markers of obesity related renal dysfunction. Thus, highlighting CB2 as a potential therapeutic target for the amelioration of the progression of obesity related renal damage.

## ***7.2 Background***

Chronic activation of CB2 for a period of 14 weeks may have a protective role in ameliorating markers of kidney damage, specifically albuminuria and glomerular podocyte loss associated with diabetic nephropathy (Barutta et al., 2011, Pacher and Mechoulam, 2011). It is still unclear however, what role the CB2 receptor may play in modulating renal tissue in the obese state. In Chapter 5, it was shown that CB2 expression was significantly reduced in renal tissue of rats with DIO after 10 weeks on a HFD. However, it is unknown if modulation of CB2 can alter the renal pathology associated with obesity.

There are a number of pathways by which obesity contributes to the development of kidney disease, including insulin resistance and activation of the sympathetic nervous system leading to hypertension (Cignarelli and Lamacchia, 2007b). Further, the obese state leads to inflammation and the activation of a number of key cytokines and growth factors which are produced by cells of the kidney and invading macrophages (Abrass, 2004). CB2 activation has been observed to attenuate inflammation via modulation of cytokine production, prevention of immune cell infiltration and abrogation of fibrotic factors in a number of pathological conditions (Pacher and Mechoulam, 2011), including in acute nephropathy induced by the chemotherapeutic drug cisplatin (Mukhopadhyay et al., 2010b). However, there is conflicting evidence as to whether this is the case in all tissue types and disease

states, where the CB2 receptor has been to shown either attenuate or promote inflammation depending on experimental protocols used (Deveaux et al., 2009, Pacher and Mechoulam, 2011). In a model of STZ induced diabetic nephropathy using C57BL6/J mice, it has been shown that CB2 agonist AM1241 significantly reduces inflammatory markers CCR2 and MCP-1 (Barutta et al., 2011). Interestingly, although a reduction in inflammatory markers were observed, AM1241 treatment was not effective in attenuating glomerular hypertrophy in diabetic animals or reducing fibrotic markers TGF-β1 or collagen mRNA levels in renal tissue (Barutta et al., 2011).

Specifically in obesity, it is yet to be elucidated how CB2 activation and blockade may modulate renal function and structure. The aim of this study was to determine metabolic and renal adaptations which occur in response to the CB2 agonist, AM1241, and antagonist, AM630, in an animal model of DIO. We also sought to identify whether potential fibrotic targets known to mediate structural renal changes are altered in response to AM1241 and AM630 compounds. Based on evidence from other models of nephropathy, it was hypothesised that pharmacological activation, but not blockade of the CB2 receptor will improve renal structure and function in a model of DIO, and lead to reductions in fibrotic proteins in the kidney of obese animals.

## ***7.3 Materials and Methods***

### *7.3.1 Animals and Experimental Protocol*

Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour light/ dark cycle (7:00 - 19:00). Rats were fed *ad libitum* a HFD (containing 21% fat, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Rats were then matched using a number of metabolic parameters and put into either obese control (n = 9), CB2 agonist, AM1241 (n = 9) or CB2 antagonist, AM630 (n = 10) treatment groups. For six weeks, rats were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80, or 3 mg/kg of AM1241 or 0.3 mg/kg AM630, with compound dissolved in vehicle saline solution and administered via IP injection. Following the conclusion of the experimental protocol, rats were deeply anesthetised and killed via cardiac puncture. Organs were removed, weighed and stored for further analysis, as outlined in Section 2.6. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036).

### *7.3.2 Metabolic Measurements*

Rat weight and food was recorded daily throughout the experimental period. Body composition and blood pressure was analyzed (as outlined in Section 2.5) for pre- and post-treatment time points. ‘Pre-treatment’ measurements were conducted the week before saline or CB2 treatments commenced and ‘post-treatment’ measurements in the final week of treatment.

### *7.3.3 Functional Measurements of Renal Parameters*

Renal measurements including creatinine clearance, urinary protein, albumin and sodium excretion were evaluated using 24 hour urine samples collected pre- and post-treatment periods and plasma collected at time of death, as outline in Section 2.7.

### *7.3.4 Histological Analysis*

To determine what structural changes occurred in response to AM1241 and AM630 treatment in this model of DIO in Sprague Dawley rats, histological analysis examining glomerular and tubular cross sectional area was performed. Following kidney dissection, a portion of kidney tissue was fresh frozen, as outlined in Section 2.6.1 and sections were stained H&E and PAS staining was performed as outlined in Section 2.6. Sections were imaged at 200 X magnification (Carl Ziess microscope) and glomerular and tubular cross sectional area was analyzed using AxioVision 4.8 software.

### *7.3.5 Cytokine Profile*

The CB2 receptor has a clearly identified role in inflammatory and immune response in health and disease (Lombard et al., 2007, Mukhopadhyay et al., 2010b, Pacher and Mechoulam, 2011, Ueda et al., 2005). To determine whether CB2 agonist and antagonist treatments affects renal cytokine profile, a number of key proteins known to mediate renal structural changes in DIO were examined. TGF-β1 plasma expression was analyzed using immunoassay techniques outlined in Section 2.8.2. Kidney tissue (40 µg) was analyzed for protein expression of TGF-β1, Collagen IV

and VEGF via Western blot analysis. Concentrations and supplier information of antibodies are outlined in Section 2.2.3 and in Table 2.2. Secondary antibodies anti-mouse and anti-rabbit were purchased from Sigma Aldrich (St Louis, MO). Band densitometry was analyzed using Image Lab software.

### *7.3.6 Statistical Analysis*

The SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Differences between treatment groups were individually analyzed compared to obese animals using independent samples T-Test for two group direct analyses or mixed model ANOVA for analysis of pre- and post-treatment measurements between treatment and obese control animals. Significance was accepted when $p < 0.05$.

## ***7.4 Results***

### *7.4.1 Metabolic Parameters in Control and CB2 Treatment Groups*

Prior to treatment, average group weights of obese control (582.9 ± 14.35 g), AM1241 (618.9 ± 21.85 g) and AM630 (626.0 ± 10.12 g) animals were not significantly different. Treatment with CB2 agonist AM1241 and CB2 antagonist AM630 did not alter weight gain during treatment period (Figure 7.1). No significant alteration in diastolic blood pressure across the treatment period was detected between obese control, AM1241 and AM630 groups (Figure 7.2A). However, compared to obese control animals (137 ± 4.89 mmHg pre-treatment, 144 ± 7.07 post-treatment mmHg), systolic blood pressure of AM1241 treated animals was significantly reduced by treatment (Figure 7.2B 153 ± 5.47 mmHg pre-treatment, 129

± 7.45 mmHg post-treatment). No significant differences in systolic blood pressure for animals treated with AM630 (159 ± 7.30 mmHg pre-treatment, 146 ± 6.88 mmHg post-treatment) were observed compared to obese control and AM1241 treated animals (Figure 7.2B).

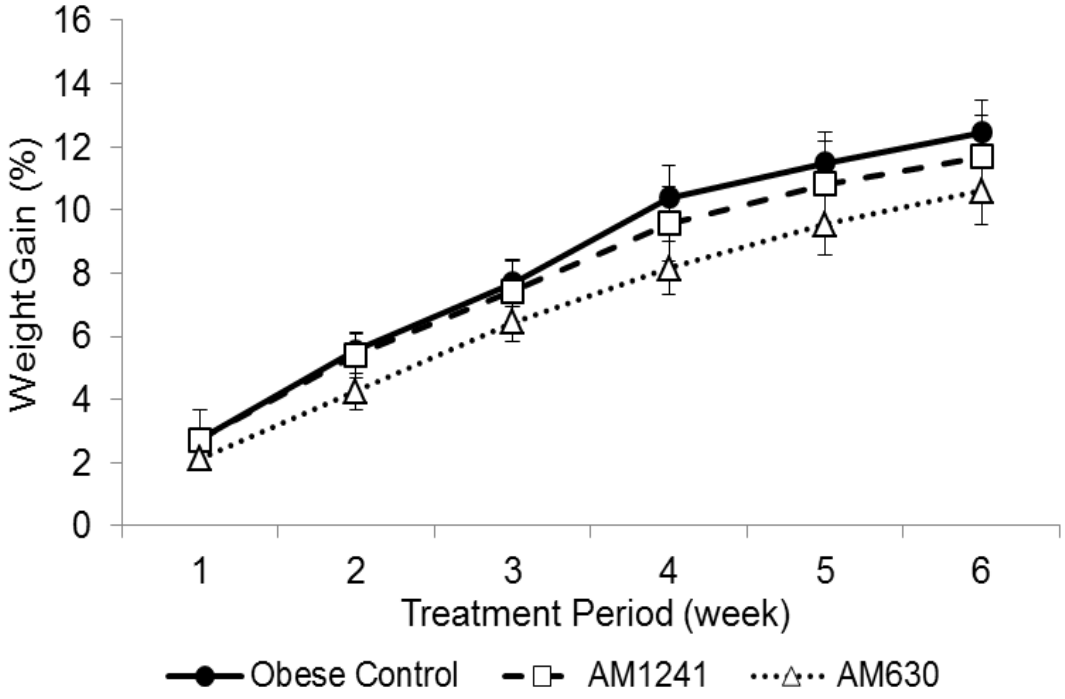



**Figure 7.1: Percentage weight gain in AM1241 and AM630 treated animals, following nine weeks of DIO compared to obese controls.** No significant differences in weight gain was observed between obese control, AM1241 and AM630 treated obese animals (n = 9 - 10).

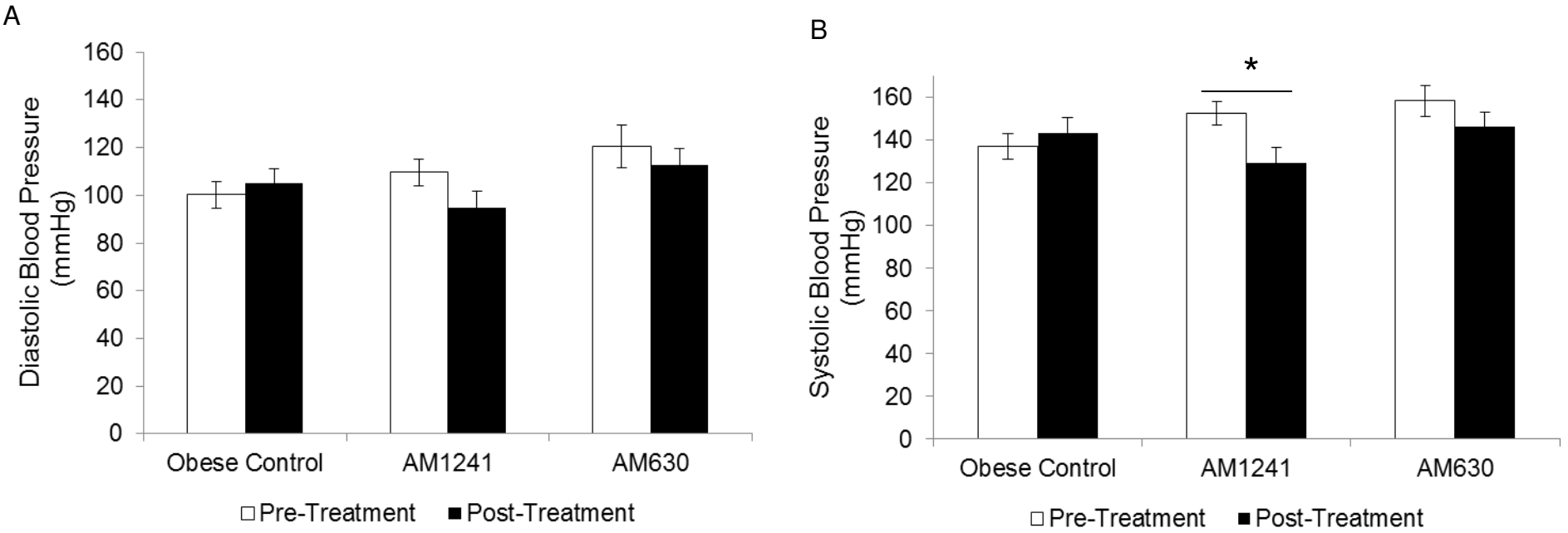



**Figure 7.2: Blood pressure of CB2 treated groups compared to obese controls.** **A.** Diastolic blood pressure was not significantly altered in obese animals by AM1241 or AM630 treatment compared to obese control. **B.** In obese animals, systolic blood pressure was significantly reduced by AM1241 treatment compared to obese control animals, significance is indicated by * ($p < 0.05$, $n = 9$). AM630 did not significantly alter systolic blood pressure in obese animals ($n = 10$) compared to obese controls.

### *7.4.2 Functional Renal Outcomes in AM1241 and AM630 Treated Obese Animals*

As outlined in Chapter 5, our model of DIO leads to significant increase in markers of kidney damage compared to lean animals. In this study, AM1241 treatment in obese animals was shown to significantly reduce urinary excretion of protein, albumin and sodium compared to obese controls across the treatment period (Figure 7.3). However, the pre-treatment level of total urinary protein was significantly higher in the AM1241 treated obese group compared to both obese controls and AM630 treated obese animals (Table 7.1). Treatment with AM630 did not significantly alter urinary protein, albumin or sodium excretion (Figure 7.3) in obese animals. Plasma creatinine (Figure 7.4A) was not altered by treatment of AM1241 or AM630 (Figure 7.4A) in obese animals. GFR, as estimated by creatinine clearance standardised to body weight was not altered in AM1241 treated obese animals, but was significantly reduced by AM630 treatment in obese animals compared to obese saline controls (Figure 7.4B). Table 7.1 outlines functional outcomes for all treatment groups.

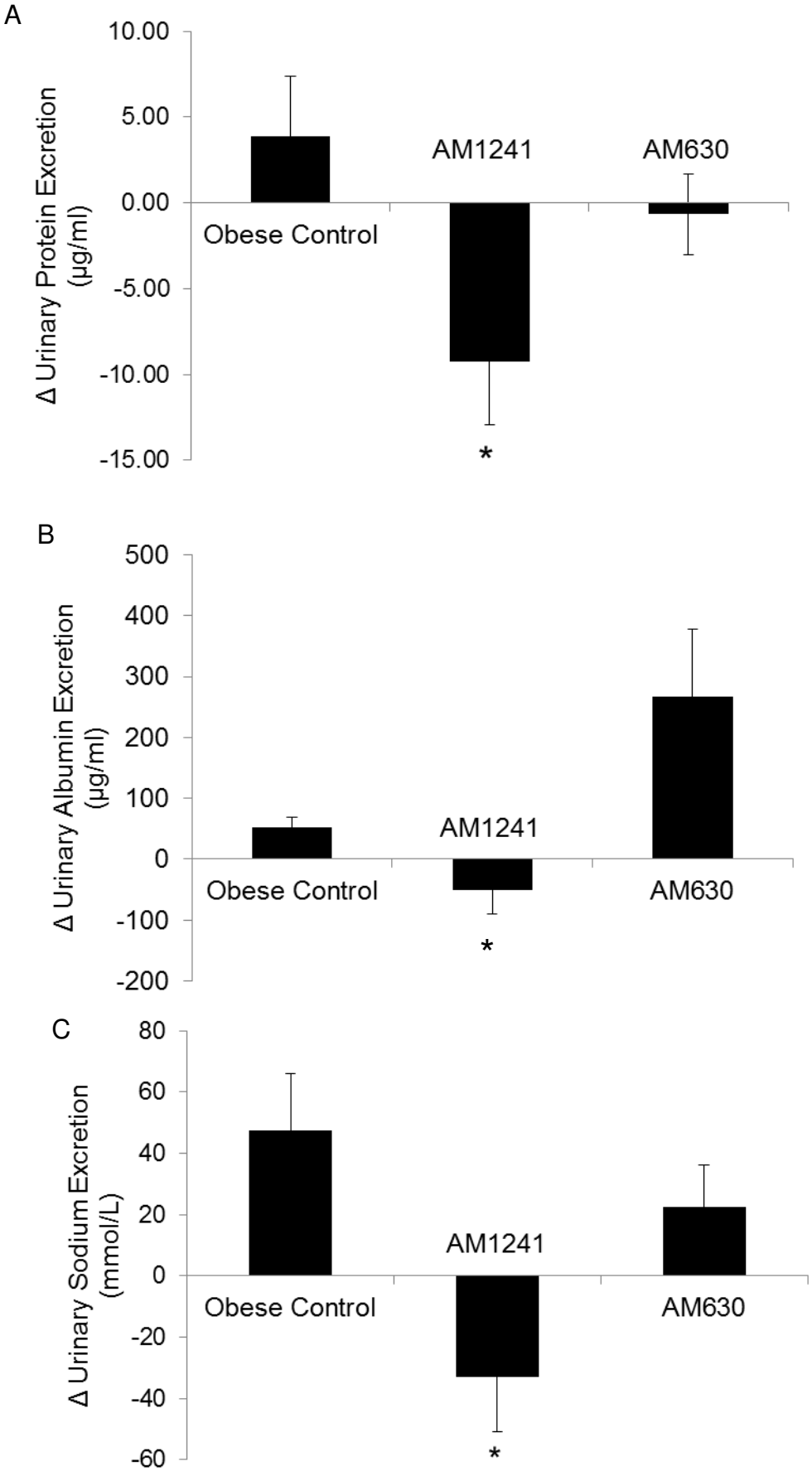

A
10.00
5.00
0.00
-5.00
-10.00
-15.00
Δ Urinary Protein Excretion (µg/ml)
Obese Control
AM1241
AM630
*
B
500
400
300
200
100
0
-100
-200
Δ Urinary Albumin Excretion (µg/ml)
Obese Control
AM1241
AM630
*
C
80
60
40
20
0
-20
-40
-60
Δ Urinary Sodium Excretion (mmol/L)
Obese Control
AM1241
AM630
*

**Figure 7.3: Functional measurements for renal parameters of AM1241 and AM630 treated obese animals compared to obese controls. A.** Urinary protein excretion was significantly reduced in obese animals treated with CB2 agonist AM1241 across the treatment period compared to obese control. No significant change in urinary protein was detected in AM630 treated obese animals. **B.** Urinary albumin excretion was significantly reduced in obese animals treated with AM1241 compared to obese control. No significant change in urinary albumin was detected in AM630 treated obese animals. **C.** Urinary sodium excretion in obese animals was significantly reduced by AM1241 treatment compared to obese control. No significant change was detected in AM630 treated obese animals. Significance is indicated by * ($p < 0.05$, $n = 9 - 10$).

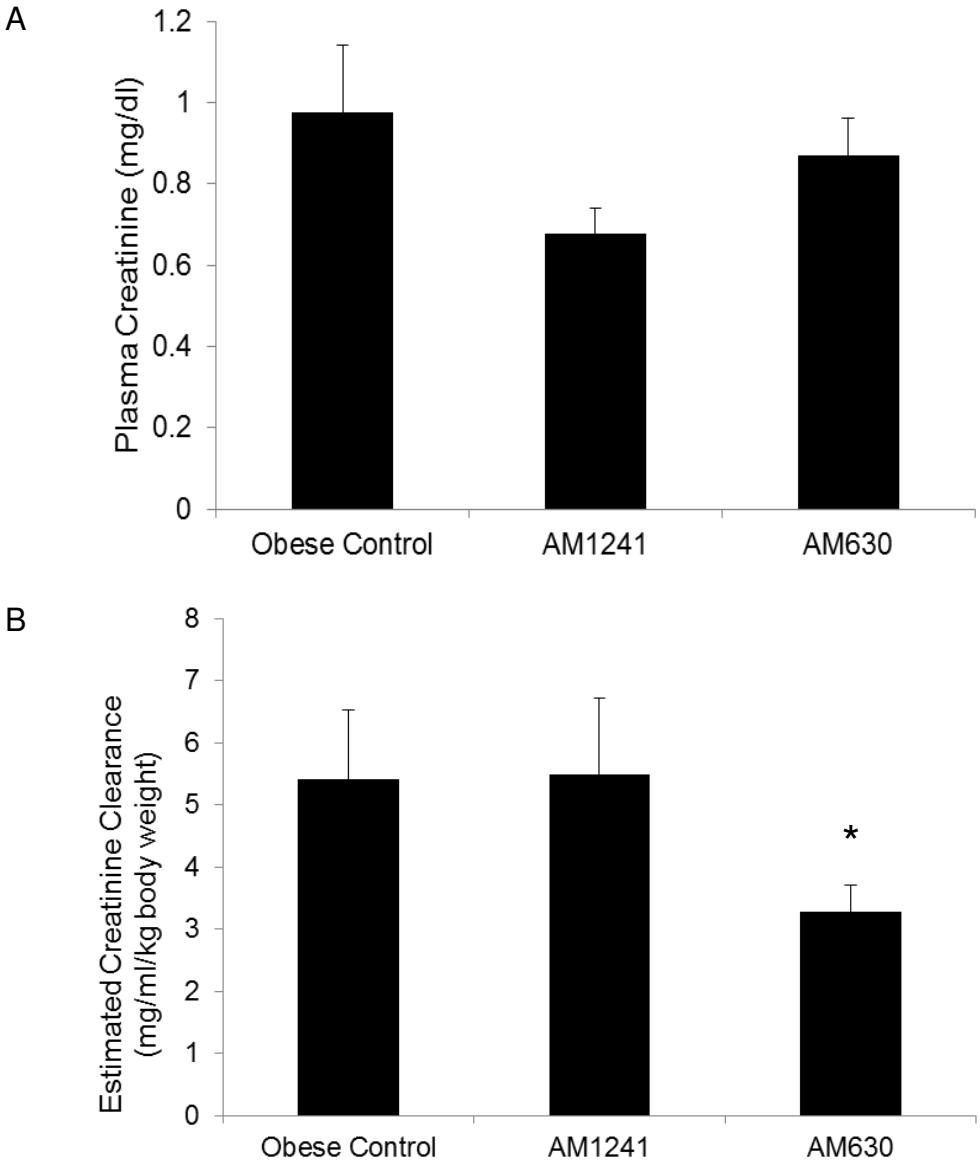



**Figure 7.4: Plasma creatinine and estimated creatinine clearance of obese animals treated with AM1241 and AM630 compared to obese controls. A.** No significant differences were observed in the plasma creatinine concentration of obese control compared to CB2 treated groups. **B.** Estimated creatinine clearance was not altered by AM1241 treatment, but was significantly reduced in AM630 treated animals compared to obese control, as indicated by * ($p < 0.05$, $n = 9 - 10$).

**Table 7.1: Functional Renal Outcomes of CB2 Treatment Groups Compared to Obese Controls.** Plasma creatinine and creatinine clearance are post-treatment measurements only. Data is shown as average ± SEM. Significance is indicated between AM1241 and AM630 treatment groups and obese controls at the same time point (pre- or post-treatment) by * ($p < 0.05$, $n = 9$ -10).

| **Measure** | **Obese Control** | | **AM1241** | | **AM630** | |
|---|---|---|---|---|---|---|
| | *Pre treatment* | *Post treatment* | *Pre treatment* | *Post Treatment* | *Pre treatment* | *Post Treatment* |
| Urinary Protein (µg/ml) | 15.34 ± 1.15 | 19.10 ± 2.96 | 23.11 ± 2.44 * | 14.09 ± 3.04 | 14.10 ± 1.46 | 13.44 ± 1.85 |
| Urinary Albumin (µg/ml) | 103.1 ± 44.0 | 226.5±118.6 | 245.3 ±85.1 | 110.3 ± 34.2 | 247.0±104.9 | 458.2 ± 200.9 |
| Urinary Sodium (mmol/L) | 146 ± 7.28 | 196 ± 20.32 | 182 ± 14.79 | 151 ± 13.79 | 147 ± 10.23 | 169 ± 16.80 |
| Plasma Creatinine (mg/dl) | - | 0.97 ± 0.16 | - | 0.67 ± 0.06 | - | 0.86 ± 0.09 |
| Creatinine Clearance (mg/ml/kg body weight) | - | 5.41 ± 1.10 | - | 5.50 ± 1.22 | - | 3.05 ± 0.45 * |

### *7.4.3 Effect of AM1241 and AM630 on Renal Structure of Obese Animals*

Histological analysis showed that in obese animals, treatment with CB2 agonist AM1241 did not affect glomerular gross sectional area, gross kidney weight or kidney weight standardised to body weight compared to obese control animals (Figure 7.5). In obese animals, treatment with AM1241 did significantly reduce tubular cross sectional size (9.8 ± 0.60 μm).compared to obese control (12.5 ± 0.23 μm, see Table 7.2). CB2 Antagonist treatment in obese animals did not show significant changes to tubular cross sectional area (11.7 ± 0.53 μm) compared to obese control animals, however glomerular cross sectional area was significantly larger (140.5 ± 3.64 μm) in obese animals treated with AM630 than both obese controls (126.2 ± 4.49 μm) and obese animals treated with AM1241 (123.1 ± 5.01 μm). Gross kidney weight was not altered by AM1241 or AM630 treatment (Table 7.2). When kidney weight was standardised for body weight (Table 7.2), AM630 animals had significantly higher kidney weight/body weight ratio (0.29 ± 0.02%) than obese control animals (0.25 ± 0.01%).

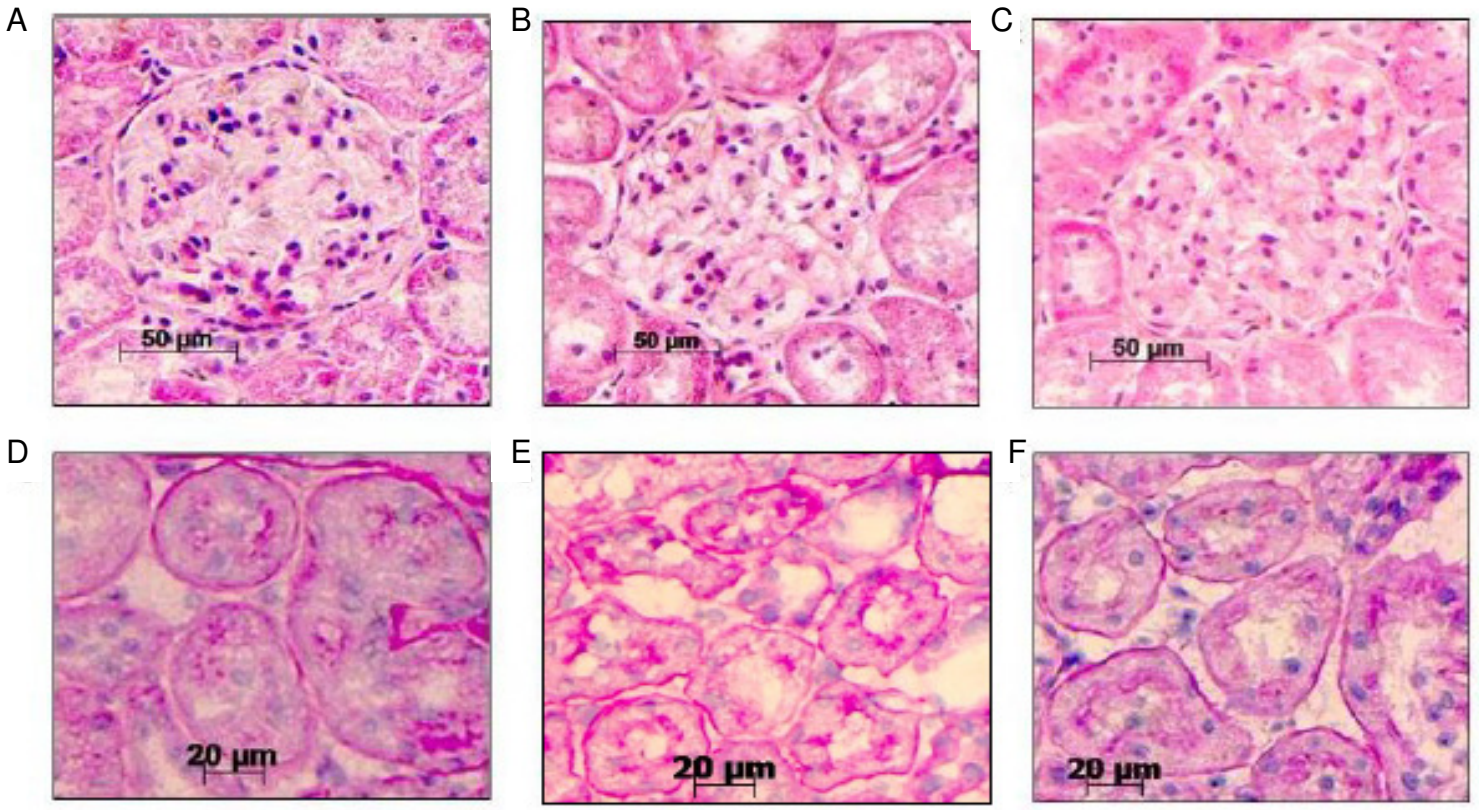



**Figure 7.5: Effect of AM1241 and AM630 treatment in obese animals on renal histology.** Glomerular H&E stained sections and PAS stained tubular sections were from 5 animals in each group; imaged at 200X magnification and analyzed using AxioVision software for cross sectional area. **A.** Glomerulus of obese control animal **B.** Glomerulus of AM1241 treated obese animal **C.** Glomerulus of AM630 treated obese animal **D.** Tubules of obese control animal **E.** Tubules of AM1241 treated obese animal **F.** Tubules of AM630 treated obese animal.

**Table 7.2: Gross and Histological Measurements of Renal Tissue in CB2 Treatment Groups and Obese Controls.** Data is shown as average ± SEM. Significance is indicated by * compared to obese control ($p < 0.05$, $n = 9 - 10$).

| Measure | Obese Control | AM1241 | AM630 |
|---|---|---|---|
| Kidney weight (g) | 1.67 ± 0.05 | 1.96 ± 0.15 | 2.00 ± 0.14 |
| Kidney weight/ Body weight (%) | 0.25 ± 0.01 | 0.28 ± 0.02 | 0.29 ± 0.02 * |
| Glomerular Cross Section (µm) | 126.2 ± 4.49 | 123.1 ± 5.01 | 140.5 ± 3.64 * |
| Tubular Cross Section (µm) | 12.5 ± 0.23 | 9.8 ± 0.60 * | 11.7 ± 0.53 |

*7.4.4 Cytokine Profile of AM1241 and AM630 Treated Obese Animals*

In obese animals, plasma TGF-β1 was not altered by treatment with AM1241 (14.75 ± 1.25 ng/mL) or AM630 (16.13 ± 1.33 ng/mL) compared to obese control (18.37 ± 3.26 ng/mL) (Figure 7.6). In whole kidney tissue, treatment with AM1241 in obese animals significantly reduced Collagen IV, TGF-β1 and VEGF protein expression compared to obese control animals (Figure 7.7). AM630 treatment in obese animals did not significant alter renal protein levels of Collagen IV or VEGF, however treatment with AM630 did significantly reduce TGF-β1 expression in obese animals compared to obese control animals (Figure 7.7).

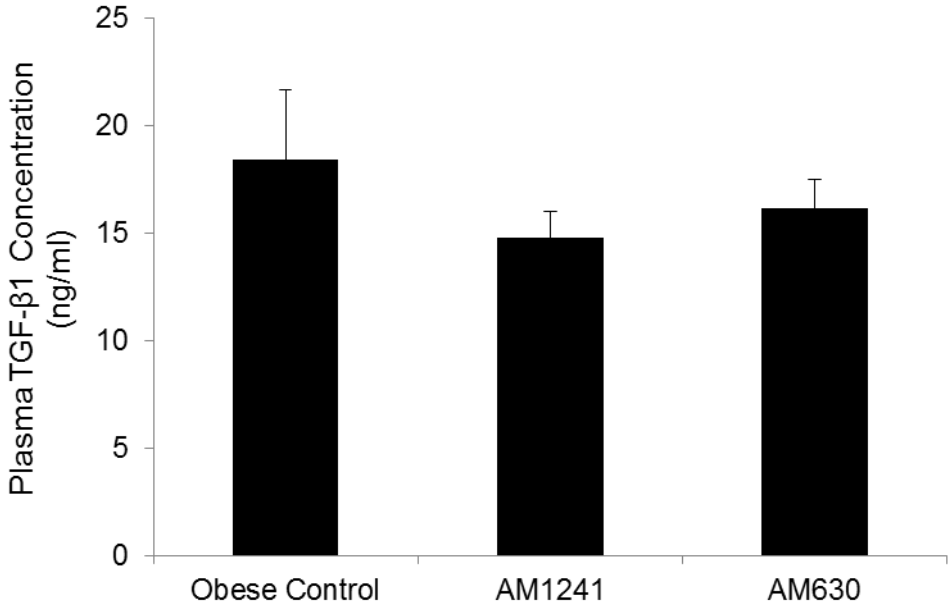



**Figure 7.6: Plasma concentrations of TGF-β1 in CB2 treatment groups compared to obese controls.** No significant differences in plasma TGF-β concentrations was observed between groups (n = 9 - 10).

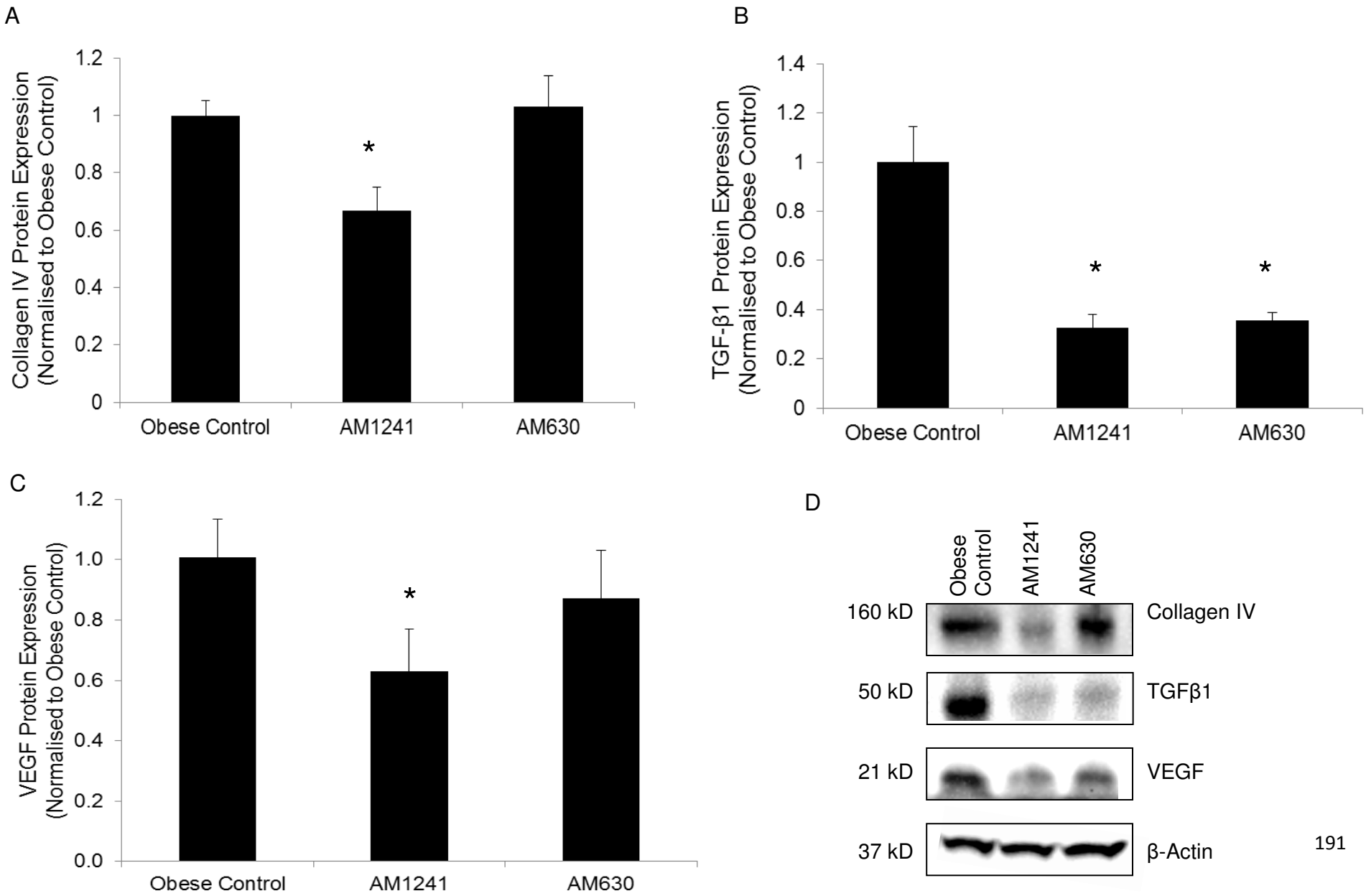

A
Collagen IV Protein Expression (Normalised to Obese Control)
1.2
1
0.8
0.6
0.4
0.2
0
*
Obese Control
AM1241
AM630
B
TGF-β1 Protein Expression (Normalised to Obese Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Obese Control
AM1241
AM630
C
VEGF Protein Expression (Normalised to Obese Control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Obese Control
AM1241
AM630
D
Obese Control
AM1241
AM630
160 kD
Collagen IV
50 kD
TGFβ1
21 kD
VEGF
37 kD
β-Actin

**Figure 7.7: Protein expression of cytokines in kidney tissue of CB2 treatment groups compared to obese controls**. **A.** Densitometry of collagen IV protein expression demonstrated that treatment with AM1241 significantly reduced collagen IV expression in obese animals compared to obese control animals. AM630 did not significantly alter renal Collagen IV expression. **B.** Densitometry of TGF-β1 protein expression demonstrated that AM1241 and AM630 treatments in obese animlas showed a significant reduction in TGF-β1 expression compared to obese control animals. **C.** Densitometry of VEGF protein expression demonstrated that treatment with AM1241 significantly reduced expression of VEGF in obese animlas compared to obese control animals. Treatment with AM630 in obese animals did not significantly alter renal VEGF expression. **E.** Representative Western blots of proteins in kidney tissue of obese control, AM1241 and AM630 treated obese animals. Significance is indiacted by * compared to obese control ($p < 0.05$, $n = 9 – 10$).

## *7.5 Discussion*

Obesity is a disease associated with a number of risk factors for chronic kidney disease including hypertension, hyperglycaemia and dyslipidaemia. However, even accounting for co-morbidities, obesity is an established independent risk factor for kidney disease (Abrass, 2004, De Jong et al., 2002). In this study we have shown for the first time in a model of DIO, CB2 activation is able to limit renal damage specifically through reduced urinary protein, albumin and sodium excretion and lowered levels of fibrotic markers including TGF-β1, collagen IV and VEGF in DIO nephropathy. These findings are consistent with data published in other models of nephropathy (Barutta et al., 2011, Mukhopadhyay et al., 2010b). Further, we have demonstrated that in obese animals, CB2 antagonism with AM630 can exacerbate markers of kidney damage, with antagonism of CB2 leading to increased albuminuria and kidney weight relative to body weight compared to obese control animals. Importantly, we have shown that after the onset of renal symptoms arising from DIO, modulation of CB2 can alter the pathophysiological phenotype. Previously it has been suggested that CB2 agonist treatment leads to improved renal function in diabetic nephropathy via a reduction in podocyte loss (Barutta et al., 2011). Here we have shown that the CB2 agonist AM1241 in a model of DIO significantly reduces fibrotic markers and tubular hypertrophy compared to obese controls.

Treatment with the CB2 agonist and antagonist did not significantly alter weight gain in DIO across the treatment period. This finding was consistent with the general understanding of how the endocannabinoid system is involved in energy homeostasis, with most effect on weight loss being mediated predominately via CB1

(De Kloet and Woods, 2009). While CB2 has been shown to mediate glucose metabolism, with activation of the receptor leading to improved glucose tolerance (Bermudez-Silva et al., 2007), its role in overall energy homeostasis and therapeutic utility in DIO has yet to be fully elucidated.

Even without significant changes to weight, we have shown that CB2 agonist AM1241 treatment was able to significantly reduce systolic blood pressure in animals with DIO compared to obese control animals. Hypertension can lead to changes in renal haemodynamics which can advance the structural remodelling and damage which occurs in the kidneys in obesity (Kramer et al., 2005). Systolic blood pressure specifically has been demonstrated to be an independent and stronger predictor of renal disease than diastolic blood pressure in adult males (He and Whelton, 1999). Currently, there is strong evidence which shows that endocannabinoids and synthetic analogues are involved in complex regulation of the cardiovascular system via central and peripheral nervous system modulation by primarily CB1 and TRPV1 (Pacher et al., 2005). The CB2 receptor is thought to play a very limited role in regulating the cardiovascular system in normal conditions but may pose a protective role in a pathophysiological setting (Pacher and Mechoulam, 2011). We have shown that treatment with the CB2 agonist, AM1241, significantly reduced blood pressure in rats with DIO, while treatment with the CB2 antagonist, AM630, had no effect. These findings are congruent with a study performed in lean normotensive mice where CB2 agonist HU-308 had acute hypotensive effects, while CB2 antagonist SR144528 were able to partially block the effect of the agonist and when SR144528 was administered alone, blood pressure was not significantly altered compared to control animals (Hanuš et al., 1999).

A reduction in systolic blood pressure can lead to improvements in markers of renal damage including reduced urinary protein, albumin and sodium excretion following AM1241 treatment in obese rats. Hypertension observed in obese animals in our study, can lead to alterations to renal haemodynamics and modifications in water and sodium handling by the kidneys (Abassi et al., 2009, Amazonas and Lopes de Faria, 2006, Dobrian et al., 2000, Kramer et al., 2005). The consequences of these changes can lead to hyperfiltration and increased intracapsular pressure in the glomerulus which can compound the reabsorptive demand of tubular cells, leading to an activation of a number of signalling pathways responsible for aspects of the microstructural remodelling (Abassi et al., 2009). AM630 treated animals did not show any significant changes to blood pressure and these animals exhibited a decline in renal function across the treatment period, with GFR, as estimated by creatinine clearance decreasing and a significant increase in urinary albumin excretion compared to both AM1241 and control animals.

It is likely that improvements in renal function in obese AM1241 treated animals are due to multiple factors in addition to reduction in systolic blood pressure. Structurally, AM1241 treatment significantly reduced tubular cross sectional area, although no changes in kidney weight (gross weight or standardised for body weight) were found. Further, we have shown that AM630 treated obese animals had a significantly higher glomerular cross sectional area compared to AM1241 treated obese animals and increased kidney weight (standardised for body weight) compared to obese control animals. This data, taken together indicates that blockade of CB2 receptor may exacerbate hypertrophic changes occurring in the kidney of obese animals (unpublished observations outlined in Chapter 5). Similar results have been

demonstrated by our group *in vitro* under normal physiological conditions, where proximal tubule cells exposed to AM630 led to significant increases in hypertrophy (Jenkin et al., 2010). Hypertrophy is a mechanism by which the kidneys are able to cope with increased filtration pressure and reabsorptive tubular demand (Declèves et al., 2011, Wolf and Ziyadeh, 1999).

Overall, AM1241 treatment in obese animals led to improvement in markers of renal damage through significantly reduced levels of proteinuria, albumin and sodium excretion. One limitation of this study is that AM1241 treated obese animals had significantly higher urinary protein excretion compared to both obese control and AM630 treated obese animals prior to treatment. Animals in this study were matched for weight, body composition and blood pressure before being placed into treatment groups. However, Sprague Dawley rats have a range of physiological responses to HFD, with some demonstrating the disease phenotype while others exhibit a less severe phenotype (Levin et al., 1997). Despite this, significant reductions in albuminuria, a measurement closely tied with proteinuria has been demonstrated by AM1241 treated obese animals in this study, as well as with models of nephropathy elsewhere (Barutta et al., 2011), indicating that reduction in proteinuria is a true outcome of CB2 agonist AM1241 in a model of DIO.

Interestingly, although AM630 treated obese animals showed signs of renal hypertrophy, the animals had significantly reduced creatinine clearance compared to obese controls. These findings indicate that CB2 antagonist treatment with AM630 may be accelerating the decline in renal structure leading to reduced renal function in DIO.

We have also showed that specifically in renal tissue of rats with DIO, markers of fibrosis including TGF-β1, collagen IV and VEGF were significantly reduced in AM1241 treated obese animals compared to obese controls. The degree of tubular interstitial fibrosis is closely associated with kidney damage, and experimental evidence suggests that proteinuria and albuminuria can lead to increased release of pro-inflammatory and fibrotic cytokines from renal cells (El Nahas and Bello, 2005). In this study AM1241 treatment in obese rats lead to both a significant reduction of fibrotic markers in renal tissue and improved proteinuria and albuminuria. Interestingly, although AM630 treated obese animals showed no significant changes in either VEGF or collagen IV protein expression, TGF-β1 was found to be significantly reduced in renal tissue compared to obese control animals. The precise mechanism for this effect is unclear; however a complex role of CB2 and TGF-β1 regulation has been highlighted in lymphocytes (Gardner et al., 2002). Here, the authors show that TGF-β1 production is both affected by and mediates CB2 receptor expression in lymphocytes (Gardner et al., 2002). This same autocrine signalling loop could also be present in the renal system, where CB2 blockade with AM630 may have led to a downregulated expression of the CB2 receptor in kidney tissue, leading to diminished renal TGF-β1 production. However, CB2 expression and immune-histological analysis of TGF-β1 in renal tissue of AM630 treated animals would be required to clarify this hypothesis.

## ***7.6 Conclusion***

This study may have important clinical implications for the treatment of obesity related renal damage. Here, we have shown that activation of CB2 has been shown

to ameliorate the progression of proteinuria, albuminuria and sodium excretion in a model of DIO. Further, CB2 agonist, AM1241, significantly reduced both systolic blood pressure and fibrotic markers in renal tissue of obese animals. It is also noteworthy that blockade of the CB2 receptor may lead to deleterious effects in the progression of obesity related renal damage. This study adds to the increasing body of evidence that CB2 activation may have a protective role in nephropathy (Barutta et al., 2011, Mukhopadhyay et al., 2010b, Pacher and Mechoulam, 2011), however further investigation is required to determine the efficacy and safety of AM1241 before it can be used in a clinical setting.

# Chapter 8 - Renal Effects of Chronic Pharmacological Manipulation of GPR55 in Rats with Diet Induced Obesity

## *8.1 Summary*

Recently, a potential role of GPR55 regulating energy homeostasis has been identified. To date, only a handful of studies have examined GPR55 in relation to the pathogenesis of obesity and related complications. Despite this, it has yet to be elucidated how GPR55 activation and blockade may modulate renal function and structure. The aim of this study was to determine metabolic and renal adaptations which occur in response to the GPR55 agonist, O-1602, and GPR55 antagonist, O-1918 in an animal model of DIO. Male Sprague Dawley consumed a HFD for nine weeks to induce obesity. Following this, rats were treated for six weeks with either GPR55 agonist O-1602 (n = 6), GPR55 antagonist O-1918 (n = 9), or saline control (n = 9), while being maintained on the HFD. Treatment with O-1602 led to significant reduction in percentage weight gain, urinary protein and albumin excretion in obese animals across the treatment period compared to obese controls. O-1602 also had significantly higher kidney to body weight ratio. Treatment with O-1918 in obese animals also significantly reduced urinary protein and albumin excretion across the treatment period compared to obese controls, but in the absence of weight gain. Histological examination showed significant reductions in renal tubular cross sectional area in O-1918 treated obese animals compared to obese controls. Neither treatment altered urinary sodium excretion, plasma creatinine, creatinine clearance,

gross kidney weight, glomerular cross sectional area or renal cytokine profile including expression of collagen IV, TGF-β1 or VEGF. The mechanism of how O-1602 and O-1918 treatment can both lead to improved proteinuria and albuminuria in a model of DIO is currently inconclusive. Certainly, the mechanism is likely to be quite different from those described for the agonism of CB2 described in Chapter 7. The results presented in this chapter indicate that GPR55 may have future clinical relevance for treated obesity related renal damage. However, O-1602 and O-1918 have both been shown to elicit physiological effects even in GPR55 knockout animal models, thus these compounds could potentially be acting upon GPCRs distinct from GPR55 (Henstridge, 2012, Johns et al., 2007, Kapur et al., 2009, Ross, 2009). Future research should be aware that both O-1602 and O-1918 could be leading to off target effects and further careful examination is required in order to elucidate the role of GPR55 in obesity related renal damage.

## ***8.2 Background***

The endocannabinoid system has an important role in both central and peripheral regulation of metabolism, which can influence appetite and body weight (Di Marzo, 2008, Di Marzo and Matias, 2005, Engeli et al., 2005). GPR55, is a G-protein coupled receptor reported to be a novel member of the cannabinoid family (Ryberg et al., 2007, Simcocks et al., 2014). Recently, it has been established that the primary endogenous ligand for GPR55, LPI, correlates with body fat percentage and BMI in humans (Moreno-Navarrete et al., 2012). GPR55 has been found in a number of metabolically active tissues, including adipose, liver, and skeletal muscle (Moreno-Navarrete et al., 2012, Simcocks et al., 2014). Further, expression of GPR55 in white

adipose tissue and plasma concentrations of LPI are increased in animals fasted for 24 – 48 hours, indicating that GPR55 may play an important role in regulating energy balance (Imbernon et al., 2013). These studies taken together suggest that GPR55 expression may be regulated by nutritional status and could be a potential physiological target for obesity and associated complications.

Currently, there is limited understanding about how GPR55 may mediate physiological processes in both health and disease. In GPR55 knockout mice, the absence of GPR55 demonstrated a reduced hyperalgesia in animals exposed to a model induced inflammation (Staton et al., 2008). In states of induced inflammation, GPR55 knockout mice have also shown to display a different cytokine profile compared to wild type mice, indicating that GPR55 may have a role in regulating cytokine production during inflammatory responses (Staton et al., 2008). Obesity and other metabolic diseases such as diabetes are associated with chronic inflammation, and currently it has yet to be explored if pharmacologically targeting GPR55 may mediate the pathophysiology of DIO renal damage.

Our group has previously demonstrated that GPR55 is expressed in renal proximal tubule cells (Jenkin et al., 2010), and this thesis has shown that after 10 weeks of DIO in rats, GPR55 expression is significantly higher in renal tissue compared to lean standard chow fed animals (Chapter 5). Given only a handful of studies have examined GPR55 in relation to the pathogenesis of obesity and related complications, it has yet to be elucidated how GPR55 activation and blockade may modulate renal function. The aim of this study was to therefore determine the metabolic and renal adaptations which occur in response to treatment with the

GPR55 agonist, O-1602, and antagonist, O-1918, in an animal model of DIO. We also sought to identify whether potential downstream signalling targets known to mediate structural renal changes are altered in response to O-1602 and O-1918 compounds. It was hypothesised that pharmacological blockade with O-1918, but not activation of GPR55 will improve renal structure and function in a model of diet induced obesity, and lead to alterations in key signalling proteins in the kidney.

## ***8.3 Materials and Methods***

### *8.3.1 Animals and Experimental Protocol*

Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24℃) with a 12 hour light/ dark cycle (7:00 - 19:00). Rats were fed *ad libitum* a HFD (containing 21% fat, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Rats were then matched using a number of metabolic parameters and put into either obese control (n = 9), GPR55 agonist O-1602 (n = 6) or GPR55 antagonist O-1918 (n = 9) treatment groups. For six weeks, rats were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80, or 5 mg/kg of O-1602 or 1 mg/kg O-1918 administered via IP injection. Stocks of O-1602 and O-1918 treatments were made using the control saline Tween 20 solution to dissolve compounds. Following the conclusion of the experimental protocol, rats were deeply anesthetised and killed via cardiac puncture. Organs were removed, weighed and stored for further analysis, as outlined in Section 2.6. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036).

### *8.3.2 Metabolic Measurements*

Rat weight and food was recorded daily throughout the experimental period. Body composition and blood pressure was analyzed (as outlined in Section 2.5) for pre- and post-treatment time points. 'Pre-treatment' measurements were conducted the week before saline or GPR55 treatments commenced and 'post-treatment' measurements in the final week of treatment.

### *8.3.3 Functional Measurements for Renal Parameters*

Renal measurements including creatinine clearance, urinary protein, albumin and sodium excretion were evaluated using 24 hour urine samples collected pre- and post-treatment periods and plasma collected at time of death, as outline in Section 2.7.

### *8.3.4 Histological Analysis*

To determine what structural changes occurred in response to treatment with GPR55 agonist and antagonist, O-1602 and O-1918, in this model of DIO in Sprague Dawley rats, histological analysis examining glomerular and tubular cross sectional area was performed. Following kidney dissection, a portion of kidney tissue was fresh frozen, as outlined in Section 2.6.1 and sections were stained for H&E and PAS staining was performed as outlined in Section 2.6. Sections were imaged at 200 X magnification (Carl Ziess microscope) and glomerular and tubular cross sectional area was analyzed using AxioVision 4.8 software.

*8.3.5 Cytokine Profile*

To determine whether GPR55 agonist and antagonist treatments affects renal cytokine profile, a number of key proteins known to mediate renal structural changes in DIO were examined. TGF-β1 plasma expression was analyzed using immunoassay techniques outlined in Section 2.8.2. Kidney tissue (40 µg) was analyzed for protein expression of TGF-β1, Collagen IV and VEGF via Western blot analysis. Concentrations and supplier information of antibodies are outlined in Section 2.2.3 and in Table 2.2. Secondary antibodies anti-mouse and anti-rabbit were purchased from Sigma Aldrich (St Louis, MO). Band densitometry was analyzed using Image Lab software.

*8.3.6 Statistical Analysis*

The SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Differences between treatment groups were individually analyzed compared to obese control animals using independent samples T-Test for two group direct analysis or mixed model ANOVA for analysis of pre- and post-treatment measurements between treatment and obese control animals. Significance was accepted when $p < 0.05$.

## ***8.4 Results***

*8.4.1 Metabolic Parameters in Control and GPR55 Treated Obese Groups*

Prior to treatment, average group weights of obese control animals (582.9 ± 14.35 g), O-1602 (605.4 ± 16.80 g) and O-1918 (604.0 ± 21.23 g) were not significantly

different. Post-treatment weights of O-1602 treated animals (609.2 ± 19.75 g), and O-1918 treated animals weights (664.4 ± 21.13 g) were not significantly different from obese controls (655.9 ± 19.23 g). However, across the treatment period, treatment with GPR55 agonist O-1602 led to significantly reduction in percentage weight gain, compared to obese control animals (Figure 8.1). The GPR55 antagonist, O-1918, did not affect weight gain across the treatment period compared to obese controls (Figure 8.1). No significant alterations in diastolic or systolic blood pressure across the treatment period were detected between obese control, O-1602 and O-1918 treated obese groups (Figure 8.2).

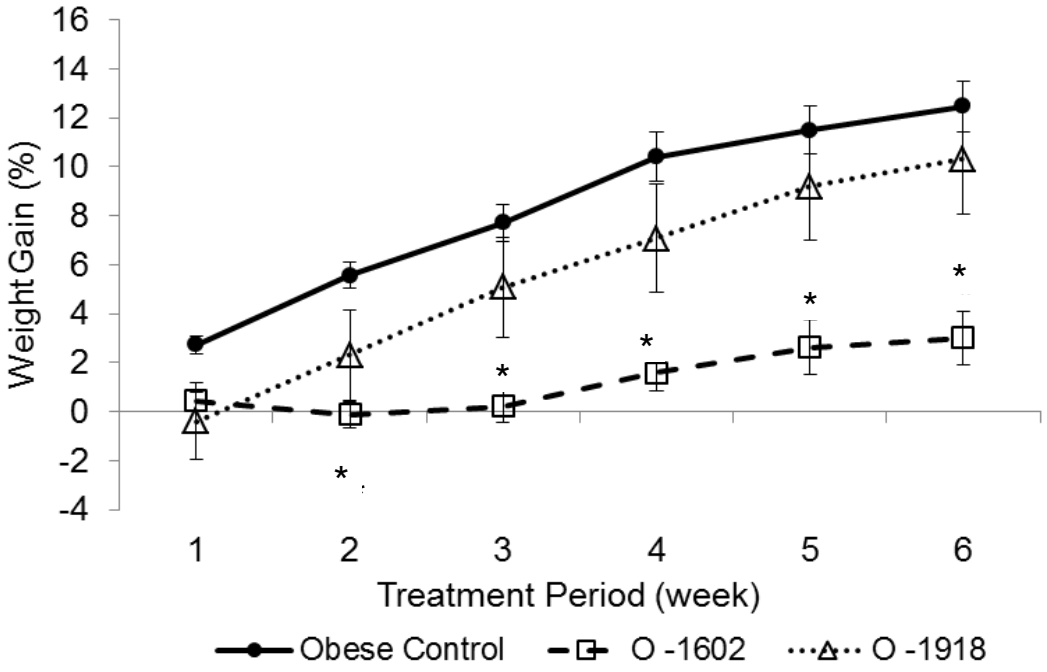



**Figure 8.1: Percentage weight gain in O-1602 and O-1918 treated animals, following nine weeks of DIO compared to obese controls.** O-1918 treatment in obese animals did not significantly affect weight gain compared to obese controls. O-1602 treated obese animals exhibited significantly reduced weight gain (% of pre-treatment weight) from Week 2 to Week 6 of treatment compared to obese control animals. Significance is indiacted by *, $p < 0.05$ ($n = 6 - 9$).

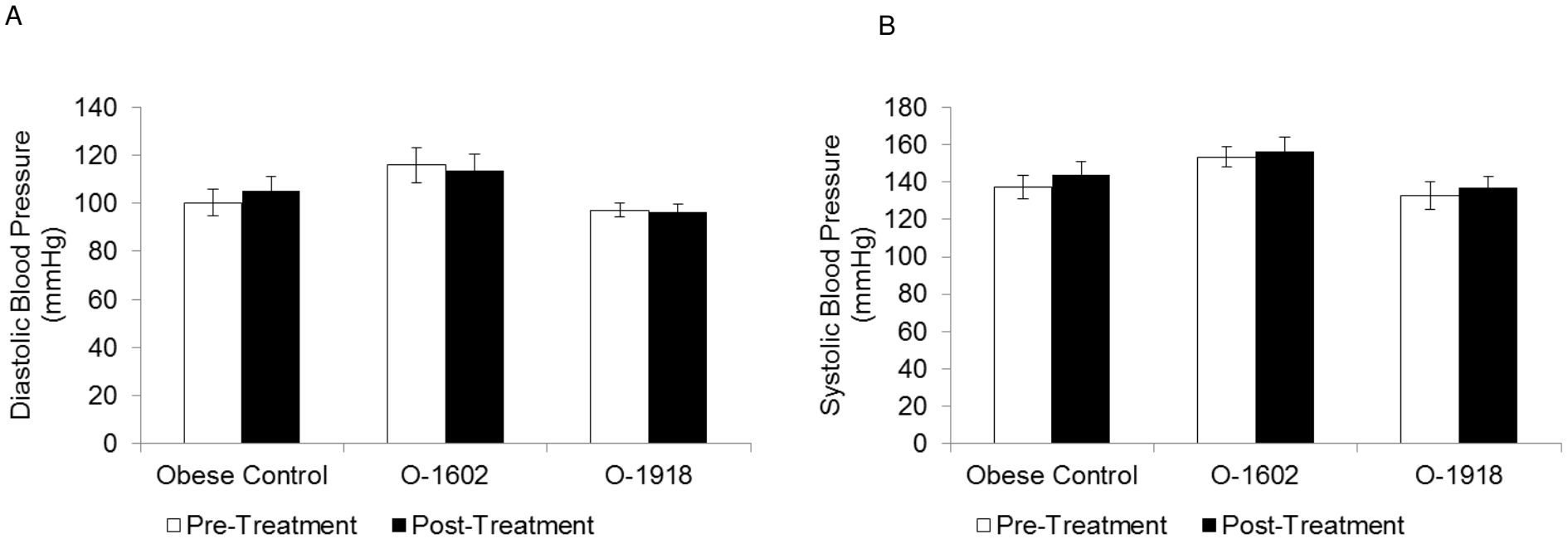



**Figure 8.2: Blood pressure of GPR55 treated groups compared to obese controls. A.** In obese animals, diastolic blood pressure was not significantly altered by O-1602 or O-1918 treatments, compared to obese control. **B.** Diastolic blood pressure was not significantly altered in obese animals by O-1602 or O-1918 treatments compared to obese control (n = 6 - 9).

*8.4.2 Functional Renal Outcomes in O-1602 and O-1918 Treated Obese Animals*

This model of experimental DIO leads to significant increases in markers of kidney damage, as outlined in Chapter 5. In this study, treatment with the GPR55 agonist O-1602 and antagonist O-1918 treatments were shown to significantly reduce urinary protein and albumin excretion in obese animals compared to obese controls ($p < 0.05$, Figure 8.3A and B). The Sprague Dawley strain of rats is a robust model used for inducing DIO in a manner which is similar to what is observed in humans. This breed exhibits a range of phenotypes in response to obesity induced by a high fat (Levin et al., 1997). One limitation of this study was that all pre-treatment measurements could not be accounted for, and as such urinary protein excretion in both O-1602 and O-1918 treated obese rats and urinary albumin in obese O-1918 treated rats were significantly higher pre-treatment compared to obese control rats ($p < 0.05$, Table 8.1). No significant changes in urinary sodium excretion were observed in O-1602 or O-1918 treated obese animals compared to obese controls (Figure 8.3C). No significant differences between GPR55 treatment groups and obese controls were found for plasma creatinine or estimated creatinine clearance (Figure 8.4). Table 8.1 outlines all functional renal outcomes for treatment groups ± SEM.

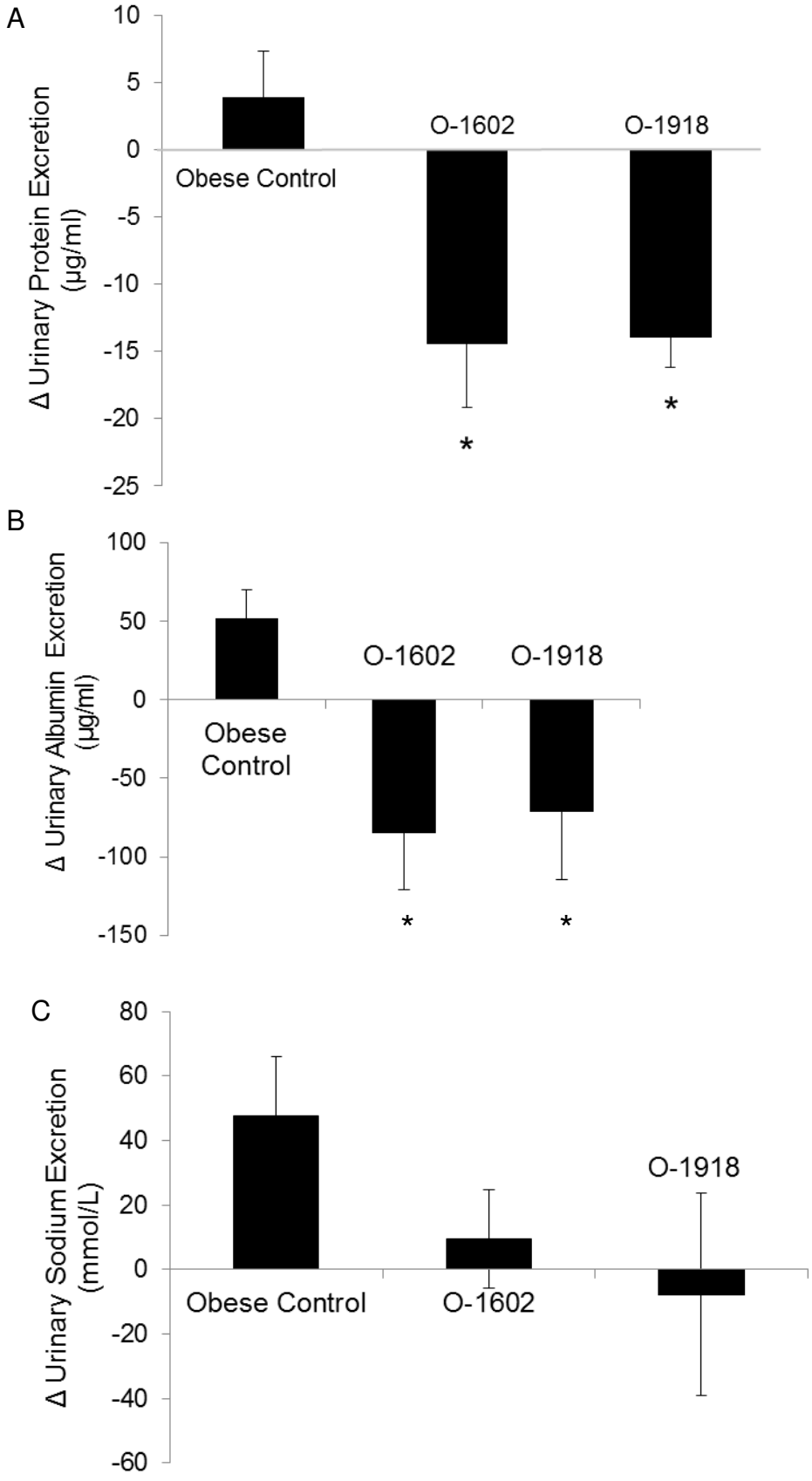

A
10
5
0
-5
-10
-15
-20
-25
Δ Urinary Protein Excretion (µg/ml)
Obese Control
O-1602
O-1918
*
*
B
100
50
0
-50
-100
-150
Δ Urinary Albumin Excretion (µg/ml)
Obese Control
O-1602
O-1918
*
*
C
80
60
40
20
0
-20
-40
-60
Δ Urinary Sodium Excretion (mmol/L)
Obese Control
O-1602
O-1918

**Figure 8.3: Functional measurements of Renal Parameters of O-1602 and O-1918 treated obese animals compared to obese control.** **A.** Urinary protein excretion was significantly reduced by treatment with O-1602 and O-1918 in obese animals compared to obese control. **B.** Urinary albumin excretion was significantly reduced in obese animals treated with O-1602 and O-1918 compared to obese control. **C.** Urinary sodium excretion was not significantly altered by O-1602 or O-1918 treatment in obese animals compared to obese control. Significance is indicated by * compared to obese control ($p < 0.05$, $n = 6 – 9$).

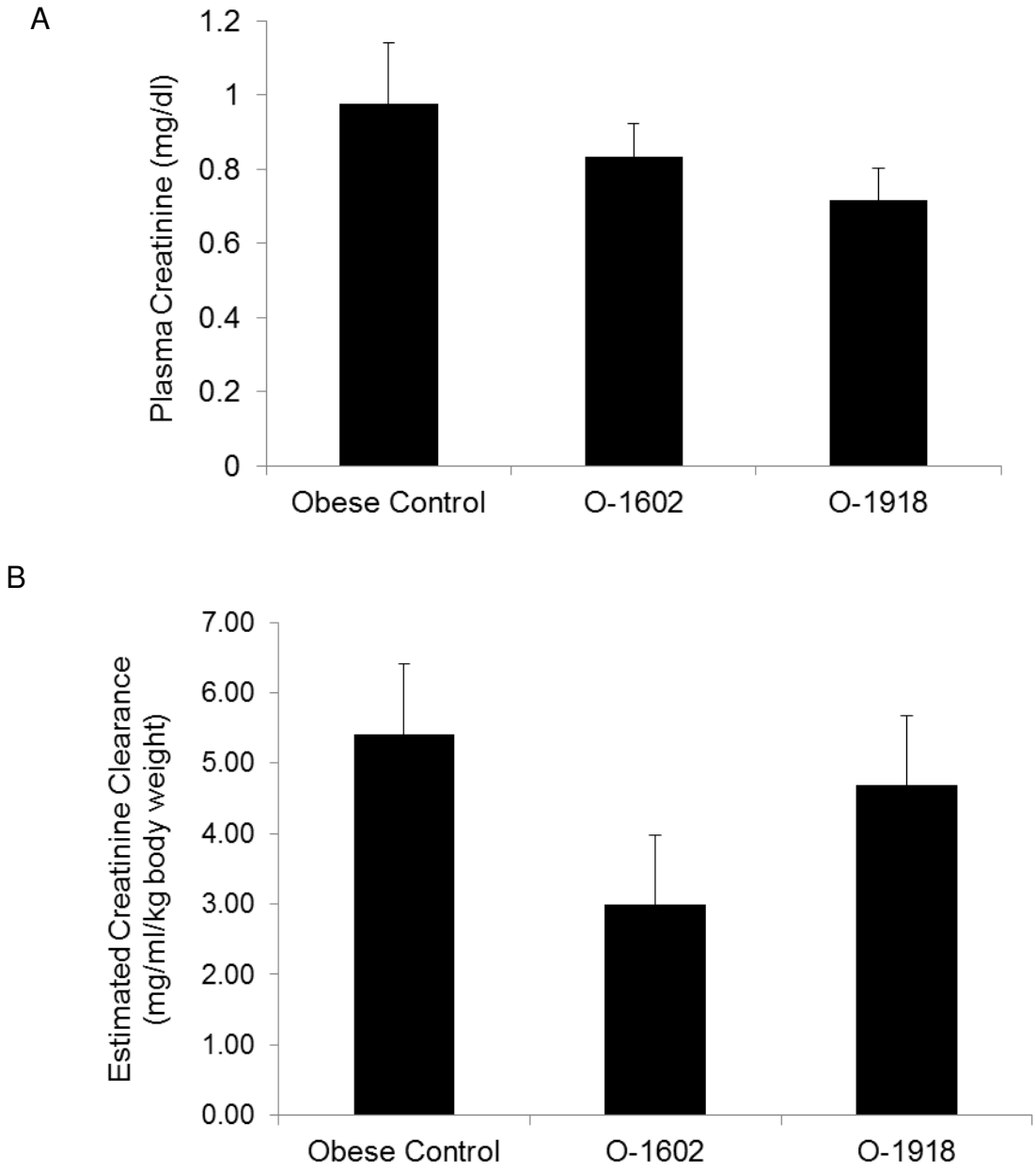



**Figure 8.4: Plasma creatinine and estimated creatinine clearance of obese animals treated with O-1602 and O-1918 compared to obese control. A.** No significant differences in plasma creatinine concentration were observed in obese control compared to GPR55 treated groups. **B.** No significant differences in estimated creatinine clearance were observed in obese control compared to GPR55 treated groups (n = 6 – 9).

**Table 8.1: Functional Renal Outcomes of GPR55 Treatment Group Compared to Obese Controls.** Plasma creatinine and creatinine clearance are post-treatment measurements only. Data is shown as average ± SEM. Significance between O-1602 and O-1918 treatment groups and obese control at the same time point (pre- or post-treatment) is indicated by * ($p < 0.05$, $n = 6 - 9$).

| Measure | Obese Control | | O-1602 | | O-1918 | |
|---|---|---|---|---|---|---|
| | *Pre treatment* | *Post treatment* | *Pre treatment* | *Post Treatment* | *Pre treatment* | *Post Treatment* |
| Urinary Protein (µg/ml) | 15.34 ± 1.15 | 19.10 ± 2.96 | 25.86 ± 3.69 * | 14.20 ± 4.26 | 24.24 ± 2.33 * | 15.85 ± 3.47 |
| Urinary Albumin (µg/ml) | 103.1 ± 44.0 | 226.5±118.6 | 329.3 ± 125.3* | 405.4 ± 123.8 | 431.3 ± 88.3* | 362.8 ± 107.2 |
| Urinary Sodium (mmol/L) | 146 ± 7.28 | 196 ± 20.32 | 185 ± 28.91 | 141.67 ± 40.04 | 185 ± 17.14 | 167.89 ± 24.34 |
| Plasma Creatinine (mg/dl) | - | 0.97 ± 0.16 | - | 0.84 ± 0.09 | - | 0.67 ± 0.10 |
| Creatinine Clearance (mg/ml/kg body weight) | - | 5.41 ± 1.10 | - | 3.11 ± 0.64 | - | 4.68 ± 0.77 |

### *8.4.3 Effect of O-1602 and O-1918 Treatment in Obese Rats on Renal Structure*

Histological analysis showed that treatment with GPR55 agonist O-1602 did not affect gross kidney weight or glomerular and tubular cross sectional area (Figure 8.5) in a model of DIO. Treatment with O-1602 did significantly increase kidney weight to body weight ratio (0.31 ± 0.01%) in obese animals compared to obese control animals (0.25 ± 0.01%). Treatment with GPR55 antagonist O-1918 in obese animals did not show significant changes to gross kidney weight, kidney weight standardised for body weight or glomerular cross sectional area (Table 8.2), however these animals did have significantly reduced tubular cross sectional area (11.0 ± 0.29 μm) compared to obese controls (12.5 ± 0.23 μm). Table 8.2 outlines all gross and histological measurements of treatment groups ± SEM.

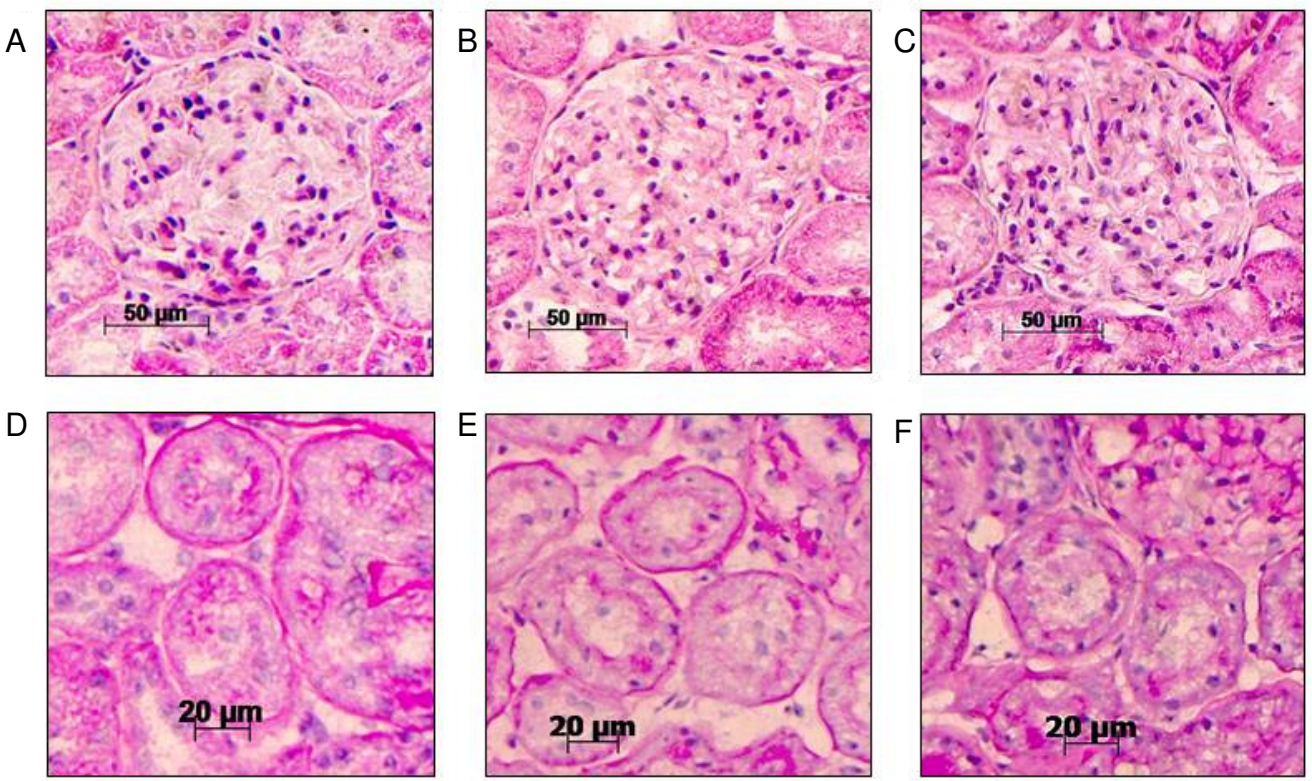



**Figure 8.5: Effect of O-1602 and O-1918 treatment in obese rats on renal histology.** Glomerular H&E stained sections and PAS stained tubular sections were from 5 animals in each group; imaged at 200X magnification and analyzed using AxioVision software for cross sectional area. **A.** Glomerulus of obese control animal **B.** Glomerulus of O-1602 treated obese animal **C.** Glomerulus of O-1918 treated obese animal **D.** Tubules of obese control animal **E.** Tubules of O-1602 treated obese animal **F.** Tubules of O-1918 treated obese animal.

**Table 8.2: Gross and Histological Measurements of Renal Tissue in GPR55 Treatment Groups Compared to Obese Controls.** Data is shown as average ± SEM, significance is indicated by * compared to obese control, ($p < 0.05$, $n = 6 - 9$).

| Measure | Obese Control | O-1602 | O-1918 |
|---|---|---|---|
| Kidney weight (g) | 1.67 ± 0.05 | 1.86 ± 0.07 | 1.67 ± 0.05 |
| Kidney weight/ Body weight (%) | 0.25 ± 0.01 | 0.31 ± 0.01 * | 0.25 ± 0.01 |
| Glomerular Cross Section (µm) | 126.2 ± 4.49 | 137.6 ± 3.84 | 135.46 ± 4.25 |
| Tubular Cross Section (µm) | 12.5 ± 0.23 | 11.4 ± 0.26 | 11.0 ± 0.29 * |

### *8.4.4 Cytokine Profile of O-1602 and O-1918 Treated Obese Animals*

Treatment with the GPR55 agonist O-1602 or antagonist O-1918 in obese animals did not significantly alter TGF-β1 plasma concentrations compared to obese control animals (Figure 8.6). No significant changes in the renal fibrotic markers collagen IV, TGF-β1 or VEGF protein were observed in O-1602 and O-1918 treated obese animals compared to obese controls (Figure 8.7).

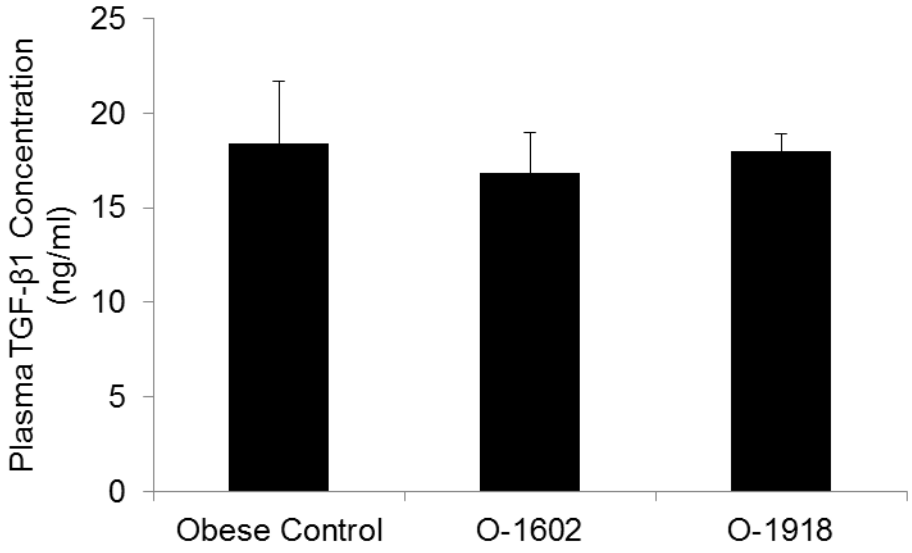



**Figure 8.6: Plasma concentrations of TGF-β1 in GPR55 treatment groups compared to obese controls.** No significant differences in plasma TGF-β1 concentrations was observed between groups (n = 6 - 9).

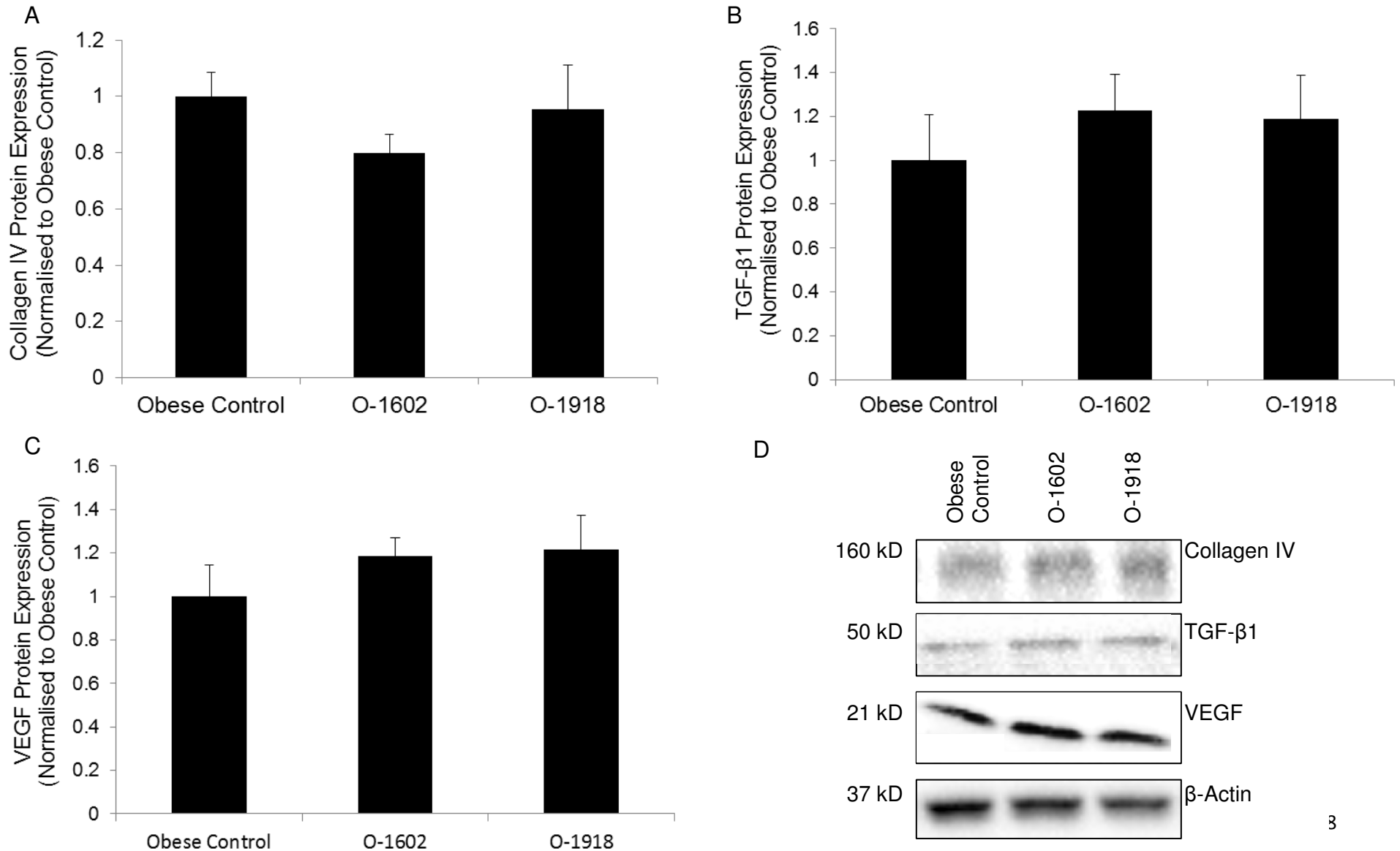

A
Collagen IV Protein Expression (Normalised to Obese Control)
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
O-1602
O-1918
B
TGF-β1 Protein Expression (Normalised to Obese Control)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
O-1602
O-1918
C
VEGF Protein Expression (Normalised to Obese Control)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Obese Control
O-1602
O-1918
D
Obese Control
O-1602
O-1918
160 kD
Collagen IV
50 kD
TGF-β1
21 kD
VEGF
37 kD
β-Actin

**Figure 8.7: Protein expression of cytokines in obese animal kidney tissue following O-1602 and O-1918 treatment. A.** Densitometry of Collagen IV protein expression demonstrated that treatment with O-1602 and O-1918 did not significantly alter renal collagen IV expression in obese animals compared to obese controls. **B.** Densitometry of TGF-β1 protein expression in obese animals demonstrated that treatment with O-1602 and O-1918 did not significantly alter renal TGF-β1 expression in obese animals compared to obese controls. **C.** Densitometry of VEGF protein expression in obese animals demonstrated that treatment with O-1602 and O-1918 did not significantly alter renal VEGF expression in obese animals compared to obese controls **E.** Representative Western blots of proteins in kidney tissue of obese control, O-1602 and O-1918 treated obese animals (n = 6 - 9).

## *8.5 Discussion*

The endocannabinoid system has been identified as a potential therapeutic target for the treatment of renal damage arising from metabolic diseases including obesity and diabetes (Barutta et al., 2010, Barutta et al., 2011, Janiak et al., 2007, Jenkin et al., 2012, Mukhopadhyay et al., 2010a, Mukhopadhyay et al., 2010b). Emerging research has indicated a potential role for GPR55 in obesity (Imbernon et al., 2013, Moreno-Navarrete et al., 2012). However, the clinical implication of GPR55 in modulating markers of renal damage in obesity has yet to be examined. This study has showed for the first time that in rats with DIO, treatment with GPR55 antagonist, O-1918, led to significant reductions in urinary protein and albumin excretion and reduced renal tubular cross sectional area compared to obese controls. Interestingly, treatment with GPR55 agonist O-1602 also showed significant reductions in urinary protein and albumin, in conjunction with abrogation of weight gain in obese animals. Blood pressure, plasma creatinine, creatinine clearance or cytokine profile in renal tissue of rats were not altered in obese animals by either treatment with O-1602 or O-1918.

Recently, it has been demonstrated that plasma concentrations of LPI, the endogenous agonist for GPR55, is significantly increased in obese patients compared lean individuals (Moreno-Navarrete et al., 2012), indicating that GPR55 may potentially have a role in mediating obesity. Here, we have shown that six weeks treatment with GPR55 agonist O-1602 led to significant reduction in percentage weight gain across the treatment period compared to obese control animals with DIO, while the antagonist, O-1918, did not alter weight gain. Given

GPR55 expression is significantly higher in visceral adipose tissue and circulating plasma LPI levels are higher in obese patients (Moreno-Navarrete et al., 2012), it was expected that GPR55 agonist treatment with O-1602 would not alter, or exacerbate weight gain in rats with DIO. Here, we have shown that six weeks of peripheral administration of O-1602 diminished weight gain in DIO male Sprague Dawley rats. The reduction in weight gain in O-1602 treated obese animals shown in this study was potentially due to the reduction in food intake, which was sustained across the treatment period, but particularly during the first three weeks of treatment (data not shown). This finding conflicts with our experimental hypothesis and a recent study published which showed male adult Sprague Dawley rats injected acutely with O-1602 demonstrated an increase in 24 hour food intake subsequent to treatment (Díaz-Arteaga et al., 2012). However, the hyperphagic effect of O-1602 reported by Diaz-Arteaga et al., was predominately observed only in animals administered treatment centrally via intracerebroventricular injection rather than peripheral delivery via IP injection (Díaz-Arteaga et al., 2012). Increased food intake and weight gain was not altered by O-1602 in animals injected sub-chronically, daily for one week, when animals were not treated acutely (Díaz-Arteaga et al., 2012). Differences in route and length of administration may partially explain the disparate results of the effect of O-1602 on weight gain and food consumption. Further, our findings were observed in a model of DIO, while the previous study used lean Sprague Dawley rats. Increased expression of GPR55 in tissues under obese conditions as previously reported (Moreno-Navarrete et al., 2012) may lead to different responses to O-1602 treatment.

Even with significant reductions in percentage weight gain in the O-1602 treated group, blood pressure was not found to be altered by either O-1602 or O-1918 treatment compared to obese controls. Early research into exploring a potential 'third' cannabinoid receptor beyond CB1 and CB2 was initiated by the hypotensive vascular effects of O-1602 in CB1 and CB2 knockout mice, which were abolished by GPR55 antagonist O-1918 (Járai et al., 1999). However, in GPR55 knockout mice, it has been shown that animals lacking GPR55 expression exhibit no differences in blood pressure and heart rate compared to wild type mice (Johns et al., 2007). Further, O-1602 and other abnormal CBD compounds which act upon GPR55 were not found to alter resistance to blood vessels in either wild type of knockout animals (Johns et al., 2007). While the study by Johns et al., (2007) is congruent with our findings, the authors examined the role of O-1602 only under normal physiological conditions. The physiology of how GPR55 may mediate cardiovascular tone has yet to be elucidated, but we have shown that six week treatment with O-1602 and O-1918 does not alter systolic or diastolic blood pressure in male Sprague Dawley rats with DIO.

A significant reduction in weight gain in O-1602 treated obese animals is one mechanism which potentially mediated the significant improvements in markers of kidney damage including reduced urinary protein and albumin. Histological examination showed there were no signs of structural alterations to the kidney induced by treatment with O-1602 in obese animals compared to obese controls and renal cytokine profile was also not altered. Kidney weight as a percentage of total body weight was the only parameter found to be significantly higher than obese control animals. The increase in the ratio of kidney to body weight observed in O-

1602 treated obese animals is likely to be the result of lower overall body weight, rather than an indication of increased renal hypertrophy, since gross kidney weight and cross sectional area of tubules and glomerulus were not altered. The reason for improved proteinuria and albuminuria in obese animals treated with O-1602 may lie beyond specific structural changes to the kidney. In obesity, leptin is an adipokine secreted by adipose tissue, and leptin plasma concentrations correlate with the level of adiposity in humans (Briffa et al., 2013, Garibotto et al., 1998). It has been shown that when leptin is infused into adult male Wistar rats, it can lead to increased proteinuria and albuminuria and other alterations to renal physiology compared to control animals (Gunduz et al., 2005). In our study, the significant reduction in weight gain exhibited by O-1602 treated obese animals may potentially lead to a reduction in circulating plasma leptin levels in DIO animals. Although the mechanism of leptin induced proteinuria has yet to elucidated, it has been postulated that changes to proteinuria in leptin infused animals may be caused by increased stimulation of renal expression of TGF-β1 expression (Gunduz et al., 2005). Given O-1602 treated obese animals in our study showed no changes to renal cytokine profile, including renal expression of TGF-β1 expression, it is unlikely that O-1602 mediates improved proteinuria and albuminuria via this pathway. In human obesity, it has been shown that plasma concentrations of LPI and leptin are significantly increased compared to lean individuals, while GPR55 expression is upregulated in adipose tissue of obese individuals (Moreno-Navarrete et al., 2012). Treatment of visceral adipose tissue *ex vivo* with LPI showed a trend for increased leptin levels although the difference was not significant (Moreno-Navarrete et al., 2012). This study, combined with our findings highlights that GPR55 agonists have a potential role in obesity, however

whether GPR55 is responsible for mediating specific functional changes in the kidney through changes to leptin or other signalling pathways has yet to be fully explored.

Interestingly, we have shown that treatment with GPR55 antagonist O-1918 also significantly reduced proteinuria and albuminuria in obese animals compared to obese control animals. These improvements in markers of kidney damage occurred in the absence of improved weight gain in DIO animals, and so it is likely that alterations to leptin levels in O-1918 animals were not responsible for improvements in protein and albumin urinary excretion. While GPR55 has been identified as having a possible role in regulating inflammatory cytokines in a model of hyperalgesia (Staton et al., 2008), renal cytokine profile of TGF-β1, collagen IV and VEGF expression was not altered in this model of DIO by O-1918 treatment. Our group has previously shown that O-1918 does not affect hypertrophy in renal proximal tubule HK2 cells *in vitro* (Jenkin et al., 2010), however we have shown in this study, six week treatment with O-1918 *in vivo* led to significant reductions in tubular cross sectional area compared to obese control animals. In Chapter 5, we identified that renal tubular cross sectional area is significantly greater in animals with DIO after 10 weeks compared to lean standard chow fed animals. Alterations to renal tubular architecture by treatment with O-1918 may contribute to reduced protein and albumin urinary excretion in animals with DIO. While many studies examining the development of renal hypertrophy under pathophysiological conditions focus on protein synthesis via TGF-β1 and increased expression of collagen, hypertrophy may also emanate from a decrease in protein catabolism (Wolf and Ziyadeh, 1999). Exploring the activity of intracellular proteases which regulate protein breakdown in

tubular cells was beyond the scope of this study, but may be a potential mechanism by which O-1918 regulates renal structure and function.

Despite O-1602 and O-1918 treatments in obese animals both exhibiting significant reductions in proteinuria and albuminuria in this model of DIO, one limitation of this study as well as an inherent problem of trying to evaluate the clinical use of pharmacologically targeting GPR55 is that both O-1918 and O-1602 have been shown to elicit effects independent of GPR55, indicating that these compounds may not be specific for GPR55 alone (Henstridge, 2012). While O-1602 and O-1918 do not have significant affinity for either CB1 or CB2 (Járai et al., 1999, Offertaler et al., 2003, Abood, 2005), the physiological effects of these compounds may be via the activation of other non-cannabinoid receptors including GPR18, PPAR and TRPV channels (Henstridge, 2012). Compounds with higher specificity to GPR55 are being developed, but currently they are not extensively available commercially (Simcocks et al., 2014). While this study has tried to identify the mechanism by which these compounds lead to improvements in markers of kidney damage, through reduced protein and albumin excretion, this study has been able to rule out that both O-1602 and O-1918 treatment alters renal cytokine profile in obese animals. It is possible that increased expression of GPR55 in kidney tissue of animals with DIO (outlined in Chapter 5) may be an outcome of obesity related renal damage rather than driving the metabolic processes which mediate renal damage in DIO. To ascertain the potential clinical relevance of targeting GPR55 for the treatment of obesity related renal damage, further investigation into the receptors' pharmacology is required.

## ***8.6 Conclusion***

Currently, there is limited understanding about how GPR55 may mediate renal physiological processes in both health and disease.This study has shown that GPR55 agonist, O-1602, and antagonist, O-1918, both lead to improvements in markers of kidney damage via reduced proteinuria and albuminuria in DIO compared to obese control animals. O-1602 treatment also improved weight gain in obese animals across the treatment period, while O-1918 lead to significant reductions in tubular cross sectional area compared to obese controls. These results indicate that GPR55 may have future clinical relevance for treating obesity related renal damage. However, O-1602 and O-1918 could potentially be acting upon GPCRs distinct from GPR55 which could be leading to off target effects. Given only a handful of studies have examined GPR55 in relation to the pathogenesis of obesity and related complications, further exploration of how GPR55 may modulate renal function and structure is required to fully understand the clinical relevance of pharmacologically targeting the receptor for obesity related renal damage.

# Chapter 9 – General Discussion, Conclusions and Recommendations for Future Research

## *9.1 General Discussion*

The studies completed within this thesis provide novel insight into pharmacologically targeting the renal cannabinoid receptors, CB1, CB2 and GPR55 in order to reduce the renal dysfunction associated with DIO. Further, this research demonstrated that CB1, CB2 and GPR55 receptor expression can be altered under diabetic and obese conditions. These findings could have potentially important clinical relevance for identifying new therapeutic targets for the treatment of nephropathy associated with obesity and also diabetes. This chapter aims to integrate and make concluding remarks about the studies contained within the thesis, as well as identifying key directions for future research regarding the role of the endocannabinoid system in renal physiology.

### *9.1.1 Role and Expression of CB1 in Renal Physiology*

Expression of CB1 was examined in cell culture and rodent experimental models of diabetes and DIO in Chapters 3 and 5. Here, we have shown that CB1 is significantly upregulated in kidney tissue of rodents and cell culture under diabetic and obese conditions. Elevated CB1 expression is also observed in diabetic nephropathy (Barutta et al., 2010, Janiak et al., 2007, Lim et al., 2010, Mukhopadhyay et al., 2010a, Nam et al., 2012), which suggests that CB1 antagonists may provide a potential therapeutic target for the treatment of obesity and diabetic related renal

damage. In support of this, in Chapter 6, this thesis demonstrated that treatment with the CB1 antagonist AM251 has beneficial effects on markers of renal damage in a model of DIO. Specifically in obese rats, treatment with AM251 reduced weight gain, systolic blood pressure, proteinuria, albuminuria, plasma creatinine levels and renal tubule cross sectional area compared to obese controls.

Growing research supports CB1 as a potential therapeutic target for the treatment of nephropathy. CB1 antagonists comparable to AM251 have been shown to directly ameliorate the pathophysiological changes which occur within the kidneys under diabetic and obese conditions. Specifically within the kidneys, treatment with CB1 antagonists have been shown to reduce podocyte loss and tubule apoptosis in models of disease (Barutta et al., 2010, Lim et al., 2010, Mukhopadhyay et al., 2010a). In this thesis, in Chapter 5, male Sprague Dawley rats fed a HFD for 10 weeks, exhibited significant increases in proteinuria, albuminuria and reduced creatinine clearance, whist histological analysis showed that animals with DIO had significantly increased tubular cross sectional area compared to lean standard chow fed animals. This shows that our model of DIO leads to a significant increase in the markers of kidney damage, in addition to signs of renal tubular hypertrophy and fibrosis in obese rats. In Chapter 6, we demonstrated that following the onset of renal dysfunction arising from DIO, treatment with the CB1 antagonist, AM251, significantly reduced urinary excretion of protein and albumin and plasma creatinine concentrations. Structurally, obese animals treated with AM251 exhibited significantly reduced renal tubule cross sectional area compared to obese controls, which is supported by previously published data from our group showing that CB1 may mediate hypertrophic changes in proximal tubule cells *in vitro* (Jenkin et al.,

2010). It has been suggested that renal hypertrophy is the precursor, rather than the response to a decline in renal function (Vallon, 2011b, Zerbini et al., 2006). Thus, ameliorating tubular hypertrophy in obesity may reduce the loss of protein leading to proteinuria and albuminuria. However, the ability of CB1 antagonists to maintain the integrity of the slit diaphragm of the glomerulus (Barutta et al., 2010, Nam et al., 2012) indicates that it is unlikely that reduced tubular size is the only contributing factor leading to improved renal function in AM251 treated animals with DIO.

The benefits of targeting CB1 to treat renal dysfunction is not limited to renal improvements alone, CB1 antagonists also deliver broader systemic benefits (Barutta et al., 2010, Janiak et al., 2007, Nam et al., 2012). Pharmacological blockade of CB1 has been shown here in this thesis and in previously published research to ameliorate a number of clinical risk factors associated with CKD (Barutta et al., 2010, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Nam et al., 2012). In Chapter 5, this thesis demonstrated that blocking CB1 activity with AM251 lead to significant reductions in weight gain and blood pressure in obese animals. These findings, in addition to treatment with CB1 antagonists in models of obesity and diabetes leading to improved glucose handling and insulin sensitivity have been extensively reported previously (Barutta et al., 2010, Després et al., 2005, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Nam et al., 2012, Rosenstock et al., 2008, Van Gaal et al., 2005). All of these systemic factors can contribute significantly to the progressive decline of renal function associated with obesity and diabetes. Thus, any treatment which reduces associated risk factors of CKD has important clinical relevance for assessing CB1 as a viable target for the treatment of renal disease.

This thesis has not been able to identify the specific signalling proteins within the kidneys by which CB1 antagonism mediates improvements in markers of renal damage. However, we have shown that treatment with the CB1 antagonist AM251 in DIO does not improve measures of kidney damage via the pro-fibrotic cytokines TGF-β1, VEGF or elicit changes to renal expression of collagen IV. It is possible that improvements in markers of kidney damage, as measured by reduced proteinuria and albuminuria is derived from alterations to any number of signalling pathways. A range of potential molecular targets are implicated in obesity related renal damage, including interleukins, nitric oxide synthase, the adipokines adiponectin and leptin, and activation of the sympathetic nervous system (Bosse et al., 1995, Eknoyan, 2007, Lambert et al., 2010, Mathew et al., 2011, Briffa et al., 2013). However, further research is required to elucidate how CB1 antagonists may be acting upon these potential regulatory pathways in order to produce beneficial effects currently being observed for the treatment of nephropathy.

### *9.1.2 Role and Expression of CB2 in Renal Physiology*

Previously, CB2 expression has been shown to become downregulated in animal and human models of diabetic nephropathy (Barutta et al., 2011). Here, we have shown in Chapters 4 and 5, that CB2 expression is significantly reduced in proximal tubule HK2 cells exposed to diabetic conditions and in whole kidney tissue of male Sprague Dawley rats with DIO. Further, we have shown that the level of CB2 expression in tubule cells is dependent on albumin uptake. Thus, glomerular damage associated with diabetes and obesity leading to increased albumin in the filtrate, may consequently contribute to the overall reduced expression of CB2 within renal

tubules. Reduced expression of CB2 in proximal tubule cells and whole kidney indicates that treatment with CB2 agonist compounds may have beneficial clinical relevance for the treatment of CKD.

It has been proposed that CB2 may play a protective role in a number of pathological conditions by reducing inflammation (Pacher and Mechoulam, 2011). This thesis showed in Chapter 7 that treatment with the CB2 agonist AM1241, in a model of DIO, led to an improvement in markers of renal damage, while CB2 antagonist, AM630, exacerbated renal damage associated with DIO. Specifically, in obese animals, treatment with AM1241 significantly reduced systolic blood pressure, urinary protein, albumin and sodium excretion and significantly lowered renal protein expression of the fibrotic markers collagen IV, TGF-β1 and VEGF compared to obese control animals. Conversely, treatment with the CB2 antagonist AM630 in obese animals led to significant increases in urinary albumin and kidney to body weight ratio, indicative of renal hypertrophy. These findings are supportive of the current literature which indicates that CB2 agonists can reduce albuminuria and podocyte loss in diabetic nephropathy (Barutta et al., 2011). Importantly, we have shown that the beneficial renal effects of treatment with the CB2 agonist AM1241, as measured by reduction in proteinuria and albuminuria, can also be applied to a model of DIO.

Obesity is a disease state associated with low grade chronic inflammation (Jung, 1997). In the kidneys, DIO can lead to increased activation of a number of key cytokines and growth factors which are produced by both cells of the kidney and invading macrophages and can induce structural changes (Abrass, 2004). CB2

activation has been shown in a variety of pathological conditions to reduce cytokines involved in inflammation and fibrosis (Pacher and Gao, 2008, Pacher and Mechoulam, 2011). Chapter 7 demonstrated that in obese rats, treatment with CB2 agonist AM1241 improved markers of kidney damage, as measured by the reduction of proteinuria, albuminuria and sodium excretion, and reduced tubular cross sectional area compared to obese controls. Treatment with AM1241 in obese animals also led to reduced expression of TGF-β1, VEGF and collagen IV in renal tissue. While the findings from this thesis cannot definitively conclude that reduced expression of pro-fibrotic factors and collagen IV directly caused reduction in tubular hypertrophy and improved markers of renal damage, the degree of tubular interstitial fibrosis is closely associated with decline of renal function (El Nahas and Bello, 2005). Indeed, obese animals treated with the CB2 antagonist, AM630 showed significantly worse albuminuria than obese controls, but did not exhibit higher expression of the pro-fibrotic factors which were affected by AM1241. Given the established relationship between fibrosis and renal function (Branton and Kopp, 1999, Christensen and Verroust, 2008, Powell et al., 2009) it is likely that reduced renal expression of TGF-β1, VEGF and collagen IV at least partially mediated the improved renal outcomes in response to AM1241 treatment in obese rodents. The ability of CB2 signalling to modulate cytokines involved in the fibrotic changes within the kidneys was not wholly unexpected, given the close relationship between CB2 and the immune system (Cabral and Griffin-Thomas, 2009, Klein et al., 2003, Tanasescu and Constantinescu, 2010). Compounds which can limit overall inflammation in diseases where cytokine production can exacerbate damage, is an area of research which has significant clinical relevance. However, our current

understanding of CB2 receptor signalling within renal physiology and its links to the production of cytokines is still incomplete and requires further investigation. This work adds to the growing body of evidence which indicates that CB2 activation may be a viable target for the clinical treatment of CKD.

### *9.1.3 Role and Expression of GPR55 in Renal Physiology*

Currently, very little is understood about the role and expression of GPR55 in normal physiology and disease states. In Chapter 3, we demonstrated that GPR55 expression is increased in HK2 cells *in vitro* exposed to high glucose alone and in conjunction with high albumin. However, also in Chapter 3, in whole kidney tissue of Sprague Dawley rats with STZ induced diabetes, GPR55 expression remained unchanged compared to non-diabetic control animals. Expression of GPCRs are highly regulated, and, recent computational modelling of GPR55 has identified a few key structural features involved in the receptor's activation, docking and distinct ligand binding sites, however its pharmacology is still not well understood (Elbegdorj et al., 2012). Thus, given the complex signalling events which occur in the diabetic kidney, modulation of GPR55 in the diabetic kidney *in vivo* must be regulated by additional factors beyond tubular exposure to high glucose and albumin. In Chapter 5, we showed that in a model of DIO, GPR55 expression was significantly increased in whole kidney tissue of rats fed a HFD for 10 weeks. Previously, it has been shown that GPR55 expression is significantly increased in visceral and subcutaneous white adipose tissue under obese and diabetic conditions (Moreno-Navarrete et al., 2012). Our group was the first to identify the presence of GPR55 in proximal tubule cells (Jenkin et al., 2010). In this thesis, we have shown for the first time that GPR55

expression is increased in the kidneys of obese rodents and in proximal tubule cells exposed to high glucose and albumin. Both obesity and diabetes are independent risk factors associated with the presence and progression of CKD (Foster et al., 2008, Kramer et al., 2005, Levey and Coresh, 2012, Afkarian et al., 2013, Colagiuri et al., 2003, Eknoyan, 2007), and increased expression of GPR55 under these conditions indicates a potential role for the receptor within renal physiology.

It was hypothesised that increased renal GPR55 expression may indicate that antagonist treatment with O-1918 may lead to beneficial renal outcomes in a model of DIO. However, in Chapter 8, we determined that treatment with the GPR55 agonist, O-1602 and antagonist, O-1918 in an obese rodent model led to significant reductions in proteinuria and albuminuria compared to obese control animals. In addition to these improvements in markers of renal damage, reduced weight gain in obese animals treated with O-1602 was also observed. In obesity related renal damage, elevated proteinuria and albuminuria is thought to be due to excess metabolic excretory load, imbalance of nephron number relative to total body weight and structural renal remodeling including glomerulosclerosis (Afshinnia et al., 2010). Thus, reduced weight gain and food consumption may contribute to lowering the amount of urinary protein and albumin excreted by O-1602 treated animals (Afshinnia et al., 2010, Díaz-Arteaga et al., 2012). However, it is unlikely that reduced weight gain is the only factor contributing to improvements to markers of kidney damage associated with DIO in O-1602 treated animals; thus further investigation is required to elucidate the mechanisms governing these observations.

Improvements in protein and albumin excretion were also demonstrated in animals treated with O-1918, without changes in weight gain, indicating that the two GPR55 compounds are likely to activate differential signalling pathways in animals with DIO. As with the CB1 antagonist, O-1918 treatment significantly reduced tubular cross sectional area in obese animals. While O-1918 treatment in obese animals did not alter other signs of renal hypertrophy, as can be measured by gross kidney weight, kidney to body weight ratio or glomerular cross sectional area, it is unclear whether reduction in tubular hypertrophy or, alternative mechanisms may modulate the improvement in proteinuria and albuminuria.

Treatment with GPR55 agonist, O-1602 and antagonist, O-1918 was found to not alter blood pressure, urinary sodium excretion, creatinine clearance, plasma TGF-β1 and plasma creatinine concentrations or renal cytokine profile. The link between fibrosis in the kidney and associated decline in renal function has been outlined in Section 9.1.2. This study has attempted to identify whether a few key markers of fibrosis, including TGF-β1, VEGF and collagen IV may be altered in the kidneys of obese animals treated with O-1602 and O-1918. Data presented in Chapter 8 demonstrated that in obese animals, treatment with both O-1602 and O-1918 does not lead to altered renal cytokine profile of TGF-β1, VEGF or collagen IV. This presents the question as to what signalling pathways are being activated in the kidneys of obese animals, in order for proteinuria and albuminuria to be ameliorated by treatment with O-1602 and O-1918. As described previously, there are a number of alternative pathways beyond the activation of pro-fibrotic markers which contributes to structural damage associated with obesity, including interleukins, nitric oxide synthase, adipokines, and activation of the sympathetic nervous system (Briffa

et al., 2013, Lambert et al., 2010, Bosse et al., 1995, Adelman, 2002). Further investigation into the potential role of GPR55 mediating these regulatory factors may provide a clearer understanding as to how the receptor modulates the process of proteinuria and albuminuria in DIO.

It is important to note that the compounds used within this study may be acting upon other GPCR's including GPR18, PPAR's and TRPV1 (Henstridge, 2012). Currently, GPR18 is not thought to be expressed in kidney tissue (McHugh and Bradshaw, 2013), however, receptors belonging to both the PPAR and TRPV families have been shown to not only to be expressed in kidney tissue, but also mediate a number of important physiological processes within renal tissue, including inflammation, fibrosis, hypertrophy and hypertension (Woudenberg-Vrenken et al., 2009, Braissant et al., 1996, Jenkin et al., 2010, Guan and Breyer, 2001, Feng et al., 2008). A number of cannabinoid ligands, including AEA and 2AG, have been shown to activate both PPARα and PPARγ receptors (Pertwee et al., 2010), while off-target effects of treatment with O-1602 and O-1918 are often attributed to PPAR activity (Henstridge, 2012). PPARα receptors are highly expressed within proximal tubule cells of the kidneys (Braissant et al., 1996, Panchapakesan et al., 2005) and are important for initiating the utilisation of fatty acids (Braissant et al., 1996), while PPARγ receptors are expressed throughout all structures of the nephron as well as vascular cells of the kidney and mediate renal sodium and water reabsorption via NHE3 (Braissant et al., 1996, Pegg et al., 2013). PPARγ agonists are a therapeutic target for the treatment of diabetes (Pertwee et al., 2010) and have been shown to have a protective role in reducing inflammation, fibrosis and sodium and water reabsorption in diabetic nephropathy (Pegg et al., 2013). It is possible that the

beneficial renal effects of treatment with O-1602 or O-1918 may be via PPARγ; however, current research is lacking data showing the specificity and potency of these compounds.

TRPV1 is abundantly expressed within renal sensory nerve fibres (Feng et al., 2008), as well as within specific renal cells, including proximal tubule cells (Jenkin et al., 2010). Physiologically, TRPV1 acts as a mechanoreceptor within the kidneys and is therefore potentially an important modulator of renal haemodynamics and urinary sodium excretion (Feng et al., 2008). In an experimental model of hypertension, TRPV1 knockout mice exhibit significantly increased albuminuria and worse structural damage and creatinine clearance compared to wild type hypertensive mice (Wang et al., 2008a). However, in normotensive animals, genetic deletion of TRPV1 does not affect renal structure or function (Wang et al., 2008a), indicating the receptor may not play a large role regulating renal physiology but may provide a protective role under hypertensive conditions. As with receptors belonging to the PPAR family, TRPV1 is known to be activated by a number of cannabinoids (Pertwee et al., 2010). However, the selectivity and potency properties of O-1602 and O-1918 in relation to the activation of TRPV1 and PPAR receptors has yet to be fully investigated, which limits our ability to draw conclusions as to whether GPR55 alone may be responsible for the reduction of proteinuria and albuminuria in DIO animals.

### *9.1.4 General Conclusions*

CB1, CB2 and GPR55 belong to the complex endogenous lipid signalling system of the endocannabinoid system. It has only been in recent years that this system has

been explored for its therapeutic potential to treat a range of diseases, including nephropathy (Atwood et al., 2012, Campbell and White, 2008, Di Marzo, 2009, Huwiler and Pfeilschifter, 2009, Barutta et al., 2010, Barutta et al., 2011, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Mukhopadhyay et al., 2010b, Nam et al., 2012). Importantly, this thesis has demonstrated that in proximal tubule cells and in whole kidney tissue of diabetic and obese rats, expression of cannabinoid receptors are significantly altered (Jenkin et al., 2013). We have also provided evidence showing that targeting cannabinoid receptors may be of significant clinical importance, as treatment with the compounds AM251, AM1241, O-1602 and O-1918 were able to ameliorate proteinuria and albuminuria in obese animals. The expression and function of cannabinoid receptors in renal physiology is an area of research which is only at its inception, however it is clear that CB1, CB2 and GPR55 could be physiological targets for the treatment of obesity and diabetic related renal disease.

## ***9.2 Recommendations for Future Research***

To extend on the observations contained within this thesis, it is suggested that future work in this area may look at the following:

### *9.2.1 Expression of Cannabinoid Receptors within Human Kidneys*

Our current understanding of cannabinoid receptors in renal physiology is almost exclusively derived from data compiled from cell culture and rodent models of nephropathy. Historically, animal and cell culture models of disease have proved to

be critical in the exploration and characterisation of disease pathology, therapeutic target identification and signalling pathways (Keenan et al., 2000, Lin and Sun, 2010), but this experimental approach can sometimes be limited when translated across to clinical applications in human pathology (McGonigle and Ruggeri, 2013). At the inception of endocannabinoid research, both CB1 and CB2 were not detected in many peripheral tissues, including the kidneys (Nieri et al., 2006, Galiègue et al., 1995). This conceivably limited further investigation into the expression and function of the endocannabinoid system within the kidneys. More recent data has showed that CB2 expression is reduced in human kidney tissue of patients with ESRD, which is also demonstrated in animals with diabetic nephropathy (Barutta et al., 2011). Future research should determine the levels of expression of CB1, CB2 and GPR55 in kidney tissue, as well as the tissue specific distribution of humans in order to ascertain whether data derived from cell culture and animal models can be translated to human renal physiology. As CB1, CB2 and GPR55 are all expressed in a range of tissues, if these receptors are to be investigated as viable physiological targets for the treatment of diabetic nephropathy, it is critical to first understand how the expression of these receptors is modified in renal cells exposed to diabetic or obese conditions. Future research should examine localisation of cannabinoid receptor expression within renal tissue using either mRNA (*in situ* hybridisation) or protein (immune-histological analysis) in both healthy and disease states. Further, if cannabinoid receptors are to be used as viable targets for the treatment of obesity and diabetic related renal disease, research needs to identify whether CB1, CB2 and GPR55 expression is also altered in humans with CKD.

On a cellular level, CB1 and CB2 are known to be highly expressed within podocyte cells in the rodent glomerulus (Barutta et al., 2010, Barutta et al., 2011, Janiak et al., 2007). The observations outlined in this thesis describe cannabinoid receptor expression in whole cell lysate of proximal tubule cells and kidney tissue. To date, limited work has investigated cannabinoid receptor expression within the tubules specifically. Proximal tubule cells play an important role in the progression of CKD in conjunction with glomerular damage (Vallon, 2011b). Localisation studies of cannabinoid receptor expression within the human renal proximal tubule are important for understanding how CB1, CB2 and GPR55 mediate renal physiology in health and disease. Within the nephron, the apical and basal aspects of proximal tubule cells are exposed to two distinct cellular environments; of the lumen containing the filtrate from the glomerulus, and the interstitial space with the surrounding capillary bed (Christensen et al., 1998). Apical localisation may indicate that the receptors are directly modulated by components within the filtrate, which may further elucidate the potential role cannabinoid receptors have in tubular function.

### *9.2.2 Functional Role of Cannabinoid Receptors within the Kidneys*

While it is clear that CB2 is involved in mediating cytokine production and reducing inflammatory response within the kidneys (Barutta et al., 2011, Mukhopadhyay et al., 2010b), it has yet to be elucidated how CB1 and GPR55 reduces proteinuria and albuminuria caused by DIO. In this respect, it is important to characterise the specific signalling pathways by which cannabinoid receptors modulate renal physiology in health and disease. This thesis has characterised the expression of three pro-fibrotic

markers known to be involved in nephropathy, in response to the pharmacological targeting of different cannabinoid receptors. However, renal damage associated with obesity is the result of a number of complex regulatory factors which drive inflammation, fibrosis, hypertrophy and changes to haemodynamics within the kidney (Vallon, 2011b, El Nahas and Bello, 2005). Novel advances in bioinformatics have shown that computational modelling systems can use RNA interference screens and input from genomic wide association studies to identify vast, complex signalling networks involved in disease progression (Gitter and Bar-Joseph, 2013). Future research using these bioinformatics methods could be an efficient and high-throughput method used to identify key regulatory pathways of cannabinoid receptors in the progression of CKD.

This thesis also showed that in DIO, treatment with the GPR55 agonist, O-1602 and antagonist, O-1918, both reduced proteinuria and albuminuria. Previously, it has been shown that it is difficult to determine whether the effects of these compounds are solely derived from GPR55 signalling, or, if O-1602 and O-1918 are eliciting effects via other receptors, such as GPR18, TRPV1 or PPAR's (Henstridge, 2012). As the GPR55 compounds O-1602 and O-1918 also target other receptors, investigation of a DIO model where there was GPR55 overexpression or knockout of GPR55, specifically within the kidneys would help characterise the physiological role of this receptor in renal system. Experimental models which target renal expression of CB1 and CB2 via knockout or overexpression transgenic animal models may also help further our current understanding of cannabinoid receptors in mediating renal physiology in both health and disease.

*9.2.3 Potential Therapeutic Benefits of Targeting Cannabinoid Receptors in CKD*

The studies within this thesis have clearly demonstrated that treatment with the CB1 antagonist, AM251 and CB2 agonist, AM1241, both reduce proteinuria and albuminuria associated with DIO. We have shown in Chapter 6, that AM251 can significantly reduce weight gain in animals with DIO, which is supported by a large body of research which highlights that CB1 antagonists are very effective at combating obesity via central and peripheral neuronal pathways which lead to reduced food intake (Rosenstock et al., 2008, Després et al., 2005, Dol-Gleizes et al., 2009, Isoldi and Aronne, 2008, Van Gaal et al., 2005). However, due to an increased risk of adverse psychological side effects including anxiety and depressive symptoms, CB1 antagonists like Rimonabant have been withdrawn from clinical use (Van Gaal et al., 2005). A new generation of CB1 antagonists which have low permeability across the blood brain barrier are currently being developed (Sink et al., 2007, Son et al., 2009, Tam et al., 2010). These compounds are predominately peripherally acting, but have been shown to reduce weight and food intake, improve glucose and lipid blood profile (Sink et al., 2007, Tam et al., 2010, Son et al., 2009). Expression of GPCRs is tightly regulated, and it is currently unclear whether pharmacological modulation of a specific cannabinoid receptor may lead to changes in renal expression of other cannabinoid receptors within the kidney. Investigating whether pharmacological interventions may alter renal expression of cannabinoid receptors will not only broaden our understanding of how the endocannabinoid signalling system within the kidneys, it could also potentially lead to opportunities to explore the simultaneous modulation of multiple cannabinoid receptors. Given our current understanding of the role of cannabinoid receptors within obesity and

diabetic related renal damage, this approach may be beneficial for the treatment of CKD. Future studies may consider using an adjunctive treatment combining new generation CB1 antagonists with CB2 agonists such as AM1241. This combined treatment would potentially be able to reduce the metabolic risk factors associated with CKD via CB1 blockade, whilst suppressing pro-fibrotic and inflammatory cytokines, which mediate renal structural changes with CB2 agonists. This dual approach may be more effective in treating CKD, compared to pharmacologically targeting either CB1 or CB2 alone.

Currently, research has shown that GPR55 may have an important role in diabetes and obesity (Simcocks et al., 2014). GPR55 has been identified in pancreatic beta cells and, *in vitro*, when beta cells are treated with O-1602, glucose stimulated insulin secretion is significantly increased (Romero-Zerbo et al., 2011). The role of GPR55 modulating glucose homeostasis has further been supported by *in vitro* experiments, where fasted rats acutely treated with O-1602 showed significant increases in glucose tolerance compared to vehicle treated animals (Romero-Zerbo et al., 2011). In obesity, GPR55 may have a role in modulating food intake and adiposity (Imbernon et al., 2013). In Chapter 8, we demonstrated that O-1602 significantly reduces weight gain in obese rats, while conflicting research indicates that O-1602 induces food intake and adipose storage (Díaz-Arteaga et al., 2012). The disparate results could be due to a number of factors, including differences in dosage, duration and route of administration of O-1602. In addition to these factors, both O-1602 and O-1918 can activate other GPCR's, as outlined in Section 9.1.3. Research should focus on the pharmacology of GPR55 signalling in order to

ascertain whether the receptor may be of potential therapeutic benefit for the treatment of CKD.

Finally, prospective studies need to examine the efficacy and safety of targeting cannabinoid receptors in human models of obese and diabetic CKD. Compounds which act as both CB1 antagonists and CB2 agonists have been shown in clinical trials to be safely administered to humans (Fernández-Ruiz et al., 2013, Pacher and Kunos, 2013). The studies contained within this thesis, and previously published observations (Barutta et al., 2010, Barutta et al., 2011, Janiak et al., 2007, Mukhopadhyay et al., 2010a, Mukhopadhyay et al., 2010b) indicate that pharmacologically targeting cannabinoid receptors to treat CKD could potentially be a powerful tool in ameliorating the progression of CKD. However, future research needs to rigorously evaluate the use of these compounds within a CKD population before the efficacy and safety can be determined.

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