REGULATION OF SKELETAL MUSCLE GLUCOSE UPTAKE DURING CONTRACTION/ EXERCISE BY NITRIC OXIDE (NO)/ NEURONAL NITRIC OXIDE SYNTHASE MU (nNOSμ)

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ABSTRACT

During exercise/ muscle contraction, large amounts of blood glucose are taken up into skeletal muscle fibers thus removing glucose from the bloodstream. This process is regulated differently to insulin-stimulated muscle glucose uptake and is normal in humans with type 2 diabetes (T2D) and in diabetic rodent models. A number of candidates have been implicated to play a role in the regulation of skeletal muscle glucose uptake during contraction and it is likely that there is some redundancy. In humans, there is convincing evidence that nitric oxide (NO) plays a role in contraction-stimulated muscle glucose uptake and may be promising from a therapeutic standpoint for people with T2D since they have a greater reliance on NO-mediated glucose uptake during exercise. However, evidence for a role of NO in regulating contraction-stimulated muscle glucose uptake in rodents is conflicting, most likely due to methodological inconsistencies. Studies have almost entirely involved the use of NO synthase (NOS) inhibitors with little data in genetically-modified models examining the role of NO in contraction-stimulated glucose uptake. Neuronal NOSμ is the major NOS isoform that activates the NO/ cGMP downstream signalling during skeletal muscle contraction. In this thesis, the role of NO/ nNOSμ in the regulation of skeletal muscle glucose uptake was examined. In rodents, NO/ nNOSμ is also involved in the regulation of skeletal muscle blood flow during contraction/ exercise, and glucose uptake is influenced by blood flow and glucose delivery. Therefore, mice lacking nNOSμ were used to investigate its role in glucose uptake during both *ex vivo* contraction and more physiological *in vivo* treadmill exercise. Also, a T2D rat model was generated and used to investigate if diabetic rats have a greater reliance on NO-mediated muscle glucose uptake during *in situ* contraction, as has previously been shown during exercise in people with T2D.

In Study 1, an *ex vivo* contraction experiment, nNOSμ$^{-/-}$ muscles had a normal contraction-stimulated muscle glucose uptake indicating that nNOSμ was not essential in regulating skeletal muscle glucose uptake during *ex vivo* contraction. Interestingly, despite this, the increase in muscle glucose uptake during contraction was significantly

attenuated by L-NMMA (a NOS inhibitor) in both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles. This attenuation was reversed by L-arginine suggesting that L-NMMA specifically inhibited NOS to attenuate muscle glucose uptake during contraction. These results showed that NO plays a role in mediating skeletal muscle glucose uptake during *ex vivo* contraction but not via nNOSμ. In Study 2, surprisingly, NOS activity was not increased with *in situ* contractions in both control and T2D Sprague Dawley (SD) rats, which differed to our previous study showing an increase in NOS activity during contraction in normal non-diabetic Wistar rats. This lack of increase in NOS activity with contraction may explain the observed lack of effect of NOS inhibition on glucose uptake during contraction in this *in situ* study in both control and T2D SD rats, again different to the attenuation of glucose uptake with NOS inhibition during contraction we observed previously in Wistar rats. This may have been due to the well documented strain differences between the two strains of rats. In Study 3, the role of nNOSμ in skeletal muscle glucose uptake during *in vivo* exercise in mice was investigated. Unexpectedly, nNOSμ$^{-/-}$ mice had a higher muscle glucose uptake during exercise than both nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ mice, which was associated with greater AMPK phosphorylation during exercise in nNOSμ$^{-/-}$ mice. On the other hand, nNOSμ$^{+/-}$ mice had normal glucose uptake during exercise.

In summary, although NO appears to play a role in skeletal muscle glucose uptake during contraction, it appears that this is not via nNOSμ. Surprisingly, it appears that unlike Wistar rats and our previous studies in mice and humans, NO is not required for skeletal muscle glucose uptake in SD rats in this thesis. Further studies are required to determine the source of NO during contraction/ exercise and how NO increases glucose uptake during contraction/ exercise.

DECLARATION

I, Yet Hoi Hong, declare that the PhD thesis entitled "Regulation of skeletal muscle glucose uptake during contraction/ exercise by nitric oxide (NO)/ neuronal nitric oxide synthase mu (nNOSμ)" is not 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 in the Preface, this thesis is my own work.

Signature: 

Date: 17 /09 /2014

PREFACE

All work carried out in the preparation of this thesis was my own except for that acknowledged below:

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[^{14}C]mannitol	D-[^{14}C] mannitol
[^{3}H]2-DG	[1,2-^{3}H]2-deoxy-glucose
[^{3}H]2-DG-6-P	[1,2-^{3}H]2-deoxy-glucose-6-phosphate/ phosphorylated [^{3}H]2-DG
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole 4-carboxamide ribonucleoside
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APS	Ammonium persulfate
AS160	Akt substrate of 160 kDa/ TBC1D4
ATP	Adenosine triphosphate
$Ba(OH)_2$	Barium hydroxide
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CaMKII	Calmodulin-dependent kinase II
CEU	Contrast-enhanced ultrasound
cGMP	Cyclic guanosine monophosphate
dNTP	Deoxynucleotide triphosphate
dpm	Disintegration per minute
DTT	Dithiothreitol
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
$eNOS^{-/-}$	eNOS knockout
$eNOS^{+/-}$	eNOS heterozygous

FAD	Flavin adenine dinucleotide
FBF	Femoral artery blood flow
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GLUT4	Glucose transporter 4
GMP	guanosine monophosphate
GTP	guanosine triphosphate
HCl	Hydrochloric acid
HKII	hexokinase II
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin-receptor substrate 1
KCl	Potassium chloride
KD	Kinase dead
KH_2PO_4	Potassium phosphate monobasic
KOH	Potassium hydroxide
L-NAME	N-G-Nitro-L-Arginine Methyl Ester
L-NMMA	N-G-Monomethyl-L-arginine
$MgSO_4$	Magnesium sulfate
Mn^{2+}	Manganese
NAC	N-Acetylcysteine
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
nNOSμ	Neuronal nitric oxide synthase mu
$nNOS\mu^{-/-}$	nNOSμ knockout
$nNOS\mu^{+/-}$	nNOSμ heterozygous
$nNOS\mu^{+/+}$	nNOSμ wild type
NO	Nitric oxide

NOS	Nitric oxide synthase
$O_2^{\bullet-}$	Superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one
$ONOO^-$	Peroxynitrite
PAS	phospho-Akt substrate
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
ROS	Reactive oxygen species
SD	Sprague Dawley
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STZ	Streptozotocin
SVL	Superficial vastus lateralis
T2D	Type 2 diabetes
TBST	Tris Buffered Saline with 0.1% Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
VO_2 peak	Peak oxygen consumption
vs	Versus
ZMP	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranotide
$ZnSO_4$	Zinc sulfate

CHAPTER ONE:

LITERATURE REVIEW

1.1 INTRODUCTION

Exercise can be an effective tool for improving glycaemic control of people with chronic hyperglycaemia states as skeletal muscle glucose uptake during exercise (Kingwell et al., 2002, Martin et al., 1995) and the increase in insulin sensitivity after training (Bruce et al., 2004) appears to be normal in people with type 2 diabetes (T2D). In fact, physical activity/ exercise is often advocated to be an effective therapy or as an important adjunct therapy in managing chronic hyperglycaemia in people with T2D (Albright et al., 2000). These people have an impairment or inadequacy in glucose uptake into tissues, in particular the skeletal muscles (plasma glucose disposal) in response to insulin (DeFronzo et al., 1985, Matthaei et al., 2000), a condition known as insulin resistance. A meta-analysis including 14 clinical trials investigated the effects of exercise on glycosylated hemoglobin (HbA_{1c}), an index for glycaemic control over the past three months, and body mass in people with T2D corroborated that regular exercise improves glycaemic control independent of body mass changes (Boule et al., 2001). In addition, a 45-min bout of cycling exercise at high intensity (70% VO_2 peak) normalised plasma glucose levels of people with T2D during the exercise (Musi et al., 2001a). Another study from our group employed a lower intensity (60% VO_2 peak) and shorter duration (25 min) cycling exercise also demonstrated a significant reduction in arterial blood glucose levels during exercise in people with T2D (Kingwell et al., 2002). These studies indicate that people with T2D have a normal contraction-stimulated glucose uptake (Kingwell et al., 2002, Martin et al., 1995), which appears to be dependent on exercise intensity and duration, despite impairment in insulin-stimulated glucose uptake. In fact, they have a normal increase in glucose transporter 4 (GLUT4) translocation (Kennedy et al., 1999), a critical process for skeletal muscle glucose uptake during exercise (Zisman et al., 2000). These studies highlight that contraction-stimulated skeletal muscle glucose uptake is at least as effective as insulin in stimulating glucose

disposal and underpin its therapeutic potential and clinical relevance in managing hyperglycaemic state in people with T2D.

Such potential leads to a great inclination to investigate the mechanisms of the contraction-mediated increase in skeletal muscle glucose uptake. In particular, current research aims to understand the molecular signalling events that lead to an increase in glucose uptake in response to muscle contraction. Such studies could provide important knowledge that serves as a biochemical entry point for the development of pharmacological exercise mimetics that could improve muscle glucose uptake without having to perform the actual exercise. This is an extremely important and beneficial strategy for people with insulin resistant or T2D as physical exercise is often not well tolerated or complied with by these populations who are either obese and/ or aged and may be physically-confined by various diabetic complications. So far, the mechanism(s) regulating contraction-stimulated skeletal muscle glucose uptake are still not well understood. There is some evidence that several signalling pathways may be involved in the regulation of contraction-stimulated skeletal muscle glucose uptake including calcium/ calmodulin-dependent protein kinase II (Ca^{2+}/CaMKII) (Witczak et al., 2007, Wright et al., 2004), AMP-activated protein kinase (AMPK) (Hayashi et al., 1998, Mu et al., 2001), protein kinase C (PKC) (Ihlemann et al., 1999a, Niu et al., 2011), reactive oxygen species (ROS) (Merry et al., 2010c, Sandstrom et al., 2006) and nitric oxide (NO) (Balon, 1998, Merry et al., 2010c, Roberts et al., 1997, Ross et al., 2007). Over the last few years even more novel proteins have been shown to at least partially regulate skeletal muscle glucose uptake during contraction such as sucrose non-fermenting AMPK-related kinase (SNARK) (Koh et al., 2010), Myo1c (Toyoda et al., 2011), PIKfyve (Liu et al., 2013) and Rac1/ PAK1 (Sylow et al., 2013).

There are likely many redundancies between these signalling pathways which lead to the increase in glucose uptake in contracting muscles (Jessen and Goodyear, 2005, Richter and Hargreaves, 2013). This literature review will focus on the acute regulation of skeletal muscle glucose uptake during contraction/ exercise, with a

particular focus on the role and possible mechanism(s) of nitric oxide (NO) and/or neuronal NO synthase mu (nNOSμ) in this process. The role of other potential signalling pathways in the regulation of skeletal muscle glucose uptake during contraction will also be briefly discussed; and readers are referred to the following reviews for further details (Fujii et al., 2006, Jensen et al., 2009b, Merry and McConell, 2009, Richter et al., 2004, Richter and Hargreaves, 2013). It is also of note that muscle contraction/ exercise can increase insulin sensitivity for up to 4-48 hours post-exercise (Richter et al., 1982, Wojtaszewski et al., 2000a). Chronic exercise can also induce training-related muscle adaptive changes that promote muscle glucose uptake. These topics, however, will not be discussed here and have been well summarised by others (Holloszy, 2005, Rockl et al., 2008, Wang et al., 2009).

1.2 DIFFERENCES BETWEEN CONTRACTION-STIMULATED AND INSULIN-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

Before proceeding with the discussion about the literature on skeletal muscle glucose uptake during contraction/ exercise, it is important to make clear that the term "contraction" will normally be used to refer to muscle contraction during *ex vivo* or *in situ* conditions; while "exercise" will exclusively be used to refer to the *in vivo* exercise setting only (e.g. treadmill exercise). The term "contraction-stimulated" can refer to either one or all of these conditions. A number of stimuli or factors have been found to have an influence on glucose uptake in skeletal muscle including, but not limited to, skeletal muscle contraction (Holloszy and Narahara, 1965), insulin (Hardwick et al., 1959). nitrate ions (Holloszy and Narahara, 1967b), leptin (Shiuchi et al., 2001), hypoxia (Cartee et al., 1991) and neuregulin (Suarez et al., 2001). Insulin is one of the most important physiological stimuli that regulates skeletal muscle glucose uptake. Skeletal muscle contraction has also long been recognised to have a stimulatory effect on skeletal muscle glucose uptake (Goldstein et al., 1953, Helmreich and Cori, 1957, Huycke and Kruhoffer, 1955).

Like insulin, skeletal muscle contraction/ exercise is a very potent physiological stimulus of skeletal muscle glucose uptake. The experiments by Holloszy et al. (1965, 1967a) demonstrated that isolated frog muscle contracted *ex vivo* led to an increase in cell membrane permeability to glucose. This contraction-stimulated glucose uptake was later demonstrated in various mammalian muscles. An early study by Berger et al. (1975) reported that contraction only increases skeletal muscle glucose uptake in perfused hindlimb muscles of severely diabetic rats when insulin is added into the perfusion medium claiming a "permissive effect" of insulin in contraction-stimulated glucose uptake. However, others have shown that contraction stimulates glucose uptake in the absence of insulin (Holloszy and Narahara, 1965, Holloszy and Narahara, 1967a). Holloszy et al. (1984, 1985) repeated the experiment applying the same methodology as that by Berger et al. and found that insulin is not necessary for the stimulation of skeletal muscle glucose uptake during contraction in rodents (Wallberg-Henriksson and Holloszy, 1984, Wallberg-Henriksson and Holloszy, 1985). A study conducted by Nesher et al. (1985) supported these observations using three different preparations of muscles that were depleted of insulin: 1) exhaustively washed muscles for 2 hours; 2) muscles from rats that were infused with anti-insulin serum; and 3) chronically streptozotocin-induced diabetic rat muscles. They demonstrated a significant increase in muscle glucose uptake in contracted muscles in the absence of insulin (Nesher et al., 1985) showing that muscle contraction alone can increase glucose uptake and insulin and contraction are likely to operate through different pathways.

Further evidence that insulin and contraction act through different mechanisms is that maximal effects of insulin and contraction on skeletal muscle glucose uptake are additive (Constable et al., 1988, Nesher et al., 1985, Wallberg-Henriksson et al., 1988, Zorzano et al., 1986). Lund et al. (1995) investigating glucose transport and GLUT4 translocation in rat soleus muscles provided evidence that combined effects of maximal insulin stimulation and muscle contraction on glucose transport and GLUT4 translocation were greater than either stimulation alone. These findings support that different mechanisms are responsible for insulin- and contraction-stimulated glucose

uptake. In fact, the finding that skeletal muscle glucose uptake in response to exercise (King et al., 1993) or contraction (Brozinick et al., 1992, Brozinick et al., 1994b) in insulin resistant obese Zucker rats was normal further supports the notion that insulin and muscle contraction stimuli operate differently. Similar evidence was demonstrated in humans where acute exercise resulted in normal leg glucose uptake (Kingwell et al., 2002) and GLUT4 translocation (Kennedy et al., 1999) in people with T2D.

Further investigation of the insulin signalling pathway provided evidence at the molecular level that the mechanisms involved in insulin and muscle contraction in stimulating skeletal muscle glucose uptake are different (Fig 1.1). Inhibition of phosphatidylinositol 3-kinase (PI3K), a critical signalling intermediate for the insulin pathway (Cheatham and Kahn, 1995, Clarke et al., 1994), with wortmannin had no effect on glucose uptake and GLUT4 translocation in isolated rat skeletal muscles contracted *ex vivo* (Lee et al., 1995, Lund et al., 1995, Yeh et al., 1995). Furthermore, muscle contraction/ exercise did not activate the proximal signalling cascade of insulin stimulation such as signal transduction of insulin receptor, insulin-receptor substrate 1 (IRS-1) or Akt/ protein kinase B (Brozinick and Birnbaum, 1998, Goodyear et al., 1995, Widegren et al., 1998). These findings indicate that exercise and insulin do not operate via the same pathway.

Nevertheless, both insulin and muscle contraction stimulate skeletal muscle glucose uptake via a same critical step – increase translocation of GLUT4 to the cell membrane allowing increased extracellular glucose diffusion into muscle fibers. Various studies, in both rodents (Brozinick et al., 1994a, Etgen et al., 1993, Goodyear et al., 1991, Lauritzen et al., 2010, Lund et al., 1995, Roy and Marette, 1996, Zisman et al., 2000) and humans (Kennedy et al., 1999, Kristiansen et al., 1996, Kristiansen et al., 1997, Thorell et al., 1999), indicated that muscle contraction signals skeletal muscle glucose uptake via increased translocation of GLUT4 to the cell membrane. It was clearly demonstrated that GLUT4 transporters are essential for glucose transport stimulated by insulin and/or muscle contraction by disrupting GLUT4 expression in

mouse skeletal muscles (Fueger et al., 2007, Zisman et al., 2000). In these studies, insulin and/ or muscle contraction/ exercise failed to stimulate glucose transport in muscles lacking GLUT4 (Fueger et al., 2007, Zisman et al., 2000).

Taken together, these results suggest that insulin and muscle contraction stimulate different proximal signalling pathways, which converge to elicit GLUT4 translocation to the membrane for glucose transport and uptake into the muscle. In the subsequent sections of this thesis the focus of discussion will be on contraction-stimulated skeletal muscle glucose uptake and its potential signalling.

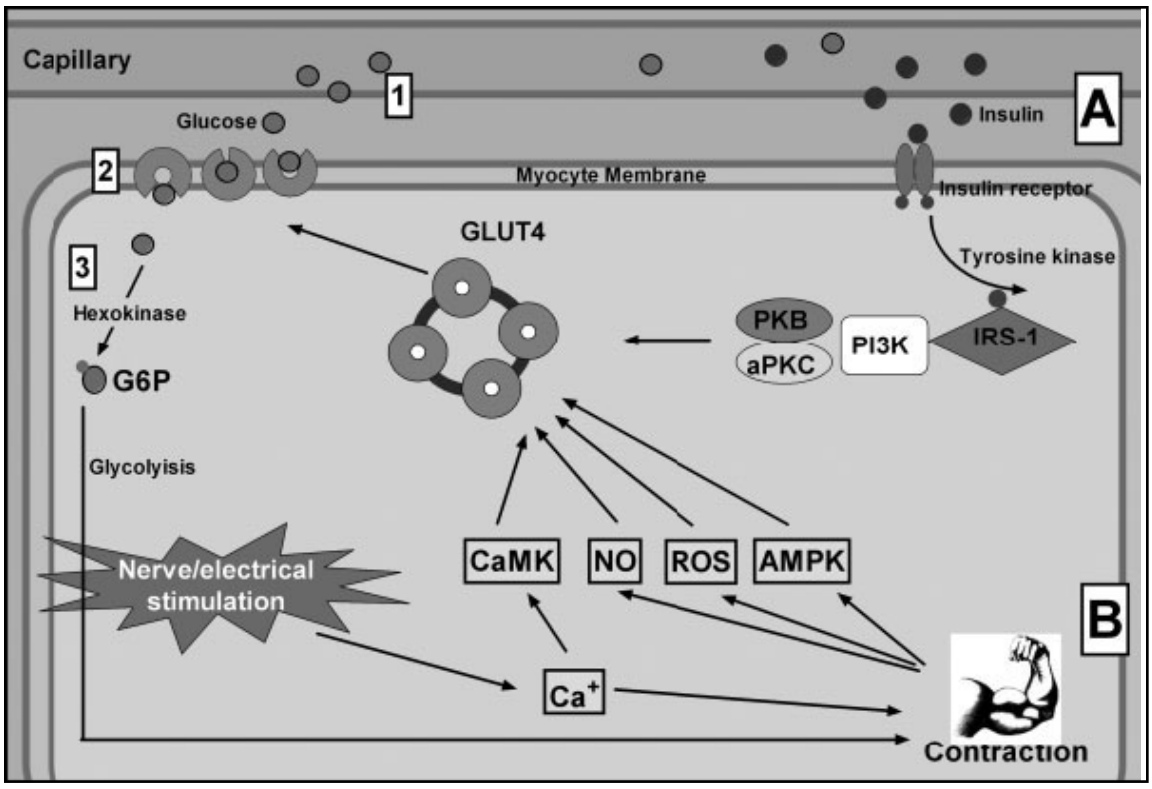


Figure 1.1: Minimal model of the mechanisms of glucose uptake into skeletal muscle. (A) Insulin-stimulated glucose uptake, (B) Potential mechanisms involved in contraction-stimulated glucose uptake.

(1) Glucose delivery to the muscle cell, (2) glucose transport through the membrane and (3) glucose phosphorylation and therefore flux through metabolism. G6P: glucose-6-phosphate, CaMK: calmodulin-dependent protein kinase, NO: nitric oxide, ROS: reactive oxygen species, AMPK: AMP-activated protein kinase, aPKC: atypical protein kinase C, PKB: protein kinase B/Akt, PI3K: phosphatidylinositol 3-kinase, IRS-1: insulin receptor substrate 1. Adapted from (Merry and McConell, 2009)

1.3 DETERMINANTS OF SKELETAL MUSCLE GLUCOSE UPTAKE DURING CONTRACTION/ EXERCISE

Glucose uptake into skeletal muscle is a complex process that involves 1) plasma glucose transport across the capillary into the interstitial space, then 2) traversing across the cell membrane into the intracellular space and then being 3) phosphorylated to glucose-6-phosphate (Fig 1.1). In this phosphorylated state, the glucose becomes impermeable to the membrane and as such will remain within the cell and committed to further metabolism either through glycolysis or glycogen formation. The rate of skeletal muscle glucose uptake can be regulated at any of all these three processes (Wasserman and Ayala, 2005, Wasserman et al., 2011). In a physiologically intact organism, the primary limiting step(s) for skeletal muscle glucose uptake is difficult to be discerned as under different circumstances the predominant barrier for glucose uptake is dynamically shifting between these three factors (Fueger et al., 2005, Halseth et al., 1998, Richter and Hargreaves, 2013, Wasserman, 2009).

Glucose delivery to skeletal muscle is a function of muscle blood flow and blood glucose concentration. Blood glucose concentration is normally regulated tightly within a narrow range. Given this skeletal muscle perfusion would then be the main determinant for muscle glucose delivery. During exercise, skeletal muscle blood flow can increase up to 20-fold (Radegran and Saltin, 1998, Sorlie and Myhre, 1978) along with, though remained controversial, the recruitment of nutritive capillaries (Clark et al., 1998, Sjoberg et al., 2011) delivering large amounts of glucose to the active muscles. As such, glucose delivery may exceed the capacity of membrane transport and/or glucose phosphorylation (Fueger et al., 2004a). The finding that there was a parallel increase in skeletal muscle interstitial glucose concentration, determined by a microdialysis technique, with increasing exercise intensity and blood flow; supported that glucose delivery can indeed exceed transport capacity during exercise (MacLean et al., 1999). If glucose delivery was limiting during exercise, then a drop in interstitial glucose level would be expected. This observation was supported by a genetic model where overexpressing muscle HK II led to an increase in glucose uptake during exercise

(Fueger et al., 2004c) indicating that glucose delivery and membrane permeability to glucose during exercise exceeded the phosphorylation capacity; thus these factors are unlikely to be the rate-limiting factors for glucose uptake during exercise.

Glucose uptake into skeletal muscle fibers is a passive facilitated transport process that requires the presence of glucose transporters docking onto the cell membrane which increase its permeability to plasma glucose. Membrane permeability is governed by the number of glucose transporters present on the cell membrane and possibly the transporter intrinsic activity (Goodyear et al., 1990, King et al., 1989, Klip, 2009, Zaid et al., 2009). Specifically, GLUT4 transporter is responsible for ferrying glucose into skeletal muscle fibers during contraction/ exercise. At rest, muscle glucose uptake is facilitated by the low level of GLUT1 expressed in the muscles (Ren et al., 1993) while most of the GLUT4 resides within vesicles in the cytoplasm (Foley et al., 2011, Ploug et al., 1998) and therefore, membrane permeability to glucose is low and limits muscle glucose uptake. Muscle contraction increases membrane permeability to glucose (Brozinick et al., 1994a, Derave et al., 1999, Goodyear et al., 1991, Karlsson et al., 2009, Lauritzen et al., 2010, Roy and Marette, 1996) through a not yet well-defined intramuscular signalling cascade that recruits and translocates intra-vesicular GLUT4 to the muscle membrane. Derave et al. (1999) demonstrated a strong positive correlation between sarcolemmal GLUT4 content and glucose uptake in a perfused contracting rat hindlimb suggesting an increase in membrane permeability increases glucose uptake when glucose supply was unrestricted. However, it was demonstrated that overexpression or partial knockout of GLUT4 protein did not affect muscle glucose uptake during exercise (Fueger et al., 2004a, Fueger et al., 2004b) indicating that GLUT4 content does not limit muscle glucose uptake during exercise and there is a great reserve for mobilisation of GLUT4 transporters, and therefore phosphorylation capacity rather than GLUT4 content may be limiting glucose uptake during exercise.

Another critical rate-limiting factor for skeletal muscle glucose uptake i.e. glucose phosphorylation appears to receive less attention than the other mentioned

factors. In skeletal muscle, glucose is phosphorylated by hexokinase II (HKII), (O'Doherty et al., 1994). At basal state, HKII could rapidly phosphorylate free glucose as such free glucose is normally not detectable in the muscle fibers (Ziel et al., 1988); and, therefore would not limit glucose uptake into skeletal muscle. This is supported by the finding that muscle HKII overexpression did not increase basal glucose uptake (Fueger et al., 2004c). Glucose phosphorylation becomes important when glucose delivery and membrane permeability increases such as during exercise. A pronounced accumulation of glucose-6-phosphate could feedback inhibit HKII activity making glucose phosphorylation a rate-limiting step (Wasserman et al., 2011). During exercise, skeletal muscle glucose uptake can be increased by overexpressing HKII indicating glucose phosphorylation can be a limiting factor during exercise (Fueger et al., 2004c). In humans, accumulation of glucose-6-phosphate was detected during intense maximal exercise (97% VO_2 peak) suggesting a possible feedback inhibition on HKII activity and thus limiting glucose uptake (Katz et al., 1986).

In summary, glucose delivery (blood flow and glucose concentration), membrane permeability and glucose phosphorylation influences the rate of glucose uptake into muscle fibers. Although GLUT4 translocation is the critical step that initiate muscle glucose uptake during exercise, glucose phosphorylation appears to be the rate-limiting factor in glucose uptake during exercise where the capacity of glucose delivery and GLUT4 translocation exceed the capacity of HKII in phosphorylating the transported glucose. The next section will discuss the potential regulation of skeletal muscle glucose uptake during contraction/ exercise.

1.4 REGULATION OF CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

The signalling mechanisms by which muscle contraction/ exercise stimulates skeletal muscle glucose uptake are not yet well defined. There is evidence that muscle contraction/ exercise activates a number of signalling mediators which in turn stimulates

TBC1D1 and/ or Akt substrate of 160 kDa (AS160 or TBC1D4), a possible convergence point for contraction- and insulin-stimulated signalling (Cartee and Funai, 2009), leading to increases in GLUT4 translocation to the sarcolemma and t-tubules (Ploug et al., 1998). Docking and fusion of GLUT4 onto the plasma membrane and t-tubule involve several GLUT4 vesicle and membrane binding proteins collectively known as SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors) (Richter and Hargreaves, 2013). There is also evidence that cytoskeleton proteins such as Rac 1 (Sylow et al., 2013) and Myo1c (Toyoda et al., 2011) are involved in GLUT4 translocation during contraction. The major potential mediators implicated in contraction-stimulated skeletal muscle glucose uptake include AMPK, Ca^{2+}/ CaMKII, PKC, ROS and NO (Richter and Hargreaves, 2013). The role of each of these mediators as well as AS160 and TBC1D1 in contraction-stimulated skeletal muscle glucose uptake will be briefly discussed here. However, the major focus of this literature review is on the potential role of NO in mediating skeletal muscle glucose uptake during contraction/ exercise which will be covered in Section 1.5.

1.4.1 Regulation of GLUT4 translocation by AS160 and TBC1D1

1.4.1.1 AS160 (TBC1D4) and TBC1D1

AS160 and TBC1D1 are paralog proteins that have been shown to be directly involved in liberating GLUT4 proteins from their intracellular vesicles and may also be the convergence point for insulin- and contraction-stimulated glucose uptake signalling pathways (Cartee and Wojtaszewski, 2007, Kramer et al., 2006a, Taylor et al., 2008). These molecules bridge the signalling gaps between the proximal signalling mediators directly activated by muscle contraction/ exercise and the translocation of GLUT4 to the sarcolemma and t-tubules.

AS160 is a member of the tre-2/USP6, BUB2, cdc16 (TBC1) domain family of proteins and is present in both skeletal muscle (Bruss et al., 2005, Karlsson et al., 2005)

and adipose tissue (Kane et al., 2002). Structurally, AS160 contains two phosphotyrosine-binding (PTB) domains and a Rab-GTPase-activating protein domain (Rab-GAP) at its NH_2 and COOH termini respectively (Kane and Lienhard, 2005). It also contains a calmodulin-binding domain (Kane and Lienhard, 2005). Six Akt-stimulated phosphorylation sites (phospho-Akt substrate, PAS) are identified on AS160 protein (Ser^{318}, Ser^{341}, Ser^{570}, Ser^{588}, Thr^{642}, Thr^{751}) (Sano et al., 2003). Except for Ser^{341}, these phosphomotif sites are responsive to insulin stimulation and are detectable by PAS antibody (Sano et al., 2003). In addition, AS160 can also be phosphorylated by AMPK (Bruss et al., 2005, Treebak et al., 2006, Treebak et al., 2014).

TBC1D1, on the other hand, is a paralog protein of AS160 sharing 50% of identity, with 79% identity in GTPase-activating protein (GAP) domains, and an identical Rab specificity of the GAP domain with AS160 (Roach et al., 2007). TBC1D1 and AS160 have a calmodulin-binding domain. In contrast to AS160, TBC1D1 has abundance of expression in skeletal muscles but little expression in adipocytes (Chavez et al., 2008, Taylor et al., 2008); suggesting a possible closer relationship between TBC1D1 and contraction-stimulated GLUT4 translocation in skeletal muscle than AS160. TBC1D1 has less insulin-responsive Akt phosphomotifs than AS160 (Chen et al., 2008, Roach et al., 2007, Sano et al., 2003); and, has AMPK phosphomotifs that are distinct from AS160 (Chen et al., 2008, Roach et al., 2007, Treebak et al., 2014, Vichaiwong et al., 2010). Thus, it appears that AS160 and TBC1D1 have, by themselves, a unique phosphosignature that may have an important regulatory function.

1.4.1.2 Mechanism of action of AS160 and TBC1D1

It is proposed that non-phosphorylated AS160 and TBC1D1 restrain mobilisation of GLUT4 vesicles to the cell membrane (Sano et al., 2003). GTP-activating protein (GAP) of AS160/ TBC1D1 promotes the hydrolysis of Rab-bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) on vesicles containing GLUT4 keeping Rab proteins in its inactive form, preventing translocation of GLUT4 to the cell surface (Sano et al., 2003). Phosphorylation at various specific phosphomotif sites,

including the PAS phosphomotifs, of AS160/ TBC1D1 inactivates Rab-GAP activity turning Rab proteins into an active form; this overcomes the inhibition of AS160/ TBC1D1 on GLUT4 vesicles allowing for translocation of GLUT4 (Sano et al., 2003). In addition, the binding of 14-3-3 protein to AS160/ TBC1D1 following their phosphorylation could play a further role in the inhibition of GAP activity and therefore promotes GLUT4 translocation (Ramm et al., 2006, Sakamoto and Holman, 2008). Studies using mice that overexpress mutant AS160 (known as AS160-4P), in which four of the Akt-responsive phosphomotifs were rendered non-phosphorylatable, demonstrated that *in vivo* insulin-stimulated muscle glucose uptake was greatly reduced indicating that phosphorylation of AS160 is essential for insulin-stimulated glucose uptake (Kramer et al., 2006b). In addition, insulin-stimulated muscle glucose uptake in muscle overexpressing AS160-4P was returned to normal when Rab-GAP activity was rendered non-functional (Kramer et al., 2006b). Together, these findings strongly support that phosphorylation of AS160/ TBC1D1 and the subsequent inhibition of Rab-GAP activity is crucial for GLUT4 translocation. Over the recent years, contraction-specific phosphomotif sites were identified on AS160/ TBC1D1 supporting their roles in contraction-stimulated GLUT4 translocation and glucose uptake (Treebak et al., 2014, Vichaiwong et al., 2010).

1.4.1.3 AS160 and TBC1D1 and contraction-stimulated skeletal muscle glucose uptake

There are many studies indicating an important role of AS160 and especially TBC1D1 in skeletal muscle glucose uptake during contraction/ exercise. Evidence of AS160 being involved in modulating GLUT4 translocation was first derived from experiment with adipocytes (Kane et al., 2002, Sano et al., 2003). Subsequently, studies using rodent skeletal muscle found increases in PAS-AS160 following muscle contraction (Bruss et al., 2005) providing an indication that AS160 phosphorylation might be involved in contraction-mediated muscle glucose uptake although unfortunately muscle glucose uptake was not evaluated in the study. On the other hand, there was evidence indicating that PAS-AS160 may not be essential for skeletal muscle

glucose uptake during contraction/ exercise. A persistent increase in PAS-AS160 in rat epitrochlearis muscle was observed 3-4 hours after endurance (2 hours) swimming exercise but by then glucose uptake would have returned to baseline (Arias et al., 2007). In addition, contraction-stimulated increases in PAS-AS160 in isolated rat epitrochlearis were abolished by wortmannin (an inhibitor of PI3K, a critical mediator in insulin signalling) without affecting glucose uptake during contraction (Funai and Cartee, 2009). PAS-AS160 levels were also unaffected by AMPK inhibition in spite of a marked attenuation in contraction-stimulated skeletal muscle glucose uptake with this AMPK inhibition (Funai and Cartee, 2009). In humans, acute (10 min) endurance exercise of moderate intensity (70% VO_2 peak) which would have increased glucose uptake did not lead to a significant increase in PAS-AS160 in vastus lateralis muscle sampled immediately after exercise (Sriwijitkamol et al., 2007). These findings suggest that PAS-160 may not play an important role in glucose uptake during acute exercise/ muscle contraction. However, it was reported that contraction-stimulated muscle glucose uptake was attenuated in muscle overexpressing AS160-4P mutant than wild type AS160 (Kramer et al., 2006b) suggesting a role of AS160 in glucose uptake during contraction. These results need to be interpreted carefully as the wild type AS160 and AS160-4P were overexpressed by up to 7-fold of that of the endogenous AS160 (Kramer et al., 2006b). This may have disturbed the normal function of other closely related proteins, in particular TBC1D1, in which overexpressed AS160 might compete and displace TBC1D1 from associating with GLUT4 vesicle, and therefore affect TBC1D1's regulation on Rab protein activity (Cartee and Funai, 2009). AS160 can also be phosphorylated at Ser^{711}, a phosphomotif site recognised by AMPK, following muscle contraction in mouse skeletal muscle and exercise in humans (Treebak et al., 2010). However, this phosphorylation was not involved in contraction-stimulated glucose uptake as muscle expressing Ser^{711} mutant had similar glucose uptake as that of wild type (Treebak et al., 2010). Recently, more AS160 phosphomotif sites (Ser^{341}, Ser^{588}, Thr^{642}, Ser^{704}, Ser^{751}) that are responsive to exercise have been identified; however, their role in glucose uptake have not been examined (Treebak et al., 2014). On the other hand, a mutation on calmodulin-binding domain of AS160 led to a reduction in contraction-

stimulated glucose uptake (Kramer et al., 2007); suggesting that AS160 could have phosphorylation sites recognised by other signalling mediators activated by muscle contraction. Overall, it appears that the role of AS160 in contraction-stimulated glucose uptake is yet to be well defined.

Interestingly, TBC1D1, like AS160, is also found to be phosphorylated at phosphomotifs that is recognised by PAS antibody in mouse muscle stimulated to contract *in situ* (Taylor et al., 2008) as well as during *ex vivo* contraction in rat epitrochlearis muscle (Funai and Cartee, 2009). In contrast to AS160, the time course of TBC1D1 phosphorylation corresponds better with skeletal muscle glucose uptake during contraction/ exercise (Funai and Cartee, 2008, Funai and Cartee, 2009, Taylor et al., 2008). This suggests TBC1D1 is a more likely than AS160 as a candidate responsible for contraction-stimulated glucose uptake. In rodents, PAS-TBC1D1 was found to consistently increase immediately with muscle contraction in both *ex vivo* (Funai and Cartee, 2008, Funai and Cartee, 2009) and *in situ* settings (Taylor et al., 2008). Likewise, an increase in PAS-150, likely to be PAS-TBC1D1, was detected immediately after resistance or moderate intensity exercise in humans (Deshmukh et al., 2006). In line with the suggestion that TBC1D1 is a potential regulator of contraction-stimulated glucose uptake, AMPK was found to be capable of phosphorylating TBC1D1 with contraction (Chen et al., 2008) and the AMPK inhibitor Compound C abolished the contraction-stimulated increase in PAS-TBC1D1 with a concomitant ~65% decrease in glucose uptake (Funai and Cartee, 2009). In addition, wortmannin had no effect on the contraction-stimulated increase in PAS-TBC1D1 and glucose uptake indicating that phosphorylated TBC1D1 is specific to muscle contraction but not insulin stimulation (Funai and Cartee, 2009). Subsequent work identified AMPK predicted phosphorylation sites on TBC1D1 (Ser^{231}, Ser^{660} and Ser^{700}) and phosphorylation at some of these sites (Ser^{231} and Ser^{660}) were significantly increased with muscle contraction and greatly reduced in AMPKα2 inactive transgenic mice (Vichaiwong et al., 2010). Contraction-stimulated skeletal muscle glucose uptake was significantly decreased in mouse muscle overexpressing the mutated TBC1D1 at these phosphomotif sites (Vichaiwong et al.,

2010) suggesting its role in muscle glucose uptake during contraction/ exercise. It is demonstrated recently that TBC1D1 Ser^{237} and Thr^{596} phosphorylation correlated significantly with activity of the α2β2γ3 AMPK trimer (Treebak et al., 2014) suggesting that AMPK-TBC1D1 signalling pathway may be important for contraction-stimulated muscle glucose uptake. An acute bout of cycling exercise in humans also led to an increase in phosphorylation of TBC1D1 on Ser^{231} and Ser^{660} (Jessen et al., 2011). These findings suggest that TBC1D1 can be regulated by signalling mediators known to be activated by exercise/ muscle contraction and this is likely to be involved in contraction-stimulated skeletal muscle glucose uptake.

1.4.2 Potential signalling of contraction-stimulated skeletal muscle glucose uptake

1.4.2.1 AMPK

AMPK is regarded as a major regulating enzyme that plays a key role in metabolism and cellular energy regulation (Hardie, 2007, Jorgensen and Rose, 2008). It functions as an intracellular energy gauge and is involved in ATP-regenerating processes in response to metabolic stress (Hardie et al., 1998). AMPK is activated in skeletal muscle of rodents during *ex vivo* and *in situ* contraction and *in vivo* exercise (Hayashi et al., 1998, Hutber et al., 1997, Musi et al., 2001b, Winder and Hardie, 1996), as well as in skeletal muscle of humans performing exercise (Chen et al., 2000, Fujii et al., 2000, Wadley et al., 2006, Wojtaszewski et al., 2000b).

AMPK was first postulated to be involved in skeletal muscle glucose uptake during contraction based on the studies that utilised a pharmacological AMPK agonist, 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR). Once taken up into skeletal muscle, AICAR is metabolised to monophosphorylated nucleotide 5-aminoimidazole-4-carboxamide-1-β-$_D$-ribofuranotide (ZMP), an AMP analogue which in turn activates AMPK. Isolated rodent skeletal muscle incubated with AICAR *ex vivo* demonstrated an increase in glucose uptake (Hayashi et al., 1998, Merrill et al., 1997). Similarly, *in vivo*

AICAR infusion in rodents increased skeletal muscle glucose uptake in the resting state (Bergeron et al., 1999, Nakano et al., 2006). GLUT4 translocation was increased following AICAR treatment in perfused rat hindlimb muscles (Kurth-Kraczek et al., 1999). The evidence of AICAR acting through AMPK activation to increase glucose uptake was demonstrated with mice lacking AMPKα2 activity that showed an abolished AICAR-induced glucose uptake (Fujii et al., 2005, Jorgensen et al., 2004, Lefort et al., 2008, Mu et al., 2001). Therefore, it is logical to assume that contraction-stimulated muscle glucose uptake is mediated by AMPK activation.

However, contraction experiments using genetic modified rodent models do not unequivocally support a role of AMPK in glucose uptake during contraction/ exercise. Contraction-stimulated skeletal muscle glucose uptake in isolated skeletal muscle from genetically-modified mouse in which AMPKα2 has been either inactivated or greatly reduced in expression demonstrated glucose uptake is either normal (Fujii et al., 2005, Jorgensen et al., 2004, Merry et al., 2010c) or partially impaired (Lefort et al., 2008, Mu et al., 2001). Likewise, rodent studies investigating skeletal muscle glucose uptake in AMPKα2 kinase dead (KD) and wild type (WT) mice during the more physiological *in vivo* exercise also provided contrasting results. One study reported that, compared with WT mice, AMPKα2 KD mice had a normal increase in glucose uptake (Maarbjerg et al., 2009) while another demonstrated a reduced but still substantial glucose uptake during exercise (Lee-Young et al., 2009). It is hard to discern the discrepancies in these studies. The reduced glucose uptake observed in Lee-Young et al. (2009) was attributed to a reduction in muscle blood flow to the contracting muscles; while, the blood flow status was unknown in Maarbjerg et al. (2009). In addition, an almost total loss of AMPKα1 and AMPKα2 activities was observed in Lee-Young et al.; while, Maarbjerg et al. detected mainly a loss in AMPKα2 activity with only a small decrease in AMPKα1 activity (Lee-Young et al., 2009, Maarbjerg et al., 2009). The combined loss of AMPKα1 and AMPKα2 activities may have a greater impact on the expression of nNOSμ and activation of NOS and NO production were reduced in Lee-Young et al. (2009). Given that nNOS can affect blood flow during exercise (Kobayashi et al., 2008),

this may explain the impairment in muscle blood flow and reduced glucose uptake in Lee-Young et al. as compared with Maarbjerg et al. (Lee-Young et al., 2009, Maarbjerg et al., 2009). In addition, the reduced activation of NOS and NO production may have also contributed to the reduced glucose uptake during exercise (Lee-Young et al., 2009).

There has also been a dissociation observed between muscle glucose uptake and AMPK activation/ activity during exercise in humans after short-term exercise training (McConell et al., 2005). Glucose disposal during exercise following the short-term exercise training was reduced compared with before training but was still substantially increased above the resting state; however, muscle AMPKα1 and AMPKα2 activities were not activated above resting levels, suggesting that activation of AMPK signalling was not required for increases in glucose disposal during exercise in humans (McConell et al., 2005). Similar training-induced prevention of AMPK phosphorylation and activity during exercise in humans has been reported by others at intensities of exercise that would substantially increase skeletal muscle glucose uptake (Mortensen et al., 2013).

It is hard to understand why AMPK appears to be essential for mediating skeletal muscle glucose uptake in some studies but not others. A possible scenario is that multiple signalling mediators could simultaneously regulate skeletal muscle glucose uptake during contraction and there is redundancy in the system (Fujii et al., 2005). Alternatively, it could be that the residual AMPK activity in mice with single mutations or deletions in muscle α or β subunits is adequate to maintain a normal contraction-stimulated muscle glucose uptake (Fujii et al., 2005, Jorgensen et al., 2004, Maarbjerg et al., 2009, Steinberg et al., 2010). Supporting this notion is a recent study that showed up to 70% attenuation of the increase in muscle glucose uptake during *ex vivo* contraction and treadmill running when AMPKα1 and α2 activities were fully abolished via muscle-specific knockout of β1 & β2 subunits (O'Neill et al., 2011). These findings support a role of AMPK in skeletal muscle glucose uptake during contraction/ exercise.

1.4.2.2 Calcium, CaMKs and PKCs

Upon muscle activation, there is a release of Ca^{2+} from sarcoplasmic reticulum leading to a transient spike of cytosolic Ca^{2+} concentration. This transient spike of Ca^{2+} concentration has been implicated as a feed-forward regulator of contraction-stimulated muscle glucose uptake (Richter et al., 2004). It was shown in isolated frog sartorius muscle that increased cytosolic Ca^{2+} concentration was associated with an enhancement in membrane permeability to plasma glucose (Holloszy and Narahara, 1967a). Subsequent studies on isolated rat muscles demonstrated that low-concentration caffeine-induced non-depolarisation release of Ca^{2+} from sarcoplasmic reticulum stimulated skeletal muscle glucose uptake (Wright et al., 2004, Wright et al., 2005, Youn et al., 1991) suggesting an independent role of Ca^{2+} in stimulating muscle glucose uptake. This is supported by the finding that incubation of isolated rat muscle with the compound N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) at a concentration that released Ca^{2+} from sarcoplasmic reticulum without eliciting muscle contraction increased glucose transport activity (Youn et al., 1991). Nevertheless, the association of caffeine-induced releases in Ca^{2+} with increased AMPK activation (Egawa et al., 2011, Jensen et al., 2007, Raney and Turcotte, 2008) and the fact that re-uptake of Ca^{2+} via sarcoplasmic reticulum Ca^{2+}-ATPase is an active process that consumes considerable energy (Norris et al., 2010) led to the hypothesis that Ca^{2+} increased glucose uptake was due to increased energy consumption that in turn activated AMPK (Jensen et al., 2007). In fact, it was demonstrated that the increase in skeletal muscle glucose uptake mediated by caffeine-induced releases of Ca^{2+} was via AMPK activation as the glucose uptake in muscle with kinase dead AMPK was greatly reduced (Jensen et al., 2007). However, it was also shown that the increases in glucose uptake induced by AICAR (an AMPK activator) and caffeine were additive and were not significantly different from contraction (Wright et al., 2004) suggesting that Ca^{2+} can mediate glucose uptake via different mechanisms in addition to AMPK activation.

A couple of signalling molecules have been postulated as the effectors of Ca^{2+} in regulating muscle glucose uptake – CaMKs and PKCs. Low concentration caffeine

incubation of rat epitrochlearis was demonstrated to elevate cytosolic Ca^{2+} level and phosphorylation of CaMKII and stimulated muscle glucose uptake, which was blocked by CaMK inhibitors KN62 and KN93 (Wright et al., 2004, Wright et al., 2005). These studies suggest that CaMKs, in particular CaMKII, may be important in contraction-stimulated muscle glucose uptake. Attenuation of contraction-stimulated glucose uptake by KN62 did not affect AMPK phosphorylation suggesting an independent role of CaMKII and AMPK in stimulating muscle glucose uptake (Wright et al., 2004). Further evidence of the involvement of CaMKII in mediating glucose uptake derived from the study that transfected CaMKII inhibitory peptide into tibialis anterior muscles (Witczak et al., 2010). An approximately 30% decrease in contraction-stimulated muscle glucose uptake was observed in that study (Witczak et al., 2010) indicating that CaMKII is one of the signalling mediators playing a role in contraction-stimulated glucose uptake while the majority of glucose uptake was accounted by other signalling mediators.

Protein kinase C, on the other hand, is also activated following an elevation in intracellular Ca^{2+} and muscle contraction (Cleland et al., 1989, Richter et al., 1987). Pharmacological inhibition of conventional PKCs in isolated skeletal muscle contracted *ex vivo* attenuated glucose uptake predominantly in fast-twitch muscle fibers (Henriksen et al., 1989, Ihlemann et al., 1999a, Wojtaszewski et al., 1998). Calphostin C (a PKC inhibitor) at a dose that did not affect the force production attenuated the increase in glucose uptake during contraction by approximately 50% (Ihlemann et al., 1999a). Isolated skeletal muscle treated with phorbol ester that down-regulates conventional and novel PKCs ablated contraction-stimulated glucose uptake (Cleland et al., 1990). Nevertheless, knockout of the conventional PKC isoform, PKCα, showed no impairment in skeletal muscle glucose uptake during contraction in mice suggesting a little role of conventional PKC in contraction-stimulated glucose uptake (Jensen et al., 2009a). There was also evidence, derived from muscle cells stimulated to contract by carbachol (an acetylcholine analog) indicating a role of novel PKCs in the regulation of contraction-stimulated translocation of GLUT4 (Niu et al., 2011). However, the findings from cell culture should be interpreted carefully due to histological and structural differences to

skeletal muscle as well as the difference in relative PKC isoform expression between cell culture and skeletal muscle (Khayat et al., 1998, Osada et al., 1992). In humans, the activity of atypical PKC in skeletal muscle was increased with exercise suggesting a possible role of atypical PKC in muscle glucose uptake (Rose et al., 2004). However, muscle specific knockout of the predominant atypical PKC isoform, PKCλ in mice did not impair glucose uptake during exercise suggesting atypical PKC may not be involved in muscle glucose uptake (Sajan et al., 2010).

1.4.2.3 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive free radical molecules (Riley, 1994) that are produced in the basal/ resting state and increase in parallel with muscle contraction/ exercise (Reid et al., 1992, Sandstrom et al., 2006). The effect of ROS on muscle cellular function appears to be multifaceted. It depends on the dynamic cellular redox status, which is governed by various antioxidant enzymes (superoxide dismutase, glutathione peroxidase or catalase) and antioxidant substrates (reduced glutathione (GSH), ascorbic acid/ vitamin C). An acute high level of oxidative stress could result in muscle dysfunction (Andrade et al., 1998, Khawli and Reid, 1994, Supinski et al., 1995) and increased fatigability during exercise (Medved et al., 2004). Chronic oxidative stress is associated with various disease development such as insulin resistance (Houstis et al., 2006) and cardiovascular disease (Kojda and Harrison, 1999). Interestingly, acute small temporary increases in ROS during contraction/ exercise may be beneficial and involved in various signalling cascades in the regulation of cellular metabolism and gene expression (Jackson, 2008, Ji, 2008, Katz, 2007). The role of ROS in stimulating muscle glucose uptake was initially observed in studies that incubated rat epitrochlearis muscle with hydrogen peroxide (H_2O_2) and demonstrated an increase in basal glucose uptake (Cartee and Holloszy, 1990, Sorensen et al., 1980). These increases are attenuated by the non-specific antioxidant, N-Acetylcysteine (NAC), which prevented the increase in oxidative stress induced by H_2O_2 (Toyoda et al., 2004). Since muscle contraction increases ROS production, it is logical that ROS may be involved in contraction-stimulated muscle glucose uptake. Indeed, Sandstrom et al. (2006) demonstrated that the

antioxidants NAC and ebselen (a glutathione peroxidase mimetic) prevented an increase in skeletal muscle oxidative stress during *ex vivo* muscle contraction and attenuated approximately 50% of the increase in muscle glucose uptake. Similar results were observed with NAC by our group (Merry et al., 2010d). Furthermore, EDL muscles, with overexpression of Mn^{2+} superoxide dismutase (SOD) which catalyses the conversion of $O_2^{\bullet-}$ to H_2O_2, were shown to have a greater glucose uptake when contracted *ex vivo*, suggesting increased H_2O_2 stimulates glucose uptake during *ex vivo* contraction. Nevertheless, our group demonstrated that ROS may not be involved in the regulation of skeletal muscle glucose uptake *in vivo* (Merry et al., 2010a, Merry et al., 2010b). Local NAC infusion had no effect on skeletal muscle glucose uptake in rat hindlimb muscles stimulated to contract *in situ* (Merry et al., 2010a) and in humans during moderate intensity exercise while receiving systemic NAC infusion (Merry et al., 2010b). Several major differences exist between the *ex vivo* model compared with the relatively more physiological perfusion-intact *in situ* contraction and *in vivo* exercise models that may make *ex vivo* models more likely to be affected by antioxidants. In the *ex vivo* model, oxidant levels could supra-physiologically be elevated and thus affect ROS signalling events as a result of: 1) a hypoxic core in the incubated muscle due to a non-uniform delivery and diffusion of oxygen into the muscle fibers despite a hyperoxic incubation medium; 2) lower antioxidant systems, which are in part derived from the blood; 3) non–physiological, supramaximal electrical stimulation of the muscle in the *ex vivo* system (Merry et al., 2010a). It is therefore possible that ROS may only play a role in glucose uptake during contraction under non-physiological conditions such as *ex vivo* muscle contraction because of the higher ROS produced under these circumstances. Nevertheless, it is worthwhile to note that the role of ROS on glucose uptake may become significant in T2D or $nNOS\mu^{-/-}$ mice in which oxidative stress is increased. It is demonstrated in $nNOS\mu^{-/-}$ skeletal muscle that lacking of NO production to scavenge superoxide increases its accumulation in muscle fibers which subsequently leads to up-regulation of SOD1 and peroxiredoxin-6 to catalyse the conversion of superoxide to hydrogen peroxide and eventually being reduced to water by other enzymes (Da Silva-Azevedo et al., 2009).

1.5 NITRIC OXIDE

1.5.1 NO, NO synthase and NO production in skeletal muscle

Nitric oxide (NO) is a gaseous signalling molecule that is implicated in various physiological processes such as vasodilatation (Lau et al., 2000, Vallance et al., 1989), neural transmission (Del-Bel et al., 2011), immune function (Schairer et al., 2012), muscle contraction (Kobzik et al., 1994) and platelet aggregation (Riddell and Owen, 1999). NO formation in mammalian tissues is catalysed by nitric oxide synthase (NOS) that convert L-arginine and NADPH to NO and L-citrulline. This process requires calmodulin and a number of co-factors such as (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD) (Stamler and Meissner, 2001). Three main isoforms of NOS have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Stamler and Meissner, 2001). In rodents, both nNOS and eNOS are constitutively expressed in skeletal muscle fibers (Kobzik et al., 1995, Lau et al., 2000) while in human skeletal muscle fibers, nNOS is the primary isoform with barely any detectable eNOS expression (McConell et al., 2007, Nakane et al., 1993). However, a small amount of eNOS, confined to the endothelial tissues, has been demonstrated in human skeletal muscle (Frandsen et al., 1996). On the other hand, expression of iNOS is inducible in skeletal muscle in disease states associated with inflammation (Torres et al., 2004) or chronic heart failure (Hambrecht et al., 1999). In skeletal muscle, the expressed nNOS isoform is nNOSμ which is a splice variant of nNOS that has an additional 34 amino acids (Larsson and Phillips, 1998, Lin et al., 1998a, McConell et al., 2007, Silvagno et al., 1996). The abundance of nNOSμ expression in skeletal muscle is dependent on the fiber types where a higher abundance of nNOSμ is found in fast-twitch muscle fibers than the slow-twitch fibers in rodents (Kobzik et al., 1994, Merry et al., 2010c) although it appears that there is similar expression in both fiber types in humans (McConell et al., 2007, Punkt et al., 2006). Other nNOS splice variants, nNOSβ and nNOSγ, are also detected in rodent skeletal muscle fibers (Baum et al., 2011, Brenman et al., 1996), no data is available from human skeletal muscle.

Evidence suggests that nNOSμ is the main isoform activated during muscle contraction (Hirschfield et al., 2000, Lau et al., 2000). It was demonstrated that electrical stimulation of isolated EDL muscle of C57Bl/6 and eNOS knockout mice resulted in an increase in cGMP content (generally considered as the major downstream signalling molecule of NO) but no increase in cGMP was observed in the contracted EDL from nNOSμ knockout ($nNOS\mu^{-/-}$) mice (Lau et al., 2000). This suggested that muscle lacking nNOSμ was unable to produce NO in response to contraction to activate its downstream signalling cascades. Studies in cremaster muscles of $nNOS\mu^{-/-}$ mice demonstrated very minimal residual NOS activity remaining in these muscles (McKinnon et al., 2006), a finding consistent with Lau et al. (2000). In addition, no difference was observed in NO production from contracting muscles of $eNOS^{+/+}$ and $eNOS^{-/-}$ mice (Hirschfield et al., 2000). Together, these findings suggest that nNOSμ is the major NOS isoform in rodent skeletal muscle responsible for NO production during contraction and that eNOS does not contribute significantly to NO production during skeletal muscle contraction.

Several different mechanisms are involved in the regulation of NOS activity and NO production. The activity of different NOS isoforms is regulated slightly differently. For example, eNOS and nNOS have a much higher Ca^{2+} dependence than iNOS for their activity and therefore for NO synthesis (Alderton et al., 2001). Since iNOS is not normally expressed in skeletal muscle and eNOS was shown to be not involved in the production of NO during contraction of skeletal muscle, this section focuses on the regulation of nNOS. Neuronal NOS activity can be regulated allosterically through Ca^{2+}/ calmodulin binding, by protein-protein interactions as well as covalently by phosphorylation (Alderton et al., 2001, Stamler and Meissner, 2001). Calmodulin is a critical protein necessary for the activation of nNOS. Interaction of Ca^{2+}/ calmodulin with nNOS is required to increase its enzymatic activity by overcoming the auto-inhibitory control element that prevents the electron transfer and thus activation of nNOS (Salerno et al., 1997). On the other hand, there is evidence suggesting that the binding of protein inhibitor of nNOS (PIN) to nNOS can inhibit its enzymatic activity (Jaffrey and Snyder, 1996). Caveolin-3 was also shown to inhibit nNOS activity in

skeletal muscle; an effect that could be reversed by Ca^{2+}/ calmodulin binding (Venema et al., 1997). Phosphorylation of nNOS by different protein kinases can also have diverse effects on its enzymatic activity. Ca^{2+}/ CaMKs phosphorylates nNOS purified from rat brain at Ser^{847} and leads to a decrease in nNOS activity (Hayashi et al., 1999, Komeima et al., 2000, Nakane et al., 1991). This is a very interesting finding in regards to the regulation of muscle glucose uptake during contraction as both Ca^{2+}/ CaMKs and NO signalling are activated during contraction and shown to enhance contraction-stimulated glucose uptake (Merry et al., 2010d, Wright et al., 2005). It could be possible that contraction leads to a stronger activation of nNOS that overcomes the inhibitory effect of Ca^{2+}/ CaMKs. Neuronal NOS phosphorylation by protein kinase C at a yet to be determined phosphorylation site, in contrast, moderately increases NOS activity (Nakane et al., 1991); whilst, the effect of AMPK phosphorylation of human nNOSμ at Ser^{1451} is unclear (Chen et al., 2000). Interestingly, the localisation of nNOS at the sarcolemma can also affect nNOS function. It was demonstrated that mice lacking the α-syntrophin or the PDZ domain, thus with a greatly reduced sarcolemmal nNOS but a preserved soluble nNOS in the cytoplasm, have impaired α-adrenergic vasoconstriction in the contracting muscles (Thomas et al., 2003). Based on the above, it appears that the regulation of nNOS is very complex and the mechanisms directly responsible for nNOS activation and the increase in NO production during muscle contraction/ exercise remains to be fully elucidated.

NO is released from skeletal muscle in the resting state and its production increases with contraction. Balon and Nadler (1994) detected the release of NO from isolated skeletal muscle at rest using a chemiluminescent system. They and others subsequently demonstrated an increase in NOS activity in response to *ex vivo* contraction (Balon and Nadler, 1994, Merry et al., 2010d, Merry et al., 2010c) and a several fold increase in NO production during muscle contraction (Balon and Nadler, 1994, Hirschfield et al., 2000). In addition, cGMP production, a major downstream mediator of NO, was also increased with contraction in isolated EDL muscle of C57Bl/6 and $eNOS^{-/-}$ mice, providing further evidence of an increase in NO production with

muscle contraction (Lau et al., 2000). Skeletal muscle NOS activity and NO degradation products (nitrite and nitrates) are also elevated with rat *in situ* contraction (Ross et al., 2007) and treadmill running exercise (Roberts et al., 1999) which are relatively more physiological contraction models than *ex vivo* contractions. Furthermore, inhibition of NOS in these conditions prevents the increase in NO production and cGMP content during contraction (Balon and Nadler, 1994, Lau et al., 1998, Pattwell et al., 2004). In humans, NOS activity is increased following 120-min of moderate intensity cycling exercise (Linden et al., 2011). Intriguingly, NO can also be produced independently of NOS pathways. The reduction of nitrite, the oxidised by-product of NO, leads to the generation of NO without the presence of NOS (Benjamin et al., 1994, Lundberg et al., 1994, Weitzberg and Lundberg, 1998, Zweier et al., 1995). Although there is evidence suggesting increase extraction of nitrite from the blood with exercise in humans during NOS inhibition, any significant *in vivo* conversion of nitrite to NO is unknown (Gladwin et al., 2000). Despite the existence of alternative NO production mechanisms, NOS-dependent mechanism likely remains the most physiological relevant pathway for the generation of NO in skeletal muscle.

1.5.2 NO and skeletal muscle glucose uptake

1.5.2.1 Basal skeletal muscle glucose uptake

NO was first implicated in skeletal muscle glucose uptake based on a study that incubated rat EDL muscle *ex vivo* (Balon and Nadler, 1994). NO concentration increased greatly in the incubation media containing the EDL compared with media alone indicating that NO was released basally from the EDL muscle (Balon and Nadler, 1994). The addition of a NOS inhibitor diminished the release of NO with a concomitant reduction in basal muscle glucose uptake indicating a role for NO in basal skeletal muscle glucose uptake (Balon and Nadler, 1994). This finding was supported by follow up studies that showed an increase in muscle glucose uptake with the NO donor, sodium nitroprusside (SNP) (Etgen et al., 1997, Higaki et al., 2001, Young et al., 1997), in a

dose-dependent manner (Balon and Nadler, 1997). A similar result was shown in muscle cell culture (Deshmukh et al., 2010, Henstridge et al., 2009) as well as in isolated human skeletal muscle strips (Deshmukh et al., 2010). Likewise, local administration of SNP into the femoral artery of healthy humans increased skeletal muscle glucose uptake (Durham et al., 2003). These studies strongly suggest that NO/ NO donors increase skeletal muscle glucose uptake in the basal state. Nevertheless, it should be noted that the NO donor dose (10 mM SNP) used in most of the above studies is likely to be well above the endogenous NO level achievable during contraction or exercise as the plasma concentration of NO in rat is in the nM range (Hernanz et al., 2004). In addition, not all studies find a reduction in basal glucose uptake with a NOS inhibitor (McConell and Kingwell, 2006).

1.5.2.2 Contraction-stimulated muscle glucose uptake in rodents

In rodents, there have been contradictory results between studies examining the potential role of NO in skeletal muscle glucose uptake during contraction/ exercise. Some of these studies supported a role of NO in the regulation of muscle glucose uptake during contraction/ exercise (Balon and Nadler, 1997, Merry et al., 2010d, Merry et al., 2010c, Roberts et al., 1997, Ross et al., 2007, Stephens et al., 2004), but others did not (Etgen et al., 1997, Higaki et al., 2001, Inyard et al., 2007, Rottman et al., 2002) (Table 1.1). These conflicting results are likely attributable to variations in the experimental protocols used by different researchers as outlined in a review by McConell et al. (2006). Differences in the intensity, duration and stimulation parameters of muscle contraction protocol; muscle with varying predominate fiber types used (which have different levels of nNOS expression); and glucose uptake measurement techniques were observed in these studies.

Based on *ex vivo* studies, NOS inhibition on rodent skeletal muscles electrically stimulated to contract have both attenuation (Merry et al., 2010d, Merry et al., 2010c, Stephens et al., 2004) and no effect (Etgen et al., 1997, Higaki et al., 2001) on muscle glucose uptake. A notable difference between these studies is the timing of the glucose

uptake measurement was done in which the studies by Merry et al. (2010d, 2010c) measured glucose uptake during contraction and 5 min into the recovery phase. These studies represent better the glucose uptake during contraction as compared with other studies that measured glucose uptake many minutes after the contraction had ceased (Etgen et al., 1997, Higaki et al., 2001, Stephens et al., 2004) (Table 1.1). Therefore, the results from the latter studies would likely to be more related to NO effect on post-contraction glucose uptake. It should be noted that cGMP content was not elevated following contraction in Etgen et al. study (1997) suggesting that NO signalling was not adequately activated and therefore would likely explain the observed lack of effect on glucose uptake of the NOS inhibitor (Etgen et al., 1997). Nevertheless, in contrast to Higaki et al. (2001), the study by Stephens et al (2004) which measured glucose uptake many minutes after contraction also demonstrated attenuation of the increase in glucose uptake by NOS inhibitor (Table 1.1). Stephens et al (2004) used isolated muscle from overnight fasted rats while in Higaki et al. study (2001) the muscles were isolated from non-fasted rats. Prolonged fasting depletes muscle glycogen levels and enhances muscle glucose uptake during contraction (Halseth et al., 1999). Low pre-contraction muscle glycogen levels are associated with an increase in glucose uptake during contraction (Hespel and Richter, 1990, Richter and Galbo, 1986) which has been postulated to be due to the release of GLUT4 from its binding with glycogen particles to facilitate glucose uptake (Richter et al., 2001). However, translocation of GLUT4 is a much more complex process and is likely to involve other signalling implicated in glucose uptake including NO signalling. Different expression of nNOS protein is found between fast-twitch and slow-twitch fibers (Kobzik et al., 1994), being higher in fast-twitch fibers, and theoretically may lead to different dependencies on NO signalling between these fibers. Generally, studies using predominantly fast-twitch muscles support a role of NO in muscle glucose uptake during contraction (Balon and Nadler, 1997, Merry et al., 2010d, Merry et al., 2010c, Stephens et al., 2004). In fact, our group demonstrated that NOS inhibition significantly attenuated muscle glucose uptake during *ex vivo* contraction in EDL but not soleus muscles (Merry et al., 2010c) (Table 1.1).

Table 1.1: Contraction-stimulated skeletal muscle glucose uptake in rodents with NOS inhibition

Study	Muscle type (species)	Contraction/ exercise protocol	Glucose uptake measurement	Inhibitor & dosing	Effect
Ex vivo					
Etgen et al. (1997)	Epitrochlearis (rats)	2 x 10 min contractions, 200 ms, 100 Hz, 10 V	≥10 min post-contraction, *ex vivo*	100 μM L-NMMA	None
Higaki et al. (2001)	EDL (rats)	2 contractions/min for 10 min, 10 s, 100 V	≥20 min post-contraction, *ex vivo*	100 μM L-NMMA	None
Stephens et al. (2004)	Epitrochlearis (rats)	1 contraction/min for 10 min, 10 s, 100 V	≥10 min post-contraction, *ex vivo*	100 μM L-NMMA	↓~70%
Merry et al. (2010d)	EDL (C57Bl/6 mice)	25 contractions/min for 10 min, 600 ms, 60 Hz	During final 5 min of contraction & 5 min of recovery, *ex vivo*	100 μM L-NMMA	↓~50%
Merry et al. (2010c)	EDL (AMPK KD mice)	12 contractions/min for 10 min, 350 ms, 60Hz	During final 5 min of contraction & 5 min of recovery, *ex vivo*	100 μM L-NMMA	↓~40%
Merry et al. (2010c)	Soleus (AMPK KD mice)	12 contractions/min for 10 min, 600 ms, 60Hz	During final 5 min of contraction & 5 min of recovery, *ex vivo*	100 μM L-NMMA	None

Table 1.1: Contraction-stimulated skeletal muscle glucose uptake in rodents with NOS inhibition (continued)

Study	Muscle type (species)	Contraction/ exercise protocol	Glucose uptake measurement	Inhibitor & dosing	Effect
In situ					
Balon et al. (1997)	EDL (rats)	2 x 5 min contraction, 500 ms, 6-8 V (sciatic nerve)	Muscle dissected, 70 min post-contraction, *ex vivo*	100 μM L-NMMA	↓~100%
Higaki et al. (2001)	EDL (rats)	2 x 5 min contraction, 500 ms, 3-10 V (sciatic nerve)	Muscle dissected, ≥20 min post-contraction, *ex vivo*	100 μM L-NMMA	None
Ross et al. (2007)	hindlimb muscles (rats)	2 Hz, 0.1 ms, 35 V for 30 min (field stimulation)	During final 10 min of contraction, *in situ*	5 μM L-NAME	↓~35%
Inyard et al. (2007)	Hindlimb muscle (rats)	0.05 – 2 Hz, 0.5 ms, 2 V for 10 min at each frequency	Arterial-venous glucose difference x FBF throughout contraction	50 μg/min/kg L-NAME	↓~50%* (NS)
In vivo					
Roberts et al. (1997)	Hindlimb muscles (rats)	Treadmill running, 15 - 25% grade at 1.3 – 1.9 km/h for 45 min	Muscle frozen immediately post-exercise, sacrolemmal vesicle, *ex vivo*	1 mg/ml, 2d L-NAME (ingestion)	↓~100%
Higaki et al. (2001)	Soleus (rats)	Treadmill running, 10% grade for 1 hour at 0.7 mph	Muscle dissected, ≥20 min post-exercise, *ex vivo*	1 mg/ml, 2d L-NAME (ingestion)	None
Rottman et al. (2002)	Gastrocnemius (mice)	Treadmill running, 0% grade for 30 min at 0.6 mph	During final 25 min of exercise, *in vivo*	1 mg/ml, 3d L-NAME (ingestion)	None

EDL: extensor digitorum longus; L-NMMA: N-G-Monomethyl-L-arginine; L-NAME: N-G-Nitro-L-arginine Methyl Ester; 2d/ 3d: two/ three days of ingestion; * at higher stimulation frequencies; NS: not significant

Conflicting results were reported in rat *in situ* contraction studies that have measured muscle glucose uptake during contraction (Inyard et al., 2007, Ross et al., 2007) (Table 1.1). In the study by Ross et al. (2007), hindlimb muscles were stimulated to contract *in situ* and local L-NAME infusion into femoral artery was started after 10 min into muscle contraction, allowing the normal increase in leg blood flow to take place before the L-NAME infusion began. Muscle glucose uptake, measured during the final 10 min of contraction, was significantly attenuated with L-NAME (Ross et al., 2007). The systemic blood pressure and capillary blood flow to the contracting muscles were not affected by the L-NAME infusion indicating that NO mediates glucose uptake via intramuscular signalling events rather than blood flow (Ross et al., 2007). In contrast, in another rat *in situ* contraction study that had the hindlimb muscle stimulated to contract over a range of low to high frequencies over 70 minutes showed no significant attenuation in glucose uptake by L-NAME although the glucose uptake at higher contraction frequencies with L-NAME appeared to be lower (~50%) than that of the saline control (Inyard et al., 2007). Indeed, it seems likely that if the ANOVA was partitioned in the Inyard et al. study (2007) there would have been a significant attenuation of the increase in glucose uptake during contraction by NOS inhibition at the higher contraction frequencies. This suggests that NO-mediated glucose uptake may be more important during higher intensity of contraction which fits with the previously reported data that intense contraction stimulation is required to increase NO production in primary rat skeletal muscle cell culture (Silveira et al., 2003) and mouse muscle NOS activity is only activated during higher running intensity (Lee-Young et al., 2009). The systemic administration of L-NAME led to significant (~30 mmHg) elevation in systemic blood pressure and remarkably an increase in femoral blood flow during contraction (Inyard et al., 2007) indicating the non-physiological effect of the systemic NOS inhibition. Therefore, the relevance of the findings of Inyard et al. (2007) needs to be interpreted with caution.

Other studies that had the muscles dissected immediately following an *in situ* contraction and glucose uptake measured *ex vivo* also demonstrated conflicting results

(Balon and Nadler, 1997, Higaki et al., 2001) (Table 1.1). A total abolishing of the increase in glucose uptake during contraction by L-NMMA was observed in the study by Balon et al. (1997) but no effect on glucose uptake was observed in Higaki et al. (2001). The reason for the discrepancy in their results was not apparent. However, it is important to highlight that L-NMMA was not administered during contraction but only presence during the post-contraction incubation in both studies. As such, these studies reflect on the effect of NO on glucose uptake after exercise and not during the contraction *per se* which is the focus of this literature review.

The *in vivo* studies examining the effects of NOS inhibition on skeletal muscle glucose uptake during exercise in rodents have also reported inconsistent results (Higaki et al., 2001, Roberts et al., 1997, Rottman et al., 2002) (Table 1.1). These studies were conducted using rats or mice running on a treadmill after ingestion of a NOS inhibitor over two or three days duration, at a dose that nearly eliminated all skeletal muscle NOS activity (Higaki et al., 2001). Different glucose uptake measurement protocols/ techniques were employed in these studies (Table 1.1). In the study by Roberts et al. (1997), hindlimb muscles were dissected and frozen immediately after exercise and glucose uptake was measured based on sarcolemmal GLUT4 content; which is more reflective of an exercise state as compared to Higaki et al. (2001) where glucose uptake was measured 20 min post-exercise. This could contribute to the different outcomes between these two studies where Roberts et al. (1997) found an attenuation on the normal increase in GLUT4 translocation with exercise by NOS inhibition but Higaki et al. (2001) found no effect of NOS inhibition. This is likely because the acute effects of the increase in NO due to muscle contraction during exercise were abolished 20 min after exercise. Another difference between these two studies was the use of soleus muscles (slow twitch muscle) by Higaki et al. (2001) compared with the mixture of muscles likely having a higher proportion of fast-twitch muscle fibers (gastrocnemius, plantaris and vastus) by Roberts et al. (1997). Neuronal NOSμ expression, the main NOS isoforms activated during contraction, and NOS activity are lower in slow-twitch fibers than fast-twitch fibers (Kobzik et al., 1994, Merry et al., 2010c). The use of soleus

muscle together with the measurement of glucose uptake 20 min after exercise may have explain the lack of effect of NOS inhibition in the study by Higaki et al. (2001).

On the other hand, despite measuring glucose uptake in C57BL/6 mice during treadmill running, Rottman et al. (2002) reported no effect of NOS inhibition on mouse skeletal muscle glucose uptake (Table 1.1). In that study, L-NAME was administered via the drinking water (~100 – 120 mg/kg/day) over 3 days. This L-NAME regimen has previously been shown to cause modest increases in systemic blood pressure (Kaikita et al., 2001). The possible increase in systemic blood pressure with a possible accompanying increase in blood flow based on systemic NOS inhibition *in situ* (Inyard et al., 2007), can increase glucose delivery to the muscle and increase skeletal muscle glucose uptake (Zinker et al., 1993). This may mask the attenuation effect of L-NAME on glucose uptake and give rise to the observed finding. In addition, ingestion of L-NAME decreased the exercise capacity of the mice (Rottman et al., 2002). Given that L-NAME-treated mice ran at the same absolute intensity as the mice that had no L-NAME ingestion, the L-NAME-treated mice were, in fact, running at higher relative exercise intensity. Therefore, they were subjected to greater metabolic stress which may have led to a greater glucose uptake and mask the possible effect of L-NAME on glucose uptake. The finding that L-NAME-treated mice had greater fatty acid uptake into the heart and skeletal muscle (Rottman et al., 2002) supported that these mice might have experienced a greater metabolic demand. Therefore, the lack of attenuation in glucose uptake during exercise in mice treated with L-NAME may be due to the greater exercise stress encountered by these mice. Nevertheless, it still could be possible that NO was not required for the increase in glucose uptake during exercise in mice as reported by the authors (Rottman et al., 2002).

So far, there was only one mouse genetic model investigating the role of NO in mediating muscle glucose uptake during exercise. Endothelial $NOS^{-/-}$ mice had higher muscle glucose uptake during exercise than wild type controls (Lee-Young et al., 2010). These mice had a lower exercise capacity and a reduction in blood flow to the

contracting muscles and it was postulated that the enhanced glucose uptake was attributable to exercise-induced hypoxia due to the absence of eNOS (Lee-Young et al., 2010) as hypoxia is a potent stimulator of skeletal muscle glucose uptake (Cartee et al., 1991). Therefore, eNOS appears to have no direct effect on muscle glucose uptake during contraction. This is in line with the finding that eNOS is not important for activating downstream signalling of NO during *ex vivo* contraction but nNOSμ is (Lau et al., 2000).

In spite of the confounding results in regards to the role of NO in glucose uptake during contraction/ exercise in rodent studies, our group has consistently demonstrated that NO is a critical mediator of this process (Merry et al., 2010d, Merry et al., 2010c, Ross et al., 2007, Stephens et al., 2004). Similar findings were also observed by our group in several human studies that will be discussed in the next section. One of the common and major issues with the rodent studies is the measurement of glucose uptake many minutes after contraction had ceased which, unfortunately, has little relevance on the regulation of muscle glucose uptake during contraction/ exercise by NO. It is also worthwhile to highlight that so far all studies to date investigating the role of NO on skeletal muscle glucose uptake during contraction/ exercise were based on NOS inhibition except one that used $eNOS^{-/-}$ mice. Experiments conducted using nNOSμ, the major NOS isoform activated during contraction, knockout mice would add great value to the existing pool of information. In particular, there is a requirement for studies examining the role of the nNOSμ isoform on contraction-stimulated glucose uptake and to address the possibility that NOS inhibitors may attenuate muscle glucose uptake via non-specific effects. Both the commonly used NOS inhibitors, L-NMMA and L-NAME, have been reported to have inhibitory effects on cytochrome C reduction, therefore they may produce non-specific effects in addition to inhibiting NOS (Peterson et al., 1992). Nevertheless, we have previously provided some evidence that L-arginine reversed the attenuation of glucose uptake by L-NMMA during exercise in humans which suggests that L-NMMA specifically inhibited NOS to attenuate glucose uptake (Bradley et al., 1999). The use of $nNOS\mu^{-/-}$ mice during exercise can also eliminate the issue that

systemic infusion of NOS inhibitors *in vivo* may result in an increase in systemic blood pressure that may affect skeletal muscle glucose uptake during exercise and complicate the interpretation of the findings.

1.5.2.3 Contraction-stimulated muscle glucose uptake in humans

Studies in humans examining the role of NO in the regulation of skeletal muscle glucose uptake during exercise have generally shown a more consistent result supporting a role of NO (Bradley et al., 1999, Kingwell et al., 2002, McConell et al., 2006, Mortensen et al., 2007). In the Bradley et al. study (1999), young, healthy participants performed 30-min of supine cycling exercise at a moderate intensity (60±2% VO_2 peak) and received intra-femoral artery infusion of L-NMMA or saline during the final 20 min of exercise. The increase in leg glucose disposal during exercise, determined from arterial-venous plasma glucose difference and femoral blood flow, was attenuated by ~60% at five min after the commencement of NOS inhibition (Bradley et al., 1999). Plasma insulin levels, systemic blood pressure, heart rate, and more importantly the femoral blood flow were not affected by L-NMMA suggesting the reduction in glucose uptake was due to a local effect within the skeletal muscle fibers (Bradley et al., 1999). In addition, L-arginine infusion, a NOS substrate that competes with and displaces L-NMMA, during the last five min of the exercise appeared to reverse the attenuation in leg glucose disposal; suggesting the attenuation by L-NMMA was due to inhibition of NOS specifically (not non-specific effects). In a similar experimental design, people with T2D had not only a normal contraction-stimulation skeletal muscle glucose uptake but, NO-mediated signalling appeared to play a greater role in stimulating muscle glucose uptake during exercise in this cohort (Kingwell et al., 2002). In that study, NOS inhibition significantly attenuated the increase in leg glucose disposal during exercise to a greater extent in people with T2D (~75%) than the healthy subjects (~35%) without affecting femoral blood flow, blood pressure, arterial plasma glucose and insulin concentrations (Kingwell et al., 2002). This finding highlighted the therapeutic potential of NO in glycaemic control of people with T2D because if we can understand how NO

regulates glucose uptake during exercise then we can try to develop pharmaceuticals to mimic this in people who cannot or will not exercise sufficiently.

L-NMMA also appeared to attenuate the increase in leg glucose uptake in another study that focused on investigating the role of NO and other substances in the regulation of blood flow during one-legged knee extension exercise at moderate intensity at 19 watts (Mortensen et al., 2007). Leg glucose uptake data was not presented by the authors in the article but using the presented femoral blood flow and arterial-venous glucose data, the calculated leg glucose uptake during exercise appeared to be much lower during the presence of L-NMMA (McConell et al., 2012, Mortensen et al., 2007). This finding is in line with the data reported by our group (Bradley et al., 1999, Kingwell et al., 2002). However, NO effects on glucose uptake may depend on exercise intensity. In a similar leg kicking study by a group that included some of the same investigators as Mortensen et al. (Heinonen et al., 2013), there was no effect of NOS inhibition on muscle glucose uptake during low intensity (10 watts) legging kicking exercise (Heinonen et al., 2013). In support of NO being more important for glucose uptake during higher intensity exercise, NO production in primary rat muscle cells occurs during intense electrical stimulation causing contractions but not during low or moderate intensity (Silveira et al., 2003). It should also be pointed out that the lack of NOS inhibition effect on leg glucose uptake in Heinonen et al. (2013) study could also be due to methodological issues. In that study, the same participants performed two consecutive exercise trials with saline and NOS inhibitor infusions separated only by 30 min and in an order that the saline infusion always preceded the NOS inhibition. Glucose uptake would be expected to be higher in the second bout due to muscle glycogen deletion (Hespel and Richter, 1990) which may have masked an effect of the NOS inhibitor. Furthermore, prior exercise also increases insulin sensitivity for several hours after the cessation of exercise (Wojtaszewski et al., 2000a) and insulin- and contraction-stimulated glucose uptake are shown to be additive (Wasserman et al., 1991). Since the second bout of exercise was always NOS inhibition and was only 30 min after the cessation of the first exercise bout, the residual signalling effects from the

prior exercise and the resulted increased insulin sensitivity would likely lead to a greater stimulation of glucose uptake during the subsequent exercise. This could mask or over-ride the effects of NOS inhibition in Heinonen et al study (2013).

The role of NO in muscle glucose uptake during exercise in humans has also been investigated using L-arginine (a NOS substrate), a strategy to increase NO production in contracting muscles (McConell et al., 2006). In this study, L-arginine was continuously infused into endurance-trained males during the final 60 min of a 120-min cycling exercise at 72±1% VO_2 peak and it significantly increased glucose disposal (tracer determined) during exercise (McConell et al., 2006). L-arginine infusion appeared to have caused no significant changes in plasma insulin among the participants and it had previously been shown to have no effect on blood flow during five min of exercise in humans (Bradley et al., 1999). Presumably, L-arginine increased skeletal muscle NOS activity and NO production and led to an increase in skeletal muscle glucose uptake. In fact, L-arginine has been shown to increase NO production, in isolated rat skeletal muscle at rest, lending further support to this hypothesis (Balon and Nadler, 1994). However, a follow-up study designed to examine the mechanism(s) by which L-arginine infusion increased glucose disposal during exercise in humans suggested that NOS might not be involved (Linden et al., 2011). NOS activity during exercise was increased similarly with and without L-arginine infusions (Linden et al., 2011). However, L-arginine infusion was associated with a significant substantial increase in plasma insulin level (Linden et al., 2011) and therefore it was postulated that the observed increase in glucose disposal during L-arginine infusion may have been due to the additive effect of muscle contraction and insulin on skeletal muscle glucose uptake (Linden et al., 2011). The discrepancy between this and the earlier studies on insulin levels may be due to a methodological difference. The possible increase in plasma insulin in the former study (McConell et al., 2006) could have been missed due to a longer blood sampling interval (every 15 min versus every 5 min) compared with the latter study (Linden et al., 2011). Alternatively, L-arginine might have indeed led to a greater glucose disposal via NO pathway despite there was no further increase in NOS

activity with L-arginine infusion. In healthy humans, plasma concentration of L-arginine (~100 μM) (Forstermann et al., 1994) is greater than the Michaelis-Menten constant (Km) for both nNOS (~1.4 μM) (Bredt and Snyder, 1990) and eNOS (2.9 μM) (Pollock et al., 1991); therefore, NOS should have been saturated provided that cellular L-arginine concentration is close or equal to plasma concentration and indeed no further increase in NOS activity during exercise in humans was observed with L-arginine infusion (Linden et al., 2011). Nevertheless, increases in leg blood flow, urinary cGMP and nitrate excretion (indirect indices of systemic NO production) have been reported with L-arginine infusion (Bode-Boger et al., 1996, Bode-Boger et al., 2003, Hickner et al., 1997) indicating that NO-mediated effects were enhanced during L-arginine infusion despite NOS enzyme being saturated and NOS activity was not increased (Linden et al., 2011) – a situation known as the L-arginine paradox (Bode-Boger et al., 2007).

Overall, studies in humans investigating the role of NO in muscle glucose uptake generally support that NO is playing a role in glucose uptake during exercise. A recent high impact review by Richter and Hargreaves (2013) summarised that NO appears to be playing a role in glucose uptake during contraction/ exercise. Meanwhile, the bulk of evidence from NOS inhibition studies indicate that when NOS is inhibited and glucose uptake is measured during contraction/ exercise, glucose uptake is also attenuated suggesting NO is an important regulator of skeletal muscle glucose uptake during muscle contraction/ exercise. However, in view of the possible non-specific effects of NOS inhibitors it is imperative that genetically-modified rodent models, in particular nNOSμ$^{-/-}$ mice, are used to examine the role of NO in the regulation of skeletal muscle glucose uptake during contraction/ exercise. In addition, further examination of why people with T2D appear to rely more on NO for glucose uptake during exercise are required.

1.5.3 Nitric oxide and skeletal muscle blood flow at rest

Nitric oxide, an important gaseous signalling messenger, is constantly released from resting skeletal muscle (Balon and Nadler, 1994). It is well established that NO is a potent vasodilator that reduces vascular resistance and reciprocally increases vascular blood flow (Hester et al., 1993, Joannides et al., 1995) via the activation of smooth muscle soluble guanylate cyclase (sGC) leading to production of cyclic guanosine monophosphate (cGMP) and activation of protein kinase G (PKG) (Denninger and Marletta, 1999, Kukovetz et al., 1979, Murad et al., 1978, Young and Leighton, 1998a). As such, NO stimulatory effect on glucose uptake may be mediated by an increase in blood flow and thus glucose delivery. Infusion of the NO donor SNP (Radegran and Saltin, 1999) and L-arginine (Hickner et al., 1997) in healthy subjects increases basal skeletal muscle blood flow. In addition, significant reductions in resting forearm blood flow (Duffy et al., 1999, Gordon et al., 2002) and basal femoral blood flow (Radegran and Saltin, 1999) are observed during infusion of a NOS inhibitor (L-NMMA) further supporting NO as an important regulator of basal muscular blood flow.

Durham et al. (2003) extended the finding that femoral SNP infusion increases basal leg blood flow and showed that basal leg glucose uptake also increased in parallel with blood flow. Thus, it appears that under basal conditions NO increases skeletal muscle glucose uptake via an increase in blood flow, if not other mechanisms as well. Nitric oxide may also have a direct effect on basal glucose uptake in addition to its effect in increasing basal blood flow and glucose delivery to skeletal muscle. Henstridge et al. (2005) found that SNP induced a small increase in basal leg glucose uptake that was significantly different from that of verapamil (calcium antagonist and potent vasodilator), used at a dose to produce matching increases in leg blood flow between the two types of infusions. However, it is worth highlighting that the difference in basal glucose uptake between the two agents appeared to be mainly driven by a drop in basal glucose uptake following verapamil infusion.

1.5.4 Nitric oxide and skeletal muscle blood flow during contraction/ exercise

During exercise, muscle blood flow can increase up to 20-fold (Sorlie and Myhre, 1978) with a concomitant increase in capillary recruitment to the active skeletal muscles (Dawson et al., 2002, Ross et al., 2007) increasing nutrient and hormone delivery to match the metabolic demands of the contracting skeletal muscles (Hamann et al., 2005). A great number of hormonal, neuronal and local factors play a role in recruiting and vasodilating blood vessels supplying the active skeletal muscle (Delp and Laughlin, 1998). NO, produced during exercise or muscle contraction (Balon and Nadler, 1994, Merry et al., 2010d, Roberts et al., 1999) may play a role in exercise hyperaemia.

In rodents, studies examining the role of NO in regulating skeletal muscle blood flow have generally shown that NOS inhibition attenuated the increase in skeletal muscle blood flow during exercise. L-NAME administration was shown to attenuate rat hindlimb blood flow and vascular conductance during treadmill exercise (Copp et al., 2010, Hirai et al., 1994). Lee-Young et al. (2010) examined the functional role of eNOS in the regulation of metabolism using $eNOS^{-/-}$ mice demonstrated that the percent of cardiac output going to the active skeletal muscle in response to exercise was proportional to total eNOS protein expression supporting a role of NO in regulating skeletal muscle blood flow during exercise. Another study using $eNOS^{-/-}$ and $nNOS^{-/-}$ mice to investigate the contribution of NO to vascular smooth muscle relaxation in contracting fast-twitch muscle also indicated both nNOS and eNOS played a role in exercise hyperaemia (Grange et al., 2001). However, that study investigated smooth muscle myosin regulatory light chain phosphorylation as an index of vascular tone but did not measure blood flow *per se* (Grange et al., 2001). Thomas et al. (2003) further demonstrated that nNOS modulation of α-adrenergic vasoconstriction in contracting muscles required the membrane localisation of nNOS via the dystrophin-associated protein - α-syntrophin. Our group also demonstrated in rats that femoral blood flow during muscle contraction *in situ* was attenuated by local infusion of L-NAME (Ross et al., 2007). However, capillary blood flow, determined by contrast-enhanced ultrasound

was not affected with L-NAME infusion during contraction (Ross et al., 2007) suggesting that NO affects only the total blood flow but not the capillary vessels that supply the contracting muscles. A similar finding was also reported by Inyard et al. (2007). These findings highlight that in rodents NO plays a role in modulating total blood flow but it is unlikely to have an effect on capillary blood flow during contraction. These capillary vessels in the muscles, also known as nutritive vessels, determine the delivery of nutrients and substrates to the active muscles (Clark et al., 2006) and therefore play an important role in regulating substrate uptake into skeletal muscle.

In humans, however, a number of studies demonstrated that NO is not important in regulating exercise hyperaemia (Bradley et al., 1999, Endo et al., 1994, Kingwell et al., 2002, Radegran and Saltin, 1999, Shoemaker et al., 1997, Wilson and Kapoor, 1993) a finding that is not universally agreeable by others (Duffy et al., 1999, Dyke et al., 1995, Gilligan et al., 1994, Hickner et al., 1997). Studies measuring leg blood flow during cycling (Bradley et al., 1999, Kingwell et al., 2002) or knee-extension exercise (Radegran and Saltin, 1999) as opposed to those measuring blood flow of forearms (Duffy et al., 1999, Dyke et al., 1995, Gilligan et al., 1994) generally show that NO is not important in the regulation of blood flow during exercise. Some possibilities for this discrepancy could be due to: 1) greater occlusion of blood vessels in skeletal muscle may be occurred during static handgrip exercise compared with the dynamic cycling or knee-extension exercise; 2) forearm exercise involved lesser muscular work at low intensities; 3) venous occlusion plethysmography blood flow measurement technique was used during forearm exercise which required a brief cessation of exercise while the measurement was being performed therefore these studies examined the role of NO in blood flow during the early recovery phase of exercise instead of during the actual exercise. In fact, Radegran et al. (1999) showed that L-NMMA reduced leg blood flow during recovery from exercise but blood flow during exercise was not affected therefore the results from these studies that involved a brief cessation of exercise need to be interpreted with caution. Despite this, Hicker et al. (1997) showed a significant decrease in muscle blood flow with L-NMMA infusion during cycling exercise. However, this

result may be questioned as the accuracy of the microdialysis blood flow measurement technique using ethanol outflow-inflow ratio used in this study has been questioned (Radegran et al., 1998). Therefore, it appears that NO does not affect blood flow during exercise. However, it needs to be considered that other neuronal and metabolic vasodilators create a level of redundancy in the regulation of exercise hyperaemia (Clifford and Hellsten, 2004) which allow NOS inhibition to have no or little effect on blood flow. Indeed, simultaneous inhibition of both NO and prostaglandins, but not one or the other, attenuates the increase in leg blood flow during exercise in humans (Mortensen et al., 2007).

In line with the above finding, our group, during the course of investigating the role of NO on skeletal muscle glucose uptake during cycling exercise demonstrated that local femoral artery infusion of L-NMMA in young healthy adults had no effect on femoral blood flow but significantly attenuated the increase in muscle glucose uptake during exercise by ~60% (Bradley et al., 1999). Similarly, NOS inhibition attenuated the increase in glucose uptake but not leg blood flow during exercise in a follow-up study performed on people with T2D (Kingwell et al., 2002). These findings strongly suggest that NO increases glucose uptake in contracting skeletal muscle independent of total blood flow. It is noteworthy to highlight that in the above studies, the L-NMMA infusion was started 10 min into the exercise at which time the maximum exercise hyperaemia had already been achieved and all vessels involved in exercise hyperaemia would have been fully recruited. This might have minimised the effect of L-NMMA on basal vascular tone and lead to a non-significant effect of L-NMMA on exercise hyperaemia.

It is not known if NOS inhibition during exercise in humans affects capillary blood flow. A redistribution of blood flow between nutritive and non-nutritive capillaries during exercise could occur with NOS inhibition without a change in total blood flow (Clark et al., 1998) and this could affect muscle glucose uptake (Clark et al., 2003). NOS inhibition could preferentially reduce nutritive blood flow with a reciprocal

increase in non-nutritive blood flow therefore maintaining total blood flow but reducing glucose uptake. Based on this hypothesis, one would expect a reduction in net oxygen uptake due to the reduction in capillary blood flow and an elevation in net lactate release (Kingwell et al., 2002), however, leg lactate production and oxygen was not changed with NOS inhibition during exercise suggesting that nutritive capillary blood flow was likely not affected (Kingwell et al., 2002). This fits with the findings in rats of no effect of NOS inhibition on capillary blood flow during *in situ* contraction (Inyard et al., 2007, Ross et al., 2007).

Taken together, in humans NO is an important contributor to vasomotor tone and blood flow at rest and in recovery after exercise but it is not critical for muscle total blood flow during exercise. These findings, together with those from *ex vivo* contraction rodent studies, suggest that NO increases glucose uptake in contracting muscle via a direct intramuscular stimulatory effect that is independent of blood flow.

1.5.5 NO and possible downstream signalling

It is surprising that the mechanisms that NO stimulates muscle glucose uptake during contraction/ exercise are still inadequately investigated despite a strong body of evidence, in both rodents and humans, indicating an important role of NO in skeletal muscle glucose uptake. GLUT4 translocation is the critical process that allows the transport of plasma glucose across the muscle membrane in response to muscle contraction, and NO has been shown to increase skeletal muscle GLUT4 translocation (Etgen et al., 1997, Roberts et al., 1997). The NO downstream signalling mechanisms that could potentially elicit GLUT4 translocation include its classical downstream target - cGMP and its downstream cascades especially PKG (Young et al., 1997), AMPK (Higaki et al., 2001), peroxynitrite, and post-translational protein modification such as S-glutathionylation and S-nitrosylation by reactive nitrogen oxide species (Merry et al., 2010d, Stamler and Meissner, 2001). So far, the downstream signalling of NO in mediating glucose uptake or GLUT4 translocation has mainly been investigated in basal non-contracting muscles. To the best of our knowledge, there is only one study to date

that has investigated the mechanisms of NO-mediated skeletal muscle glucose uptake during contraction (Merry et al., 2010d), which demonstrated preliminary evidence that the NO mechanism regulating skeletal muscle glucose uptake during contraction differed from that during basal conditions. In the following sections, the possible downstream signalling whereby NO regulates muscle glucose uptake will be discussed.

1.5.5.1 NO/cGMP/PKG pathway

From a series of comprehensive experiments, Young et al. (1997, 1998b, 1998a) provided evidence that NO donors stimulated basal muscle glucose uptake via a cGMP/ PKG pathway. In the experiments, SNP significantly increased cGMP content and glucose oxidation, which the authors assumed was equivalent to or indicative of glucose uptake (Young et al., 1997). LY-83583, which inhibits the action of sGC (Fig 1.2), decreased muscle glucose oxidation with a parallel reduction in the cGMP level (Young et al., 1997). However, it should be noted that LY-83583 could also have a direct inhibitory effect on nNOS activity (Luo et al., 1995) and therefore the reduction in cGMP level and glucose oxidation could have resulted from the inhibition of nNOS in addition to sGC alone. Nevertheless, basal muscle glucose oxidation was increased when muscles were treated with a cGMP analogue (8-bromo-cGMP) (Young and Leighton, 1998a) or a cGMP phosphodiesterase 5 inhibitor (zaprinast) that prevents breakdown of cGMP and thus results in elevated cGMP levels (Fig 1.2) (Young and Leighton, 1998b). These findings support the notion that NO-induced increases in cGMP are important for stimulating glucose uptake/ oxidation in the basal (none-contracting) state. Using a more specific sGC inhibitor than LY-83583, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (Garthwaite et al., 1995) (Fig 1.2), our group similarly demonstrated that the increase in muscle glucose uptake induced by a NO donor is attenuated by ODQ (Merry et al., 2010d) providing further support that cGMP mediates NO effect of stimulating muscle glucose uptake in the basal state. Interestingly, in the same study ODQ had no effect on glucose uptake when the muscle was stimulated to contract *ex vivo* (Merry et al., 2010d). It is worth noting that in that study cGMP formation following contraction was unable to be measured to verify that ODQ had adequately inhibited sGC activity

during muscle contraction, probably due to not freezing the muscles fast enough (Merry et al., 2010d). However, in line with this finding, a PKG inhibitor, also had no effect on glucose uptake in mouse muscle contracted *ex vivo* (Merry et al., 2010d). These findings suggest that, in contrast to basal muscle glucose uptake, NO-mediated glucose uptake during contraction may occur via a cGMP/PKG-independent mechanism. A possible reason for these discrepancies in NO's mechanism(s) to regulate glucose uptake could be due to the supratherapeutic doses of NO donors that are used to stimulate basal muscle glucose uptake; while, NO signalling during contraction is derived from the physiological level of endogenous NO.

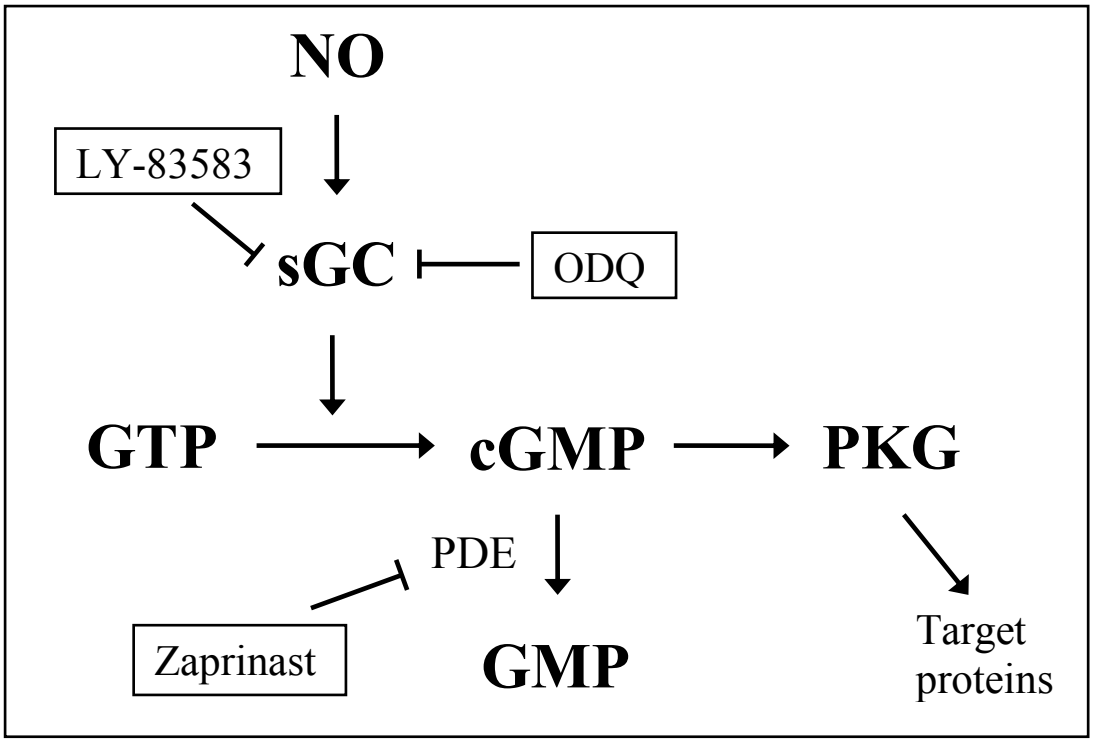


Figure 1.2: "Classic" NO signalling via cGMP/PKG which appears to be involved in glucose uptake basally but perhaps not during contraction/ exercise.

NO: nitric oxide, sGC: soluble guanylate cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G, GMP: guanosine monophosphate, PDE: phosphodiesterase, ODQ: 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one. Zaprinast is a PDE 5 inhibitor.

1.5.5.2 NO/cGMP/PKG-independent pathways

During muscle contraction/ exercise, both superoxide ($O_2^{\bullet-}$) and NO production in skeletal muscle are greatly increased (Balon and Nadler, 1994, Reid et al., 1992, Roberts et al., 1999, Sandstrom et al., 2006). These molecules can interact to form peroxynitrite ($ONOO^-$), rather than $O_2^{\bullet-}$ being scavenged by superoxide dismutase to form hydrogen peroxide, due to a kinetic predisposition for the former reaction (Halliwell, 1989). Alternatively, the highly unstable and reactive NO can react easily with other molecules to produce a variety of compounds known collectively as "reactive nitrogen oxide species" (RNOS). RNOS can react with cysteine residues of proteins leading to different post-translational modifications including S-nitrosylation, S-glutathionylation and tyrosine nitration which could alter protein function (Broillet, 1999, Martinez-Ruiz and Lamas, 2009). The reactions and mechanisms of formation for these post-translational modifications have been discussed in detail in a review paper (Martinez-Ruiz and Lamas, 2009).

Peroxynitrite, at high concentrations, is associated with various disease states (Pacher et al., 2007) and can cause irreversible nitration of tyrosine residues on proteins (Pacher et al., 2007) and therefore prevent tyrosine phosphorylation or inactivation of a particular protein (Gow et al., 1996). However, at low levels (10 – 200 μM), $ONOO^-$ can promote transient and reversible phosphotyrosine signalling which has been shown to mediate various metabolic effects (Pacher et al., 2007). Peroxynitrite was shown to activate proteins associated with glucose uptake such as PI3K, AMPK (Zou et al., 2002, Zou et al., 2003) and PKC (Balafanova et al., 2002) suggesting a possible role of peroxynitrite in muscle glucose uptake during contraction. In line with these findings, incubation of isolated mouse EDL muscle contracted *ex vivo* with urate (a peroxynitrite scavenger) significantly attenuated the increase in contraction-stimulated muscle glucose uptake (Merry et al., 2010d) suggesting that peroxynitrite may mediate NO's effect on muscle glucose uptake. That said, muscles incubated with urate were associated with lower peak force that may affect glucose uptake and urate may have other effects in addition to scavenging $ONOO^-$.

S-nitrosylation is a covalent post-translational modification on cysteine residues of a protein where a nitrosothiol or a thionitrite bond is formed in a thiol group (Stamler et al., 1992). Nitric oxide has been postulated to increase GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes via protein S-nitrosylation (Kaddai et al., 2008). In that study, a NO donor stimulated an increase in GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes and these were not attenuated by various pharmacological agents that inhibit sGC and PKG suggesting a cGMP/PKG-independent pathway may be responsible for inducing glucose uptake. After excluding the involvement of other important signalling involved in glucose uptake including AMPK and insulin signalling in mediating glucose uptake in these cells, it was postulated that the associated increase in S-nitrosylated proteins with glucose uptake may be responsible for this NO-mediated glucose uptake (Kaddai et al., 2008). It should be noted however that no causal relationship between the S-nitrosylated proteins and NO-mediated glucose uptake was established in that study. On the other hand, another study found that white light from a dissecting lamp, which should break S-nitrosylation bonds (Borutaite et al., 2000) had no effect on contraction-stimulated skeletal muscle glucose uptake (Merry et al., 2010d). This suggests that S-nitrosylation may not be involved in muscle glucose uptake during contraction. However, S-nitrosylation levels were not measured to verify that they were increased with contraction or that adequate breakdown of S-nitrosylation occurred with white light treatment (Merry et al., 2010d). Therefore, although there is some evidence that S-nitrosylation is associated with NO-mediated glucose uptake a causal relationship is yet to be established and there is not enough information as yet as to whether s-nitrosylation is involved in NO signalling to glucose uptake during contraction/ exercise.

S-glutathionylation is another example of post-translational modification by NO and ROS which involves the incorporation of a glutathione molecule to a protein thiol via a disulfide bridge (Dalle-Donne et al., 2009). Our group have some emerging preliminary data that S-glutathionylation may be involved in muscle glucose uptake during contraction/ exercise (Merry et al., 2010d, Merry et al., 2010c). NAC, a non-specific antioxidant, was shown to attenuate *ex vivo* contraction-stimulated glucose

uptake and S-glutathionylation of a protein with a molecular weight of 270 kDa in mouse EDL muscles (Merry et al., 2010d, Merry et al., 2010c). However, further work needs to be done to evaluate the identity and function of this protein band and whether it is involved in muscle glucose uptake.

1.5.5.3 NO and AMPK

NO donors can induce phosphorylation and activation of AMPKα1 in L6 myotubes and an AMPK inhibitor can ablate NO-induced increases in GLUT4 mRNA expression in these cells, suggesting that AMPKα1 mediates the effect of NO (Lira et al., 2007). Since NO can inhibit cytochrome synthase (Cleeter et al., 1994) and creatine kinase (Gross et al., 1996) activities to affect cellular energy status, NO is postulated to indirectly activate AMPKα1 via an increase in AMP/ ATP ratio (Lira et al., 2007). NO was also postulated to modulate the activity of AMPK kinases or AMPK phosphatases leading to activation of AMPKα1 (Lira et al., 2007). Other studies also found that NO-mediated increase in basal muscle glucose uptake was associated with an increase in AMPKα1 activity (Deshmukh et al., 2010, Higaki et al., 2001). These findings suggest that AMPKα1 lies downstream of NO and may mediate the effect of NO in stimulating muscle glucose uptake. Nevertheless, AMPKα1 activity may not be important for stimulating muscle glucose uptake during contraction because AMPKα2 appears to be the major isoform activated during contraction and it is likely to be the putative AMPK isoform involved in the regulation of skeletal muscle glucose uptake during contraction (Jorgensen et al., 2004). Dissociation between NO and AMPK phosphorylation is also observed where NOS inhibition attenuated the increase in skeletal muscle glucose uptake during contraction without affecting AMPK phosphorylation (Ross et al., 2007). Furthermore, AICAR-induced rat muscle glucose uptake was not prevented by NOS inhibition despite attenuation in glucose uptake with a NOS inhibition alone (Stephens et al., 2004) suggesting that NO and AMPK stimulate glucose uptake via independent pathways. It was also demonstrated that AMPKα2 KD mouse muscle which had no activation of AMPK following contraction had normal contraction-stimulated glucose uptake that was attenuated by NOS inhibition (Merry et al., 2010c) indicating that NO

does not stimulates glucose uptake via AMPK activation. Taken together, it appears that NO does not stimulate skeletal muscle glucose uptake during contraction via AMPK signalling.

In summary, preliminary evidence suggests that NO appears to regulate glucose uptake during contraction/ exercise through cGMP/PKG-independent mechanisms but more work is required to confirm this and to determine what these mechanisms are.

1.6 SUMMARY

Exercise/ muscle contraction is a potent stimulus to increase skeletal muscle glucose uptake. This process is normal/ intact in people with insulin resistance or T2D and a single bout of intense exercise can normalise plasma glucose levels during exercise in people with T2D. Exercise/ muscle contraction activates several signalling mediators that lead to an increase in GLUT4 translocation to the cell surface to increase glucose uptake. These mediators include AMPK, Ca^{2+}/ CaMK, PKC, ROS and NO and these appear to act in a redundant fashion to regulate skeletal muscle glucose uptake during contraction. This thesis focuses on the role of NO in this process.

Evidence indicating NO plays a critical role in stimulating skeletal muscle glucose uptake during contraction/ exercise is promising from a therapeutic standpoint for people with insulin resistance or T2D. However, some conflicting results exist in rodents in regards to the potential role of NO in the regulation of glucose uptake during contraction/ exercise. Methodological inconsistency and systemic administration of NOS inhibitors contributed to most of these conflicting findings. Besides refining the experimental protocol to measure muscle glucose uptake during contraction/ exercise, the use of a genetic model that lacking the main NOS isoform activated during contraction, nNOSμ, is imperative to determine the role of NO/ nNOSμ in muscle glucose uptake during contraction/ exercise.

Neuronal NOSμ$^{-/-}$ mice would, therefore, be important for examining the role of NO in muscle glucose uptake during contraction/ exercise. Furthermore, the apparent greater role of NO on glucose uptake during exercise in people with T2D has not been followed up to better understand the underlying mechanisms responsible for this phenomenon. The findings from these studies may provide the knowledge for the development of targeted pharmaceutical treatments and therefore improve the current management of diabetes mellitus which involves total healthcare cost of $0.8 billion for Australia in 2000-01.

1.7 AIMS

Therefore, the general aims of this thesis were to examine the role of NO and/ or nNOSμ in skeletal muscle glucose uptake during contraction/ exercise. The specific aims were:

1. To examine if NO/ nNOSμ is essential in mediating contraction-stimulated skeletal muscle glucose uptake. To do this a nNOSμ$^{-/-}$ mouse model was used. EDL muscles were isolated and stimulated to contract *ex vivo* to investigate the role of nNOSμ in contraction-stimulated muscle glucose uptake without the influence of blood flow.

2. To investigate whether T2D rats demonstrate a greater reliance on NO to increase muscle glucose uptake during *in situ* contraction, like people with T2D during exercise, and if so, to investigate the mechanism(s) that may be responsible for this phenomenon. We combined four weeks of high fat feeding and low dose streptozotocin injections to create a T2D rat model, and infused a NOS inhibitor locally into the femoral artery during muscle contraction while measuring capillary recruitment and glucose uptake.

3. To examine the effect of total and partial loss of nNOSμ in mice on muscle glucose uptake using a conscious, *in vivo*, and therefore physiological exercise model. To do this a chronic jugular cannulation was put into place in nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ mice, and they were subjected to treadmill running while glucose uptake was determined.

1.8 HYPOTHESES

The hypotheses tested were that:

1. Neuronal NOSμ$^{-/-}$ mice, due to a reduction in NO production in skeletal muscle, would have an attenuated increase in skeletal muscle glucose uptake during *ex vivo* contraction.

2. T2D rats would have a greater attenuation of glucose uptake with NOS inhibition during *in situ* contraction than control rats and that this would be independent of capillary blood flow.

3. Neuronal NOSμ$^{-/-}$ mice would have an attenuated increase in muscle glucose uptake during *in vivo* exercise and mice with partial loss of nNOSμ protein will have a normal glucose uptake as seen in people with T2D who have reduced nNOSμ expression

With respect to the above-mentioned aims and hypotheses, three research chapters detailing three distinct experiments testing these hypotheses will be presented. Preceded these chapters is a methodology chapter (Chapter Two) that details the general methods and some specific methods used in each research chapter with greater detail in order to simplify the respective research chapters. Chapter Three to Five as outlined in the aims and hypotheses: Aims 1 (Chapter Three), Aim 2 (Chapter Four), Aim 3 (Chapter Five). Chapter Six will summarise these findings in light of each other and discuss some interpretations, limitations and conclusions as well as some future directions that this research may take.

CHAPTER TWO:

METHODS

This chapter describes the general experimental and analytical techniques that were used in more than one study of this thesis. The rest of the experimental procedures that are specific to a particular study project are described in the method sections of the relevant study chapter. In addition, some methods that are described in only one study are being elaborated on within this chapter in order to simplify the respective study chapters.

2.1 GLUCOSE UPTAKE DETERMINATION

2.1.1 Glucose uptake determination during ex vivo contraction (Chapter Three)

In this method, the radioactive glucose analogue, [1,2-^{3}H]2-deoxy-glucose ([^{3}H]2-DG), and radioactive mannitol, D-[^{14}C] mannitol ([^{14}C]mannitol), were used to determine glucose uptake into skeletal muscle during *ex vivo* muscle contractions (Hansen et al., 1994). Being a glucose analogue, [^{3}H]2-DG can be transported into skeletal muscle and phosphorylated by hexokinase II to [^{3}H]2-DG-6-phosphate ([^{3}H]2-DG-6-P). [^{3}H]2-DG-6-P cannot undergo glycolysis or oxidation nor efflux out from muscle fibers (Jenkins et al., 1986) and therefore it accumulates in the fiber and serves as an index of glucose uptake (Hansen et al., 1994). Phosphorylated [^{3}H]2-DG has no measurable inhibitory action on hexokinase as such its accumulation does not limit glucose transport until an extremely high concentration of [^{3}H]2-DG-6-P (> 30 mM) is achieved (Hansen et al., 1994). On the other hand, [^{14}C]mannitol is not permeable to the plasma membrane and not readily transported into cells but remains in the extracellular space (Ford and Harrison, 1983). As such, [^{14}C]mannitol is used as a marker of extracellular space (Ford and Harrison, 1983, Young et al., 1986). To calculate skeletal muscle glucose uptake, [^{14}C]mannitol counts (beta counter) are subtracted from the [^{3}H]2-DG counts in muscle homogenates to provide an index for intracellular [^{3}H]2-DG-

6-P accumulation and an estimate of skeletal muscle glucose uptake (Hansen et al., 1994). It is, however, needs to be clarified that 2-DG uptake is not equal to glucose uptake since the transport and phosphorylation rates and the volume of distribution of 2-DG and glucose in the muscles are different. The calculation of the glucose metabolic rate needs to be adjusted for a proportionality constant, the lump constant, which determines the accuracy of measurement of glucose metabolic rate.

Since nNOS is predominantly expressed in glycolytic muscles, and oxidative muscles (soleus) was shown to have no significant increase in NO-mediated glucose uptake during *ex vivo* contraction (Merry et al., 2010c), EDL muscles which have a good diffusion capacity were used in this study. Muscle was either rested or stimulated to contract for 10 min and glucose uptake was measured during the final 5 min of contraction or basal incubation. It was demonstrated previously that a significant increase in muscle glucose uptake during contraction occurs after a delay of approximately 5 min from the beginning of contraction (Lauritzen et al., 2010, Mossberg et al., 1993). Thus, this protocol captured the period when we would expect the highest glucose uptake during contraction and did not include the glucose uptake when contraction has ceased. Some studies examine glucose uptake during the last 5 min of contraction and the first 5 min after contraction has ceased and use this value for the contraction-stimulated glucose uptake (Jorgensen et al., 2004). We believe that our procedure more accurately reflects the situation during muscle contractions. This also allows a standardised experimental protocol between experiments performed to obtain samples for muscle glucose uptake and samples for immunoblotting and enzymatic activity assays. Each experiment was performed thrice, with the muscles from one experiment used for glucose uptake measurements and the others for biochemical analysis. This was necessary due to the small size of the EDL. Initial attempts were made to measure glucose uptake using half of the EDL muscles but the radioactivity counts recovered at the concentration of [^{3}H]2-DG and [^{14}C]mannitol used were too low to be reliable. This is why it was necessary to repeat the experiment so that we could have an entire EDL muscle for glucose uptake, and other EDLs for the same conditions

for biochemical analysis. To measure glucose uptake, the incubation buffer [(in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 8 mannitol, 2 sodium pyruvate, 0.01% BSA, pH 7.4] was rapidly exchanged with the buffer containing 1 mM [^{3}H]2-DG (0.128 μCi/ml) and 8 mM [^{14}C]mannitol (0.083 μCi/ml) (PerkinElmer, Boston, MA, USA). At the end of the experiment, muscles were immediately washed in ice-cold buffer, blotted on filter paper and snap frozen in liquid nitrogen. The muscle was frozen within approximately 10 second after the cessation of the muscle contractions. Muscle weight was recorded and the entire frozen muscle was digested with 125 μl of 1 M NaOH for 10 min at 80 ^{o}C which was then neutralised with an equal volume of 1 M HCl, vortex-mixed and centrifuged at 13,000 *g* for 2 min. The supernatant was recovered and 175 ul of this was added with 4.5 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA, USA). Radioactivity was determined by β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA) and muscle glucose uptake was calculated based on [^{14}C]mannitol and [^{3}H]2-DG counts and muscle weight.

2.1.2 Glucose uptake determination during in situ contraction (Chapter Four)

The principle of the skeletal muscle glucose uptake during *in situ* contraction was essentially the same as the *ex vivo* study discussed above (Section 2.1.1), however, the labelled phosphorylated 2-deoxy-glucose in the muscles in this *in situ* study was extracted using an anion-exchange resin, therefore, unlike the *ex vivo* study an indicator for the glucose concentration in the extracellular space was not needed in this *in situ* study. This procedure was performed as previously described (Ross et al., 2007). Twenty minutes into the *in situ* contraction, i.e. 10 min before the completion of the experiment, a bolus of [1-^{14}C]2-deoxy-glucose ([^{14}C]2-DG) (20 μCi) (American Radiolabeled Chemicals, St Louis, MO, USA) in isotonic saline was administered via a jugular vein. Then, an arterial blood sample was withdrawn continuously at 30 μl/min over the final 10 min using an automated syringe pump. An aliquot of plasma (25 μl) from this blood sample was used to determine average plasma radioactivity of [^{14}C]2-DG that to be used in the calculations of muscle glucose uptake. At the end of the

experiment, hindlimb muscles (gastrocnemius, plantaris and soleus) were excised and immediately freeze-clamped using liquid nitrogen-cooled tongs and stored at -80 ^{o}C. The whole hindlimb muscles (gastrocnemius, plantaris and soleus) was used because it allows for better matching and interpretation of the glucose uptake and capillary blood flow data since the hindlimb muscles was electrically stimulated as a group during the *in situ* contraction and the capillary blood flow of the mid-plane of the whole hindlimb muscles was measured by the CEU technique. Frozen muscles were pulverised under liquid nitrogen and ~100 mg of muscle sample was homogenised with 1.5 ml of MilliQ water using a Heidolph^{TM} silent crusher M (Schwabach, Germany). The homogenate was centrifuged at 13,000 *g* at 4 ^{o}C for 10 min. One ml of the lysate was used to extract the phosphorylated [^{14}C]2-DG ([^{14}C]2-DG-6-P) using an anion exchange resin column (AG1-X8; Bio-Rad Laboratories, CA, USA). The column was washed with 2.5 ml of MilliQ water to elute non-phosphorylated [^{14}C]2-DG (free [^{14}C]2-DG) that was not bound to the resin and was collected into a scintillation vial. Then, the [^{14}C]2-DG-6-P bound to the resin was eluted with 4 ml of 2 M HCl and collected for radioactivity counting. A separate 100 µl of lysate was used directly (no column) to estimate the total radioactivity in the sample. Biodegradable counting scintillant (16 ml) (BCS: Amersham Biosciences) was added to each of the sample collected above (25 µl of plasma sample from arterial withdrawal, free [^{14}C]2-DG from the resin column following elution with MilliQ water, [^{14}C]2-DG-6-P from the resin column following elution with HCl, and 100 µl of lysate from homogenised sample) and radioactivity was determined using a β-counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL, USA). The rate of muscle glucose uptake (R'g) was calculated based on the below formula as previously described (Ross et al., 2007). The free [^{14}C]2-DG, [^{14}C]2-DG-6-P and total sample radioactivity counts were used to determine the recovery of the extraction procedure. A recovery between 90 to 110% was considered satisfactory; otherwise the extraction procedure would be repeated.

$$R'g = \frac{\text{Muscle } [^{14}C]2\text{-DG-6-P (dpm/g) x plasma [glucose] (}\mu\text{g/ml)}}{\text{Average plasma } [^{14}C]2\text{-DG (dpm/ml) x 10 (min)}}$$

2.1.3 Glucose uptake determination during treadmill exercise (Chapter Five)

The principle of this procedure was similar to that described for the *in situ* contraction experiments discussed above (Section 2.1.2). This procedure was performed as previously described (Halseth et al., 1999). [1,2-^{3}H]2-deoxy-glucose ([^{3}H]2-DG) was used in this study to determine muscle glucose uptake. A bolus of [^{3}H]2-DG (13 μCi reconstituted in normal saline) was injected into a jugular vein 5 min into the experiment. At the end of the experiment, gastrocnemius and superficial vastus lateralis muscles and brain tissues (excluding the hypothalamus and brainstem) were excised and immediately freeze-clamped using liquid nitrogen-cooled tongs and stored at -80 ^{o}C. Gastrocnemius (primary glycolytic muscle fibers) was used in this study as it is more suitable for examining the role of nNOSμ in glucose uptake during exercise compared with oxidative muscles due to the higher nNOS expression in glycolytic muscles. Frozen muscles were pulverised under liquid nitrogen. Muscle sample and brain tissue (~30 mg) were homogenised with 1.5 ml of MilliQ water using a polytron. The homogenates were centrifuged at 13,000 *g* at 4 ^{o}C for 10 min. An aliquot (0.5 ml) of the lysate was loaded into an anion exchange resin column (AG1-X8, Bio-Rad) to extract phosphorylated [^{3}H]2-DG ([^{3}H]2-DG-6-P). The column was washed with 6 ml of MilliQ water and an aliquot of eluate (2 ml) was collected for radioactivity determination. A separate aliquot (0.5 ml) of lysate was added directly into scintillation vials for counting of total radioactivity. Ten ml of scintillant fluid (IRGA-Safe Plus, Perkin Elmer) was added into each vial and radioactivity was determined using a β-counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL, USA). [^{3}H]2-DG-6-P radioactivity in the muscle and brain tissues (dpm/mg/min) were calculated. Glucose uptake for each muscle was expressed as an index of [^{3}H]2-DG-6-P accumulation in the muscle normalised to [^{3}H]2-DG-6-P in the

brain of that mouse (Halseth et al., 1999, He et al., 2012). Brain glucose uptake was used as a control for the integrated plasma [^{3}H]2-DG concentration differences over the duration of the experiments (Halseth et al., 1999). Brain glucose uptake is a valid representative of the integrated plasma [^{3}H]2-DG concentration because: 1) glucose uptake into most parts of the central nervous system excluding hypothalamus is a passive process that is exclusively determined by the concentration of blood glucose (Mayer, 1953) and not directly affected by insulin (Hom et al., 1984); 2) there is little accumulation of free 2-DG in brain tissues under normoglycaemic conditions indicating that glucose phosphorylation does not limit glucose uptake into brain tissue (Hom et al., 1984); 3) there is also insignificant expression of hexokinase II in the brain (Printz et al., 1993).

2.2 NOS ACTIVITY

This assay was employed in all experimental chapters (Chapter Three, Four and Five). Muscle NOS activity was measured as previously described (Lee-Young et al., 2009). The principle of this assay is based on the catalytic reaction of NOS which converts radioactive L-arginine to radioactive L-citrulline and NO (Moncada and Higgs, 1993). The formation of L-citrulline is proportional to the activity of NOS in the muscle samples. At pH 5.5 (the pH of stop buffer), the newly formed L-citrulline has a neutral charge while L-arginine is positively charged. The L-citrulline is separated from the excess L-arginine using a cation-exchange resin which binds the excess L-arginine but not the L-citrulline. Radioactive counts in the eluate provide a measurement of the newly formed L-citrulline and thereby a marker of NOS activity. In this assay, frozen muscles were homogenised in 10 volume of ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μl/ml protease inhibitor mixture, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate]. Samples were incubated on ice for 20 min and then centrifuged at 10,000 *g* for 20 min at 4 ^{o}C. The lysate was collected for determination of protein concentration and NOS activity assay. Protein

concentration was determined as described in Section 2.3.2. To measure NOS activity, 15 µl of lysate (~90 µg of total protein) was added to 15 µl of pre-heated (37 ^{o}C) assay buffer [50 mM Tris HCl (pH 7.5), 4 µM BH_4, 100 nM calmodulin, 0.7 mM $CaCl_2$, 0.63 µM FAD, 1.15 mM NADPH, 2.5 µCi/ml L-[^{14}C]arginine]. The assay was conducted at 37 ^{o}C for 10 min (previously shown to be within linear range by our group) (Lee-Young, 2006) and terminated by adding 200 µl of ice-cold stop buffer containing 20 mM HEPES (pH 5.5), 2 mM EDTA and 2 mM EGTA. For each sample, one reaction was done in the presence of 1 mM L-NAME to block NOS activity, and duplicates were performed without L-NAME. The radioactive counts from these reactions allowed the calculation of radioactive counts that were attributed to NOS reaction and eliminated the background counts. Equilibrated resin (300 µl, Cayman Chemical Company, Michigan, USA) was added to the reactions to trap the excess L-arginine which was then transferred to spin cups/ column, and then centrifuged for 30 s at 13,000 *g*. Another 200 µl of stop buffer was added to the columns and were spun again. The eluate (400 µl) containing radioactive L-citrulline was transferred to scintillation vials along with 5 ml of scintillation fluid (PerkinElmer, Boston, MA, USA), vials were vortexed, left to rest for an hour and then radioactivity was counted using a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA). NOS activity was determined from the difference between samples incubated with and without L-NAME and was expressed as picomoles of L-[^{14}C]citrulline formed per min, per mg of protein. This assay was validated using muscle samples containing different nNOS expression levels. It was demonstrated that NOS activity corresponded with nNOSµ expression levels where EDL had higher NOS activity than gastrocnemius muscles (Fig 3.8 and Fig 5.10). NOS activity in nNOSµ$^{+/+}$ muscles was approximately double that of nNOSµ$^{+/-}$ and only minimal residual activity detected in nNOSµ$^{-/-}$ muscles (Fig 3.8 and Fig 5.10). It is to acknowledged that some NOS activity may be lost in the pellet during sample homogenisation; however, it is expected that equal proportion lost of NOS activity in the pellet occurred between the comparisons groups (treatment vs control). If the NOS activity including those in the pellet were measured the absolute reading maybe different

but the relative results between treatment and control groups would be expected to be the same. Therefore, the overall results and conclusion are expected not to be changed.

2.3 IMMUNOBLOTTING

This procedure was used in all experimental chapters (Chapter Three, Four, and Five). Immunoblotting was performed according to an immunoblotting method as previously described (Murphy, 2011) with some modifications as mentioned below. The rationale and advantages of this immunoblotting technique has been discussed in detail in a recent review (Murphy and Lamb, 2013). The main principle of this technique is by not centrifuging the homogenised samples and using a very sensitive chemiluminescence substrate, one is able to detect proteins by only loading a small amount (2 to 8 μg) of total protein. This became extremely useful in the *ex vivo* experiments because each EDL muscle weighs only approximately 10 mg and was needed for several different muscle analyses. In the study shown in Chapter Three, experiments were repeated to obtain three sets of mouse EDL muscle samples to perform several biochemical analyses. Unfortunately, a whole set of these samples was lost during a freezer breakdown in our laboratory. Therefore, the samples would have been insufficient if immunoblotting using the standard procedures of sample processing and homogenisation methods as previously described were used (Mahmood and Yang, 2012). Nevertheless, we feel that the current sample preparation method is preferable to the conventional sample preparation for immunoblotting as samples that are fractionated (centrifuged and then the pellet discarded) can give rise to incorrect results due to a proportion of the protein of interest being inadvertently discarded during sample preparation (Murphy and Lamb, 2013).

2.3.1 Muscle homogenisation

Frozen muscles were cryosectioned at 20 μm thickness or pulverised under liquid nitrogen. Muscle samples were then homogenised (no homogeniser used) with

solubilising buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.001% bromophenol blue) for 1 hour at room temperature with occasional vortex-mixing as modified from described (Murphy, 2011). The homogenates were kept at -80 ^{o}C before performing a protein concentration assay and immunoblotting. Aliquots of whole (non-fractionated) muscle homogenate were used for protein loading and separation without prior centrifugation. A concern regarding this sample preparation method was the possibility of degradation of phosphorylation of proteins due to a lack of phosphatases and proteases inhibitors in the solubilising buffer and the fact that the homogenates were incubated at room temperature during homogenisation. However, using a similar sample preparation method, Thomassen et al. (2013) detected a significant increase in FXYD1Ser^{68} phosphorylation following exercise. We also detected a large, significant increase in AMPKα Thr^{172} phosphorylation in skeletal muscles following *ex vivo* contraction (Fig 3.4B) indicating that the phosphorylation status of these proteins appears to have remained intact. It is likely that the high concentration of denaturing agent (4% of SDS) and reducing agent (0.1 M DTT) in the solubilising buffer completely denatured the 3D conformation and reduced all disulfide bonds of all proteins including phosphatases and proteases, therefore inactivating them and prevented the degradation of phosphorylation bonds. Also, the high concentration of EGTA, a strong Ca^{2+} chelator, will prevent the necessary Ca^{2+} activation of proteases and phosphatases.

2.3.2 Protein concentration assay

Protein concentration of the homogenates was determined using the RED 660 Protein Assay Kit (G-Biosciences, St Louis, MO, USA) as per manufacturer's instruction. This is a calorimetric assay based on a single proprietary dye-metal complex reagent. The interaction between the positively charged amino acid groups of protein and the dye-metal complex under acidic conditions causes deprotonation of the dye-metal complex and leads to a change in color density that is proportional to the protein concentration. The single most important advantage of this assay kit compared to other commercial protein assay kits such as Bradford or Coomassie protein assays is that it is

compatible, when used together with its Neutralizer™, with the high concentration of detergent (4% of SDS) and reducing agent (0.1 M of DTT) that were used in the solubilising buffer for muscle homogenisation. The Neutralizer™ is a unique chemical that sequesters ionic detergents and makes the muscle homogenate compatible with the RED 660 Protein assay.

Briefly, 10 µl of muscle homogenate or bovine serum albumin (BSA) standards were added into a 96-well plate in triplicate and 200 µl of the RED 660 protein assay reagent containing Neutralizer™ (50 mg/ml) was added into each well and mixed with repeated pipetting several times. The mixture was then incubated at room temperature for 5 min and absorbance was measured at 660 nm using xMark Microplate Spectrophotometer (Bio-Rad). The mean absorbance of the blank standards was subtracted from the mean absorbance of the triplicate BSA standards and muscle homogenates. Protein concentration of each muscle homogenate was then calculated from the absorbance versus BSA concentration standard curve.

2.3.3 Immunoblotting procedures

Homogenates containing 5 µg of protein were heated at 95 ^{o}C for 5 min (homogenates that used for GLUT4 detection were not heated) and then separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Hand-cast gels consisted of a stacking gel [0.5 M Tris-HCl pH 6.8, 10% SDS, 30% bisacrylamide (Bio-Rad, Hercules, CA, USA), 10% APS, TEMED] and an 8% resolving gel (1.5 M Tris-HCl pH 8.0, 10% SDS, 30% bisacrylamide, 10% APS, TEMED) were used. Total proteins were separated through the gels in an electrophoresis buffer (25 mM Tris-HCl, 190 mM glycine, 0.1% SDS) at 100 V until the dye front was eluted from the gels (~100 min). Molecular weight markers containing 10 to 250 kDa protein markers (Precision Plus Protein Western C, Bio-Rad) were loaded onto each gel for estimation/ validation of the size of the proteins of interest. An internal standard was loaded into each gel to account for the variability between gels/ membranes. The separated proteins on the gel were then wet transferred onto a polyvinylidene difluoride (PVDF) membrane

(Millipore, Billerica, MA, USA) in a Bio-Rad Trans-blot electrophoresis transfer tank filled with ice-cold transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol) at 100 V for 100 min. Membranes were blocked with 5% skim milk dissolved in TBS containing 0.1% Tween-20 (TBST) for one hour at room temperature. The membranes were then incubated with respective primary antibodies overnight at 4 ^{o}C on a rocker after four washes of 5 min each with TBST. The following morning, the primary antibody was removed and membranes were washed (4 x 5 min in TBST) and incubated with the appropriate HRP-conjugated secondary antibody diluted in 5% skim milk in TBST for 1 hour at room temperature. Another series of washes (4 x 5 min in TBST) followed and then chemiluminescent signal was developed using SuperSignal West Femto substrate (Thermo Scientific, Rockford, IL, USA). Blot images were taken with Molecular Imager VersaDoc MP 4000 fitted with a charge-couple device (CCD) camera using Quantity One software (Bio-Rad, Hercules, CA, USA). Pre-stained molecular weight markers on the membrane were imaged under white light source with the membrane in the same position to superimpose the images onto the molecular weight marker. When quantifying both phosphorylated and total protein abundance, protein phosphorylation was first determined then the membrane was stripped (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.8% β-mercaptoethanol) for 30 min, re-blocked and re-probed with primary antibody for the total protein. Stripping efficiency was determined prior to primary antibody incubation by incubating stripped and re-blocked membrane with appropriate secondary antibodies and membrane imaging was performed as described above. The primary antibodies used in this thesis included: phospho-AMPKα Thr^{172} (1: 1000), phospho-TBC1D1 Ser^{660} (1:1000), AMPKα (1:1000), TBC1D1 (1:500), α-tubulin (1:1000) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1:10,000), eNOS (1:10,000), iNOS (1:2000) (BD Biosciences, San Jose, California, USA); GLUT4 (1:8000) (Thermo Scientific, Rockford, IL, USA); and, actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Loading control proteins (actin and α-tubulin) were always probed using non-stripped membranes. Actin was used as a loading control for all proteins except GLUT4 where α-tubulin was used as the loading control because actin and GLUT4 have similar molecular weights and it was not possible to probe both of these

proteins without undertaking the stripping process. For data analysis, the signal of band of interest of each sample was normalised against its loading control to account for loading variability between samples. Then, the data of each sample on the same membrane was normalised against the internal standard to account for the variability between membranes. Since this was the first time that the Murphy et al. (2011) immunoblotting technique was performed in our laboratory, various optimisation and validation on the immunoblotting conditions, including determination of the optimum antibody concentrations and protein loading, and the specificity of the antibodies used were performed prior to sample analysis. These data are presented in Appendices A and B.

2.4 GENOTYPING

This procedure was used in Chapter Three. The genotyping was performed based on the protocol from The Jackson Laboratory. Mouse tail samples were digested in 500 µl of digestion buffer containing 100 mM NaCl, 50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.5% SDS and 0.2 mg/ml of Proteinase K (Promega, Madison, WI, USA) at 55 ^{o}C overnight with gentle shaking. Proteinase K was inactivated at 95 ^{o}C for 15 min and the mixture was centrifuged at 15,000 *g* for 5 min at room temperature. Supernatant (300 µl) was transferred to a new autoclaved tube and added with equal volume of phenol: chloroform: isoamyl alcohol solution (Sigma Aldrich). The mixture was rotated end over end for 60 min and then centrifuged at 15,000 *g* for 5 min at room temperature. The upper layer aqueous solution (250 µl) was transferred into a new autoclaved tube and added with an equal volume of 100% isopropanol. The mixture was centrifuged (15,000 *g*, 4 ^{o}C) for 20 min and the supernatant was discarded with the pellet containing DNA remained in the tube. The pellet was added with 70% cold ethanol, centrifuged again (15,000 *g*, 4 ^{o}C) for 5 min and the supernatant was discarded. The pellet containing DNA was air dried for about 10 min. It was then re-suspended in 100 µl of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and incubated at 37 ^{o}C for 1 hour. PCR was performed to amplify DNA for gel separation and band detection. DNA extract (2 µl)

was added with PCR mixture solution containing PCR buffer, $MgCl_2$, dNTP mixture, DNA polymerase (all from Promega, Madison, WI, USA) and DNA primers for nNOS$\mu^{+/+}$ or nNOS$\mu^{-/-}$ alleles (Invitrogen, Grand Island, NY, USA). The DNA primers used for nNOS$\mu^{+/+}$ alleles were 5'GCA GAT CCA ACC CAA CGT CAT 3' and 5'GCG CTG TCA TAG CTG AGG TCT 3'. The nNOS$\mu^{-/-}$ DNA primers were: 5'CTT GGG TGG AGA GGC TAT TC 3' and 5'AGG TGA GAT GAC AGG AGA TC 3'. PCR was ran in a thermal cycler (Bio-Rad, Hercules, CA, USA) at 94 ^{o}C for 2 min and then 35 cycles of repeated temperature changes from 94 ^{o}C to 55 ^{o}C and then to 72 ^{o}C for a 30 s duration for each temperature, respectively. At the end of the 35 cycles, temperature was maintained at 72 ^{o}C for 2 min and then it was cooled to 4 ^{o}C until the DNA was retrieved for gel separation. The DNA was separated electrophoretically at 110 V for 20 min using 2.0% Agarose gel. DNA band was stained with ethidium bromide and detected using VersaDoc MP4000 imaging system (Bio-Rad). Mice with tail sample containing both nNOS$\mu^{+/+}$ and nNOS$\mu^{-/-}$ DNA were identified as nNOS$\mu^{+/-}$ mice; while, nNOS$\mu^{+/+}$ and nNOS$\mu^{-/-}$ mice only contain nNOS$\mu^{+/+}$ and nNOS$\mu^{-/-}$ DNA, respectively.

2.5 PLASMA INSULIN

This assay was used in Chapter Four and Five. Plasma insulin levels were determined using enzyme-linked immunosorbent assay (Mercodia, AB, Uppsala, Sweden). This method utilises the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. In this method, insulin from the sample reacts with the anti-insulin antibodies that are bound to microtiter wells and the peroxidase-conjugated anti-insulin antibodies added into the incubation solution. Following the reaction between the conjugated peroxidase and 3,3',5,5'-tetramethylbenzidine (TMB) substrate, the bound conjugate gives a colorimetric endpoint that is optimally detected at 450 nm wavelength. The plasma insulin assay was performed according to manufacturer's instruction. Briefly, 10 µl of samples or insulin standards were added into microtiter wells containing bound anti-

insulin antibodies. Then, enzyme conjugate (100 µl) containing peroxidase-conjugated anti-insulin antibodies were added and the mixture was incubated on a plate shaker (700 – 900 rpm) for 2 hours at room temperature. It was then washed six times with washing solution to remove the unbound peroxidase-conjugated anti-insulin antibodies. The microtiter well plate was tap dried before TMB substrate (200 µl) was added into each well and incubated at room temperature for 15 min. The reaction was stopped by adding stop solution (50 µl) and the optical density was read at 450 nm. Plasma insulin was calculated based on an absorbance versus insulin concentration standard curve.

2.6 PLASMA LACTATE

This assay was used in Chapter Five. The principle of this assay was based on the enzymatic method of Lowry and Passonneau (Lowry and Passonneau, 1972). In the presence of NAD^+, lactate is oxidised by lactate dehydrogenase (LDH) to form pyruvate and NADH. Pyruvate will then react with hydrazine to form pyruvate hydrazone, an end product that assists the full oxidation of lactate to pyruvate. The lactate concentration is determined from the formation of NADH that is detected with a fluorometer at ~365 nm absorption and ~455 nm emission. NADH and lithium L-lactate were used as the standard and internal control respectively in this assay. For the determination of plasma lactate, plasma samples were diluted 1:3 (v/v) with 3 M perchloric acid, vortex-mixed and centrifuged at 13,000 *g* for 2 min at 4 ^oC. The supernatant was retrieved and kept on ice. The supernatant was further diluted with 1:4 and 1:7 with MilliQ water for plasma samples from sedentary and exercised mice, respectively, and kept on ice for later analysis. The higher dilution factor was used for the exercised plasma samples, which are expected to have higher concentration of lactate, to ensure that their readings would fall within the calibration range. These dilution factors were corrected during the calculation for determination of plasma lactate concentration. The standards and plasma samples were run in triplicate on a microplate. The reaction cocktail (0.1 M hydrazine, 0.1 M glycine, 0.5 mM NAD^+ and 5 µl/ ml LDH) required for the assay was freshly prepared and left for 30 min before use to allow the complete oxidation of lactate that

might possibly be present in the cocktail. The concentration of NADH standards used was determined spectrophotometrically at 340 nm. The fluorescence reading of the cocktail (R1) was first determined by adding 200 µl of cocktail into each well. Then, 10 µl of the supernatant was added, mixed and incubated in the dark for one hour to ensure complete oxidation of lactate before a second fluorescence reading (R2) was taken. Plasma lactate was calculated based on the constructed NADH standard curve after subtracting the second fluorescence reading (R2) from the background fluorescence reading (R1).

2.7 MICROBUBBLES PREPARATION FOR CONTRAST-ENHANCED ULTRASOUND

This solution was prepared for the experiment in Chapter Four for determination of capillary blood flow using contrast-enhanced ultrasound (CEU). Microbubbles stock solution (100 ml) was prepared from the following ingredients as shown in Table 2.1. Propylene glycol was first heated at 75 ^{o}C in a water bath and then added with the lipid blend and the mixture was kept in the water bath until the lipids were dissolved. Sodium chloride was dissolved in distilled water and glycerol was added and they were then heated at 75 ^{o}C. The sodium chloride/glycerol and propyelene glycol/lipid blend solutions were mixed together and remained at 75 ^{o}C until fully dissolved. The solution was adjusted to pH 6.5 and topped up to 100 ml with distilled water. It was then filtered through a 0.45 µm filter membrane. The stock solution was aliquotted (1.5 ml) into small glass vials. Air in the vial was gas-exchanged with octafluoropropane (OFP) gas before sealed off using an aluminum seal on a rubber cap and then stored at 4 ^{o}C. Microbubbles were generated via mechanical agitation just before use. Microbubbles were diluted (1:10) with heparinised saline that was gassed with OFP gas for 10 min.

Table 2.1: Chemical compositions of microbubbles stock solution (100 ml)

Chemicals	**Weight (g)**
Propylene glycol (1,2-propanediol)	10.35
Glycerol	12.62
Sodium chloride	0.66
Lipid blend solution	
• 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid, monosodium salt (DPPA)	0.0042
• 1,2-dipalmitoyl-sn-glycero-3-phosphatidychloline (DPPC)	0.0390
• N-(methoxypholyethylene glycol 500 carbonyl)-1,2-dipalmitoyl-sn-glycero-3-phosphatidylanolamine, monosodium salt (MPEG5000-DPPE)	0.0307

CHAPTER THREE:

NOS INHIBITION ATTENUATES THE INCREASE IN MOUSE SKELETAL MUSCLE GLUCOSE UPTAKE DURING *EX VIVO* CONTRACTION INDEPENDENTLY OF nNOSμ

3.1 INTRODUCTION

Contraction-stimulated skeletal muscle glucose uptake is an important regulator of plasma glucose levels in people with type 2 diabetes (T2D) (Musi et al., 2001a) and therefore could be a great therapeutic avenue for managing glycaemic control of these people. Skeletal muscle contraction stimulates muscle glucose uptake via an increase in GLUT4 translocation (Fueger et al., 2007, Zisman et al., 2000) and its signalling mechanism is at least partially independent from insulin signalling (Cartee and Funai, 2009). Understanding the signalling mechanism(s) involved in contraction-stimulated muscle glucose uptake may provide important information for exploring new therapeutics for managing glycaemic control. Some of the known signalling intermediates involved in contraction-stimulated glucose uptake appear to include AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent protein kinase II (Ca2+/CaMKII), protein kinase C (PKC), reactive oxygen species (ROS) and nitric oxide (NO) (McConell et al., 2012, Richter and Hargreaves, 2013). Over the last few years even more novel proteins have been shown to at least partially regulate skeletal muscle glucose uptake during contraction such as sucrose non-fermenting AMPK-related kinase (SNARK) (Koh et al., 2010), Myo1c (Toyoda et al., 2011) and PIKfyve (Liu et al., 2013), Rac1 and PAK1 (Sylow et al., 2013). There are clearly multiple sites and levels of regulation, and embedded within this multiple levels of redundancy.

Skeletal muscle contraction activates NO synthase (NOS) leading to an increase in NO production (Balon and Nadler, 1994, Lau et al., 2000). Balon and colleagues demonstrated that NO was involved in mediating contraction-stimulated muscle glucose uptake (Balon and Nadler, 1997, Roberts et al., 1997). Our group has accumulated evidence from a wide range of experimental models, both in rodents and humans, that

NOS inhibition attenuates the increase in skeletal muscle glucose uptake during contraction/ exercise. These include mouse muscle stimulated to contract *ex vivo* (Merry et al., 2010d, Merry et al., 2010c), rat muscles contracting *in situ* (Ross et al., 2007) and humans *in vivo* during cycling exercise in both healthy (Bradley et al., 1999, Kingwell et al., 2002) and people with T2D (Kingwell et al., 2002). The attenuation of the increase in glucose uptake with NOS inhibition in these models tends to indicate that NO plays a critical role in the regulation of skeletal muscle glucose uptake during contraction/ exercise. Interestingly, the role of NO in contraction-stimulated glucose uptake appears to be greater in people with T2D. Local NOS inhibition caused a significantly greater attenuation of the increase in leg glucose uptake in people with T2D performing cycling exercise compared with their healthy counterparts (Kingwell et al., 2002). These findings position NO as a candidate with great clinical and therapeutic potential in diabetes management.

Nevertheless, there are some discrepancies in rodent studies in regards to the involvement of NO in contraction-stimulated muscle glucose uptake. Some studies have found that NOS inhibition has no effect on glucose uptake in rat skeletal muscle contracted *ex vivo* (Etgen et al., 1997, Higaki et al., 2001). One of the reasons for these contradictory findings could be the differences in experimental protocols. These studies measured glucose uptake many minutes after contraction had ceased (Etgen et al., 1997, Higaki et al., 2001) while our studies measured glucose uptake during muscle contraction (Merry et al., 2010d, Ross et al., 2007). Secondly, in the study by Etgen et al. (1997), NO signalling did not appear to be activated as muscle cGMP content, a major NO downstream signalling molecule, was not elevated during muscle contraction. In another study that measured rat hindlimb muscle glucose uptake during *in situ* contraction found no significant attenuation in glucose uptake by L-NAME although the glucose uptake at higher stimulation frequencies appeared to be much lower than that of saline control (Inyard et al., 2007). It was also reported that short-term ingestion of NOS inhibitor had no effect on skeletal muscle glucose uptake in rats (Higaki et al., 2001) and mice (Rottman et al., 2002) performing treadmill exercise. This was in contrary to a

previous similar study in rats that found an almost complete attenuation in the increase in skeletal muscle glucose uptake with NOS inhibitor ingestion for two days before treadmill running (Roberts et al., 1997). The discrepancy in findings between these studies was not readily apparent and difficult to resolve. Nevertheless, the administration of NOS inhibitors into systemic circulation increases blood pressure (Higaki et al., 2001, Inyard et al., 2007) and femoral blood flow (Inyard et al., 2007). The increased systemic blood pressure and blood flow could increase glucose delivery to the muscle and therefore affect muscle glucose uptake during contraction/ exercise (Richter and Hargreaves, 2013, Zinker et al., 1993) complicating the interpretation of NO effects on skeletal muscle glucose uptake during contraction/ exercise.

One of the concerns of using non-specific NOS inhibitors is their inhibition on all isoforms of NOS (Vitecek et al., 2012). These inhibitors compete with L-arginine to bind to NOS and inhibit NOS activity (Vitecek et al., 2012). Neuronal NOS (nNOS) is constitutively expressed in rodent and human skeletal (Kobzik et al., 1994, McConell et al., 2007). Endothelial NOS (eNOS) is also expressed in rodent muscle (Kobzik et al., 1995) but it is mainly found in the vasculature within human muscle bundles and not in the muscle fibers (Frandsen et al., 1996). Under pathological conditions, inducible NOS (iNOS) can also be induced to express in these muscles (Adams et al., 2002, Hambrecht et al., 1999, Torres et al., 2004) but in normal conditions is undetectable. The NO involved in the regulation of skeletal muscle glucose uptake during contraction in healthy models could potentially be derived either from nNOS or eNOS or both. N-G-Nitro-L-Arginine Methyl Ester (L-NAME) and N-G-Monomethyl-L-arginine (L-NMMA), the non-specific competitive NOS inhibitors most commonly used in the investigation of contraction-stimulated skeletal muscle glucose uptake, inhibit all NOS isoforms (Vitecek et al., 2012). Therefore, the putative NOS isoform(s) involved in contraction-stimulated muscle glucose uptake cannot be ascertained with these inhibitors and remains unknown. Neuronal NOSμ is believed to be the main isoform activated during skeletal muscle contraction (Lau et al., 2000), but the potential involvement of other NOS isoforms during contraction is not known. A study using $eNOS^{-/-}$ mice

demonstrated that whole-body absence of eNOS is associated with higher skeletal muscle glucose uptake during treadmill exercise (Lee-Young et al., 2010). These mice are exercise intolerant and have significantly lower exercise-induced hyperaemia (Lee-Young et al., 2010). The elevated glucose uptake was postulated to be attributed to exercise-induced hypoxia due to the absence of eNOS (Lee-Young et al., 2010) as hypoxia is a potent stimulator of skeletal muscle glucose uptake (Cartee et al., 1991). There is, however, no information on the role of nNOSμ in the regulation of skeletal muscle glucose uptake during contraction/ exercise. Given that our previous *ex vivo* studies indicate that NO mediates glucose uptake via intramuscular signalling mechanism (Merry et al., 2010d), and data from $eNOS^{-/-}$ mice suggests that eNOS may not be important for contraction-stimulated glucose uptake (Lee-Young et al., 2010), we postulated that nNOSμ is important in regulating skeletal muscle glucose uptake during contraction. Therefore, the purpose of this study was to investigate the role of nNOSμ in the regulation of skeletal muscle glucose uptake during *ex vivo* contraction. We tested the hypothesis that mice lacking nNOSμ would show an attenuated increase in skeletal muscle glucose uptake during *ex vivo* contraction.

3.2 METHODS

3.2.1 Animals

All procedures were approved by The University of Melbourne Animal Ethics Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th Edition). Male and female nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ littermates were generated by mating C57Bl/6 nNOSμ$^{+/-}$ breeding pairs originally obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping was performed using standard PCR techniques (Chapter Two: Section 2.4) on genomic DNA extracted from tail samples taken at day 21. Mice were housed in standard cages and maintained under constant temperature of 21 ± 1 ^{o}C with 12-hour light/ dark cycle in the Biological Research Facility at The University of Melbourne. They had access to standard rodent chow and water ad libitum. All mice were at 14 to 17 weeks of age when experiments were conducted.

3.2.2 Muscle dissection and experimental procedure

The surgical and experimental procedures were as previously described (Merry et al., 2010d). Extensor digitorum longus (EDL) muscles were used as it was previously shown that nNOSμ expression is greater in fast-twitch muscles (Kobzik et al., 1994), and NO inhibition had a greater effect on glucose uptake in EDL than in soleus muscles when contracted *ex vivo* (Merry et al., 2010c). EDL muscles were excised from deeply anaesthetised mice (Avertin: 0.25 mg/g of body weight, intraperitoneally, Sigma Aldrich Chemicals, St Louis, MO). Both tendons were tied with 5-0 silk suture with one tendon attached to a fixed hook and the other to a force transducer (PanLab, Barcelona, Spain). Muscles were suspended vertically at optimal length, determined by progressively increasing the muscle length in small increments until maximum twitch contraction force was obtained, in incubation solution [(in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, pH 7.4] with the addition of 0.01% BSA, 8 mM mannitol and 2 mM sodium pyruvate. This solution was maintained at 30 ^{o}C and continuously oxygenated with 95% O_2 and 5% CO_2 throughout the experiment. Muscles

were incubated for 40 min in the presence or absence of L-NMMA (NOS inhibitor, 100 μM, Sigma Aldrich) or L-NMMA + L-arginine (NOS substrate, 1 mM, Sigma Aldrich). L-NMMA is a modification of L-arginine that competitively inhibits NOS (Vitecek et al., 2012). Muscles either remained rested or were stimulated to contract during the final 10 min of incubation. Contractions were elicited by square wave electrical pulses (350 ms train; 0.2 ms; 6 V; 12 contractions/ min) generated by a Grass S48 stimulator (Grass Instruments, Warwick, RI) and delivered via two platinum plate electrodes that flanked, without touching, the muscle. Contraction force was acquired with PowerLab Chart 4.0 software (AD Instruments, Castle Hill, NSW, Australia). At the end of the basal or contraction incubation, muscles were rapidly cut from the suture attachment, washed in ice-cold buffer, blotted on filter paper, snap frozen in liquid nitrogen and kept at -80 ^{o}C for biochemical analyses later. The experiments were repeated thrice to obtain an EDL for glucose uptake measurement, another for immunoblotting and NOS activity and a third muscle for cGMP measurement. However, cGMP analysis was unable to be performed as we loss a whole set of muscles during a freezer breakdown.

3.2.3 Muscle glucose uptake measurement

Muscle glucose uptake was measured during the final 5 min of contraction or basal incubation. The procedures for muscle glucose uptake determination are described in Chapter Two (Section 2.1.1). Muscle glucose uptake was calculated as previously described (Merry et al., 2010d).

3.2.4 Immunoblotting

Immunoblotting was performed using frozen muscle sections. Frozen EDL muscles were cryosectioned (10 sections, 20 μm thickness) and homogenised with 100 μl of solubilising buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.001% bromophenol blue) as described in Chapter Two (Section 2.3). The primary antibodies used in this chapter were phospho-AMPKα Thr^{172} (1:1000), AMPKα (1:1000), α-tubulin (1:1000) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1:10,000), eNOS (1:10,000), iNOS (1:2000) (BD Biosciences, San

Jose, California, USA); GLUT4 (1:8000) (Thermo Scientific, Rockford, IL, USA), and actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Two different loading control proteins (actin and α-tubulin) were used in this chapter and they were always probed using non-stripped membranes. Actin was used for all except GLUT4 where α-tubulin was used as the loading control as actin and GLUT4 have similar molecular weights and it was not possible to probe both of these proteins without undertaking the stripping process.

3.2.5 NOS activity assay

Skeletal muscle NOS activity was determined as described in Chapter Two based on the conversion of radioactive L-arginine to radioactive L-citrulline as previously described (Lee-Young et al., 2009). For each sample, one reaction was done in the presence of 1 mM L-NAME to block NOS activity, and duplicates were performed without L-NAME. Subtracting the radioactive counts from these reactions allowed the calculation of radioactive counts that were attributed to NOS reaction and eliminated the background counts. NOS activity was determined from this calculated count and was expressed as picomoles of L-[^{14}C]citrulline formed per min, per mg of protein.

3.2.6 Statistical analysis

All data are expressed as means ± SEM. Statistical testing was performed using SPSS statistical package using t-test (nNOSμ$^{+/+}$ vs nNOSμ$^{-/-}$: body and muscle weights, expressions of total AMPKα, eNOS and GLUT4), two-factor ANOVA (between factor: genotype and treatment condition - glucose uptake, contraction force, NOS activity) or two-factor repeated measures ANOVA (between factor: genotype and treatment condition; within factor: time - decrease in contraction force over time), if there was a significant interaction, specific differences between mean values were identified using Fisher's least significance test. The significance level was set at $p < 0.05$. No sex-specific difference in glucose uptake was observed; therefore, the data from both female and male mice were pooled and analysed together.

3.3 RESULTS

3.3.1 Morphological characteristics of nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice

Body weight was significantly lower in the age matched nNOSμ$^{-/-}$ mice compared with their nNOSμ$^{+/+}$ littermates. Accordingly, their EDL muscle mass was also significantly smaller than nNOSμ$^{+/+}$ muscle (Table 3.1). Body composition was not measured in these mice and to our best knowledge, there was no such data available from the literature.

Table 3.1: Morphological characteristics of nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice

	nNOSμ$^{+/+}$	nNOSμ$^{-/-}$
Age (weeks)	15.2 ± 0.2	15.3 ± 0.2
Body weight (g)	28.6 ± 0.7	26.3 ± 0.6 #
EDL muscle mass (mg)	10.9 ± 0.2	9.9 ± 0.3 #

Data are means ± SEM, n = 22 – 36 per group; EDL = extensor digitorum longus. # $P < 0.05$ vs nNOSμ$^{+/+}$.

3.3.2 Contraction force

The peak contraction force normalised to muscle weight was not different between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles (Fig 3.1A). Inhibition of NO production by L-NMMA did not affect peak contraction force in either genotype (Fig 3.1A). Reversal of inhibition of L-NMMA by L-arginine (a NOS substrate which competes with and overcomes the inhibition of L-NMMA) had no effect on peak force production in nNOSμ$^{+/+}$ muscle (Fig 3.1A). Throughout the 10 min of contraction, force production decreased to a similar extent over time in both genotypes in the presence or absence of L-NMMA or L-arginine (Fig 3.1B).

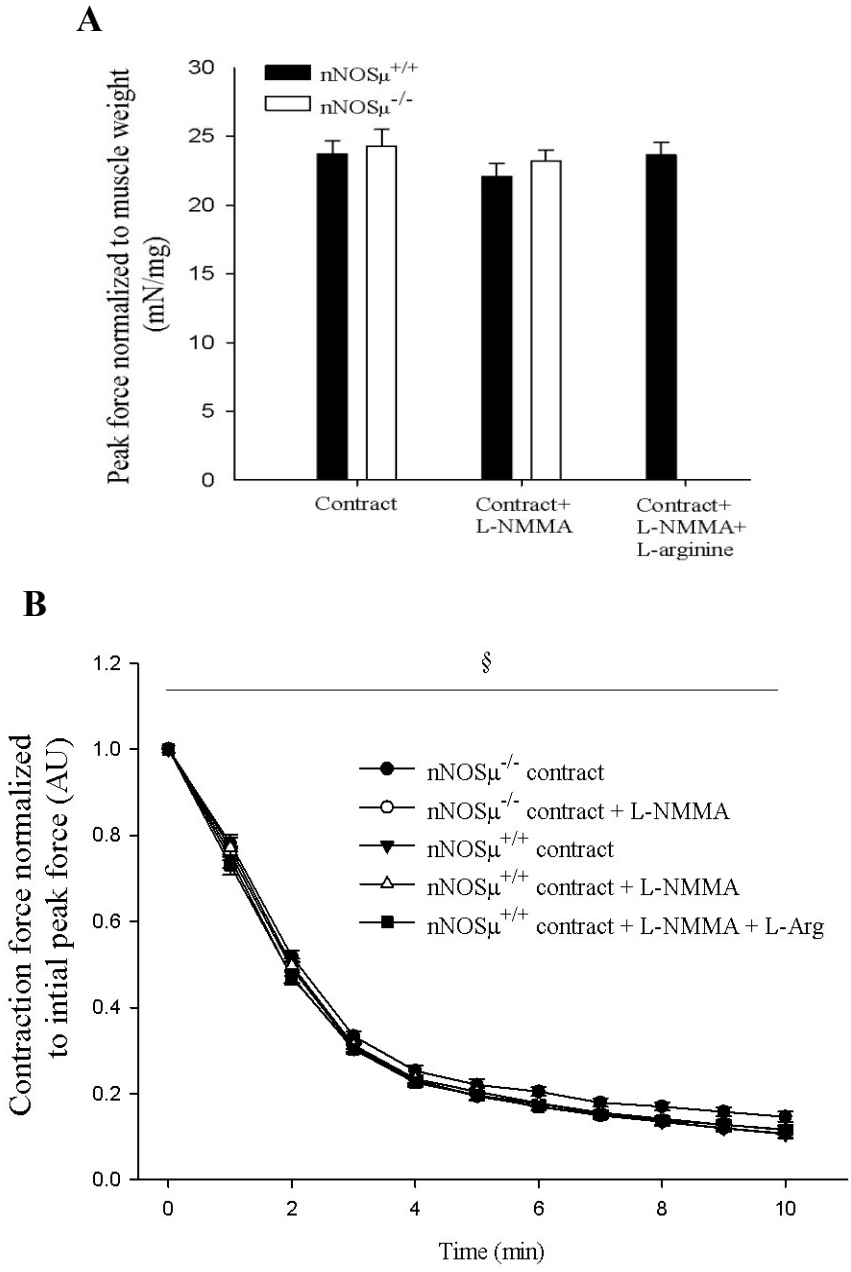


Figure 3.1: Peak contraction force normalised to EDL muscle weight (A), decrease in force production from initial peak force during contraction (B), in the presence or absence of N^G-monomethyl-L-arginine (L-NMMA) and/or L-arginine (L-Arg) in $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ EDL muscles.

Data are means ± SEM, n = 8 – 19 per group; § $P < 0.05$ main effect for time. Contract = contraction.

3.3.3 Skeletal muscle glucose uptake

Basal muscle glucose uptake was not different between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ (Fig 3.2). The presence of L-NMMA had no impact on basal glucose uptake (Fig 3.2). Contraction significantly increased glucose uptake in nNOSμ$^{+/+}$ by approximately four-fold (Fig 3.2). There was a similar increase in glucose uptake during contraction in nNOSμ$^{-/-}$ which was not statistically different from the glucose uptake of nNOSμ$^{+/+}$ (Fig 3.2). L-NMMA significantly attenuated the increase in glucose uptake during contraction in both genotypes with no difference between genotypes (Fig 3.2). This represented about 21% and 24% attenuation in the increase in glucose uptake during contraction in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscle, respectively. L-arginine fully reversed the attenuation of glucose uptake during contraction by L-NMMA in nNOSμ$^{+/+}$ muscle (Fig 3.2) suggesting that the attenuation of the increase in glucose uptake during contraction by L-NMMA was due to its inhibition on NOS, and not due to non-specific effect of the inhibitor. It is important to note that glucose uptake was not measured in nNOSμ$^{-/-}$ mice during contraction with L-arginine and L-NMMA due to several unavoidable issues. Seeing that both nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$ mouse muscles responded similarly to contraction and contraction with L-NMMA, we expected that nNOSμ$^{-/-}$ mice would response similarly as with nNOSμ$^{+/+}$ during contraction with L-arginine and L-NMMA. Therefore, it is believed that the data from nNOSμ$^{-/-}$ mice under this condition (if available) would not change the overall result and conclusion of this study.

3.3.4 Loading control protein expression

The expression of actin (Fig 3.3A) and α-tubulin (Fig 3.3B) proteins were found to be not different between genotypes indicating that they were suitable to be used as a loading control.

3.3.5 AMPKα signalling

Total AMPKα expression was similar between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles, indicating no compensatory induction of AMPKα in nNOSμ$^{-/-}$ muscle (Fig 3.4A). In the

resting state, both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles had similar AMPKα Thr^{172} phosphorylation relative to AMPKα abundance (Fig 3.4B). As expected, contraction significantly increased AMPKα Thr^{172} phosphorylation with an approximately 10-fold increase observed in both genotypes and no difference between genotypes was observed (Fig 3.4B). As previously shown in rats (Ross et al., 2007) and C57Bl/6 mice (Merry et al., 2010d), NOS inhibition during contraction had no impact on AMPKα Thr^{172} phosphorylation in both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles (Fig 3.4B).

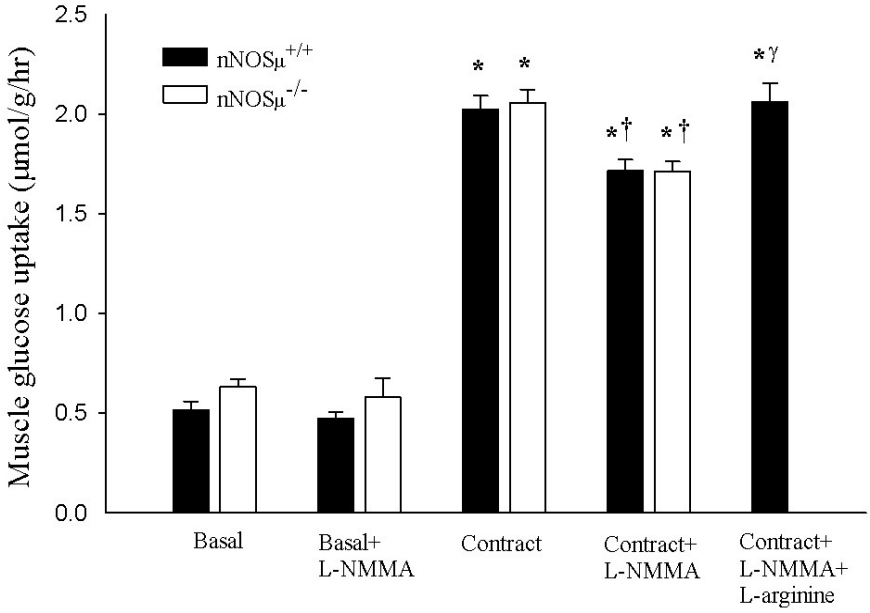


Figure 3.2: Muscle glucose uptake of nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ at rest or during contraction with the presence or absence of L-NMMA and L-arginine.

Data are means ± SEM, n = 7 – 19 per group. * $P < 0.05$ vs basal of the same genotype; † $P < 0.05$ vs contract of the same genotype; γ $P < 0.05$ vs contract + L-NMMA of the same genotype.

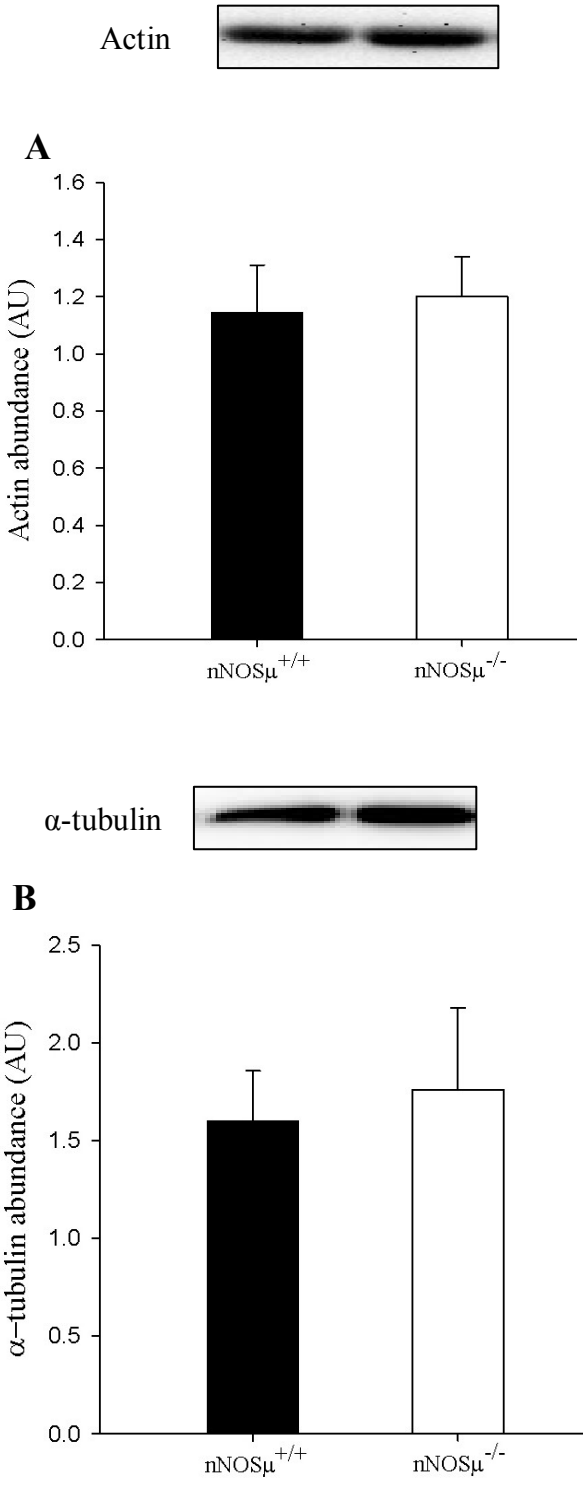


Figure 3.3: Actin (A) and α-tubulin (B) protein expressions in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles.

Data are means ± SE; n = 6 & 9 for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively.

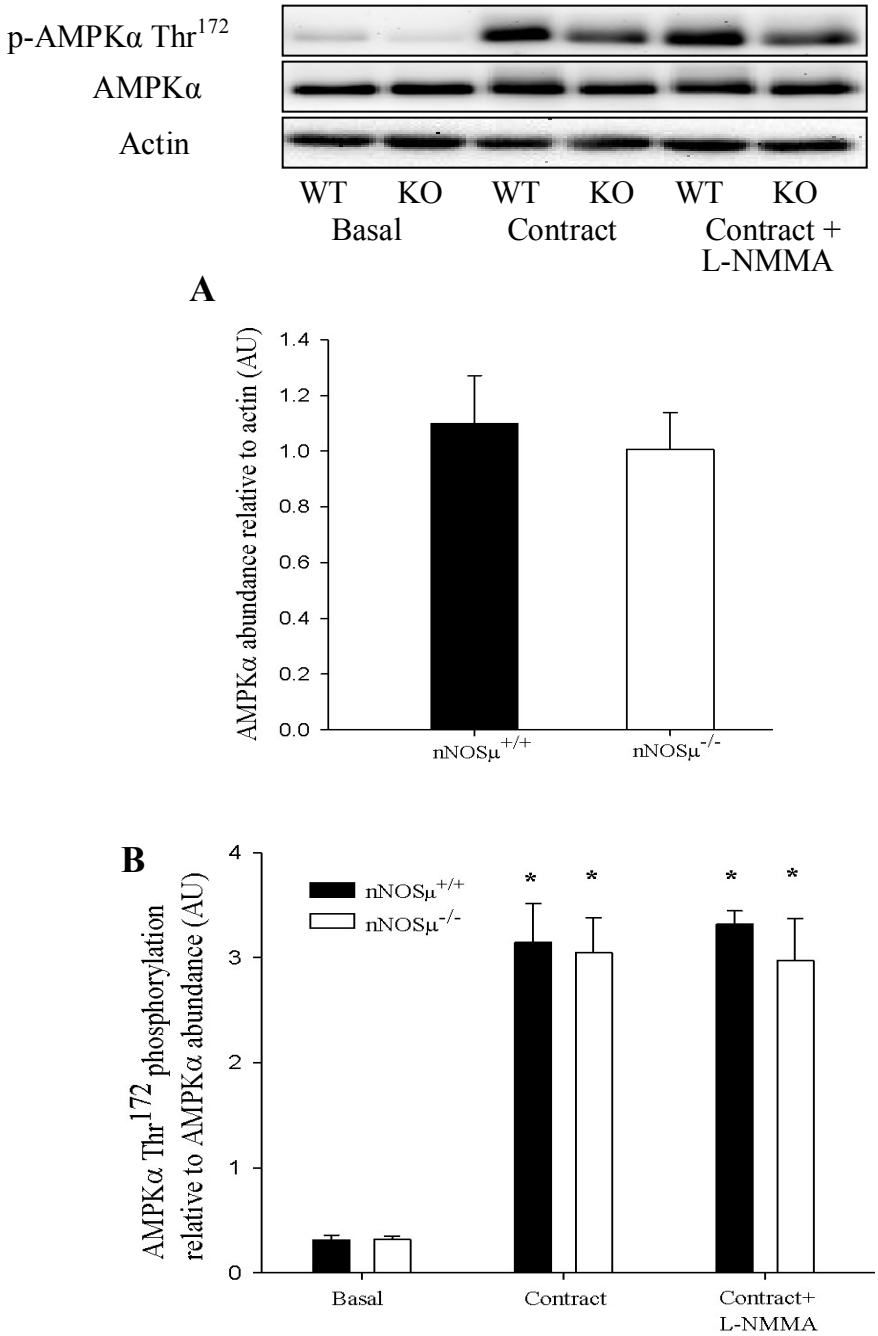


Figure 3.4: AMPKα abundance in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles at basal state (A), AMPKα Thr^{172} phosphorylation relative to AMPKα abundance in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles at basal state and during contraction with the presence or absence of L-NMMA (B).

Data are means ± SEM, n = 5 – 9 per group. * $P < 0.05$ vs basal of the same genotype. WT = nNOSμ$^{+/+}$ & KO = nNOSμ$^{-/-}$.

3.3.6 Inducible NOS, eNOS and GLUT4 expressions

In line with our previous report (Wadley et al., 2007), we were unable to detect any iNOS in the muscles of both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles (data not shown). Endothelial NOS expression was not different between nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles (Fig 3.5), indicating that the absence of nNOSμ in skeletal muscle did not cause an up-regulation of eNOS protein expression to compensate for a reduction of NO production at resting state. Also, GLUT4 protein expression was similar in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ (Fig 3.6).

3.3.7 Expression of nNOSμ and nNOS splice variants

The nNOSμ band is expected to be detected at 160 kDa and it was detected in this position in nNOSμ$^{+/+}$ muscle and expectedly, not seen in the nNOSμ$^{-/-}$ muscle (Fig 3.7A). There was also another band at 140 kDa, presumably the nNOSβ splice variant (Baum et al., 2011), detected in the nNOSμ$^{+/+}$ but not nNOSμ$^{-/-}$ muscles (Fig 3.7A). In order to better compare the expressions of nNOSμ and nNOS splice variants, homogenates from nNOSμ$^{+/-}$ mouse skeletal muscle was run together with nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ homogenates (n = 1) (Fig 3.7A). Both the nNOSμ and 140 kDa bands were detected in nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ but not in nNOSμ$^{-/-}$ muscles (Fig 3.7A). There were bands at 60 kDa and 52 kDa detected in all the genotypes (Fig 3.7A). As previously reported, the 140 kDa band, presumably nNOSβ, was also detected in muscle of C57Bl/6 (Baum et al., 2011) and Sprague Dawley rat and the brain tissue of nNOSμ$^{-/-}$ (Brenman et al., 1996), nNOSμ$^{+/-}$ and nNOSμ$^{+/+}$ mice (Fig 3.7B).

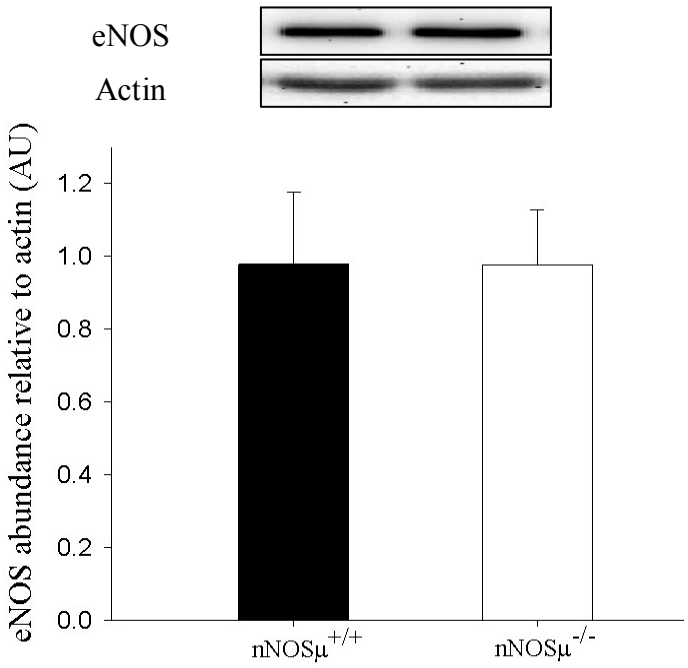


Figure 3.5: Endothelial NOS protein expression in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles relative to actin abundance.

Data are means ± SEM; n = 6 & 9 for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively.

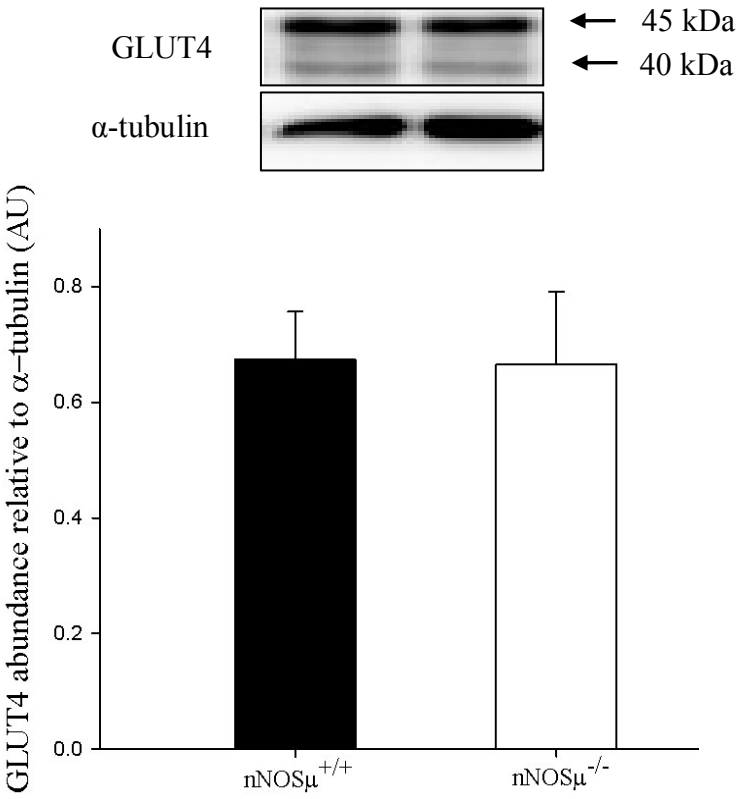


Figure 3.6: GLUT4 protein expression in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles relative to α-tubulin abundance.

Data are means ± SEM; n = 6 & 9 for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively. Bands at 45 and 40 kDa represented glycosylated and de-glycosylated GLUT4 respectively (see Appendix B for more detail). Both bands were used for data analysis.

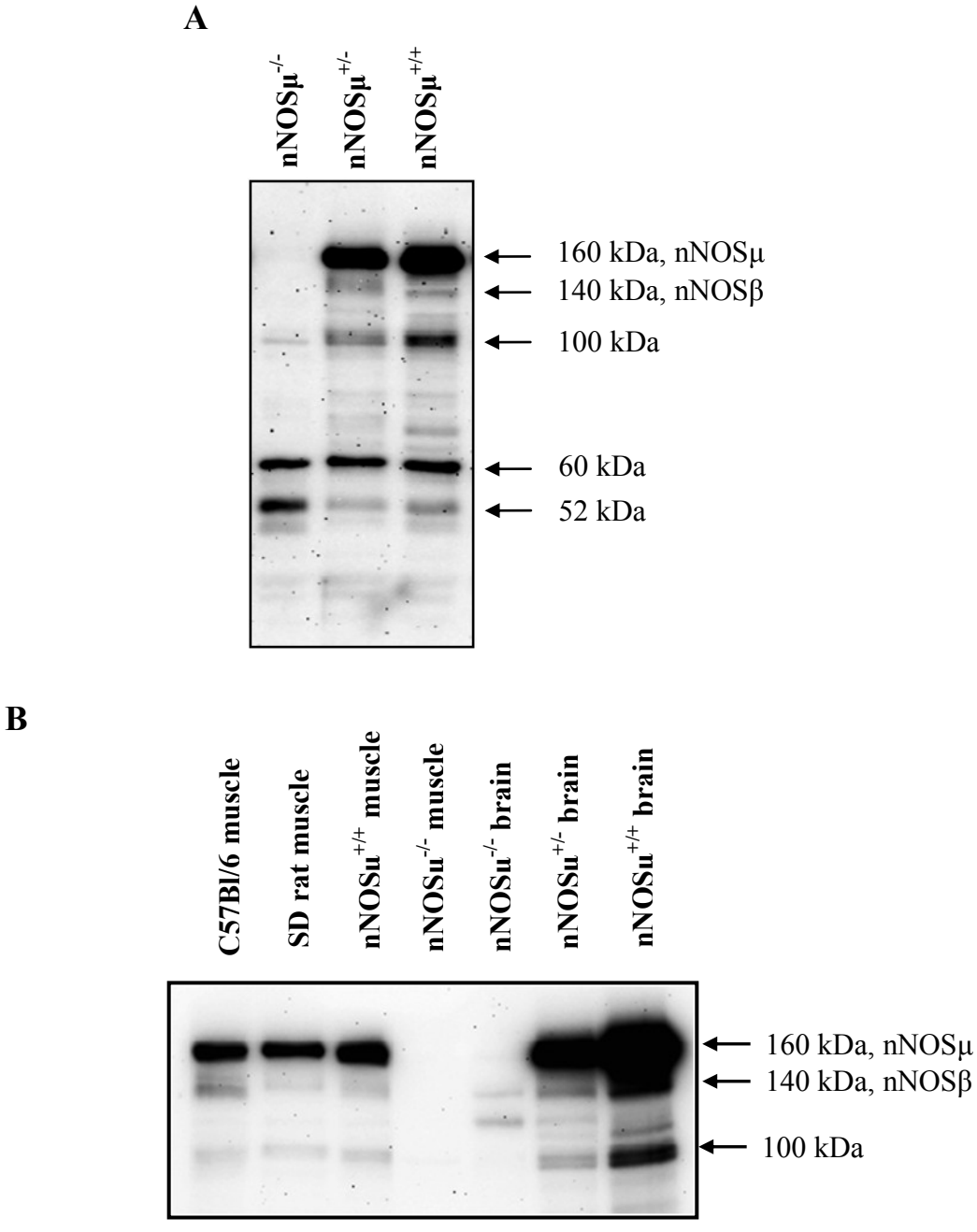


Figure 3.7: Representative blot showing nNOSμ and nNOSβ expressions and the appearance of other unknown bands in nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ muscles, each lane was loaded with 5 μg of total protein (A), nNOSμ and nNOSβ expressions in brain and muscle of nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ mice and muscle of C57Bl/6 and Sprague Dawley rat, each lane was loaded with 7 μg of total protein (B).

3.3.8 NOS activity

In nNOSμ$^{+/+}$ muscle, contraction significantly increased NOS activity and L-NMMA inhibited it to a level significantly below the basal state (Fig 3.8). This is in line with our previously reported data from C57Bl/6 mice (Merry et al., 2010d). There was some, albeit very small NOS activity detected in nNOSμ$^{-/-}$ muscles which represented approximately 4% of nNOSμ$^{+/+}$ value in the basal state (Fig 3.8). A similar degree of residual NOS activity was also reported in the brain tissue of nNOSμ$^{-/-}$ mice (Huang et al., 1993).

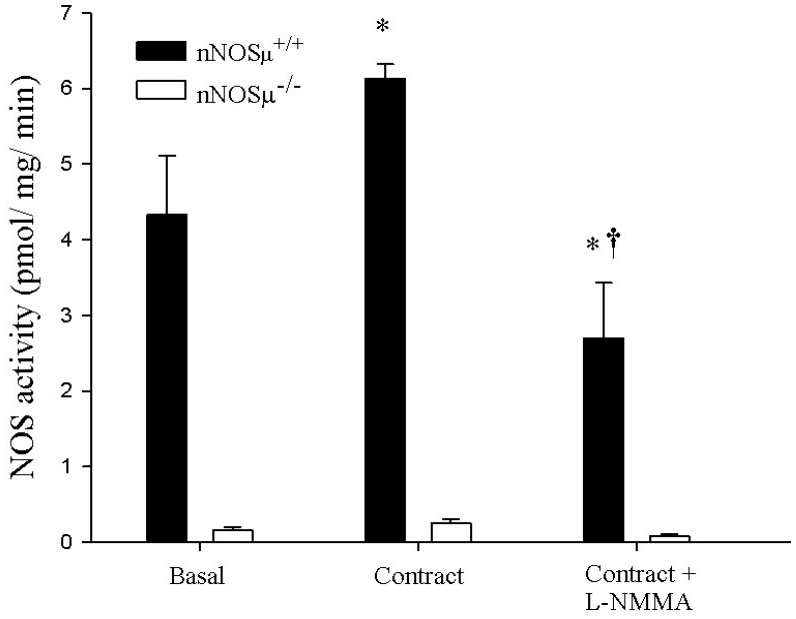


Figure 3.8: NOS activity of nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles at basal state and during contraction in the presence or absence of L-NMMA.

Data are means ± SEM; n = 5 - 8 per group. † $P < 0.05$ vs contraction of the same genotype & * $P < 0.05$ vs basal of the same genotype.

3.4 DISCUSSION

In this study, we report for the first time that nNOSμ is not essential for the regulation of skeletal muscle glucose uptake during *ex vivo* contraction in mice. Interestingly, NOS inhibition significantly attenuated the increase in skeletal muscle glucose uptake during contraction to a similar extent in both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles.

Since it has been shown that muscles contracted *ex vivo* have a reduced glucose uptake in the presence of a NOS inhibitor (Merry et al., 2010d, Merry et al., 2010c), and there is no blood flow, we hypothesised that the effect of NO is from NOS expressed within the muscle. Given the evidence that nNOSμ is the most important and prominent isoform during contraction in muscle (Lau et al., 2000, Lee-Young et al., 2010, McConell et al., 2007), we suspected that nNOSμ would be responsible for this effect. Contrary to our hypothesis, contraction stimulated a normal and comparable increase in glucose uptake in nNOSμ$^{-/-}$ compared with nNOSμ$^{+/+}$ muscles (Fig 3.2). This indicates that nNOSμ was not required for normal glucose uptake during *ex vivo* contraction of mouse skeletal muscle. Our lab has previously shown that basal muscle glucose uptake in nNOSμ$^{-/-}$ was higher compared with C57Bl/6 control mice (Unpublished data, Linden, K.C. and McConell, G.K.). However, in the current study basal muscle glucose uptake was normal and comparable with nNOSμ$^{+/+}$ littermates. It is hard to discern the reason for this discrepancy but it highlights the importance of using nNOSμ$^{+/+}$ littermate mice as a proper experimental control.

The regulation of skeletal muscle glucose uptake during contraction is a very complex integrated process that involves multiple signalling cascades and can be affected by several external factors such as stimulation protocol and contractile force production (Ihlemann et al., 2000, Ihlemann et al., 2001). In the current study, nNOSμ$^{-/-}$ mice had lower body and muscle masses compared with nNOSμ$^{+/+}$ littermates (Table 3.1). However, it was unlikely that these affected muscle glucose uptake during contraction in nNOSμ$^{-/-}$ muscles because similar to previous reports (Church et al., 2011,

Percival et al., 2010), the smaller muscle did not affect contraction force normalised to per unit of muscle weight (Fig 3.1A). The same was found for fatigability in that force production decreased to a similar extent over time in both genotypes in the presence or absence of L-NMMA or L-arginine (Fig 3.1B). It is expected that both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles were activated equally and the metabolic challenge was also equal, and therefore had the same stimulatory impact on contraction-stimulated glucose uptake in both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles. This was supported by similar increases in AMPK phosphorylation (Fig 3.4B) which is a sensitive marker for metabolic status/ perturbation.

The finding that NOS inhibitor significantly attenuated skeletal muscle glucose uptake during contraction in both nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles (Fig 3.2) was very surprising. Some critical concerns are the pharmacology and specificity of L-NMMA on NOS action. L-NMMA competitive inhibits all NOS by binding to the L-arginine binding site. It could easily be transported into the cells/ tissues via cationic amino acid transporters since it has a similar affinity with L-arginine for arginine transport system (McDonald et al., 1997). The effective concentration of L-NMMA range from 0.1 to 10 mmol/ L (Vitecek et al., 2012). Though, L-NMMA was reported to inhibit electron transfer by inhibiting cytochrome C reduction (Peterson et al., 1992), its biological effects were rather specific through inhibiting NOS. It is shown that the effect of L-NMMA is enantiomer-specific where D-stereoisomer (D-NMMA) is unable to elicit the same effect as their L-stereoisomer (Lin et al., 1998b), and we showed that the attenuation of glucose uptake by L-NMMA was completely reversed by L-arginine (Fig 3.2). This strongly suggests that L-NMMA attenuated contraction-stimulated muscle glucose uptake by inhibiting NOS and not via non-specific effect of the inhibitor. In fact, our results imply that there were other NOS isoforms or splice variants in nNOSμ$^{-/-}$ muscles that generated NO and stimulated muscle glucose uptake during contraction. Endothelial NOS was the other NOS isoform detected in these muscles. However, it has been demonstrated that mice lacking eNOS do not have an attenuation of muscle glucose uptake during treadmill exercise and actually have higher contraction-stimulated glucose

uptake (Lee-Young et al., 2010). Furthermore, eNOS was shown to be not involved in the production of NO during contraction (Hirschfield et al., 2000) suggesting that eNOS is not essential for contraction-stimulated muscle glucose uptake. Studies using a nNOS specific inhibitor will help reconcile the different results regarding NOS inhibitions and nNOSμ$^{-/-}$ mice results and verify if there are non-specific effects of general NOS inhibitors.

As nNOSμ$^{-/-}$ mice were developed by targeted deletion of exon 2 (Huang et al., 1993), the alternative splice variants, nNOSβ and nNOSγ which do not contain exon 2, can still be transcribed and remain intact in various tissues in these mice (Eliasson et al., 1997, Gyurko et al., 2002). Neuronal NOSβ is catalytically active with about 80% of the catalytic activity as nNOSμ; while, nNOSγ is considered functionally inactive (Brenman et al., 1996). The expression of nNOSβ in skeletal muscle of nNOSμ$^{-/-}$ mice is controversial. Some studies demonstrated indirect evidence from immunohistochemistry staining suggesting the presence of nNOS splice variants, presumably nNOSβ, in skeletal muscle of nNOSμ$^{-/-}$ mice (Percival et al., 2010, Rothe et al., 2005). However, we and other (Baum et al., 2002) did not detect any nNOSβ in skeletal muscles of nNOSμ$^{-/-}$ mice (Fig 3.7A). We performed immunoblotting on skeletal muscle homogenates of nNOSμ$^{-/-}$ mice and found no band at 140 kDa – the expected molecular weight for nNOSβ. A band at 140 kDa, presumably nNOSβ, was detected in nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ muscles (Fig 3.7A). Since nNOSβ has been observed with immunoblotting in the brain tissue of nNOSμ$^{-/-}$ mice (Brenman et al., 1996), we performed immunoblotting using the brain homogenates from nNOSμ$^{-/-}$ mice and detected the similar 140 kDa band in the brain tissue of nNOSμ$^{-/-}$ mice (Fig 3.7B) supporting that this band was indeed nNOSβ. In addition, we also detected this nNOSβ band in skeletal muscle of rat and C57Bl/6 mice (Fig 3.7B) (Baum et al., 2011) as previously reported. The absence of nNOSβ in nNOSμ$^{-/-}$ muscle does not seem to fit well with the earlier reports by others (Percival et al., 2010, Rothe et al., 2005). It is worthwhile to highlight though that in these studies the presence of nNOSβ in skeletal muscle of nNOSμ$^{-/-}$ mice were derived from immunohistochemistry staining and have not been evaluated with (Percival et al.,

2010) or detected with immunoblotting (Rothe et al., 2005). Therefore, the identity and molecular weight of the stained protein(s) were unknown. The indirect evidence for nNOSβ in skeletal muscle of nNOSμ$^{-/-}$ mice from immunohistochemistry (Percival et al., 2010, Rothe et al., 2005) may be resulted from non-specific binding.

Indeed, we detected two additional bands at 60 kDa and 52 kDa respectively in nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ muscles (Fig 3.7A). The identity and functional significance of these bands are yet to be determined. It is unknown if these proteins are involved in NO production or in fact could be the proteins that represented the immunohistochemistry staining in the above studies (Percival et al., 2010, Rothe et al., 2005). A protein database search in NCBI Protein Blast Search Database using amino acid sequences of the immunogen for the nNOS primary antibody used in this study revealed no known protein at approximate 50 – 60 kDa that may be associated with NO production (Altschul et al., 2005). Mass spectrometry analysis may be helpful in identifying these protein bands and understand their relevance and importance in regards to contraction-stimulated muscle glucose uptake.

In support of the indication that NO was produced in nNOSμ$^{-/-}$ muscles, there was residual NOS activity detected in these muscles. It is however difficult to imagine this low level of NOS activity (~4% of nNOSμ$^{+/+}$) could be sufficient to account for the normal increase in muscle glucose uptake during contraction. Nevertheless, the small residual NOS activity may be of significant importance in these nNOSμ$^{-/-}$ mice. NO has been implicated in a number of important neurological functions such as neurotransmitter release (Montague et al., 1994), motor neuron development (Kalb and Agostini, 1993), synaptic plasticity and memory formation (Bohme et al., 1993); however, nNOSμ$^{-/-}$ mice with a maximum of 7% remaining NOS activity in the central nervous system, appear grossly normal and lack of obvious histopathological abnormalities (Huang et al., 1993) suggesting that this remaining NOS activity might be adequate to protect them from severe pathology (Eliasson et al., 1997). In isolated skeletal muscle of nNOSμ$^{-/-}$ mice, muscle integrity and performance also appears to be normal (Percival et al., 2010); in contrast, skeletal muscles with total loss of all nNOS

splice variants (KN2 mice) fatigued faster and have severe disruption of the microtubule cytoskeleton (Percival et al., 2010). These results suggest that the small residual NOS activity is critical in preserving most, if not all of, the normal neurological and muscular functions in nNOSμ$^{-/-}$ mice. Despite its effectiveness in preserving neurological and muscular functions, it is difficult to reconcile how this small amount of NOS activity can be responsible for normal glucose uptake during contraction. During NOS inhibition at the dose that we used (100 μM), NOS activity was never inhibited to zero and in fact was between 10% to just slightly below basal levels as shown in Fig 3.8 and by others (Higaki et al., 2001, Merry et al., 2010d, Merry et al., 2010c). Relative to nNOSμ$^{-/-}$ muscle, there appears to be plenty of remaining NOS activity in nNOSμ$^{+/+}$ muscles (Fig 3.8) and the other studies during contraction with NOS inhibition, yet there is a significant attenuation of glucose uptake during contraction in the current (Fig 3.2) and the other studies (Merry et al., 2010d, Merry et al., 2010c). If absolute NOS activity is the determining factor for muscle glucose uptake during contraction, then the observed attenuation in contraction-stimulated glucose uptake during NOS inhibition with more than 10% remaining NOS activity in this (Fig 3.2) and the other studies (Merry et al., 2010d, Merry et al., 2010c) would have not be seen. Perhaps, the spike/ fluctuation in NOS activity during contraction rather than the absolute level might be more important for transduction of NO signalling and therefore stimulation of glucose uptake during contraction.

Another possibility for the normal contraction-stimulated glucose uptake in nNOSμ$^{-/-}$ muscle is changes in ROS signalling due to a lack of NO to combine with superoxide ($O_2^{\bullet-}$) to form peroxynitrite. ROS are produced during contraction (Reid et al., 1992, Sandstrom et al., 2006) and the excess $O_2^{\bullet-}$ could lead to an increase production in hydrogen peroxide (H_2O_2) which then activates glucose uptake. Our group (Merry et al., 2010c) and others (Sandstrom et al., 2006) have shown that ROS is involved in the regulation of muscle glucose uptake during *ex vivo* contraction. Overexpression of Mn^{2+} superoxide dismutase that catalyses the conversion of $O_2^{\bullet-}$ to H_2O_2 significantly increases muscle glucose uptake during *ex vivo* contraction

(Sandstrom et al., 2006). The non-specific antioxidant, N-Acetylcysteine (NAC), which prevents the increase in oxidative stress induced by H_2O_2 (Toyoda et al., 2004), significantly attenuated contraction-stimulated muscle glucose uptake (Merry et al., 2010c, Sandstrom et al., 2006). These results suggest that ROS may have been involved in the normal muscle glucose uptake during *ex vivo* contraction in nNOSμ$^{-/-}$ muscle. However, the results implicating ROS are in a mouse *ex vivo* model with intense contraction protocols (Merry et al., 2010c, Sandstrom et al., 2006), and it has been shown in more physiological models *in situ* contraction and *in vivo* exercise that NAC has no effect on glucose uptake during contraction/ exercise (Merry et al., 2010a, Merry et al., 2010b) but NOS inhibition significantly attenuated glucose uptake during contraction/ exercise (Bradley et al., 1999, Ross et al., 2007). In the current study, we observed no compensatory increase in AMPK phosphorylation following contraction in nNOSμ$^{-/-}$ muscle that could possibly explain the normal glucose uptake during *ex vivo* contraction. Genetic knockout of nNOSμ could potentially lead to an up-regulation of eNOS and iNOS in skeletal muscle; however, expression of eNOS was similar in nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ muscles and no iNOS was detected indicating no compensatory increase in these NOS isoforms. Total muscle GLUT4 content was not elevated in nNOSμ$^{-/-}$ muscles either. Although other signalling such as CaMKII and PKC has not been assessed, our data suggest that the normal contraction-stimulated muscle glucose uptake in nNOSμ$^{-/-}$ muscles was unlikely to be attributed to a compensatory effect of other potential signalling involved in muscle glucose uptake during contraction. A total nNOS knockout/ knockdown rodent model (KN2 mouse) could be valuable model for further investigation of the role of nNOS in skeletal muscle glucose uptake during contraction although their ability to contract is compromised and they have cytoskeletal changes that would make interpretation difficult given recent evidence that the cytoskeleton may be playing a role in glucose uptake during contraction (Sylow et al., 2013).

In summary, nNOSμ is not essential for skeletal muscle glucose uptake during *ex vivo* contraction of mouse muscle. Given that NOS inhibition attenuated contraction-

stimulated muscle glucose uptake, and lines of evidence suggest eNOS is not important in this regard, we suggest that other nNOS splice variants could be the putative NO-producing enzymes that are responsible for the normal contraction-stimulation muscle glucose uptake. It will be important that future studies examine the effects of nNOS specific inhibitors on muscle glucose uptake during contraction.

CHAPTER FOUR:

NO EFFECT OF NOS INHIBITION DURING *IN SITU* HINDLIMB CONTRACTION ON SKELETAL MUSCLE GLUCOSE UPTAKE IN HEALTHY AND DIABETIC SPRAGUE DAWLEY RATS

4.1 INTRODUCTION

Exercise/ muscle contraction stimulates uptake of blood glucose into skeletal muscle fibers (Richter and Hargreaves, 2013). Muscle contraction is able to stimulate normal glucose uptake in skeletal muscle of insulin-resistant obese Zucker rats (Brozinick et al., 1992) and in people with type 2 diabetes (T2D) (Kingwell et al., 2002), by inducing a normal increase in skeletal muscle GLUT4 translocation (Kennedy et al., 1999, Ploug et al., 1998). Indeed, an acute bout of intense cycling exercise has been shown to reduce plasma glucose levels of people with T2D to that of healthy controls by the end of the exercise (Musi et al., 2001a). These data suggest that the skeletal muscle of people with insulin-resistant/ T2D has functional machinery that responds appropriately to increase glucose uptake during muscle contractions despite an impairment in the insulin signalling mechanism (Yeh et al., 1995). Ironically, many studies investigating new pharmaceutical therapy for diabetic subjects and the mechanism(s) of contraction-stimulated skeletal muscle glucose uptake employ healthy non-diabetic rodents or non-diabetic humans in their experiments. The need to examine diabetic models is clear since the diabetic state is associated with various pathophysiological changes including up- or down-regulation of signalling proteins which are important in glucose metabolism (Bradley et al., 2007, Bravard et al., 2011, Farese et al., 2005, Torres et al., 2004).

Nitric oxide (NO) appears to play an important role in regulating glucose uptake in skeletal muscle during contraction/ exercise (McConell et al., 2012, Richter and Hargreaves, 2013). Various studies by us and others, using NO synthase (NOS) inhibitors, demonstrate that NO mediates muscle glucose uptake during *ex vivo* mouse contraction (Merry et al., 2010d, Merry et al., 2010c), *in situ* rat contraction (Balon and

Nadler, 1997, Ross et al., 2007), *in vivo* rat treadmill exercise (Roberts et al., 1997) and during exercise in humans (Bradley et al., 1999, Kingwell et al., 2002). However, some studies show no effect of NOS inhibition on glucose uptake in isolated rat skeletal muscle contracted *ex vivo* (Etgen et al., 1997, Higaki et al., 2001), *in situ* rat contraction (Inyard et al., 2007) and rat and mice treadmill exercise (Higaki et al., 2001, Rottman et al., 2002). Various methodological differences may explain these conflicting results, in particular that glucose uptake was measured many minutes after contraction was completed rather than during contraction in these *ex vivo* studies as discussed in Chapter One. Studies in people with T2D have shown that local infusion of the NOS inhibitor, N-G-Monomethyl-L-arginine (L-NMMA) into the femoral artery of participants during 25 min of cycling exercise led to a greater attenuation of the increase in leg glucose uptake in people with T2D compared with controls (~75% versus ~30% respectively) (Kingwell et al., 2002). This suggests that people with T2D have a greater reliance on NO-mediated muscle glucose uptake during exercise, revealing a potential therapeutic target to aid glycaemic control in these subjects. It is important to note that the attenuation of glucose uptake occurred independently of femoral artery blood flow (FBF) suggesting a direct intra-muscular effect of NO on glucose uptake (Kingwell et al., 2002). The intra-muscular effects however were not investigated as muscle biopsies were not obtained during the experiment. Also, it was not examined whether exercise increases NOS activity to a greater extent in skeletal muscle of people with T2D, which could explain the greater effect of NOS inhibition on glucose uptake in the diabetics.

Contraction/ exercise increases blood flow to the contracting muscle and can also increases muscle capillary recruitment (Clark et al., 1998). The increased capillary recruitment and blood flow during muscle contraction/ exercise may stimulate muscle glucose uptake (Clark et al., 2003, Richter and Hargreaves, 2013). This capillary response has been shown to be normal in response to contraction in insulin-resistant rats (St-Pierre et al., 2012). However, diabetic subjects with microvascular complications have impaired capillary recruitment during hand-grip exercise (Womack et al., 2009). While, we have previously demonstrated that local NOS inhibition does not affect

muscle capillary recruitment during contraction in healthy rats (Ross et al., 2007), it remains unknown whether NOS inhibition reduces capillary recruitment during contraction in diabetic rats.

Therefore, the purpose of the current study was to examine if local NOS inhibition results in a greater attenuation of the increase in contraction-stimulated muscle glucose uptake in T2D rats compared with healthy controls, as we have previously shown in individuals with T2D (Kingwell et al., 2002) and if so, to determine the mechanisms involved. We generated a cohort of diabetic rats using a combination of high fat diet feeding and low-dose streptozotocin (STZ) injections based on previously published methodologies (Ramadan et al., 2006, Srinivasan et al., 2005, Zhang et al., 2008). We hypothesised that local NOS inhibition would cause greater attenuation of the increase in muscle glucose uptake during contraction in T2D rats compared with healthy controls and that this effect would be due to intramuscular signalling alterations rather than changes in capillary blood flow in T2D rats.

4.2 METHODS

4.2.1 Animals

All procedures were approved by The University of Tasmania Animal Ethics Committee, and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th Edition). Male Sprague Dawley (SD) rats were obtained from either Monash Animal Services or The University of Tasmania Animal House. Animals were housed in standard cages and maintained at constant temperature of 21 ± 1 ^{o}C and on a 12 h light/ dark cycle.

4.2.2 Diet/ streptozotocin injections

Prior to diet manipulation, rats were allowed ad libitum access to standard rat chow (4.8% fat wt/wt; Specialty Feeds, Glen Forest, Western Australia, Australia) and water. A modified protocol from Zhang et al. (2008) was followed to generate a cohort of T2D rats. Briefly, at six weeks of age, rats were randomly divided into two groups: control group (control) and high-fat diet with STZ (T2D). The control group continued on the standard chow while the T2D group received a high-fat diet (23% fat wt/wt; Specialty Feeds) for four weeks. Please refer to Appendix C for the compositions of the high-fat diet. Two weeks into high-fat diet feeding, T2D rats were injected intraperitoneally with a low dose STZ (35 mg/kg) in citrate buffer (pH 4.4) and a second, equivalent dose was administered three days later. Control rats were injected with vehicle citrate buffer. Both groups continued on their respective diets until experimentation. This level of high-fat diet feeding causes insulin resistance in animal models but not frank hyperglycaemia or the diabetic state (Kraegen et al., 1986, Storlien et al., 1991, Tanaka et al., 2007, Zhao et al., 2008). Multiple low-dose STZ injections induce gradual and lessened pancreatic β-cells destruction (Kim et al., 2006) leading to decrease insulin secretion, which mimics the development of a diabetic state (Festa et al., 2006). This is in contrast to high dose STZ (~60 mg/kg) which causes abrupt and massive pancreatic destruction and therefore Type 1 diabetes. It is of note that the dose of STZ (35 mg/kg) used in this study has been shown to cause T2D only in conjunction

with high-fat diet-induced insulin resistance but not in chow fed rats (Srinivasan et al., 2005). This resembles the situations in humans where people with obesity and insulin resistance are predisposed to T2D development (Srinivasan et al., 2005). Non-fasting plasma samples were collected for measurement of plasma glucose and insulin levels prior to the contraction experiments, to assess the level of insulin resistance.

4.2.3 Surgical procedure

The surgical procedures have been described previously (Ross et al., 2007, St-Pierre et al., 2012). Briefly, rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight; Ilium, Troy Laboratory, Australia). Body temperature was maintained throughout the surgery and experiment at 37 ^{o}C using a water-jacketed platform and a heating lamp. A tracheotomy tube was inserted to facilitate respiration and jugular veins were cannulated (PE60, Microtube Extrusions, North Rocks, NSW, Australia). Continuous anesthesia was maintained by infusion of sodium pentobarbital (0.6 mg/min/kg body weight) via one of the jugular veins. A carotid artery was cannulated for arterial blood pressure and heart rate monitoring (Transpac IV; Abbott Critical Systems, Morgan Hill, CA, USA) as well as for arterial blood sampling. Both femoral arteries were exposed via a small incision (~1.5 cm) in the overlying skin to allow for measurement of femoral blood flow using ultrasonic probes (VB series 0.5 mm; Transonic systems, Ithaca, NY, USA) placed around the exposed femoral artery of each leg. The probes were connected to a flow meter (Model T106 ultrasonic volume flow meter; Transonic systems) interfaced with a computer. Blood pressure, heart rate and FBF were recorded using Windaq Data Acquisition software (DATAQ Instruments, Akron, OH, USA). The epigastric artery in the stimulated (contracted) leg was also cannulated with an insulin needle (30 G) connected to PE30 tubing for local (retrograde) infusion of N-G-Nitro-L-Arginine Methyl Ester (L-NAME) directly into the femoral artery.

4.2.4 Experimental protocols

The experimental protocols were as previously described (Fig 4.1) (Ross et al., 2007). Hindlimb muscle contractions were achieved by attaching electrodes to the skin at the knee and the Achilles tendon of the contracting leg while the contralateral leg served as a resting control. There were two protocols in the experiments. Protocol A was designed to assess the effect of contraction on FBF and force development, and protocol B was for measurement of capillary recruitment and capillary blood flow in the muscle using contrast-enhanced ultrasound (CEU). It was necessary to do these as two separate experiments because the microbubbles interfere with the Doppler signal of the transonic flow probe and therefore it is not possible to measure FBF during microbubble infusion. In both protocols, twitch contractions were elicited with 0.1 ms pulses at 2 Hz and 35 Volt at t = 0 min (Fig 4.1) (Grass SD9 Stimulator; Grass Products, Natus Neurology, WI, USA). To measure force of contraction, the contracted leg was stabilised in a jig and an isometric force transducer (Harvard Apparatus, MA. USA) was attached to the Achilles tendon. Contraction force was acquired using Windaq Data Acquisition software. Local L-NAME (or saline) infusion was commenced at t = 10 min via the epigastric artery of the contracting leg to achieve 5 μM concentration based on concentration of infusate, rate of infusion and FBF, as per our previous study (Ross et al., 2007). A bolus of [1-^{14}C]2-deoxy-glucose ([^{14}C]2-DG) was injected into a jugular vein at t = 20 min to assess muscle specific glucose uptake over the final 10 min of muscle contraction. Arterial blood samples were taken as indicated in Figure 4.1. In protocol A, FBF was recorded throughout the experiment. These data were later used to guide the adjustment of the infusion rate of L-NAME in protocol B to maintain 5 μM in the hindleg. In protocol B, capillary blood flow was determined using CEU with microbubble infusion. Microbubbles were infused as indicated in Fig 4.1 and capillary blood flow was measured immediately before contraction (basal, t = 0 min), during contraction but before L-NAME infusion (t = 10 min), and during contraction and with L-NAME infusion (t = 30 min). Contrast-enhanced ultrasound determination was performed as previously described (Ross et al., 2007, St-Pierre et al., 2012). Briefly, a linear array transducer (L9-3) interfaced with an iU22 ultrasound machine (Philips

Ultrasound, Bothell, WA, USA) was positioned over the lower leg muscles (gastrocnemius, plantaris and soleus) of the contracting hindlimb. Real-time imaging was performed at low-mechanical index (0.08) with the acoustic focus set at the mid-portion of the muscles. Gain settings were optimised and held constant throughout the experiment. Octafluoropropane-gassed microbubbles were infused through the right jugular vein at 40 μl/min. A steady state of systemic microbubble concentration was achieved before imaging. A high-energy destructive ultrasound pulse was delivered to destroy microbubbles within the volume of muscle tissues being imaged. Immediately after, data was acquired for 30 s in real time to image the replenishment of microbubbles within the muscle vasculature. Three repeated loops were performed and the acoustic intensity was analysed offline using QLab (Phillips Ultrasound, Bothell, WA, USA). Images were background subtracted for 1.0 s frames to eliminate signal from larger blood vessels and tissues per se. Background subtracted acoustic intensity versus time was plotted to allow calculation of capillary blood volume (A) and capillary flow rate (A*β) according to the equation $y = A(1 - e^{-\beta t})$, where y is acoustic intensity at a given time t.

4.2.5 Muscle glucose uptake

Muscle glucose uptake was measured during the final 10 min of contraction and the procedures for muscle glucose uptake determination are described in Chapter Two (Section 2.1.2). Muscle glucose uptake (R'g) was calculated as previously described (Ross et al., 2007).

4.2.6 Plasma biochemistry

Plasma glucose and lactate were determined using an automated analyser (Model 2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH, USA). Plasma insulin levels were determined using enzyme-linked immunosorbent assay (Mercodia, AB, Uppsala, Sweden) as per manufacturer's instructions and the procedures are described in Chapter Two (Section 2.5).

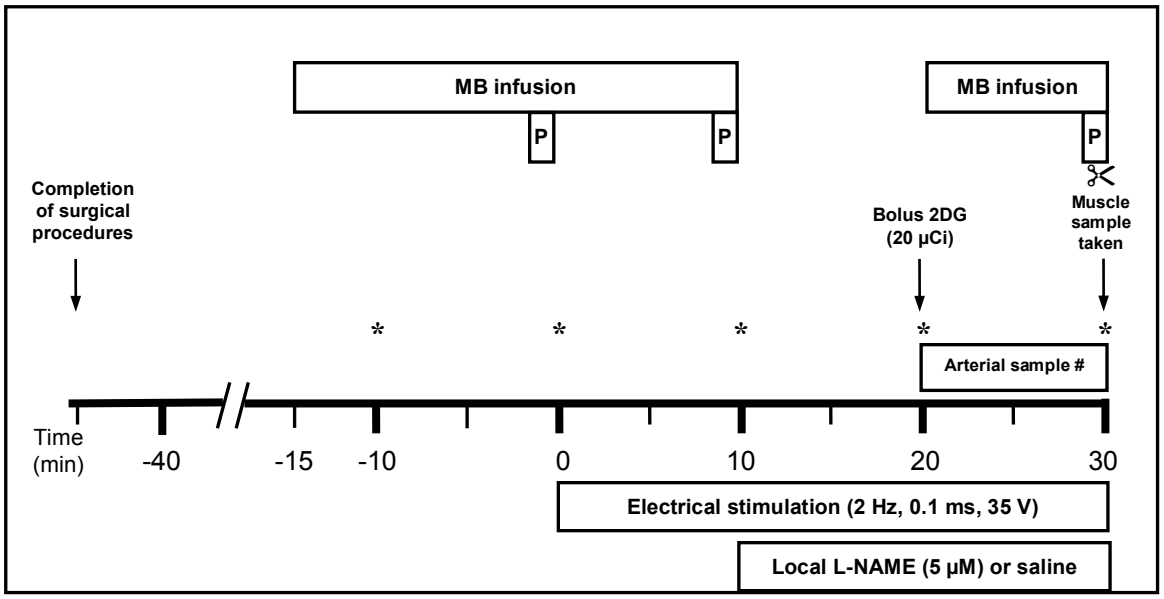


Figure 4.1: Protocol for *in situ* rat hindlimb muscle contraction.

Twitch contractions were elicited by electrical stimulation (2 Hz, 0.1 ms, 35 V). MB: microbubbles; P: pulsing interval; 2DG: [1-^{14}C]2-deoxy-glucose; L-NAME: N-G-Nitro-L-Arginine Methyl Ester; # continuous withdrawal (30 µl/min) from the carotid artery; * arterial blood sampling for blood glucose or insulin levels. Capillary recruitment measurements were done immediately before contraction, during contraction and during contraction with saline or L-NAME infusion; requiring MB infusion over the durations as indicated. Microbubbles were only infused in Protocol B as described in the method section above.

4.2.7 Immunoblotting

Immunoblotting was performed using ground frozen muscle homogenised with 200 volume of solubilising buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) as described in Chapter Two (Section 2.3). The primary antibodies used in this chapter were phospho-AMPKα Thr^{172} (1:1000), AMPKα (1:1000), α-tubulin (1:1000) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1:10000) and eNOS (1:1000) (BD Biosciences, San Jose, California, USA); GLUT4 (1: 8000) (Thermo Scientific, Rockford, IL, USA), and actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Two different loading control proteins (actin and α-tubulin) were used in this chapter and they were always probed using non-stripped membranes. Actin was used for all except GLUT4 where α-tubulin was used as the loading control as actin and GLUT4 have similar molecular weights and it was not possible to probe both of these proteins without undertaking the stripping process.

4.2.8 NOS activity assay

Skeletal muscle NOS activity was determined as described in Chapter Two (Section 2.2) based on the conversion of radioactive L-arginine to radioactive L-citrulline as previously described (Lee-Young et al., 2009). For each sample, one reaction was done in the presence of 1 mM L-NAME to block NOS activity, and duplicates were performed without L-NAME. Subtracting the radioactive counts from these reactions allowed the calculation of radioactive counts that were attributed to NOS reaction and eliminated the background counts. NOS activity was determined from this calculated count and was expressed as picomoles of L-[^{14}C]citrulline formed per min, per mg of protein.

4.2.9 Statistical analysis

All data are presented as mean ± SEM. Data were analysed by SPSS statistical package using independent Student's t-test (control vs T2D), two (between factors: control vs T2D, saline vs L-NAME) or three factor ANOVA (between factors: control

vs T2D, saline vs L-NAME; within factors: contraction or time). If a significant interaction was detected, specific differences between mean values were located using Fisher's least significant difference test. The level of significance was set at $p < 0.05$.

4.3 RESULTS

4.3.1 Baseline characteristics of control and T2D rats

Body weight and epididymal fat (a measure of adiposity) of T2D rats were significantly lower than control rats (Table 4.1). When normalised to body weight, epididymal fat was not different between the two groups of rats (Table 4.1). Body lean mass data was not available. The lower body weight for T2D rats though is unusual but is not uncommon as it was shown that high fat fed rats had significantly higher serum glucose levels and lower body weight when injected with low dose STZ (Wang et al., 2007). T2D rats had significantly elevated plasma glucose concentration and a decreased plasma insulin concentration compared with their control counterparts (Table 4.1). These features indicated a partial decompensation of pancreatic capacity to secrete higher levels of insulin in response to the insulin-resistant state induced by high fat feeding and are consistent with the manifestation of T2D and reported data (Zhang et al., 2008).

Table 4.1: Baseline characteristics of control and type 2 diabetes rats

Variables	Control	T2D
Body weight (g)	340 ± 5	304 ± 4 #
Epididymal fat (g)	2.2 ± 0.10	1.7 ± 0.09 #
Epididymal fat per body weight (%)	0.63 ± 0.03	0.57 ± 0.03
Plasma glucose (mmol/l)	9.4 ± 0.1	16.7 ± 1.2 #
Plasma insulin (pmol/l)	718 ± 69	424 ± 30 #

Values are means ± SEM, n = 25 to 30 and 35 to 42 in control and T2D groups, respectively; # $P < 0.05$ vs control. T2D: Type 2 Diabetes.

4.3.2 Contraction force production

The peak force normalised to body weight (Fig 4.2A) were similar between control and T2D rats. Local infusion of saline or L-NAME into the femoral circulation did not affect the force production in either group (Fig 4.2A). Both groups of rats experienced a similar reduction in force over time during the contractions irrespective of the type of infusion received (Fig 4.2B). At the end of the contraction protocol, contraction force was reduced to approximately half of the peak force in all groups (Fig 4.2B).

4.3.3 Systemic blood pressure during contraction

Systemic blood pressures at rest were not different between the experimental groups (Fig 4.3). A small (~10 mmHg) but significant increase in systemic blood pressure across all groups was observed during muscle contraction (Fig 4.3). Local L-NAME infusion did not lead to a significant further increase in the systemic blood pressure implying no spillover of L-NAME into systemic circulation took place (Fig 4.3).

4.3.4 Plasma lactate and insulin changes during contraction

Plasma lactate at rest was not different between control and T2D rats (Fig 4.4). There was a small but significant increase in plasma lactate concentrations during contraction, with no difference between the two groups and was not affected by L-NAME (Fig 4.4). At basal state, plasma insulin concentrations were significantly lower in T2D groups (Fig 4.5). A main effect of contraction in increasing plasma insulin during contraction was observed (Fig 4.5). We have observed plasma insulin to increase during contraction in previous studies involving single hindlimb contraction (unpublished observation). A tendency of higher plasma insulin levels in exercising mice was also reported previously (Rottman et al., 2002).

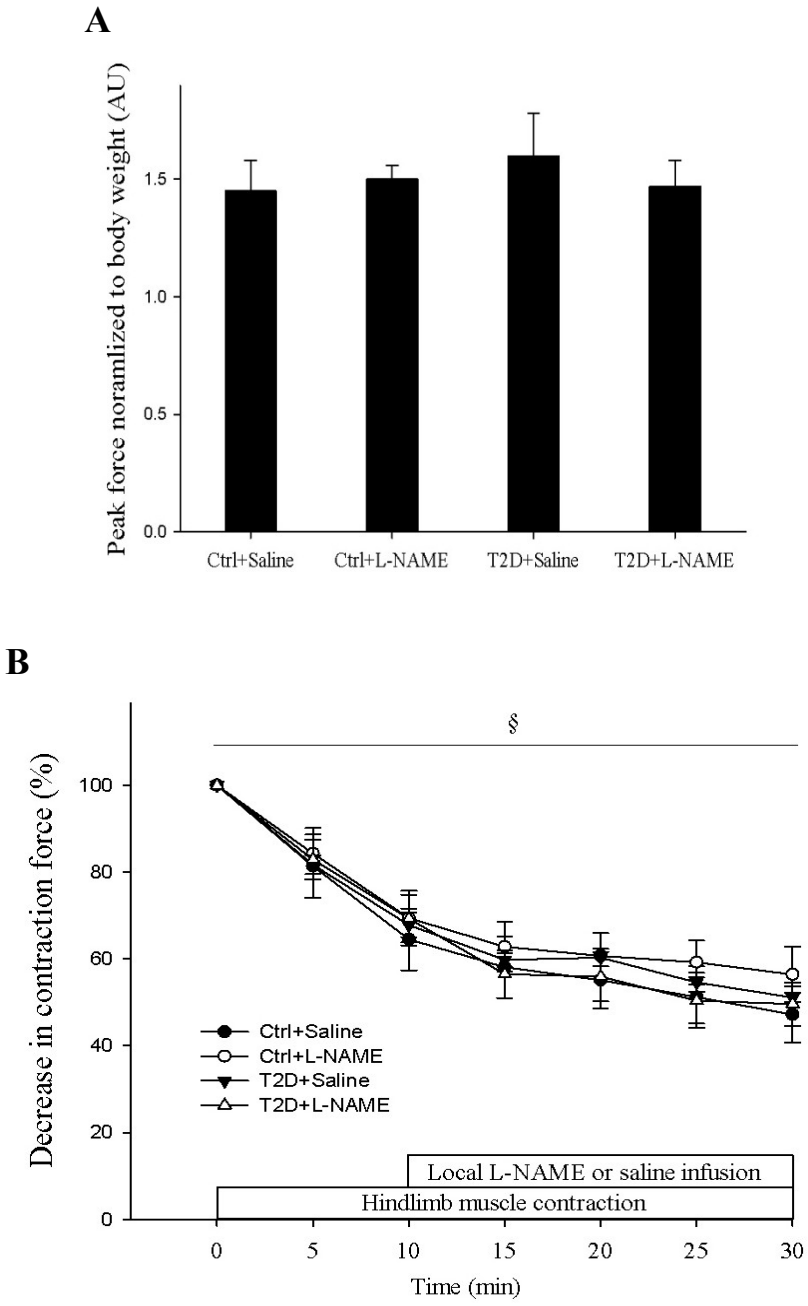


Figure 4.2: Peak contraction force normalised to body weight (A) and percent of decrease in contraction force from the initial maximum force over time (B).

Values are means ± SEM, n = 5 – 7 per group. § $P < 0.05$ main effect for time. Ctrl = control rats; T2D = type 2 diabetic rats

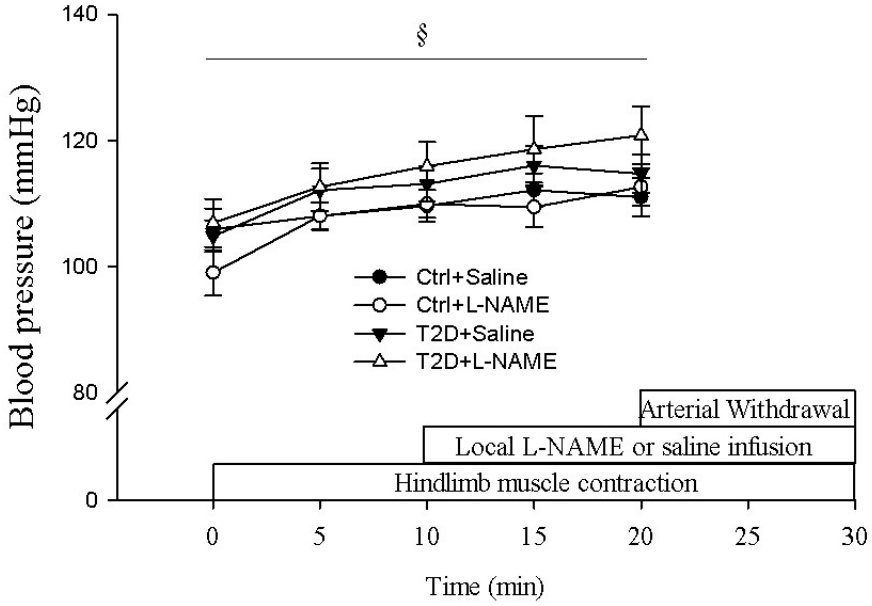


Figure 4.3: Systemic blood pressure changes at rest and during contraction.

Note: Blood pressure measurement could not be obtained during the final 10 min because the same carotid artery line was necessary for the continuous arterial withdrawal for glucose uptake measurement. Values are means ± SEM, n = 8 – 12 per group. § $P < 0.05$ main effect for time.

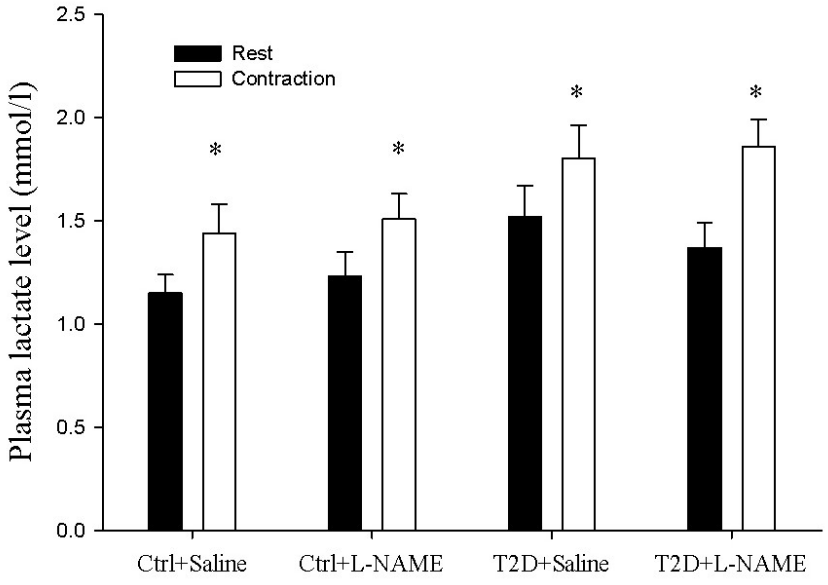


Figure 4.4: Plasma lactate levels at rest and during contraction.

Values are means ± SEM, n = 13 – 21 per group. * $P < 0.05$ vs basal of the same group.

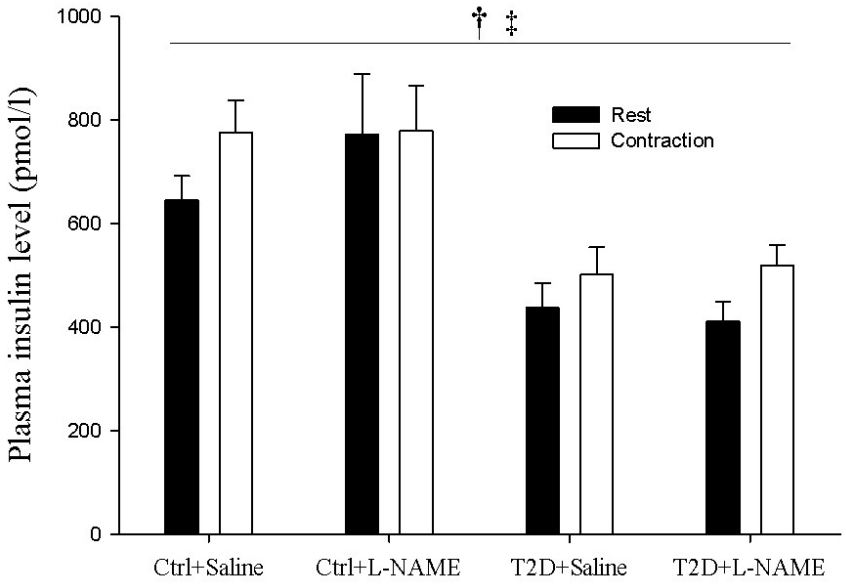


Figure 4.5: Plasma insulin levels at rest and at the end of contraction.

Values are means ± SEM, n = 12 – 21 per group. † $P < 0.05$ main effect for contraction, ‡ $P < 0.05$ main effect for T2D.

4.3.5 Hindlimb muscle glucose uptake

Contraction greatly increased (> 10 fold) hindlimb muscle glucose uptake in both groups (Fig 4.6). T2D rats had a significantly greater (37 - 41%) muscle glucose uptake compared with control rats (Fig 4.6). Local infusion of L-NAME did not affect muscle glucose uptake during contraction in either control or T2D groups (Fig 4.6). Muscle glucose uptake of the contralateral rested leg was similar between both groups of rats with or without L-NAME infusion (Fig. 4.6).

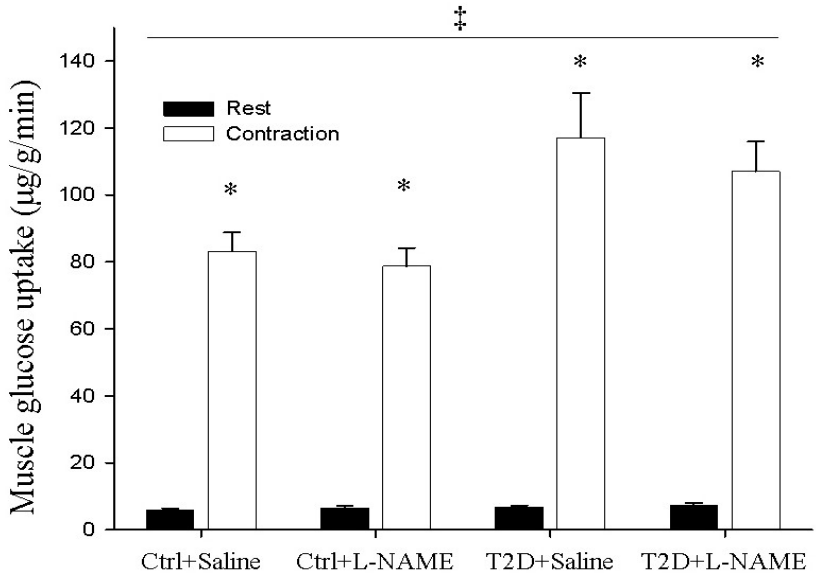


Figure 4.6: Rest and contracted leg muscle glucose uptake in control and T2D rats with local infusion of saline or L-NAME.

Values are means ± SEM, n = 12 – 14 per group. * $P < 0.05$ vs rest of the same group; ‡ $P < 0.05$ main effect for T2D.

4.3.6 Femoral and capillary blood flows

Contraction significantly increased FBF between 3.5 to 4.4 fold during the first five min; after which, femoral blood flow slowly decreased throughout the contraction and remained at 2.4 to 3.4 fold higher than basal (Fig 4.7A). Local L-NAME infusion into the contracting hindlimb led to a significant greater reduction in FBF compared with saline infusion in both control and T2D rats (Fig 4.7A and Fig 4.7B). This effect was seen within 5 min of L-NAME infusion and continued throughout the rest of the experiment (Fig 4.7A). At rest, capillary blood volume (A value) (1.36 ± 0.23, 0.94 ± 0.20, 1.20 ± 0.27, 0.84 ± 0.24 for control + saline, control + L-NAME, T2D + saline and T2D + L-NAME, respectively) and capillary flow rate (A*β) (0.14 ± 0.03, 0.14 ± 0.05, 0.18 ± 0.08, 0.08 ± 0.04 for control + saline, control + L-NAME, T2D + saline and T2D + L-NAME, respectively) were not significantly different between experimental groups. In view of the variations in basal values between individual rats, capillary blood volume and capillary flow rate during contraction with and without saline or L-NAME infusion were normalised against its basal values. Contraction significantly increased capillary blood volume (recruitment) (Fig 4.8A) and capillary flow rate (Fig 4.8B). These indices were similar between control and T2D rats and were not significantly affected by L-NAME infusion (Fig 4.8A and Fig 4.8B) indicating local NOS inhibition did not affect muscle capillary blood flow responses during contraction in both control and T2D rats.

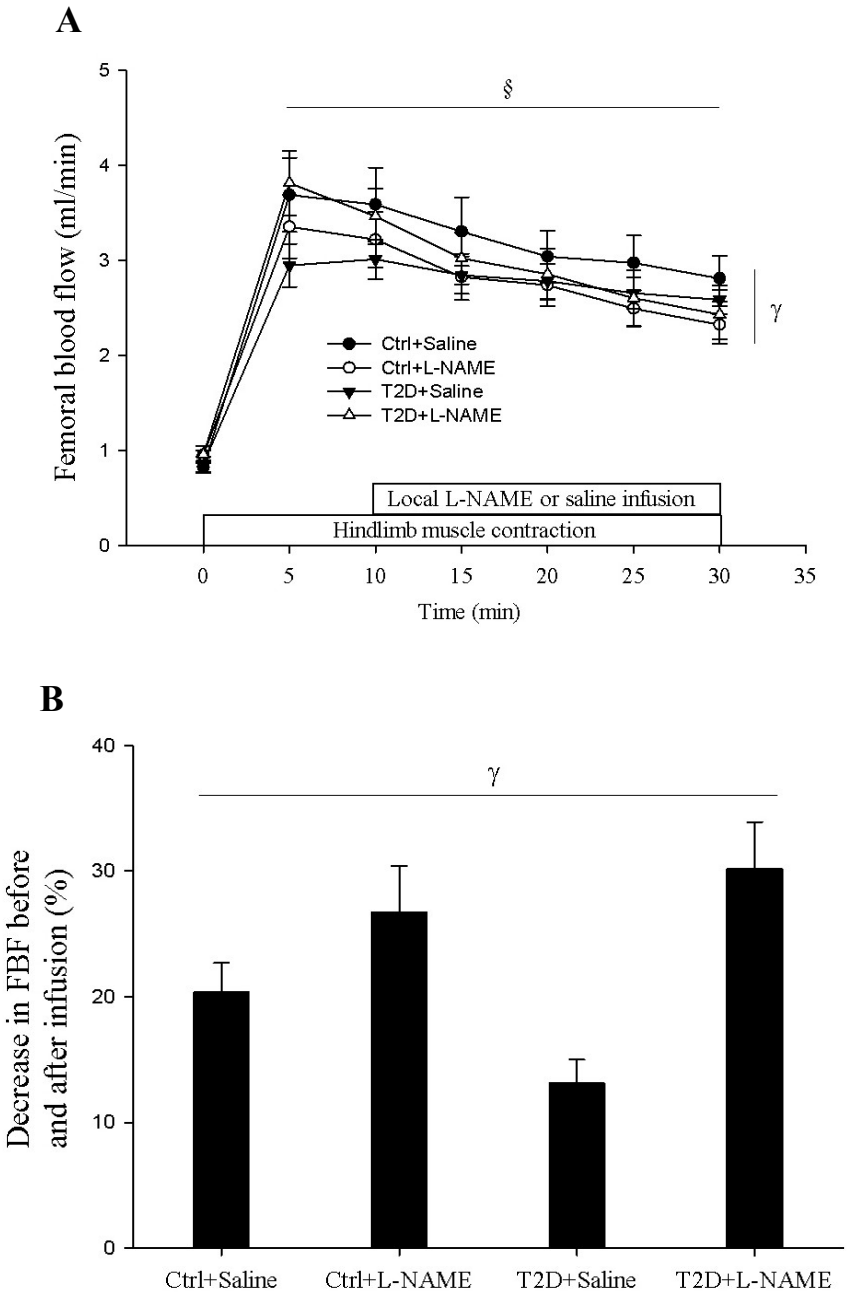


Figure 4.7: Femoral blood flow of contracted hindlimb during contraction (A) and percent of decrease in femoral blood flow in the contracted hindlimb before and at the end of local infusion of saline or L-NAME (B).

Values are means ± SEM, n = 8 – 12 per group. § $P < 0.05$ main effect for time, γ $P < 0.05$ interaction between L-NAME and saline.

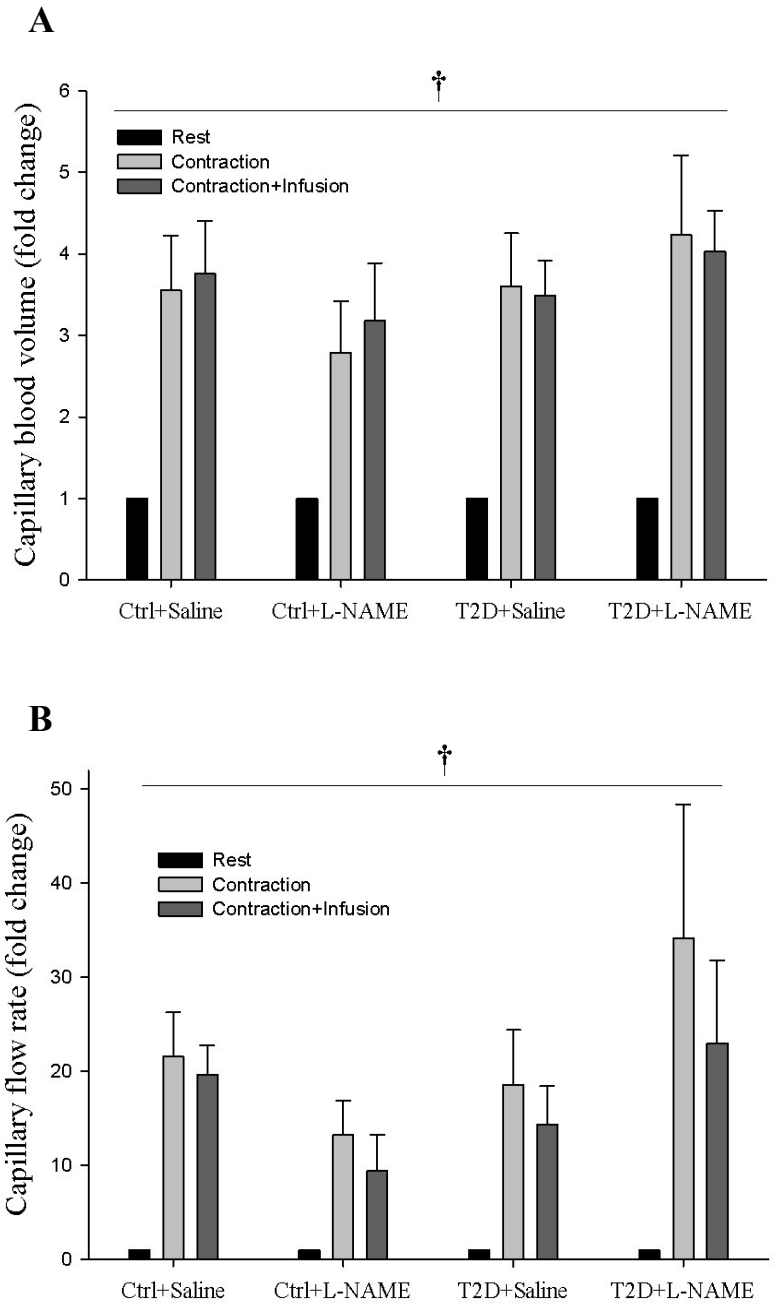


Figure 4.8: Changes in capillary blood volume (A) and capillary flow rate (B) of contracted hindlimb during contraction and during contraction with saline or L-NAME infusion relative to resting values.

Values are means ± SEM, n = 3, 4, 5 & 5 for T2D+L-NAME, control+L-NAME, T2D+saline and control+saline respectively. † $P < 0.05$ main effect for contraction and contraction + infusion.

4.3.7 Loading control protein expression

The expression of actin (Fig 4.9A) and α-tubulin (Fig 4.9B) proteins were found to be not different between control and T2D rats indicating that they were suitable to be used as a loading control.

4.3.8 AMPK signalling

The induction of T2D in these rats did not significantly change the expression of AMPKα (Fig 4.10A). Contraction significantly increased skeletal muscle AMPKα phosphorylation in all experimental groups (> 2 fold) (Fig 4.10B) and no difference was observed between control and T2D rats (Fig 4.10B). L-NAME had no effect on AMPK phosphorylation during exercise in either group (Fig 4.10B).

4.3.9 GLUT4, eNOS and nNOS protein expressions

Skeletal muscle protein expression of eNOS (Fig 4.11) and GLUT4 (Fig 4.12) were similar in both control and T2D rats. However, a significant reduction (~27%) in nNOSβ (a nNOS splice variant) protein expression was observed in T2D rats (Fig 4.13B). A similar trend (~16%) was also observed for nNOSμ protein ($P = 0.057$) between control and T2D rats (Fig 4.13A).

4.3.10 Muscle NOS activity

T2D rats tended to have a lower NOS activity compared with control rats ($P = 0.051$) (Fig 4.14), in line with their tendency to have lower nNOS protein expressions (Fig 4.13A and Fig 4.13B). Unexpectedly, contraction did not increase NOS activity and NOS inhibition did not significantly reduce NOS activity in either control or T2D rats (Fig 4.14).

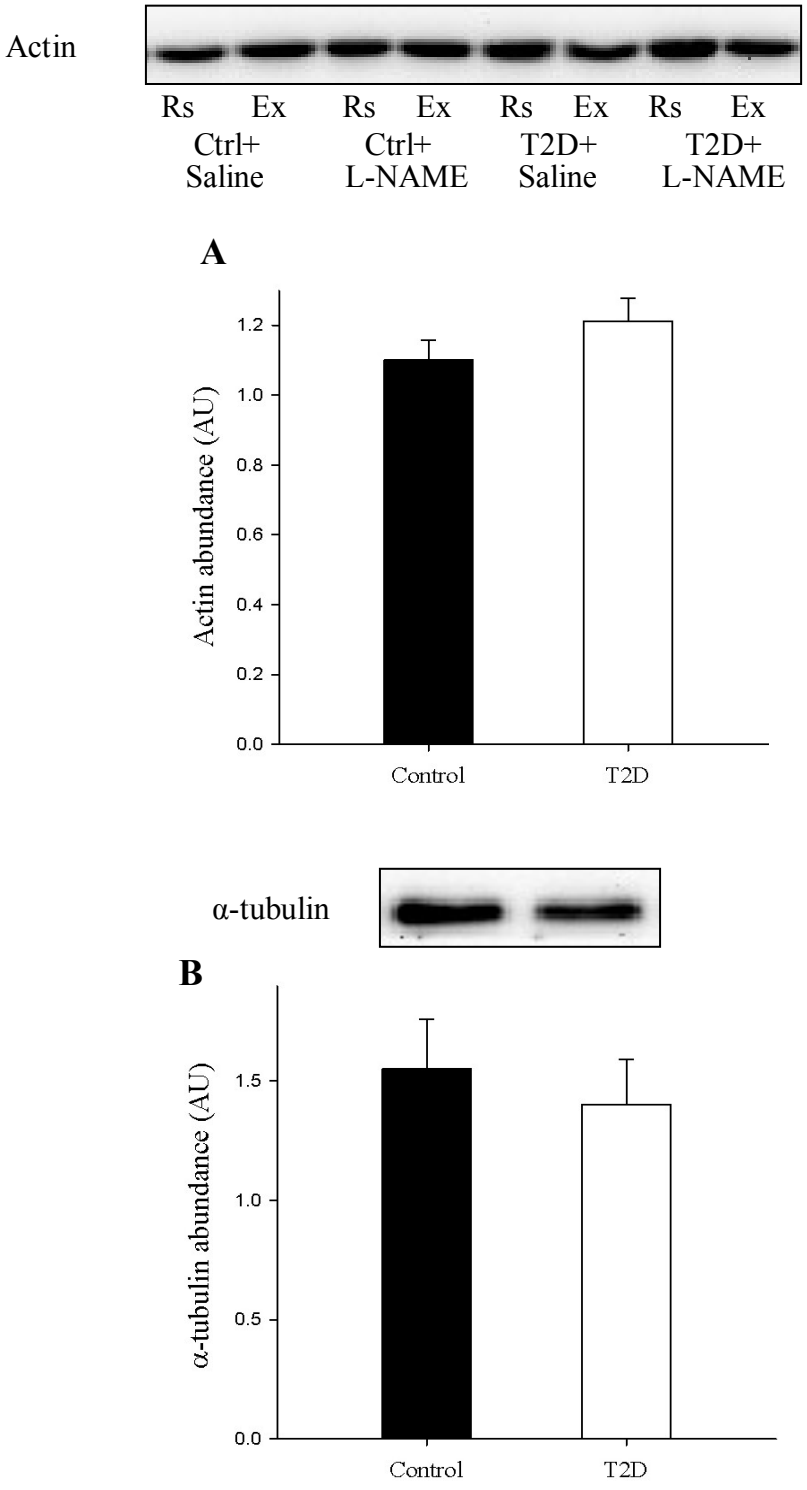


Figure 4.9: Actin (A) and α-tubulin (B) protein expressions in resting muscles of control and T2D rats.

Data are means ± SEM, n = 8.

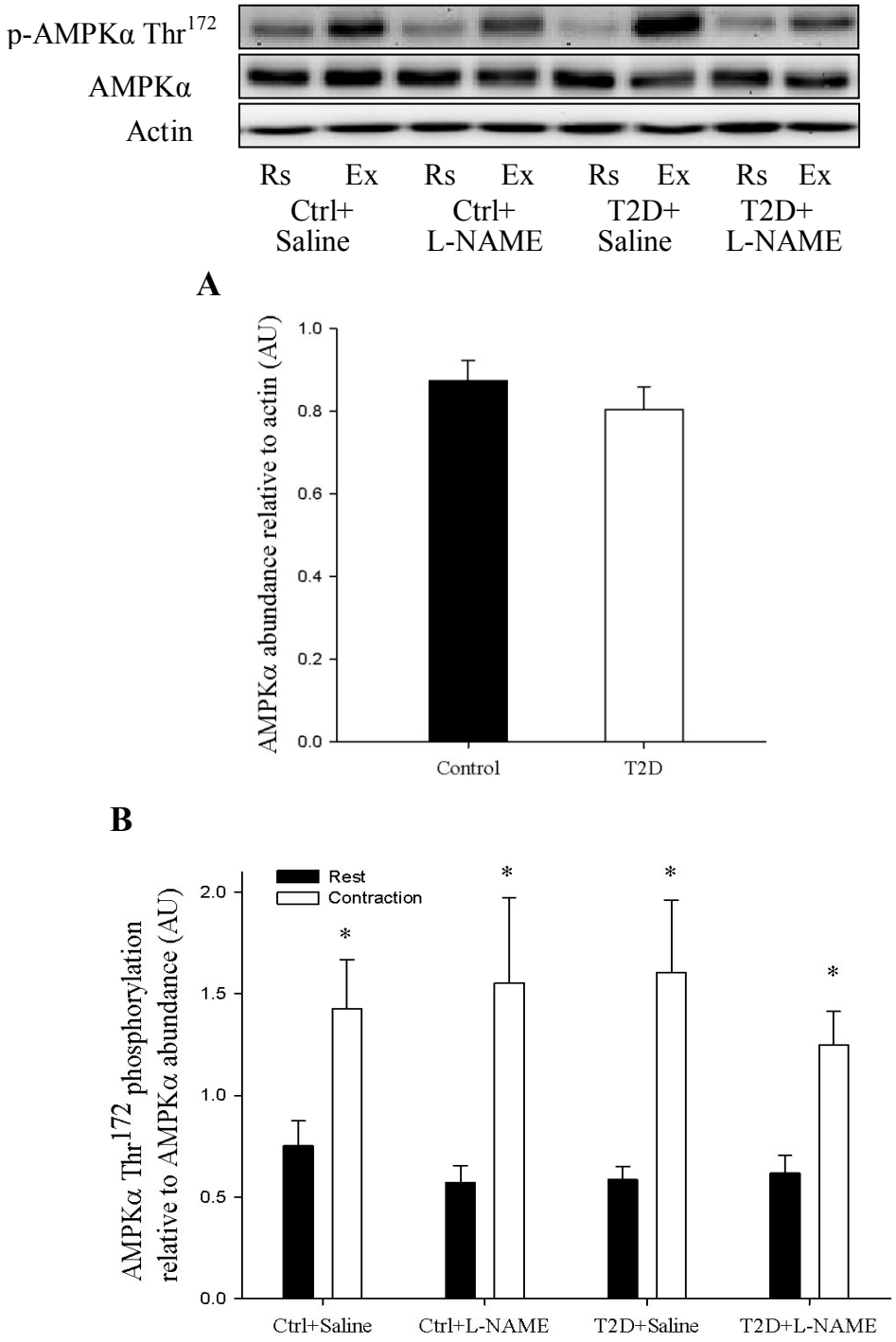


Figure 4.10: AMPKα abundance in control and T2D rats at resting state (A), AMPKα Thr^{172} phosphorylation relative to AMPKα abundance in control and T2D rats during *in situ* contraction with saline or local L-NAME infusion (B).

Data are means ± SEM, n = 8 per group. * $P < 0.05$ vs rest of the same genotype.

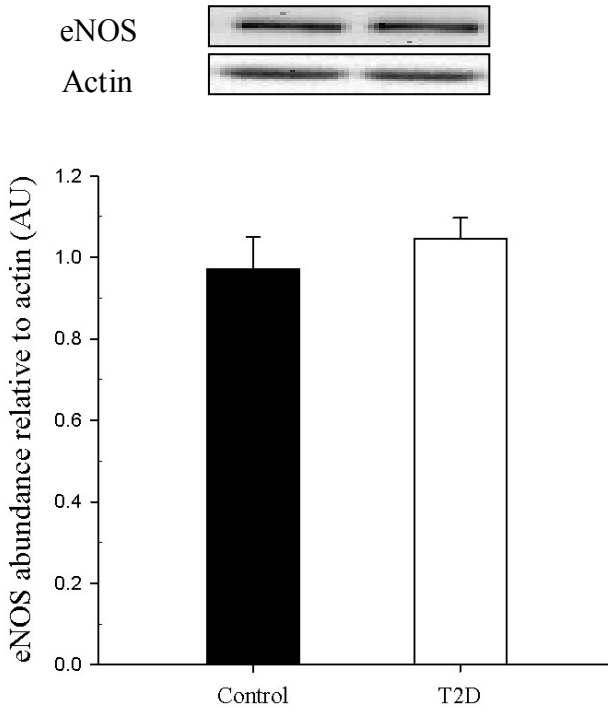


Figure 4.11: eNOS protein expression in control and T2D rats relative to actin abundance.

Data are means ± SEM, n = 8 per group.

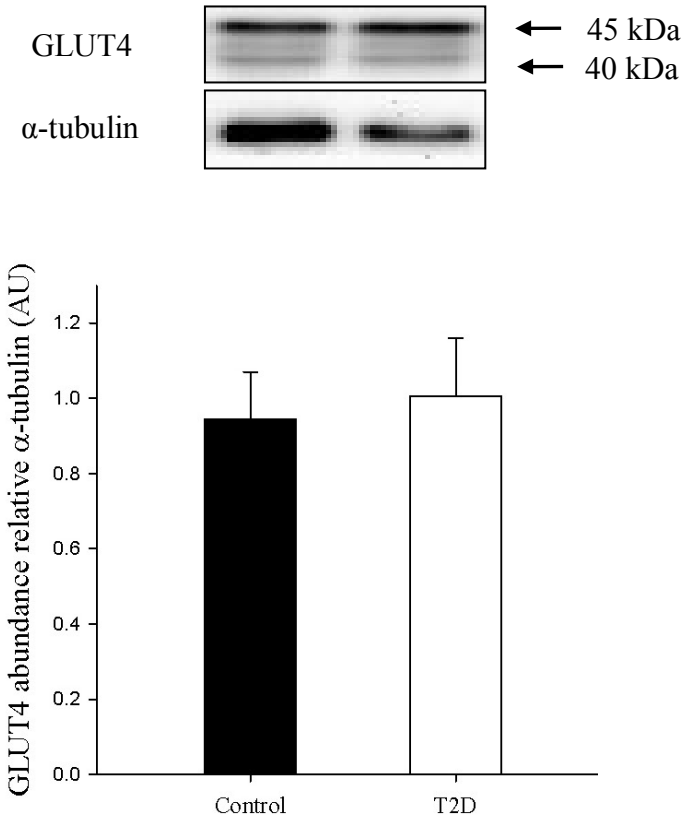


Figure 4.12: GLUT4 protein expression in control and T2D rats relative to α-tubulin abundance.

Data are means ± SEM, n = 8 per group. Bands at 45 and 40 kDa represented glycosylated and de-glycosylated GLUT4 respectively (see Appendix B for more detail). Both bands were used for data analysis.

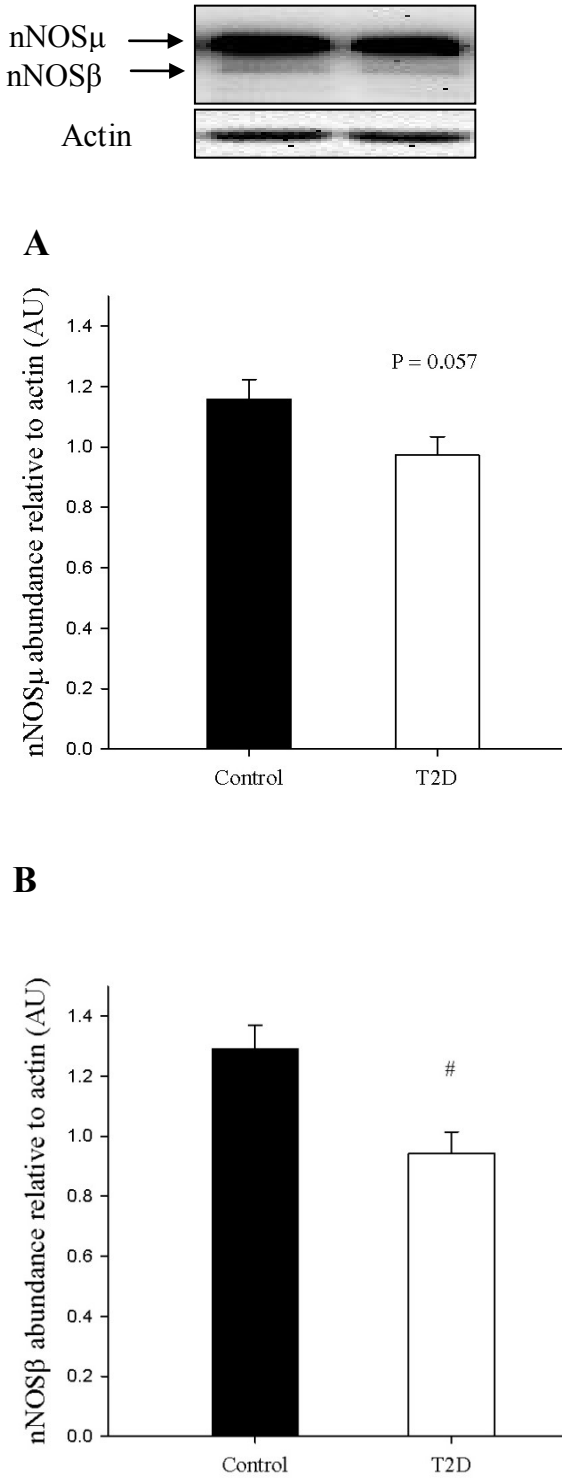


Figure 4.13: nNOSμ (A) and nNOSβ (B) protein expressions in control and T2D rats relative to actin abundance.

Data are means ± SEM, n = 8 per group. # $P < 0.05$ vs control.

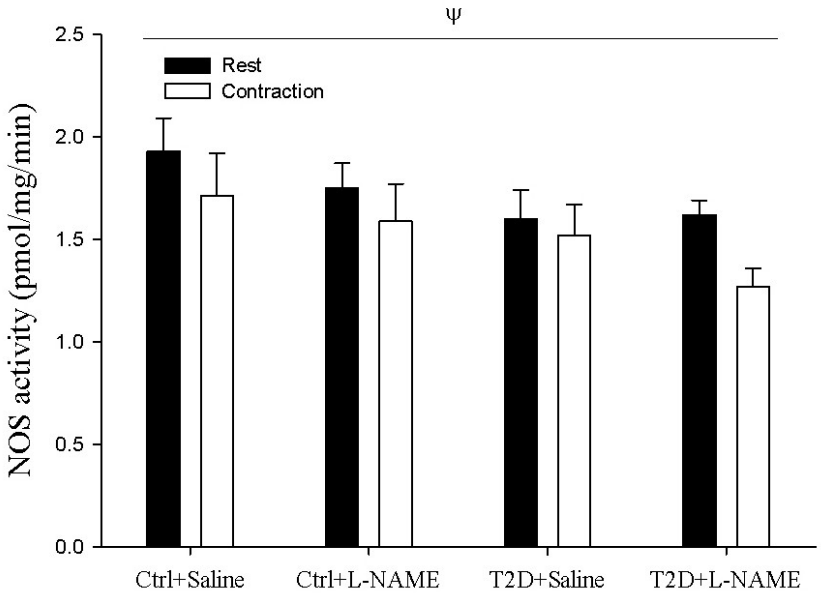


Figure 4.14: Rest and contracted muscle NOS activity in control and T2D rats with local infusion of saline or L-NAME.

Data are means ± SEM, n = 8 per group. ψ trend (P = 0.051) for main effect of T2D

4.4 DISCUSSION

In this study, contraction significantly increased skeletal muscle glucose uptake, in SD rats, however, local infusion of the NOS inhibitor, L-NAME, did not attenuate this response in control or T2D rats. This was contradictory to our previous findings in Wistar rats (Ross et al., 2007) and may be attributed to a different strain of rat used. The contraction protocol used failed to activate NOS in skeletal muscle of these SD rats unlike our previous study using Wistar rats that showed a significant increase in NOS activity with the same contraction protocol that was inhibited with NOS inhibition (Ross et al., 2007). The combination of four weeks high fat diet (23% wt/wt) and two low dose (35 mg/kg) STZ injections in healthy SD rats was successful in achieving a T2D phenotype.

In this study, single hindlimb *in situ* contraction increased skeletal muscle glucose uptake and, there was a significantly greater increase in glucose uptake during contraction in T2D rats compared with control ($P<0.001$). This could be due to the higher blood glucose concentrations (Table 4.1) in these rats as glucose uptake across muscle fibers during contraction/ exercise follows saturation kinetics with higher plasma glucose concentrations leading to greater increases in muscle glucose uptake during exercise in dogs (Zinker et al., 1993). Unexpectedly, local NOS inhibition had no effect on contraction-stimulated muscle glucose uptake in control or T2D rats. This finding does not correspond with our previous similar study in Wistar rats in which we showed that local NOS inhibition significantly attenuates contraction-stimulated muscle glucose uptake by approximately 35% (Ross et al., 2007). The reasons for the lack of effect of NOS inhibition in glucose uptake during contraction in the current study are unclear. Skeletal muscle glucose uptake is dependent on frequency of stimulation and force of contraction (Ihlemann et al., 1999b, Ihlemann et al., 2000, Ihlemann et al., 2001); however, these parameters were comparable in both studies. Also, the same concentration of L-NAME was used in both studies (5 μM) which had previously been optimised to elicit a local effect without systemic spillover effects on blood pressure (Bradley et al., 2013, Ross et al., 2007). We observed no increase in systemic blood

pressure induced by L-NAME during contraction in the current study; supporting that L-NAME effects were confined to the specific hindlimb. The use of L-NAME in this study compared with L-NMMA as per the *ex vivo* contraction study in Chapter Three allowed for better comparison of the data between the current and our previous studies (Ross et al., 2007). It is important to note that L-NAME needs to be metabolised to become bioactive inhibitor of NOS (Pfeiffer et al., 1996). As discussed below, L-NAME appears to be rapidly metabolised since it has an effect on femoral blood flow.

A small but significant greater reduction in FBF during contraction was observed following L-NAME infusion compared with saline infusion (with similar results in both control and T2D rats), a finding consistent with our previous data (Ross et al., 2007). This proves that the NOS inhibition was having effects, despite no effects on muscle glucose uptake. Capillary blood flow, which could affect glucose delivery to the muscle and therefore affects muscle glucose uptake (Richter and Hargreaves, 2013), can change independently of FBF (Clark et al., 1998, Vincent et al., 2006). In this study, we showed that capillary recruitment and capillary blood flow increased normally during muscle contraction in T2D rats. These findings are in line with previous studies showing that insulin-resistant rats (St-Pierre et al., 2012) and people with T2D without microvascular complications (Womack et al., 2009) have normal capillary recruitment and capillary blood flow in response to muscle contraction/ exercise. We also found that L-NAME infusion had no effect on capillary recruitment and capillary blood flow during contraction in either group of rats, which is in line with our (Ross et al., 2007) and others (Inyard et al., 2007) previous findings in non diabetic rats. To our best knowledge, we showed for the first time that L-NAME infusion had no effect on capillary recruitment and capillary blood flow during contraction in T2D rats. It is important to note that tissue movement may give higher contrast enhancement during the early phase of replenishment (Weber et al., 2006). Nevertheless, with the probe being kept stationary at the identical position, it is assumed that the signals obtained would mainly due to the inflow of new microbubbles but not the false signal enhancement. Also, the short half-life of microbubbles in the circulation (several minutes) was expected not to affect the

result of this study as the measurement was done after a stabilisation of microbubble infusion was achieved.

The regulation of skeletal muscle glucose uptake during contraction/ exercise involves a number of signalling mechanisms that can simultaneously and synergistically contribute to an increase in skeletal muscle glucose uptake (Richter and Hargreaves, 2013). AMPK is one of the potential regulators in this process. An up-regulation of AMPK protein or increased activation of AMPK could potentially compensate for a loss of other signalling, including NO, in regulating skeletal muscle glucose uptake during contraction. However, in this study, there was no difference in total AMPK protein expression between groups and contraction increased AMPK phosphorylation to a similar extent in both groups of rats receiving either saline or L-NAME infusion. Therefore, it appears unlikely that AMPK compensated for NOS inhibition to maintain glucose uptake during contraction. Similarly, the expression of GLUT4 was not different between control and T2D rats, however GLUT4 translocation with contraction was not examined. Endothelial NOS (eNOS) isoform expressed in rat skeletal muscle may also be involved in skeletal muscle glucose uptake during contraction. It has, however, been shown, at least in mice, that eNOS is not required in the normal regulation of skeletal muscle glucose uptake during exercise (Lee-Young et al., 2010). In addition, we found no difference in skeletal muscle eNOS expression between the two groups of rats. Taken together, we have no evidence to suggest compensatory signalling events in the proteins examined that could explain the lack of effect of NOS inhibition on contraction-stimulated skeletal muscle glucose uptake in this study. In addition, these data also suggest that the higher skeletal muscle glucose uptake in T2D rats was not due to altered expression of total AMPK, GLUT4 or eNOS proteins. Nevertheless, it might be that the subcellular compartmentation of these proteins rather than the total expression is more important in determining their role in a physiological process (Gorlich and Kutay, 1999) such as muscle glucose uptake during contraction/ exercise. For example, GLUT4 needs to be translocated from intracellular vesicle to cellular membrane to facilitate glucose

uptake across membrane. The subcellular compartmentation of GLUT4, AMPK and eNOS were, however, not examined in this study.

Studies have shown that muscle contraction/ exercise activate NOS in skeletal muscle and lead to an increase in NO production (Balon and Nadler, 1994, Lee-Young et al., 2009, Roberts et al., 1999, Ross et al., 2007, Tidball et al., 1998). Inhibition of NOS using pharmacological agents has been shown previously to attenuate this increase in NOS activity and NO production induced by muscle contraction (Merry et al., 2010d, Ross et al., 2007). In the present study, however, skeletal muscle NOS activity was not increased in the contracted muscles of either control or T2D rats. T2D muscles tended ($P = 0.051$) to have overall lower NOS activity compared to controls; and, this could be due to the reduced nNOS protein expressions in these rats. It is hard to reconcile why NOS activity was not increased during muscle contraction in the current study. Nevertheless, it fits with the current observation that NOS inhibition had no effect on skeletal muscle glucose uptake during contraction, most likely because NO/ NOS signalling was not increased to stimulate muscle glucose uptake during contraction. In our previous study (Ross et al., 2007), muscle contraction elicited by the same electrical stimulation protocol as the current study led to a significant, albeit small, increase in NOS activity. In that study (Ross et al., 2007), local L-NAME infusion in the contracting leg prevented the activation of NOS and led to a significant attenuation in glucose uptake during contraction. This may reflect strain differences as will be discussed below.

The activation of NOS during contraction/ exercise appears to be at least partly influenced by contraction/ exercise intensity. In a mouse study it was shown that low intensity treadmill running at 45% maximum running speed did not increase NOS activity but NOS activity increased significantly at 70% maximum speed (Lee-Young et al., 2009). Similarly, NO production was only increased with intense stimulation but not moderate stimulation in primary rat skeletal muscle cell culture (Silveira et al., 2003). Activation of NOS also appears to be dependent on factors other than contraction intensity. A mild *ex vivo* contraction stimulation (15 ms trains, 2 Hz & 20 V)

significantly increased NOS activity in Wistar rat muscles (Tidball et al., 1998) while no activation of NOS was observed in SD rat muscle stimulated to contract *ex vivo* with a relatively much greater stimulation protocol (200 ms trains, 100 Hz & 10 V) (Etgen et al., 1997) suggesting that the strain of rat could influence NOS activation during contraction. Consistent with this finding, in this experiment muscle NOS activity in SD rats was not elevated during contraction despite the same stimulation protocol significantly increased NOS activity in the muscle of Wistar rats in our previous experiment (Ross et al., 2007). Thus, it appears that, in addition to contraction intensity, rodent strain differences could also affect activation of NOS in contracting skeletal muscle. When the contraction stimulation/ exercise intensity is strong enough NOS can also be activated in the SD rats. A significant but small increase in NOS activity (~37% above sedentary) was detected in SD rats performed 45 min of exhaustive treadmill running (Roberts et al., 1999). SD rats stimulated to contract *in situ* with a stepwise increase in frequencies (0 to 2 Hz) over 70 min also appeared to have increase in NOS activity as evidenced by a tendency of L-NAME in attenuating muscle glucose uptake during higher frequencies of muscle contraction (Inyard et al., 2007). Collectively, these results suggest that there is interplay between contraction/ exercise intensity and strain of rats in the activation of NOS during contraction/ exercise. It is to note that SD rats is the common rat strain used in our facility for glucose uptake and CEU measurements since several years ago. These rats are equally susceptible with Wistar rats to the induction of T2D using combination of high fat diet and low doses of STZ. As mentioned above, SD rats were also shown to have increased NOS activity and skeletal muscle glucose uptake during exhaustive exercise. Therefore, SD rats were used in the current study.

Rat strain biological differences and susceptibility to pharmacological agents between SD and Wistar rats have been widely reported and well summarised (Kacew and Festing, 1996). These differences span across a wide range of tissues and organs and it highlights that a difference in response to an intervention between these strains of rats is not uncommon. The NOS/ NO signalling-related differences between these rats have

been reported in nervous tissue but no information was previously available from skeletal muscle, which could be a result of better recognised roles of NOS/ NO in nervous tissues. For example, mechanically-evoked withdrawal threshold, which is at least partly modulated by NO signalling, is significantly higher in injured nerve from SD than Wistar rats (Wei et al., 2007). There is no effect of NOS inhibition on long-term potentiation of synaptic transmission in SD rats but a complete blocking of this is observed in Wistar rats (Holscher, 2002). In addition, the percentage of NOS-immunoreactive neurons presence in the spinal cord is noted to be significantly different between SD and Wistar Kyoto rats, an inbred rat derived from the same ancestral of the outbreed Wistar rats (Hinrichs and Llewellyn-Smith, 2009). Another inbred rat strain developed from a Wistar stock - Wistar Furth rats exhibit higher renal nNOS abundance, NO production and a preserved renal cortex NOS activity after renal insult which protects them from developing chronic renal disease (Erdely et al., 2003). In contrast, SD rats have a marked decrease in renal nNOS abundance leading them to develop chronic renal disease following renal injury (Erdely et al., 2003). Furthermore, strain differences in basal glucose uptake and insulin sensitivity have also been observed between SD and Wistar rats (Gaudreault et al., 2001). Extensor digitorum longus muscles of Wistar rats have lower *ex vivo* basal and *in vivo* insulin-stimulated glucose uptakes than SD rats (Gaudreault et al., 2001). *Ex vivo* insulin-stimulated glucose uptake of soleus muscle of Wistar rats is also lower than SD rats (Gaudreault et al., 2001). Collectively, these results suggest that there may be a rat strain difference in skeletal muscle glucose metabolism, in particular, NO-mediated skeletal muscle glucose uptake between SD and Wistar rats, which fits with the differences that we have observed between SD rats (current study) and Wistar rats (Ross et al., 2007).

In conclusion, SD T2D rats had normal muscle capillary recruitment and actually greater skeletal muscle glucose uptake during contraction than control rats and NOS inhibition had no effect on skeletal muscle capillary recruitment or glucose uptake during contraction in control of T2D rats. Strain differences in the regulation of contraction-stimulated muscle glucose uptake between SD and Wistar rats may exist

given that, unlike our previous study in Wistar rats, contraction did not increase NOS activity and NOS inhibition had no effect on NOS activity or contraction-stimulated glucose uptake in SD rats.

CHAPTER FIVE:

SKELETAL MUSCLE GLUCOSE UPTAKE DURING TREADMILL EXERCISE IN nNOSμ$^{-/-}$ MICE

5.1 INTRODUCTION

Skeletal muscle glucose uptake during exercise is an important physiological process for blood glucose and cellular energy homeostasis. It is regulated by both haemodynamic factors and intramuscular signalling that modulate blood flow and membrane permeability to glucose, respectively (Richter and Hargreaves, 2013). Nitric oxide (NO) is a signalling mediator that is involved in both the haemodynamic regulation (Tschakovsky and Joyner, 2008) and alteration of membrane permeability to glucose via modulation of GLUT4 translocation (Etgen et al., 1997, Roberts et al., 1997). The production of NO increases with skeletal muscle contraction/ exercise (Balon and Nadler, 1994, Lau et al., 2000, Ross et al., 2007) and it has been shown in a series of studies using NOS inhibition to mediate skeletal muscle glucose uptake during contraction/ exercise (Balon and Nadler, 1997, Bradley et al., 1999, Kingwell et al., 2002, Merry et al., 2010d, Roberts et al., 1997, Ross et al., 2007).

However, conflicting results have been observed in rodent studies with some studies finding no effect of NOS inhibition on muscle glucose uptake during contraction/ exercise (Etgen et al., 1997, Higaki et al., 2001, Inyard et al., 2007, Rottman et al., 2002). The confounding findings are likely a result of methodological differences between studies. For example, in the studies by Etgen et al. (1997) and Higaki et al. (2001) glucose uptake was measured some time after the contractions had ceased, not during the contractions, in which case in those studies production of NO was likely much attenuated compared with during contractions. Furthermore, in some of these studies the NOS inhibitor was not present during contraction but was only added during measurement of glucose uptake some time after the contraction had ceased (Higaki et al., 2001). Other differences including the use of muscles of primarily different fiber types could have also affected the outcome of the studies. There is a higher expression

of neuronal NO Synthase mu (nNOSμ), the major NOS isoform activated during contraction (Lau et al., 2000), in fast-twitch fibers than slow-twitch fibers (Kobzik et al., 1994, Merry et al., 2010c) suggesting a differential role of NO with different fiber types. This may explain the lack of or lessened effect of NOS inhibition observed on glucose uptake with contraction in slow-twitch muscles (Higaki et al., 2001, Merry et al., 2010c). Our group measures muscle glucose uptake during contraction (Merry et al., 2010d, Merry et al., 2010c, Ross et al., 2007) and administers NOS inhibitor locally to the contracting muscles, (Ross et al., 2007), and has demonstrated consistent results that NOS inhibition attenuated the increase in glucose uptake during contraction.

Adding further complexity to the regulation of skeletal muscle glucose uptake by NO is the fact that NO can be derived from several NOS isoforms, including endothelial NOS (eNOS) and nNOS, which are constitutively expressed in skeletal muscle (Kobzik et al., 1994, Kobzik et al., 1995). Inducible NOS (iNOS) is expressed under inflammatory or disease states (Adams et al., 2002, Hambrecht et al., 1999) and is not likely to be involved in acute contraction-mediated events. The most commonly used NOS inhibitors in studies investigating the role of NO in contraction-stimulated glucose uptake, N-G-Monomethyl-L-arginine (L-NMMA) and N-G-Nitro-L-Arginine Methyl Ester (L-NAME), are non-specific competitive inhibitors that inhibit all these NOS isoforms (Vitecek et al., 2012). Therefore, these NOS inhibitors cannot isolate the role of different NOS isoforms on muscle glucose uptake during contraction/ exercise. As such, genetically modified rodent models are imperative in this regard.

Surprisingly, eNOS knockout ($eNOS^{-/-}$) mice were found to have higher glucose uptake during treadmill exercise. This may have been because of the lower exercise-induced increase in blood flow to the contracting muscle which likely led to a hypoxic state in the muscles which, in turn, may have stimulated a greater muscle glucose uptake (Lee-Young et al., 2010). Indeed, hypoxia is a potent stimulator of skeletal muscle glucose uptake (Cartee et al., 1991). It was shown that NO production was not increased in skeletal muscle of $eNOS^{-/-}$ mice contracted *ex vivo* (Hirschfield et al., 2000)

suggesting that eNOS may not be directly involved in NO-mediated intramuscular signalling. Given that nNOSμ is the major NOS isoform activated during contraction (Lau et al., 2000), it was surprising that it was shown, in this thesis (Fig 3.2), that nNOSμ is not essential in regulating muscle glucose uptake during *ex vivo* contraction. Nevertheless, that study suggested that NO played a role in muscle glucose uptake during contraction as NOS inhibition of isolated nNOSμ knockout ($nNOS\mu^{-/-}$) and wild type ($nNOS\mu^{+/+}$) muscle still attenuated the increase in skeletal muscle glucose uptake during contraction (Fig 3.2). It should be considered that *ex vivo* contraction lacks the complex integrated interactions underlying *in vivo* exercise conditions such as neural input, blood flow and hormonal changes. Highly relevant to this context is that nNOS has been shown to mediate arterial relaxation in contracting skeletal muscle (Lau et al., 2000). Thus, *in vivo* studies are essential to define the role of nNOSμ in muscle glucose uptake during exercise.

Despite their insulin resistance, people with T2D have normal increases in muscle glucose uptake during exercise (Kingwell et al., 2002, Martin et al., 1995). Surprisingly, the regulation of glucose uptake in these people appears to involve a greater reliance on NO given that NOS inhibition resulted in a greater attenuation of the increase in glucose uptake during exercise in people with T2D than controls (Kingwell et al., 2002). This is despite people with insulin resistance/ T2D having only ~60% of normal nNOSμ protein expression in skeletal muscle (Bradley et al., 2007). The mechanism(s) responsible for the greater response in people with T2D during exercise is unknown and it is important to examine the effect of nNOSμ protein expression per se by examining nNOSμ heterozygous ($nNOS\mu^{+/-}$) mice that have ~50% of normal nNOSμ protein expression (Fig 5.10). It would be interesting to examine if partial loss of nNOSμ may lead to an increase in NOS activity that may help explain the higher dependency on NO-mediated muscle glucose uptake during exercise in people with T2D.

In this study, $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ and $nNOS\mu^{-/-}$ mice were used to investigate the effect of nNOSμ on skeletal muscle glucose uptake in conscious and unrestrained

chronically catheterised mice running on a treadmill. This allows examination of the role of nNOSμ in skeletal muscle glucose uptake in a physiological unstressed condition with intact haemodynamic and intramuscular signalling responses. We hypothesised that the increase in muscle glucose uptake during treadmill running would be attenuated in $nNOS\mu^{-/-}$ mice but $nNOS\mu^{+/-}$ mice would have normal contraction-stimulated muscle glucose uptake as seen in people with T2D (Kingwell et al., 2002).

5.2 METHODS

5.2.1 Animals

All procedures were approved by The Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee, and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th Edition). Neuronal NOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ littermates were generated by mating C57Bl/6 nNOSμ$^{+/-}$ mice originally obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping was performed using tail samples obtained at day 21 by a commercial vendor (Transnetyx Inc, Cordova, TN). Mice were housed in standard cages and maintained under constant temperature of 21 ± 1 ^{o}C with 12-hour light/ dark cycle in the AMREP Animal Facility. Animals had access to standard rodent chow and water ad libitum. Both male and female mice were used for experiments at 16 weeks of age.

5.2.2 Exercise stress test

Mice were subjected to an incremental exercise stress test as previously described (Lee-Young et al., 2009) to determine their maximum exercise capacity. Briefly, mice commenced running at a speed of 10 m/min on a 0% incline treadmill. Running speed was increased by 4 m/min every 3 min until mice were exhausted, which was defined as the point whereby mice continuously remained at the back of the treadmill for more than five seconds despite tail prodding.

5.2.3 Surgery and experimental procedures

Surgery procedures were performed as previously described (Ayala et al., 2006) except that only jugular vein cannulation was performed due to an intolerance of nNOSμ$^{-/-}$ mice to carotid cannulation that was observed over several years with several different people conducting the cannulations. Briefly, mice were anaesthetised with 5% isoflurane in oxygen and maintained with 2% isoflurane in oxygen throughout the cannulation procedure. Carprofen was given subcutaneously for pain relief prior to the skin incision for jugular cannulation. The right jugular vein was cannulated with a

silastic catheter (0.3 mm internal diameter (ID)). The free end of the catheter was tunnelled under the skin to the back of the neck where it was exteriorised. The catheter was kept patent with saline containing 200 U/ml of heparin and sealed with stainless steel plugs. Mice were housed individually after surgery and body weight was monitored. Mice were used for experiments three days post-surgery when they had fully recovered as indicated by normal activity, healthy appearance and weight regained after surgery.

On the day of the experiment, the exteriorised jugular catheter was connected, via a stainless steel connector, with Micro-Renathane tubing (0.35 mm ID) approximately one hour prior to the experiment. Mice were then placed in a single lane treadmill to acclimate to the environment. During the experiment, mice remained sedentary or began a single bout of exercise (t = 0 min). Exercise started at 15 m/min (0% incline) for three min and then increased to 17 m/min throughout the rest of the experiment until t = 30 min (Lee-Young et al., 2010, Rottman et al., 2002). Sedentary mice were allowed to move freely on the stationary treadmill for 30 min. In all mice, a bolus of 13 μCi of [1,2-^{3}H]2-deoxy-glucose ([^{3}H]2-DG) was injected into the jugular vein at t = 5 min for evaluation of tissue-specific glucose uptake. At the end of the experiment, mice were anaesthetised with a jugular vein injection of sodium pentobarbital (3 mg). A tail blood sample was immediately obtained for determination of blood glucose levels and plasma [^{3}H]2-DG radioactivity. The gastrocnemius and superficial vastus lateralis muscles from each limb and the brain were rapidly excised, frozen with liquid nitrogen-cooled tongs and stored at -80 ^{o}C. A blood sample was collected via cardiac puncture after exercise and used for plasma insulin and lactate determination.

Initial attempts were made to cannulate the carotid artery together with the jugular vein, however, although nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ mice generally survived this surgery nNOSμ$^{-/-}$ mice did not tolerate the carotid cannulation and did not have a high survival rate. Therefore, only jugular cannulation was performed for all the rats.

Unfortunately, to assess the percentage of cardiac output to the exercising muscles ($\%Q_M$) as an index of muscle blood flow, using microspheres, the microspheres need to be injected into the carotid artery or into the systemic blood circulation but cannot be injected into the pulmonary circulation (via jugular vein) because then almost all of the microspheres would become trapped in the capillaries of the lungs without reaching the systemic circulation. We isolated the lungs and extracted microspheres from two mice, that had been injected with microspheres via jugular vein, as previously described (Lee-Young et al., 2009) and found that ~97% of the injected microspheres were trapped in the lungs. Hence, muscle blood flow was unable to be assessed in this experiment.

5.2.4 Muscle glucose uptake and plasma radioactivity determination

The procedures for muscle glucose uptake determination are described in Chapter Two (Section 2.1.3). Muscle glucose uptake was expressed as an index of phosphorylated [^{3}H]2-DG ([^{3}H]2-DG-6-P) accumulation in the muscle normalised to [^{3}H]2-DG-6-P in the brain of that animal (Halseth et al., 1999, He et al., 2012). Plasma [^{3}H]2-DG concentrations were determined following deproteinisation of the tail blood with 0.3 N $Ba(OH)_2$ and 0.3 N $ZnSO_4$ (Ayala et al., 2007). Plasma [^{3}H]2-DG concentrations were normalised against [^{3}H]2-DG concentrations of infusate and body weight (as an indicator of total blood volume) to provide an index to compare the whole body glucose disposal between individual mouse.

5.2.5 Blood and plasma biochemistry

Plasma insulin concentrations were determined using enzyme-linked immunosorbent assay (Mercodia, AB, Uppsala, Sweden) as per manufacturer's instructions (described in Chapter Two, Section 2.5). Plasma lactate concentrations were analysed with enzymatic method of Lowry and Passonneau (Lowry and Passonneau, 1972) as described in Chapter Two (Section 2.6). Blood glucose levels were determined directly from the tail blood using an ACCU-CHEK Advantage monitor (Roche Diagnostics).

5.2.6 Immunoblotting

Immunoblotting was performed using ground frozen muscle homogenised with 200 volume of solubilising buffer as described in Chapter Two (Section 2.3). The primary antibodies used in this chapter were phospho-AMPKα Thr^{172} (1:1000), phospho-TBC1D1 Ser^{660} (1:1000), AMPKα (1:1000), TBC1D1 (1:500), α-tubulin (1:1000) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1:10,000), eNOS (1:10,000), iNOS (1:2000) (BD Biosciences, San Jose, California, USA); GLUT4 (1:8000) (Thermo Scientific, Rockford, IL, USA), and actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Two different loading control proteins (actin and α-tubulin) were used in this chapter and they were always probed using non-stripped membranes. Actin was used for all except GLUT4 where α-tubulin was used as the loading control as actin and GLUT4 have similar molecular weights and it was not possible to probe both of these proteins without undertaking the stripping process.

5.2.7 NOS activity assay

Skeletal muscle NOS activity was determined as described in Chapter Two (Section 2.2).

5.2.8 Statistical analysis

All data are expressed as means ± SEM. Statistical testing was performed using SPSS statistical package using one factor ANOVA (genotype) or two-factor ANOVA (genotype and exercise), if there was a significant interaction, specific differences between mean values were identified using Fisher's least significance test. The significance level was set at $p < 0.05$. No sex-specific differences were observed in muscle glucose uptake and therefore, data from male and female mice were pooled and analysed together. It should be noted that the sample sizes for $nNOS\mu^{-/-}$ groups were relatively small, i.e. 4 and 6 for sedentary and exercise, respectively due to loss of mice following carotid catheterisation and slower breeding rate of this genotype. In $nNOS\mu^{-/-}$ sedentary group, one of the mice was excluded from glucose uptake analysis as leaking

of [^{3}H]2-DG during injection was noted; therefore, there was only 3 mice in nNOSμ$^{-/-}$ sedentary group for glucose uptake data. There were at least 8 or more mice in each of the sedentary and exercise groups of nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ mice.

5.3 RESULTS

5.3.1 Body weight and exercise capacity of nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ mice

At 16 weeks of age, the body weight of nNOSμ$^{-/-}$ mice was significantly lower than that of nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ littermates while no difference was observed between nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ littermates (Table 5.1). The maximum running speeds achievable during exercise stress test were similar across genotypes (Table 5.1). Similarly, the maximum running times were not different between these mice although nNOSμ$^{-/-}$ mice tended (P = 0.09) to run for a shorter time than nNOSμ$^{+/+}$ littermate (Table 5.1).

Table 5.1: Body weight and exercise capacity of nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ mice

	nNOSμ$^{+/+}$	nNOSμ$^{+/-}$	nNOSμ$^{-/-}$
Body weight (g)	29.0 ± 0.8	30.1 ± 1.2	23.6 ± 1.0 ‡
Max running speed (m/min)	31.5 ± 0.9	30.6 ± 0.6	29.4 ± 1.2
Max running time (min)	17.7 ± 0.6	17.0 ± 0.5	15.9 ± 0.9

Values are means ± SEM, n = 30, 37 and 14 for nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ respectively. ‡ P < 0.05 vs nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$.

5.3.2 Blood glucose level

At the end of experiment, blood glucose concentration from the sedentary mice was not significantly different between all genotypes (7.9 ± 0.5; 8.6 ± 0.5 & 7.3 ± 0.8 mmol/l for $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ & $nNOS\mu^{-/-}$ respectively, $P > 0.05$). Exercise had no effect on the blood glucose concentration compared with the sedentary state and remained similar between genotypes (8.7 ± 1.0; 8.8 ± 0.7 & 7.0 ± 0.3 for $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ & $nNOS\mu^{-/-}$ respectively, $P > 0.05$).

5.3.3 Skeletal muscle glucose uptake

Gastrocnemius muscle glucose uptake at rest (sedentary state) was not different between genotypes. Exercise significantly increased glucose uptake in gastrocnemius muscle (~3 to 6-fold) (Fig 5.1A) while a significantly higher exercise-induced glucose uptake was observed in $nNOS\mu^{-/-}$ mice compared with both $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ mice (Fig 5.1A). A similar muscle glucose uptake pattern was observed in the superficial vastus lateralis (SVL) muscle although muscle glucose uptake in $nNOS\mu^{-/-}$ mice was not significantly higher than $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ (interaction $P = 0.09$) (Fig 5.1B). There was no significant different in DPM count of the brain between genotypes during sedentary or exercise (19.5±10.8; 13.9±7.7; 13.2±6.9; 13.0±4.1; 17.7±6.6 & 17.4±10.2 dpm/mg/min for sedentary and exercise of WT, HET and KO, respectively).

5.3.4 Plasma [^{3}H]2-DG concentration normalised to infusate and body weight

Plasma [^{3}H]2-DG concentration at the end of experiment normalised to [^{3}H]2-DG of infusate and body weight represents an index for the disposal of [^{3}H]2-DG from plasma into tissues i.e. an indicator of whole body glucose disposal. In line with the increased muscle glucose uptake during exercise in all genotypes, this index was significantly lower in all genotypes at the end of exercise compared with sedentary state (Fig 5.2). Disposal of plasma [^{3}H]2-DG at the end of exercise was, however, not significantly lower in $nNOS\mu^{-/-}$ than $nNOS\mu^{+/+}$ or $nNOS\mu^{+/-}$ mice (interaction $P = 0.59$) (Fig 5.2) suggesting that the whole body glucose uptake was similar between genotypes.

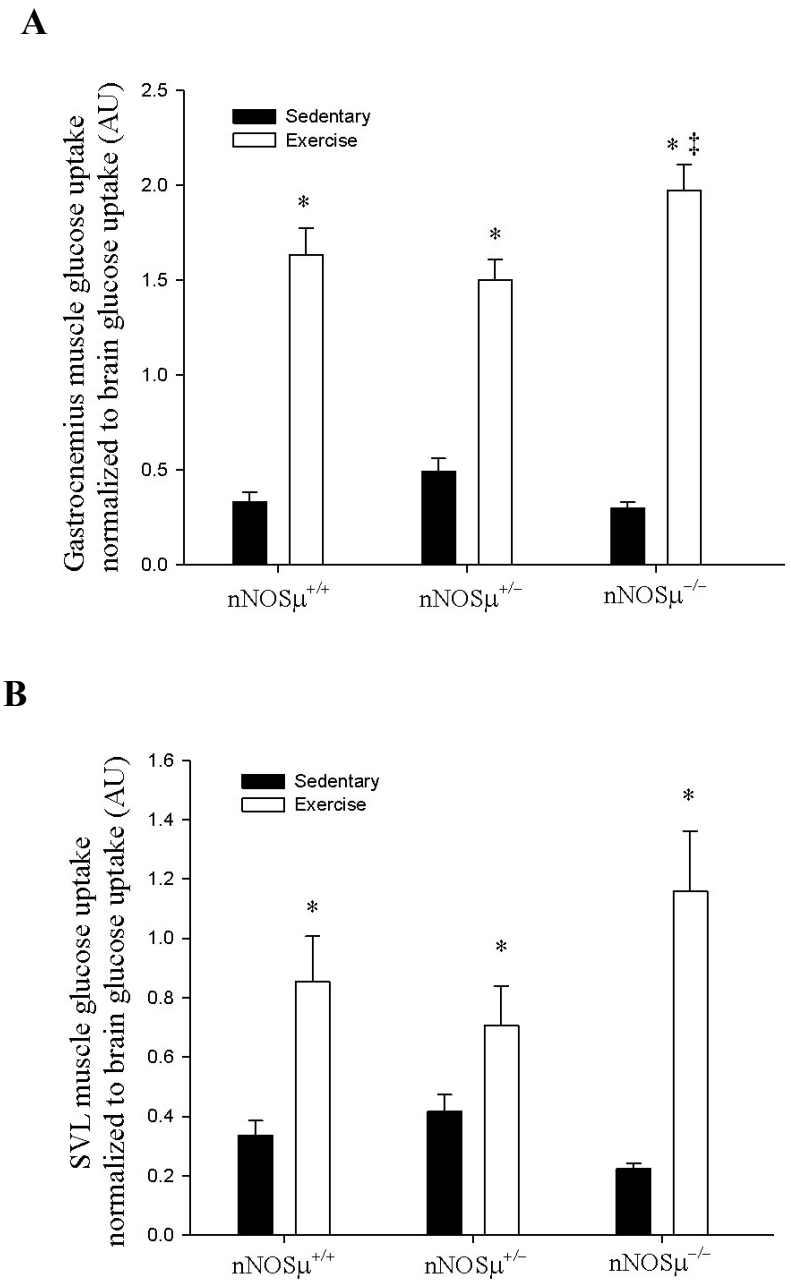


Figure 5.1: Gastrocnemius muscle (A), superficial vastus lateralis muscle (B) glucose uptake normalised to brain glucose uptake of that animal.

Data are means ± SEM, n = 11, 12 & 3 for sedentary nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ respectively, and 10, 8 & 6 for exercise nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ respectively. * $P < 0.05$ vs sedentary of the same genotype; ‡ $P < 0.05$ vs exercise nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$.

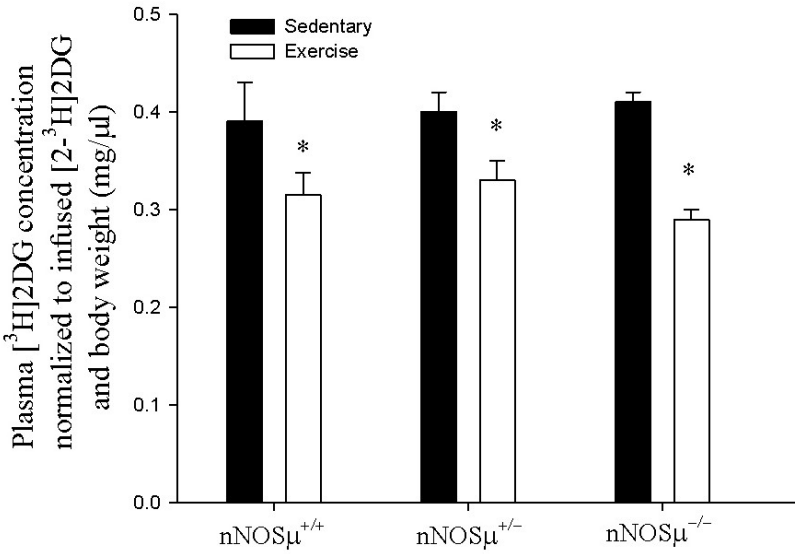


Figure 5.2: Plasma [^{3}H]2-DG at the end of experiment normalised to total infusate [^{3}H]2-DG and body weight.

Data are means ± SEM, n = 8, 10 & 3 for sedentary nNOSµ$^{+/+}$, nNOSµ$^{+/-}$ and nNOSµ$^{-/-}$ respectively & 9, 11 & 6 for exercise nNOSµ$^{+/+}$, nNOSµ$^{+/-}$ and nNOSµ$^{-/-}$ respectively. * $P < 0.05$ vs sedentary of the same genotype.

5.3.5 Plasma insulin and lactate levels

At the end of the exercise, plasma insulin was not different between genotypes (1.00 ± 0.16; 0.97 ± 0.14 & 0.89 ± 0.17 μg/l for nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ & nNOSμ$^{-/-}$ respectively, $P > 0.05$). Plasma lactate was significantly elevated following exercise compared to sedentary state, and its levels following exercise were not significantly different between genotypes (6.0 ± 0.5; 5.2 ± 0.6 & 5.4 ± 0.7 mmol/l for nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ & nNOSμ$^{-/-}$ respectively, $P > 0.05$).

5.3.6 Loading control protein expression

The expression of actin (Fig 5.3A) and α-tubulin (Fig 5.3B) proteins were found to be not different between genotypes indicating that they were suitable to be used as a loading control.

5.3.7 AMPKα protein expression and phosphorylation

Total AMPKα expression in gastrocnemius muscle was similar between all genotypes (Fig 5.4A). In sedentary muscles, AMPKα Thr^{172} phosphorylation relative to AMPKα abundance was not different between genotypes. These findings were in line with that of EDL muscles of nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$ mice from Chapter Three (Fig 3.4). Exercise significantly increased skeletal muscle AMPKα Thr^{172} phosphorylation of nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$ but not the nNOSμ$^{+/-}$ mice (Fig 5.4B) compared to their respective sedentary groups. Exercise-induced AMPKα Thr^{172} phosphorylation was significantly higher in nNOSμ$^{-/-}$ than nNOSμ$^{+/-}$ muscle ($P < 0.05$), but was not significantly different from that of nNOSμ$^{+/+}$ (Fig 5.4B). In view of a possible difference in fold changes of AMPKα Thr^{172} phosphorylation between genotypes following exercise, exercise-induced AMPKα Thr^{172} phosphorylation was normalised to their respective sedentary level to assess the fold changes of AMPKα Thr^{172} phosphorylation during exercise. Exercise stimulated more than 2-fold of AMPKα Thr^{172} phosphorylation in the nNOSμ$^{-/-}$ mice and it was significantly higher than that in both nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ mice (Fig 5.4C). This suggested that a compensatory increase or a stronger

stimulation of AMPKα Thr^{172} phosphorylation occurred in $nNOS\mu^{-/-}$ mouse skeletal muscle during exercise.

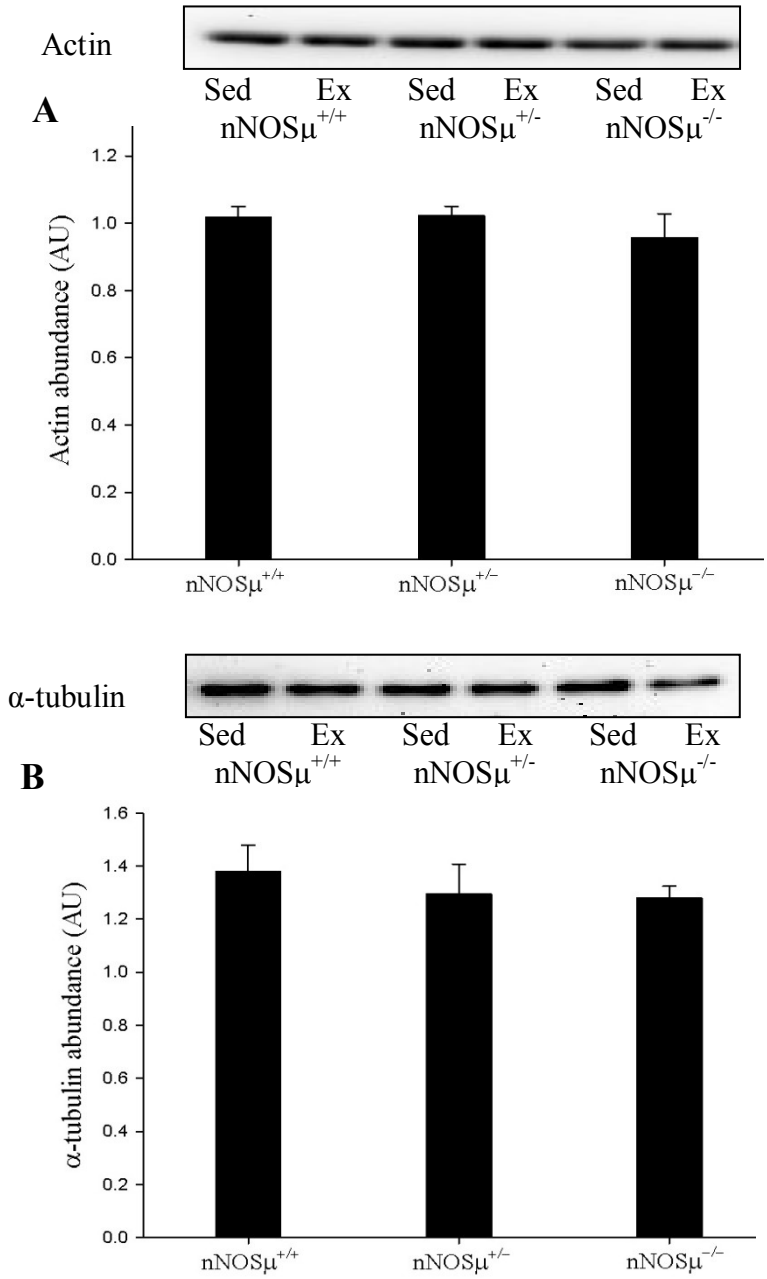
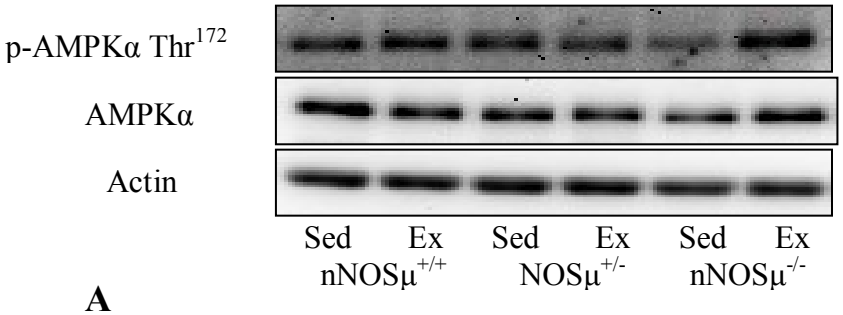
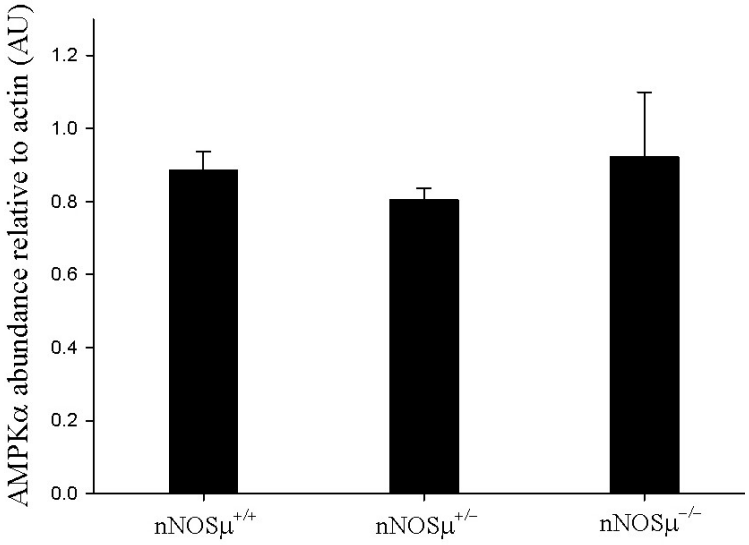


Figure 5.3: Gastrocnemius muscle actin (A) and α-tubulin (B) protein expressions in sedentary mice.

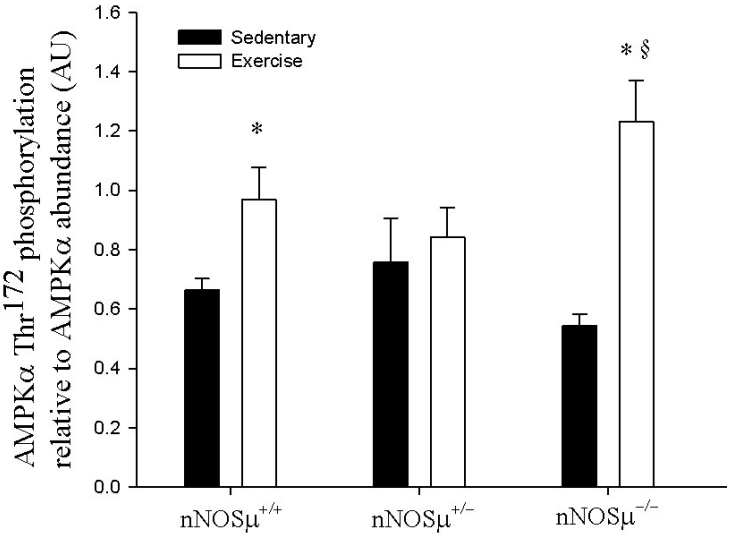
Data are means ± SEM; n = 9 each for both $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ and, 4 for $nNOS\mu^{-/-}$


p-AMPKα Thr172
AMPKα
Actin
Sed
Ex
Sed
Ex
Sed
Ex
nNOSμ+/+
NOSμ+/-
nNOSμ-/-

A


AMPKα abundance relative to actin (AU)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
nNOSμ+/+
nNOSμ+/-
nNOSμ-/-

B


AMPKα Thr172 phosphorylation relative to AMPKα abundance (AU)
Sedentary
Exercise
*
* §
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
nNOSμ+/+
nNOSμ+/-
nNOSμ-/-

C

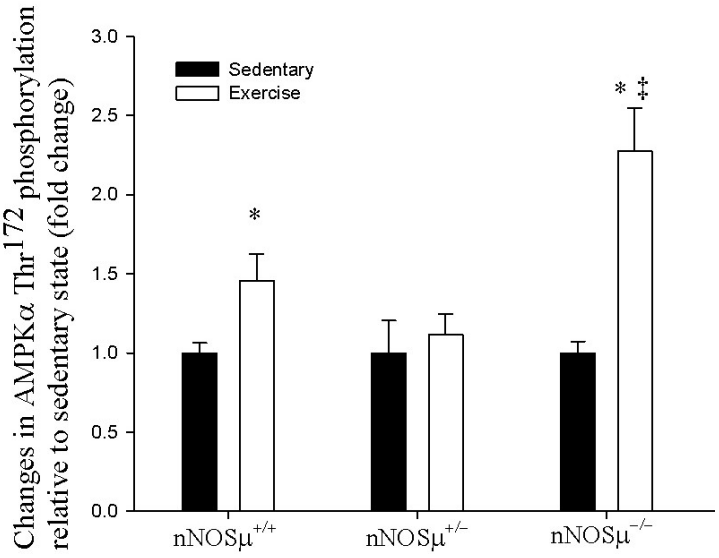


Figure 5.4: Gastrocnemius muscle AMPKα abundance in sedentary muscles (A), AMPKα Thr^{172} phosphorylation relative to AMPKα abundance (B), fold changes of AMPKα Thr^{172} phosphorylation during exercise relative to sedentary state (C).

Data are means ± SEM. For AMPKα abundance, n = 9, 9 and 4 for $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ and $nNOS\mu^{-/-}$ respectively. For AMPKα Thr^{172} phosphorylation, n = 9 each for sedentary and exercise for both $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$; 4 & 5 for $nNOS\mu^{-/-}$ sedentary and exercise respectively. * $P < 0.05$ vs sedentary of the same genotype; § $P < 0.05$ vs exercise $nNOS\mu^{+/-}$; ‡ $P < 0.05$ vs exercise $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$.

5.3.8 TBC1D1 protein expression and phosphorylation

Expression of TBC1D1 in gastrocnemius muscle was similar between all genotypes (Fig 5.5A) and there was no difference in sedentary TBC1D1 Ser^{660} phosphorylation relative to TBC1D1 abundance between genotypes (Fig 5.5B). Exercise had a main effect in increasing TBC1D1 Ser^{660} phosphorylation (Fig 5.5B). Exercise-induced TBC1D1 Ser^{660} phosphorylation was normalised to their respective sedentary level to assess the fold changes of TBC1D1 Ser^{660} phosphorylation during exercise. Exercise had a main effect in increasing fold change in TBC1D1 Ser^{660} phosphorylation. In contrast to AMPKα Thr^{172} phosphorylation, TBC1D1 Ser^{660} phosphorylation, a phosphomotif site of AMPK, was not increased in $nNOS\mu^{-/-}$ mice during exercise compared with sedentary muscles (Fig 5.5C) (interaction P = 0.50).

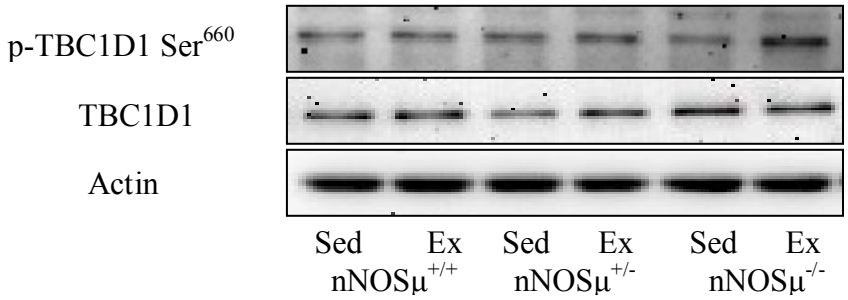
5.3.9 Inducible NOS, eNOS and GLUT4 expression

No iNOS was detected in gastrocnemius muscle of $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ or $nNOS\mu^{-/-}$mice, a finding that is in line with that in EDL muscles of $nNOS\mu^{-/-}$ and $nNOS\mu^{+/+}$ mice in Chapter Three. Endothelial NOS (eNOS) protein expression was not different between genotypes (Fig 5.6). GLUT4 protein expression was also similar across the genotypes (Fig 5.7).

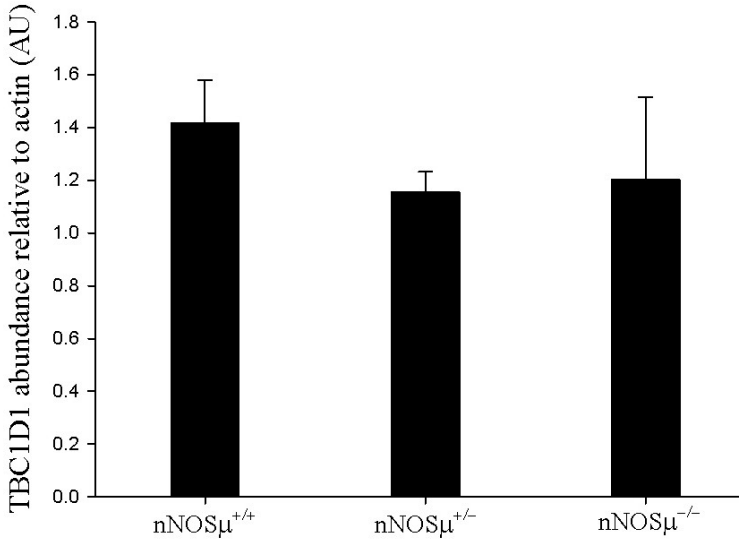
5.3.10 Expression of nNOSμ and nNOS splice variants

As expected, a nNOSμ band at 160 kDa was detected in gastrocnemius muscle of $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ but not in $nNOS\mu^{-/-}$ mice. In $nNOS\mu^{+/-}$ muscle, nNOSμ protein expression was about half of that in $nNOS\mu^{+/+}$ (Fig 5.8A). In order to visualise better the much weaker nNOSβ band at 140 kDa, the membranes were re-imaged with longer exposure time which overexposed the strong nNOSμ band (Fig 5.8 & Fig 5.9). Neuronal NOSβ was detected in $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ gastrocnemius muscles but was not present in $nNOS\mu^{-/-}$ muscle (Fig 5.8B). Neuronal $NOS\mu^{+/-}$ gastrocnemius muscle had less than half of nNOSβ of that in $nNOS\mu^{+/+}$ (Fig 5.8B). Bands at 60 kDa and 52 kDa that were detected in EDL muscle (Fig 3.7A) were also detected in gastrocnemius

muscles of all genotypes (Fig 5.9). All these nNOS protein expression findings observed in the gastrocnemius muscles were similar with that was observed in the EDL muscles in Chapter Three (Fig 3.7A).



A



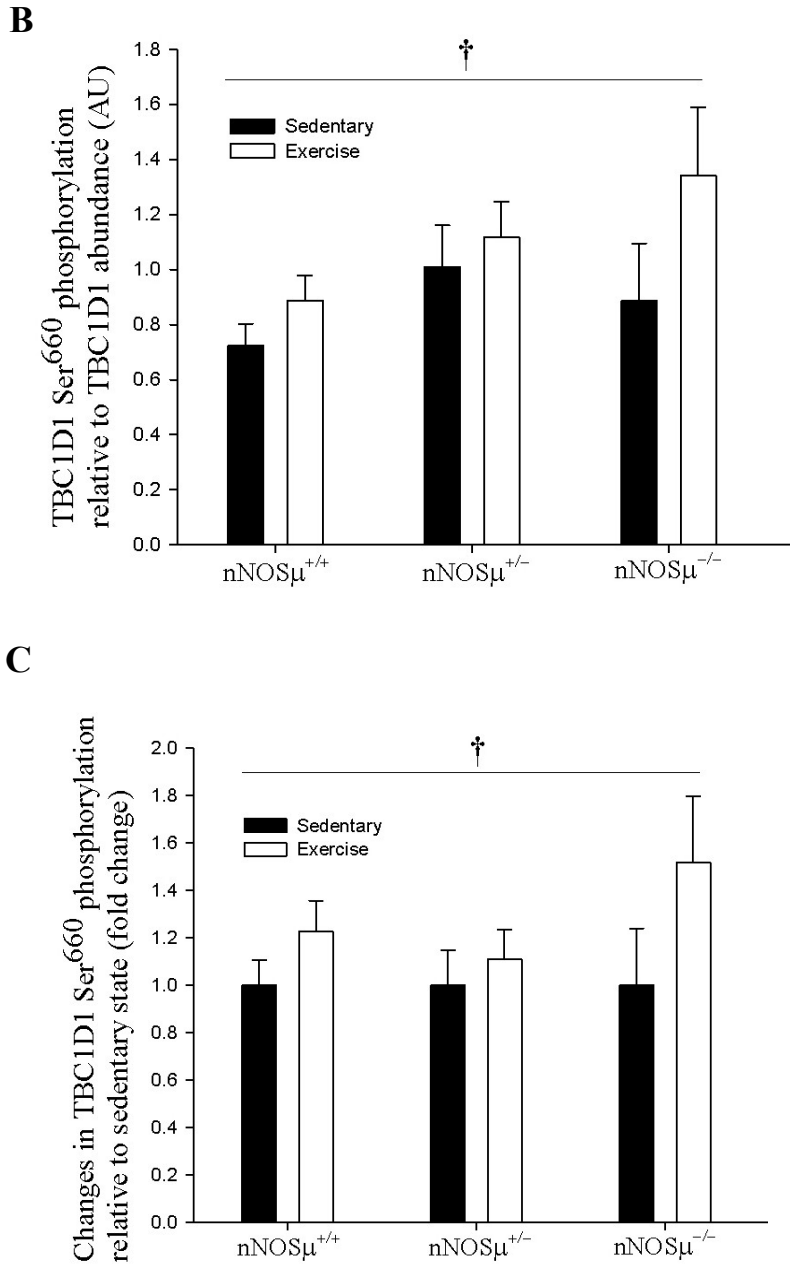


Figure 5.5: Gastrocnemius muscle TBC1D1 abundance in sedentary muscles (A), TBC1D1 Ser^{660} phosphorylation relative to TBC1D1 abundance (B), fold changes of TBC1D1 Ser^{660} phosphorylation during exercise relative to sedentary state (C).

Data are means ± SEM. For TBC1D1 abundance, n = 9, 9 and 4 for $nNOS\mu^{+/+}$, $nNOS\mu^{+/-}$ and $nNOS\mu^{-/-}$ respectively. For TBC1D1 Ser^{660} phosphorylation, n = 9 each for sedentary and exercise for both $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$; 4 & 5 for $nNOS\mu^{-/-}$ sedentary and exercise respectively. † $P < 0.05$ main effect for exercise.

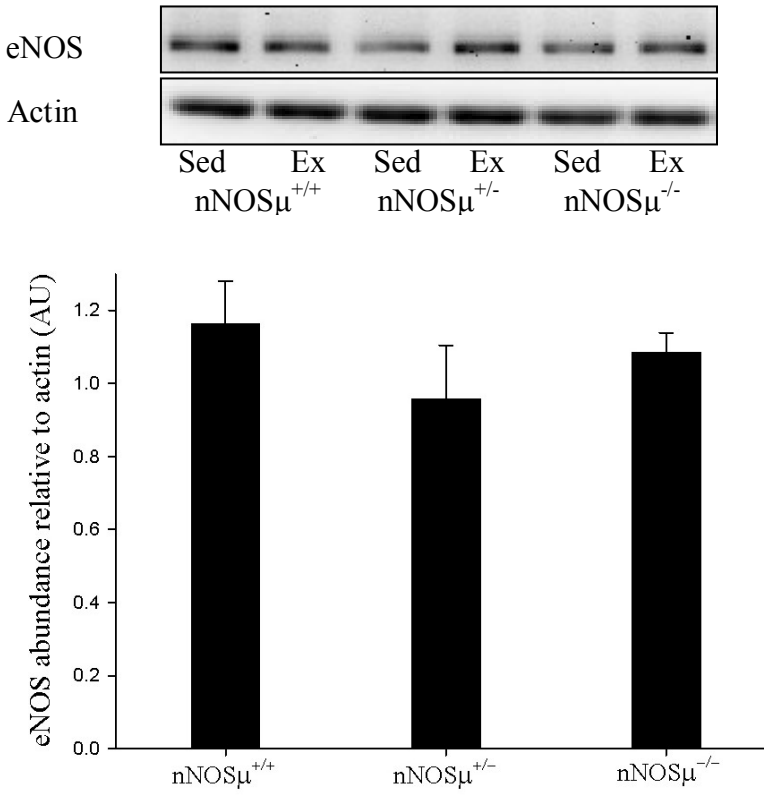


Figure 5.6: Gastrocnemius muscle eNOS protein expression in sedentary muscles relative to actin abundance.

Data are means ± SEM; n = 9 each for both nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ and 4 for nNOSμ$^{-/-}$.

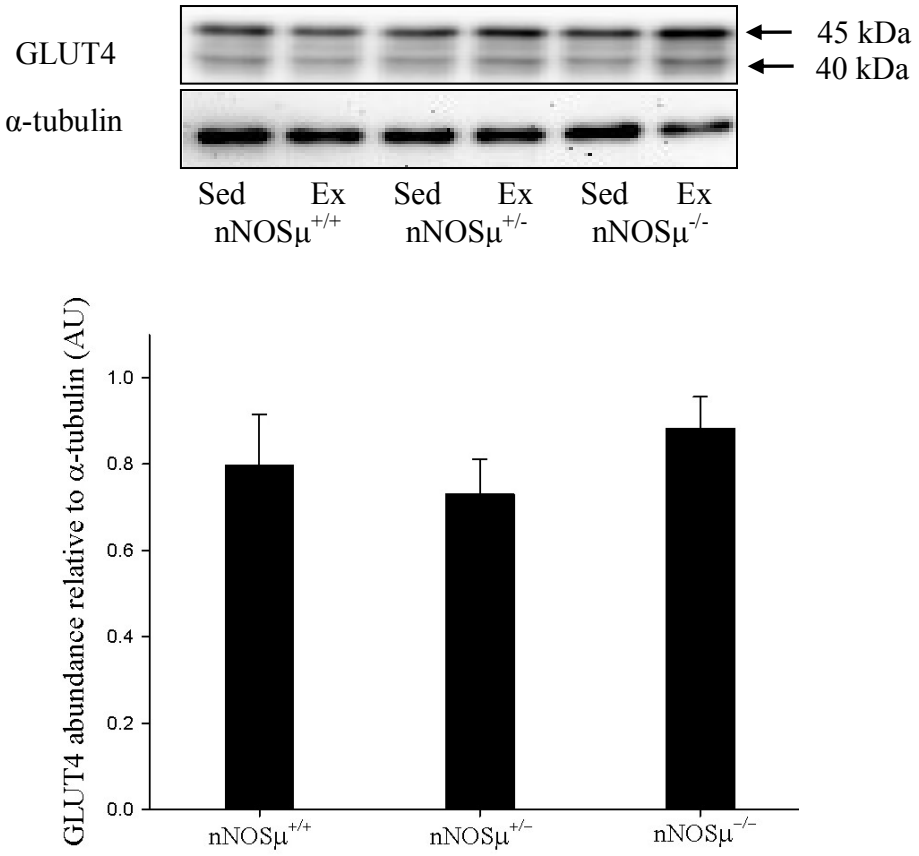


Figure 5.7: Gastrocnemius muscle GLUT4 protein expression in sedentary muscle relative to actin abundance.

Data are means ± SEM; n = 9 each for both nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ and 4 for nNOSμ$^{-/-}$. Bands at 45 and 40 kDa represented glycosylated and de-glycosylated GLUT4 respectively (see Appendix B for more detail). Both bands were used for data analysis.

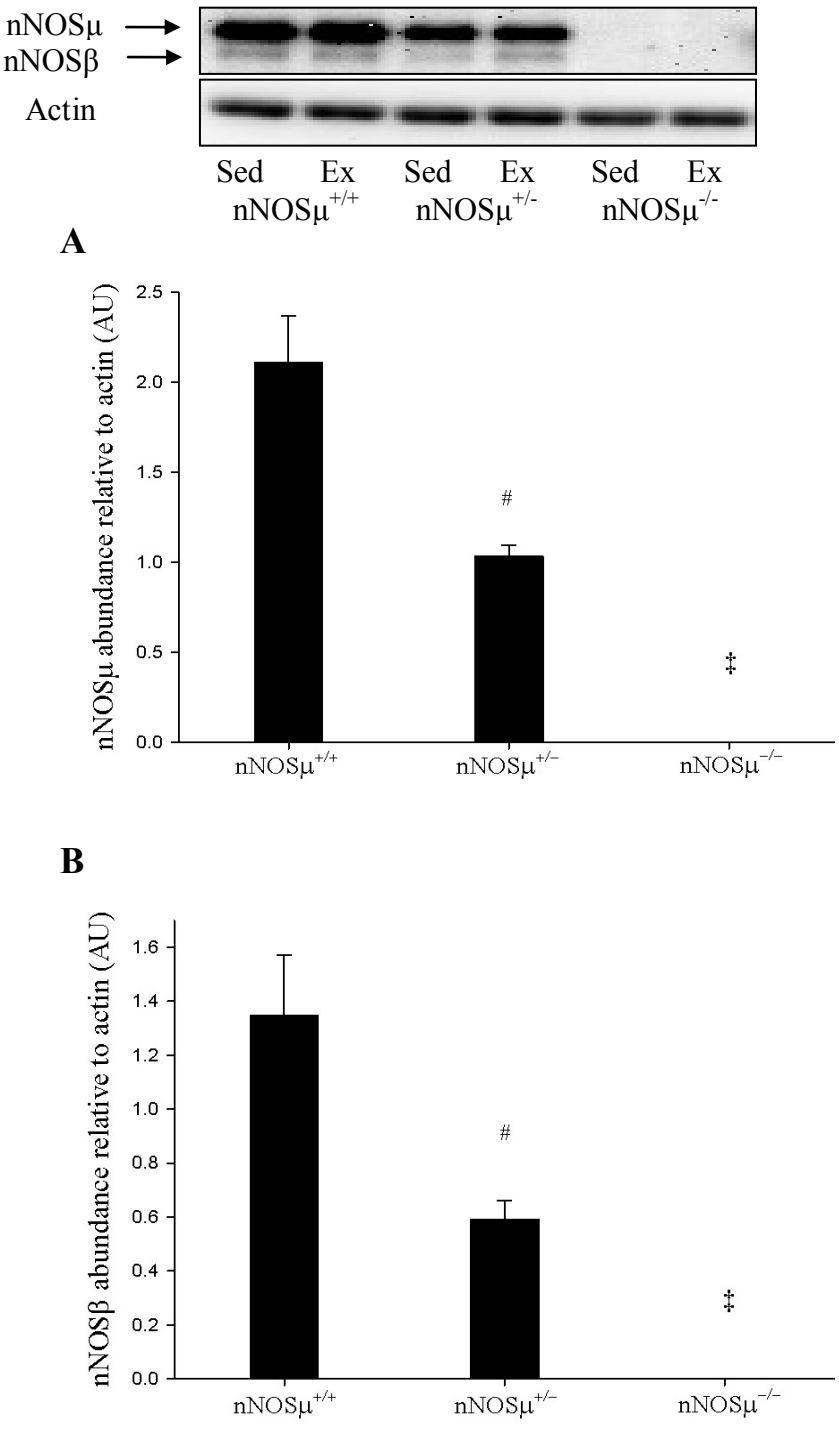


Figure 5.8: Gastrocnemius muscles nNOSμ (A) and nNOSβ (B) protein expressions in sedentary state relative to actin abundance.

Data are means ± SEM; n = 9 each for both $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$ and 4 for $nNOS\mu^{-/-}$. # $P < 0.05$ vs $nNOS\mu^{+/+}$; ‡ $P < 0.05$ vs $nNOS\mu^{+/+}$ and $nNOS\mu^{+/-}$.

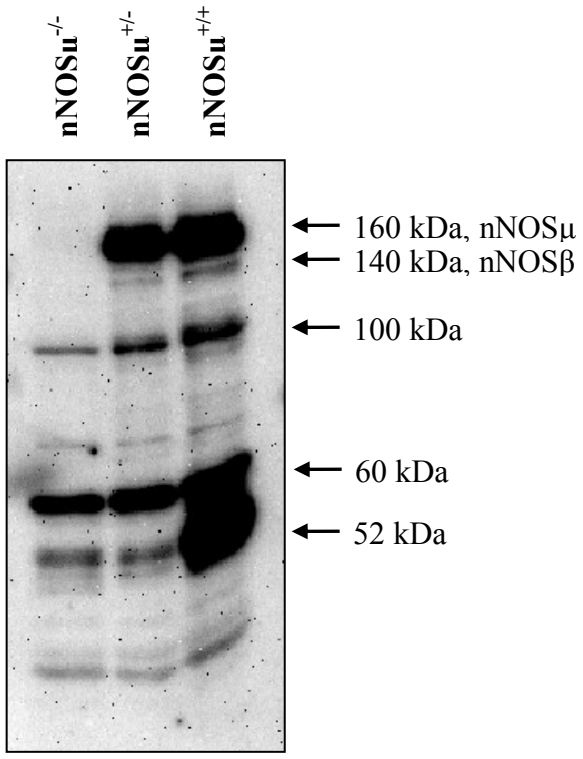


Figure 5.9: Representative blot showing nNOSμ and nNOSβ protein expressions and the appearance of other unknown bands in $nNOS\mu^{-/-}$, $nNOS\mu^{+/-}$ and $nNOS\mu^{+/+}$ gastrocnemius muscles (each lane had 5 μg of total protein).

5.3.11 Muscle NOS activity

In the resting (sedentary) state gastrocnemius muscle NOS activity was significantly different across genotypes (Fig 5.10) with nNOSμ$^{+/-}$ muscle having only approximately half of the NOS activity of nNOSμ$^{+/+}$ while a very small amount of residual NOS activity (~3% of nNOSμ$^{+/+}$) was observed in nNOSμ$^{-/-}$ muscle (Fig 5.10). Similar residual NOS activity was reported in EDL muscle in Chapter Three (Fig 3.8) and brain tissue of nNOSμ mice (Huang et al., 1993). The NOS activity being approximately half in nNOSμ$^{+/-}$ versus nNOSμ$^{+/+}$ was in line with the differences in nNOS protein abundance (Fig 5.8). Exercise significantly increased NOS activity in nNOSμ$^{+/+}$ gastrocnemius muscles ($P < 0.05$) but not in nNOSμ$^{-/-}$ or nNOSμ$^{+/-}$ muscles (Fig 5.10).

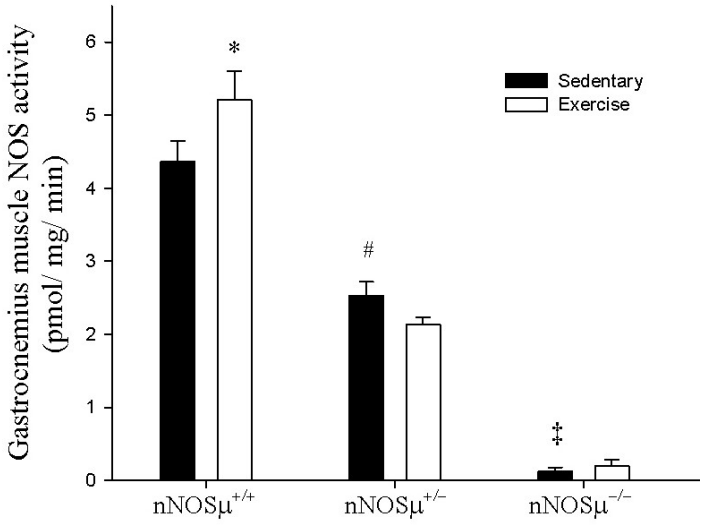


Figure 5.10: Gastrocnemius muscle NOS activity at rest (sedentary) or during exercise. Data are means ± SEM; n = 7, 9 & 3 for sedentary nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ respectively, and 7, 9 & 5 for exercise nNOSμ$^{+/+}$, nNOSμ$^{+/-}$ and nNOSμ$^{-/-}$ respectively. * $P < 0.05$ vs sedentary of the same genotype; # $P < 0.01$ vs sedentary nNOSμ$^{+/+}$; ‡ $P < 0.05$ vs sedentary nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$.

5.4 DISCUSSION

The main finding of this study was that surprisingly nNOSμ$^{-/-}$ mice had a higher, not lower, skeletal muscle glucose uptake than nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ littermates during 30-min of moderate intensity treadmill running. The higher muscle glucose uptake in nNOSμ$^{-/-}$ mice was associated with a greater increase in AMPK phosphorylation during exercise. Neuronal NOSμ$^{+/-}$ mice, on the other hand, had a normal exercise-induced glucose uptake and no significant increase in AMPK phosphorylation during exercise.

We have demonstrated earlier in this thesis that nNOSμ was not essential for skeletal muscle glucose uptake during *ex vivo* contraction although NO signalling was still shown to be present to mediate muscle glucose uptake in muscles with and without nNOSμ as evidenced by the attenuation of contraction-stimulated glucose uptake by NOS inhibition (Fig 3.2). Here, we showed that exercise-induced muscle glucose uptake in nNOSμ$^{-/-}$ mice was elevated above nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ under a physiological *in vivo* exercise condition. The interpretation of muscle glucose uptake under *in vivo* conditions has to be considered beyond the signalling events within the muscle and should consider the muscle glucose uptake in an integrated context where various endocrine, vascular, neural and internal millieu inputs could affect skeletal muscle glucose uptake.

Neuronal NOSμ$^{-/-}$ mice were generally comparable with their nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ littermates in a number of phenotypic features that may directly or indirectly influence muscle glucose uptake. The blood glucose level at rest (sedentary) or during exercise was similar between genotypes implying that the higher glucose uptake in nNOSμ$^{-/-}$ mice was not due to higher blood glucose levels. Similarly, plasma insulin levels after exercise were not different between genotypes suggesting that the observed higher glucose uptake in nNOSμ$^{-/-}$ mice was not due to a potential additive effect of insulin on contraction-stimulated glucose uptake.

Exercise stimulated a greater muscle AMPK phosphorylation in nNOSμ$^{-/-}$ mice compared with nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ littermates. AMPK is a metabolic fuel sensor that can be activated following metabolic stress/ perturbations in which the degradation of ATP and the consequent accumulation of ADP and AMP increase AMP/ ATP ratio which leads to an increase in phosphorylation of AMPK (Towler and Hardie, 2007). The higher AMPK phosphorylation in nNOSμ$^{-/-}$ mice suggested that they may have endured a higher metabolic stress. However, the plasma lactate data did not support this with similar increases in plasma lactate concentrations between genotypes suggesting that similar metabolic stress/ perturbation occurred across all genotypes. Indeed, this is in line with the finding that different mice groups had similar maximum exercise capacity (maximal running speed and time). Alternatively, AMPK can also be activated under hypoxic conditions (Hayashi et al., 2000, Wadley et al., 2006). Neuronal NOS has been shown to be involved in mediating arteriolar relaxation in contracting muscles (Lau et al., 2000, Thomas et al., 2003). Therefore, it is plausible that nNOSμ$^{-/-}$ mice might have attenuated blood flow during exercise leading to muscle hypoxia and subsequent phosphorylation of AMPK. However, eNOS$^{-/-}$ mice with lower exercise-induced increase in blood flow to the contracting muscle and a likely hypoxic state in the muscles have no increase in AMPK phosphorylation during exercise (Lee-Young et al., 2010). This finding does not support the speculation of hypoxia-induced increase in AMPK phosphorylation in nNOSμ$^{-/-}$ mice during exercise. Therefore, the stimulus for the increased AMPK phosphorylation in nNOSμ$^{-/-}$ mice remained unclear. Though the higher muscle glucose uptake in nNOSμ$^{-/-}$ mice could be due to the increased AMPK phosphorylation, we have no direct evidence to prove a causal relationship between the increased AMPK phosphorylation and elevated glucose uptake in nNOSμ$^{-/-}$ mice as we have not assessed the glucose uptake in these mice while preventing the increased of AMPK phosphorylation.

TBC1D1 has been implicated in the regulation of translocation of GLUT4 during contraction/ exercise and TBC1D1 Ser^{660} phosphorylation is one of the downstream effectors of AMPK (Vichaiwong et al., 2010) to stimulate GLUT4 translocation and

glucose uptake. TBC1D1 Ser^{660} phosphorylation increased with exercise suggesting that the higher glucose uptake in nNOSμ$^{-/-}$ mice may be mediated by an AMPK-TBC1D1 mechanism. However, unlike AMPK phosphorylation, TBC1D1 Ser^{660} phosphorylation did not increase significantly more in nNOSμ$^{-/-}$ mice than nNOSμ$^{+/+}$. Perhaps other AMPK phosphomotif sites on TBC1D1 (Treebak et al., 2014, Vichaiwong et al., 2010) may be more important for mediating AMPK's effect on GLUT4 translocation and glucose uptake. Alternatively, AMPK can also phosphorylate other downstream mediators such as AS160 to stimulate muscle glucose uptake (Kramer et al., 2006b) although recent evidence does not support a role of AMPK-mediated AS160 phosphorylation in muscle glucose uptake with contraction (Treebak et al., 2010).

It is unfortunate that we have no data on the blood flow to the contracting muscle during exercise to assess if there was any change to the blood flow that may have affected muscle glucose uptake in these mice. Assessment of blood flow to the muscle using microspheres was not possible because we were unable to cannulate the carotid artery of nNOSμ$^{-/-}$ mice. Carotid artery cannulation was initially attempted on these mice; however, due to unknown reasons nNOSμ$^{-/-}$ mice usually did not survive the carotid catheterisation procedure.

A caveat to the interpretation of data using genetically-modified mice must be considered. The loss of a protein of interest during development that spans the entire life could possibly induce secondary adaptations including compensatory overexpression of closely related proteins (Meeson et al., 2001). These changes could mask the effects elicited by the loss of the protein of interest. In this study, no compensatory increase in iNOS, eNOS, nNOS splice variants, GLUT4 or NOS activity, all of which could directly or indirectly affect muscle glucose uptake, were detected in nNOSμ$^{-/-}$ mice. The increase AMPK phosphorylation was also not a result of a compensatory increase in total AMPK expression. Likewise, no overexpression of TBC1D1 was seen. These data, together with the finding that nNOSμ$^{-/-}$ and nNOSμ$^{+/-}$ mice had greater and normal glucose uptake during exercise respectively, suggest that nNOSμ may not play a role in muscle

glucose uptake during *in vivo* exercise as total or partial loss of nNOSμ did not attenuate glucose uptake nor elicit a compensatory response in the proteins examined. It should be considered, however, that there may have been compensatory increases in the many other potential proteins that may regulate skeletal muscle glucose uptake including Ca^{2+}/ calmodulin-dependent protein kinase (CaMKII), protein kinase C, Rac1/PAK1 and also changes in reactive oxygen species (ROS).

Alternatively, although it would seem unlikely, the small remaining residual NOS activity/ signalling as shown in *ex vivo* contraction in Chapter Three (Fig 3.2 and 3.7) may have been adequate to stimulate normal glucose uptake during exercise. Indeed, studies investigating the role of AMPK in contraction-stimulated muscle glucose uptake using mice with single mutations or deletions in muscle α or β subunit generally have normal glucose uptake with these mice demonstrating a varying degree of residual contraction-induced AMPK phosphorylation or activity (Fujii et al., 2005, Jorgensen et al., 2004, Maarbjerg et al., 2009, Steinberg et al., 2010). When these residual contraction-induced AMPK phosphorylation or activity were eliminated via double knockout of muscle AMPKβ1β2 subunits, about 55 - 70% attenuation of the increase in muscle glucose uptake during contraction was observed (O'Neill et al., 2011). These results suggest that residual AMPK phosphorylation or activity in genetically-modified AMPK mice is adequate to maintain a normal muscle glucose uptake during contraction/ exercise. Something similar may be occurring in nNOSμ$^{-/-}$ muscle with residual NOS activity/ NO signalling.

Unlike nNOSμ$^{-/-}$ mice, glucose uptake during exercise in nNOSμ$^{+/-}$ mice, with ~50% reduction of nNOSμ, was normal. This is line with the finding that people or rodents with insulin resistant/ T2D and an accompanied reduction of nNOSμ protein expression (Bradley et al., 2007, Perreault et al., 2000, Sasaki et al., 1998) have normal glucose uptake during exercise (Brozinick et al., 1992, Brozinick et al., 1994b, Kingwell et al., 2002). However, it is unknown if nNOSμ$^{+/-}$ mice were more dependent of NO signalling to maintain the normal glucose uptake as previously shown in people with

T2D (Kingwell et al., 2002). We observed no paradoxical increase in NOS activity in nNOSμ$^{+/-}$ mice, as previously reported with eNOS$^{+/-}$ and eNOS$^{-/-}$ mice (Lee-Young et al., 2010), to suggest a possible greater NO-dependent muscle glucose uptake during exercise in these mice. In fact, nNOSμ$^{+/-}$ mice had no significant increase in NOS activity and AMPK phosphorylation during exercise raising the question about the putative signalling pathways that may involved in mediating muscle glucose uptake in these mice. Again, other potential signalling pathways including ROS and Ca^{2+}/ CaMKII may be involved. It is unfortunate that our attempts to assess the involvement of CaMKII signalling in this process via immunoblotting were unsuccessful. Multiple weak-intensity bands were detected across a wide range of molecular weights despite using three different types of primary antibodies. We were not satisfied and cannot be sure that the correct bands were quantified, thus these data were not included.

In summary, skeletal muscle glucose uptake in nNOSμ$^{-/-}$ mice is not reduced and is actually greater during a moderate intensity treadmill exercise than nNOSμ$^{+/+}$ and nNOSμ$^{+/-}$ mice, possibly due to a compensatory increase in AMPK activation. The normal glucose uptake during exercise in mice with a partial loss of nNOSμ is unlikely to be mediated via AMPK phosphorylation.

CHAPTER SIX:

DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

A major finding of this thesis was that nNOSμ did not appear to be essential in the regulation of skeletal muscle glucose uptake during *ex vivo* contraction/ exercise (Fig 3.2 and Fig 5.1). However, NO was involved in the regulation of glucose uptake in nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$ muscles during contraction as evidenced by the attenuation of glucose uptake by L-NMMA (Fig 3.2). The attenuation was reversed by L-arginine indicating that L-NMMA specifically inhibited NOS to attenuate muscle glucose uptake (Fig 3.2). During *in vivo* exercise, total loss of nNOSμ (nNOSμ$^{-/-}$) was actually associated with a higher skeletal muscle glucose uptake (Fig 5.1A) and a greater increase in AMPK phosphorylation during exercise (Fig 5.4C); however, whether there was a causal relationship between increased AMPK phosphorylation and glucose uptake during exercise was not determined in that study. On the other hand, mice with partial loss of nNOSμ (nNOSμ$^{+/-}$) had normal muscle glucose uptake during exercise (Fig 5.1A) but had no increase in AMPK phosphorylation during exercise (Fig 5.4C). In rat *in situ* contraction, NOS activity was not increased with contraction in Sprague Dawley (SD) rats (Fig 4.14), as compared with our previous study showing an increase in NOS activity during contraction in Wistar rats (Ross et al., 2007), and this may explain why no effect of NOS inhibition on glucose uptake during contraction was observed. This may have been due to the well documented strain differences between the two strains of rats (Gaudreault et al., 2001, Holscher, 2002, Kacew and Festing, 1996, Wei et al., 2007).

The primary aim of this thesis is to investigate the role of NO and/ or nNOSμ (the major NOS isoform activated during contraction (Lau et al., 2000)) in the regulation of skeletal muscle glucose uptake during contraction/ exercise. The regulation of skeletal muscle glucose uptake is a very complex process influenced by both haemodynamic factors and intramuscular signalling (Richter and Hargreaves, 2013). A number of potential signalling pathways have been implicated in this process including Ca^{2+}/

CaMKII (Witczak et al., 2007, Wright et al., 2004), AMPK (Hayashi et al., 1998, Mu et al., 2001), PKC (Ihlemann et al., 1999a, Niu et al., 2011), ROS (Merry et al., 2010c, Sandstrom et al., 2006) and NO (Balon, 1998, Merry et al., 2010c, Roberts et al., 1997, Ross et al., 2007), which likely work in a redundant manner. The investigation of the role of NO in muscle glucose uptake during contraction/ exercise is made more complicated given that in skeletal muscle NO can be derived from various NOS isoforms such as eNOS, nNOSμ and iNOS (Stamler and Meissner, 2001) and NO is also involved in the regulation of blood flow during contraction/ exercise in rodents (Hirai et al., 1994). Therefore, investigating the role of NO in contraction-stimulated glucose uptake using both *ex vivo* and *in situ* contraction and *in vivo* exercise models is necessary. Under *ex vivo* contractions, the intramuscular role of NO and nNOSμ in regulating muscle glucose uptake can be assessed without the influence of blood flow. On the other hand, *in situ* contraction and *in vivo* exercise models allow assessment of the role of NO/ NOSμ in this process under more integrated physiological conditions with intact blood flow.

In this thesis, we have modified the timing of glucose uptake measurement as compared to previous studies (Etgen et al., 1997, Higaki et al., 2001, Merry et al., 2010d) for investigating skeletal muscle glucose uptake during contraction in rodent models. As discussed in Chapter One, a delay in muscle glucose uptake measurement some time after the contraction had ceased and the systemic administration of NOS inhibitors have been identified (McConell and Kingwell, 2006) as among the main reasons contributing to the conflicting results found in the rodent literature investigating the role of NO in muscle glucose uptake during contraction/ exercise. Here, skeletal muscle glucose uptake measurement for all the experiments was done during contraction/ exercise, rather than afterwards. Therefore, this is more of a true reflection of the actual muscle glucose uptake during contraction/ exercise. This also allows for a better time-course matching of intramuscular signalling with contraction-stimulated muscle glucose uptake. Ideally, to measure the contraction-activated signalling that mediates muscle glucose uptake during contraction muscle samples need to be collected

and freeze-clamped immediately after contraction has ceased. If this is not done the timing of the signalling events does not truly reflect the timing of the glucose uptake, thus making interpretation of the effects of signalling events on glucose uptake during contraction challenging, if not erroneous. In this thesis we measured skeletal muscle glucose uptake during contraction/ exercise and collected muscle samples for biochemical analyses as soon as possible after the contraction/ exercise.

In this thesis we also avoided the use of systemic NOS inhibition (systemic intravenous injection or oral ingestion of NOS inhibitors) to investigate the role of NO in muscle glucose uptake during *in situ* and *in vivo* exercise by the use of local NOS inhibition and nNOSμ genetically-modified mouse model, respectively. Systemic administration of NOS inhibitors is associated with an increase in systemic blood pressure (Higaki et al., 2001, Inyard et al., 2007, Kaikita et al., 2001) and blood flow (Inyard et al., 2007) and increases in blood flow can lead to an increase in glucose uptake (Zinker et al., 1993) which complicates the interpretation of the role of NO in muscle glucose uptake (Inyard et al., 2007). Neuronal NOSμ$^{-/-}$ mice, on the other hand, have normal blood pressure (Kuhlencordt et al., 2006). Systemic administration of a NOS inhibitor is also associated with other side effects including elevation in body temperature (Bernstein et al., 1996) and inhibition of brain NOS activity (Iadecola et al., 1994) which may affect the general well being of the mice performing exercise.

The *ex vivo* experiment using nNOSμ$^{-/-}$ muscles (Chapter Three) indicated that NO is involved in skeletal muscle glucose uptake during contraction, the evidence from the other studies in this thesis were, however, less apparent. The complex regulation of NOS/ NO signalling during contraction complicates the interpretation of the findings in these experiments. One of the main unanswered questions is relates to the source of NO during *ex vivo* contraction in nNOSμ$^{-/-}$ muscles. Since nNOSμ was not present in these muscles it suggests that other NOS isoforms or splice variants were involved in NO production during *ex vivo* contraction. This is unexpected as nNOSμ has been previously shown to be the major NOS isoform activated during *ex vivo* contraction as evidence by

a lack of increase in cGMP, the major downstream signalling of NO, during *ex vivo* contraction in nNOSμ$^{-/-}$ muscle (Lau et al., 2000). However, it should be considered that NO/ cGMP/ PKG pathway may not be the mechanism that NO signals glucose uptake during contraction since our group has shown that ODQ, which prevents sGC production of cGMP, and PKG inhibition, had no effect on muscle glucose uptake during *ex vivo* contraction (Merry et al., 2010d). Taken together these findings suggest that a cGMP-independent pathway likely mediates skeletal muscle glucose uptake during *ex vivo* contraction and that nNOSμ is not necessarily the NOS isoform that is important to elicit NO's effect during contraction/ exercise. On the other hand, eNOS is also unlikely to mediate muscle glucose uptake during contraction as eNOS appears to not contributing to the production of NO in skeletal muscle during *ex vivo* contraction (Hirschfield et al., 2000, Lau et al., 2000). In addition, mice lacking eNOS have a higher muscle glucose uptake during exercise that is postulated to due to exercise-induced hypoxia (Lee-Young et al., 2010) suggesting that eNOS has no direct effect on muscle glucose uptake. This is, in fact, in line with the human studies that demonstrated a role of NO in mediating muscle glucose uptake during exercise (Bradley et al., 1999, Kingwell et al., 2002). Since little or no eNOS is expressed in human skeletal muscles (McConell et al., 2007), this suggests that eNOS is unlikely to be involved in mediating muscle glucose uptake during exercise in humans. It is possible that eNOS from the vascular compartment was activated during exercise and produced great amount of NO that may modulate intramuscular signalling to stimulate GLUT4 translocation and glucose uptake. However, two factors may limit the effect of NO, produced in vascular compartment, on surrounding cells. Haemoglobin is a good chelator of NO (Young et al., 1997), chelation of NO in the circulation by haemoglobin could prevent NO from having an effect beyond vascular compartment. Also, NO is a highly reactive molecule that has a short diffusion range before it is converted to a more stable NO intermediate (Martinez-Ruiz and Lamas, 2009); this may prevent the NO from vascular compartment to have an effect on surrounding cells. The similar response in exercise-induced muscle glucose uptake in both eNOS$^{-/-}$ (Lee-Young et al., 2010) and nNOSμ$^{-/-}$ (Chapter Five) mice suggests that both eNOS and nNOSμ might have an identical role in muscle glucose

uptake, i.e. both are either involve in or have no role in modulating muscle glucose uptake. Since nNOSμ and eNOS were showed to be not essential for stimulating glucose uptake and does not contribute to NO production, respectively during *ex vivo* contraction, it is likely that both nNOSμ and eNOS are not involved in glucose uptake during contraction/ exercise. Although a previous study detected an increase in iNOS mRNA during exercise suggesting a role of iNOS in adaptation to exercise (Gomez-Cabrera et al., 2005) we did not detect any iNOS protein expression in the mouse muscles during contraction/ exercise in this thesis suggesting that if the elevated iNOS mRNA during contraction/ exercise were to be translated into iNOS protein, then its function would be more related to post-exercise state. Taken together, these data suggest that all the major NOS isoforms (nNOSμ, eNOS and iNOS if any) in the skeletal muscle may not be involved in NO-mediated glucose uptake during contraction.

Despite seemingly no role of the major NOS isoforms in contraction-stimulated muscle glucose uptake, evidence from this thesis (Fig 3.2) and others support the concept that NO regulates skeletal muscle glucose uptake during contraction (Balon and Nadler, 1997, Bradley et al., 1999, Merry et al., 2010d, Roberts et al., 1997, Ross et al., 2007). Perhaps, other nNOS splice variants such as nNOSβ, which has been reported to be present in skeletal muscle (Percival et al., 2010, Rothe et al., 2005), may be responsible for NO-mediated muscle glucose uptake during contraction. Nevertheless, we (Fig 3.7A) and others (Baum et al., 2002) detected no nNOSβ protein expression in nNOSμ$^{-/-}$ skeletal muscles raising more questions about the source and downstream signalling of contraction-NO-mediated glucose uptake. Two nNOS-immunoreactive bands (60 and 52 kDa proteins) were detected in nNOSμ$^{-/-}$ muscles (Fig 3.7A) and to our knowledge, they have not been reported before. However, the role and significance of these proteins are unknown and remain to be investigated.

Neuronal NOSμ$^{-/-}$ skeletal muscle was associated with a small residual NOS activity (~4% of nNOSμ$^{+/+}$) (Fig 3.8). However, it is hard to imagine that such a small amount of NOS activity is all that is required to regulate NO's contribution to

contraction-stimulated glucose uptake. Yet, this residual NOS activity appears to be of importance in nNOSμ$^{-/-}$ mice since residual NOS activity of up to 7% in the central nervous system (CNS) is apparently adequate to protect nNOSμ$^{-/-}$ mice from suffering severe histopathological abnormalities in the CNS (Huang et al., 1993). This is despite NO is being implicated in a number of important neurological functions such as neurotransmitter release (Montague et al., 1994), motor neuron development (Kalb and Agostini, 1993), synaptic plasticity and memory formation (Bohme et al., 1993). In isolated skeletal muscle of nNOSμ$^{-/-}$ mice (KN1 mice), muscle integrity and performance also appears to be normal (Percival et al., 2010); in contrast, skeletal muscles with total loss of all nNOS splice variants (KN2 mice) fatigue faster and have severe disruption of the microtubule cytoskeleton (Percival et al., 2010). These results suggest that the residual NOS activity may be critical in preserving most, if not all of, the normal neurological and muscular functions of nNOSμ$^{-/-}$ mice and could possibly in some way be responsible, at least in part, for the normal increase in skeletal muscle glucose uptake during contraction. Despite its effectiveness in preserving neurological and muscular functions, it is difficult to reconcile how this small amount of NOS activity can be responsible for normal glucose uptake during contraction. During NOS inhibition at the dose that we used (100 μM), NOS activity was never inhibited to zero and in fact was between 10% to just slightly below basal levels as shown in this thesis (Fig 3.8) and by others (Higaki et al., 2001, Merry et al., 2010c, Merry et al., 2010b). Relative to nNOSμ$^{-/-}$ muscle, there appears to be plenty of remaining NOS activity in nNOSμ$^{+/+}$ muscles (Fig 3.8) and the other studies during contraction with NOS inhibition, yet there is a significant attenuation of glucose uptake during contraction in this (Fig 3.2) and the other studies (Merry et al., 2010c, Merry et al., 2010b). If absolute NOS activity is the determining factor for muscle glucose uptake during contraction, then the observed attenuation in contraction-stimulated glucose uptake during NOS inhibition with more than 10% remaining NOS activity in this (Fig 3.2) and the other studies (Merry et al., 2010c, Merry et al., 2010b) would have not be seen. Perhaps, the spike/ fluctuation in NOS activity during contraction rather than the absolute level might be more important

for transduction of NO signalling. It should also be considered that the NOS activity measured *in vitro* may differ to the actual NOS activity *in vivo*.

It should be considered that there is evidence from other genetically-modified rodent models that residual signalling may be adequate for normal muscle glucose uptake during contraction/ exercise. For example, studies investigating the role of AMPK in contraction-stimulated muscle glucose uptake using mice with single mutations or deletions in muscle α or β subunit generally have normal glucose uptake with these mice have a varying degree of residual contraction-induced AMPK phosphorylation or activity (Fujii et al., 2005, Jorgensen et al., 2004, Maarbjerg et al., 2009, Steinberg et al., 2010). When these residual contraction-induced AMPK phosphorylation or activity were eliminated via double knockout of muscle AMPKβ1β2 subunits, up to 70% attenuation of the increase in muscle glucose uptake during contraction was observed (O'Neill et al., 2011). These results suggest that the residual AMPK phosphorylation or activity in genetically-modified AMPK mice is adequate to maintain a normal muscle glucose uptake during contraction/ exercise. Something similar may be occurring in nNOSμ$^{-/-}$ muscle with residual NOS activity/ NO signalling.

NO could also regulate muscle glucose uptake during contraction/ exercise via modulation of blood flow. Data from the rat *in situ* study was in agreement with our previous study (Ross et al., 2007) that NOS inhibition attenuates total (femoral) blood flow to the contracting muscles (Fig 4.7) but has no effect on muscle capillary blood flow (Fig 4.8). Unfortunately, we were unable to assess the effect of total loss of nNOSμ (nNOSμ$^{-/-}$) on muscle blood flow during exercise due to nNOSμ$^{-/-}$ mice being unable to tolerate the carotid cannulation procedures. Based on the findings from the rat *in situ* study, NO is unlikely to regulate muscle glucose uptake during contraction via blood flow as glucose delivery and the extraction efficiency, which affect glucose uptake, are influenced by capillary blood flow but not total blood flow to the muscles. We have shown that NOS inhibition attenuated the increase in glucose uptake during exercise in people with and without T2D and this occurred without any reduction in leg blood flow with NOS inhibition (Bradley et al., 1999, Kingwell et al., 2002). Given that capillary

blood flow and capillary flow rate were not affected by NOS inhibition in rats (Fig 4.8), it is possible that similar response occurs in humans but this needs to be investigated.

The inability to activate skeletal muscle NOS in SD rats (Fig 4.14) with a well established contraction protocol (Ross et al., 2007) was surprising and it showcased the complexity of the regulation of NOS activity and its function. As such, we were unable to assess the modulation of intramuscular signalling by NO to stimulate muscle glucose uptake in rats with T2D stimulated to contract *in situ* since no activation of NOS was detected in the muscle during contraction (Fig 4.14). This may explain why no effect of NOS inhibition on glucose uptake during contraction was observed (Fig 4.6). The attenuation in total blood flow by NOS inhibition provided evidence that the NOS inhibitor was having effects, despite no effects on muscle glucose uptake. A possibility for the inability to activate NOS in muscle of SD rats is that the contraction stimulation protocol was not strong enough, though we observed an approximately 50% reduction in force during the contraction protocol (Fig 4.2B). Previous studies showed that low intensity treadmill running at 45% maximum running speed did not increase NOS activity but NOS activity increased significantly at 70% maximum speed (Lee-Young et al., 2009) indicating that greater contraction intensity is needed to activate NOS. Similarly, NO production was increased with intense stimulation but not moderate stimulation in primary rat skeletal muscle cell culture (Silveira et al., 2003). However, activation of NOS appears to be dependent on factors other than contraction intensity. A mild *ex vivo* contraction stimulation increased NOS activity in Wistar rats (Tidball et al., 1998) while no activation of NOS was observed in SD rat muscle stimulated to contract *ex vivo* with a relatively much greater stimulation protocol (Etgen et al., 1997) suggesting that the strain of rat could influence NOS activation during contraction. Consistent with this, the current experiment showed no increase in muscle NOS activity in SD rats during contraction (Fig 4.14) despite the same stimulation protocol significantly increased NOS activity in muscle of Wistar rats in our previous experiment (Ross et al., 2007). In support to the different activation of NOS between SD and Wistar rats, there are a number of studies demonstrated the strain differences between SD and

Wistar rats in particular related to NOS signalling (Gaudreault et al., 2001, Holscher, 2002, Wei et al., 2007), and other general biological differences between these rats were well summarised in a review (Kacew and Festing, 1996). In fact, it appears likely that SD rats require a stronger electrical stimulation protocol or exercise intensity to activate NOS. A study that demonstrated a small increase (~37%) in NOS activity in SD rats during exercise employed a prolonged exhaustive treadmill running protocol (Roberts et al., 1999). SD rats stimulated to contract *in situ* with a stepwise increase in frequencies (0 to 2 Hz) over 70 min also appeared to have an increase in NOS activity only at the higher frequencies (intensities) based on a tendency for L-NAME to attenuate muscle glucose uptake during contraction only at the higher frequencies of contraction (Inyard et al., 2007). Collectively, these findings suggest that both the strain of rat and contraction intensity can influence the activation of NOS enzyme in skeletal muscle and stronger contraction stimulation may be needed to activate NOS in SD rats which was likely the case in the current study of Chapter Four.

It has been suggested that various signalling effectors act in a redundant manner to regulate skeletal muscle glucose uptake during contraction/ exercise. Our findings are in support to this notion in several ways. Firstly, NOS inhibition only partially attenuated muscle glucose uptake during contraction (Fig 3.2) suggesting a substantial portion of skeletal muscle glucose uptake was mediated by other signalling pathways. The association of higher glucose uptake with higher AMPK phosphorylation during exercise in nNOSμ$^{-/-}$ mice compared with nNOSμ$^{+/+}$ mice suggests that AMPK could be a possible redundant candidate that co-regulates muscle glucose uptake during contraction/ exercise. Nevertheless, a casual relationship behind the higher glucose uptake and AMPK phosphorylation during exercise in nNOSμ$^{-/-}$ mice was not investigated in this thesis. Such a study would require combined use of nNOSμ$^{-/-}$ mice and AMPK inhibitor or mating nNOSμ$^{-/-}$ mice with AMPK DN/KO mice. Interestingly, AMPK pathway appeared to be not involved in stimulating the normal glucose uptake in nNOSμ$^{+/-}$ mice since no increase in AMPK phosphorylation during exercise was detected (Fig 5.4C). These findings suggest that under different circumstances the

signalling pathways involved in mediating muscle glucose uptake during contraction/ exercise could be different. This scenario is more likely if every/ several involved signalling pathways are present in excess to stimulate muscle glucose uptake. In fact, as discussed above, AMPK and NO signalling may appear to be exist in excess given that the residual signalling from AMPK and nNOSμ genetically-modified mice are able to maintain normal muscle glucose uptake during contraction under some circumstances. In line with this notion, it has previously been shown that as little as 5% of Akt phosphorylation is adequate to elicit maximum effects of insulin on GLUT4 translocation in L6 myotubes (Hoehn et al., 2008). This highlights that, at least with insulin-stimulated glucose uptake, signalling transduction may not be a linear cascade in which changes in stimulus are being transduced proportionally to the downstream effectors. Similarly, cellular GLUT4 pools also appeared to have a great reserve capacity as a 50% reduction in GLUT4 has no effect on exercise-induced muscle glucose uptake (Fueger et al., 2004b). These studies clearly indicated that there is considerable reserve in the capacity of signalling intermediates for signalling transduction in mediating glucose uptake. More importantly, these findings require a change in interpretation on the importance of residual signalling in mediating biological effects and thus our attention to refine experimental methodology to ensure complete inhibition/ knockout of a particular signalling for assessing its role in a biological effect.

In summary, the *ex vivo* contraction study provides evidence that NO is involved in muscle glucose uptake during contraction, though nNOSμ appears to be not required in this process. The role of NO in muscle glucose uptake in healthy and SD rats with T2D was inconclusive due to the lack of activation of NOS in the muscle during contraction. In the mouse *in vivo* exercise condition, nNOSμ also appeared to be not essential for muscle glucose uptake during exercise; however, its role in regulating muscle blood flow and potential this influence on muscle glucose uptake was unable to be determined. Overall, although this thesis provides some support for NO playing a role in regulating skeletal muscle glucose uptake during contraction/ exercise, it also suggests that it may not be as important as our previous results have suggested, given

that some inconclusive results were reported in this thesis. The complex regulation of NOS/ NO signalling complicate further the interpretation of some of these findings. It should be kept in mind, however, that studies in humans by our group clearly demonstrate a role for NO in glucose uptake during exercise in humans (Bradley et al., 1999, Kingwell et al., 2002).

It should be noted that some limitations or confounding factors may have affected the outcome of the studies in this thesis. As the regulation of skeletal muscle glucose uptake during contraction/ exercise is likely to involve multiple signalling pathways working in a redundant and dynamic manner, it is important to control for the role of other potential signalling pathways in order to allow accurate assessment of the role of a particular signalling pathway in this process. In this thesis, we have assessed the potential compensatory effect from AMPK signalling while investigating the role of NO in muscle glucose uptake during contraction. We have also attempted to assess any alteration in CaMKII signalling following NOS inhibition or loss of nNOSμ but were unable to acquire a satisfactory immunoblotting outcome to evaluate CaMKII signalling despite using three different types of primary antibodies. Nevertheless, the better strategy to assess the role of a particular signalling in a biological effect is to prevent the activation of other potential redundant signalling pathways rather than assessing their compensatory effect as these effects may not be easily detected under a complex integrated signalling interaction condition. As such, experiments involving a combination of genetically-modified mouse models with various inhibitors could be a useful model to isolate the role of the signalling pathway of interest (for example $nNOS\mu^{-/-}$ mice combined with an AMPK and or CaMKII inhibitor). It should be kept in mind that knockout model can have compensations so ideally a conditional knockout model should be used. Also, inhibitors can have non-specific effects that should be kept in mind.

The residual NOS activity observed in $nNOS\mu^{-/-}$ muscles also makes interpretation of the role of NO in glucose uptake during, in particular, *in vivo* exercise

more difficult. Local administration of a NOS inhibitor into running mice is impractical and systemic infusion of a NOS inhibitor can lead to increased systemic blood pressure and an increase in skeletal muscle blood flow during *in situ* contraction (Inyard et al., 2007), which complicates the interpretation of NO effects on glucose uptake during contraction. Because of these difficulties it will be difficult to block the residual NOS activity during exercise in nNOSμ$^{-/-}$ mice to determine the potential role of residual NOS activity on glucose uptake in an *in vivo* exercise study. Nevertheless, this nNOSμ$^{-/-}$ mouse *in vivo* exercise model provided important information that nNOSμ is not essential for mediating glucose uptake during exercise. This is the first study to examine the role of nNOSμ on glucose uptake during exercise. It is unfortunately that due to experimental limitation the potential role of nNOSμ in regulating blood flow during exercise was unable to be assessed. Future studies with the use of total nNOS knockout mice (KN2 mice) (Percival et al., 2010) without the expression of any nNOS splice variants could serve as a good model for investigating the role of NO/ nNOS splice variants in regulating muscle glucose uptake and the possible downstream signalling involved in muscle glucose uptake during *ex vivo* contraction or during exercise. However, these mice have disruption of their skeletal muscle microtubule cytoskeleton and are more susceptible to muscle fatigue (Percival et al., 2010). Therefore, proper matching of force production/ running speed between the total nNOS knockout (KN2 mice) and wild type mice needs to be carefully considered as muscle force production (Fujii et al., 2005, Ihlemann et al., 2001) and muscle tension (Ihlemann et al., 1999b) affect glucose uptake during *ex vivo* muscle contraction. In addition, factors associated with the muscle cytoskeleton may itself regulate skeletal muscle glucose uptake during contraction/ exercise via Rac1/PAK1 (Sylow et al., 2013)

The KN2 mouse muscle would also be valuable in regards to detection of nNOSβ protein in skeletal muscle of nNOSμ$^{-/-}$ mice. There has been controversial about the existence or not of nNOSβ in nNOSμ$^{-/-}$ mouse muscle; some studies showed immunostaining evidence for nNOSβ (Percival et al., 2010, Rothe et al., 2005) but other (Baum et al., 2002) studies including this thesis not detecting any nNOSβ in nNOSμ$^{-/-}$

muscles. The KN2 mouse muscle could serve as a good negative control sample for the detection of nNOSβ in skeletal muscle of nNOSμ$^{-/-}$ mice with the use of nNOSβ-specific primary antibody which, to our knowledge, has been developed recently. Confirmation of the presence of nNOSβ in these muscles would help increase understanding of the role of NO in mediating muscle glucose uptake during contraction/ exercise.

Given that nNOS splice variants could possibly be present in skeletal muscle and be involved in muscle glucose uptake during contraction, it will be important that future studies examine the effects of nNOS specific inhibitors on muscle glucose uptake during contraction. This will help reconcile the different results regarding NOS inhibitions and nNOSμ$^{-/-}$ mice and verify if there are non-specific effects of general NOS inhibitors.

One of the issues leading to some inconclusive results on the involvement of NO in muscle glucose uptake during contraction/ exercise is the complex regulation of NOS activity in rat muscle. It appears that strain differences in rats and the response of rat strains to a particular contraction stimulation protocol may influence the activation of NOS during contraction. A follow up study to directly compare the activation of NOS during contraction using different contraction stimulation protocols in SD and Wistar rats could provide more knowledge on the regulation of NOS activity and its biological effects in skeletal muscle. This would help with the planning and design of future studies for investigating any specific role of NO in skeletal muscle during contraction/ exercise.

CONCLUSIONS

In summary, the major conclusions of this thesis are:

1. Neuronal NOSμ is not essential for the regulation of skeletal muscle glucose uptake during *ex vivo* contraction. However, NO plays a role in the regulation of skeletal muscle glucose uptake during *ex vivo* contraction.

2. A rat strain difference may exist between SD and Wistar rats in the activation of NOS and NO signalling during *in situ* contraction. The well established contraction protocol used in the *in situ* contraction experiment failed to activate NOS in SD rats used in this thesis and that may explain why skeletal muscle glucose uptake was not attenuated by NOS inhibition during contraction in these rats.

3. The total loss of nNOSμ ($nNOS\mu^{-/-}$) is associated with greater skeletal muscle glucose uptake during exercise than in $nNOS\mu^{+/+}$ mice perhaps due to the greater increase in AMPK phosphorylation observed during contraction.

4. Partial loss of nNOSμ ($nNOS\mu^{-/+}$), on the other hand, does not affect exercise-stimulated skeletal muscle glucose uptake despite no increase in NOS activity and AMPK phosphorylation during exercise.

Overall, this thesis provided some support for NO playing a role in regulating skeletal muscle glucose uptake during contraction/ exercise. Nevertheless, the complexity of the system and the nature of the redundancy in skeletal muscle glucose uptake during contraction/ exercise complicate the interpretation of the data. Future works to investigate the role of NO should be carried out under the conditions where the involvement of other potential signalling is being controlled.

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APPENDIX A:

Immunoblotting images that indicate the specificity of the primary antibodies used to detect eNOS, nNOS and iNOS. Primary antibodies were used at an optimised concentration and condition based on a method modified from previously published (Murphy, 2011).

Endothelial NOS (eNOS)

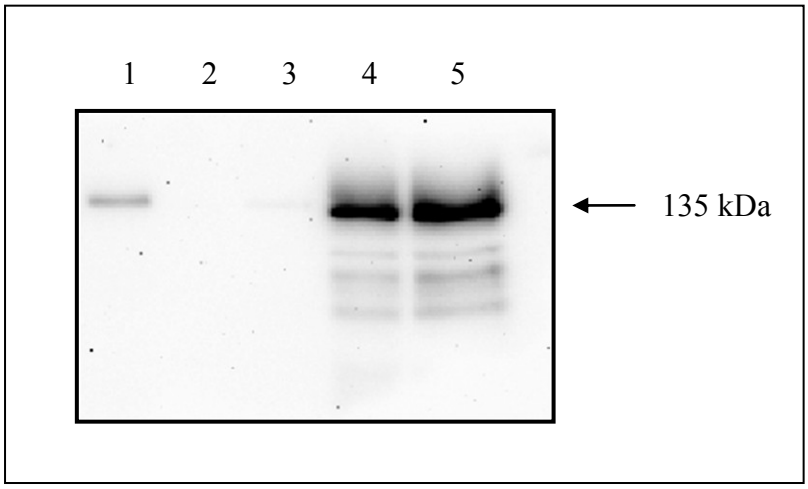

Figure A.1: Specificity of eNOS antibody.

Expected molecular weight for eNOS protein is 135 kDa. Human skeletal muscle and aorta were used as negative and positive control samples, respectively to determine the specificity of eNOS antibody used. Human skeletal muscle has been shown to have minimal eNOS expression (McConell et al., 2007) while it is expressed in rodent skeletal muscle (Kobzik et al., 1995) and in rodent aorta tissue (Pollock et al., 1991). Lane 1 = rat skeletal muscle (5 µg); lane 2 = human skeletal muscle (5 µg); lane 3 = human skeletal muscle (10 µg); lane 4 = mouse aorta tissue (5 µg) and lane 5 = mouse aorta tissue (10 µg).

Inducible NOS (iNOS)

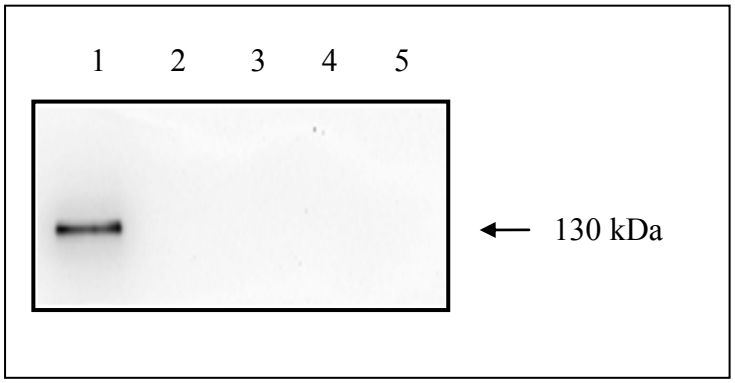

Figure A.2: Specificity of iNOS antibody.

Expected molecular weight for iNOS protein is 130 kDa. Inducible NOS does not express in healthy skeletal muscle. Lane 1 = iNOS positive control (Cayman Chemical); lane 2 - 5 = healthy mouse skeletal muscle of 2, 4, 6 & 7.5 µg.

Neuronal NOS (nNOS)

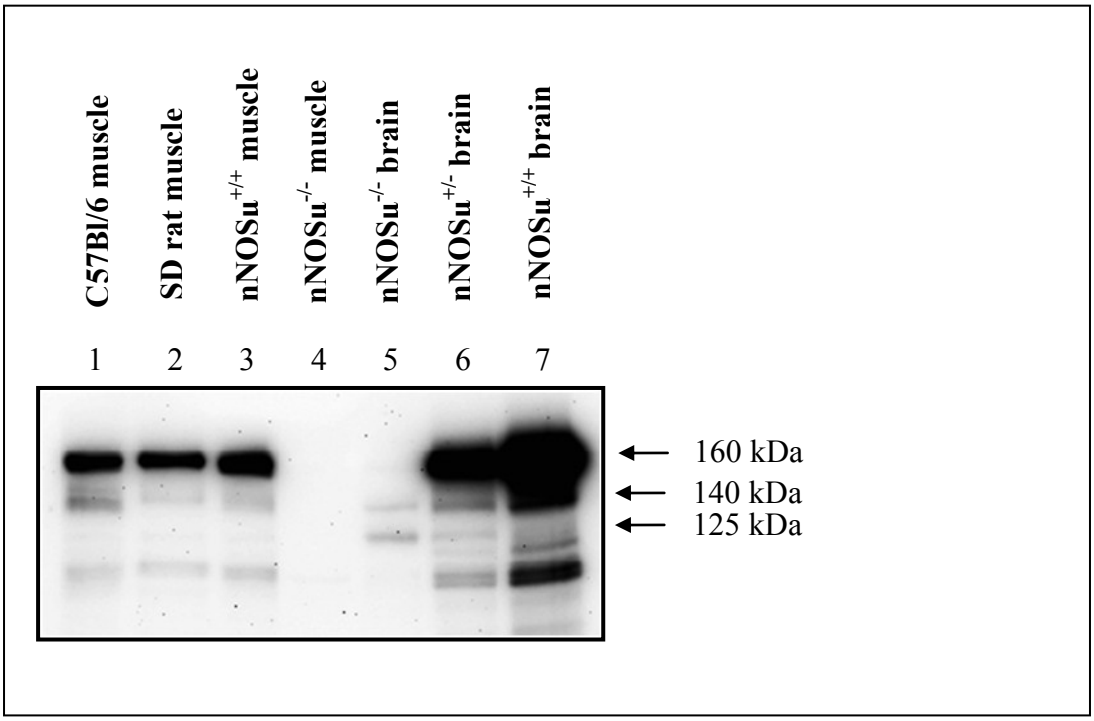

Figure A.3: Specificity of nNOS antibody.

Expected molecular weight for nNOSμ, nNOSβ and nNOSγ proteins are 160, 140 kDa and 125 kDa respectively. Each lane loaded with 7 μg of total protein. From this immunoblot, the band at 140 kDa (presumably nNOSβ) was absence in nNOSμ$^{-/-}$ mice (lane 4 from the left) but consistently found in skeletal muscle of C57Bl/6 mice and SD rat and the brain tissue of nNOSμ$^{-/-}$, nNOSμ$^{+/-}$ and nNOSμ$^{+/+}$ mice. These results are in agreement with previous report that nNOSβ and nNOSγ were present in the brain tissue of nNOSμ$^{-/-}$ mice but no nNOSβ was found in the skeletal muscle (Brenman et al., 1996). Neuronal NOSβ has also been immunoblotted in skeletal muscle of C57Bl/6 mice (Baum et al., 2011) but not in nNOSμ$^{-/-}$ mice (Baum et al., 2002).

APPENDIX B:

Chemiluminescent signal linearity over increasing total protein loadings for various proteins of interest immunoblotted under optimised conditions.

Endothelial NOS

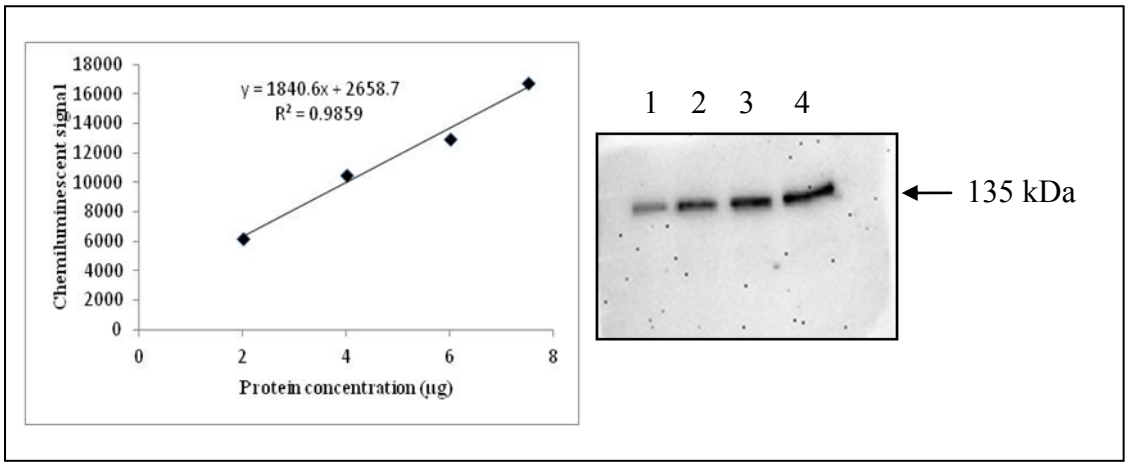

Figure B.1: Chemiluminescent signal of eNOS against total protein loading.
Expected molecular weight for eNOS protein is 135 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

Neuronal NOSµ

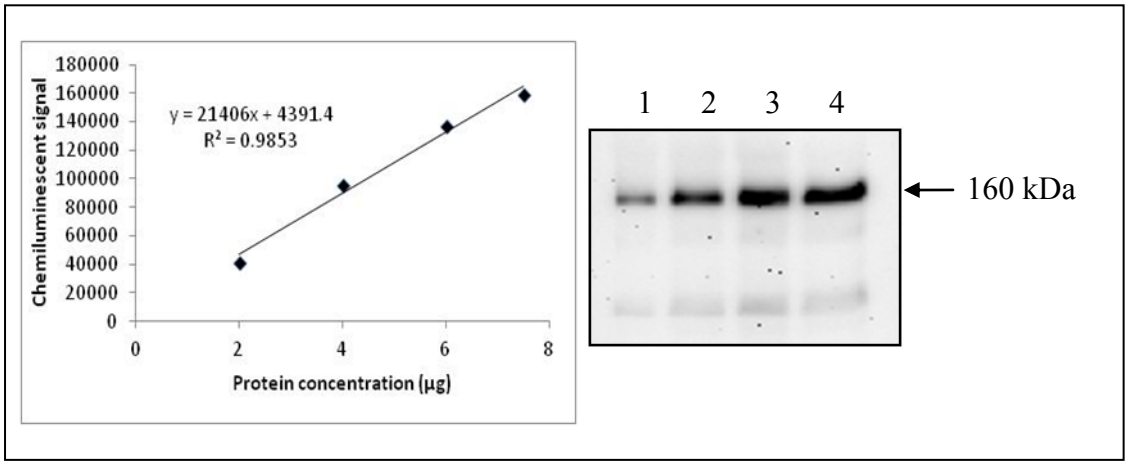

Figure B.2: Chemiluminescent signal of nNOSµ against total protein loading.
Expected molecular weight for nNOSµ protein is 160 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

Neuronal NOSβ

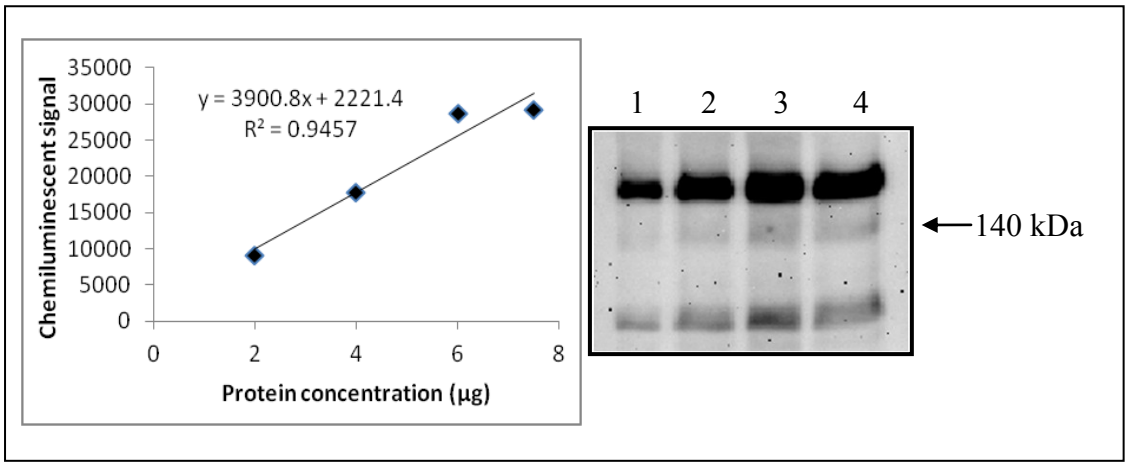

Figure B.3: Chemiluminescent signal of nNOSβ against total protein loading.
Expected molecular weight for nNOSβ protein is 140 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

AMPKα Thr^{172} phosphorylation

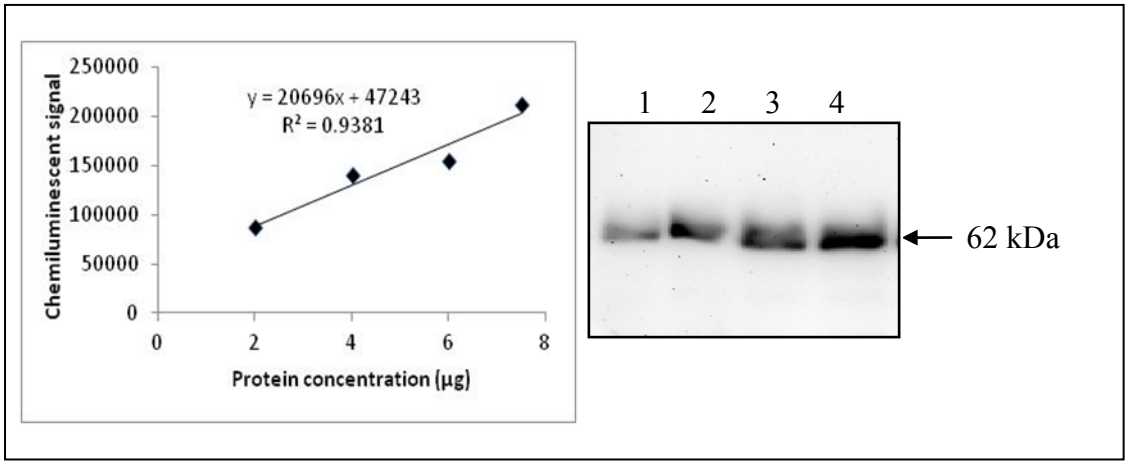

Figure B.4: Chemiluminescent signal of AMPK Thr^{172} phosphorylation against total protein loading.
Expected molecular weight for phospho-AMPK protein is 62 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

AMPKα

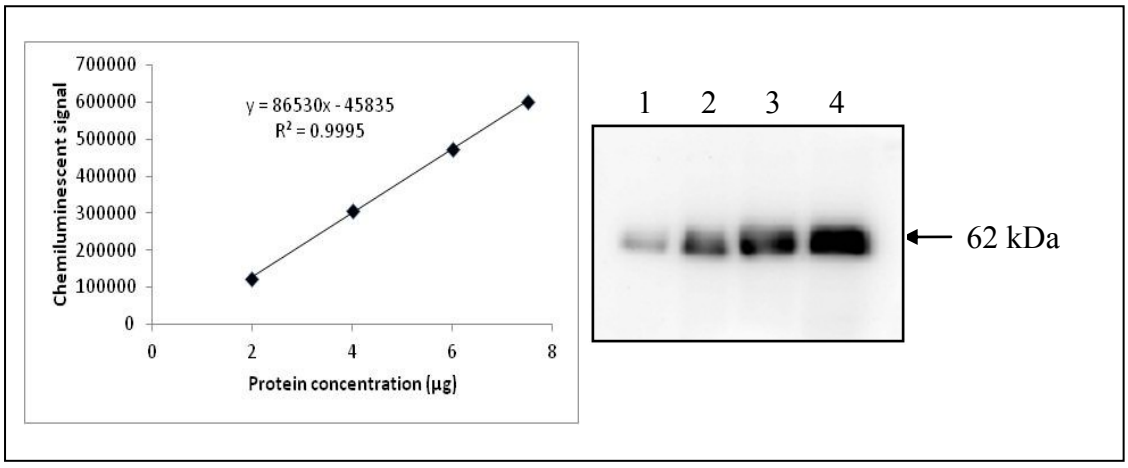

Figure B.5: Chemiluminescent signal of AMPK against total protein loading.
Expected molecular weight for AMPK protein is 62 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

TBC1D1 Ser660 phosphorylation

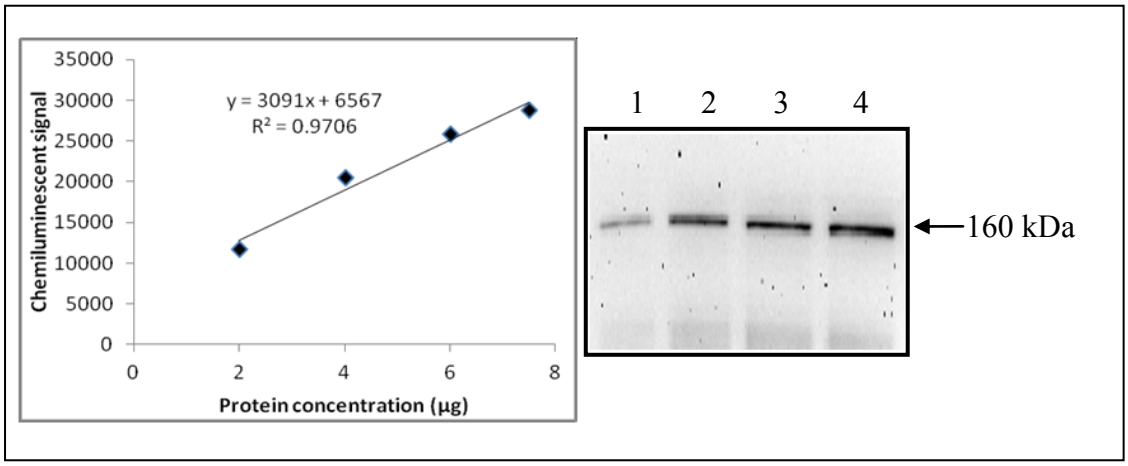

Figure B.6: Chemiluminescent signal of TBC1D1 Ser^{660} phosphorylation against total protein loading.
Expected molecular weight for TBC1D1 Ser^{660} phosphorylation is 160 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

TBC1D1

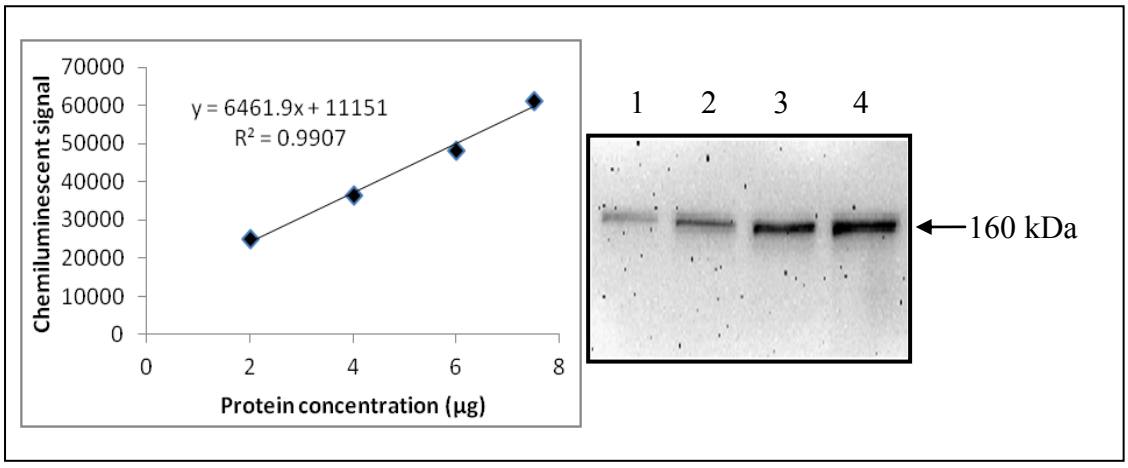

Figure B.7: Chemiluminescent signal of TBC1D1 against total protein loading. Expected molecular weight for TBC1D1 is 160 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

Actin

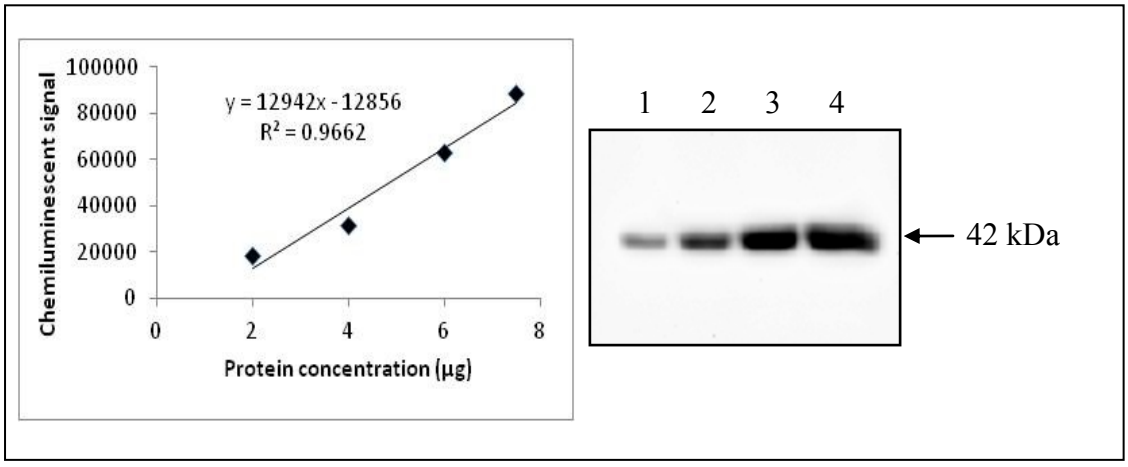

Figure B.8: Chemiluminescent signal of actin against total protein loading. Expected molecular weight for actin protein is 42 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

GLUT4

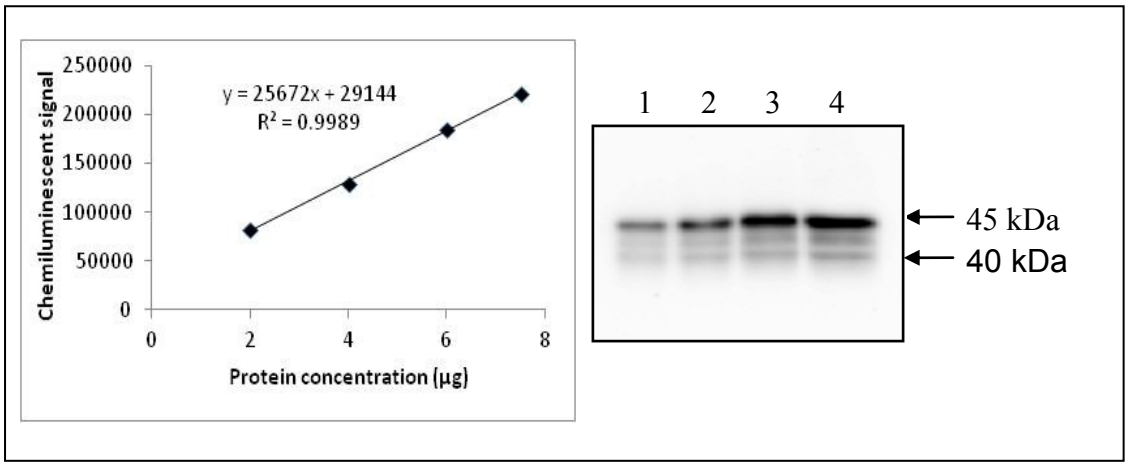

Figure B.9: Chemiluminescent signal of GLUT4 against total protein loading.

Expected molecular weight for glycosylated and deglycosylated GLUT4 proteins are 45 & 40 kDa respectively. This immunoblot was developed using the same GLUT4 antibody under similar conditions as used by Dr Robyn Murphy from La Trobe University, Melbourne who has previously verified the specificity of the antibody using PNGase to deglycosylate GLUT4. It was shown that PNGase-treated sample had reduced band intensity at 45 kDa and an increase signal at 40 kDa (personal communication). Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

α-tubulin

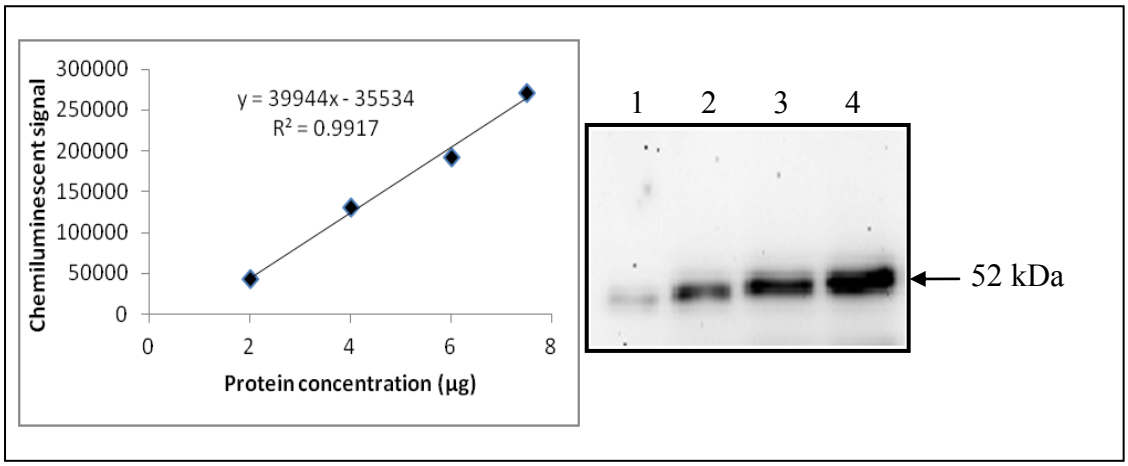

Figure B.10: Chemiluminescent signal of α-tubulin against total protein loading. Expected molecular weight for α-tubulin is 52 kDa. Lane 1 to 4 = 2, 4, 6 & 7.5 µg of total protein, respectively.

APPENDIX C:

Nutrient compositions of high-fat diet used to induce T2D in Sprague Dawley rats in Chapter Four.

Specialty Feeds

3150 Great Eastern Hwy
Glen Forrest
Western Australia 6071
p: +61 8 9298 8111
F: +61 8 9298 8700
Email: info@specialtyfeeds.com

Diet
SF01-028

23% Fat Semi-Pure Rodent Diet
43% of Energy From Fat

A high fat semi-pure modification of AIN93G.

- Fat content has been increased from around 7% in AIN93G to 23%.
- Calculated energy has increased by around 24% over the base diet. 40% of the total calculated energy is from lipids.
- The triglyceride profile has an increased proportion of saturated and mono-unsaturated fatty acids over the standard diet.
- Other nutritional parameters have remained unchanged.
- The high fat content has resulted in a significant reduction in pellet hardness. The pellets must be handled with great care to avoid breakage.

Calculated Nutritional Parameters

Protein	19.00%
Total Fat	22.60%
Crude Fibre	4.70%
AD Fibre	4.70%
Digestible Energy	19.9 MJ / Kg
% Total calculated digestible energy from lipids	43.00%
% Total calculated digestible energy from protein	17.00%

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 1.5 Kg, trays vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients

Casein (Acid)	200 g/Kg
Sucrose	388 g/Kg
Canola Oil	48 g/Kg
Cocoa Butter	180 g/Kg
Cellulose	50 g/Kg
Wheat Starch	90 g/Kg
DL Methionine	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg
Sodium Chloride	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg
Potassium Citrate	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg
AIN93 Vitamins	10 g/Kg

Calculated Amino Acids	
Valine	1.10%
Leucine	1.70%
Isoleucine	1.00%
Threonine	0.70%
Methionine	0.70%
Cystine	0.05%
Lysine	1.50%
Phenylanine	0.90%
Tyrosine	1.00%
Tryptophan	0.10%

Calculated Total Minerals	
Calcium	0.45%
Phosphorous	0.30%
Magnesium	0.09%
Sodium	0.11%
Chloride	0.16%
Potassium	0.40%
Sulphur	0.23%
Iron	70 mg/Kg
Copper	6.8 mg/Kg
Iodine	0.2 mg/Kg
Manganese	18 mg/Kg
Cobalt	No data
Zinc	50 mg/Kg
Molybdenum	0.15 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	No data
Chromium	1.0 mg/Kg
Fluoride	1.0 mg/Kg
Lithium	0.1 mg/Kg
Boron	3.4 mg/Kg
Nickel	0.5 mg/Kg
Vanadium	0.1 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	4 000 IU/Kg
Vitamin D (Cholecalciferol)	1 000 IU/Kg
Vitamin E (a Tocopherol acetate)	75 mg/Kg
Vitamin K (Menadione)	1 mg/Kg
Vitamin C (Ascorbic acid)	None added
Vitamin B1 (Thiamine)	6.1 mg/Kg
Vitamin B2 (Riboflavin)	6.3 mg/Kg
Niacin (Nicotinic acid)	30 mg/Kg
Vitamin B6 (Pyridoxine)	7 mg/Kg
Pantothenic Acid	16.5 mg/Kg
Biotin	200 ug/Kg
Folic Acid	2 mg/Kg
Inositol	None added
Vitamin B12 (Cyancobalamin)	100 ug/Kg
Choline	1 700 mg/Kg

Calculated Fatty Acid Composition	
Staurated Fats C12:0 and less	0.09%
Myristic Acid 14:0	0.04%
Palmitic Acid 16:0	4.79%
Stearic Acid 18:0	6.55%
Arachidic Acid 20:0	0.21%
Palmitoleic Acid 16:1	0.04%
Oleic Acid 18:1	8.73%
Gadoleic Acid 20:1	0.08%
Linoleic Acid 18:2 n6	1.50%
a Linolenic Acid 18:3 n3	0.55%
Arachadonic Acid 20:4 n6	No data
EPA 20:5 n3	Trace
DHA 22:6 n3	No data
Total n3	0.58%
Total n6	1.50%
Total Mono Unsaturated Fats	8.85%
Total Polyunsaturated Fats	2.09%
Total Saturated Fats	11.80%

Calculated data uses information from typical raw material composition. **Diet post treatment by irradiation or auto clave could change these parameters**. It could be expected that individual batches of diet will vary from this figure. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.