THE ROLE OF REACTIVE OXYGEN SPECIES AND NITRIC OXIDE IN THE REGULATION OF SKELETAL MUSCLE GLUCOSE UPTAKE DURING CONTRACTION

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ABSTRACT

There is evidence that reactive oxygen species (ROS) and nitric oxide (NO) are involved in the regulation of skeletal muscle glucose uptake during contraction. This thesis examined the role of ROS in regulating skeletal muscle glucose uptake during contractions *ex vivo* and *in situ* in rodents and during *in vivo* exercise in humans and the potential downstream mechanisms through which NO signals skeletal muscle glucose uptake during contractions *ex vivo*. Since there has been some evidence that both ROS and NO may signal through AMPK, the relationships between NO, ROS and AMPK with contraction were examined.

Isolated muscles from mice that express a muscle specific kinase dead AMPKα2 isoform (AMPK KD) were stimulated to contract *ex vivo*. Despite no increases in AMPK activity, muscles (soleus and extensor digitorum longus; EDL) from AMPK KD mice showed normal contraction-stimulated increases in glucose uptake which was attenuated by the antioxidant N-acetylcysteine (NAC) and the NOS inhibitor N^G-Monomethyl-L-Arginine (L-NMMA) to a similar extent as in muscles of wild type mice. Furthermore, the co-treatment of EDL muscles from C57Bl/6 mice with L-NMMA and NAC did not have an additive effect on the attenuation of skeletal muscle glucose uptake during contraction. These results indicate that ROS and NO are involved in regulating skeletal muscle glucose uptake during contractions *ex vivo*, through a similar pathway that is independent of AMPK.

To examine the downstream mechanisms through which NO regulates glucose uptake, EDL muscles from C57Bl/6 mice were contracted *ex vivo* in the presence and absence of inhibitors of potential NO signalling intermediates. In contrast to NOS inhibition with L-NMMA, the inhibition of soluble guanylate cyclase (sGC; which prevents the formation of cGMP) and PKG (cGMP dependent protein kinase) did not affect skeletal muscle glucose uptake during contraction. This suggests that during contraction NO regulates skeletal muscle glucose uptake through a cGMP-PKG independent pathway. To investigate alternative mechanisms through which NO regulates skeletal muscle glucose uptake during contraction, while light was used to prevent S-nitrosylation, and the antioxidants urate and DTT were used to scavenge peroxynitrite and prevent protein S-glutathionylation, respectively. It was found that urate and DTT, but not white light, attenuated contraction-stimulated increases in skeletal muscle glucose uptake. Furthermore, like L-NMMA and NAC, DTT and urate attenuated contraction-stimulated increases in S-glutathionylation and tyrosine nitration of a protein band

at ~37 kDa. This suggests that during contractions *ex vivo*, NO and ROS regulate glucose uptake through a similar pathway that may involve signalling through peroxynitrite and/or S-glutathionylation.

The results of these *ex vivo* studies suggest that ROS are regulating skeletal muscle glucose uptake during contraction, perhaps via a similar mechanism(s) as NO. To examine this further in more physiological models, NAC was infused locally into the hindlimb of rats contracted *in situ* and systemically in humans during exercise. Surprisingly, NAC did not affect the increase in glucose uptake during hindlimb contractions *in situ* in rats, or the increase in glucose disposal during exercise in humans. It is possible the discrepancy between results obtained in *ex vivo* preparations and the *in situ* and *in vivo* models is related to an unphysiological increases in ROS in *ex vivo* preparations as a result of supramaximal contraction protocols, non-uniform delivery of oxygen to muscle fibres, hyperoxic incubation medium and an absence of antioxidant systems surrounding muscle (such as that supplied by blood flow).

Therefore, the findings of this thesis provide evidence that ROS and NO regulate skeletal muscle glucose uptake during contractions *ex vivo* through a similar pathway that is independent of AMPK and cGMP/PKG, but may involve signalling through peroxynitrite and/or S-glutathionylation. However, unlike our previous observations in regards to NO, ROS do not appear to be essential for the regulation of skeletal muscle glucose uptake during moderate intensity contractions/exercise in intact physiological preparations.

DECLARATION

This is to certify that:

1. This thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the Preface.

2. Due acknowledgement has been given in text to all other materials used.

3. This thesis is less than 100,000 words in length, exclusive of tables, figures and references.

Troy L. Merry

PREFACE

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LIST OF ABBREVIATIONS

ACCβ	Acetyl-CoA carboxylase-β
ADP	Adenosine diphosphate
AICAR	Aminoimidazole 4-carboxamide ribonucleoside
Akt	Protein kinase B (PKB)
AMP	Adenosine-5'-monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMPK kinase
ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
APS	Ammonium persulfate
AS160	TBC1D4/Akt substrate of 160 kDa
ATP	Adenosine triphosphate
BPM	Beats per minute
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaMK	Calmodulin Kinase
CaMKK	CaMK Kinase
CAT	Catalase
CBD	Calmodulin binding domain
cGMP	Cyclic guanosine monophosphate
CK	Creatine kinase
CON	Control
CR	Clearance rate
Cr	Creatine
d.m	Dry mass

DCF	2',7'-dichlorofluorescin
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
Deta/NO	Diethylenetriamine nitric oxide adduct
DTT	Dithiothreitol
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
FBF	Femoral blood flow
G-6-P	Glucose-6-phosphate
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanylate cyclase
GLUT	Glucose transporter
GPx	Glutathione peroxidise
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HK	Hexokinase
HPLC	High performance liquid chromatography
HR	Heart rate
HX	Hypoxanthine
IRS	Insulin receptor substrate
KD	Kinase dead
LDH	Lactate dehydrogenase
L-NAME	L-nitro-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase

Mhy	Myosin heavy chain
NAC	N-acetylcysteine
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NaF	Sodium fluoride
NaPP	Sodium pyrophosphate
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	NO synthase
$O_2^{\bullet-}$	Superoxide
ODQ	1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one
•OH	Hydroxyl radicals
$ONOO^-$	Peroxynitrite
PAS	Phosphorylation (Ser/Thr)-Akt/PKB substrate
PBS	Phosphate buffered saline
PCA	Perchloric acid
PCr	Phosphocreatine
PDE	Phosphodiesterases
PEP	Phosphoenolpyruvate
PI3K	Phosphoinositide-3 kinase
PK	Pyruvate kinase
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidine difluride

R_a	Rate of appearance
R_d	Rate of disappearance
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
Rp-8-Br-PET-cGMPS	p-8-bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt
RPE	Rate of perceived exertion
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
Ser	Serine
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase
SNF-1	Sucrose non-fermenting-1 protein kinase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TBC1D1	Akt substrate of 150 kDa
TEMED	N,N,N',N'-tetramethylethylenediamine
TA	Tibialis anterior
Thr	Threonine
Tyr	Tyrosine
$\dot{V}O_2$	Oxygen consumption
VR	Vascular resistance
WT	Wild type
XO	Xanthine oxidase
2-DG	2-deoxy-D-glucose

CHAPTER ONE

LITERATURE REVIEW

1.0 INTRODUCTION

Following a meal whole body glucose homeostasis is largely dependent on the transport of glucose into skeletal muscle cells (Bryant *et al.*, 2002). Insulin and contraction both regulate skeletal muscle glucose uptake by signalling the translocation of the GLUcose Transporter 4 (GLUT4) protein to the cell surface, which facilitates the transport of glucose through the cell membrane into the cell (Bryant *et al.*, 2002). However, insulin and contraction signal skeletal muscle GLUT4 translocation and glucose uptake through discrete mechanisms (Ploug *et al.*, 1984; Yeh *et al.*, 1995). Although the insulin signalling pathway of skeletal muscle glucose uptake is relative well described (Bryant *et al.*, 2002; Khan & Pessin, 2002; Leney & Tavare, 2009) the signalling intermediates regulating glucose uptake during contraction are yet to be fully elucidated. However, there is evidence to suggest that calcium/calmodulin-dependent protein kinase (CaMK) (Witczak *et al.*, 2007), AMP-activated protein kinase (AMPK) (Hayashi *et al.*, 1998), nitric oxide (NO) (Bradley *et al.*, 1999; Ross *et al.*, 2007), protein kinase C (PKC) (Wojtaszewski *et al.*, 1998) and reactive oxygen species (ROS) (Sandstrom *et al.*, 2006) may act, with some level of redundancy, as discrete or integrated signalling intermediates in the regulation of skeletal muscle glucose uptake during contraction.

Insulin resistance is characterised by impaired insulin-stimulated glucose uptake into skeletal muscle (Eriksson *et al.*, 1999; Matthaei *et al.*, 2000). Insulin resistance is the major contributing factor to the onset of a variety of metabolic disorders including Type 2 diabetes (Reaven, 1993). The prevalence of Type 2 diabetes in the western world is increasing at an alarming rate, with almost one in four Australians aged over 25 now suffering from diabetes or impaired glucose tolerance (Zimmet *et al.*, 2001). Type 2 diabetes accounts for over 85% of diabetic patients. Although people with Type 2 diabetes have impaired insulin-stimulated skeletal muscle glucose uptake, their increase in GLUT4 translocation (Kennedy *et al.*, 1999) and muscle glucose uptake during exercise (Kingwell *et al.*, 2002) and enhancement of insulin action following physical activity (Kjaer *et al.*, 1990) is comparable to that of non-diabetics.

Therefore, exercise is an effective preventative and treatment option for people with Type 2 diabetes and sufferers of insulin resistance (Borghouts & Keizer, 2000). Unfortunately, many people with Type 2 diabetes cannot, or will not, exercise regularly and therefore other strategies are required. The current pharmacological treatments are limited in their effectiveness and specificity (Tupper & Gopalakrishnan, 2007). Since diabetes is beginning to be described as an epidemic (Zimmet *et al.*, 2001) it is imperative that alternative therapies for the effective management and prevention of this disease be investigated. Gaining a better understanding of the signalling pathway(s) through which contraction-stimulates skeletal muscle GLUT4 translocation and glucose uptake may allow for the development of new targeted pharmaceutical treatments to aid in the treatment of this disease. This literature review will focus on the potential mechanisms regulating skeletal muscle glucose uptake during contraction.

1.1 SKELETAL MUSCLE GLUCOSE UPTAKE: INSULIN VS. CONTRACTION

Glucose is an essential substrate for the metabolism and homeostasis of all eukaryotic cells. Skeletal muscle is critical for glucose disposable and blood glucose regulation in response to insulin and exercise (DeFronzo *et al.*, 1981; Rose & Richter, 2005). Glucose cannot passively diffuse into cells, but requires transportation through the cell membrane by GLUT's. Under normal basal conditions skeletal muscle glucose uptake is determined by the concentration of glucose in the interstitial fluid, and is mediated primarily by GLUT1 (Pessin & Bell, 1992). GLUT1 resides in low abundance in the plasma membrane and this limits its capacity to transport glucose into the cell (Burant *et al.*, 1992; Holloszy & Hansen, 1996). In contrast, the translocation of GLUT4, the major glucose transporter expressed in skeletal muscle, to the cell membrane can readily increase glucose transport across the cell membrane through facilitative diffusion. GLUT4 is contained in vesicles located in various intracellular locations (Klip *et al.*, 1990), and the signalling of its translocation to the cell surface is essential to facilitate glucose transport and maintain whole-body glucose homeostasis in response to acute perturbations in blood glucose (Bryant *et al.*, 2002).

Insulin-stimulation and contraction both increase glucose uptake into muscle (Ploug *et al.*, 1984). This occurs via an increase in capillary recruitment and total muscle blood flow (glucose delivery) (Vincent *et al.*, 2006; Newman *et al.*, 2007), as well as, and of focus here, the signalling of GLUT4 translocation to the cell surface (glucose transport) (Holloszy & Hansen, 1996; Holman & Kasuga, 1997; Zisman *et al.*, 2000). Like insulin, skeletal muscle contraction requires GLUT4 translocation to increase glucose transport (Stenbit *et al.*, 1997; Ryder *et al.*, 1999; Zisman *et al.*, 2000), however the pathway through which contraction signals GLUT4 translocation and insertion into the cell membrane (Roy & Marette, 1996) differs from that of insulin (Ploug *et al.*, 1984; Nesher *et al.*, 1985; Brozinick *et al.*, 1994a; Gao *et al.*, 1994; Lee *et al.*, 1995; Lund *et al.*, 1995). Indeed, during skeletal muscle contraction glucose uptake can occur in the absence of insulin (Ploug *et al.*, 1984) and the affects of insulin and contraction are synergistic (Nesher *et al.*, 1985; Brozinick *et al.*, 1994a; Gao *et al.*, 1994; Lund *et al.*, 1995). Furthermore, the knocking out of muscle insulin receptors (Wojtaszewski *et al.*, 1999) or inhibition of PI3K with wortmannin (Lee *et al.*, 1995; Yeh *et al.*, 1995) prevents insulin-stimulated skeletal muscle glucose uptake without affecting glucose uptake during contraction. In addition, and what may be the most convincing evidence that contraction regulates skeletal muscle glucose uptake independent of insulin, people with Type 2 diabetes (Zierath *et al.*, 1996) and insulin-resistant rodents (King *et al.*, 1992) have impaired GLUT4 recruitment to the cell membrane and glucose uptake in response to insulin stimulation (Shepherd & Kahn, 1999), but during exercise their GLUT4 translocation (Kennedy *et al.*, 1999) and glucose uptake is normal (Minuk *et al.*, 1981; Martin *et al.*, 1995; Kingwell *et al.*, 2002) or supernormal (Colberg *et al.*, 1996; Giacca *et al.*, 1998; Kang *et al.*, 1999).

Briefly, insulin signals GLUT4 translocation by binding to the α-subunit of the insulin receptor located on the cell surface. This allows autophosphorylation of tyrosine residues in the receptor β-subunit and activation of tyrosine kinase (Cheatham & Kahn, 1995). Tyrosine kinase then phosphorylates the insulin-receptor-substrate family (IRS1 and IRS2) and IRS1 binds to the regulator subunit (p85) of phosphatidylinositol 3-kinase (PI3K) (White, 1998) which leads to PI3K-mediated activation of Akt (PKB; protein kinase B) (Bae *et al.*, 2003; Thong *et al.*, 2005), GLUT4 translocation and glucose uptake. Although insulin signalling downstream of PI3K is not clear, it is known that PI3K and Akt activation are integral for normal insulin-mediated increases in skeletal muscle glucose uptake (Cheatham *et al.*, 1994; Cho *et al.*, 2001).

More recently, Akt phosphorylation of Akt substrate of 160 kDa (AS160/TBC1D4) has been implicated in the regulation of GLUT4 translocation through its GTPase-activating protein (GAP) domain. Sano *et al.* (2003) purposed that GLUT4 vesicle Rab proteins are bound in the GAP domain of AS160, maintaining it as an inactive GDP. It is believed that when AS160 becomes phosphorylated by Akt, GAP activity towards the Rab protein is inhibited increasing the active form of the Rab protein, GTP. This allows AS160 to dissociate from the GLUT4 vesicles (Larance *et al.*, 2005) promoting GLUT4 translocation and the facilitation of glucose uptake (Kane *et al.*, 2002; Sakamoto & Holman, 2008). Indeed, the mutation of four Akt-phosphorylation substrates of AS160 to prevent phosphorylation impairs skeletal muscle glucose uptake in response to a glucose bolus (Kramer *et al.*, 2006b). Interestingly, TBC1D1, which shares 79% GAP domain identity with AS160 (Roach *et al.*, 2007), has recently been identified as an Akt substrate at 150 kDa (Ishikura & Klip, 2008), and furthermore its over-expression in L6 myotubes increases insulin-stimulated GLUT4 translocation (Stone *et al.*, 2006). Therefore, like AS160, TBC1D1 may also play an important role in facilitating the translocation of GLUT4 and glucose uptake in response to insulin stimulation.

While the insulin signalling pathway is reasonably well defined, how contraction regulates skeletal muscle glucose uptake is less well understood. Since insulin and contraction stimulate GLUT4 translocation though independent pathways it has been suggested that GLUT4 is located in various distinct intracellular compartments (Slot *et al.*, 1991; Aledo *et al.*, 1997), and the mobilisation of these discrete pools may depend on the respective stimulus (Douen *et al.*, 1990a; Douen *et al.*, 1990b; Ploug *et al.*, 1998). Partial ablation of GLUT4 expression in mice does not impair basal, insulin-stimulated or contraction-stimulated glucose uptake (Fueger *et al.*, 2004b; Fueger *et al.*, 2004c), and the overexpression of GLUT4 in skeletal muscle does not enhance skeletal muscle glucose uptake during contraction (Fueger *et al.*, 2004a). This suggests that regardless of stimulus only a small proportion of the total GLUT4 pool is required to fully enhance glucose transport, and that the signalling pathway for GLUT4 translocation rather than muscle GLUT4 concentration or specific GLUT4 pool recruited is the primary regulator of facilitated skeletal muscle glucose uptake.

1.2 SKELETAL MUSCLE GLUCOSE UPTAKE DURING EXERCISE

During dynamic physical exercise skeletal muscle energy demands increase dramatically. In order to meet the elevated energy requirements of working muscle ATP is synthesised by the oxidative and anaerobic catabolism of carbohydrates (muscle glycogen and blood glucose) and fatty acids (intramuscular triglycerides and blood lipid). Blood glucose use by skeletal muscle can increase >50-fold during exercise (Katz *et al.*, 1986). Its contribution to total ATP synthesis, although relatively small compared with other sources such as glycogen or fat, is dependent on exercise intensity and duration. In general as exercise continues and/or intensity increases blood glucose contribution to total energy turnover is increased proportionally (Wahren *et al.*, 1971; Coyle *et al.*, 1986; Romijn *et al.*, 1993).

Skeletal muscle glucose uptake during exercise is the function of three important regulatory steps; glucose delivery to the muscle cell, glucose transport through the cell membrane and glucose phosphorylation (Wasserman & Fueger, 2006) and therefore flux through intracellular metabolism (Figure 1.1). Increasing the supply of glucose during exercise increases skeletal muscle glucose uptake (Zinker *et al.*, 1993). Delivery of glucose to the muscle cell is a product of muscle blood flow/capillary blood flow and blood glucose concentration. Muscle blood flow can increase up to 20-fold during exercise (Andersen & Saltin, 1985), and under normal exercise conditions of hydration and heat strain the increased perfusion of muscle vascular beds provides ample capacity for the delivery of substrate (Clifford & Hellsten, 2004; Rattigan *et al.*, 2005). Conversely, elevations in extracellular glucose concentration during *ex vivo* contractions (Nesher *et al.*, 1985; Ploug *et al.*, 1987) or during *in vivo* exercise (Zinker *et al.*, 1993) result in a proportional increase in skeletal muscle glucose uptake. Thus, delivery of glucose to the muscle plays an important regulatory role in skeletal muscle glucose uptake during exercise, with blood glucose concentration generally having a greater influence on glucose delivery than blood flow (Rose & Richter, 2005). Describing determinates of blood glucose concentration (i.e. the balance between the rate of blood glucose appearance and disappearance) during exercise is beyond the scope of this review, and readers are referred to the following articles (Coyle & Montain, 1992; Rehrer, 1994; Kjaer, 1998) for comprehensive reviews regarding carbohydrate supplementation, gastric emptying, intestinal absorption of glucose during exercise as well as liver glucose production during exercise.

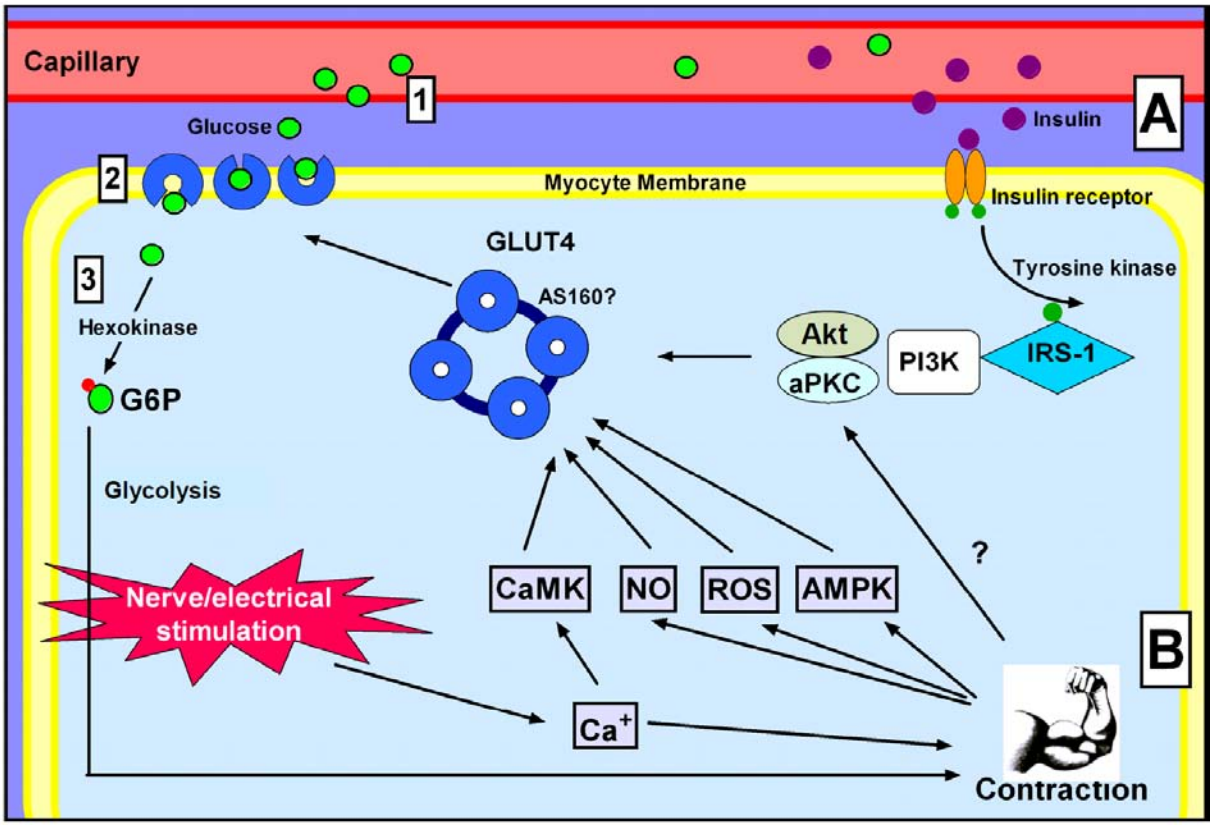

Figure 1.1 Factors determining glucose uptake; glucose (1) delivery, (2) transport through the cell membrane and (3) phosphorylation. Potential mechanisms of (A) insulin- and (B) contraction-stimulated signalling of GLUT4 translocation. G6P= Glucose-6-phosphate; Ca^{2+}= Calcium; NO= Nitric oxide; ROS= Reactive oxygen species; AMPK= AMP-activated protein kinase; Akt= Protein kinase B (PKB); aPKC= atypical protein kinase C; PI3K= Phosphoinositide-3 kinase; IRS-1= insulin receptor substrate 1.

The separate contribution of glucose transport and phosphorylation in limiting glucose uptake during contraction is complex given their close coupling. Recently, in a series of studies using *in vivo* rodent models (for details see Wasserman & Fueger, 2006) Wasserman and colleagues have shown that a) during exercise glucose accumulates intracellularly (Halseth *et al.*, 1998) (i.e. after membrane transport), b) partial knock out of hexokinase II (HKII; an essential enzyme in glucose phosphorylation, Figure 1.1) reduces muscle glucose influx (Fueger *et al.*, 2003), and c) the overexpression of HKII in skeletal muscle increases the ability of working muscle to consume glucose independent of GLUT4 expression (Fueger *et al.*, 2004a; Fueger *et al.*, 2004b; Fueger *et al.*, 2004c). Despite these findings being limited to oxidative type muscle fibres (Fueger *et al.*, 2003) they suggest that phosphorylation may be limiting glucose

utilisation during conditions of high glucose flux through the cell membrane, such as during exercise.

Indeed, unphosphorylated glucose appears to accumulate at the onset of exercise and during supra-maximal exercise in human muscle, however, this does not occur during submaximal exercise (Katz *et al.*, 1986; Katz *et al.*, 1991). In human muscles of heterozygous fibre population there is little evidence that HK activity is up-regulated in response to contraction (Koval *et al.*, 1998; Wasserman & Halseth, 1998). In addion, people with Type 2 diabetes have normal glucose uptake during exercise despite lower skeletal muscle HKII expression (Pendergrass *et al.*, 1998). Therefore, it appears that under most exercise conditions glucose transport into the muscle cell limits glucose utilisation in human skeletal muscle, but when glucose flux is high, glucose phosphorylation may become limiting.

In summary, the individual influence of glucose delivery, glucose transport and glucose phosphorylation on the rate of blood glucose utilisation during exercise is difficult to discern due their highly integrated nature. It is unlikely that each works in isolation, but rather they interact and collectively contribute to regulating glucose uptake during exercise. The relative contribution of each may depend on numerous factors including metabolic state, muscle fibre type, contraction parameters (exercise duration and intensity) and, nutritional and training status. However, during normal exercise conditions human skeletal muscle glucose uptake appears to be limited by glucose transport though the cell membrane. Importantly, glucose transport is also the primary aspect of insulin-stimulated glucose uptake that is impaired with diabetes (Brozinick *et al.*, 1994b), but remains functional during exercise. The potential pathway(s) though which contraction signals skeletal muscle glucose transport and the resulting increase in glucose uptake will be the focus of the remainder of this literature review.

1.3 REGULATION OF CONTRACTION-STIMULATED GLUCOSE TRANSPORT

The mechanism(s) by which contraction/exercise stimulates GLUT4 translocation and the resulting increase in glucose uptake are poorly understood (Rose & Richter, 2005). However, it is generally accepted that the mechanism(s) arise from local factors within skeletal muscle because contraction of isolated skeletal muscle *ex vivo* increases glucose utilisation above that of basal (Ploug *et al.*, 1984).

1.3.1 MUSCLE GLYCOGEN CONCENTRATION

There are conflicting views regarding the involvement of muscle glycogen content in the regulation of skeletal muscle glucose uptake during exercise. Glucose uptake during exercise in humans has been reported to be enhanced (Richter *et al.*, 2001) and unaffected (Hargreaves *et al.*, 1995; McConell *et al.*, 2005) by low muscle glycogen levels. *Ex vivo* studies have shown that glucose transport is elevated in response to low muscle glycogen content in fast-twitch (glycolytic) (Richter & Galbo, 1986; Hespel & Richter, 1990), but not slow-twitch (oxidative) muscle fibres (Derave *et al.*, 1999). Unlike rodent muscle where one fibre type tends to predominate, the prime mover muscles during exercise in humans tend to comprise of a mix of fibre types. Since GLUT4 is structurally bound to glycogen particles (Coderre *et al.*, 1994), it has been speculated that depleting glycogen releases GLUT4 to facilitate glucose transport (Richter *et al.*, 2001). However, this process and the overall regulatory role of muscle glycogen content on regulating skeletal muscle glucose uptake during contraction are yet to be comprehensively defined. If muscle glycogen has some influence on contraction-stimulated glucose uptake it is likely to be via one or more of the signalling pathways discussed below.

1.3.2 AMP-ACTIVATED PROTEIN KINASE (AMPK) IN SKELETAL MUSCLE

1.3.2.1 AMPK activation during exercise: a cellular fuel gauge?

AMPK: structure and expression

Skeletal muscle contraction dramatically increases muscle cell energy turnover, lowering ATP:ADP ratio, muscle glycogen concentration and elevating free cytosolic AMP. The magnitude of intracellular energy depletion is closely related to the increase in skeletal muscle glucose uptake during contraction (Aslesen *et al.*, 2001), regardless of stimulation frequency and therefore presumably Ca^{+2} concentration (Ihlemann *et al.*, 2000; Ihlemann *et al.*, 2001). This suggests that metabolic stress is monitored by the muscle during contraction and contributes to the regulation of glucose metabolism.

AMP-activated protein kinase (AMPK) is a ubiquitously expressed multisubstrate serine/threonine protein kinase which has been purposed, amongst the regulation of various

other cell processes (Richter & Ruderman, 2009; Steinberg & Kemp, 2009), to function as a intracellular fuel sensor; reducing ATP-consumption and initiating ATP-regenerating pathways during times of metabolic stress (Hardie *et al.*, 1998). The AMPK heterotrimeric protein consists of a catalytic (α) and two regulatory (β and γ) subunits (Hardie *et al.*, 1998; Kahn *et al.*, 2005). Each subunit has several isoforms (α1, α2, β1, β2, γ1 γ2 and γ3) which are expressed in a tissue specific manner with $\alpha_1\beta_1\gamma_1$ being the predominate combination in most cell types (Hardie & Sakamoto, 2006). The major isoforms expressed in skeletal muscle are α_2, β_2 and γ_1 or γ_3, (Stapleton *et al.*, 1996; Thornton *et al.*, 1998; Cheung *et al.*, 2000; Wojtaszewski *et al.*, 2005). The main complex expressed in skeletal muscle is $\alpha_2\beta_2\gamma_1$, with $\alpha_2\beta_2\gamma_3$ and $\alpha_1\beta_2\gamma_1$ also being highly abundant (Wojtaszewski *et al.*, 2005).

AMPK regulation

AMPK activity can be modulated both allostericaly by AMP and covalently via phosphorylation at αThr^{172} (Carling *et al.*, 2008), as well as through dephosphorylation by protein phosphatase 2Cα (PP2C) (Davies *et al.*, 1995). In brief, the depletion of high energy phosphate compounds reduce intracellular ATP and increase AMP, promoting binding of AMP to the two bateman domains of the γ-subunit (Cheung *et al.*, 2000; Kahn *et al.*, 2005). The β_2-subunit then acts as scaffolding for the γ-subunit to bind to the catalytic α-subunit, directly activating AMPK (Hawley *et al.*, 2003; Woods *et al.*, 2003b) and preventing the α subunits auto-inhibition of its catalytic domain (Sanders *et al.*, 2007). Furthermore, the binding of AMP to AMPK is proposed to reduce the complexes affinity for PP2C and therefore its dephosphorylation (Davies *et al.*, 1995; Sanders *et al.*, 2007). However, for complete AMPK activation the α-subunit of the AMPK-AMP complex must be phosphorylated at Thr^{172} by an AMPK Kinase (AMPKK) (Crute *et al.*, 1998; Adams *et al.*, 2004; Kahn *et al.*, 2005).

Phosphorylation can increase AMPK activity 200-fold greater than that of AMP allosteric activation alone (Suter *et al.*, 2006) making phosphorylation of major importance to the functional significance of AMPK. For many years the identity of AMPKK(s) have remained elusive. It wasn't until the identification of Sac1, Tos3 and Elm1 as upstream kinases of sucrose non-fermenting-1 protein kinase (SNF-1) (Hong *et al.*, 2003; Sutherland *et al.*, 2003), a yeast form of AMPK, that the mammalian equivalents, tumor suppressor LKB1 kinase (LKB1) and the Calcium-calmodulin dependent protein kinase kinase (CaMKK) family of kinases were identified as potential AMPKKs. Indeed, the deletion of LKB1 in some cell lines

including skeletal muscle of mice prevents AMPK agonists increasing AMPKα2 activity, and severely blunts any increases in AMPKα1 activity (Hawley *et al.*, 2003; Sakamoto *et al.*, 2005). Therefore, LKB1 is now generally accepted as the predominate upstream kinase of AMPK in skeletal muscle (Carling *et al.*, 2008). Since LKB1 is constitutively active (Woods *et al.*, 2003a; Sakamoto *et al.*, 2004) and elevated AMP is accompanied by increased AMPK phosphorylation (Sanders *et al.*, 2007), it has been proposed that AMP inhibits the dephosphorylation of AMPK by PPC2 allowing uninhibited LKB1 phosphorylation and full activation of AMPK (Sanders *et al.*, 2007).

Interestingly, some increases in AMPK activity can still be induced in a number of cell lines that lack LKB1 (Hawley *et al.*, 2005; Woods *et al.*, 2005). CaMKK have been shown to phosphorylate AMPK *in vitro*, and this phosphorylation appears to be mediated by CaMKKβ rather than CaMKKα (Hawley *et al.*, 2005; Woods *et al.*, 2005). Furthermore, increasing the concentration of Ca^{2+} in cells activates CaMKKβ and increases AMPK activity independent of LKB1 and without affecting intracellular AMP concentration (Stahmann *et al.*, 2006), suggesting that CaMKKβ may also act as a AMPKK. Indeed, Jensen *et al.* (2007b) showed that the CaMKK inhibitor, STO-609, attenuates increases in mouse EDL and soleus muscle AMPK activity during low intensity titanic twitch *ex vivo*. This provides some evidence that CaMKK may activate AMPK in skeletal muscle. Recent evidence suggests a third mammalian member of the AMPKK family, with Tak1 being shown to phosphorylate and activate AMPK in cell free assay (Momcilovic *et al.*, 2006). However, whether Tak1 is involved in the regulation of AMPK activity in more physiological conditions is unclear (Momcilovic *et al.*, 2006).

Contraction activates AMPK

AMPK is sensitive to the metabolic state of the cell, and therefore numerous pathological and pharmacological stimuli which generally deplete or interfere with cellular ATP production can activate AMPK including; ischemia (Russell *et al.*, 2004; Li *et al.*, 2006), hypoxia (Hayashi *et al.*, 2000; Mu *et al.*, 2001), oxidative stress (Toyoda *et al.*, 2004; Sandstrom *et al.*, 2006), hyperosmotic stress (Fryer *et al.*, 2000; Hayashi *et al.*, 2000), inhibition of the mitochondrial respiratory chain (Adams *et al.*, 2004; Jing & Ismail-Beigi, 2006) or inhibition of mitochondrial ATP synthesis (Adams *et al.*, 2004; Bertrand *et al.*, 2006) and uncoupling of oxidative phosphorylation (Pelletier *et al.*, 2005). Indeed, exercise or muscle contraction causes metabolic stress and accordingly AMPK is activated in rodent skeletal muscle during

in vivo exercise (Winder & Hardie, 1996; Rasmussen & Winder, 1997; Musi *et al.*, 2001b), nerve stimulation causing contraction *in situ* (Hutber *et al.*, 1997; Vavvas *et al.*, 1997), contractions *ex vivo* (Hayashi *et al.*, 1998; Musi *et al.*, 2001b), and in human skeletal muscle during exercise (Chen *et al.*, 2000; Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000; Chen *et al.*, 2003; Wadley *et al.*, 2006). The level of AMPK activation is dependent on metabolic stress of the contracting muscle (Ihlemann *et al.*, 1999a; Ihlemann *et al.*, 2001; Sandstrom *et al.*, 2007), as such AMPK activity increases with exercise duration (Stephens *et al.*, 2002; McConell *et al.*, 2005) and exercise intensity (Rasmussen & Winder, 1997; Wojtaszewski *et al.*, 2000; Chen *et al.*, 2003). Furthermore, skeletal muscle AMPK activity and free intracellular AMP is largely determined by absolute rather than relative exercise intensity (Wadley *et al.*, 2006).

The α2-containing complex of AMPK is the primary catalytic isoform activated during physiological *in situ* contractions (Vavvas *et al.*, 1997; Sakamoto *et al.*, 2004) and *in vivo* exercise in rodents (Musi *et al.*, 2001b), as well as during exercise in humans (Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000; Stephens *et al.*, 2002; McConell *et al.*, 2005). Interestingly, a relatively small (compared to AMPKα2) increase in skeletal muscle AMPKα1 activity is also observed during *ex vivo* contractions in rodents (Musi *et al.*, 2001b; Dzamko *et al.*, 2008; Jensen *et al.*, 2008) and sometimes during exercise in humans (Chen *et al.*, 2000; Musi *et al.*, 2001a; Roepstorff *et al.*, 2004; McConell *et al.*, 2005; Wadley *et al.*, 2006). However, AMPKα1 activity during contraction seem to be derived largely from non-muscle cells (Jorgensen *et al.*, 2004) and is less sensitive to allosteric regulation by AMP (Salt *et al.*, 1998; Cheung *et al.*, 2000) and PP2C dephosphorylation (Suter *et al.*, 2006). This would make AMPKα1 less likely to be regulated by LKB1 (the major AMPKK in skeletal muscle (Carling *et al.*, 2008)), and potentially the target of CaMKK or Tak1 activation (McGee *et al.*, 2008). Therefore, it appears that in skeletal muscle the α1 and α2 isoforms of AMPK are differentially regulated during contraction and may, therefore, have unique regulatory roles. Regardless, there is strong evidence that AMPK is activated in both human and rodent skeletal muscle in response to changes in intracellular energy status, and therefore may play a signalling role for pathway(s) which regulate cellular fuel status.

1.3.2.2 AMPK and glucose uptake: insights from AICAR

The first targets of AMPK identified were 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase and acetyl-CoA carboxylase (ACC), which are key regulatory enzymes for steroid and fatty

acid synthesis (Hardie *et al.*, 1998). Consequently, early research concerning AMPK and the regulation of cellular metabolism concentrated on steroid and fatty acid metabolism in working muscle (reviewed in Hardie *et al.*, 1998). More recently AMPK has been associated with insulin-independent skeletal muscle glucose uptake. Close parallels between glucose uptake and AMPK activity have been reported in contracting isolated rat skeletal muscle (Hayashi *et al.*, 2000; Ihlemann *et al.*, 2000; Ihlemann *et al.*, 2001; Musi *et al.*, 2001b), during exercise in humans (Chen *et al.*, 2003; Wadley *et al.*, 2006) and in isolated skeletal muscle exposed to stimuli that enhance glucose uptake including hypoxia, hyperosmolarity, and incubation in rotenone, sorbitol, and dinitrophenol (Hayashi *et al.*, 2000).

Although these parallels suggest a relationship between skeletal muscle AMPK activation and glucose uptake, they do not prove a cause and effect relationship between AMPK and contraction-stimulated glucose uptake. Many studies which have investigated the relationship between AMPK and glucose uptake into skeletal muscle have utilised the pharmacological AMPK agonist adenosine analogue 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR). AICAR is taken up into skeletal muscle and converted by adenosine kinase to the monophosphorylated nucleotide 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranotide (ZMP) (Merrill *et al.*, 1997). ZMP is an AMP mimetic, although it is less potent than AMP itself (Corton *et al.*, 1995), it induces a time- and dose-dependent increase in AMPK activity (Merrill *et al.*, 1997). Increasing AMPK activation by treating isolated rodent skeletal muscle with AICAR *ex vivo* (Merrill *et al.*, 1997; Hayashi *et al.*, 1998; Kurth-Kraczek *et al.*, 1999; Koistinen *et al.*, 2003) or *in vivo* AICAR infusion (Bergeron *et al.*, 1999; Nakano *et al.*, 2006) facilities glucose uptake by increasing GLUT4 translocation to the cell membrane (Kurth-Kraczek *et al.*, 1999; Russell *et al.*, 1999). Moreover, combining AICAR and insulin, but not AICAR and contraction, has an additive effect on skeletal muscle glucose uptake (Hayashi *et al.*, 1998; Bergeron *et al.*, 1999), and PI3K inhibition abolishes insulin but not contraction or AICAR-stimulated skeletal muscle glucose uptake (Hayashi *et al.*, 1998; Bergeron *et al.*, 1999). Therefore, it would appear that AICAR stimulates skeletal muscle glucose uptake through a similar insulin-independent pathway as contraction, however AICAR- and contraction-stimulated skeletal muscle glucose uptake cannot be assumed to be synonymous.

AMPK activation is required for AICAR-stimulated skeletal muscle glucose uptake (Musi *et al.*, 2001b). Indeed, mice that have the α2 isoform of AMPK knocked out (AMPKα2 KO) or express a kinase dead (AMPK KD) form in their skeletal muscle (Fryer *et al.*, 2002a; Sakoda

et al., 2002) have abolished AICAR-stimulated, but normal contraction-stimulated skeletal muscle glucose uptake. Furthermore, AICAR stimulation preferentially increases glucose uptake and AMPK activity in skeletal muscles containing a higher percentage of fast twitch muscle fibres (Balon & Jasman, 2001; Kaushik *et al.*, 2001; Ai *et al.*, 2002), whereas contraction activates glucose uptake in both glycolytic and oxidative fibre types (Rasmussen & Winder, 1997; Jorgensen *et al.*, 2004). This may be attributed to AICAR preferential activation of AMPKα2 (Musi *et al.*, 2001b; Jorgensen *et al.*, 2004) and the higher abundance of AMPKα2 in glycolytic muscles (Ai *et al.*, 2002). Interpreting AICAR studies is also hampered by AICAR not only targeting AMPK, but affecting all enzymes and proteins sensitive to AMP concentration. This causes numerous non-specific effects when administrated *in vivo* (Gruber *et al.*, 1989; Young *et al.*, 1996). Therefore, AICAR may only partially (if at all) mimic the contraction-induced activation of AMPK and glucose uptake in skeletal muscle.

Regardless, AICAR is still an effective substitute stimulus for insulin-independent skeletal muscle glucose uptake, and similar molecules may prove to be an important therapeutic device for both the prevention and treatment of Type 2 diabetes. While it is evident that acute AICAR treatment can reduce blood glucose in rodents (Hayashi *et al.*, 1998; Bergeron *et al.*, 2001) and humans (Cuthbertson *et al.*, 2007; Boon *et al.*, 2008), chronic AICAR treatment has also been reported to attenuate hyperglycaemia in rats (Jessen *et al.*, 2003), and obese (Song *et al.*, 2002) and insulin resistant mice (Fiedler *et al.*, 2001). This appears to occur via the suppression of endogenous glucose production (Fiedler *et al.*, 2001; Song *et al.*, 2002; Boon *et al.*, 2008) and some enhancement of insulin-stimulated glucose uptake (Bergeron *et al.*, 2001; Jessen *et al.*, 2003) rather than the activation of insulin-independent skeletal muscle glucose uptake. Similarly, the pharmacological agents metformin, rosiglitazone and phenformin, which are commonly prescribed to people with Type 2 diabetes to improve glucose homeostasis, increase basal skeletal muscle AMPK activity and reduce blood glucose levels partly by increasing glucose disposal (Zhou *et al.*, 2001a; Fryer *et al.*, 2002b; Hawley *et al.*, 2002; Musi *et al.*, 2002; Hardie & Sakamoto, 2006). Interestingly, recent studies have shown that chronic AICAR treatment in rodents may induce similar beneficial adaptations as endurance exercise training, such as increased muscle glycogen, GLUT4 and mitochondrial enzyme concentrations (Holmes *et al.*, 1999; Winder *et al.*, 2000), muscle signalling during exercise (McConell *et al.*, 2008), endurance performance and metabolic gene expression (Narkar *et al.*, 2008). Surprisingly, combining AICAR treatment and endurance training

appears to have some synergistic effects (Narkar *et al.*, 2008). Thus, the potential role of AICAR and similar molecules as therapeutic agents is likely to prove to be an exciting area of research in the future, with its influence likely not being limited to AMPK activation alone.

AICAR as an AMPK activator has provided substantial insight into the metabolic regulatory function of AMPK, but the stimulation of skeletal muscle AMPK activity and glucose uptake by AICAR is not analogous with contraction. Regardless, AICAR has provided evidence to suggest that AMPK has the potential to mediate insulin-independent skeletal muscle glucose uptake, and its role as a therapeutic has only begun to be investigated.

1.3.2.3 AMPK and contraction-stimulated glucose uptake

A lack of AMPK-specific pharmacological inhibitors and activators has made characterising the role of AMPK in regulating cellular metabolism difficult. Despite links between exercise-induced skeletal muscle AMPK activation and glucose uptake, disassociations between AMPK activation and glucose uptake have been observed in humans during hypoxic exercise (Wadley *et al.*, 2006), glycogen-loaded *ex vivo* muscle contractions (Derave *et al.*, 2000), low intensity exercise (Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000; Wojtaszewski *et al.*, 2002) and during exercise following short-term training (McConell *et al.*, 2005). These results have called into question the importance of AMPK in regulating skeletal muscle glucose uptake during contraction. Indeed, during the first 2 hr of low intensity exercise (45% $VO_{2\ peak}$) in humans AMPK activity is not increased, despite leg glucose uptake being increased ~10-fold (Wojtaszewski *et al.*, 2002). Furthermore, 10 days of exercise training in humans abolished the 9-fold increase in AMPK α2 activity observed during the pre-training exercise, but only attenuated the increase in glucose uptake by ~10-15% (McConell *et al.*, 2005).

The development of mice models with genetically manipulated expression and activation of AMPK in skeletal muscle has provided a viable non-pharmacological approach to studying the relationship between skeletal muscle contraction-stimulated AMPK activation and glucose uptake. Mu *et al.* (2001) and Fujii *et al.* (2005) developed transgenic mice that over express a muscle specific kinase dead dominate negative form of AMPKα2 (AMPK KD), which is not activated by AICAR-, hypoxia-, hyperosmotic- or contraction-stimulation (Mu *et al.*, 2001; Fujii *et al.*, 2005). These mice have abolished skeletal muscle AICAR-stimulated and hypoxia-stimulated glucose uptake, but surprisingly, glucose uptake during *ex vivo* contractions (Mu *et al.*, 2001; Fujii *et al.*, 2005) and *in situ* (Mu *et al.*, 2001) was only

attenuated by 20-40%. This reduction in glucose uptake was associated with compromised maximal contraction force when stimulated at high frequencies (Fujii *et al.*, 2005; Lefort *et al.*, 2008), and reduced cell surface GLUT4 content after simulation (Mu *et al.*, 2001). Since skeletal muscle glucose uptake may be dependent on the degree of force development or work done by the muscle (Ihlemann *et al.*, 1999b; Ihlemann *et al.*, 2001; Fujii *et al.*, 2005), it is possible that the reduction in glucose uptake observed in AMPK KD muscles was a result of reduced maximal contraction force and not the impairment of AMPKα2 activity (Fujii *et al.*, 2005). Indeed, when wild type (WT) contraction force is matched to AMPK KD contraction force by reducing the stimulation voltage, AMPK KD mice have similar increases in skeletal muscle glucose uptake during contraction as WT (Fujii *et al.*, 2005). However, despite workload remaining similar, reducing WT stimulation voltage may alter muscle fibre recruitment and signalling, and therefore potentially reduce glucose uptake in itself (Sandstrom *et al.*, 2007; Lefort *et al.*, 2008).

Several studies have shown disassociations between skeletal muscle contraction force and glucose uptake (Holloszy & Narahara, 1965; Sandstrom *et al.*, 2007). In an elegant study by Sandstrom *et al.* (2007) the treatment of isolated EDL muscles with the cross-bridge force production inhibitor, N-benzyl-p-toluene sulphonamide (BTS), abolished mechanical force development during stimulation, but only had minor reducing effects on glucose uptake and AMPK phosphorylation. This indicates that skeletal muscle glucose uptake signalling in response to metabolic stress occurs independent of, or prior to contractile force production. Recently, Lefort *et al.* (2008) has shown that during a less intense, shorter duration *ex vivo* contraction protocol, where AMPK KD and WT mice muscle have similar contractile force development, the increase in AMPK KD mice muscle glucose uptake is reduced by ~50%. In contrast, neither whole body AMPKα2 knockout nor AMPKα1 knockout affects *ex vivo* EDL or soleus muscle glucose uptake during contraction (Jorgensen *et al.*, 2004). Importantly, however AICAR-induced glucose uptake is abolished by AMPKα2 but not α1 ablation. Although AMPKα2 knockout mice had a 2-3-fold increase in AMPKα1 expression compared to wild type mice (Jorgensen *et al.*, 2004), AMPKα1 appears to play little role in the regulation of skeletal muscle glucose uptake during contraction (see above: *Contraction activates AMPK*), therefore making it unlikely that the upregulation of AMPKα1 activity could compensate for the loss in AMPKα2 (Sakamoto *et al.*, 2005). Interestingly, a recent study by Jensen *et al.* (2008) reported that during low intensity *ex vivo* twitch-contractions (0.1 ms, 2 Hz, 2 min) AMPK α1 KO soleus muscles have impaired glucose uptake compared

to WT. Whether AMPKα1 is important in regulating skeletal muscle glucose uptake during very low intensity stimulation in glycolytic muscle types or during *in vivo* exercise is yet to be established. It should be considered, however, that in human skeletal muscle AMPKα1 is not activated at low exercise intensities (Wojtaszewski *et al.*, 2000; Chen *et al.*, 2003; Wadley *et al.*, 2006) although glucose uptake increases substantially.

The two studies which have directly investigated the role of AMPK in regulating glucose uptake during *in vivo* exercise in rodents have provided conflicting results (Lee-Young *et al.*, 2009; Maarbjerg *et al.*, 2009). While both studies measured skeletal muscle glucose uptake in AMPK KD and WT mice during treadmill running, Maarbjerg *et al.* (2009) reported that AMPK KD had similar increases in skeletal muscle glucose uptake as WT type mice, but Lee-Young *et al.* (2009) reported that AMPK KD had reduced skeletal muscle glucose uptake during exercise. The discrepancies between these findings are difficult to discern, but may relate to methodological differences with Lee-Young *et al.* (2009) using catheters to administer glucose tracer 5 min into exercise, while Maarbjerg *et al.* (2009) used I.P injection immediately prior to exercise. Interestingly, in addition to both studies reporting that AMPK KD mice have severely impaired exercise tolerance, Lee-Young *et al.* (2009) showed that AMPK KD mice have substantially reduced substrate delivery to muscle during exercise as shown by a ~50% lower proportion of cardiac output to going to the contraction muscle than WT mice. They suggest that this contributed to the attenuation of skeletal muscle glucose uptake during exercise in the AMPK KD mice, questioning whether AMPK signalling is required for the transport of glucose into the muscle cell during exercise *in vivo*. Indeed, Maarbjerg *et al.* (2009) showed that AMPK KD mice have normal GLUT4 translocation to the muscle cell surface during exercise. Therefore, taken with the findings that at low exercise intensities (Wojtaszewski *et al.*, 2000; Wojtaszewski *et al.*, 2002) and during exercise following short-term exercise training (McConell *et al.*, 2005) AMPK is not activated despite increases in glucose uptake these *in vivo* studies in AMPK KD mice suggest that AMPK is required for the signalling of glucose transport during *in vivo* exercise.

The generation of mice with reduced or deficient expression of LKB1, an upstream kinase of AMPK (Hawley *et al.*, 2003), has provided further insight into the regulation of skeletal muscle contraction-stimulated glucose uptake (Sakamoto *et al.*, 2005). Reduced expression of LKB1 lowered AMPKα2 activity by ~2-fold but did not affect *ex vivo* skeletal muscle glucose uptake during contraction (Sakamoto *et al.*, 2005), while the ablation of LKB1 abolished

AMPKα2 activation and severely blunted contraction-stimulated skeletal muscle glucose uptake. This may provide some support for to a pivotal role of AMPK activation in signalling skeletal muscle glucose uptake during contraction. However, LKB1 is not only an upstream kinase for AMPK but regulates up to 12 other AMPK-related kinases (Sakamoto *et al.*, 2004), suggesting one or more of these may also be necessary for normal increases in skeletal muscle glucose uptake during contraction independent of AMPK.

In summary, findings from mice with deficient AMPK signalling *ex vivo* lend some support to the notion that AMPKα2 is involved in regulating skeletal muscle glucose uptake during contraction. However, AMPK appears not to be a critical mediator of skeletal muscle glucose uptake during *ex vivo* contractions or *in vivo* exercise. Indeed, it may play a redundant role, where under some conditions it is more important in signalling skeletal muscle glucose uptake during contraction than others. In contrast AMPKα1 does not appear to be significantly involved in the regulation of contraction-stimulated glucose uptake. Interestingly, there is evidence to suggest that LKB1 is critical for normal contraction-stimulated skeletal muscle glucose uptake, but it may be involved in AMPK-independent signalling and this is worthy of further investigation.

1.3.2.4 Downstream targets of AMPK involved with skeletal muscle glucose regulation

AMPK-NO

While it is known that AMPK activation increases the trafficking of GLUT4 vesicles to the cell membrane to facilitate glucose uptake (Kurth-Kraczek *et al.*, 1999), the mechanism by which this occurs is not completely understood (Hardie, 2007). AICAR has been shown to phosphorylate endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase in rat cardiac muscle and human skeletal muscle (Chen *et al.*, 1999; Li *et al.*, 2004). The production of nitric oxide (NO) by NOS has been implicated in the facilitation of insulin-independent skeletal muscle GLUT4 translocation and glucose uptake (see section 1.3.4 Nitric oxide and McConell & Kingwell, 2006). Indeed, the inhibition of NOS in H-2k^{b} cells (Fryer *et al.*, 2000), rat cardiac muscle (Li *et al.*, 2004) and *in vivo* in rats (Shearer *et al.*, 2004) has been shown to attenuate AICAR-stimulated increases in glucose uptake. However, it is not known whether AMPK phosphorylation of nNOSμ in skeletal muscle increases its activity like eNOS (Chen *et al.*, 1999). Interestingly, there is some evidence that AMPK may also act downstream of NO in regulating skeletal muscle glucose uptake, and this is discussed in section *1.3.4 Nitric oxide.*

AMPK-AS160 (TBC1D4) and TBC1D1

While there is strong evidence that Akt phosphorylation of AS160 is required for insulin stimulated GLUT4 trafficking and glucose uptake (see section *1.1 Skeletal muscle glucose uptake: insulin vs contraction* and Sano *et al.*, 2003) there is also some evidence that AS160 may be involved in regulating AMPK-stimulated and contraction-stimulated skeletal muscle glucose uptake. The treatment of isolated skeletal muscle with the AMPK activator, AICAR, increases AS160 phosphorylation without altering Akt phosphorylation (Bruss *et al.*, 2005; Kramer *et al.*, 2006a), and similar to glucose uptake, mice with deficient AMPK signalling have severely diminished or completely abolished AICAR-stimulated AS160 phosphorylation (Treebak *et al.*, 2006). Furthermore, recombinant AMPK phosphorylates AS160 in cell free assays (Treebak *et al.*, 2006) and combining insulin and AICAR, as with glucose uptake, has an additive effect on skeletal muscle AS160 phosphorylation (Kramer *et al.*, 2006a). Taken together this suggests that AMPK can directly phosphorylate AS160, and is likely to be the mechanism though which AMPK stimulates GLUT4 translocation and glucose uptake.

Contraction of rodent skeletal muscle *ex vivo* (Bruss *et al.*, 2005; Kramer *et al.*, 2006a; Funai & Cartee, 2008), *in situ* (Kramer *et al.*, 2006a; Kramer *et al.*, 2006b) and during *in vivo* exercise in humans and rodents (Kramer *et al.*, 2006a; Treebak *et al.*, 2007) can increase AS160 phosphorylation. However, this is not a universal findings with AS160 phosphorylation not increasing during short duration (30s, 2 min and 20 min), high intensity (maximal sprint, 110% and 80% $VO_{2\ peak}$, respectively) exercise, and not occurring until >60 min of moderate intensity (67% $VO_{2\ peak}$) exercise in humans (Treebak *et al.*, 2006). Similarly, low-intensity twitch contractions in mouse soleus muscles *ex vivo* do not increase AS160 phosphorylation but does increase AMPKα1 activity and glucose uptake (Jensen *et al.*, 2008). When contraction does increase AS160 phosphorylation it is independent of Akt signalling in some (Kramer *et al.*, 2006a; Funai & Cartee, 2008, 2009), but not all (Bruss *et al.*, 2005; Kramer *et al.*, 2006a) studies. Indeed, Akt2 KO mice show no increase in gastrocnemius AS160 phosphorylation with insulin stimulation, but normal increases with contraction (Kramer *et al.*, 2006a). This indicates that contraction can increase AS160 phosphorylation independent of Akt. AMPK appears to be the major upstream kinase phosphorylating AS160 during contraction since contraction-stimulated AS160 phosphorylation is either partially or completely inhibited in skeletal muscle of mice with deficient AMPK signalling (Kramer *et al.*, 2006a; Treebak *et al.*, 2006). However, some contraction-stimulated increases in AS160 phosphorylation is evident in muscles from AMPK KD mice treated with wortmannin (which

prevents Akt signalling) (Kramer *et al.*, 2006a), and the AMPK inhibitor, compound C, does not affect the Akt-independent increases in AS160-phosphorylation during muscle contraction (Funai & Cartee, 2009). This suggests that during contraction a kinase(s) other than AMPK and Akt is phosphorylating AS160. Since AS160 has a calmodulin binding domain (CBD) (Kane & Lienhard, 2005) and calmodulin (CaM) binds to AS160 *in vitro* (Kane & Lienhard, 2005; Kramer *et al.*, 2007), it appears that during contraction CaM kinases may also regulate AS160 signalling.

There have only been a handful of studies which have directly assessed the role of AS160 in regulating skeletal muscle glucose uptake during contraction. Kramer *et al.* (2006b) used *in vivo* electroporation to over-express a mutant AS160 which prevents phosphorylation at four Akt/AMPK phosphorylation sites (4P) in mouse tibialis anterior (TA) muscles. They showed 4P mice had reduced TA muscle glucose uptake during contraction. In a separate study, using the same technique, this group were also able to show that the mutation of the AS160 CBD also attenuated TA muscle contraction-stimulated glucose uptake, but combined CBD and 4P mutations did not have an additive effect (Kramer *et al.*, 2007). This is suggestive of a mutual role for the CBD and AMPK/Akt phosphorylation sites of AS160 on regulating GLUT4 translocation and glucose uptake during skeletal muscle contraction. Importantly, Kramer *et al.* (2006b; 2007) also showed that the co-expression of AS160 mutated to prevent Rab GTPase activity (thus acting as activated AS160) with either 4P or CBD mutation restored TA muscle contraction-stimulated glucose uptake. This supports the hypothesis that AS160 regulates glucose uptake via the alteration of Rab GTPase activity (see section 1.1 *Skeletal muscle glucose uptake: insulin vs contraction* and Sano *et al.*, 2003).

The majority of studies (Kane *et al.*, 2002; Bruss *et al.*, 2005; Kramer *et al.*, 2006a; Treebak *et al.*, 2006; Kramer *et al.*, 2007; Funai & Cartee, 2008) assessing AS160 signalling in skeletal muscle use an anti-phosphorylation-(Ser/Thr) Akt Substrate (PAS) antibody that recognises several Akt phosphorylation motif peptide sequences on proteins. The protein band detected at 160 kDa with this PAS-antibody represents AS160. Therefore, this antibody shows the cumulative phosphorylation of several Akt sites (some of which are also phosphorylated by AMPK) of AS160, and does not recognise CBD activation of AS160 (Kramer *et al.*, 2007). Interestingly, recent work has shown that during contraction AS160 is phosphorylated outside of the Akt consensus motif at AMPK regulated Ser^{711} (Treebak *et al.*, 2009b). Therefore, it is important that other Akt-independent phosphorylation sites on AS160

be investigated. Furthermore, phosphorylation site specific AS160 antibodies should be used since the Akt phosphorylation sites of AS160 may be differentially regulated during contraction, and contribute in isolation to AS160 regulation of skeletal muscle glucose uptake during contraction. Indeed, such phosphorylation site specific antibodies have been developed by Jorgen Wojtaszewski's group, and have been used to show that insulin-stimulation of human muscle causes differential phosphorylation of AS160 Akt phosphorylation sites 4 hr post exercise, compared to that of rested muscle (Treebak *et al.*, 2009a).

Recently, another Akt substrate has been shown to exist in skeletal muscle very close to AS160, at 150 kDa (TBC1D1) (Funai & Cartee, 2008; Taylor *et al.*, 2008). Like AS160, skeletal muscle TBC1D1 is recognised by the PAS antibody (Funai & Cartee, 2008; Taylor *et al.*, 2008; Pehmoller *et al.*, 2009). Furthermore, in muscles with a higher proportion of glycolytic fibres TBC1D1 is much higher expressed than AS160, and it accounts for the majority of Akt substrate recognised by PAS around 160 kDa (Taylor *et al.*, 2008). Therefore, care must be taken when interpreting results from the PAS antibody as it may not solely represent phosphorylation of AS160.

TBC1D1 and AS160 share similar GAP domains (Roach *et al.*, 2007), therefore it is not surprising that TBC1D1 is also suspected to be involved in the regulation of skeletal muscle GLUT4 translocation and glucose uptake (Chen *et al.*, 2008). In skeletal muscle PAS-TBC1D1 phosphorylation is increased by insulin-stimulation, AICAR-stimulation and contraction (Funai & Cartee, 2008; Taylor *et al.*, 2008; Funai & Cartee, 2009; Pehmoller *et al.*, 2009). Similar to contraction-stimulated glucose uptake, PAS-TBC1D1 phosphorylation is not affected by the inhibition of the insulin signalling pathway (with wortmannin), but the AMPK inhibitor compound C prevents contraction-stimulated PAS-TBC1D1 phosphorylation and attenuates glucose uptake during *ex vivo* contraction (Funai & Cartee, 2009). This suggests that during contraction AMPK may be responsible for the activation of TBC1D1. In support, Pehmoller *et al.* (2009) has recently shown that the contraction-stimulated increases in skeletal muscle TBC1D1 Thr^{596} and Ser^{237} phosphorylation or 14-3-3 protein binding (the mechanisms through which TBC1D1 is proposed to regulate GAP function (Chen *et al.*, 2008)) are abolished in muscles from AMPK KD mice. Furthermore, insulin-stimulation does not affect TBC1D1 Ser^{237} phosphorylation or 14-3-3 protein binding. This suggests that TBC1D1 signalling during contraction may be regulated by AMPK, and through a pathway(s) independent to insulin TBC1D1 signalling. Whether TBC1D1 is essential for normal

increases in skeletal muscle glucose uptake during contraction will be an interesting area for further research.

To summarise, the Akt substrate of 160 kDa (AS160) is phosphorylated by AMPK, and during skeletal muscle contraction AS160 phosphorylation appears to occur through an Akt independent mechanism that may involve signalling from AMPK and CaMK's. Although AS160 facilities skeletal muscle insulin-stimulated GLUT4 translocation and glucose uptake, the role of AS160 in regulating skeletal muscle contraction-stimulated glucose uptake is less clear and would be aided by studies which identify how specific phosphorylation sites of AS160 are regulated during contraction. Like AS160, Akt substrate of 150 kDa, TBC1D1, can regulate GLUT4 translocation and glucose uptake, and appears to be activated by AMPK during skeletal muscle contraction. However its role in regulating contraction-stimulated glucose uptake is just beginning to be investigated. There is some evidence to suggest, however, that AS160 and TBC1D1 may act as convergent points for insulin-dependent and -independent signalling of skeletal muscle glucose uptake. Furthermore AS160 and TBC1D1 regulation of GLUT4 translocation are likely to be the primary, but not only, mechanism through which AMPK can increase glucose uptake independent of insulin.

1.3.3 CALCIUM, PKC AND CAMK'S

Skeletal muscle cell plasma membrane and t-tubule depolarisation initiates contraction by causing the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in the muscle cell. This transient increase in myocellular Ca^{2+} concentration may be involved in the signalling of skeletal muscle contraction-stimulated glucose uptake (Holloszy & Hansen, 1996). Early studies using caffeine to induce non-depolarisation release of Ca^{2+} from the SR of isolated frog sartorius and rat epitrochlearis muscles showed that rising skeletal muscle intracellular Ca^{2+} concentrations increases glucose uptake *ex vivo* (Holloszy & Narahara, 1967; Youn *et al.*, 1991) without altering high energy phosphate levels or inducing contraction (Youn *et al.*, 1991; Terada *et al.*, 2003). Furthermore, the inhibition of SR release of Ca^{2+} prevents caffeine-stimulated glucose uptake (Youn *et al.*, 1991; Nolte *et al.*, 1995), and muscle cell depolarisation alone (i.e. without contraction) increases glucose uptake (Wijesekara *et al.*, 2006).

Intracellular concentrations of Ca^{2+} and it is likely that Ca^{2+} activates downstream kinases which then facilitate glucose uptake during skeletal muscle contraction, rather than directly stimulating GLUT4 translocation and glucose uptake itself. Protein kinase C (PKC) is a kinase with 12 different isoforms expressed in skeletal muscle, some of which are activated by increases in cellular Ca^{2+} and contraction (Jessen & Goodyear, 2005). The down regulation of conventional and novel PKC isoforms by long-term phorbol ester treatment in perfused rat hindlimb (Cleland *et al.*, 1989), and inhibition using calphostin C (Wojtaszewski *et al.*, 1998; Ihlemann *et al.*, 1999a) and polymyxin B (Henriksen *et al.*, 1989; Young *et al.*, 1991) has been shown to attenuate increases in skeletal muscle glucose uptake during *ex vivo* contraction of rat muscle with predominantly glycolytic fibres, but not in muscle with predominantly oxidative fibres (Wojtaszewski *et al.*, 1998). Furthermore, evidence of this pathway in regards to contraction-stimulated glucose uptake is lacking in humans (Rose *et al.*, 2004).

The calmodulin family of kinases are also calcium activated substrates which have been suggested to play a role in the signalling of skeletal muscle glucose uptake during contraction. Increases in cytosolic concentration of Ca^{2+} cause the phosphorylation of calmodulin-dependent protein kinase's (CaMK) and the CaMK inhibitors KN62 and KN93 block caffeine-stimulated skeletal muscle glucose uptake (Rose & Hargreaves, 2003; Wright *et al.*, 2004; Wright *et al.*, 2005). Furthermore, the inhibition of CaMK and CaMKK (an upstream kinase of the CaMK's) reduces skeletal muscle *ex vivo* contraction-stimulated glucose uptake in rat and mouse epitrochlearis, EDL and soleus muscles (Wright *et al.*, 2004; Wright *et al.*, 2005; Jensen *et al.*, 2007a; Jensen *et al.*, 2007b). Since the inhibition of CaMK kinase shows little effect on glucose uptake in muscles with a higher proportion of glycolytic fibres (EDL) during twitch contractions (Jensen *et al.*, 2007b), the relationship between CaMK activation and skeletal muscle glucose uptake during contraction seems to depend on contraction duration and/or intensity, as well as muscle fibre composition.

Whether the relationship between Ca^{2+}, CaMK's and glucose uptake requires the activation of AMPK has been the focus of several studies. Wright and colleagues have provided evidence that AMPK and Ca^{2+} work in parallel to increase skeletal muscle glucose uptake during contraction (Wright *et al.*, 2004). They suggest that in muscles containing predominantly glycolytic fibres AMPK and Ca^{2+}/CaMK both contribute 50% to the increase in skeletal muscle glucose uptake during contraction, whereas muscles containing predominantly

oxidative fibres depend largely on Ca^{2+} signalling (Wright *et al.*, 2004; Wright *et al.*, 2005). This is based on the observation that 1) the AMPK activator, AICAR, and caffeine have an additive affect on skeletal muscle glucose uptake in glycolytic type muscles, 2) in muscles containing predominantly glycolytic fibres the inhibition of CaMK during contraction reduces skeletal muscle glucose uptake (by ~50%) without affecting AMPKαThr^{172} phosphorylation, and 3) AICAR (and therefore presumably AMPK activation) does not increase glucose uptake in muscles containing predominantly oxidative fibres.

In contrast to Holloszy's laboratory (Wright *et al.*, 2004; Wright *et al.*, 2005), Erik Richter's group have provided evidence of interactions between AMPK and CaMK signalling in the regulation of skeletal muscle glucose uptake during contraction. They have shown that isolated skeletal muscle from AMPK KD mice have an attenuated caffeine-stimulated increase in glucose uptake (Jensen *et al.*, 2007a), and that the inhibition of CaMKK or CaMK does not further attenuate contraction-stimulated glucose uptake in AMPK KD mice muscle (Jensen *et al.*, 2007b). Furthermore, the inhibition of CaMKK attenuates glucose uptake and AMPKα1 and α2 activity following 10 min of contraction in both predominantly slow and fast type muscles (Jensen *et al.*, 2007b). However, although the inhibition of CaMK initially (at 2 min) attenuates the increase in AMPKα1 and α2 activity during contraction in muscles containing both predominantly oxidative and glycolytic fibres, this attenuation is reversed by 10 min of contraction despite CaMK inhibition attenuating skeletal muscle glucose uptake (Jensen *et al.*, 2007b). This suggests some degree of disassociation between AMPK and CaMK signalling of skeletal muscle glucose uptake during contraction. In agreement with this disassociation, the overexpression of constitutively active CaMKKα increases *in vivo* resting muscle glucose uptake in WT and AMPK KD mice (Witczak *et al.*, 2007), indicating that AMPK is not required for CaMKKα-stimulated skeletal muscle glucose uptake. It is worthy noting, however, that CaMKKβ is also expressed in skeletal muscle and may play a role in the regulation of contraction stimulated glucose uptake (Jensen *et al.*, 2007b).

Taken together this data indicates that Ca^{2+} signalling contributes, in part, to the regulation of skeletal muscle glucose uptake during contraction through CaMKK and CaMK's, but this relationship is somewhat dependent on contraction duration and/or intensity as well as muscle fibre type. Furthermore, the Ca^{+}/CaMKK/CaMK pathway interacts to some extent with AMPK, but AMPK activation may not be required for Ca^{2+}-stimulated skeletal muscle glucose uptake during contraction. Therefore, pathways other than Ca^{2+} and AMPK must be

required for glucose uptake during contraction, and it is possible that Ca^{2+} may also signal glucose uptake though CaMK-independent pathway(s). Indeed, there is evidence that Ca^{+}/CaMK signalling can increase NOS activity (Fleming *et al.*, 2001), and therefore presumably NO production, and activate AS160, both of which have been implicated in the regulation of skeletal muscle glucose uptake during contraction (see sections *1.3.2 AMP-activated protein kinase (AMPK) in skeletal muscle*, and *1.3.4 Nitric oxide*).

1.3.4 NITRIC OXIDE

Nitric oxide (NO) is a biological gas produced by a wide range of cells and is a member of a group of labile molecules known as reactive nitrogen species (RNS). NO has been implicated in the signalling of a diverse range of physiological processes such as smooth muscle relaxation (Warner *et al.*, 1994), immune function (Bogdan, 2001), platelet aggregation (Buechler *et al.*, 1994) and neural transmission (Warner *et al.*, 1994). NO has generally been considered to exert its downstream affects by binding to soluble guanylate cyclase, promoting the formation of cyclic guanosine monophosphate (cGMP) and activating cGMP-dependent protein kinase (PKG) (Denninger & Marletta, 1999). However, it is increasingly becoming recognised that NO can also act as a signalling molecule though several cGMP-independent pathways that are discussed below (*1.3.4.4 Potential downstream targets of nitric oxide in relation to glucose uptake* and Foster *et al.*, 2009).

1.3.4.1 NO production

The synthesis of NO and L-citrulline from L-arginine and oxygen is catalysed by nitric oxide synthase (NOS) in a complex series of reactions which require tight binding of CaM with cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) and iron protoporphin (heam) group, as well as the co-substrates NADPH and molecular oxygen (Stamler & Meissner, 2001). There are three NOS isoform's; 1) neuronal NOS (nNOS), 2) endothelial NOS (eNOS), and 3) inducible NOS (iNOS), which are also known as NOS I, II and III respectively. nNOSμ which has an extra 34 amino acids compared to normal nNOS (nNOSα) is the primary isoform expressed in skeletal muscle and is more abundant in muscles with a high proportion of glycolytic fibres (Balon & Nadler, 1994; Kobzik *et al.*, 1994; Tidball *et al.*, 1998; Lau *et al.*, 2000). nNOS is likely responsible

for NO-mediated signalling in skeletal muscle (Silvagno *et al.*, 1996; Lau *et al.*, 2000; McConell *et al.*, 2007) and will be the focus of the remainder of this review.

nNOS is constitutively active in skeletal muscle (Balon & Nadler, 1994), and its activity level is regulated both allosterically through Ca^{2+}, and covalently by phosphorylation (Chen *et al.*, 2000; Stamler & Meissner, 2001). Ca^{2+} has been proposed to increase nNOS activity (Bredt & Snyder, 1990) by reducing the autoinhibition of nNOS through increasing CaM binding to its FMN subdomain (Alderton *et al.*, 2001). CaM binding to nNOS FMN subdomain facilitates FAD and FMN transfer of electrons from NADPH to its haem group which is required for an increase in NOS activity (Klatt *et al.*, 1995; Gachhui *et al.*, 1996). Furthermore, there is some evidence to suggest that catalytic inhibition of NOS by protein inhibitor of nNOS (PIN) (Jaffrey & Snyder, 1996; Guo *et al.*, 2001) and caveolin (Venema *et al.*, 1997) may contribute to the regulation of NOS activity. Moreover, several proteins including CaMKII (Nakane *et al.*, 1991; Komeima *et al.*, 2000), PKC (Hayashi *et al.*, 1999) and AMPK (Chen *et al.*, 2000; Chen *et al.*, 2003) have been shown to phosphorylate nNOS. While *in vitro* nNOS activity is increased by PKC phosphorylation and reduced by CaMKII phosphorylation, the affect of AMPK phosphorylation on nNOS activity is not known, and the biological significance of skeletal muscle nNOS phosphorylation by any of these proteins is unclear. However, similar to CaMK (Wright *et al.*, 2004), AMPK (Winder & Hardie, 1996) and PKC (Rose *et al.*, 2004) signalling, NOS phosphorylation, activity and NO production is increased during skeletal muscle contraction (Balon & Nadler, 1994; Chen *et al.*, 2000; Ross *et al.*, 2007).

Interestingly, *in vivo* NO can also be produced through a largely L-arginine/NOS independent pathway. This pathway involves the reduction of the oxidised by-product of NO, nitrite and nitrate (or collectively NOx), to generate NO (Benjamin *et al.*, 1994; Benjamin & Vallance, 1994; Lundberg *et al.*, 1994). Although the exact mechanisms though which NOx is reduced to generate NO is not clear, there is some evidence that NO generation from NOx is important during exercise since NOS inhibition increases NOx uptake from the blood during cycling (Kingwell *et al.*, 2002) and forearm exercise (Gladwin *et al.*, 2000).

1.3.4.2 Nitric oxide and resting skeletal muscle glucose uptake

Balon and Nadler (1994) were the first to show that skeletal muscle produces NO at rest and production of NO by skeletal muscle is increased with contraction. They also observed that in addition to the NOS inhibitor L-NMMA reducing resting muscle NO production, it also

slightly diminished basal skeletal muscle glucose uptake. In a subsequent study by the same group, the NO donor sodium nitroprusside (SNP) was shown to increase resting skeletal muscle glucose uptake (Balon & Nadler, 1997). NO donors stimulate skeletal muscle glucose uptake in a dose-dependent manner (Young *et al.*, 1997; Higaki *et al.*, 2001) by increasing GLUT4 translocation to the cell membrane (Etgen *et al.*, 1997). In humans, femoral artery infusion of SNP increases resting glucose uptake (Durham *et al.*, 2003). Of note however, very high concentrations of donors (ie >15 mM) attenuate increases in skeletal muscle glucose uptake (Higaki *et al.*, 2001). Since NOS inhibition does not affect *ex vivo* insulin-stimulated glucose uptake, and combining NO donors and insulin have an additive effect on skeletal muscle glucose uptake, it is generally accepted that NO donors stimulate glucose uptake independent of insulin signalling (Balon & Nadler, 1997; Roberts *et al.*, 1999; Higaki *et al.*, 2001).

1.3.4.3 Is nitric oxide involved in signalling contraction-stimulated glucose uptake?

In Rodents:

The role of NO in signalling rodent skeletal muscle glucose uptake during contraction is controversial (Table 1). NOS inhibition has been shown by some to substantially attenuate or abolish the increase in rodent skeletal muscle glucose uptake during contractions *ex vivo* and *in vivo* (Balon & Nadler, 1997; Roberts *et al.*, 1997; Stephens *et al.*, 2004; Ross *et al.*, 2007) while others have reported no affect (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002). Although it is difficult to explain these conflicting findings they are likely due to methodological inconsistencies. In particular, a number of studies that have found NOS inhibition not to effect skeletal muscle glucose uptake measured *>20 min following* contraction/exercise (Etgen *et al.*, 1997; Higaki *et al.*, 2001) and/or the have not inhibited NOS until after contraction (Balon & Nadler, 1997; Higaki *et al.*, 2001). Consequently, these studies have investigated whether NO is involved in regulating the enhanced rate of glucose uptake seen *following* exercise/contraction, rather than glucose uptake *during* contraction. Interestingly, however, several studies that have also measured skeletal muscle glucose uptake *following* contraction have shown NOS inhibition to attenuate (Stephens *et al.*, 2004) or abolish (Balon & Nadler, 1997) the increase in skeletal muscle glucose uptake. These inconsistencies may be attributed to seemingly minor disparities in methodologies, including muscle fibre type, contraction parameters, glucose uptake measurement techniques (ie, capping of the incubation chambers vs. open top chambers) and variability in gender and age of rodents (McConell & Kingwell, 2006).

Table 1.1 The effect of NOS inhibition on contraction-stimulated glucose uptake in rats

Study	Muscle	Dose and inhibitor	Effect
Ex vivo			
Stephens *et al.* (2004)	Epitrochlearis	100 μM L-NMMA	↓ ~70%
Higaki *et al.* (2001)	EDL	100 μM L-NMMA	None
Etgen *et al.* (1997)	Epitrochlearis	100 μM L-NMMA	None
In situ/ex vivo			
Ross *et al.* (2007)	Lower Hind Limb	5 μmol/L L-NAME	↓ ~35%
Higaki *et al.* (2001)	EDL	100 μM L-NMMA	None
Balon *et al.* (1997)	EDL	100 μM L-NMMA	↓ ~100%
In vivo			
Rottman *et al.* (2002)	Not stated	1 $mg \cdot mL^{-1}$, 3d L-NAME	None
Higaki *et al.* (2001)	Soleus	1 $mg \cdot mL^{-1}$, 2d L-NAME	None
Roberts *et al.* (1997)	Fast twitch	1 $mg \cdot mL^{-1}$, 2d L-NAME	↓ ~100%

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

The three studies that have investigated the effect of NOS inhibition on skeletal muscle glucose uptake during/immediately following *in vivo* exercise have also reported conflicting results. Rottman *et al.* (2002) showed that 3 days of NOS inhibitor (N^G-nitro-L-arginine methyl ester; L-NAME) ingestion did not affect glucose uptake of rat soleus, superficial vastus lateralis or gastrocnemius muscles immediately following treadmill running to exhaustion. Similarly Higaki *et al.* (2001) found that soleus muscles glucose uptake during treadmill running was not affected by 2 days L-NAME ingestion. In contrast Roberts *et al.* (1997) found that 2 days of L-NAME ingestion abolished *in vivo* exercise-stimulated, but not insulin-stimulated increases in glucose transport in sarcolemmal vesicles of EDL muscle. Since short-term L-NAME ingestion elevates blood pressure and causes insulin resistance (Balon *et al.*, 1999; De Angelis Lobo d'Avila *et al.*, 1999), it is conceivable that the longer L-NAME ingestion protocol employed by Rottman *et al.* (2002) (3 vs 2 days) may have influenced contraction-stimulated glucose uptake and account for the this discrepancy. Although Higaki *et al.* (2001) used only 2 day L-NAME ingestion, glucose uptake was only measured in soleus which contain predominantly oxidative fibres and have substantially lower NOS activity than muscles containing glycolytic fibres (Kobzik *et al.*, 1994). Furthermore,

glucose uptake was measured *ex vivo*, following exercise rather than *during* exercise which may have confounded findings.

Inyard *et al.* (2007) found that infusion of L-NAME tended to attenuate rodent skeletal muscle glucose uptake during *in situ* contractions independent of bloodflow. In support a recent study from our laboratory showed that the infusion of L-NAME locally (via the epigastric artery) into one contracting hindlimb of an anesthetised rat attenuated the increase in glucose uptake during contraction by ~35% compared with saline infusion (Ross *et al.*, 2007). Importantly, although L-NAME infusion caused a slight reduction in femoral blood flow; muscle capillary blood flow, blood pressure and heart rate were not affected (Ross *et al.*, 2007). This suggests that in rodents NO is an important regulator of skeletal muscle glucose uptake *during* contraction, and that NO affects on glucose uptake are independent of blood flow and involve intramuscular factors, most likely to be associated with GLUT4 signalling.

In humans

In contrast to the equivocal results of NOS inhibition on skeletal muscle glucose uptake in rodents, our group have shown that the inhibition of NOS attenuates the increase in glucose disposal during exercise in humans (Bradley *et al.*, 1999; Kingwell *et al.*, 2002). In these studies participants completed 25-30 min of supine cycling (~60% VO_2 peak) while either the NOS inhibitor L-NMMA (5 $mg \cdot kg^{-1}$ body weight) or saline was administered via the femoral artery during the final 15-20 min of exercise. It was observed that in healthy young males L-NMMA administration attenuated the increase in leg glucose disposal by ~48% (at 15 min) compared with saline, without effecting leg blood flow. Furthermore, this L-NMMA-induced reduction in glucose disposal during exercise was seemingly reversed by the administration of the NO precursor L-arginine (Bradley *et al.*, 1999). Interestingly, we have also shown that NOS inhibition during exercise attenuates the increase in leg glucose disposal to a greater extent in people with Type 2 diabetes than in matched controls (Kingwell *et al.*, 2002). This suggests that people with Type 2 diabetes have a greater reliance on the NO-mediated glucose transport pathway during exercise (Kingwell *et al.*, 2002). Furthermore, our group have also shown that infusion of L-arginine (which is a substrate for the production of NO by NOS) into healthy males during the final 60 min of 135 min of cycle ergometery augments glucose disposal without affecting heart rate, respiratory exchange ratio, oxygen consumption, rating of perceived exertion or performance (McConell *et al.*, 2006). In support, calculation of leg glucose uptake from arterial and venous blood glucose concentrations presented in Mortensen

et al. (2007) provides evidence that the infusion of L-NMMA during one-legged knee extensions attuates inreases in leg glucose uptake.

To summarise, NO donors increase resting skeletal muscle glucose uptake. NOS is found in skeletal muscle and its activity and resulting production of NO is increased with exercise/contraction. There is building evidence that inhibition of NOS during contraction/exercise attenuates normal increases in both rodent and human skeletal muscle glucose uptake. This suggests that NO is involved in the regulation of skeletal muscle glucose uptake during contraction, but also that other mechanisms are involved.

1.3.4.4 Potential downstream targets of nitric oxide in regulating glucose uptake

Our group and others have provided strong evidence in humans and rodents that the NOS-NO pathway is important for normal increases in skeletal muscle glucose uptake during exercise/contraction. However, the mechanisms through which NO regulates skeletal muscle glucose uptake during contraction have not yet been investigated. There are a number of potential candidates and reactions through which NO may be signalling GLUT4 translocation and the resulting increase in glucose uptake during contraction. These include, but by no means are limited to, the activation of AMPK (Higaki *et al.*, 2001), production of peroxynitrite ($ONOO^-$) (Halliwell, 1991), the promotion of protein or enzyme S-nitrosylation (Stamler & Meissner, 2001) and through the formation of cyclic guanosine monophosphate (cGMP) (Young *et al.*, 1997) (Figure 1.2). Currently there have been only a handful of studies which have investigated the downstream mechanism(s) of NO donor-stimulated skeletal muscle glucose uptake, and none which have investigated the mechanisms of NO-mediated glucose uptake during skeletal muscle contraction.

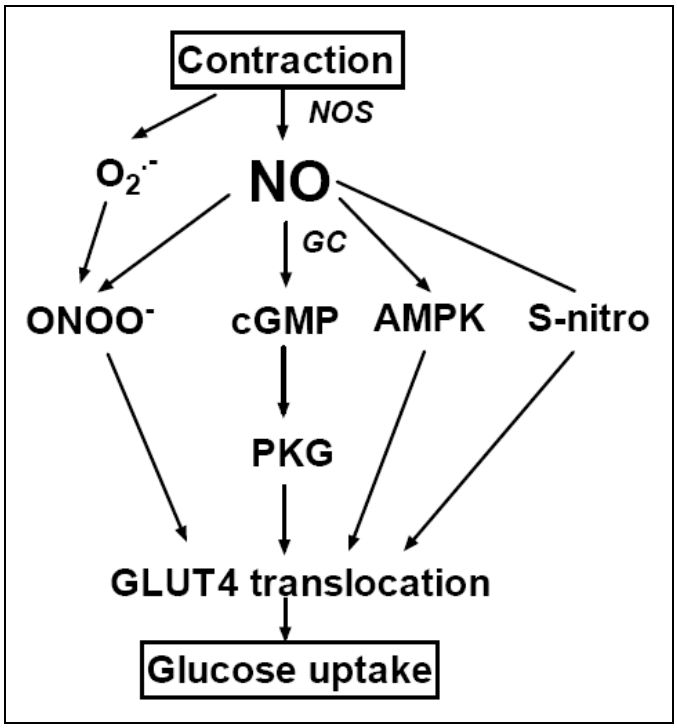

Figure 1.2 Potential intermediates of NO-mediated glucose uptake during contraction. NOS= nitric oxide synthase, NO= nitric oxide, $O_2^{\bullet-}$= superoxide, $ONOO^-$= peroxynitrite, GC= guanylate cyclase, cGMP= cyclic guanosine monophosphate, AMPK= AMP activated protein kinase, S-nitro= S-nitrosylation, PKG= cGMP-dependent protein kinase.

NO-cGMP-PKG pathway

In a series of experiments, Young and Leighton (1997; 1998a, b) provided strong evidence that the traditiobally considered major downstream target of NO, cGMP, is involved in the regulation of NO donor-stimulated glucose uptake in resting skeletal muscle. They showed that the cGMP analogue, 8-bromo-cGMP, and the cGMP phosphodiesterase (PDE) inhibitor, zaprinast (which increases muscle cGMP content by preventing its rapid breakdown by PDE's) increased rat soleus and EDL muscle basal glucose oxidation (Young & Leighton, 1998a, b). Furthermore, the inhibition of guanylate cyclase (GC; an enzyme that interacts with NO to catalyze the formation of cGMP) with LY-83583 dramatically attenuated the increase in skeletal muscle cGMP content and glucose oxidation (by ~60 and ~50%, respectively), and prevented increases in PKG activity stimulated by 15 mM of SNP (Young & Leighton, 1998b). This suggests that in resting muscle NO-donors stimulate GLUT4 translocation and glucose uptake through a cGMP-dependent pathway that involves PKG activation (Figure 1.2). Unfortunately, and seeming unbeknown to the authors, LY-83583 is also a potent

inhibitor of NOS activity (Luo *et al.*, 1995). Therefore, Young and Leighton's findings based on LY-83583 being a specific inhibitor of GC may in fact be the result of concurrent NOS inhibition. In contrast, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a more recently developed pharmacological inhibitor of GC seems to be more specific (Garthwaite *et al.*, 1995) and may give a better understanding of the role of cGMP in NO donor-stimulated glucose uptake.

It is notable that contraction increases cGMP content to a similar extent as NO-donors in muscles of C57Bl6 and $eNOS^{-/-}$ mice. However, this contraction-induced increase in cGMP content does not occur in muscles of $nNOS^{-/-}$ mice or when muscles of C57Bl/6 (wild type) are treated with a NOS inhibitor (Lau *et al.*, 2000). Since NO donor-stimulated skeletal muscle glucose uptake appears to require the formation of cGMP (see above: *NO-cGMP-PKG pathway*), it is likely that NO increases skeletal muscle glucose uptake during contraction via a cGMP-dependent pathway. However, this is yet to be examined. This is an important area of investigation since NO is known to have numerous cGMP-independent effects (discussed below and see Stamler & Meissner, 2001).

NO-AMPK

NO donors have also been hypothesised to increase glucose uptake in resting skeletal muscle through the activation of AMPK (Higaki *et al.*, 2001). However, this seems unlikely to be important during contraction since the NO donors, SNP and N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate), primarily activate AMPKα1 (Higaki *et al.*, 2001; Deshmukh *et al.*, 2010), whereas AMPKα2 is the major isoform activated by contraction and most likely to be involved in the regulation of skeletal muscle glucose uptake during contraction (Jorgensen *et al.*, 2004). Furthermore, inhibition of NOS during contraction attenuates the increase in skeletal muscle glucose uptake during contraction without affecting AMPK signalling (Ross *et al.*, 2007).

NO-$ONOO^-$

In biological systems when both NO and superoxide ($O_2^{\bullet-}$) are present they react to form peroxynitrite ($ONOO^-$), a reaction that is three times more efficient than superoxide dismutase (SOD) scavenging of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) (Halliwell, 1989). $ONOO^-$ production is generally considered to be associated with disease states (Pacher *et al.*, 2007). High physiological, or pathological concentrations of $ONOO^-$ cause irreversible nitration of protein

tyrosine residuals (tyrosine nitration) (Pacher *et al.*, 2007), and this can prevent normal protein phosphorylation (Gow *et al.*, 1996). As such tyrosine nitration is commonly used as a marker of the deleterious effects of $ONOO^-$ on muscle function during chronic stimulation (Klebl *et al.*, 1998), sepsis (Barreiro *et al.*, 2002) and its role in the pathophysiology of insulin resistance (Nomiyama *et al.*, 2004), cardiac disease (Turko & Murad, 2002), cancer (Coussens & Werb, 2002) and various others disease states (Pacher *et al.*, 2007).

In contrast, however, at low levels (10-200μM) $ONOO^-$ can promote transient and reversible phosphotyrosine signalling (Pacher *et al.*, 2007) which has been shown to activate various mitogen-activated protein kinase's (MAPK) (Gutkind, 2000), the Akt (Schroeder *et al.*, 2001) and PKC pathways (Chakraborti *et al.*, 2005), as well as the transcription factor nuclear factor-κB (NF-κB) (Matata & Galinanes, 2002). Furthermore, in cultured bovine aortic endothelial cells $ONOO^-$ increases the activity of proteins associated with glucose uptake such as PI3K and AMPK (Zou *et al.*, 2002; Zou *et al.*, 2003), independent of tyrosine nitration. Therefore, because both $O_2^{\bullet-}$ and NO production is increased in skeletal muscle during contraction (Jackson *et al.*, 2007), they are likely to interact resulting in acute physiological increases in muscle $ONOO^-$ and this may act as a signalling molecule during exercise and regulate such processes as glucose uptake. Indeed, $ONOO^-$ has already been shown to affect metabolic pathways in human ethrocytes, activating band 3-Tyr phosphorylation and promoting glycolysis at low concentrations (10-100μM), but at high concentrations (200-1000μM) causing nitration of band 3-Tyr, blocking phosphorylation and preventing glycolysis (Mallozzi *et al.*, 1997).

S-nitrosylation signalling

NO can act as a signalling molecule through the addition of a nitric oxide group to protein thiol groups and cysteine residuals (S-nitrosylation) (Stamler *et al.*, 1992; Broillet, 1999; Stamler & Meissner, 2001). Importantly, S-nitrosylation occurs in the basal state, in response to physiological increases in NO production, and, like phosphorylation, it is an acute reversible process that alters protein activity (Hess *et al.*, 2005). S-nitrosylation is becoming increasingly recognised as one of the major mechanisms of NO cellular signal transduction (Hess *et al.*, 2005). Since the increase in rodent skeletal muscle cGMP content in response to NO-donors is modest compared to that of other tissues (Katsuki *et al.*, 1977), NO signalling through cGMP-independent means may be more important in skeletal muscle (Stamler & Meissner, 2001). Interestingly, however, the increase in cGMP content of human vastus

lateralis muscle strips in response to a NO donor (spermine NONOate) appears to be substantial (80-fold) (Deshmukh *et al.*, 2010). S-nitrosylation has been implicated in the regulation of numerous enzymes, proteins, membrane receptors, and transcription factors (Hess *et al.*, 2005), some of which play a regulatory role in skeletal muscle metabolism such MAPK's (Park *et al.*, 2006), Akt (Yasukawa *et al.*, 2005) NF-κB (DelaTorre *et al.*, 1997; delaTorre *et al.*, 1999; Marshall & Stamler, 2001) and G-proteins (Lander *et al.*, 1995; Yun *et al.*, 1998). Interestingly, protein S-nitrosylation has been proposed as the mechanism through which NO increases GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes independent of the cGMP/PKG pathway (Kaddai *et al.*, 2008). Since NO production is increased during skeletal muscle contraction (Balon & Nadler, 1994) it is likely that signalling via S-nitrosylation is also elevated, however this has yet to be examined, and potential S-nitrosylation targets during muscle contraction identified.

To summarise, there is building evidence that NO plays a critical role in the signalling of skeletal muscle glucose uptake during contraction, however the mechanisms through which NO mediates skeletal muscle glucose uptake during contraction have yet to be investigated. There is strong evidence that NO-donors increase glucose uptake in resting skeletal muscle through a cGMP-dependent pathway, and that skeletal muscle cGMP content is increased with contraction. However, NO also acts as a signalling molecule through cGMP-independent mechanisms which include S-nitrosylation and peroxynitrite formation. Therefore, to better understand the mechanisms through which contraction regulates skeletal muscle glucose uptake, it is important to determine whether NO regulates skeletal muscle glucose uptake during contraction through a cGMP-dependent or independent pathway(s).

1.3.5 REACTIVE OXYGEN SPECIES

1.3.5.1 Generation and function of reactive oxygen species in skeletal muscle

Reactive oxygen species (ROS) are molecules that have an unpaired electron in their outer shell making them highly reactive (Riley, 1994). Skeletal muscle is a major contributor to the basal production of ROS (Reid *et al.*, 1992a; Reid *et al.*, 1992b), and this production is increased substantially with contraction (Reid *et al.*, 1992b; Medved *et al.*, 2004b; Sandstrom *et al.*, 2006). ROS, NO and other reactive nitrogen species (RNS) are free radicals and intimately interrelated (Brown & Borutaite, 2006) (Figure 1.3), and therefore many authors

use the term ROS (Jackson *et al.*, 2007) to include both ROS and RNS, For the purpose of this thesis ROS will refer to superoxide ($O_2^{\bullet-}$) and its derivatives and not RNS.

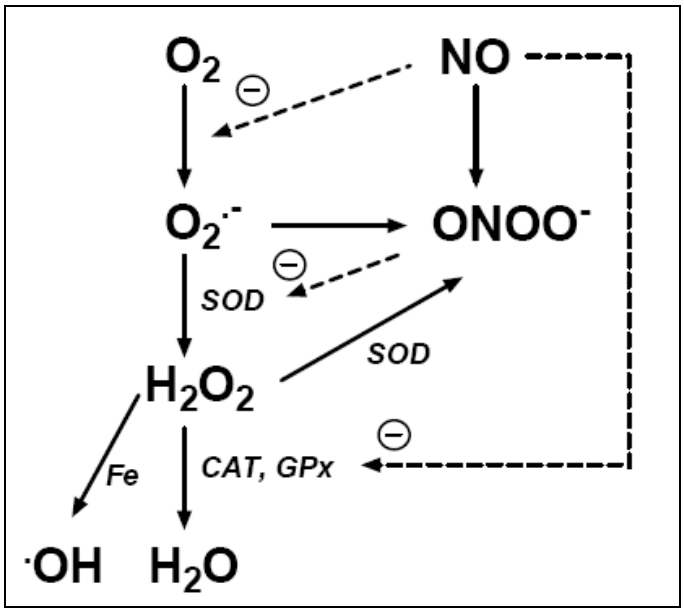

Figure 1.3 Interactions between ROS and NO. Adapted from Brown et al.(2006) NO= nitric oxide, $O_2^{\bullet-}$= superoxide, $ONOO^-$= peroxynitrite, H_2O_2= hydrogen peroxide, SOD= superoxide dismutase, CAT= catalase, GPx= glutathione peroxidise, Fe= iron, $^{\bullet}OH$= hydroxyl radicals, H_2O= water

Superoxide serves as the precursor for the formation of numerous other ROS (Figure 1.3). As discussed above (see *1.3.4 Nitric oxide*), $O_2^{\bullet-}$ can combined with NO to form $ONOO^-$. Alternatively, however, SOD (superoxide dismutase; mitochondrial, MnSOD; cytosolic, Cu,ZnSOD) catalyzes $O_2^{\bullet-}$ dismutation to the less reactive H_2O_2 which can then be reduced to water in a reaction catalyzed by either glutathione peroxidase (GPx) or catalase (CAT), or interact with iron and $O_2^{\bullet-}$ to form highly damaging hydroxyl radicals ($^{\bullet}OH$) (Jackson, 2008). These reactions describe the primary ROS thought to be produced by skeletal muscle (Jackson, 2008), but this list is by no means comprehensive, and therefore it must be acknowledged that other ROS exist and are likely to be produced by skeletal muscle (Jackson *et al.*, 2007; Powers & Jackson, 2008).

The mitochondrial electron transport chain is the major source of skeletal muscle $O_2^{\bullet-}$ production in the basal state (Reid *et al.*, 1992a; Reid *et al.*, 1992b). Approximately 2-5% of

oxygen molecules consumed by mitochondria undergo a one electron reduction to form $O_2^{\bullet-}$ (Loschen *et al.*, 1974). Since oxygen consumption increases >100-fold during exercise, the increase in O_2 flux through the mitochondria is often assumed to account for the increase in ROS generation during contractile activity (Halliwell & Gutteridge, 1989). However, during exercise mitochondria are primarily in state III (ADP-stimulated respiration) mitochondrial respiration, whereas electron leaking that generates $O_2^{\bullet-}$ is most common when the ATP:ADP ratio is greatest (state IV) (Boveris *et al.*, 1976; Cadenas & Davies, 2000). Moreover, ROS generation during exercise increases a mere 2-4-fold above basal, a fraction of the increase in total oxygen consumption (McArdle *et al.*, 2005). These disassociations between mitochondrial activity and $O_2^{\bullet-}$ generation during contractile activity suggest that ROS are generated from non-mitochondrial sources during exercise (St-Pierre *et al.*, 2002; Vasilaki *et al.*, 2006a). Possible primary sources of skeletal muscle ROS generation during exercise/contraction exist both extra- and intra-cellularly and may include NAD(P)H oxidase, phospholipase A_2-dependent (PLA_2) and xanthine oxidase enzymes (Nethery *et al.*, 1999; Nethery *et al.*, 2000; Javesghani *et al.*, 2002; Gomez-Cabrera *et al.*, 2003; Xia *et al.*, 2003; Pattwell *et al.*, 2004). Indeed, Malcolm Jackson's laboratory (Vasilaki *et al.*, 2006a; Vasilaki *et al.*, 2006b) and others (Gomez-Cabrera *et al.*, 2010) have provided strong evidence to support non-mitochondrial sources being responsible for ROS production during skeletal muscle contraction. It is beyond the scope of this review to discuss the evidence for these as sources of ROS generation during contraction, and readers are referred to the following excellent reviews (Jackson *et al.*, 2007; Powers & Jackson, 2008).

Skeletal muscle has specific antioxidant enzymes (SOD, GPx and CAT) and antioxidant substrates (e.g. reduced glutathione (GSH), ascorbic acid (vitamin C) and uric acid) which act as defence mechanisms to prevent deleterious effects of high concentrations of ROS on cell function (Powers & Jackson, 2008). However, when ROS production increases above the cellular antioxidant capacity there is a pro-oxidant shift in cell redox status which is indicative of an increase in cellular oxidative stress (Powers & Jackson, 2008). Chronic low levels of oxidative stress are typically associated with the pathophysiology of various disease states including insulin resistance (Houstis *et al.*, 2006), inflammation (Chapple, 1997) and cardiovascular disease (Kojda & Harrison, 1999), and acute high levels of oxidative stress result in deleterious muscle function (Khawli & Reid, 1994; Supinski *et al.*, 1995; Andrade *et al.*, 1998) and fatigue during exercise (Reid *et al.*, 1994; Medved *et al.*, 2004b). Recent evidence, however, suggests acute physiological increases in ROS during contraction may

play a significant role in the normal regulation of cellular metabolism and gene expression (Katz, 2007; Jackson, 2008; Ji, 2008) through the activation of several signalling cascades which include MAPK's (Allen & Tresini, 2000; Gomez-Cabrera *et al.*, 2005), NF-κB (Zhou *et al.*, 2001b), PI3K/Akt (Zou *et al.*, 2002; Zou *et al.*, 2003) and heat shock proteins (Broome *et al.*, 2006). Furthermore, a small oxidative shift in muscle cell redox is required for optimal muscle function during contraction (Reid, 2001). These seemingly contrasting dynamics of ROS are likely to be a product of duration and extent of exposure, with acute physiological increases in ROS levels having advantageous signalling affects, but chronic elevation or acute large increases in ROS levels being detrimental to cellular function. This is analgous to the situation with NO.

During exercise there is an acute increase in ROS generation in skeletal muscle which stimulates adaptive responses to training such as increases in antioxidant enzymes and substrates (Powers *et al.*, 1992a; Powers *et al.*, 1992b) and markers of mitochondrial biogenesis (Gomez-Cabrera *et al.*, 2005). However, ROS may not only initiate adaptive responses to exercise training, but may also regulate acute responses during exercise. Indeed, antioxidants attenuate increases in p38 MAPK (Gomez-Cabrera *et al.*, 2005) and AMPK (Sandstrom *et al.*, 2006) signalling during exercise, and therefore ROS may contribute to the regulation of muscle metabolism during exercise. Indeed, and as will be discussed in the following section, there is evidence to suggest that ROS are involved in the regulation of skeletal muscle glucose uptake during contractions *ex vivo* (Sandstrom *et al.*, 2006).

1.3.5.2 Reactive oxygen species and glucose uptake

Recent interest in the involvement of ROS in stimulating glucose uptake has stemmed from early observations that exogenous H_2O_2 can increase basal glucose uptake in adipocytes (Lavis & Williams, 1970; Czech & Fain, 1972) and isolated rat epitrochlearis muscle (Sorensen *et al.*, 1980; Cartee & Holloszy, 1990). More recently, Toyoda *et al.* (2004) were able to replicate the early findings of Cartee and Holloszy (1990) that the addition of exogenous ROS (H_2O_2) or a superoxide generation system (hypoxanthine + xanthine oxidase; HXXO) to the incubation medium of isolated skeletal muscle enhances glucose uptake. Moreover, Toyoda *et al.* (2004) reported that H_2O_2 treatment increased skeletal muscle AMPK activity, and that preventing the increase in oxidative stress induced by H_2O_2 with the non-specific antioxidant NAC attenuated both the increase in skeletal muscle glucose uptake and AMPK activity.

Sandstrom *et al.* (2006) have provided evidence that increases in H_2O_2 production during skeletal muscle contraction may contribute to the regulation of glucose uptake. They showed that the antioxidants NAC and ebselen (a GPx mimetic) prevented increases in skeletal muscle oxidative stress during contraction *ex vivo* and attenuated contraction- but not insulin-stimulated glucose uptake *ex vivo* by ~50%. Furthermore, EDL muscles from mice that over express Mn^{2+}-dependent superoxide dismutase were shown to have elevated contraction-stimulated glucose uptake compared to WT controls. The overexpression of Mn^{2+} SOD increases the capacity of $O_2^{\bullet-}$ to be reduced to H_2O_2, and therefore presumably increases intracellular H_2O_2 production during contraction. This suggests that H_2O_2 is likely to be the ROS involved in regulating skeletal muscle glucose uptake during contractions *ex vivo*. In support, the antioxidant catalase (which reduces H_2O_2 to H_2O) but not SOD (catalyses the dismutation of $O_2^{\bullet-}$ to H_2O_2) inhibits the increase in rat EDL muscles glucose uptake stimulated by the superoxide generating system HXXO (Higaki *et al.*, 2008).

Importantly, the only evidence that ROS are involved in the regulation of skeletal muscle contraction-stimulated glucose uptake has come from *ex vivo* models which rely on diffusion gradients for substrate delivery and clearance (Allen *et al.*, 2008), unlike *in vivo* where muscle and capillary blood flow are involved in the regulation of glucose uptake (Wheatley *et al.*, 2004; Rattigan *et al.*, 2005). Furthermore, *ex vivo* models of muscle contraction generally involve non-uniform delivery of oxygen to muscle fibres, hyperoxic incubation medium, an absence of antioxidant systems surrounding muscle (such as that supplied by blood flow) and supramaximal highly fatiguing stimulation protocols (Allen *et al.*, 2008), which are all likely to artificially inflate oxidative stress and alter ROS signalling (Reid, 2008). Therefore, it is important to now determine the role of ROS in regulating skeletal muscle glucose uptake during contraction using more physiologically relevant (intact) preparations and contraction protocols.

1.3.5.3 Potential downstream targets of ROS in regulating glucose uptake during contraction

ROS-AMPK

While there is some evidence that ROS may be involved in the regulation of glucose uptake during *ex vivo* contractions, studies examining the mechanism(s) through which ROS act have largely focused on exogenous ROS-stimulated glucose uptake in resting muscle (Toyoda *et*

al., 2004; Higaki *et al.*, 2008; Jensen *et al.*, 2008). The observation that the increase in both glucose uptake and total AMPK activity (Sandstrom *et al.*, 2006) in contracting, or AMPKα1 activity in H_2O_2 treated (Toyoda *et al.*, 2004) skeletal muscles is diminished by NAC has lead to the hypothesis that ROS stimulate increases in skeletal muscle glucose uptake through the activation of AMPK (Katz, 2007). In contrast, however, Jensen *et al.* (2008) has recently shown that although the treatment of mouse soleus muscles with a very high concentration of H_2O_2 (3mM) increases AMPKα1 and AMPKα2 activity, the soleus muscles from AMPK KD, AMPKα1 and AMPKα2 knockout mice have similar H_2O_2-simulated glucose uptake as WT. In agreement, Higaki *et al.* (2008) found that the treatment of rat EDL muscles with a lower (600 μM) concentration of H_2O_2 increased glucose uptake without affecting AMPK α1 or α2 activity.

Therefore, in resting skeletal muscle it appears that at high concentrations exogenous H_2O_2 activates AMPK, but AMPK activation is not required for exogenous ROS-stimulated glucose uptake. Since there is building evidence to suggest that AMPK is not essential for normal increases in skeletal muscle glucose uptake during contraction (See section *1.3.2 AMP-activated protein kinase (AMPK) in skeletal muscle*), it is likely that ROS regulate skeletal muscle glucose uptake during contraction through an AMPK-independent pathway. However, because a variety of different ROS are produced from several different internal sources at lower concentrations during contraction (Jackson, 2008), exogenous H_2O_2 may activate different signalling cascades to that of ROS produced endogenously during contraction (Chambers *et al.*, 2009). Therefore, the effect of exogenous H_2O_2 on muscle signalling may not reflect that of endogenously produced ROS during contraction, as such further studies are required to identify the mechanisms through which ROS regulate skeletal muscle glucose uptake during contractions.

ROS-insulin pathway

In adipocytes ROS appear to be involved in regulating insulin-stimulated glucose uptake (Hayes & Lockwood, 1987; Heffetz *et al.*, 1990). Similarly, in skeletal muscle H_2O_2 activates the insulin PI3K/Akt pathway (Higaki *et al.*, 2008; Jensen *et al.*, 2008), and the PI3K inhibitor wortmannin completely abolishes H_2O_2-simulated glucose uptake (Higaki *et al.*, 2008). However, co-treatment of skeletal muscle with insulin and H_2O_2 has a partially additive effect on skeletal muscle glucose uptake (Higaki *et al.*, 2008), and NAC does not affect insulin-stimulated skeletal muscle glucose uptake (Sandstrom *et al.*, 2006). This suggests that the

pathway through which ROS and insulin stimulate glucose uptake in resting skeletal muscle interact to some extent, but one may not be completely reliant on the other. It is unlikely that ROS are regulating skeletal muscle glucose uptake during contraction via a PI3K-Akt dependent pathway because PI3K and Akt phosphorylation is not required for glucose uptake during contraction (Ploug *et al.*, 1984; Lee *et al.*, 1995). However, it is plausible that during contraction ROS may act below PI3K and Akt in the insulin pathway to simulate glucose uptake.

ROS-p38 MAPK

H_2O_2-stimulated and contraction-stimulated skeletal muscle glucose uptake is attenuated by the inhibition of p38 MAPK (p38) with SB203580 (Somwar *et al.*, 2000; Kim *et al.*, 2006), suggesting that ROS may regulate contraction-stimulated glucose uptake through a p38 dependent pathway. However, SB203580 also reduces GLUT4 activity questioning whether the attenuation of ROS- and contraction-stimulated skeletal muscle glucose uptake is entirely attributable to the inhibition of p38 (Ribe *et al.*, 2005). However, since the xanthine oxidase inhibitor, allopurinol prevents exercise-induced increases in oxidative stress and p38 phosphorylation (Gomez-Cabrera *et al.*, 2005), this suggests that ROS are required for normal increases in skeletal muscle p38 signalling during contraction. Furthermore, Chambers *et al*, (2009) have shown that skeletal muscle stretch-stimulated glucose uptake is blocked with antioxidants (NAC, ebselen, SOD-catalase, MnTBAP) and p38 inhibitors (SB203580 and A304000), proposing that ROS signalling via p38 is involved in the regulation of skeletal muscle stretch-induced glucose uptake (Chambers *et al.*, 2009). Therefore, the role of p38 in regulating skeletal muscle glucose uptake, and its possible stimulation by ROS during exercise requires further experimental investigation.

Other possible ROS pathways; S-glutathionylation and interactions with NO

S-glutathionylation is the oxidative stress induced reversible formation of mixed disulfides between protein sulfide groups and glutathione (Dalle-Donne *et al.*, 2009). S-glutathionylation is now recognised as the redox equivalent of phosphorylation in the regulation of protein function (Martinez-Ruiz & Lamas, 2007; Dalle-Donne *et al.*, 2009). Like peroxynitrite and S-nitrosylation signalling, S-glutathionylation post translational modification of target proteins plays a critical role in regulating physiological cellular processes ranging from protein folding (Demasi *et al.*, 2008) to energy metabolism (Cotgreave *et al.*, 2002). Although S-glutathionylation is primarily promoted by physiological

increases in ROS production, $ONOO^-$ and nitrosative stress can promote S-glutathionylation both directly (Townsend, 2007), and indirectly, as a result of reduced glutathione being S-nitrosylated to form S-nitrosoglutathione (GSNO) and then GSNO causing S-glutathionylation of proteins (Figure 1.4) (Dalle-Donne *et al.*, 2007; Martinez-Ruiz & Lamas, 2007).

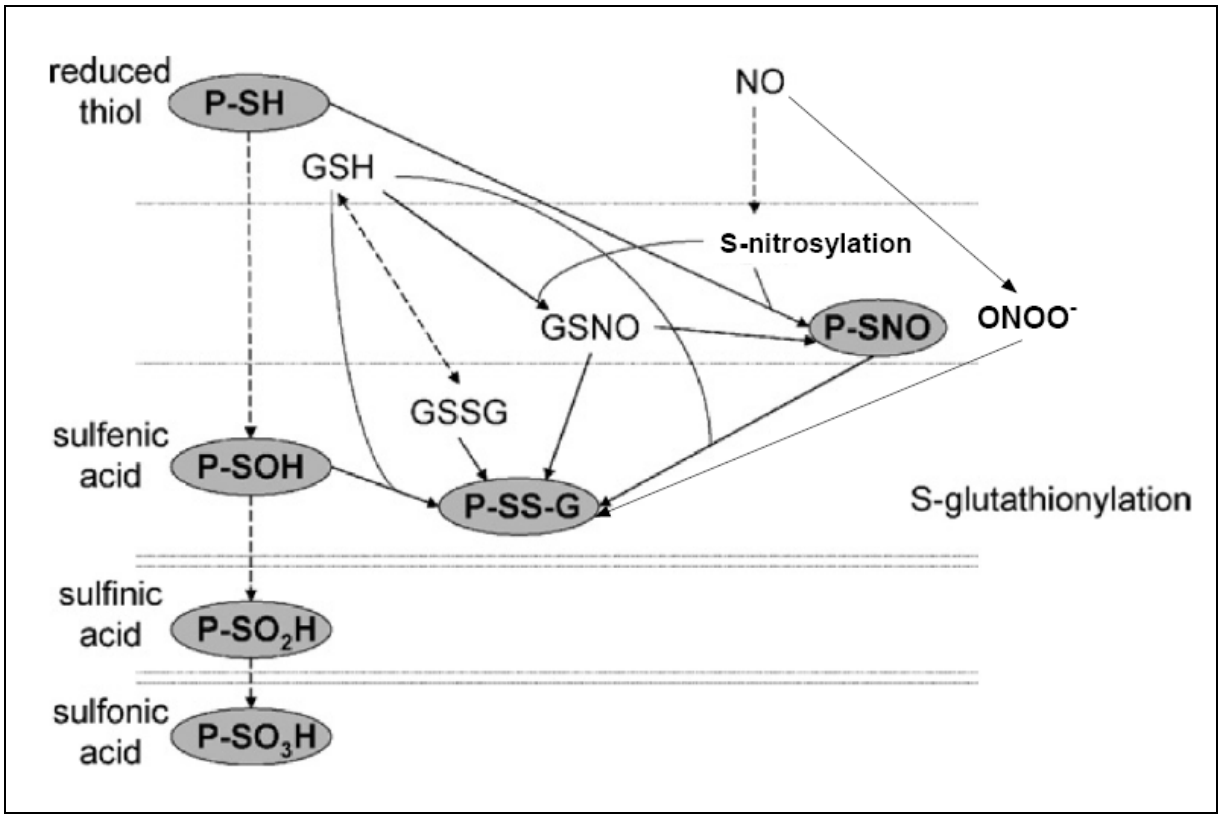

Figure 1.4 Nitric oxide-related species and oxidative stress cause modifications of cysteine thiols resulting in S-nitrosylation (P-SNO) and/or S-glutathionylation (P-SS-G), both of which are important signalling events. Horizontal dotted lines separate one electron oxidative states (induced by oxidative stress). Adapted from Martinez-Ruiz & Lamas (2007). NO= nitric oxide, $ONOO^-$= peroxynitrite.

Since exercise acutely increases both oxidative and nitrosative stress it is not surprising that skeletal muscle protein S-glutathionylation has been shown to increase during exercise, and this increase can be prevented with antioxidant (allopurinaol) supplementation (Gomez-Cabrera *et al.*, 2005). However, the protein targets or regulatory function of S-glutathionylation during exercise have yet to be identified. Interestingly, p21*ras* is activated by S-glutathionylation (Sethuraman *et al.*, 2007) and p21*ras* activation influences glucose transport in fat (Kozma *et al.*, 1993) and muscle (Manchester *et al.*, 1994) by increasing

GLUT4 translocation to the cell membrane. It appears that there is no direct requirement for p21*ras* in insulin-stimulated glucose uptake (Czech & Corvera, 1999). This is interesting since insulin and contraction activate GLUT4 translocation in skeletal muscle by two distinct pathways (Yeh *et al.*, 1995). Since p21*ras* may be involved in GLUT4 translocation but not via the insulin pathway, it is quite possible that S-glutathionylation of p21*ras* might be involved in regulating glucose uptake during skeletal muscle contraction.

PKG has recently been recognised as a redox sensor, independent of its function as a cGMP-dependent kinase (Burgoyne *et al.*, 2007). As discussed above (see section *1.3.4 Nitric oxide*), PKG activation has been associated with NO donor-stimulated glucose uptake (Young & Leighton, 1998b). Therefore, in addition to the possible interaction between $O_2^{\bullet-}$ and NO to form $ONOO^-$ and its role of as ROS/NO signalling molecule (see section *1.3.4 Nitric oxide*), PKG might also act as a related downstream target for both ROS and NO signalling of glucose uptake. Similarly, H_2O_2 has also been implicated in the activation of sGC (Griendling & Harrison, 1999), which NO binds to, resulting in the formation of cGMP and signalling of NO donor-stimulated glucose uptake (see section *1.3.4 Nitric oxide*).

In summary, there are numerous potential pathways through which exogenous ROS, and ROS produced during contraction may act as intermediate signalling molecules to increase skeletal muscle glucose uptake. Indeed, some of the targets of ROS signalling during contraction (p38, AMPK and PKG) have already been implicated in the regulation of skeletal muscle contraction-stimulated glucose uptake. However, the mechanisms through which ROS regulate skeletal muscle glucose uptake during contraction have yet to be comprehensively investigated. Furthermore, given the close interaction between ROS and NO, it is possible that ROS and NO regulate skeletal muscle glucose uptake during contraction through a related pathway(s).

1.4 SUMMARY

Whole body glucose disposal increases during exercise and this is largely the result of a substantial increase in the capacity for glucose to be transported from the blood into skeletal muscle fibres. The magnitude of the increase in skeletal muscle glucose uptake during exercise/contraction is comparable to that of insulin-stimulated glucose uptake, but is mediated via a distinct pathway(s) that also requires GLUT4 translocation to the cell membrane. Indeed, people with Type 2 diabetes have impaired insulin-stimulated skeletal muscle glucose transport, but during exercise their glucose uptake is normal. The mechanism(s) by which contraction/exercise increases glucose uptake into skeletal muscle are unclear. At present Ca^{2+} (via CaMK's), AMPK, ROS, and NO are the major signalling intermediates implicated in the regulation of skeletal muscle glucose uptake during contraction (Figure 1.5). It is likely that, at one time, more than one single pathway is simultaneously involved in the signalling of skeletal muscle glucose uptake during contraction, and several pathways may work on a redundancy basis; if one pathway is inadequate or prevented another pathway will be upregulated.

AMPK and Ca^{2+}/CaMK have been hypothesised to be critical regulators of contraction-stimulated glucose uptake because they are activated during contraction and have the ability to increase glucose uptake in resting skeletal muscle. However, the genetic manipulation of AMPK signalling in mice and the development of specific CaMK inhibitors have shown that although these proteins are involved in regulating skeletal muscle glucose uptake, they may not be essential for normal increases in glucose uptake during contraction. NOS inhibition studies in exercising humans and during *in situ* and *ex vivo* contractions of rat skeletal muscle from our laboratory and others have provided evidence that NO is a critical regulator of skeletal muscle contraction-stimulated glucose uptake. However, the mechanisms through which NO regulates skeletal muscle glucose uptake during contraction have yet to be examined. Exciting new, yet limited, evidence suggests that ROS may also play a role in regulating skeletal muscle glucose uptake during contractions *ex vivo*, potentially via AMPK. However, whether ROS are involved in the regulation of skeletal muscle glucose uptake during more physiologically relevant contraction conditions is not known. Furthermore, the mechanisms through which ROS may regulate skeletal muscle glucose uptake during contraction requires further investigation. Given that ROS and NO are highly interrelated molecules it is also important to establish any possible signalling interaction and/or

convergences between these molecules in the signalling of skeletal muscle glucose uptake during contraction/exercise.

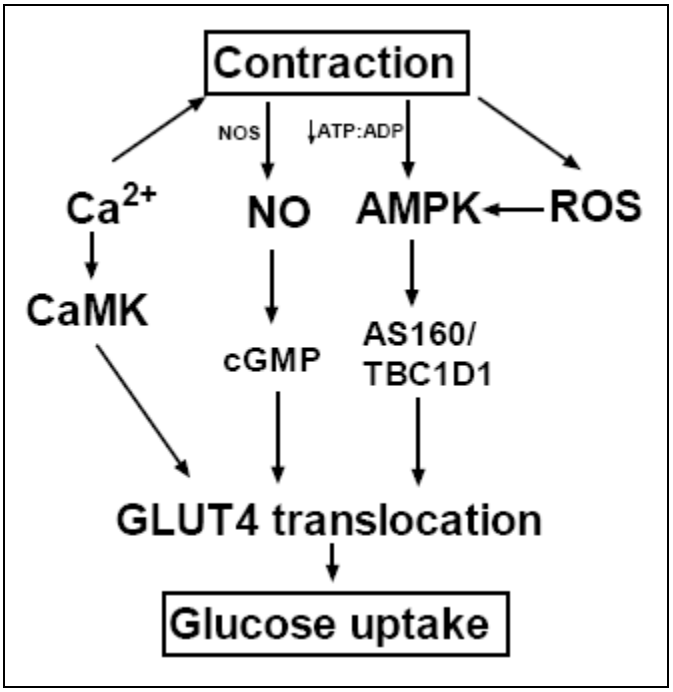

Figure 1.5 Summary of the major signalling intermediates thought to be involved with the regulation of skeletal muscle glucose uptake during contraction. For sake of simplicity interrelations between these intermediates, and intermediates that are less well supported in the literature have been omitted. Ca^{2+}= Calcium, NOS= nitric oxide synthase, NO= nitric oxide, ROS- reactive oxygen species, cGMP= cyclic guanosine monophosphate, AMPK= AMP activated protein kinase, CaMK= Calmodulin Kinase's, AS160= TBC1D4/Akt substrate of 150 kDa, TBC1D1= Akt substrate of 150 kDa.

1.5 AIMS

Therefore, the general aim of this thesis was to examine the role of NO and ROS in regulating skeletal muscle glucose uptake during contraction, with the specific aims being:

1. To determine whether NO and ROS regulate skeletal muscle glucose uptake via an AMPK-dependent pathway during contractions *ex vivo* using AMPK kinase dead mice.
2. To examine the mechanisms through which NO regulates skeletal muscle glucose uptake during contractions *ex vivo*.
3. To investigate possible interactions between NO and ROS in regulating skeletal muscle glucose uptake during contractions *ex vivo*.
4. To determine whether ROS are involved in the regulation of hindlimb skeletal muscle glucose uptake and AMPK signalling during contractions in rats *in situ.*
5. To determine whether ROS are involved in the regulation of glucose disposal and AMPK signalling during exercise in humans.

1.6 HYPOTHESES

The hypotheses tested were that:

1. NO and ROS regulate skeletal muscle glucose uptake during contractions *ex vivo* via an AMPK independent pathway.
2. NO increases skeletal muscle glucose uptake during contractions *ex vivo* via cGMP-dependent and -independent pathways that involve signalling through interaction with ROS.
3. ROS are involved in regulating glucose uptake and AMPK signalling during hindlimb contractions in rats *in situ*, and during exercise in humans.

CHAPTER TWO

METHODS

2.0 GENERAL OVERVIEW

This chapter describes the general experimental models and analytical techniques used in this thesis. Detailed experimental procedures for each study are described in the methods section of the relevant chapters. To simplify study chapters some methods that were only used once in this thesis are also described in detail in this chapter.

2.1 *EX VIVO* MOUSE STUDIES: Chapters 3 and 4

Two studies were conducted using isolated hindlimb muscle from mice. In Chapter 3 extensor digitorum longus (EDL) and soleus muscles were used, however because NOS inhibition was shown to have a greater effect on glucose uptake in EDL than in soleus muscles (Figure 3.11) only EDL muscles were used in Chapter 4. Muscles were excised from the hindlimbs of anaesthetised mice and incubated at rest or stimulated to contract. Details on experimental protocols can be found in Chapters 3 and 4. For both studies mice were maintained in an environmentally controlled room at 21°C with 12 hour light-dark cycle (light 06:30-18:30). Mice were given *ad libitum* access to standard rodent chow and water. All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne, and conformed to the Australian code of practice for the care and use of animals for scientific purposes, as described by the National Health and Medical Research Council (Australia).

2.1.1 Muscle dissection and incubation

On the day of experimentation mice were anesthetised with sodium pentobarbital (Nembutal, Rhone Merieux, Pinkenba, Queensland, Australia; 60 $mg \cdot kg^{-1}$ i.p.) and the proximal and distal tendons of EDL and/or soleus muscles of both hindlimbs were tied with 5/0 silk suture. Muscles were carefully excised tendon-to-tendon, the proximal tendon was tied to a force transducer (PanLab, Barcelona, Spain) and the distal to a fixed hook. The muscles were suspended in incubation chambers (Radnoti, Monrovia, CA, USA) filled with Ringer solution

((in mM): NaCl 118.5, $NaHCO_3$ 24.7, KCl 4.74, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, pH 7.4) containing 0.01% BSA, 8 mM mannitol and 2 mM sodium pyruvate. Chambers were oxygenated with 95% O_2 and 5% CO_2 (Carbogen; BOC Gases; Preston, VIC, Australia) and maintained at 30°C. This incubation temperature has been shown previously to preserve contractile properties during 45 min of *ex vivo* incubation, minimising fatigue during contraction (Segal *et al.*, 1986) and maximising glucose uptake (Higaki *et al.*, 2001; Jorgensen *et al.*, 2004). After all the muscles had been excised the mice were killed by cervical dislocation while still anesthetised deeply. Separate muscles were used for the measurement of glucose uptake and all other analysis.

2.1.2 Muscle contractions

Muscles were stimulated to contract with square wave electrical pulses generated by a Grass S48 stimulator (model DC-300A Series II; Crown International, MA, USA), amplified by an EP500B power amplifier (Audio Assemblies, Cambellfield, VIC, Australia) and delivered to two platinum plate electrodes that flanked the muscle preparation but did not touch it. Optimal muscle length (L_o) was determined from isometric twitch contractions (Schertzer *et al.*, 2007), and forces were recorded on a PowerLab running Chart 5.0 software (AD Instruments, Castle Hill, NSW, Australia). Contraction protocols were of 10 min duration and are described in methods section of Chapters three and four.

2.1.3 2-Deoxy-D-glucose uptake

2-deoxy-D-glucose (2-DG) accumulation in muscle was measured as an estimation of glucose uptake (Hansen *et al.*, 1994). 2-DG is transported into the muscle cell and phosphorylated to form 2-DG-6-phosphate which cannot undergo further oxidation and therefore becomes trapped in the cell (Jenkins *et al.*, 1986). Since mannitol has a very low affinity to all glucose transporters (and therefore is not readily transported into cells) it was used as a marker of extracellular glucose (Young *et al.*, 1986). Accumulation of labelled mannitol was subtracted from 2-DG-6-phosphate to estimate intracellular 2-DG accumulation and therefore skeletal muscle glucose uptake (Jorgensen *et al.*, 2004; Stephens *et al.*, 2004).

Skeletal muscle glucose uptake was measured during the final 5 min of contraction and for 5 min of recovery in contracted muscles (Jorgensen *et al.*, 2004), this protocol incorporates the measurement of glucose uptake *during* contraction, and the maximal increase in glucose uptake that occurs following contraction. Although it is acknowledged that while this

approach has been used previous to estimate contraction-stimulated glucose uptake (Jorgensen *et al.* 2004), in reality this method may not only reflect the recruitment of glucose transporters to the cell membrane during contraction but also the movement of glucose transporters back into the cell during recovery. In non-contracted muscles glucose uptake was measured during the final 10 min of incubation. The measurement of glucose uptake involved exchanging the muscle incubation buffer for a buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.128 μCi·ml^{-1}) and 8 mM D-[^{14}C] mannitol (0.083 μCi·ml^{-1}) (Amersham Bioscience, Piscataway, NJ, USA) (Higaki *et al.*, 2001; Jorgensen *et al.*, 2004) . Following incubation in this buffer, muscles were washed in ice cold Ringer's solution, blotted on filter paper and then snap frozen in liquid nitrogen. Whole (intact) muscles were digested in 125 μl of 1 M NaOH for 10 min at 80°C, and then neutralised with 125 μl of 1 M HCl, mixed and centrifuged at 13,000 g for 2 min. The supernatant (175 μl) was recovered and transferred to 4.5 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA, USA). Radioactivity of both tracers was determined using dual isotope analysis by a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA).

2.1.4 Nitric oxide synthesis (NOS) activity assay

NOS activity in muscle was determined using a commercially available NOS activity assay kit (Cayman Chemicals, Ann Arbor, MI, USA) which measured the conversion of labelled L-arginine to labelled L-citrulline; a reaction that is catalysed by NOS (Moncada & Higgs, 1993). Frozen muscles were homogenised in ice cold homogenising buffer (20 μl·mg^{-1} of tissue; 25 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA), incubated for 20 min on ice and then centrifuged at 13,000 g for 20 min at 4°C. Five μl of supernatant was used to determine protein concentration (see section *2.6.2 Total protein assay*) and the NOS activity assay was performed on the remaining supernatant according to manufacturer's instructions.

Briefly, 40 μl of reaction buffer (25 mM Tris-HCl, pH 7.4, 3 μM tetrahydrobiopterin (BH_4), 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 1.25 mM NADPH, 0.75 mM $CaCl_2$ and 3 μM L-[^{14}C]arginine, 0.1 μM calmodulin) was added to 30 μl of supernatant and incubated at 37°C for 40 min. Four hundred μl of stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA) was then added to each sample to stop the reaction. Samples were then transferred to spin cups containing resin, centrifuged for 30 s at 13,000 g to remove any labelled L-aginine, and the elutate was transferred to scintillation vials containing 4 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA, USA). Sample radioactivity

was counted on a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA). The assay was run in triplicate with 5 µl of 10 mM N^G-nitro-L-arginine (L-NA; NOS inhibitor) being added with the reaction buffer to the third triplicate to act as an internal negative control. NOS activity was expressed as the pmol of L-[^{14}C]citrulline formed·min^{-1}·mg^{-1} protein, and calculated by subtracting sample counts from counts of the internal negative control.

2.1.5 Oxidant levels assay

The fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) was used to measure muscle oxidant levels (Reid *et al.*, 1992a). DCFH-DA passively diffuses into cells and its diacetate moiety is cleaved by intercellular esterases to form DCFH. DCFH is oxidised to its fluorescent derivative, 2′,7′-dichlorofluorescein (DCF), by a variety of reactive species including NO (Murrant *et al.*, 1999), H_2O_2 (Murrant *et al.*, 1999), $ONOO^-$ (Kooy *et al.*, 1997) and ·OH (Zhu *et al.*, 1994).

Oxidant levels were determined in skeletal muscle cross-sections. Following incubation, muscles were mounted in OCT and frozen in isopentane cooled by liquid nitrogen. Muscles were sectioned (10 µm muscle cross-sections), treated with DCFH-DA (5 µM) covered with a cover-slip and allowed to dry overnight at room temperature. DCF fluorescence was measured using confocal microscopy (480 nm excitation, 520 nm emissions; Zeiss LSM-510 Meta confocal microscope, Carl Zeiss Microimaging, Thornwood, NY, USA) and emission intensity determined using Carl Zeiss LSM-510 acquisition and analysis software. Since this assay had not been conducted previously in our laboratory several control experiments were preformed. EDL muscles from mice that were not treated with DCFH-DA did not show any fluorescence (Figure 2.1A). EDL muscle incubated with 5 mM H_2O_2 or EDL muscle sections treated directly with 500 µM H_2O_2 showed greater fluorescence than EDL muscles incubated in the absence of H_2O_2 (Figure 2.1). Furthermore, none of the inhibitors used in Chapters 3 and 4 emitted their own fluorescence.

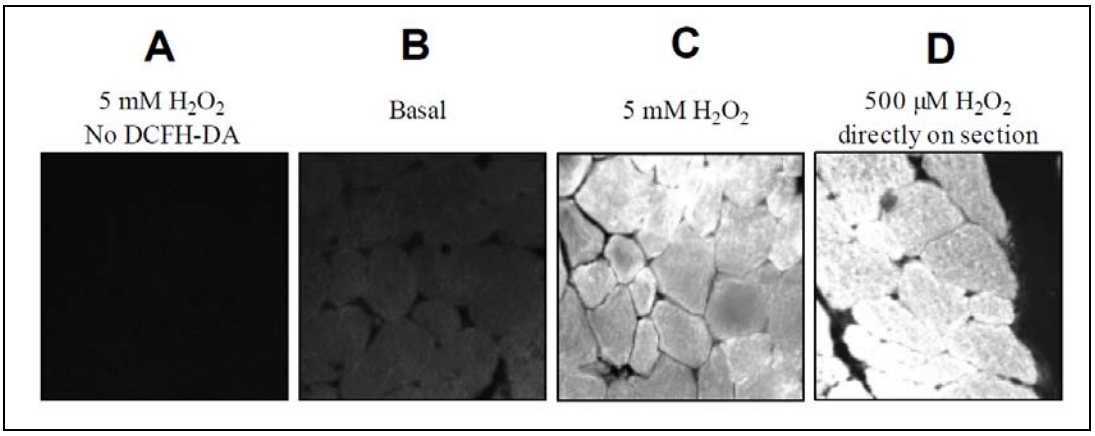

Figure 2.1 Representative images of cross-sections from extensor digitorum longus (EDL) muscles either treated with H_2O (A) or 5µM DCFH-DA (B, C and D). Muscles were incubated for 30 min in the presence (A and C) or absence (B and D) of 5 mM H_2O_2 before being mounted in OCT and frozen in isopentane cooled in liquid nitrogen and sectioned. Muscle cross-section C was treated directly with 500 µM of H_2O_2 prior to DCFH-DA treatment.

It is acknowledged that interpreting the biological significance of oxidative levels determined by this method is complicated because muscle cross-sections were treated with DCFH-DA *following* contraction. As such, it is likely that muscle cells experience some degree of artificial alterations in cell oxidative balance during this assay, and ideally ROS production would be measured in intact muscles during contraction. In an effort to measure muscle oxidant levels during contraction, muscles were pre-incubated in DCFH-DA (10-50 µM) for 30 min prior to contraction. Following contraction muscles were mounted in OCT and frozen in isopentane cooled in liquid nitrogen, sectioned and DCF fluorescence was measured using confocal microscopy as described above. However, it was found that this method did not give a reliably measureable fluorescence signal, and therefore oxidant levels were determined by directly treating muscle sections with DCFH-DA as described above.

2.1.6 AMPK activity assay

Frozen muscles were homogenised and protein concentration was determined as described in section *2.6 Immunoblotting*. Fifteen µl of homogenates were incubated with AMPKα1 (amino acid sequence 373-390 of rat AMPKα1) or AMPKα2 (amino acid sequence 351- 366 and 490-516 of rat AMPKα2) antibody bound Protein A sepharose beads for 2h at 4°C. AMPK antibodies were a gift from Prof. Kemp (St. Vincent's Institute, Fitzroy, Victoria, Australia).

Immunocomplexes were washed with PBS and suspended in 50 mM Tris-HCl buffer (pH 7.4) for performance of the AMPK activity assay which was conducted by Dr Gregory Steinberg at St. Vincent's Institute, Fitzroy, Victoria, Australia.

Briefly, after the addition 25 µl of reaction buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 0.05% Triton-X, 0.1 M DTT, 100 µM substrate for AMP-activated Protein Kinase (SAMS) peptide (Upstate, Lake Placid, NY, USA), 200 µM AMP, 0.1 mM [^{32}P]ATP (~200 cpm·pmol^{-1}, PerkinElmer, Boston, MA, USA)) to immunocomplexes, samples were incubated at 30°C for 20 min. Twenty five µl of the sample was transferred onto P81 chromatography paper (Whatman, Maidstone, UK) and washed 3 times for 10 min in 75 mM H_3PO_4, once in 100% ethanol, and air dried with a hair dryer. P81 paper was place in 10 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA, USA) and radioactivity was measured by a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA). SAMS peptide has the same AMPK phosphorylation site as ACC-β. Thus, AMPK will phosphorylate SAMS peptide using γ-[^{32}P]-ATP. The phosphorylated SAMS peptide is then trapped onto P81 chromatography paper, with any excess γ-[^{32}P]-ATP being washed away. The greater the activity of AMPK, the more γ-[^{32}P]-ATP that becomes incorporated into the SAMS peptide. Therefore, AMPK activities were calculated as picomoles of phosphate incorporated into the SAMS peptide (acetyl-CoA carboxylase (ACC)α(73-87)A^{77})·min^{-1}·mg total protein subjected to immunoprecepitation.

2.2 IN SITU RAT STUDY: Chapter 5

One study was conducted using an *in situ* rat model. This experiment and sample collection was conducted by Dr Renee Dywer at the University of Tasmania, Hobart, Australia, and I assisted in some of experiments and sample collection, and performed signalling analysis on the muscle samples collected. Therefore, the experimental model will only be described in brief, and details on the experimental protocol will be given in Chapter 5. Prior to experimentation male hooded Wistar rats were maintained in an environmentally controlled room at 21°C with 12 h light-dark cycle (light 06:30-18:30) at The University of Tasmania, and given *ad libitum* access to standard rodent chow and water. This study was approved by The University of Tasmania Ethics Committee, and conformed to the guidelines for the care and use of experimental animals, as described by the National Health and Medical Research Council (Australia).

2.2.1 Experimental model

The anesthetised rat model utilised in this study has been described previously (Rattigan *et al.*, 1999; Wheatley *et al.*, 2004; Ross *et al.*, 2007). Briefly, rats were anesthetised using sodium pentobarbital (1.5 μl·g bwt^{-1} i.p.) and cannulas were inserted into the carotid artery and jugular veins for arterial sampling and continuous administration of anaesthetic, respectively. A cannula was also inserted into the epigastric artery of one hindlimb for local muscle infusions, and this hindlimb was electrically stimulated to contract (0.1 ms impulse at 2 Hz and 35 V) (Ross *et al.*, 2007). The knee was secured by the tibiopatellar ligament and the Achilles tendon was attached to a Harvard Apparatus (MA, USA) isometric transducer thereby allowing measurement of tension development from the gastrocnemius-plantaris-soleus muscle group during contraction. As described previously (Rattigan *et al.*, 1999; Wheatley *et al.*, 2004), blood pressure and heart rate were measured by the insertion of a pressure transducer (Transpac IV, Abbott Critical Systems, South Pasadena, CA, USA) into the carotid artery, and femoral blood flow was monitored with an ultrasonic flow probe (Transonic Systems, VB series, 0.5mm, Ithaca, NY, USA) positioned around the femoral artery of each hindlimb and connected to a flow meter (model T106 ultrasonic volume flowmeter, Transonic Systems, Ithaca, NY, USA). Blood pressure, heart rate, and femoral blood flow were recorded on a PC with WINDAQ data acquisition software (DATAQ Instruments, Akron, OH, USA)

2.2.2 2-Deoxy-D-glucose uptake

Similar to the *ex vivo* muscle preparations described in *2.1.3 2-Deoxy-D-glucose uptake*, 2-deoxy-D-glucose (2-DG) accumulation in muscle was measured as an estimation of skeletal muscle glucose uptake since it becomes trapped in the cell after transport and phosphorylation (Jenkins *et al.*, 1986; Hansen *et al.*, 1994). This procedure was performed by Dr Renee Dywer at the University of Tasmania, Hobart, Australia, and I went to the University of Tasmania and assisted with some of this analysis. At t= 20 min (10 min before the completion of the experiment) a 1.85 MBq bolus of labeled 2DG in isotonic saline was administered via the right jugular vein. Immediately following the 2DG bolus, an arterial blood sample (0.5 ml) was withdrawn by an automated syringe pump at 50 $\mu l \cdot min^{-1}$ for 10 min before hindlimb muscles were excised (soleus, plantaris, gastrocnemius red and gastrocnemius white). From the blood sample a plasma sample (25µl) was collected to determine the average plasma specific radioactivity of 2-DG. Excised muscle samples were ground under liquid nitrogen and 100 mg was homogenised with 1.5 ml water before free and phosphorylated glucose were separated by ion exchange chromatography using anion exchange resin (AG1-X8; Bio-Rad, Hercules, CA, USA). Inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA, USA) was added to samples and radioactivity was measured by a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA, USA). As described previously (Rattigan *et al.*, 1999; Wheatley *et al.*, 2004; Ross *et al.*, 2007) average plasma glucose (see section *2.4.3 Plasma analysis*) and the plasma radioactivity of 2-DG (determined using the same procedure as for muscle) from the arterial blood sample drawn continuously following 2-DG bolus administration were used to calculate muscle 2-DG glucose uptake as follows:

$$R'g = \frac{\text{muscle}[^3H]2DG6\text{-}P(dpm/g) \times \text{plasma[glucose]}(\mu g/ml)}{\text{average plasma}[^3H]2DG(dpm/ml) \times 10(mins)}$$

2.3 HUMAN EXERCISE STUDY: Chapter 6

One study was conducted using adult male human participants. Participants were recruited via advertisements, informed of the all experimental procedures and provided written consent (Appendix A). All participants completed a medical questionnaire (Appendix A) and were only included in the study if they were non-smokers, not taking any medication and had no history of blood clotting, cardiovascular, cerebrovascular, metabolic or respiratory disease. This study was approved by The University of Melbourne Human Ethics Committee and conducted in accordance with the Declaration of Helsinki.

2.3.1 VO_2 Peak test and familiarisation

Upon reporting to the laboratory participants provided descriptive information including height, weight and age. Participants' aerobic fitness was determined using a peak oxygen consumption ($\dot{V}O_{2\ peak}$) test. The $\dot{V}O_{2\ peak}$ test was conducted in normal ambient laboratory conditions (~20°C) using a graded cycle ergometer (electronically braked ergometer; Lode, Groningen, The Netherlands) protocol to voluntary exhaustion. Participants were fitted with a nose clip and adjustable headset (Hans Rudolph Inc., Kansas City, MO, USA) which supported a one-way non-rebreathing valve (Hans Rudolph Inc., Kansas City, MO, USA) and was connected to douglas bags to collect expired air. Participants completed three submaximal workloads starting at 75 W and increasing by 50 W every 3 min, after which point the workload was increased in 25 W increments every minute until voluntary exhaustion. Expired respiratory gas was analysed for volume (dry gas air flow meter; Vacuumed, Ventura, CA, USA), O_2 and CO_2 content (S-3A O_2, and ametek CO_2 analyser; Applied Electrochemistry, Sunnyvale, CA, USA). Heart rate was monitored throughout the test (Polar Favor, Polar Elector OY, Finland).

On a separate day following $\dot{V}O_{2\ peak}$ test and 1-2 weeks before the first experimental trial, participants completed a familiarisation session. In this session participants were required to cycle at ~60% $\dot{V}O_{2\ peak}$ for 20-30 min. The purpose of the familiarisation ride was to familiarise participants with laboratory conditions and procedures, as well as confirm the accuracy of the workload calculated to be equivalent to 60% $\dot{V}O_{2\ peak}$ from the regression line between $\dot{V}O_2$ and workload from the $\dot{V}O_{2\ peak}$ test. Detials on experiment a trial are given in Chapter 6, *6.1 Method*.

2.3.2 Blood and muscle sampling

Participants had a 22-gauge Teflon cannula (Optiva, Ethicon Endo-Surgery, Cincinnati, OH, USA) inserted into an antecubital forearm vein of both arms. Cannula's were protected and secured with Tegaderm (3M Health Care, St Paul, MN, USA). A one-way valve (Braun Medical Inc., Behelhem, PA, USA) and 0.25 m extension tube (Terumo, Somerset, NJ, USA) was connect to one cannula for the collection of blood. The cannula in the contralateral arm was used for infusions via a Discofix three-way stopcock (B.Braun Medical Inc., Bethlehem, PA, USA) and 1.5 m extension tube (Terumo, Somerset, NJ, USA). All blood collected was transferred immediately to tubes containing lithium-heparin (Sarstedt, Postfach, Nümbrecht, Germany), except for blood used for non-esterified fatty acid (NEFA) analysis which was preserved by tubes containing EDTA (Sarstedt, Postfach, Nümbrecht, Germany). These tubes were placed on ice until the end of the trial when they were centrifuged at 3,000 g for 20 min and plasma was removed and stored at -80°C for later analysis.

The percutaneous needle biopsy technique with suction (Bergstrom, 1975) was used for the sampling of participants vastus lateralis muscle. Muscle biopsies were performed by experienced medical practitioners (Dr. Andrew Garnham, Deakin Universtiy, VIC, Australia, and Prof. Benedict Canny, Monash University, VIC, Australia). Prior to exercise, the skin over the vastus lateralis muscle of the one leg to be biopsied was shaved and sterilised with 5% chlorohexidine and cetrimide solution (Betadine®, Pharmacia Pty Ltd, Bently, WA, Australia). Under local anaesthesia (Xylocaine; 1% Lignocaine, AstraSenca Pty. Ltd., Welshpool, WA, Australia) three separate ~0.5 cm incisions were made with a surgical scalpel (Swann-Morton, Sheffield, UK) for sampling in a distal to proximal order. All muscle samples were obtained with the participant in the supine position. To obtain exercising muscle samples the participant ceased exercise and immediately slid backwards off cycle ergometer and lay on a bench placed behind the ergometers seat. Muscle samples were frozen in liquid nitrogen while still in the biopsy needle within 4-6 s of sampling at rest, and 6-12 s following the cessation of exercise. Muscle samples were then removed from the needle while still frozen stored in liquid nitrogen for later analysis. Muscle samples were obtained from the contralateral leg during the second trial.

2.3.3 Plasma analysis

Plasma lactate

Plasma lactate was measured using an automated L-lactate oxidase method (YSI 2300 Stat, Yellow Springs, OH, USA). In this method lactate is immobilised between a polycarbonate and cellulose acetate membrane where it is oxidised by lactate oxidase producing H_2O_2. H_2O_2 then passes through the cellulose acetate membrane to a platinum electrode where it is oxidised. This results in a current proportional to the concentration of lactate in the sample (Williams *et al.*, 1970).

Plasma glucose

Glucose is broken down in a two step reaction to produce NADPH. This involves the production of glucose-6-phophate (G-6-P) and ADP from glucose and ATP in a reaction catalyzed by hexokinase. G-6-P can then react with NADP in the presence of G-6-P dehydogenase to produce NADPH and 6-phosphoate-gluconolate (Figure 2.3). Plasma glucose was determined using an enzymatic fluorometric assay which detects the production of NADPH (Lowry & Passonneau, 1972).

Two hundred and fifty µl of plasma was first deproteinised by adding 250 µl of 3 M perchloric acid (PCA), mixed and centrifuged at 13,000 g for 3 min at 4°C. Three hundred µl of supernatant was recovered and added to 75 µl of 6 M potassium hydroxide to neutralise. Samples were mixed, centrifuged for 3 min at 13,000 g and the supernatant was stored at -80°C until analysis. The glucose assay was performed by adding 2.5 µl of glucose standards (1, 2, 4, 6, 8 and 10 mM), blank (neutralised PCA) or deproteinised sample and 300 µl of cocktail reagent (100mM Tris-HCl, pH 8.1, 50 mM HCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM ATP, 0.05 mM NADP, 0.1 $U \cdot ml^{-1}$ G-6-P dehydrogenase, 1 $U \cdot ml^{-1}$ hexokinase) to a 96 well plate in triplicate, mixing and incubating in the dark at room temperature for 30 min. Fluorescence was then measured on a multiskan fluorescent plate reader (Thermo Electron Corporation, Waltham, MA, USA) at 365 nm absorption and 455 nm emission. Plasma glucose concentration was determined from the standard curve.

Plasma non-esterified fatty acids (NEFA)

Plasma NEFAs were measured by an enzymatic colorimetric procedure (NEFA-C test; Wako, Osaka, Japan) as per the manufacturer's instructions. In the presence of coenzyme A (CoA), and ATP, NEFA are converted to Acyl-CoA, AMP and pyrophosphoric acid (PPi) in a

reaction catalyzed by Acyl-CoA Synthetase (ACS). Acyl-CoA oxidase (ACOD) then oxidises Acyl-CoA to produce 2,3-transenoyl-CoA and H_2O_2. In the presence of peroxidase (POD), 3-methyl-N-ethyl-N(β-hydroxyethyl)-aniline (MEHA) and 4-aminoantipyrine, H_2O_2 produced yields a blue purple pigment. The H_2O_2 produced and therefore absorbance of the blue purple colour is proportional to the NEFA concentration.

The NEFA assay was performed by incubating 5 µl of standards (0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2 $mEq \cdot l^{-1}$) or plasma in a 96 well plate with 90 µl of reagent A (50 mM phosphate buffer, pH 7.0, 0.27 $U \cdot ml^{-1}$ ACS, 0.73 $units \cdot L^{-1}$ CoA, 4.5 $mmol \cdot L^{-1}$ ATP, 1.5 $mmol \cdot L^{-1}$ 4-aminoantipyrine, 2.7 units ascrobate oxidase) at 37°C for 10 min. One hundred and eighty µl of reagent B (1.2 $mmol \cdot L^{-1}$ MEHA, 5.5 $U \cdot ml^{-1}$ ACOD, 6.8 $U \cdot ml^{-1}$ POD) was then added to each well and incubated at 37°C for a further 10 min before absorbance was measured at 560 nm using a multiskan EX plate reader (Thermo Electron Corporation, Waltham, MA, USA). Plasma NEFA concentration was determined from the standard curve.

Plasma Insulin

Plasma insulin concentration was determined using an ultra sensitive human radioimmunoassay kit (Linco Research, St Charles, MO, USA) as per the manufacturer's instructions. Briefly, 100 µl of standards (0.2, 0.5, 1.0, 2, 5, 10, 20 $\mu U \cdot ml^{-1}$ human insulin) or plasma, 300 µl of assay buffer (50 mM phosphosaline, pH 7.4, 25 mM EDTA, 0.08% sodium azide, 1% BSA), and 100 µl of guinea pig anti-sensitive human insulin antibody were incubated overnight at room temperature. 100 µl of ^{125}I labelled insulin (specific activity 367 $\mu Ci \cdot \mu g^{-1}$) was then added, and samples were again incubated overnight at room temperature. The following day 1 ml of precipitating reagent (goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 50 mM phosphosaline, 25 mM EDTA, 0.08% sodium azide) was added and samples were incubated for 20 min at 4°C, centrifuged at 3,000 g for 40 min at 4°C and the supernatant was decanted. Radioactivity of the pallet was then measured on a gamma counter (PerkinElmer, Boston, MA, USA). Since the insulin antibody has limited binding sites, the higher the concentration of insulin in the sample the less binding sites there will be available for ^{125}I labelled insulin binding (i.e. competitive binding between sample and tracer insulin). Therefore, the higher the concentration of insulin in the sample, the lower the radioactivity of antibody bound in pallet. Plasma insulin concentrations were determined from the standard curve.

2.3.4 Glucose kinetics

To determine glucose kinetics a 40.2 ± 0.5 μmol·kg^{-1} bolus, followed by a continuous constant infusion (0.39 ± 0.02 μmol·kg^{-1}·min^{-1}; for 90 min at rest and throughout the 80 min of exercise) of a stable isotope glucose tracer (6,6-^{2}H-glucose; Cambridge Isotope Laboratories, MA) was administered via a cannula inserted into an antecubital forearm vein. The rates of plasma glucose appearance (glucose R_a), disappearance (glucose R_d) and clearance (glucose CR) were estimated from the changes in % enrichment of 6,6-^{2}H-glucose and plasma glucose concentration. To determine plasma 6,6-^{2}H-glucose concentration plasma samples were first deproteinised by adding 50 μl $Ba(HO)_2$ to 50 μl of plasma, mixing, adding 50 μl $ZnSO_4$, mixing, and centrifuging at 6,000 g for 3 min at 4°C. To remove $^{2}H_2O$, 80 μl of deproteinised supernatant was transferred to a limited volume glass insert and dried in an oven at 60°C. One hundred μl of derivitisation solution (2:3, pyridine:acetic anhydride) was added to each sample, and samples were capped in glass vials. The labelled to non-labelled glucose abundance was measured using selected-ion-monitoring gas chromatography-mass spectrometry (SIM-GC-MS) by Dr. Vince Murone, Victoria University, VIC, Australia. Percent enrichment was calculated from a 6,6-^{2}H-glucose standard curve (0, 1, 2, 4 and 6% enrichment).

Glucose R_a, R_d and CR were estimated using a modified one-pool, non-steady state model (Steele *et al.*, 1956; Radziuk *et al.*, 1978). The rapidly mixing portion of the glucose pool was assumed to be 0.65, and the apparent glucose space was estimated to be 25% of body mass. Over 95% of tracer determined glucose R_d is oxidised by skeletal muscle at power outputs requiring ~60% $\dot{V}O_{2\,peak}$ (Jeukendrup *et al.*, 1999). Figure 2.2 gives the formulas used for the estimation of plasma glucose R_a, R_d and CR.

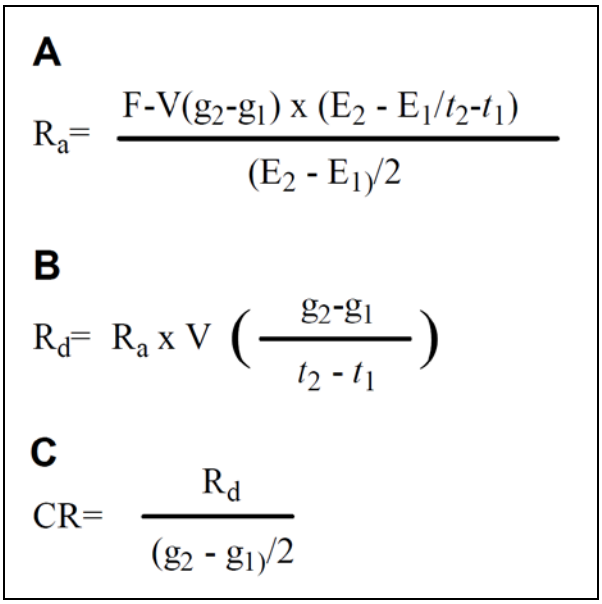

Figure 2.2 Formulas for the estimation of plasma glucose appearance (R_a; A), glucose disappearance (R_d; B) and glucose clearance rate (CR; C). F= 6,6-^{2}H-glucose infusion rate (µmol·min^{-1}), V= volume of distribution (162.5 ml·kg^{-1}), g= plasma glucose concentration at times t_1 and t_2. E= plasma 6,6-^{2}H-glucose concentration at times t_1 and t_2.

2.3.5 Muscle metabolites

Approximately 25 mg of muscle was freeze-dried and ground to a powder, and any visible connective tissue was removed. For the determination of muscle glycogen, ~1 mg of freeze-dried muscle was incubated at 95°C for 2 h in 250 µl of 2 M HCl, neutralised with 750 µl of 0.67 M NaOH and the extracts were stored at -80°C until analysis. Muscle ATP, creatine, PCr and lactate were determined by extracting ~2 mg of freeze-dried muscle in 250 µl of 0.5 M PCA and 1 mM EDTA. Samples were mixed every 30 s for 10 min, centrifuged at 15,000 g for 2 min at 0°C, and 200 µl of the supernatant was transferred to pre-cooled tubes containing 50 µl of 2.1 M $KHCO_3$, mixed and incubated on ice for 5 min. Samples were then centrifuged at 15,000 g for 2 min at 0°C and supernatant was stored at -80°C until analysis.

Muscle glycogen

Glucosyl units from muscle glycogen are broken down it a two step reaction (Figure 2.3A) yielding one NADPH molecule for each glucosyl unit. Muscle glycogen was determined using an enzymatic fluorometric assay which detects the production of NADPH (Passonneau & Lauderdale, 1974).

In triplicate 10 µl of sample, glucose standards (250 and 500 µM), NADPH standards (50, 100, 200 and 400 µM) or water blank was combined with 1 ml of cocktail reagent (50 mM Tris, pH 8.1, 1 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM ATP, 50 µM $NADP^+$, 25 $\mu l \cdot ml^{-1}$ glucose-6-phosphate dehydogenase (1000 $U \cdot ml^{-1}$)) in a test tube. Background fluorescence was measured on a fluorimeter (Turner biosystems, Sunnyvale, CA, USA) at 365 nm absorption and 455 nm emission. Twenty five µl of hexokinase (1000 $U \cdot ml^{-1}$ diluted 1/40 in cocktail reagent) was added to each test tube and samples were mixed and incubated in the dark at room temperature for 60 min. Fluorescence of each sample was again determined, and muscle glycogen was calculated from NADPH standard curve and expressed per kg dry muscle mass ($mmol \cdot kg^{-1}$ d.m).

Muscle lactate

Lactate is converted to pyruvate by lactate dehydrogenase in a reaction that yields one NADH molecule for each lactate molecule (Figure 2.3B). Muscle lactate was determined using an enzymatic fluorometric assay which detects the production of NADH (Lowry & Passonneau, 1972).

The background fluorescence of test tubes containing 1 ml of cocktail reagent (100 mM hydrazine, 100 mM Glycine, 0.5 mM NAD^+, 5 $\mu l \cdot ml^{-1}$ lactate dehydrogenase (550 $U \cdot ml^{-1}$)) was determined using a fluorimeter (Turner biosystems, Sunnyvale, CA, USA) at 365 nm absorption and 455 nm emission. Hydrazine was included in the cocktail reagent to scavenge pyruvate, preventing the reformation of lactate (Figure 2.3B). In triplicate 10 µl of sample, lactate standards (50 and 500 µM), NADPH standards (50, 100, 200 and 400 µM) or water blank was added to each test tube, and samples were mixed and incubated in the dark at room temperature for 60 min. Fluorescence of each sample was again determined, and muscle lactate was calculated from NADPH standard curve and expressed per kg dry muscle mass ($mmol \cdot kg^{-1}$ d.m).

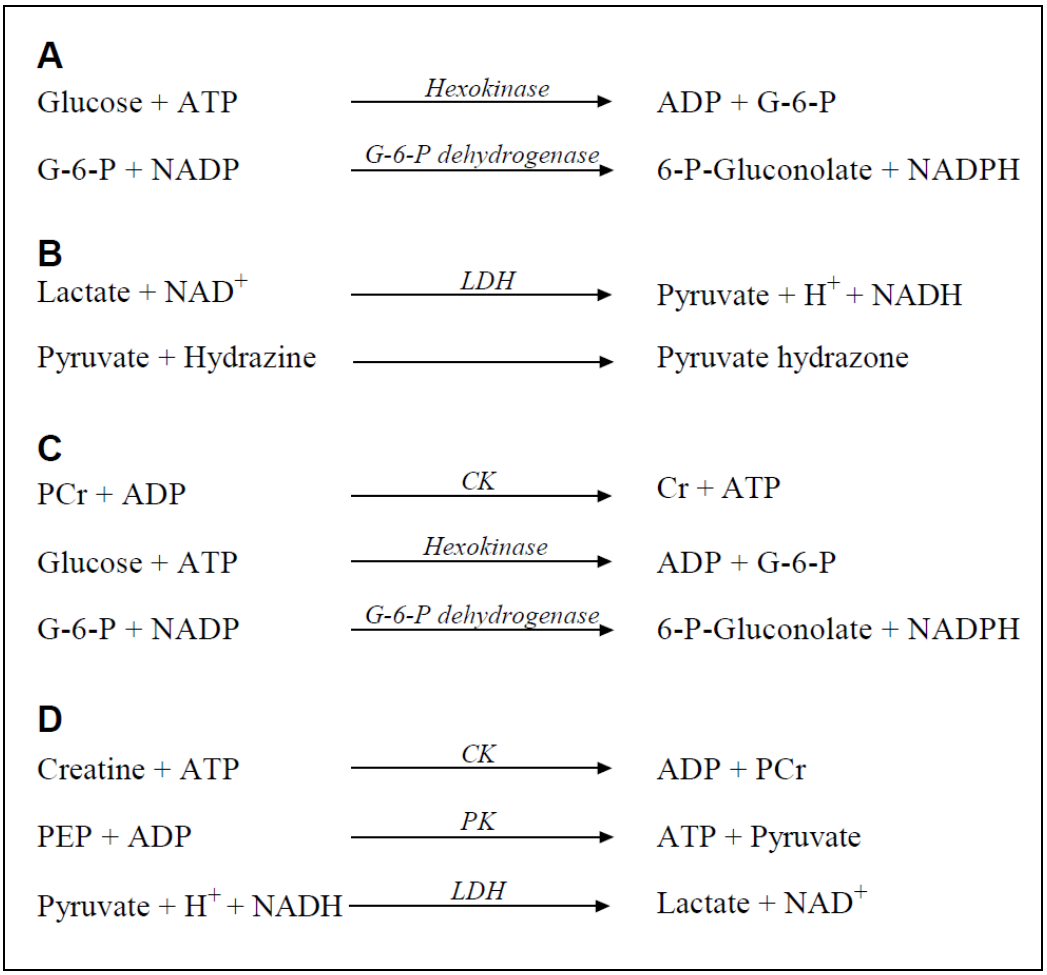

Figure 2.3 Principles for the determination of glycogen (glucose) and adenosine triphosphate (ATP) (A), lactate (B), creatine phosphate (PCr; C), and creatine (Cr; D). ADP= Adenosine diphosphate, G-6-P= Glucose-6-phosphate, NADPH= Reduced nicotenamide adenine dinucleotide, NAD^+= Nicotenamide adenine dinucleotide, LDH= Lactate dehydrogenase, H^+= Hydrogen CK= Creatine kinase, PEP= Phosphoenolpyruvate, PK= Pyruvate kinase.

Muscle ATP and creatine phosphate

ATP is required for glucose to be broken down by hexokinase to G-6-phosphate then to NADPH (Figure 2.3A). Muscle ATP was determined using an enzymatic fluorometric assay which detects production of NADPH (Passonneau & Lauderdale, 1974). Creatine phosphate (PCr) is broken down by creatine kinase to rephosphorylate ADP to ATP, which is required for the glucose to be broken down by hexokinase to produce NADPH (Figure 2.3C). Muscle PCr was determined using an enzymatic fluorometric assay which detects production of NADPH (Passonneau & Lauderdale, 1974).

In triplicate 10 µl of sample, ATP standards (100 and 200 µM), PCr standards (100 and 200 µM) NADPH standards (50, 100, 200 and 400 µM) or water blank was combined with 1 ml of cocktail reagent (50 mM Tris, pH 8.1, 1 mM $MgCl_2$, 0.5 mM DTT, 100 µM glucose, 50 µM $NADP^+$, 25 $\mu l \cdot ml^{-1}$ glucose-6-phosphate dehydogenase (1000 $U \cdot ml^{-1}$)) in a test tube. Background fluorescence was measured using a fluorimeter (Turner biosystems, Sunnyvale, CA, USA) at 365 nm absorption and 455 nm emission (R1), 25 µl of hexokinase (1000 $U \cdot ml^{-1}$ diluted 1/40 in cocktail reagent) was added to test tubes, and samples were mixed and incubated in the dark at room temperature for 60 min. Fluorescence of each sample was then determined (R2), and 20 µl of ADP-creatine phosphokinase (CK; 2 mg ADP, 2 mg CK, 10% BSA in 1 ml of cocktail reagent) was added to each test tube and samples were mixed and incubated in the dark at room temperature for a further 60 min. Fluorescence of each sample was again determined (R3). Muscle ATP was calculated from the change in fluorescence from R1 to R2, and muscle PCr from the change in fluorescence from R2 to R3 based on the NADPH standard curve and expressed per kg dry muscle mass ($mmol \cdot kg^{-1}$ d.m).

Muscle creatine

Creatine (Cr) is broken down in a two strep reaction to produce pyruvate (Figure 2.3D). NADH is required for lactate dehydrogenase reduction of pyruvate to lactate (Figure 2.3D). Muscle Cr was determined using an enzymatic fluorometric assay which detects reduction in NADH (Lowry & Passonneau, 1972).

In triplicate 30 µl of sample, Cr standards (200 and 500 µM), NADPH standards (50, 100, 200 and 400 µM) or reagent blank was combined with 1 ml of cocktail reagent (50 mM imadazole, 5 mM $MgCl_2$, 30 mM KCl, 0.1 mM phosphoenolpyruvate, 0.2 mM ATP, 0.03 mM NADH 0.2 $\mu l \cdot ml^{-1}$ lactate dehydrogenase (550 $U \cdot ml^{-1}$), 5 $\mu g \cdot ml^{-1}$ pyruvate kinase) in a test tube. Background fluorescence was measured using a fluorimeter (Turner biosystems, Sunnyvale, CA, USA) at 365 nm absorption and 455 nm emission, 20 µl of creatine kinase (10 $mg \cdot ml^{-1}$ in 0.05% BSA) was added to each test tube and samples were mixed and incubated in the dark at room temperature for 60 min. Fluorescence of each sample was again determined and muscle creatine was calculated from NADPH standard curve and expressed per kg dry muscle mass ($mmol \cdot kg^{-1}$ d.m). To account for any non-muscle contamination, final values were corrected to the highest muscle total creatine content (total Cr = PCr + Cr) for each participant.

Calculation of free ADP and free AMP

The near equilibrium nature of the creatine kinase and adenylate kinase reactions allow for the calculation of ADP_{free} and AMP_{free} concentration (Golding *et al.*, 1995). ADP_{free} was calculated from measured muscle ATP, Cr, PCr levels and estimated H^+ concentration (from measured muscle lactate (Mannion *et al.*, 1993)), using 1.66×10^9 as the equilibrium constant (K_{obs}) for creatine kinase (Lawson & Veech, 1979). AMP_{free} was calculated from measured muscle ATP and calculated ADP_{free} using a K_{obs} of 1.05 for adenylate kinase (Lawson & Veech, 1979). Calculated muscle ADP_{free} and AMP_{free} were expressed per kg dry muscle mass ($\mu mol \cdot kg^{-1}$ d.m and $mmol \cdot kg^{-1}$ d.m, respectively).

2.4 MUSCLE AND PLASMA THIOLS AND N-ACETYLCYSTEINE: Chapters 5 and 6

Muscle cysteine, glutathione and N-acetylcysteine (NAC), and plasma cysteine and NAC levels were determined using high performance liquid chromatography (HPLC) by myself at the University of Tasmania, Hobart, Australia. Muscle glutathione levels were measured as a marker of cellular oxidative state (Powers & Jackson, 2008) since under conditions of oxidative stress reduced glutathione (GSH) is more rapidly oxidized to oxidized glutathione (GSSG). NAC is rapidly deacylated to produce cysteine (Deneke, 2000), and like NAC, cysteine can directly scavenge ROS (Aruoma *et al.*, 1989; Cotgreave, 1997). Therefore, cysteine levels were measured as a marker of NAC-enhanced antioxidant defenses.

Muscle samples for NAC and thiol analysis were extracted by homogenising 30 mg of frozen muscle in 300 µl of ice-cold 0.42 M PCA. Forty µl of 2.5 M K_2CO_3 was then added to neutralise samples before spinning at 13,000 g for 5 min at 4°C and recovering supernatant. For analysis of total thiols and NAC, 10 µl of 1:10 tributylphosphine (Sigma-Aldrich Chemicals, St. Louis, MO, USA) was added to 50 µl of supernatant, and following a 30 min incubation on ice 25 µl of 4-Fluoro-7-sulfamoylbenzofurazan (Sigma-Aldrich Chemicals, St. Louis, MO; ADB-F; 5 $mg \cdot ml^{-1}$ in borate buffer: 0.2M boric acid, 2 mM sodium EDTA, pH 8.0) was added. Samples were then incubated at 50ºC for 10 min, 10 µl of 2 M PCA was added and they were spun at 13,000 g for 5 min. The supernatant was recovered and 40 µl was injected into a reverse-phase HPLC Gemini column (5 µm C18 110Å, phenomenex®) with 0.1 M sodium acetate buffer (pH 4.0) in 10% methanol as a mobile phase, at a flow rate of 1.5 $ml \cdot min^{-1}$ and detection wavelength of 386 nmm excitation and 516 nm emission. For the determination of reduced thiols and NAC tributylphosphine was replaced with H_2O and the protocol was repeated. Oxidised thiols were calculated from the difference between total and reduced thiols.

For the determination of plasma cysteine and NAC, muscle supernatant was replaced with 50 µl of plasma and the same protocol described above for muscle thiols was used.

2.5 IMMUNOBLOTTING: Chapter 3-6

2.6.1 Muscle extraction

Frozen muscle was homogenised (10-20 $\mu l \cdot mg^{-1}$ tissue; 50 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% v/v glycerol, 1% v/v Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 100 μM DTT, 1 mM PMSF and 5 $\mu l \cdot ml^{-1}$ Protease Inhibitor Cocktail (P8340, Sigma)), incubated for 20 min on ice and spun at 13,000 g for 20 min at 4°C. The supernatant was recovered and 5 μl was used for the determination of total protein content (see section *2.6.2 Total protein assay*), with the remaining supernatant being diluted 1:3 in sample buffer (1.5M Tris-HCL, pH 6.8, 30% glycerol, 10% SDS, 0.6M DTT, 0.0012% bromophenol blue), boiled for 10 min at 100°C and then stored in the freezer at -20°C for western blot analysis.

2.6.2 Total protein assay

In an alkaline environment protein peptide bonds reduce Cu^{2+} to Cu^{1+}, with this reduction being proportional to protein concentration. Bicinchoninic acid (BCA) reacts with Cu^{1+} ions forming a purple-coloured product which absorbs light at a wavelength of 562 nm. Total protein concentration was determined using a commercially available BCA protein assay kit (Pierce, Rockford, Il, USA) as per the manufacturer's instructions.

Ten μl of sample diluted 1:20 in double distilled H_2O or BSA standards (150, 200, 300, 400, 600, 1000 $\mu g \cdot ml^{-1}$) was transferred to a 96 well plate in triplicate and 200 μl of BCA reagent was added. Samples were incubated at 37°C for 30 min and absorbance was measured at 560 nm using a multiskan EX plate reader (Thermo Electron Corporation, Waltham, MA, USA). Total protein content of samples was calculated from BSA standard curve.

2.6.3 Western blots

Sixty to one hundred μg of total protein was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels consisted of a stacking gel (0.5M Tris-HCl, pH 6.8, 10% SDS, ddH_2O, 30% bisarylamide (Bio-Rad, Hercules, CA, USA), 10% ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED)) and a resolving gel (1.5M Tris-HCl, pH 8., 10% SDS, ddH_2O, 30% bisarylamide (Bio-Rad, Hercules, CA, USA), 10% APS, TEMED). Samples were run through gels in a electrophoresis buffer (25 mM Tris-HCl, 1M glycine, 5% SDS) at 90 V through stacking gel,

then at 120 V through resolving gel (Bio-Rad electrophoresis running tank, Hercules, CA, USA). The resolving gel was transferred to polyvinylidine difluride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) in a Bio-Rad Trans-blot electrophoresis transfer tanks (Bio-Rad, Hercules, CA, USA) filled with ice cold transfer buffer (150 mM glycine, 37 mM Tris-HCl, pH 8.3, 20% methanol) and run at 95 V for 90 min at 4°C. To estimate size of proteins of interest a 10-250 kDa molecular marker (Bio-Rad, Hercules, CA, USA) was run on every gel.

Membranes were blocked in PBS containing 5% nonfat milk for 1 h at room temperature, washed (4 x 5 min in PBS containing 0.1% Tween-20 (PBST)) and incubated overnight on a rocker at 4°C with a primary antibody. The primary antibody was then removed and membranes were washed (4 x 5 min in PBST) and incubated for 1 h at room temperature with a fluorescent secondary antibody (IRDye™ 800-conjugated anti-mouse or 700-conjugated anti-rabbit IgG, Rockland Inc., PA, USA), washed (4 x 5 min in PBS and 1 x 5 min in PBS) and direct fluorescence was determined and quantified using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NB, USA). Primary antibodies were diluted 1:1000 in PBS, and secondary anti-bodies were diluted 1:5000 in PBST containing 50% blocking buffer (Licor Biosciences, Lincoln, NB, USA) and 0.01% SDS. When both protein phosphorylation and total protein content were measured, membranes were first probed with the phosphorylation specific antibody then stripped (2% SDS (w/v) in 25mM Glycine, pH 2.0) for 40 min at room temperature, blocked and re-probed with primary antibody for total protein. However, for AMPK, membranes were first probed with AMPKα (for total protein) primary antibody before being stripped and re-probed with AMPKα Thr^{172} phosphorylation-specific antibody. This is because in our hands we find that AMPKα Thr^{172} cannot be stripped effectively. Protein phosphorylation was expressed relative to the total protein abundance of the protein of interest.

The following primary antibodies for total protein were used in this thesis: AMPKα, p38 MAPK, AS160, α-tubulin, Akt (all from Cell Signaling Technology; Hartsfordshire, UK), nNOS (BD Transduction Laboratories, NSW, Australia), nitrotyrosine (Chemicon, CA, USA) and glutathione (Abcam, Cambridge, UK). The following phosphorylation-specific primary antibodies were used in this thesis: anti-phospho-ACCβ Ser^{222} and anti-phospho-AMPK Thr^{172} (Upstate Biotechnology, NY, USA), anti-phospho-NOSμ Ser^{1446} (gift from Prof. Kemp, St. Vincent's Institute, Fitzroy, Victoria, Australia (Chen *et al.*, 2000)), and anti-

phospho-p38 MAPK Thr^{180}/Tyr^{182}, anti-phospho-Akt Substrate (PAS) and anti-phospho-Akt Ser^{473} (all from Cell Signaling Technology; Hartsfordshire, UK).

S-glutathionylation

S-glutathionylation is the addition of glutathione to protein cysteine residues (Dalle-Donne *et al.*, 2005). S-glutathionylation is a post-translational signalling mechanism which is enhanced during times of nitrosative and oxidative stress (Dalle-Donne *et al.*, 2009). As such, protein S-glutathionylation can be measured by probing membranes with a glutathione antibody (Abcam, Cambridge, UK). Since reducing agents break the S-glutathionylation bonds between proteins and GSH, muscle samples examined for S-glutathionylation were extracted and run under non-reducing conditions (i.e. no DTT in homogenisation or sample buffer). To help preserve S-glutathionylation of proteins and alkylate free thiol groups, homogenisation and sample buffers contained 5 mM and 10 mM of N-ethylmaleimide, respectively. All other western blotting procedures were performed as described above.

Since S-glutathionylation had not been measured in our laboratory previously several control experiments were performed. Figure 2.4A shows that incubating tibialis anterior (TA) muscle from mice in 5 mM H_2O_2 (positive control) for 10 min increases muscle S-glutathionylation of protein bands ranging from ~15 kDa to ~270 kDa. Furthermore, when mouse TA muscle was incubated in 5 mM H_2O_2 and extracted and run under normal reducing conditions (homogenisation and sample buffer containing 100 μM and 0.6 M DTT, respectively) S-glutathionylation of protein bands between ~15 kDa to ~270 kDa is almost completely lost (Figure 2.4B). The glutathione antibody also showed good linearity; increasing band intensity with increased protein loading.

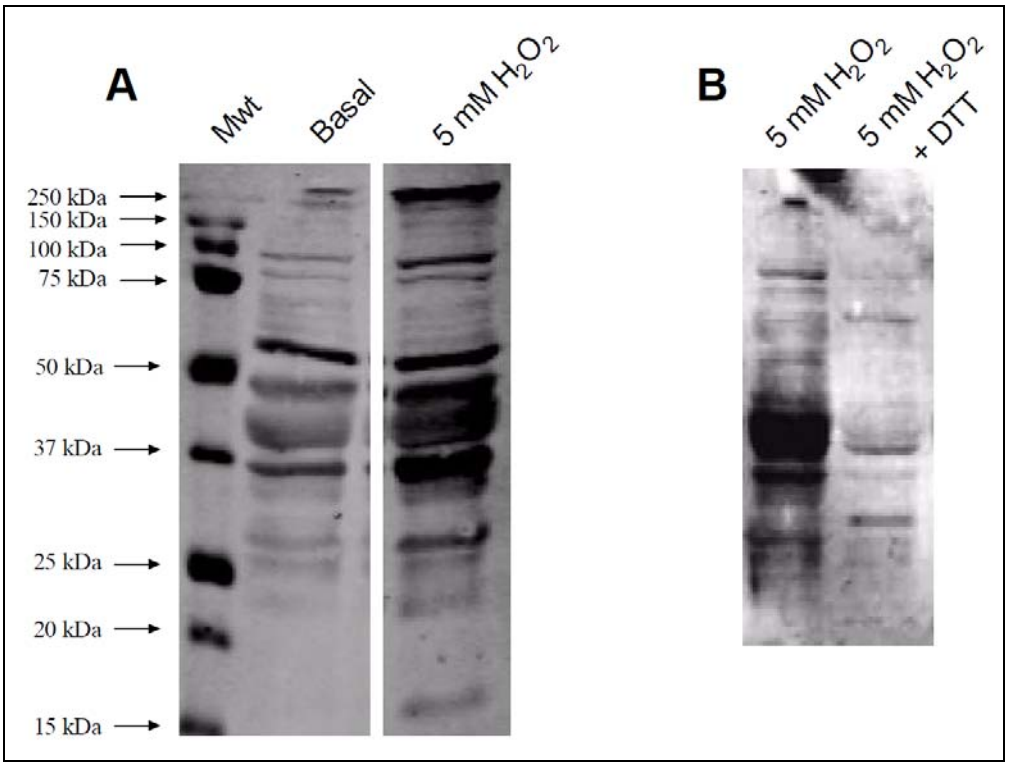

Figure 2.4 Representative S-glutathionylation blots of tibialis anterior (TA) muscle from mice. A) TA muscles incubated for 10 min in the presence or absence (basal) of 5 mM H_2O_2. Blots for basal and 5 mM H_2O_2 treated TA muscle presented in A are from the same membrane. B) TA muscles incubated for 10 min in the presence of 5 mM H_2O_2 were extracted under non-reducing (5 mM H_2O_2) or reducing (5 mM H_2O_2 + DTT) conditions. Mwt= molecular weight marker.

Tyrosine nitration

Peroxynitrite ($ONOO^-$) nitration of protein tyrosine residuals (tyrosine nitration) produces nitrotyrosine which can be detected with an anti-nitrotyrosine antibody (Chemicon, CA, USA). Muscle extraction and western blotting procedures were performed as described above, except that muscle homogenates were heated at 37°C for 10 min rather than boiled at 100°C. This is because boiling of samples reduces nitrotyrosine to aminotyrosine (Balabanli *et al.*, 1999). Since nitrotyrosine had not been measured in our laboratory previously a positive control experiment was performed. Incubating TA muscle form mice for 10 min in the presence of 2.5 mM $ONOO^-$ increased tyrosine nitration of protein bands at ~72 kDa, 42 kDa, 35 kDa and 26 kDa (Figure 2.5). The tyrosine nitration antibody also showed good linearity; increasing band intensity with increased protein loading.

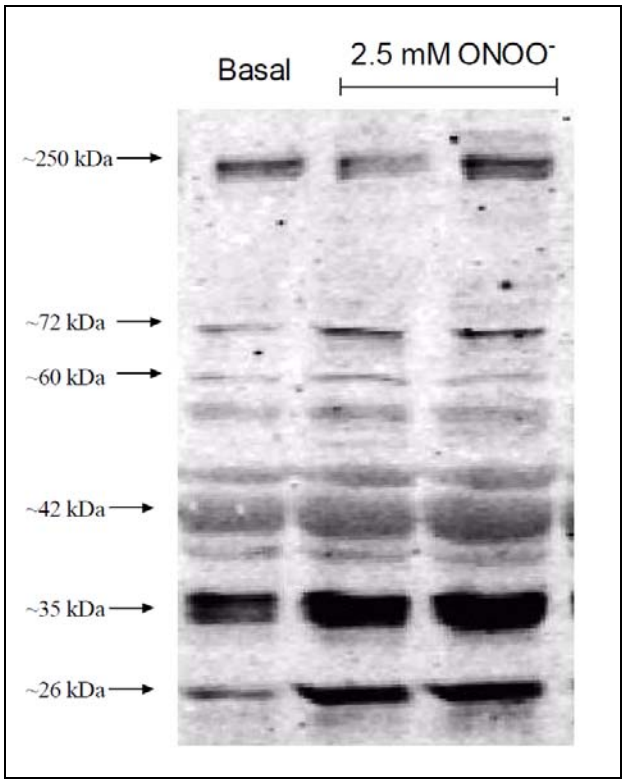

Figure 2.5 Representative tyrosine nitration blots of tibialis anterior (TA) muscles from mice. TA muscles were incubated for 10 min in the presence or absence (basal) of 2.5 mM peroxynitrite ($ONOO^-$).

PAS-AS160

AS160 phosphorylation is commonly measured using the anti-phospho-Akt Substrate (PAS) antibody that recognises several Akt phosphorylation motif peptide sequences [RXRXX(T*/S*)] of proteins (Cell Signaling Technology; Hartsfordshire, UK) (Kane *et al.*, 2002; Bruss *et al.*, 2005; Kramer *et al.*, 2006a; Treebak *et al.*, 2006; Kramer *et al.*, 2007; Funai & Cartee, 2008). Therefore, this antibody shows the cumulative phosphorylation of several Akt sites on various proteins, and the protein band detected at 160 kDa represents AS160 phosphorylation. However, the protein band detected at ~160 kDa may also represent TBC1D1 phosphorylation, another Akt substrate that PAS recognises at ~150 kDa (Taylor *et al.*, 2008). Although PAS 160 kDa may also represent TBC1D1 phosphorylation, the PAS antibody was used in this thesis because no phosphorylation site specific TBC1D1 or AS160 antibodies were commercially available. As such, results of the PAS antibody in this thesis refer to a protein band at ~160 kDa (PAS-160) rather than AS160 or TBC1D1 phosphorylation specifically as we cannot be sure which protein this band represents due to their close proximity. However, AS160 total protein levels were still used as a loading control.

Since the PAS antibody has not been used in our laboratory previously a positive control experiment was performed. Incubating soleus muscles from mice for 10 min in the presence or absence of 1 μM insulin increased PAS-160 phosphorylation (Figure 2.6).

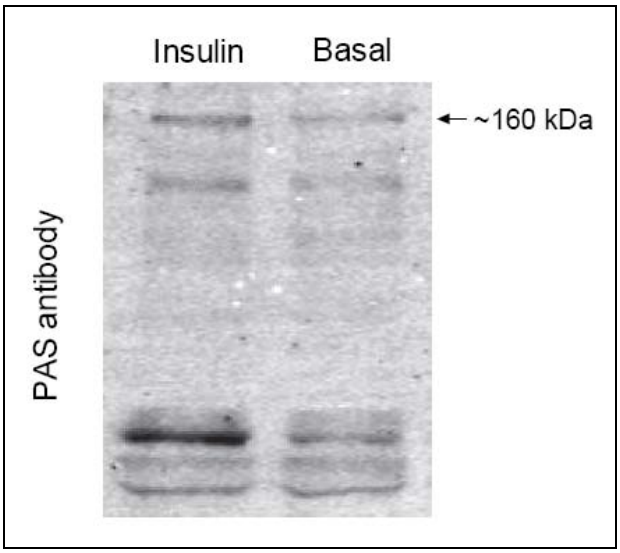

Figure 2.6 Representative PAS-160 phosphorylation blots in soleus muscle from mice. Soleus muscles were incubated for 10 min in the presence or absence (basal) of 1 μM insulin.

CHAPTER THREE

SKELETAL MUSCLE GLUCOSE UPTAKE DURING CONTRACTION IS REGULATED BY NITRIC OXIDE AND REACTIVE OXYGEN SPECIES INDEPENDENT OF AMPK

3.1 INTRODUCTION

AMP-activated protein kinase (AMPK) is one of the most well studied signalling intermediates proposed to be involved in the regulation of skeletal muscle glucose uptake during contraction. It is well recognised that that the AMPK activator 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) increases skeletal muscle glucose uptake (Merrill *et al.*, 1997; Hayashi *et al.*, 1998; Kurth-Kraczek *et al.*, 1999; Koistinen *et al.*, 2003) through an insulin independent pathway (Hayashi *et al.*, 1998; Bergeron *et al.*, 1999). Furthermore, the increase in AMPK activity during contraction correlates with increases in skeletal muscle glucose uptake (Ihlemann *et al.*, 2001; Musi *et al.*, 2001b; Chen *et al.*, 2003). However, disassociations between skeletal muscle glucose uptake and contraction-stimulated AMPK activity have also been reported during hypoxic exercise (Wadley *et al.*, 2006), glycogen-loaded *ex vivo* muscle contractions (Derave *et al.*, 2000), low intensity exercise (Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000) and following short-term training (McConell *et al.*, 2005). In some (Mu *et al.*, 2001; Jensen *et al.*, 2007b; Lefort *et al.*, 2008) but not all studies (Fujii *et al.*, 2005; Fujii *et al.*, 2007; Maarbjerg *et al.*, 2009) mice that over express a muscle specific kinase dead AMPKα2 isoform (AMPK KD) have reduced skeletal muscle glucose uptake with contraction. Furthermore, mice which have AMPKα1 or α2 knocked out, have normal increases in skeletal muscle glucose uptake during contraction (Jorgensen *et al.*, 2004). Therefore, the role of AMPK in regulating skeletal muscle glucose uptake during contraction is controversial.

Recent studies have focused on the role of free radical signalling in regulating skeletal muscle glucose uptake, with both donors of nitric oxide (NO) and exogenous reactive oxygen species (ROS) increasing glucose uptake in resting skeletal muscle (Higaki *et al.*, 2001; Higaki *et al.*,

2008; Jensen *et al.*, 2008). Indeed, acute physiological increases in ROS production, NO synthase (NOS) activity and NO production occur during contraction (Sandstrom *et al.*, 2006; Ross *et al.*, 2007; Jackson, 2008; Reid, 2008), and antioxidant treatment (Sandstrom *et al.*, 2006) and NOS inhibition (Balon & Nadler, 1997; Roberts *et al.*, 1997; Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Ross *et al.*, 2007) attenuate increases in skeletal muscle glucose uptake during contraction. However, it must be acknowledged that not all studies report that NOS inhibition during contraction attenuates skeletal muscle glucose uptake (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002),

Since exogenous ROS increase AMPK activity (Toyoda *et al.*, 2004; Jensen *et al.*, 2008) and antioxidant treatment during contraction attenuates increases in AMPK activity (Sandstrom *et al.*, 2006), it has been proposed that ROS, particularly hydrogen peroxide (H_2O_2), may regulate skeletal muscle glucose uptake during contraction via an AMPK-dependent mechanism (Sandstrom *et al.*, 2006). However, it appears that increases in AMPK activity are not required for exogenous ROS-simulated glucose uptake in resting skeletal muscle (Higaki *et al.*, 2008; Jensen *et al.*, 2008), but PI3K signalling may be involved (Higaki *et al.*, 2008). Therefore, because exogenous ROS can increase resting skeletal muscle glucose uptake through an AMPK-independent but seemingly PI3K-dependent pathway, and PI3K is not involved in regulating skeletal muscle glucose uptake during contraction (Lee *et al.*, 1995; Yeh *et al.*, 1995), it is important to investigate whether ROS regulate skeletal muscle glucose uptake during contraction via AMPK.

Similarly, NO has been proposed to act both upstream and downstream of AMPK, with AMPK phosphorylating NOS (Chen *et al.*, 1999; Chen *et al.*, 2000; Shearer *et al.*, 2004), and NO-donors increasing AMPK activity (Higaki *et al.*, 2001; Lira *et al.*, 2007). Indeed, some (Fryer *et al.*, 2000), but not others (Stephens *et al.*, 2004) report that NOS inhibition attenuates AICAR-stimulated glucose uptake in skeletal muscle. Therefore, it is possible that NO regulates skeletal muscle glucose uptake during contraction through interaction with AMPK. However, NOS inhibition during contraction has been shown to attenuate glucose uptake without affecting AMPK signalling (Ross *et al.*, 2007).

Based on these equivocal findings, the aim of the present study was to determine whether NO and ROS regulate glucose uptake during contraction via an AMPK-dependent pathway by contracting isolated skeletal muscle from AMPK KD and wild type (WT) mice in the

presence of a NOS inhibitor (L-NMMA) or an antioxidant (NAC). We tested the hypothesis that ROS and NO regulate skeletal muscle glucose uptake during contraction via an AMPK independent mechanism.

3.2 METHODS

3.2.1 Animals

This study used male C57Bl/6 mice, and male and female mice with muscle-specific overexpression of a kinase dead form of the AMPKα2 isoform (AMPK KD), which have been described in detail previously (Mu *et al.*, 2001; Dzamko *et al.*, 2008). C57Bl/6 mice were used at 12-14 weeks of age for non-contration (basal) experiments using AICAR. AMPK KD mice and WT litter mate controls were supplied at ~15-18 weeks and were used at 22-24 weeks of age (Table 3.1). Since no differences were found between male and female mice for muscle glucose uptake during contraction the results were pooled. All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne, and conformed to the Australian code of practice for the care and use of animals for scientific purposes, as described by the National Health and Medical Research Council (Australia).

3.2.2 Experimental procedure

As described in Chapter 2 (*2.1 Ex vivo mouse studies*) hindlimb EDL and soleus muscles of anaesthetised mice were excised and incubated *ex vivo.* Muscle optimal length was determined before incubating for 40 min in the presence or absence of the AMPK activator 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR; 2 mM) (Jorgensen *et al.*, 2004), the NOS inhibitor N^G-monomethyl-L-Arginine (L-NMMA; 100 μM) (Higaki *et al.*, 2001), the non-specific antioxidant N-acetylcysteine (NAC; 20 mM) (Sandstrom *et al.*, 2006), AICAR+NAC or AICAR+L-NMMA. NAC and L-NMMA were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and AICAR from Toronto Chemicals Inc. (Toronto, Canada). AICAR and inhibitors were added as the same to standardise total incubation times between contraction and AICAR experiments.

3.2.3 Muscle contraction

Following 30 min of incubation at rest in the presence or absence of L-NMMA or NAC, muscles were stimulated to contract for 10 min (pulse durations: 350 ms (EDL) and 600 ms (soleus) at a frequency of 60 Hz, 12 contractions·min^{-1}). This stimulation protocol has been previously shown to be sufficient to recruit all motor units within the contracting muscle (Lynch *et al.*, 2001). The contraction protocol was designed minimise fatigue but still

maintain a large increase in glucose uptake. Non-contracted muscles were treated the same as contracted muscles except that they were not eclectically stimulated.

3.2.4 Analytical techniques

Muscle 2-deoxy-D-glucose (2-DG) uptake was measured during the final 5 min of contraction and over 5 min of recovery in contracted muscles (Jorgensen *et al.*, 2004), and during the final 10 min of incubation in non-contracted (resting) muscles as described in Chapter 2 (*2.1 Ex vivo mouse studies*). Muscles were analysed for AMPKα1 and α2 activity, AMPKα expression, nNOS expression, AMPKα Thr^{172} phosphorylation, ACCβ Ser^{221} phosphorylation, PAS-160 phosphorylation, S-glutathionylation. Seperate muscles were analysed for oxidant levels and NOS activity and glucose uptake. The methodology used for these analytical procedures is described in detail in Chapter 2.

3.2.5 Statistical analysis

All data are expressed as means ± SEM. Results were analysed by SPSS statistical package using one factor (condition) ANOVA for experiments using C57Bl/6 mice, two factor (genotype and condition) ANOVA for experiments using AMPK KD and WT mice, and three factor (genotype, condition and time) ANOVA for muscle contraction forces of AMPK KD and WT mice. If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher's least significance difference test. Morphological properties for AMPK KD and WT mice were compared using an unpaired Students t-test. The level of significance was set at $P < 0.05$.

3.3 RESULTS

3.3.1 AMPK KD mice morphological properties

Body mass, EDL mass or soleus mass was not different between age and sex matched AMPK KD and WT mice (Table 3.1).

3.3.2 Muscle contraction

Neither treatment nor genotype affected initial peak force (normalised to muscle mass) during contraction of either the EDL or soleus muscles from AMPK KD or WT mice (Figure 3.1A and 3.2A). Initial peak force of both muscles was obtained within the first 10 s of the contraction protocol and declined thereafter ($P<0.001$; Figure 3.1B and 3.2B). The rate of decline from initial peak force was not affected by genotype or any treatment ($P>0.05$; Figure 3.2B and 3.2B).

3.3.3 AMPK signalling

EDL and soleus muscles from AMPK KD mice showed a 2-fold greater expression of AMPKα than muscles from WT mice (Figure 3.3A and B). Despite this EDL muscles from AMPK KD mice showed lower ($P<0.01$) basal AMPKα1 and AMPKα2 activity than EDL muscles from WT mice (Figure 3.4A and B). AMPKα1 activity in EDL muscle from WT and AMPK KD mice did not increase with contraction (Figure 3.4A). Contraction increased ($P=0.001$) AMPKα2 activity in EDL muscles from WT mice by 3.5-fold, but did not increase AMPKα2 activity in EDL muscles from AMPK KD mice (Figure 3.4B).

EDL and soleus muscles from WT mice showed greater AMPKα Thr^{172} phosphorylation relative to AMPKα expression than EDL and soleus muscles from AMPK KD mice ($P<0.05$, Figures 3.5A and B). Contraction increased ($P<0.05$) AMPKα Thr^{172} phosphorylation by 4-fold and 7-fold above basal in EDL and soleus muscles of WT mice, respectively, but did not increase AMPKα Thr^{172} phosphorylation in EDL or soleus muscles of AMPK KD mice ($P>0.05$). AMPKα Thr^{172} phosphorylation in EDL and soleus muscles of both genotypes was not affected by NAC or L-NMMA (Figure 3.5A and B).

ACCβ Ser^{221} phosphorylation in EDL and soleus muscles was greater in WT than in AMPK KD mice ($P<0.005$; Figures 3.6A and B). Contraction increased ACCβ Ser^{221} phosphorylation

in EDL and soleus muscles of WT mice ($P<0.05$), and in EDL ($P=0.05$) but not soleus ($P=0.17$) muscles of AMPK KD mice (Figures 3.6A and B). NAC treatment prevented the contraction-stimulated increase in ACCβ Ser^{221} phosphorylation in soleus ($P=0.17$ vs. rest) but not EDL muscle of WT mice, and did not affect ACCβ Ser^{221} phosphorylation in the EDL or soleus muscles of AMPK KD mice (Figures 3.6A and B). L-NMMA treatment did not affect ACCβ Ser^{221} phosphorylation during contraction in EDL or soleus muscles of either genotype (Figures 3.6A and B).

3.3.4 PAS-160

EDL muscles from AMPK KD mice showed lower PAS-160 phosphorylation than EDL muscles from WT mice ($P=0.01$; Figure 3.7A). Soleus muscle from AMPK KD and WT showed similar PAS-160 phosphorylation ($P=0.54$; Figure 3.7B). Contraction did not affect PAS-160 phosphorylation in EDL or soleus muscles of either genotype (Figure 3.7A and B).

3.3.5 Oxidant levels and S-glutathionylation

Contraction increased ($P<0.05$) oxidant levels by ~50% in the EDL muscles of both AMPK KD and WT mice, and this increase was prevented by NAC (Figure 3.8A and B).

Contraction increased ($P<0.05$) S-glutathionylation of protein bands at ~270 kDa and ~37 kDa by ~2-fold in EDL muscles of WT mice, and these increases were prevented by NAC (Figure 3.9A and B). In EDL muscles from AMPK KD mice contraction did not increase S-glutathionylation at ~270 kDa (Figure 3.9B), but increased ($P<0.05$) S-glutathionylation at ~37 kDa (Figure 3.9A) by ~2.3-fold ($P<0.05$), and this increase was prevented by NAC. The nature of these protein bands are investigated in Chapter 4. Neither contraction nor NAC affected protein S-glutathionylation of any other protein bands.

3.3.6 NOS activity and expression

EDL muscles from AMPK KD mice showed ~30% lower NOS activity than EDL muscles from WT mice ($P<0.05$) (Figure 3.10A). Contraction increased ($P<0.05$) NOS activity ~2-fold in the EDL muscles of both WT and AMPK KD mice, and this increase was prevented with L-NMMA (Figure 3.10A).

nNOS protein was 3-fold more abundant in EDL than in soleus muscles (P<0.001), and there was no difference in nNOS protein expression in the EDL and soleus muscles of WT and AMPK KD mice (Figure 3.10B).

3.3.7 Basal glucose uptake

Basal (resting) glucose uptake of EDL muscles from C57Bl/6 mice was not affected by L-NMMA, but was reduced (P=0.02) from 1.1 ± 0.1 to 0.7 ± 0.1 μmol·g·hr^{-1} by NAC (Chapter 4, Figure 4.11).

Contraction increased (P<0.05) glucose uptake in the EDL (2.3-fold) and soleus (1.6-fold) muscles from both WT and AMPK KD mice to a similar extent (Figure 3.11A and B). NAC attenuated the increase in glucose uptake during contraction in EDL muscles from WT mice by ~50% (P=0.01), and prevented increases in glucose uptake in EDL muscles of AMPK KD mice during contraction (Figure 3.11A). In the soleus muscles of both WT and AMPK KD mice NAC prevented the increase in glucose uptake during contraction (Figure 3.11B).

L-NMMA attenuated (by ~40%; P<0.05) the increase in glucose uptake during contraction in EDL muscles of WT and AMPK KD mice, but did not affect glucose uptake during contraction in the soleus muscles of either WT (P=0.34) or AMPK KD mice (P=0.51) (Figure 3.11A and B).

3.3.8 AICAR-stimulated glucose uptake and AMPK phosphorylation

AICAR increased glucose uptake 2.6-fold (P<0.01) in the EDL muscles of C57Bl/6 mice, and this increase was prevented by NAC (Figure 3.12A). L-NMMA had no effect on AICAR-stimulated glucose uptake in EDL muscles from C57Bl/6 mice (Figure 3.12A). NAC had no effect on basal AMPK phosphorylation in EDL muscles from C57Bl/6 mice, but prevented the 2.3-fold increase in AMPKα Thr^{172} phosphorylation in response to AICAR (P=0.01) (Figure 3.12B).

3.3.9 AICAR-stimulated oxidant levels

AICAR did not affect oxidant levels in EDL muscles of C57Bl/6 mice, but the addition of NAC to AICAR tended to reduce oxidant levels in EDL muscles (P=0.09; Figure 3.13A and B).

Table 3.1 Morphologic characteristics of AMPK KD and WT mice.

	WT	**AMPK KD**
Age (weeks)	23 ± 0.2	23 ± 0.2
Body mass (g)	24.5 ± 0.6	24.1 ± 0.4
EDL muscle mass (mg)	10.8 ± 0.5	10.1 ± 0.5
Soleus muscle mass (mg)	8.8 ± 0.4	8.7 ± 0.4

Values are means ± SEM, n=28-30 per group; EDL = extensor digitorum longus.

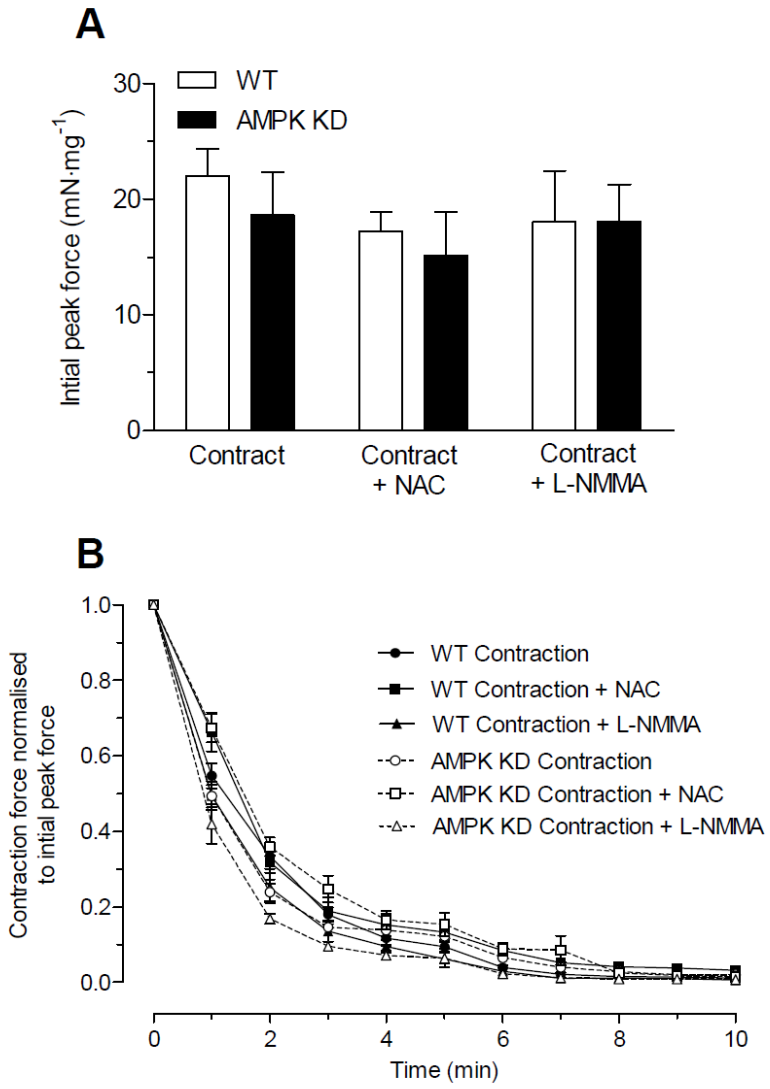

Figure 3.1 Initial peak contraction force (A) of EDL muscles from WT and AMPK KD mice, and drop in force production from the initial peak force (B) during each minute of the 10 minute contraction protocol in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-12 per group.

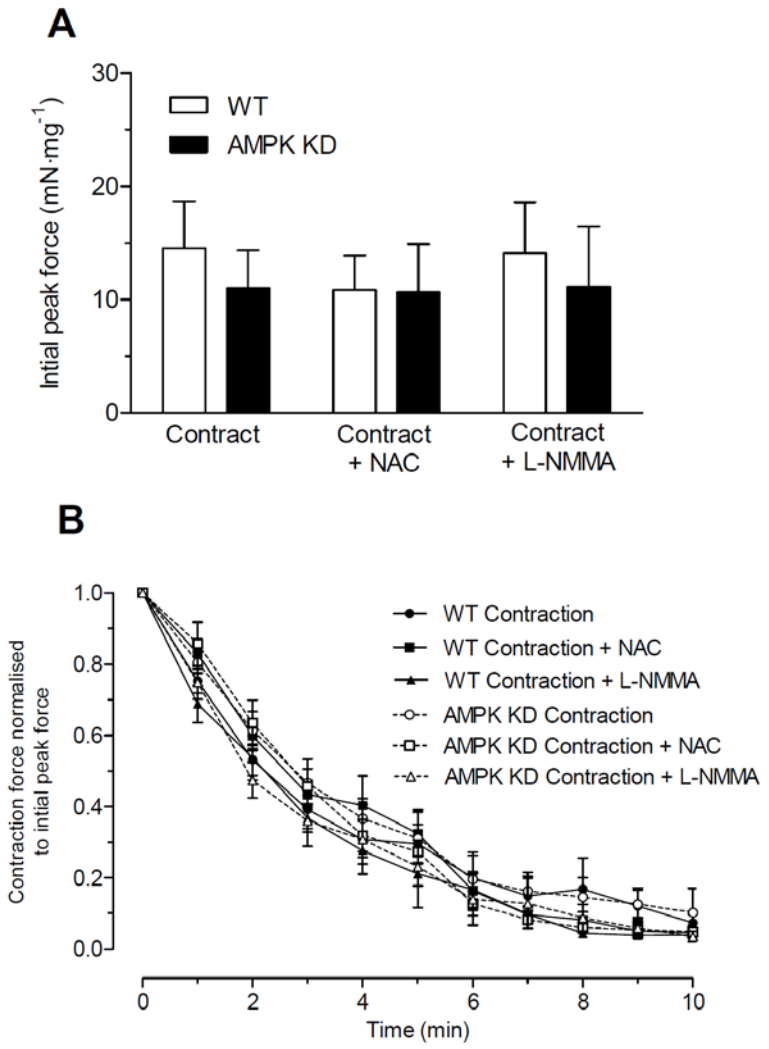

Figure 3.2 Peak contraction force (A) of soleus muscles from WT and AMPK KD mice, and drop in force production from the initial peak force (B) during each minute of the 10 min contraction protocol in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-12 per group.

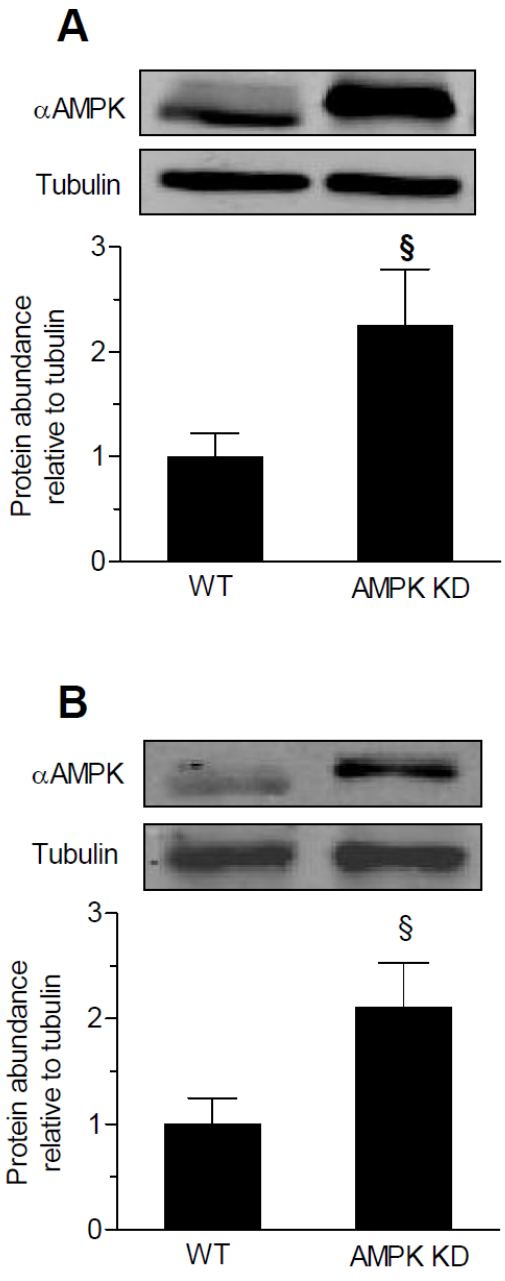

Figure 3.3 AMPKα expression in EDL (A) and soleus (B) muscles of WT and AMPK KD mice. Data are means ± SEM, n=12 per group. Presented blots are representative. §P<0.05 for genotype effect.

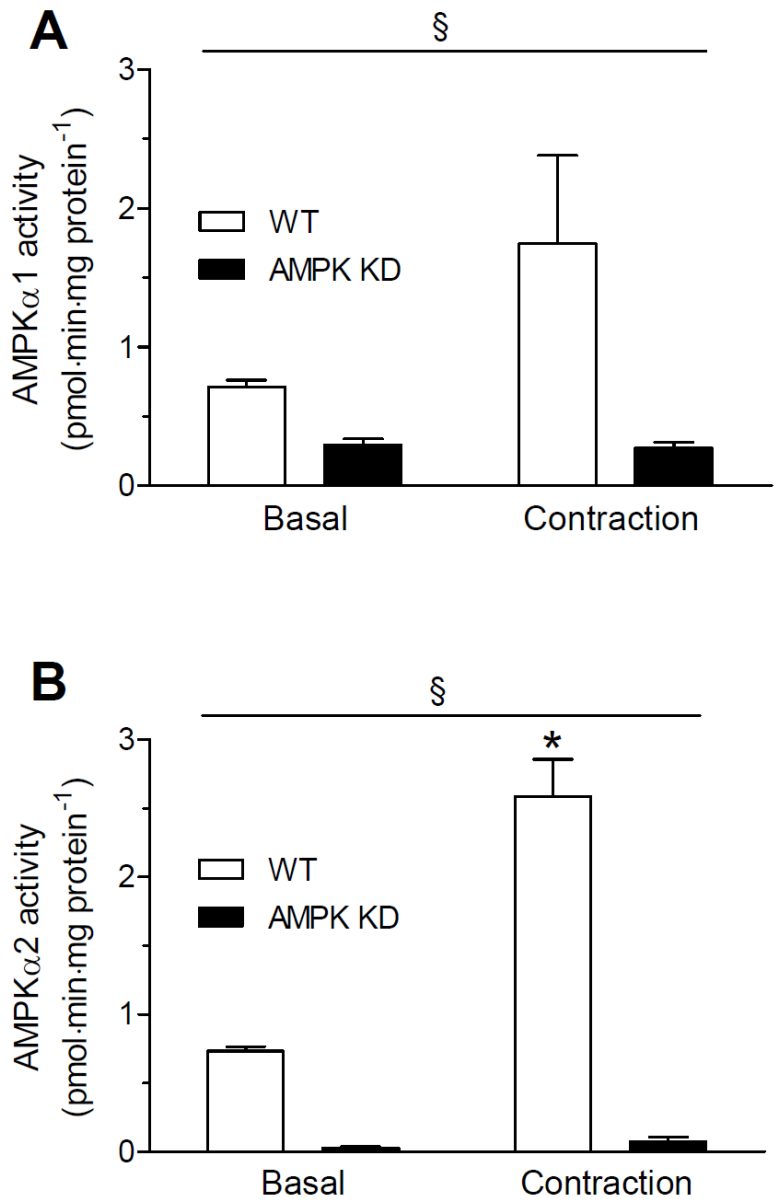

Figure 3.4 AMPKα1 (A) and AMPKα2 (B) activity in EDL muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contact for 10 min. Data are means ± SEM, n=3-4 per group. *P<0.05 vs basal group of the same genotype, §P<0.05 for genotype effect.

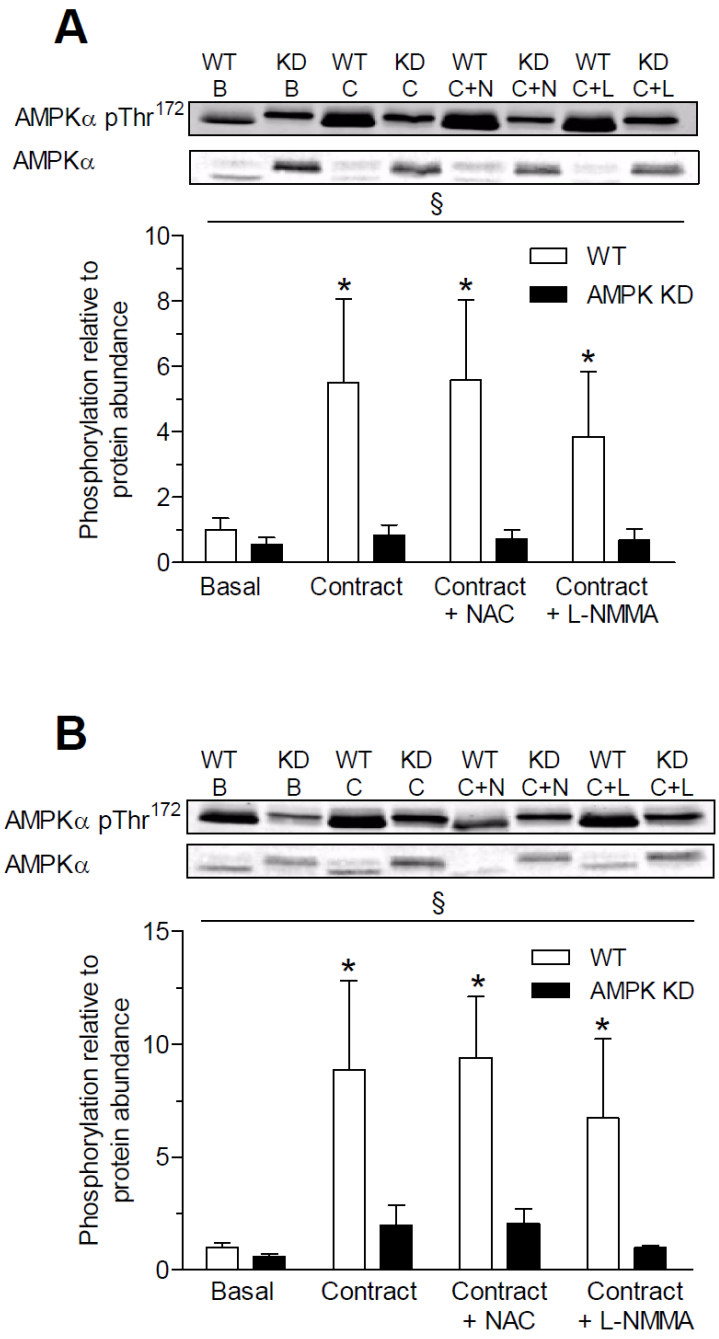

Figure 3.5 AMPKα Thr^{172} phosphorylation in EDL (A) and soleus (B) muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-7 per group, *P<0.05 vs basal group of the same genotype, §P<0.05 for genotype effect. Presented blots are representative. For representative blots; KD= AMPK KD mice, B= basal, C= contraction, N= contraction+NAC, L= contraction+L-NMMA.

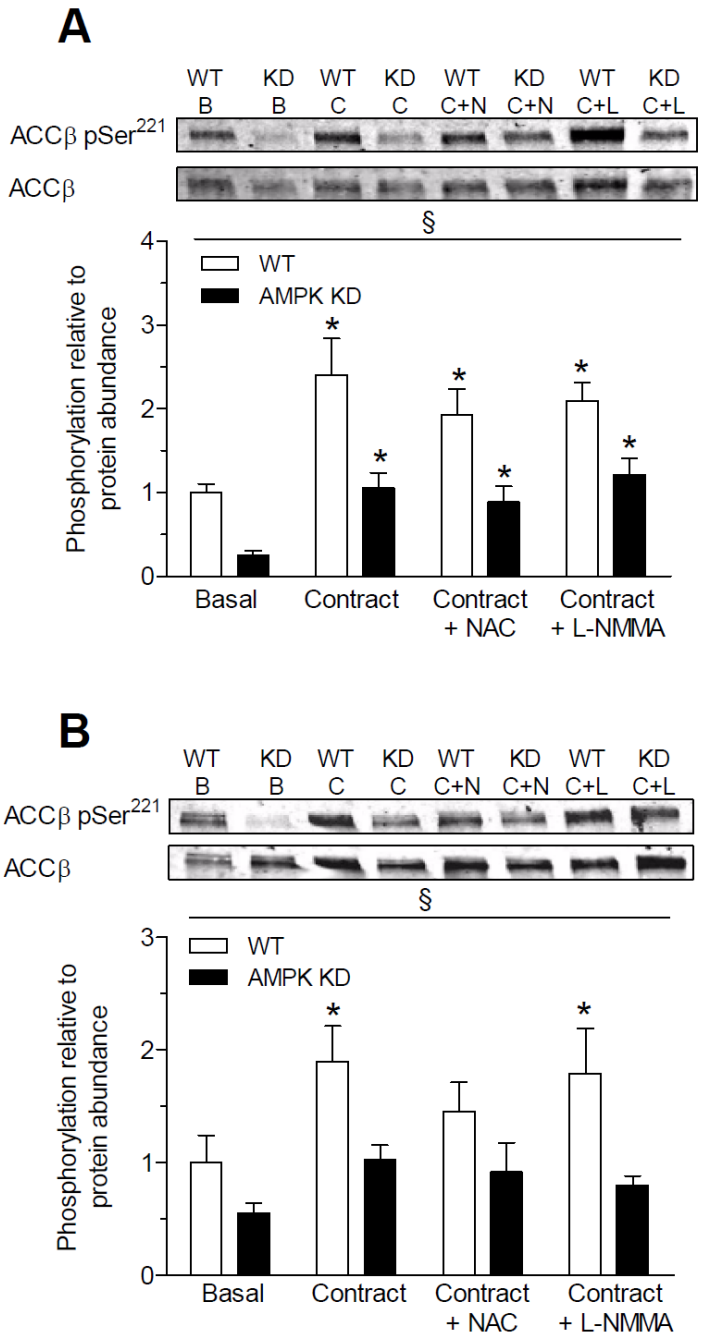

Figure 3.6 ACCβ Ser^{221} phosphorylation in EDL (A) and soleus (B) muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-7 per group, *P<0.05 vs basal group of the same genotype, §P<0.05 for genotype effect. Presented blots are representative. For representative blots; KD= AMPK KD mice, B= basal, C= contraction, N= contraction+NAC +, L= contraction+L-NMMA.

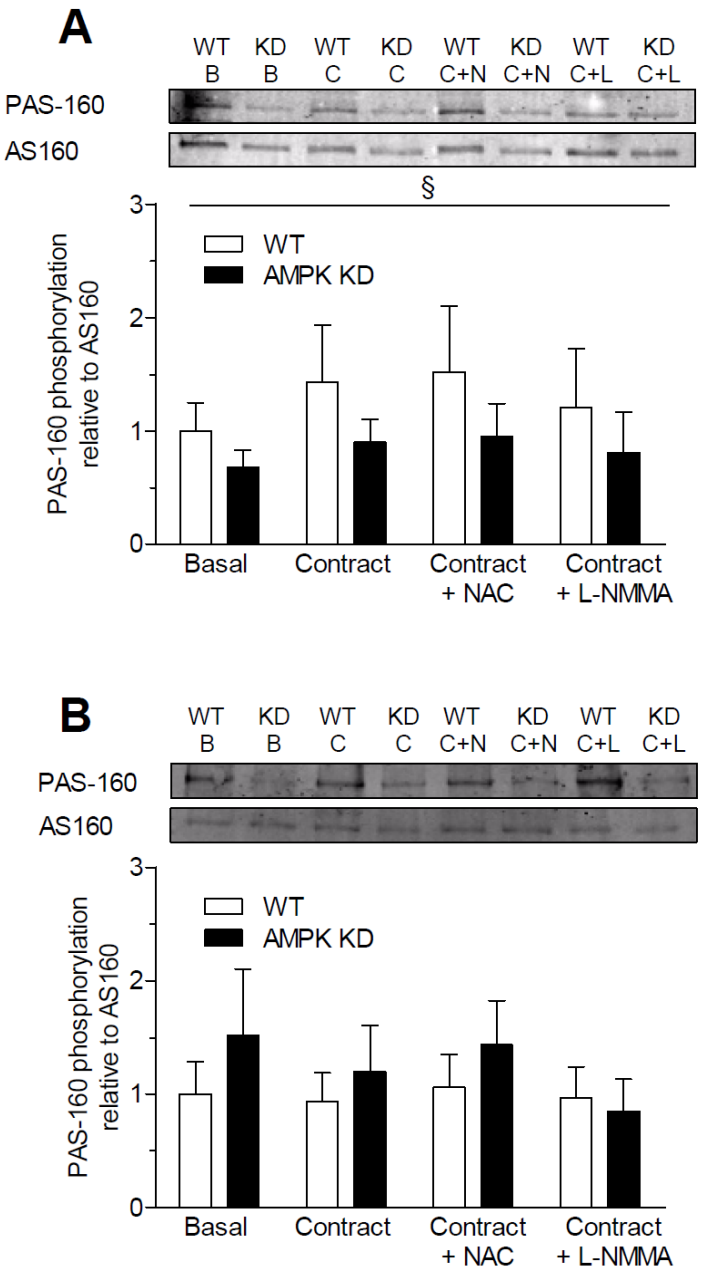

Figure 3.7 PAS-160 phosphorylation in EDL (A) and soleus (B) muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-7 per group, §P<0.05 for genotype effect. Presented blots are representative. For representative blots; KD= AMPK KD mice, B= basal, C= contraction, N= contraction+NAC, L= contraction+L-NMMA.

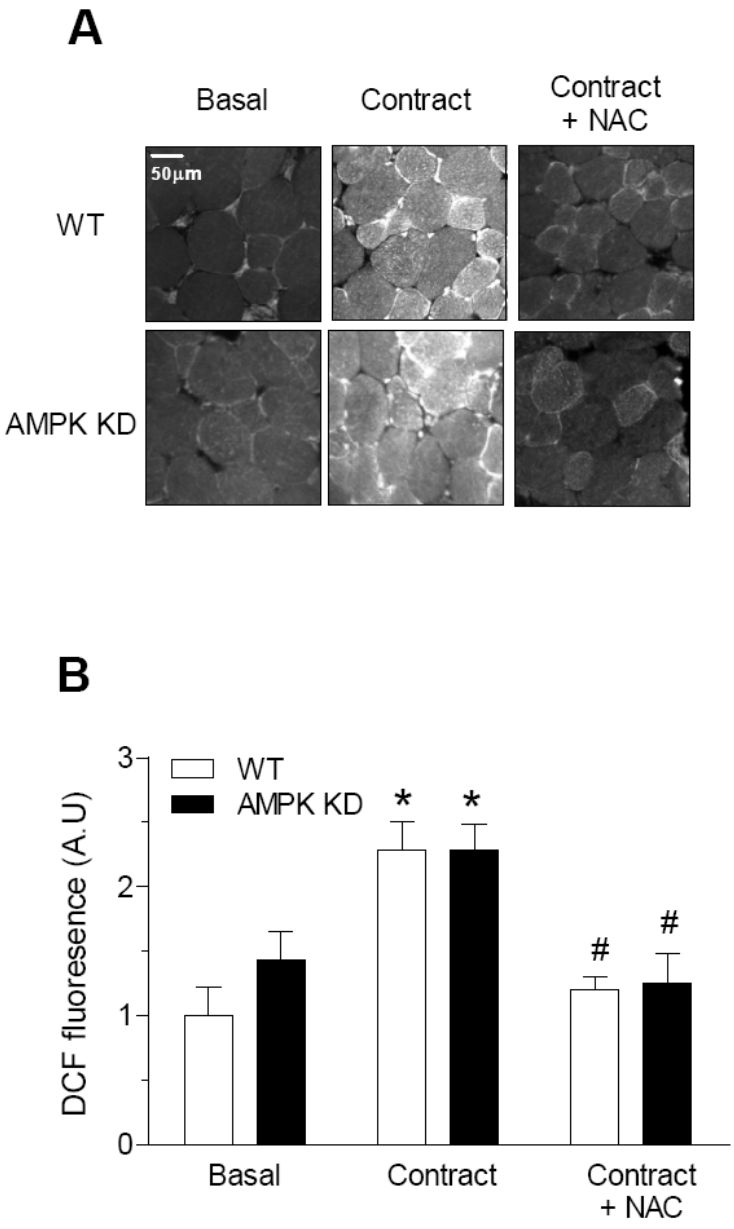

Figure 3.8 Representative images (A) and emission intensity (B) of DCF fluorescence in cross-sections of EDL muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contract for 10 min in the presence or absence of N-acetylcysteine (NAC). Data are means ± SEM, n=4-6 per group, *P<0.05 vs basal group of the same genotype. #P<0.05 vs contraction group of the same genotype.

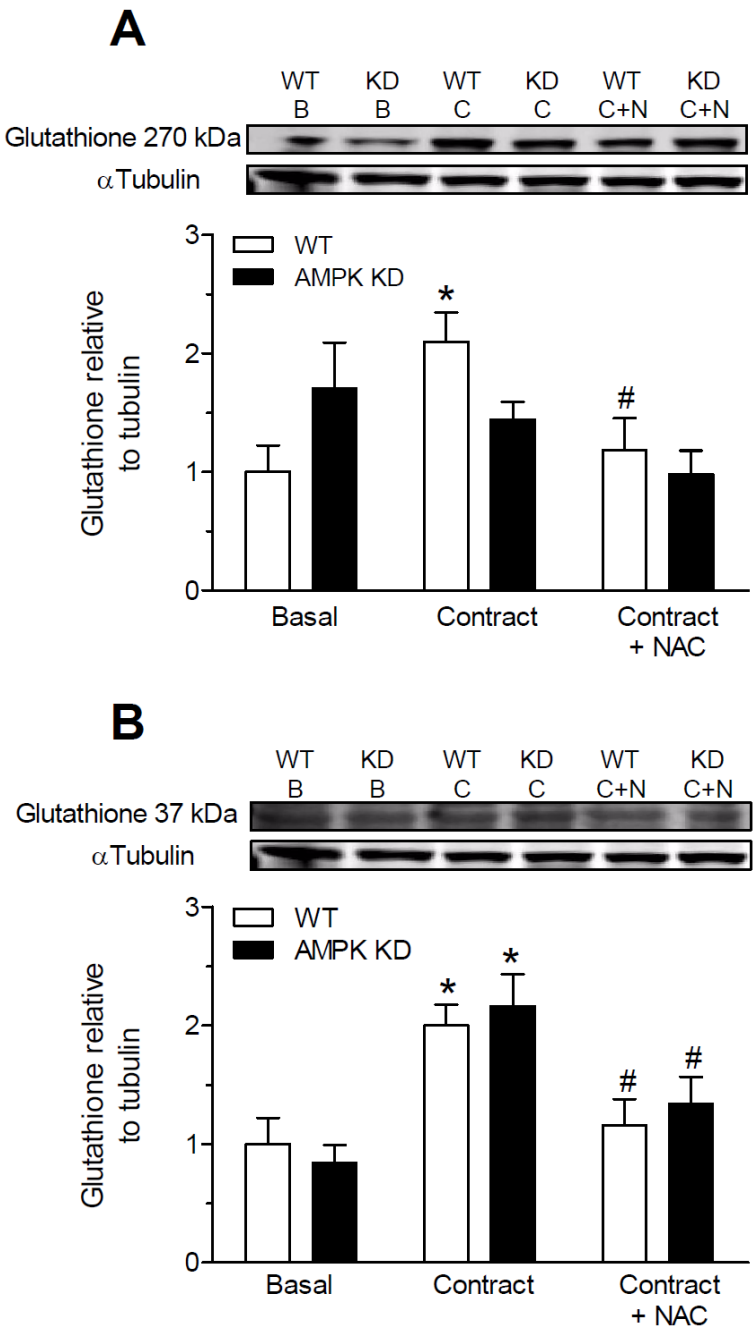

Figure 3.9 S-glutathionylation of a protein band at ~270 kDa (A) and 37 kDa (B) in EDL muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contract for 10 min in the presence or absence of N-acetylcysteine (NAC). Data are means ± SEM, n=4-6 per group, *P<0.05 vs basal group of the same genotype, #P<0.05 vs contraction group of the same genotype. Presented blots are representative. For representative blots; KD= AMPK KD mice, B= basal, C= contraction, N=contraction+NAC.

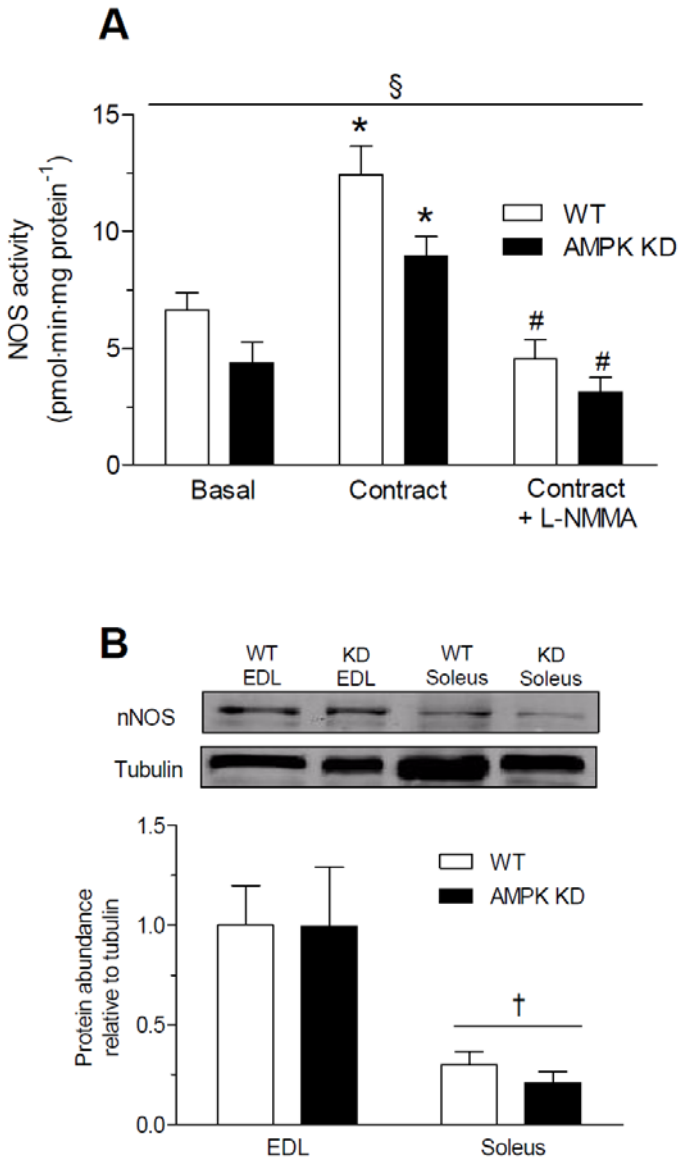

Figure 3.10 Nitric oxide synthase (NOS) activity in EDL muscles from WT and AMPK KD mice with basal incubation or when stimulated to contract for 10 min in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) (A), and nNOS expression in EDL and soleus muscles from WT and AMPK KD mice (B). Data are means ± SEM, n=6-8 per group. *P<0.05 vs basal group of same genotype, #P<0.05 vs contraction of the same genotype, §P<0.05 for genotype effect, †P<0.05 vs EDL. Presented blots are representative. For representative blots; KD= AMPK KD mice.

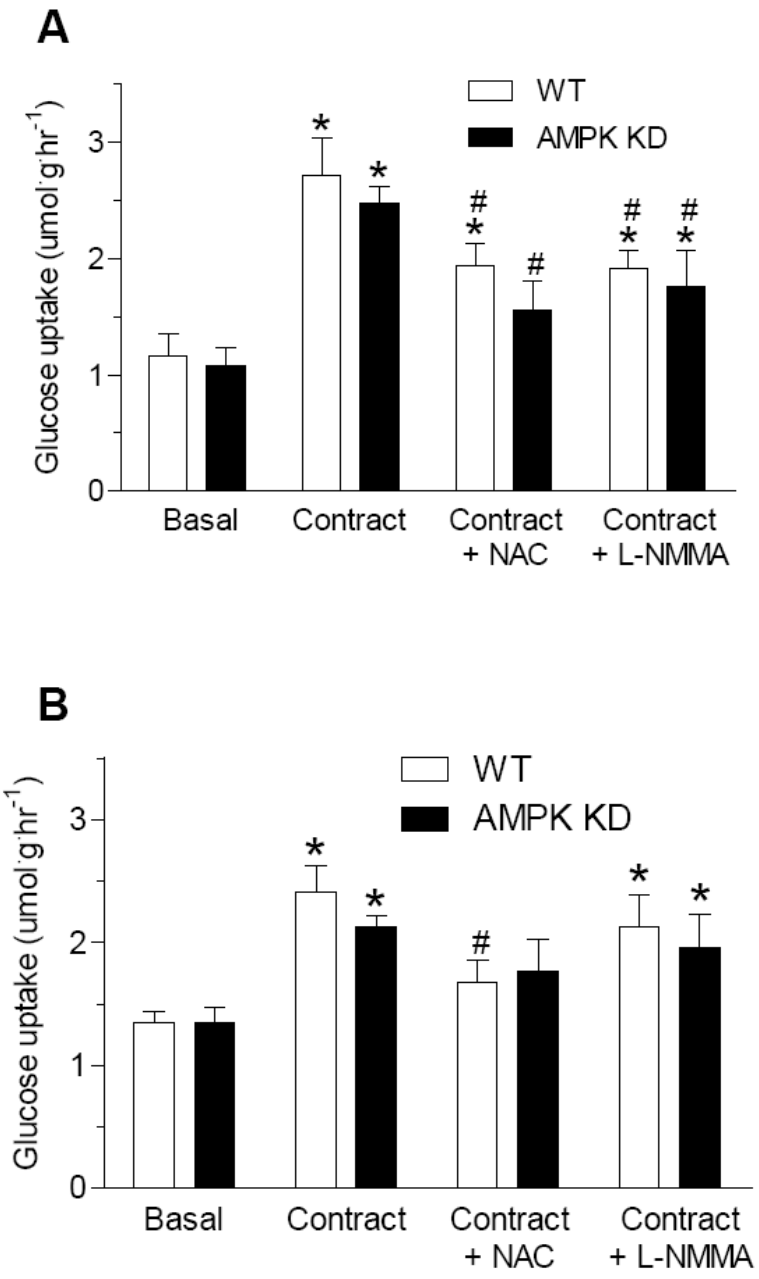

Figure 3.11 Glucose uptake in EDL (A) and soleus (B) muscles from WT and AMPK KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA) and N-acetylcysteine (NAC). Data are means ± SEM, n=6-12 per group, *P<0.05 vs basal group of the same genotype, #P<0.05 vs contraction of the same genotype.

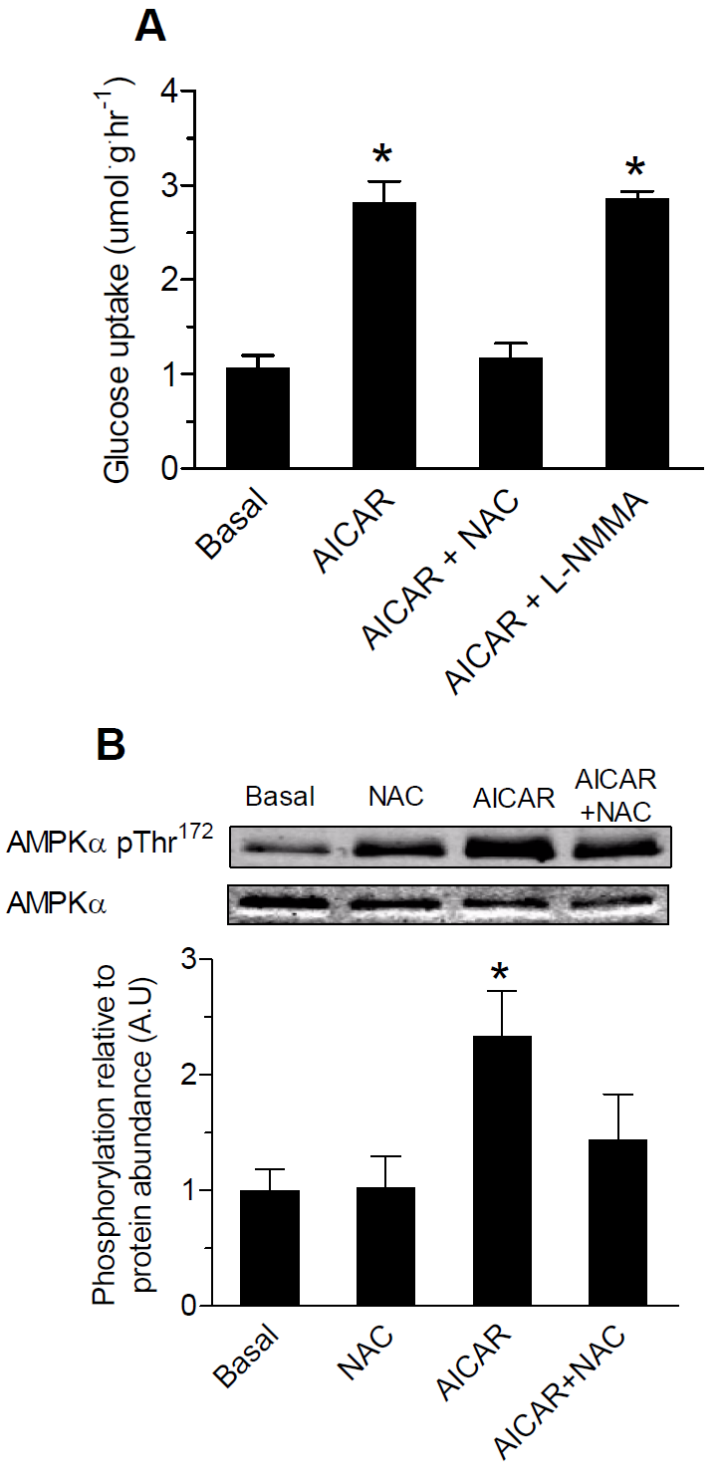

Figure 3.12 Glucose uptake (A) and AMPKα Thr^{172} phosphorylation (B) in EDL muscles from C57Bl/6 mice when incubated for 40 min in the presence and absence of N-acetylcysteine (NAC), 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) , NAC + AICAR or AICAR + N^G-monomethyl-L-Arginine (L-NMMA). Data are means ± SEM, n=4-6 per group. Presented blots are representative. *P<0.05 vs basal.

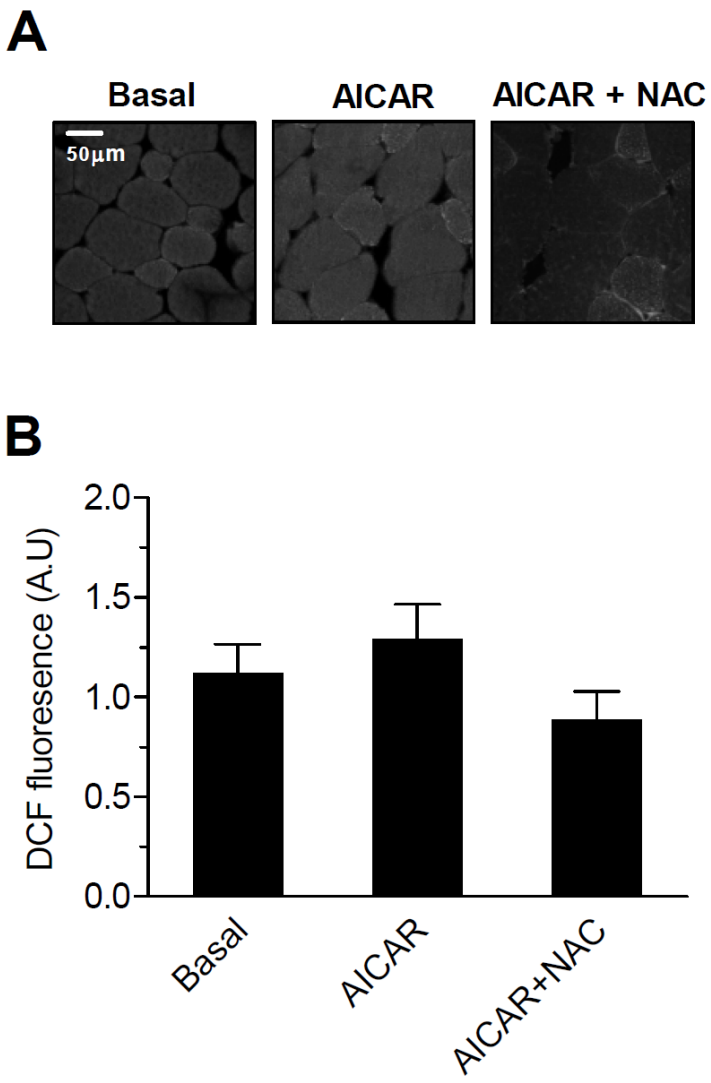

Figure 3.13 Representative images (A) and emission intensity (B) of DCF fluorescence in cross-sections of EDL muscles from C57Bl/6 mice when incubated for 40 min in the presence and absence of 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) or N-acetylcysteine (NAC) + AICAR. Data are means ± SEM, n=4-6 per group.

3.4 DISCUSSION

The most important finding of this study was that ROS and NO are involved in regulating glucose uptake during contraction via a mechanism that is independent of AMPK. In addition, we also provide some preliminary evidence to suggest that ROS, but not NO, may be required for AICAR-stimulated increases in resting skeletal muscle AMPK phosphorylation and glucose uptake.

As reported previously (Dzamko *et al.*, 2008; Lefort *et al.*, 2008), AMPK KD mice have reduced AMPKα1 and α2 activity in EDL muscles, and unlike WT mice, AMPK KD mice show no increases in AMPKα2 activity in EDL muscles during contraction (Figure 3.4). Despite this, glucose uptake increased to a similar extent with contraction in the EDL and soleus muscles of WT and AMPK KD mice (Figure 3.11). Previously, isolated muscles from AMPK KD mice have been reported to have a slight but significant attenuation of the increase in glucose uptake during contraction (Mu *et al.*, 2001; Jensen *et al.*, 2007b; Lefort *et al.*, 2008). However, when the force produced by muscles from WT mice, which is greater than that in muscles from AMPK KD mice at high stimulation frequencies (≥75 Hz) (Lefort *et al.*, 2008), is matched to the force of muscles from AMPK KD mice by reducing the stimulation voltage, muscles from AMPK KD mice have similar increases in glucose uptake during contraction as muscles from WT mice (Fujii *et al.*, 2005). Despite a similar workload, reducing stimulation voltage in muscles of WT mice may alter muscle fibre recruitment and signalling, thus potentially reducing glucose uptake in itself (Sandstrom *et al.*, 2007; Lefort *et al.*, 2008). Furthermore, recent evidence suggests that skeletal muscle contraction force is not necessarily synonymous with glucose uptake (Sandstrom *et al.*, 2007).

In the present study we employed a more physiologically relevant contraction protocol than used previously (Hayashi *et al.*, 1998; Higaki *et al.*, 2001; Jorgensen *et al.*, 2004; Sandstrom *et al.*, 2006), specifically by employing lower stimulation frequencies, train durations and contraction rates. In agreement with findings of Lefort *et al.* (2008), we found no difference in force production between muscles of AMPK KD and WT mice (Figures 3.1 and 3.2) using a low (60 Hz) frequency stimulation. In contrast to our finding that muscles from AMPK KD and WT mice had similar increases in glucose uptake during contraction, Lefort *et al.* (2008) reported that contraction-stimulated glucose uptake was reduced by ~50% in muscles from

AMPK KD mice. It is difficult to discern the reason for the discrepancy between our findings and those of Lefort *et al.* (2008), but it may be attributed to differences in the contraction protocols. Lefort *et al.* (2008) stimulated muscles at a higher intensity for a shorter duration at one train per second for 2 min, compared with our protocol of 12 contractions per minute for 10 min. This is consistent with the fact that AMPK activation is higher during more intense contractions/exercise (Ihlemann *et al.*, 1999b; Chen *et al.*, 2003) and therefore may play a greater role in regulating glucose uptake during exercise at higher intensities.

As reported previously (Reid *et al.*, 1992a; Reid *et al.*, 1992b; Sandstrom *et al.*, 2006), we show that skeletal muscle oxidant levels are increased during contraction and this increase is prevented by the non-specific antioxidant NAC (Figure 3.8). As discussed in Chapter 2 (*2.1.5 Oxidant levels assay*), we measured DCF fluorescence as a marker of oxidant levels in cross-sections of EDL muscles. Although DCFH can be oxidised to DCF by both reactive nitrogen species (Murrant *et al.*, 1999) and ROS (Murrant & Reid, 2001), because NAC prevented contraction induced increases in DCF we are confident of this method as a marker of muscle ROS levels. Furthermore, oxidative stress promotes the addition of reduced glutathione to protein residuals (S-glutathionylation) (Dalle-Donne *et al.*, 2009) and, indeed, protein S-glutathionylation has been shown to increase during exhaustive exercise in rats, and this increase is prevented with antioxidant treatment (allopurinol, a xanthine oxidase inhibitor) (Gomez-Cabrera *et al.*, 2005). In agreement, we show that contraction-stimulated increases in S-glutathionylation of protein bands at ~37 kDa in EDL muscles of AMPK KD, and ~270 kDa and 37 kDa in EDL muscles of WT mice, and this increase was prevented by NAC (Figure 3.9). Experiments to identify the nature of the proteins that are S-glutathionylated during contraction were preformed and are reported in Chapter 4. Regardless, these S-glutathionylation results provide further evidence that our contraction protocol increased muscle ROS levels, and this increase was prevented by NAC.

In addition to reducing contraction-stimulated increases in oxidative stress, NAC attenuated the increase in glucose uptake during contraction (Figure 3.11A) of glycolytic (EDL) muscles from WT mice to a similar extent as reported previously (Sandstrom *et al.*, 2006). Sandstrom *et al.* (2006) additionally reported that NAC attenuated increases in AMPK activity and AMPK phosphorylation during contraction, suggestive of a causative relationship between ROS, AMPK activation and glucose uptake during contraction. In the present study we show that in addition to NAC attenuating increases in glycolytic muscle glucose uptake during

contraction, NAC also prevented increases in glucose uptake during contraction in oxidative (soleus) muscles (Figure 3.11B). Furthermore, we found that NAC attenuated increases in skeletal muscle glucose uptake during contraction even in the absence of any increases in skeletal muscle AMPKα1 or α2 activity during contraction (Figure 3.4). Therefore, our findings suggest that ROS are involved in regulating glucose uptake in both glycolytic and oxidative muscles independent of AMPK.

In support of this finding, recent studies have reported that exogenous ROS can increase glucose uptake in isolated glycolytic and oxidative skeletal muscles without activating AMPK (Higaki *et al.*, 2008; Jensen *et al.*, 2008). Higaki *et al.* (2008) showed that the PI3K inhibitor, wortmannin, prevented exogenous H_2O_2-stimulated skeletal muscle glucose uptake in resting muscle. However, contraction increases skeletal muscle glucose uptake via a PI3K-independent pathway (Yeh *et al.* 1995). Therefore, it is likely that during contraction ROS regulate skeletal muscle glucose uptake through a pathway that differs from that of exogenous H_2O_2. Interestingly, ROS appear to regulate p38 MAPK (p38) phosphorylation during exercise (Gomez-Cabrera *et al.*, 2005), and p38 has been implicated in the regulation of skeletal muscle contraction-stimulated glucose uptake (Somwar *et al.*, 2000). Furthermore, ROS have been proposed to increase stretch-stimulated skeletal muscle glucose uptake via p38 MAPK (Chambers *et al.*, 2009). Given the numerous pathways beginning to be recognised as being redox-sensitive (Jackson, 2008), it is important to investigate whether ROS regulate skeletal muscle glucose uptake during contraction via p38 MAPK or alternative pathways. Alternative pathways are investigated in Chapter 4.

As mentioned above, in contrast to Sandstrom *et al.* (2006), we found that NAC did not affect the increase in muscle AMPK Thr^{172} phosphorylation during contraction. The reason for this discrepancy is difficult to discern, but it could be attributed to minor differences in methodology such as the incubation temperature (25°C (Sandstrom *et al.*, 2006) vs 30°C used in the present study), longer NAC pre-incubation period (60 min (Sandstrom *et al.*, 2006) vs 30 min used in the present study) and differences in contraction protocol as discussed above. Regardless, our finding that NAC reduced glucose uptake did not affect AMPK Thr^{172} phosphorylation during contraction in muscles from WT mice supports the involvement of ROS in regulating skeletal muscle contraction-stimulated glucose uptake independent of AMPK. In further support, increases in ACCβ phosphorylation, the major downstream target of AMPK, during contraction were not affected by NAC in EDL muscles of both genotypes,

and only showed a minor reduction in soleus muscles from WT mice. Interestingly, we found that despite muscles from AMPK KD showing no increases in AMPK activity or AMPK Thr^{172} phosphorylation during contraction, ACCβ Ser^{221} phosphorylation was increased in the EDL and tended to be increased in soleus muscle of AMPK KD mice during contraction (Figure 3.6). This supports the recent finding by Dzamko *et al.* (2008) that a kinase other than AMPK is likely to be phosphorylating ACCβ during contraction.

The role of NO in the regulation of skeletal muscle glucose uptake during contraction is controversial, with some studies reporting that inhibition of NOS during contraction attenuates the increases in glucose uptake (Balon & Nadler, 1997; Roberts *et al.*, 1997; Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Ross *et al.*, 2007) while others report no effect (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002). These differences are also likely attributed to inconsistencies in methodology such as the measurement of glucose uptake and the inhibition of NOS taking place *following* rather than *during* contraction (for discussion see McConell & Kingwell, 2006). In the present study, L-NMMA treatment attenuated the increase in glucose uptake during contraction in EDL, but not soleus muscles of AMPK KD and WT mice (Figure 3.11). Since NOS activity and glucose uptake in EDL muscles of WT and AMPK KD mice was increased with contraction, and L-NMMA prevented these increases (Figure 3.10A), this suggests that NO is involved in the regulation of skeletal muscle glucose uptake during contraction *ex vivo*, independent of AMPK, in muscles with a higher proportion of glycolytic fibres. This was supported by the finding that L-NMMA also did not affect contraction-stimulated increases in AMPK Thr^{172} or ACCβ Ser^{221} phosphorylation in EDL muscles (Figure 3.5 and 3.6). nNOS protein abundance was similar between genotypes, and lower in the soleus than in EDL muscles (Figure 3.10B), consistent with reports that soleus muscles produce less NO than EDL muscles during contraction (Kobzik *et al.*, 1994). This may explain why L-NMMA did not affect glucose uptake during contraction in the soleus muscles of WT or AMPK KD mice, and suggests that NO plays a greater role in regulating glucose uptake during contraction in glycolytic vs. oxidative muscles.

Interestingly, and as reported previously (Lee-Young *et al.*, 2009), EDL muscles from AMPK KD mice showed ~30% lower NOS activity during contraction than EDL muscles from WT mice. This supports evidence that AMPK phosphorylates NOS (Chen *et al.*, 1999) and suggests that the phosphorylation of NOS by AMPK increases its activity. Interestingly, the

lower absolute NOS activity in EDL muscles of AMPK KD mice (Figure 3.10) did not affect glucose uptake during contraction. This suggests that because muscles from AMPK KD and WT mice showed similar increases in NOS activity and glucose uptake during contraction (Figure 3.10 and 3.11), it may be that the contraction-stimulated increase in NO production from basal, rather than the absolute NO concentration, is more important in regulating glucose uptake during contraction. Alternatively, a threshold level of NO may need to be reached during contraction to activate glucose uptake, with further increases in NO levels not being able to increase glucose uptake further. As discussed above, it has been reported previously that during intense stimulation protocols *ex vivo,* muscles of AMPK KD mice have attenuated increases in glucose uptake during contraction compared with muscles from WT mice (Mu *et al.*, 2001; Jensen *et al.*, 2007b). Therefore, whether this lower glucose uptake is a result of lower AMPK or lower NOS activity requires further examination.

Since both AS160 and TBC1D1 are Akt substrates that have been suggested to facilitate the translocation of GLUT4 and act as a convergence point between contraction and insulin stimulated glucose uptake (Funai & Cartee, 2008, 2009), we measured PAS phosphorylation at ~160 kDa. As discussed in Chapter 2 (*2.6.3 Western blots*) PAS recognises Akt phosphorylation sites of both TBC1D1 and AS160, and both these proteins appear around 150-160 kDa. Since we cannot be certain whether TBC1D1 or AS160, or a combination of both is responsible for PAS-phosphorylation we report it simply as PAS-160. Nevertheless, we found that PAS-160 phosphorylation was not increased following 10 min of contraction (Figure 3.7), a finding that is in contrast with some (Bruss *et al.*, 2005; Kramer *et al.*, 2006a) but not other (Treebak *et al.*, 2007; Jensen *et al.*, 2008) studies. During moderate intensity exercise in humans, PAS-160 phosphorylation does not increase until 60 min of exercise (Treebak *et al.*, 2006), and low-intensity twitch contractions in mouse soleus muscles *ex vivo* does not increase PAS-160 phosphorylation (Jensen *et al.*, 2008). Therefore, since our contraction protocol was less strenuous than some (see above) it is possible that our protocol did not provide a sufficient stimulus to increase PAS-160 phosphorylation within 10 min. Regardless, our results indicate that PAS-160 phosphorylation may not be required for normal increases in glucose uptake during contraction of mouse skeletal muscle *ex vivo* since contraction increased glucose uptake without increasing PAS-160 phosphorylation. This is supported by the observation that the PI3K inhibitor, wortmannin, abolishes PAS-160 phosphorylation during skeletal muscle contraction without affecting glucose uptake (Bruss *et al.*, 2005; Kramer *et al.*, 2006a). Since PAS-160 only recognises Akt phosphorylation sites

(Taylor *et al.*, 2008), it is important to now investigate whether other phosphorylation sites of TBC1D1 or AS160 are involved in the regulation of glucose uptake during contraction, and are sensitive to signalling by NO and/or ROS. Consistent with previous observations (Kramer *et al.*, 2006a), we also report that EDL muscles from AMPK KD mice had lower PAS-160 phosphorylation than muscles from WT mice. This further supports recent findings that AMPK phosphorylates AS160 and TBC1D1 (Pehmoller *et al.*, 2009; Treebak *et al.*, 2009b).

To further investigate the role of ROS and NO in regulating AMPK activation and glucose uptake, we examined the affects of NAC and L-NMMA on AICAR-stimulated glucose uptake in skeletal muscle from C57Bl/6 mice. Interestingly, AICAR-stimulated EDL muscle AMPK phosphorylation and glucose uptake were prevented by NAC. Although AICAR-stimulation did not increase basal EDL oxidant levels, NAC tended to reduce AICAR oxidant levels (Figure 3.13). It is possible that basal ROS levels may be required for AICAR-stimulated AMPK phosphorylation and glucose uptake, but NO is not involved. This is in contrast to a previous report by Sandstrom *et al.* (2006) that NAC does not affect skeletal muscle AICAR-stimulated glucose uptake. This is likely the result of treatment order, since Sandstrom *et al.* (2006) incubated muscle in the presence of NAC for 30 min prior to the addition of AICAR for 80 min, we treated muscle with the combination of NAC and AICAR for 40 min. Therefore, the relationship between AICAR-activation of AMPK and ROS requires further attention. However, it is important to acknowledge that the mechanisms through which AICAR activates AMPK and stimulates glucose uptake are considerably different to that of contraction. Indeed, AMPKα2 knockout mice have abolished AICAR- but normal contraction-stimulated glucose uptake (Jorgensen *et al.*, 2004). Therefore care must be taken when applying activators, donors or exogenous stimuli in an attempt to simulate contraction conditions and signalling pathways.

In conclusion, this study provides evidence that ROS and NO are involved in regulating skeletal muscle glucose uptake during contraction, independent of AMPK. In addition, our findings provide further evidence that AMPK activity is not essential for normal increases in glucose uptake during skeletal muscle contraction.

CHAPTER FOUR

DOWNSTREAM MECHANISMS OF NITRIC OXIDE MEDIATED SKELETAL MUSCLE GLUCOSE UPTAKE DURING CONTRACTION

4.1 INTRODUCTION

Our group have shown that NOS inhibition attenuates increases in skeletal muscle glucose uptake during exercise in humans (Bradley *et al.*, 1999; Kingwell *et al.*, 2002), during *in situ* contractions in rats (Ross *et al.*, 2007) and during *ex vivo* contractions in mice (Figure 3.11). Although NO has also been shown to be involved in the regulation of contraction-stimulated skeletal muscle glucose uptake/transport in other rodent studies (Balon & Nadler, 1997; Roberts *et al.*, 1997), this is an equivocal finding (Etgen *et al.*, 1997; Higaki *et al.*, 2001; Rottman *et al.*, 2002). The contradictory results are likely due to methodological inconsistencies (for discussion see McConell & Kingwell, 2006), particularly the examination of glucose uptake up to 20 min *following*, rather than during contraction (Etgen *et al.*, 1997; Higaki *et al.*, 2001). Like NO, the attenuation of contraction-stimulated increases in skeletal muscle ROS levels with antioxidants attenuates increases in glucose uptake during contraction (Figure 3.11; Sandstrom *et al.*, 2006). This suggests that NO and ROS are both involved in the regulation of skeletal muscle glucose uptake during contractions. Given that the origins, chemistry, metabolism and biological actions of NO and ROS are highly interrelated (Brown & Borutaite, 2006), for example NO and superoxide ($O_2^{\bullet -}$) interact to form peroxynitrite ($ONOO^-$), it is possible that they may interact to increase skeletal muscle glucose uptake during contraction.

Studies investigating the mechanism(s) through which NO stimulates glucose uptake have focused largely on NO donor-stimulated glucose uptake in resting skeletal muscle (Young *et al.*, 1997; Young & Leighton, 1998a, b; Toyoda *et al.*, 2004; Higaki *et al.*, 2008; Jensen *et al.*, 2008), rather than NO produced endogenously during contraction. The prevention of NO donor-mediated increases in cyclic guanosine monophosphate (cGMP) formation (through the inhibition of guanylate cyclase (GC)) and cGMP-dependent protein kinase (PKG) activity,

prevents NO donor-stimulated increases in resting (basal) skeletal muscle glucose uptake (Young *et al.*, 1997). Similarly, the cGMP analog, 8-bromo-cGMP, can increase resting skeletal muscle glucose uptake (Young & Leighton, 1998a), suggesting that NO mediates glucose uptake in skeletal muscle via the traditional/classic (Denninger & Marletta, 1999) NO-cGMP/PKG signaling pathway (see Figure 1.2). Like NO-donors, contraction-stimulated increases in skeletal muscle NO production elevates muscle cGMP concentration (Lau *et al.*, 2000), and therefore cGMP is likely to contribute to the regulation of skeletal muscle glucose uptake during contraction.

However, NO can often exert its effects independently of the cGMP-PKG signalling pathway, via various post-translational signalling events such as the addition of a NO group (S-nitrosylation) or glutathione (S-glutathionylation) to cysteine sulfhydryls on proteins, or peroxynitrite signalling through nitration of protein tyrosine residuals (tyrosine nitration) or tyrosine phosphorylation (Stamler & Meissner, 2001; Zou *et al.*, 2002; Dalle-Donne *et al.*, 2009). Importantly all of these signalling events require, or have the potential to be modulated by ROS (Brown & Borutaite, 2006; Martinez-Ruiz & Lamas, 2007). Therefore, it is important to determine whether NO produced endogenous during contraction stimulates skeletal muscle glucose uptake through a pathway similar to that of NO-donors in resting muscle (cGMP/PKG) or through alternative mechanism(s). Furthermore, whether reactive oxygen and nitrogen (such as NO) species regulate skeletal muscle glucose uptake through a similar pathway during contraction requires examination.

Therefore, the purpose of this study was to investigate the mechanism(s) through which NO regulates glucose uptake during skeletal muscle contractions *ex vivo*. We tested the hypothesis that NO increases skeletal muscle glucose uptake during contractions via cGMP-dependent and cGMP-independent pathways that involve signalling through interaction with ROS.

4.2 METHODS

4.2.1 Inhibitors and scavengers

Isolated EDL muscles from mice were treated with the following inhibitors and scavengers *ex vivo*. To prevent NO production and reduce ROS levels in muscle the NOS inhibitor N^G-monomethyl-L-Arginine (L-NMMA; 100 µM (Higaki *et al.*, 2001)), and the non-specific antioxidant N-acetylcysteine (NAC; 20 mM, (Toyoda *et al.*, 2004; Sandstrom *et al.*, 2006)) were used, respectively. The GC inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ) was used to prevent the formation of cGMP (10 µM (Garthwaite *et al.*, 1995)), and PKG activity was inhibited with Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt (Rp-8-Br-PET-cGMPS, 5 µM (Dhanakoti *et al.*, 2000)). The antioxidants dithiothreitol (DTT, 10 mM (Aracena *et al.*, 2003)), and urate (10 mM (Reiter *et al.*, 2000; Sawa *et al.*, 2000)) were used to reduce thiols and scavenge peroxynitrite, respectively. To break S-nitrosylated bonds (Borutaite *et al.*, 2000; Benhar *et al.*, 2009), a dissection lamp emitting white light was placed 10 cm from incubating muscle (Novaflex, world precision instruments, Sarasota, FL, USA)

4.2.2 Experimental procedure

As described in Chapter 2 (*2.1 Ex vivo mouse studies*) C57Bl/6 mice aged 12-14 weeks were anaesthetised and hindlimb EDL muscles were excised and incubated *ex vivo.* EDL muscles were used in this study because it was shown in Chapter 3 (Figure 3.11) that NO plays a greater role in regulating glucose uptake during contraction in muscles containing predominantly glycolytic fibres. Muscle optimal length was determined then muscles were incubated for 40 min in the presence or absence of L-NMMA, NAC, L-NMMA+NAC, ODQ, Rp-8-Br-PET-cGMPS, DTT, urate or white light. Non-contracted (resting) muscles were incubated in the presence or absence of the NO-donor diethylenetriamine/NO (Deta/NO, 500µM), Deta/NO+ODQ, L-NMMA, NAC, DTT, urate, or peroxynitrite ($ONOO^-$; 500 µM), for 40 min. Deta/NO was used as a NO donor as it releases NO slower than other NO-donors like SNP (Artz & Thatcher, 1998), and 500 µM was found to be the lowest concentration that simulated skeletal muscle glucose uptake (data not shown). All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne, and conformed to the Australian code of practice for the care and use of animals for scientific purposes, as described by the National Health and Medical Research Council (Australia).

4.2.3 Muscle contraction

Following 30 min of incubation at rest, contracted muscles were stimulated for 10 min (pulse durations: 600 ms at a frequency of 60 Hz for 25 contractions·min^{-1}). This stimulation protocol has been shown previously to be sufficient to recruit all motor units within the contracting muscle (Lynch *et al.*, 2001). Non-contracted muscles were treated the same as contracted muscles except that they were not stimulated during the contraction period.

4.2.4 Analytical techniques

Muscle 2-deoxy-D-glucose (2-DG) glucose uptake was measured during the final 5 min of contraction and over 5 min of recovery in contracted muscles (Jorgensen *et al.*, 2004), and during the final 10 min of incubation in non-contracted (resting) muscles as described in Chapter 2 (*2.1 Ex vivo mouse studies*). Separate muscles were snap frozen in liquid nitrogen immediately following incubation/contraction and analysed for AMPKα Thr^{172} phosphorylation, ACCβ Ser^{221} phosphorylation, PAS-160 phosphorylation, nNOSμ Ser^{1451} phosphorylation, p38 MAPK Thr^{180}/Tyr^{182} phosphorylation, Akt Ser^{473} phosphorylation, S-glutathionylation, tyrosine nitration, oxidant levels and NOS activity. The methodology used for these analytical procedures is described in detail in Chapter 2.

4.2.5 Mass spectrometry

The nature of protein bands S-glutathionylated during contraction was investigated using mass spectrometry. The tibialis anterior muscle from C57Bl/6 mice were incubated for 10 min in 5 mM H_2O_2, extracted using the S-glutathionylation extraction method described in Chapter 2 (*2.6.3 Western blots*) and protein was separated using SDS-PAGE. The gel was then stained with coomassie dye, destained, and protein bands corresponding to the molecular weight of protein bands that were S-glutathionylated during contraction were cut out. The bands were digested and subjected to mass spectrometry analysis to identify peptide sequences in the band. Proteins were identified from peptide sequences using NCBI blast search. Mass spectrometry protein identification was performed Dr Rohan Steel at St. Vincent's Institute, Fitzroy, Victoria, Australia.

4.2.6 Statistical analysis

All data are expressed as means ± SEM. Results were analysed using SPSS statistical package using one-factor ANOVA (treatment) and one-factor repeated measures ANOVA for contraction forces (treatment and time). If the ANOVA revealed a significant interaction,

specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

4.3 RESULTS

4.3.1 Muscle contraction

Treatment of muscles with the peroxynitrite scavenger, urate, reduced initial peak tetanic force (normalised to muscle mass) by ~37% ($P<0.05$; Figure 4.1B). Initial peak force was not affected by any other treatment ($P>0.05$; Figure 4.1A and B). Peak force was obtained within the first 10 s of the contraction protocol and declined thereafter ($P<0.001$; Figure 4.2A and B). The rate of decline from peak force was not affected by any treatment (Figure 4.2A and B).

4.3.2 Oxidant levels and NOS activity

Oxidant levels in muscles were increased ~95% with contraction (Figure 4.3A and B). Urate tended ($P=0.06$) to attenuate this increase (Figure 4.3B), and the non-specific antioxidant, NAC, and the thiol reducing agent, DTT, attenuated the contraction-stimulated increase in oxidant activity ($P>0.05$ vs basal; Figure 4.3B). Contraction increased ($P<0.05$) NOS activity in muscles by ~40% and the NOS inhibitor, L-NMMA, reduced ($P<0.05$) NOS activity during contraction to ~35% of basal activity (Figure 4.3C).

4.3.3 Contraction-stimulated and Deta/NO-stimulated glucose uptake

Contraction increased muscle glucose uptake by 1.8-2.3-fold ($P<0.05$, Figure 4.4, 4.5 and 4.6). NAC and L-NMMA attenuated the increase in glucose uptake during contraction, similarly, by ~50% ($P<0.05$). The co-treatment of L-NMMA and NAC did not have an additive effect on the magnitude of the reduction in glucose uptake during contraction (Figure 4.4). The GC inhibitor, ODQ, which prevents cGMP formation at concentration used (10 µM) (Garthwaite *et al.*, 1995), prevented the 1.5-fold increase ($P=0.01$) in glucose uptake stimulated by the NO-donor Deta/NO (Figure 4.5A), but did not affect contraction-stimulated muscle glucose uptake (Figure 4.5B). Furthermore, muscle glucose uptake during contraction was not affected by the PKG inhibitor, Rp-8-Br-PET-cGMPS (Figure 4.5C). White light treatment, which breaks S-nitrosylation bonds (Borutaite *et al.*, 2000; Benhar *et al.*, 2009), did not affect contraction-stimulated increases in muscle glucose uptake (Figure 4.6). DTT and urate attenuated ($P<0.05$) the increase in muscle glucose uptake during contraction by ~70% and ~40%, respectively (Figure 4.6).

4.3.4 S-glutathionylation and tyrosine nitration

Contraction increased S-glutathionylation of protein bands at ~270 kDa and ~37 kDa by 1.8-, and 1.5-fold ($P<0.05$, Figure 4.7A), respectively. The contraction-induced increase in S-glutathionylation at ~270 kDa was increased 2.4-fold by urate ($P<0.001$), but this increase was prevented by NAC, NAC+L-NMMA and white light, and reduced to almost zero with DTT ($P<0.02$ vs basal; Figure 4.7A). L-NMMA, NAC, L-NMMA+NAC, DDT and urate attenuated the contraction-stimulated increase in S-glutathionylation at ~37 kDa ($P<0.05$; Figure 4.7A). Contraction increased tyrosine nitration of a protein band at ~37 kDa by 1.7-fold ($P=0.01$), and this increase was prevented by L-NMMA, NAC, L-NMMA+NAC, DDT and urate (Figure 4.7B).

Tibialis anterior muscles were treated with H_2O_2 and protein bands that corresponded with the S-glutathionylated bands at ~270 kDa and ~37 kDa were subjected to mass spectrometry analysis to identify the nature of the proteins in these bands. The most abundant proteins found in the protein band at ~270 kDa were the contractile protein myosin heavy chain polypeptide 4 (Myh4) and sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase 1 (SERCA1). Given that SERCA is already recognised as a target for S-glutathionylation in vascular smooth muscle (Adachi *et al.*, 2004) it is likely to be the protein S-glutathionylated at 270 kDa. Indeed, in Chapter 6, a protein band at ~270 kDa was found to be S-glutathionylated in human muscle, and probing membranes with a SERCA1 antibody showed SERCA1 was present at this molecular weight (see Figure 6.8). It is important to note that SERCA1 is only 110 kDa and since SERCA1 was found here to be present at ~270 kDa it suggests SERCA is a diamer and/or forming a heterodimer with another protein, possibly Myh4, to be present at 270 kDa.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was in the protein band at ~37 kDa, making it likely to be the protein S-glutathionylated at ~37 kDa. Indeed, exogenous ROS have been shown to promote S-glutathionylation of GAPDH (Cotgreave *et al.*, 2002; Rodriguez-Pascual *et al.*, 2008).

4.3.5 AMPK, ACCβ, nNOS, PAS-160, p38 MAPK phosphorylation

Contraction increased muscle AMPKα Thr^{172} phosphorylation 4.5-fold ($P=0.004$), and this increase was not significantly affected by any treatment (Figure 4.8A). The ~2-fold contraction-stimulated increase ($P=0.02$) in ACCβ Ser^{221} phosphorylation was attenuated by

urate but not significantly affected by any other treatment (Figure 4.8B). The ~3-fold increase ($P<0.001$) in muscle nNOS Ser^{1451} phosphorylation during contraction was not significantly affected by any treatment (Figure 4.9A). PAS-160 phosphorylation was not increased by contraction or any other treatment (Figure 4.9B). p38 MAPK Thr^{180}/Tyr^{182} phosphorylation was increased ~3-fold ($P=0.04$) with contraction and this increase was prevented by white light and urate, but not affected by any other treatment (Figure 4.10).

4.3.6 Basal glucose uptake, resting tension, change in contraction stimulated glucose uptake from basal, and AMPK signalling

Resting (basal) muscle glucose uptake was reduced ~25% by NAC ($P<0.05$) (Figure 4.11A) and this was independent of Akt Ser^{473} phosphorylation (Figure 4.12B). Although NAC reduced basal glucose uptake, the delta glucose uptake for NAC (the subtraction of basal+NAC glucose uptake from contraction + NAC glucose uptake) shows that NAC was attenuating the increase in glucose uptake during contraction independent of its affects on basal glucose uptake (Figure 4.11C). Neither L-NMMA nor DTT affected resting muscle glucose uptake ($P>0.05$; Figure 4.11A). Urate increased ($P<0.05$) resting (passive) muscle tension (from 1 min; Figure 4.11B) and this was accompanied by a ~3-fold and ~1.8-fold increase ($P<0.05$) in resting muscle AMPKα Thr^{172} phosphorylation (Figure 4.12A) and glucose uptake ($P<0.001$; Figure 4.11A), respectively. The delta glucose uptake for basal+Urate and contraction+Urate showed that urate prevented any increase in glucose uptake during contraction (Figure 4.11C). There was a slight but significant increase ($P<0.05$) in passive tension with DTT treatment which appeared to be transient (Figure 4.11B). Exogenous $ONOO^-$ treatment increased resting muscle AMPK Thr^{172} phosphorylation ~3-fold ($P=0.001$; Figure 4.12A) without affecting muscle glucose uptake (Figure 4.11A).

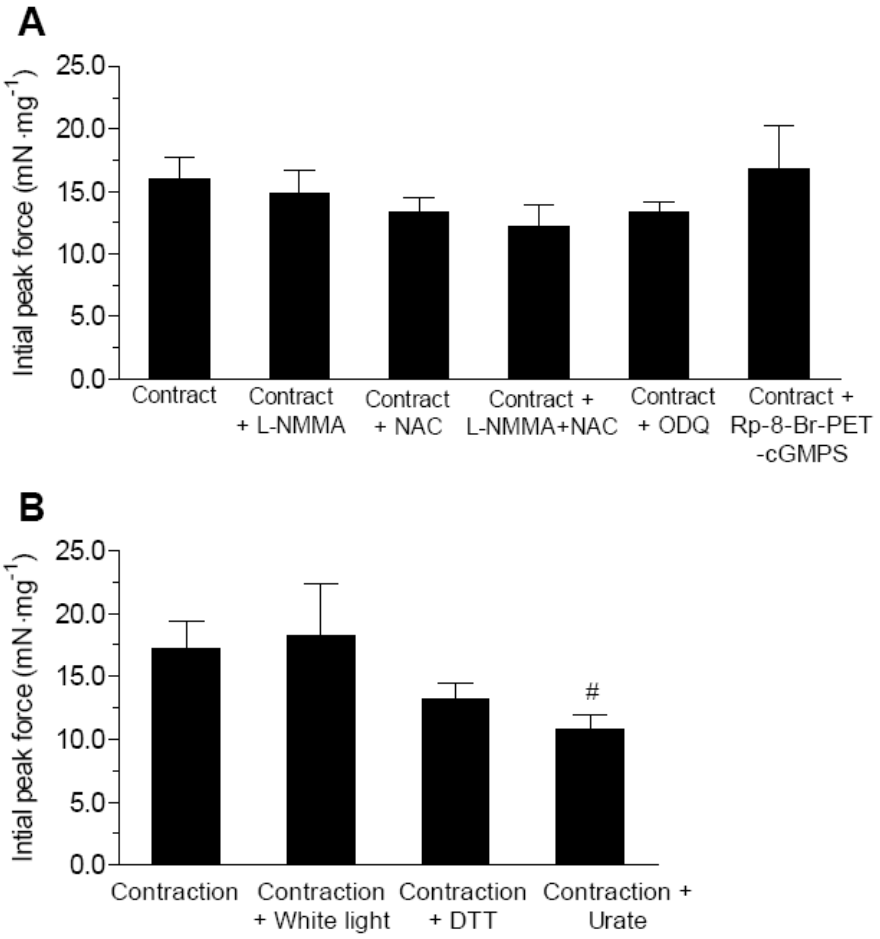

Figure 4.1 Initial peak contraction force of isolated EDL muscles in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), L-NMMA+NAC, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt (Rp-8-Br-PET-cGMPS) (A), white light, dithiothreitol (DTT) or urate (B). Data are means ± SEM; n=6-12 per group. #P<0.05 vs. contraction.

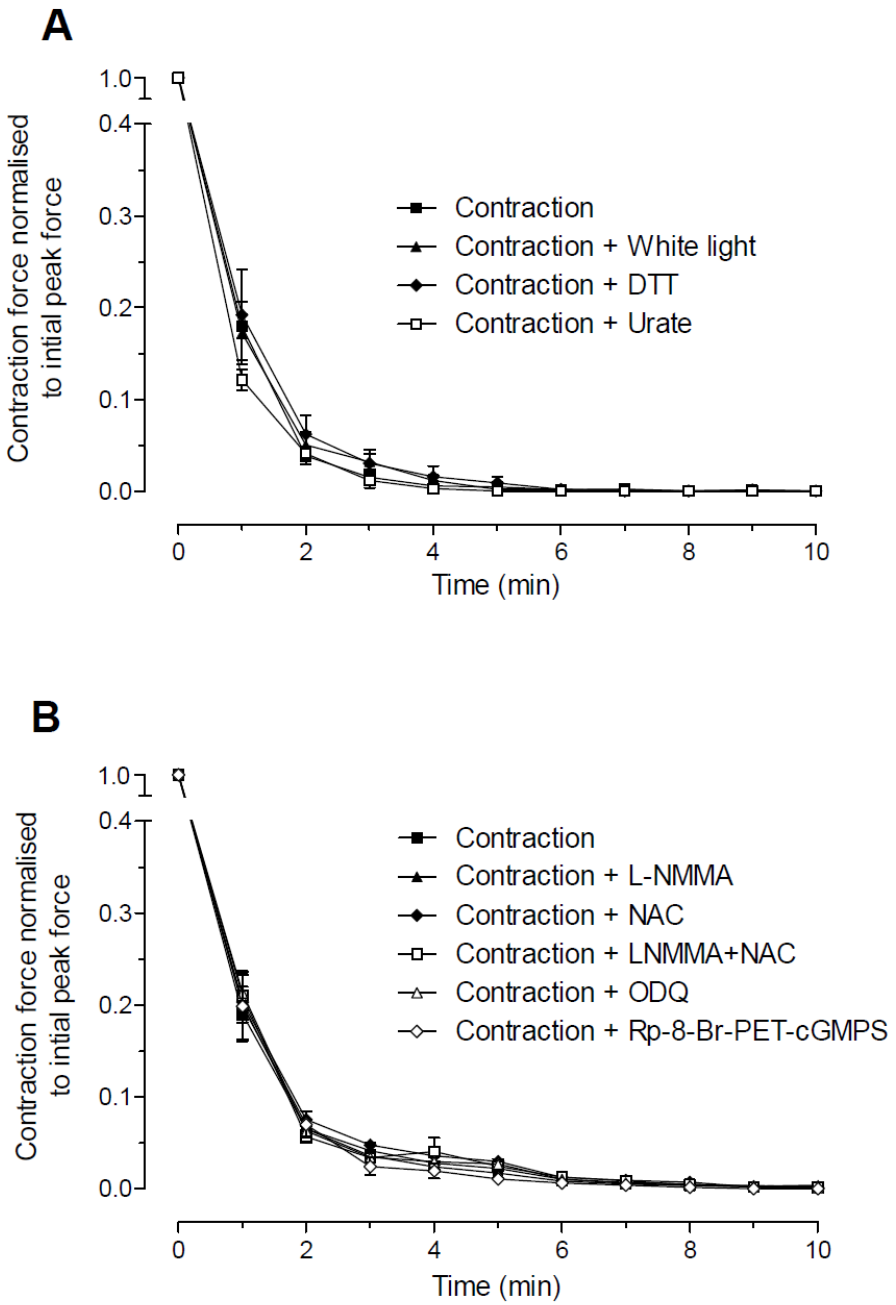

Figure 4.2 Drop in EDL muscle force production from the initial peak force during each minute of the 10 min contraction protocol in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), L-NMMA+NAC, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt (Rp-8-Br-PET-cGMPS) (A), white light, dithiothreitol (DTT) or urate (B). Data are means ± SEM; n=6-12 per group.

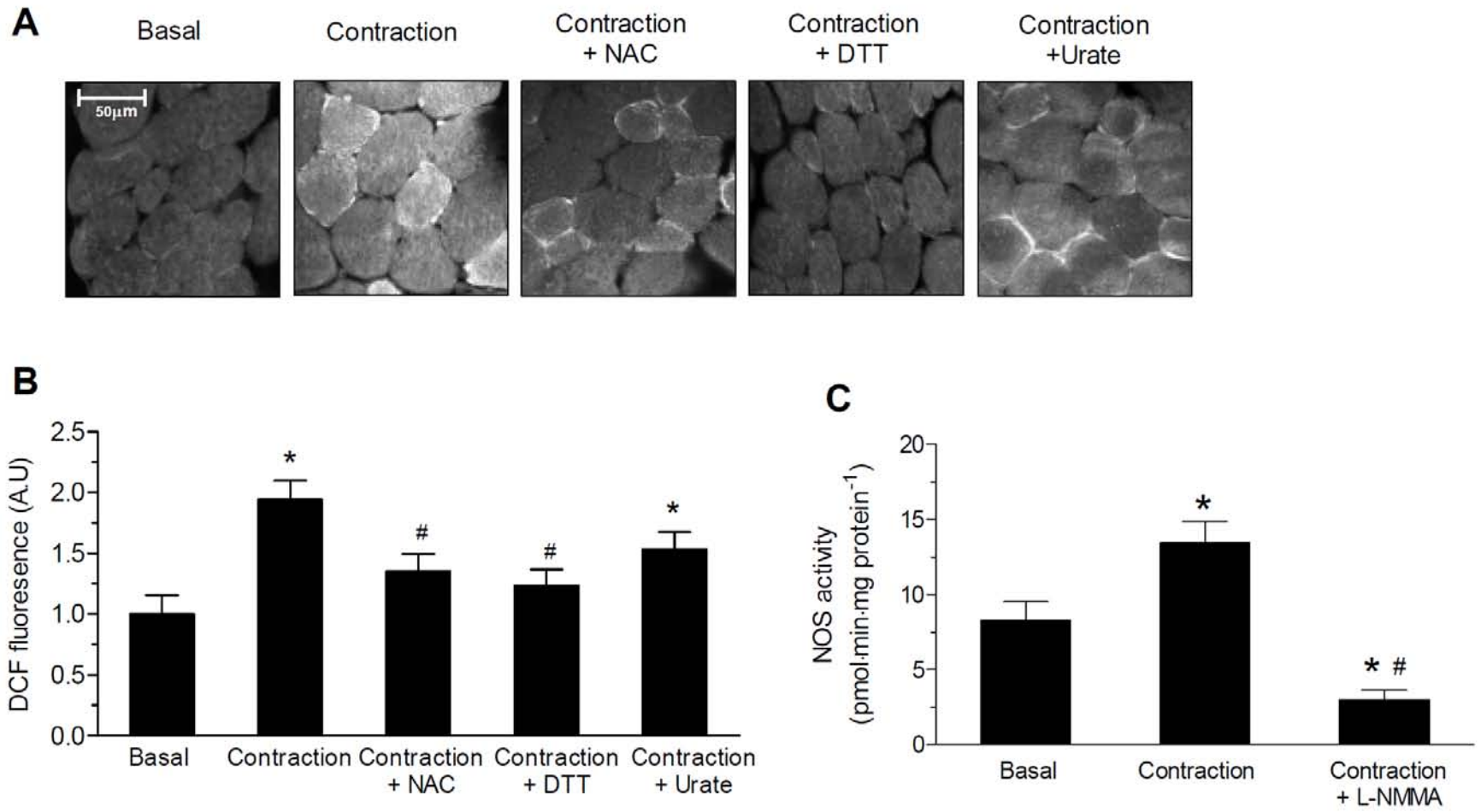

Figure 4.3 Representative images (A) and emission intensity (B) of DCF fluorescence, and nitric oxide synthase (NOS) activity (C) in EDL muscles after 40 min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), dithiothreitol (DTT) or urate. Data are means ± SEM; n=6-7 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction.

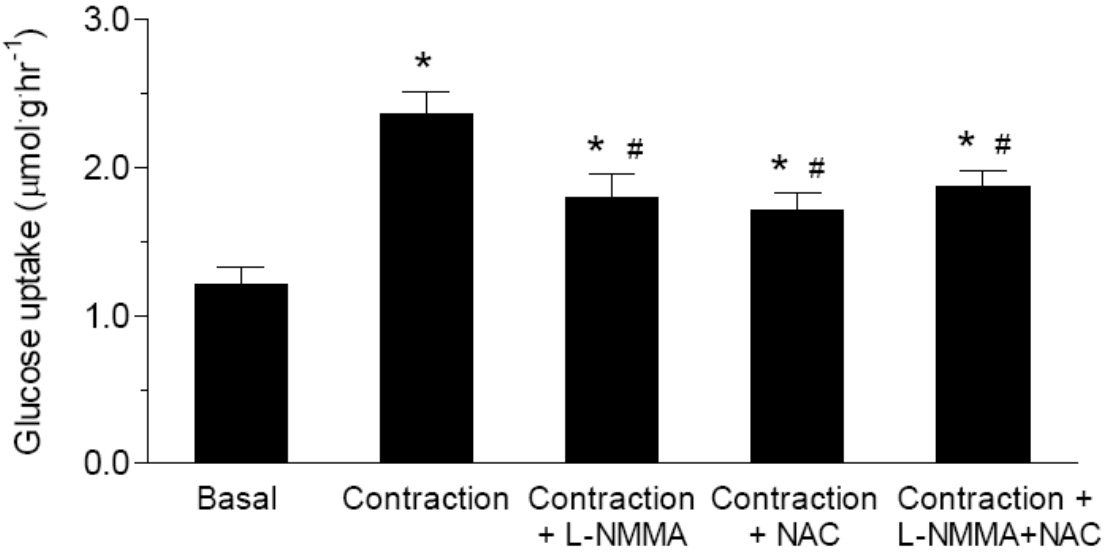

Figure 4.4 Glucose uptake in EDL muscles during the final 10 min of basal incubation or 10 minutes of contraction in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC) or NAC+L-NMMA. Data are means ± SEM; n=6-12 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction.

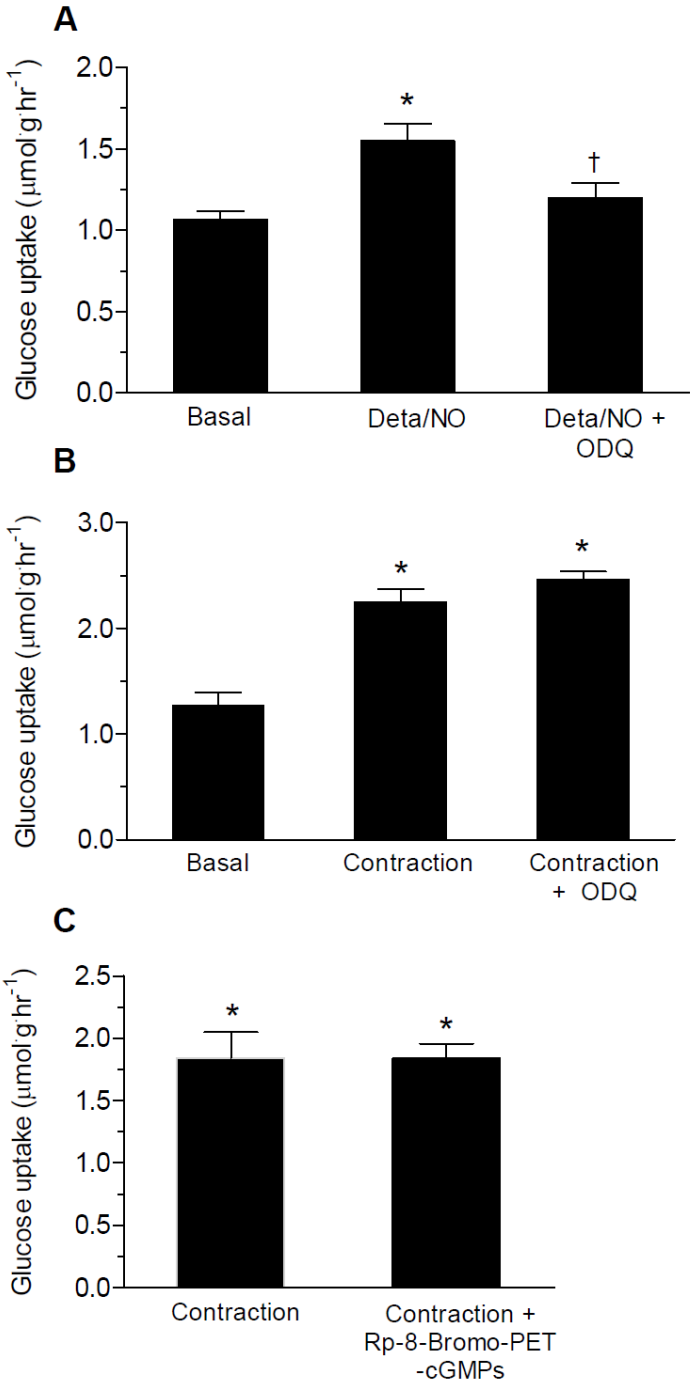

Figure 4.5 Glucose uptake in EDL muscles during the final 10 min of incubation in the presence or absence of diethylenetriamine/NO (Deta/NO, 500µM) or Deta/NO+1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (A), or during the final 10 min of basal incubation or 10 min of contraction in the presence or absence ODQ (B), or Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3',5'-cyclic monophosphorothioate sodium salt (Rp-8-Br-PET-cGMPS) (C). Data are means ± SEM; n=6-12 per group. *P<0.05 vs. basal; †P<0.05 vs. Deta/NO.

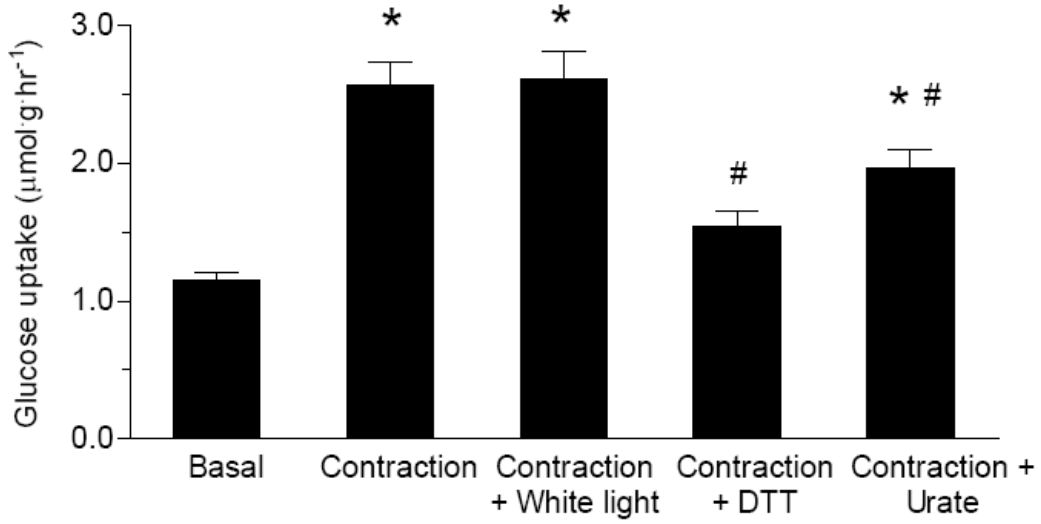

Figure 4.6 Glucose uptake in EDL muscles during the final 10 minutes of basal incubation or 10 min of contraction in the presence or absence of white light, dithiothreitol (DTT) or urate. Data are means ± SEM; n=6-10 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction.

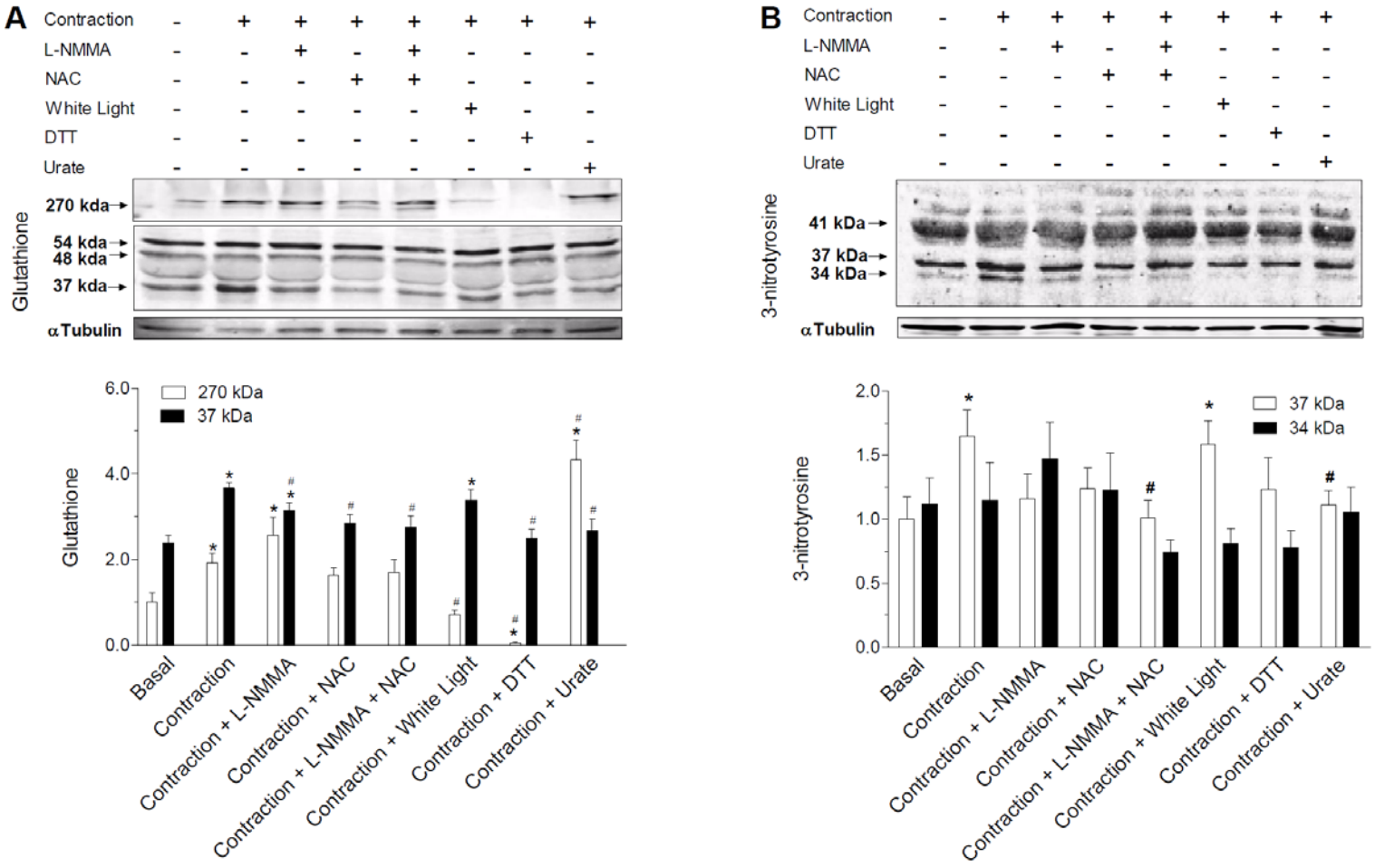

Figure 4.7 S-glutathionylation (A) and tyrosine nitration (B) in EDL muscles after 40 min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), NAC+L-NMMA, white light, dithiothreitol (DTT) or urate. Data are means ± SEM; n=8 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction. Presented blots are representative.

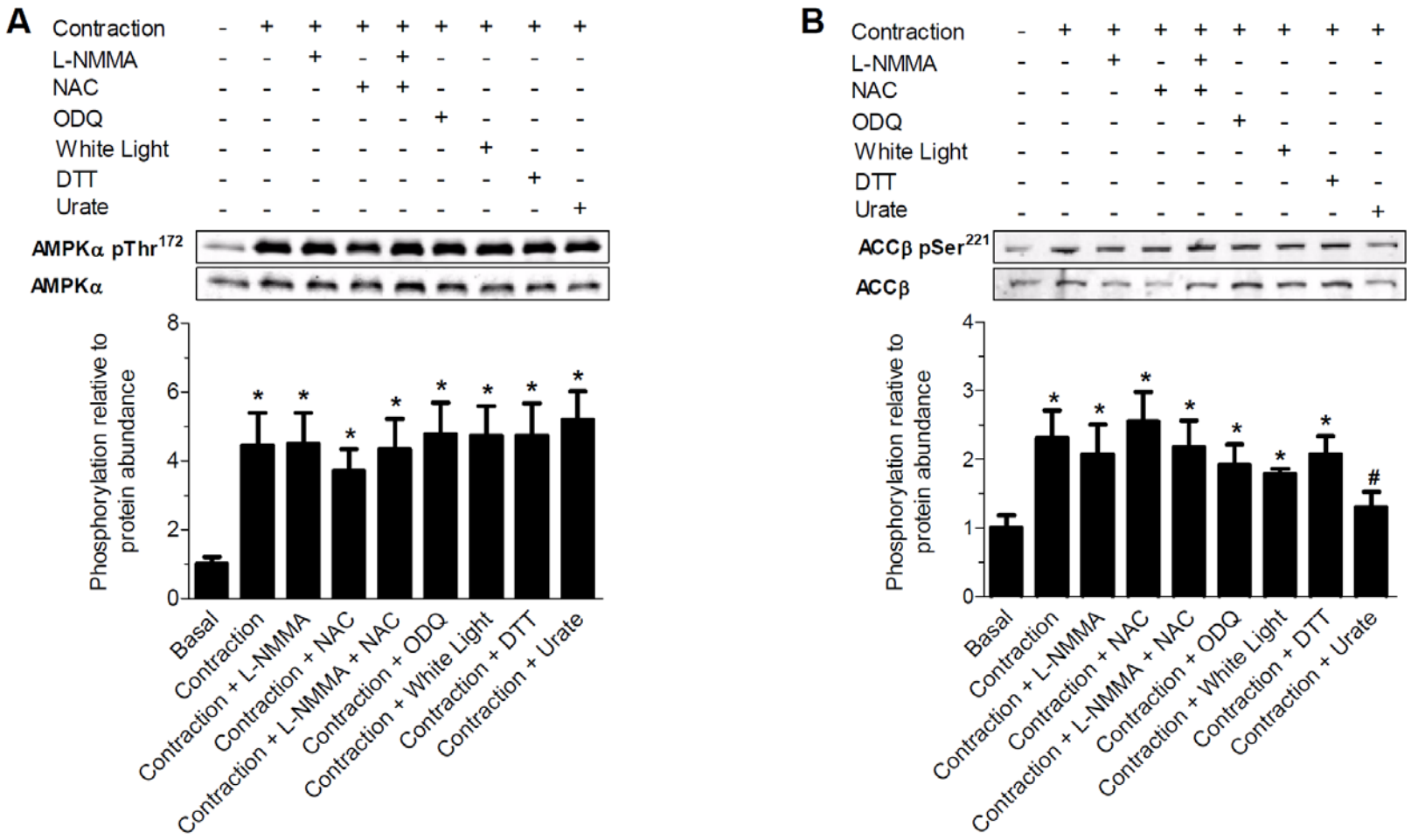

Figure 4.8 AMPK Thr^{172} phosphorylation (A) and ACCβ Ser^{221} phosphorylation (B) in EDL muscles following 40 min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of N^{G}-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), NAC+L-NMMA, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), white light, dithiothreitol (DTT) or urate. Data are means ± SEM; n=8 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction. Presented blots are representative.

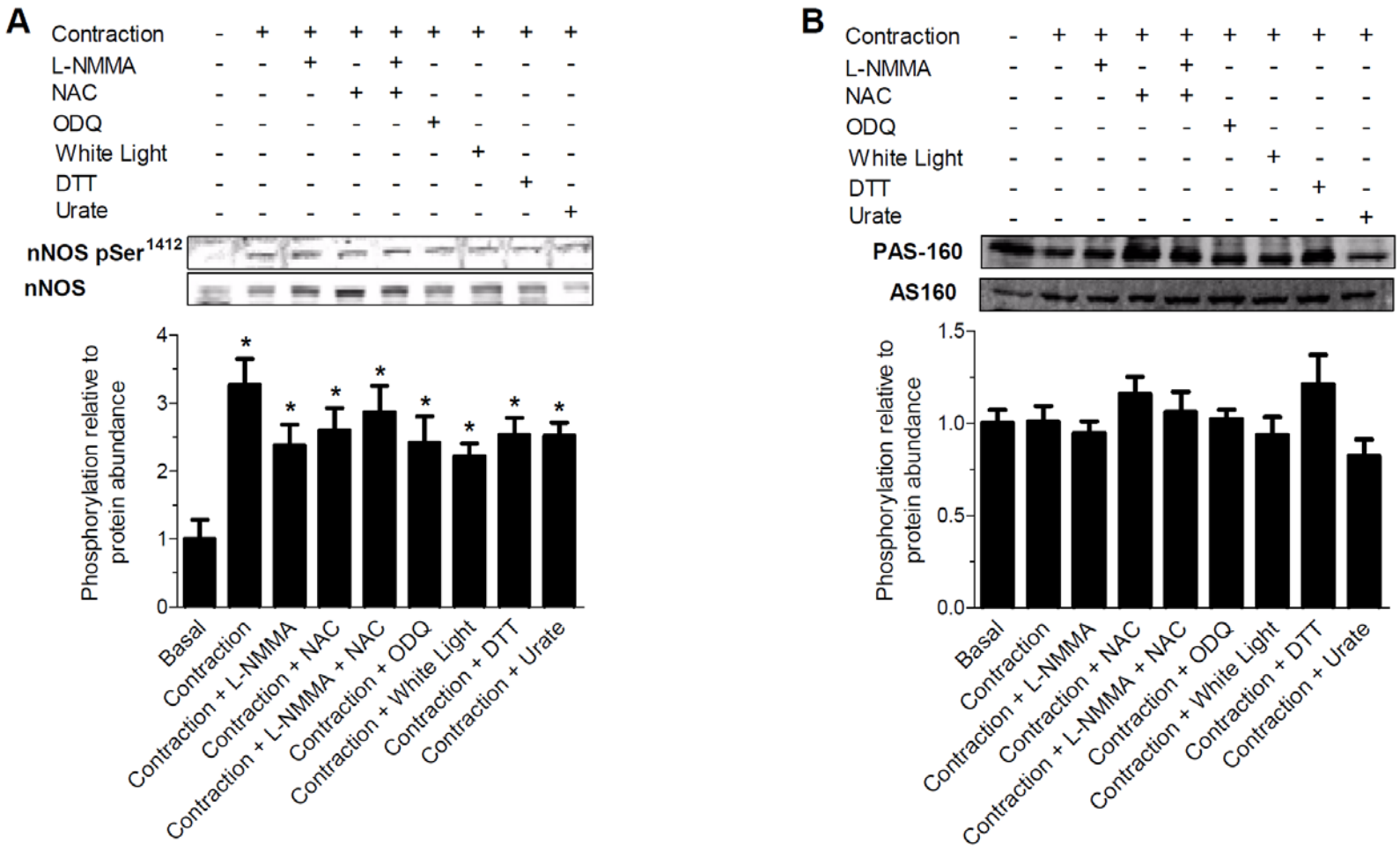

Figure 4.9 nNOSμ Ser^{1412} phosphorylation (A) and PAS-160 phosphorylationin in EDL muscles following 40 min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), NAC+L-NMMA, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), white light, dithiothreitol (DTT) or urate. Data are means ± SEM; n=8 per group. *P<0.05 vs. basal; #P<0.05 vs. contraction. Presented blots are representative.

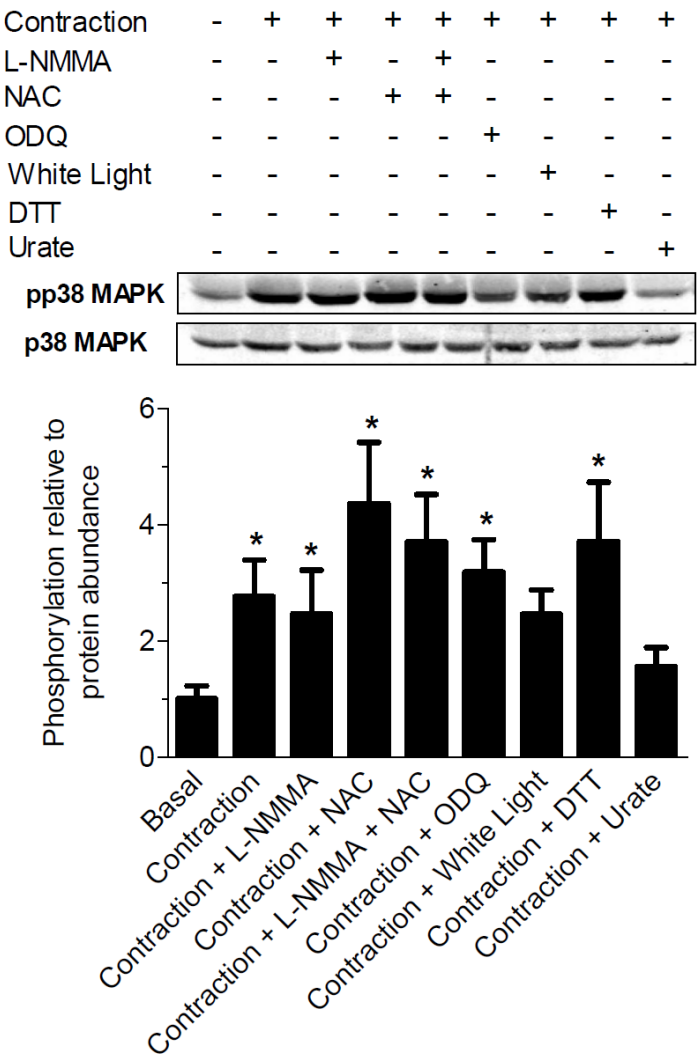

Figure 4.10 p38 MAPK kinase Thr^{180}/Tyr^{182} phosphorylation in EDL muscles following 40 min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of N^{G}-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), NAC+L-NMMA, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), white light, dithiothreitol (DTT) or urate. Data are means ± SEM; n=8 per group. *P<0.05 vs. basal;

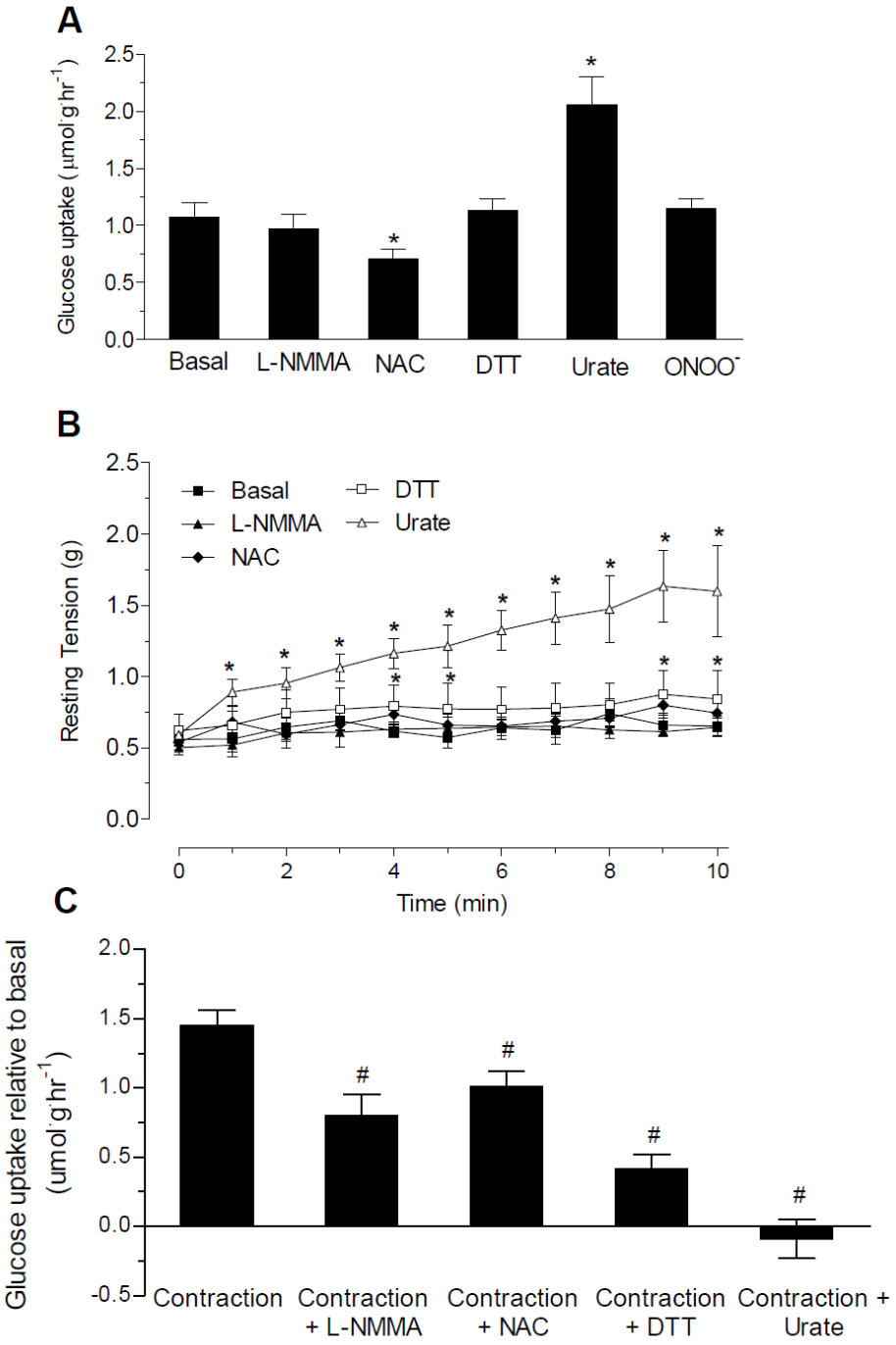

Figure 4.11 Glucose uptake in EDL muscles during the final 10 min of basal incubation in the presence or absence of N^G-monomethyl-L-Arginine (L-NMMA), N-acetylcysteine (NAC), dithiothreitol (DTT), urate or peroxynitrite ($ONOO^-$) (A). Resting (passive) tension of EDL muscles during the final 10 min of basal incubation in the presence or absence of L-NMMA, NAC, DTT, urate or $ONOO^-$ (B). Glucose uptake in EDL muscles during the final 10 min of contraction in the presence or absence of L-NMMA, NAC, DTT, urate relative to basal glucose uptake of the same condition (delta: contraction-basal). Data are means ± SEM; n=4-5 per group. *P<0.05 vs. basal.

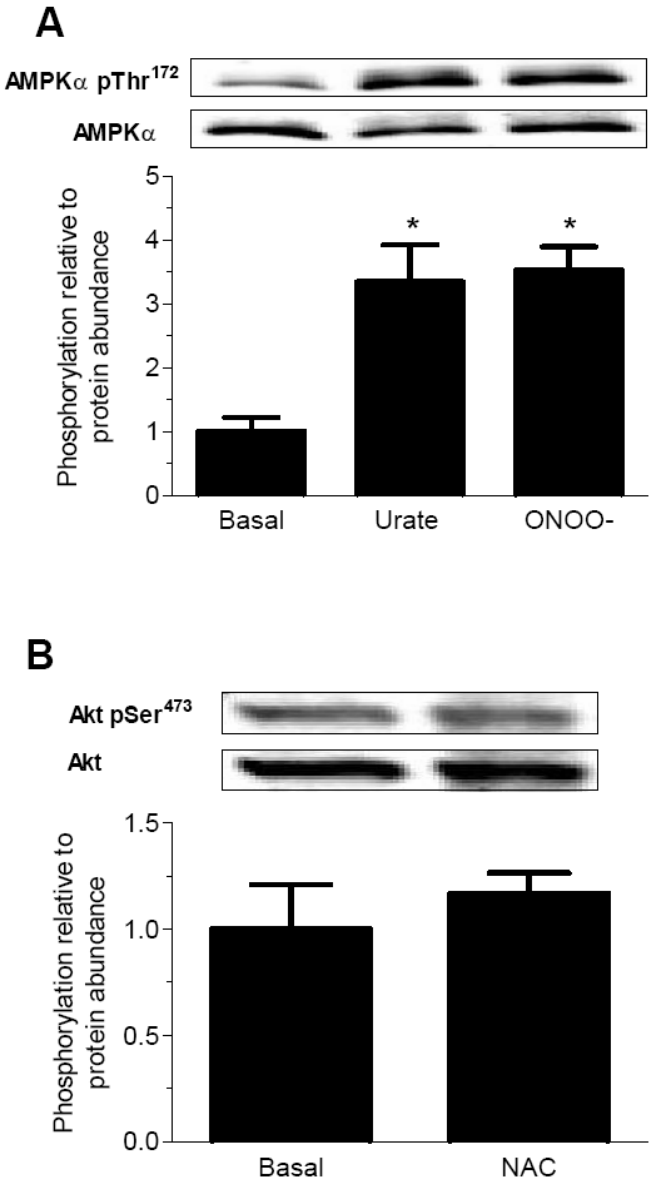

Figure 4.12 AMPK Thr^{172} phosphorylation in EDL muscles following 40 min of basal incubation in the presence or absences of urate or peroxynitrite ($ONOO^-$) (A). Akt Ser^{473} phosphorylation in EDL muscles during the final 10 min of basal incubation in the presence or absence of NAC (B). Data are means ± SEM; n=4-5 per group. Presented blots are representative.

4.4 DISCUSSION

The major findings of this study were that (a) the co-treatment of skeletal muscle with L-NMMA and NAC during contractions *ex vivo* does not have an additive affect on the attenuation of glucose uptake; (b) the guanylate cyclase inhibitor, ODQ, attenuates NO donor-stimulated glucose uptake in resting muscle, but neither ODQ nor the PKG inhibitor, Rp-8-Br-PET-cGMPS, affect skeletal muscle glucose uptake during contraction; (c) L-NMMA and NAC attenuate contraction-stimulated increases in muscle protein S-glutathionylation and tyrosine nitration at ~37 kDa without affecting AMPK or p38 MAPK phosphorylation; and (d) the thiol reducing agent (DTT) and peroxynitrite scavenger (urate) attenuate contraction-stimulated increases in skeletal muscle glucose uptake, protein S-glutathionylation (at ~37 kDa) and tyrosine nitration (at ~37 kDa). Taken together these findings suggest that NO regulates skeletal muscle glucose uptake during *ex vivo* contractions via a cGMP-independent pathway. In addition, ROS and NO regulate skeletal muscle glucose uptake during *ex vivo* contractions through a similar pathway that does not require AMPK or p38 MAPK but may involve signalling through peroxynitrite and S-glutathionylation.

In support of findings from Chapter 3 (Figure 3.11), preventing increases in NOS activity during *ex vivo* contractions of mouse EDL muscles attenuated increases in glucose uptake (Figure 4.4). In Chapter 3 evidence is provided that NO mediates glucose uptake during contraction independent of AMPK (Figure 3.11), therefore this study investigated alternative mechanism(s) through which NO may mediate glucose uptake during contraction. There is strong evidence to suggest that NO-donors increase resting muscle glucose uptake through a cGMP/PKG dependent pathway (Young *et al.*, 1997; Young & Leighton, 1998a, b). In support of this the specific GC inhibitor, ODQ (Garthwaite *et al.*, 1995), prevented the increase in resting muscle glucose uptake stimulated by the NO donor, Deta/NO (Figure 4.5A). However, surprisingly, ODQ or the PKG inhibitor (Rp-8-Br-PET-cGMPS) did not affect skeletal muscle glucose uptake during contraction (Figure 4.5B and C). Unfortunately, and despite several attempts, we were unable to measure skeletal muscle cGMP formation following contraction. Since cGMP is broken down extremely quickly by cGMP-specific phosphodiesterases (PDE) in the absence of PDE inhibitors (Bellamy & Garthwaite, 2001), we believe we did not freeze the muscle fast enough following contraction to prevent the breakdown of cGMP and observe an increase in cGMP content. However, when Lau *et al.* (2000) freeze clamped muscles following contraction they were able to show that contraction

increases muscle cGMP to a similar extent as NO-donors. Since ODQ inhibited NO donor-stimulated glucose uptake (Figure 4.5), a pathway that requires cGMP signalling (Young & Leighton, 1998b), we are confident that ODQ prevented any increases in cGMP formation that may have occurred during contraction. Therefore, this data suggests that during skeletal muscle contraction NO regulates glucose uptake through a cGMP-PKG-independent pathway.

Nitric oxide has been shown to regulate glucose uptake in adipocytes through a cGMP-independent mechanism that possibly involves signalling via S-nitrosylation (Kaddai *et al.*, 2008). However, S-nitrosylation does not appear to be involved in regulating skeletal muscle glucose uptake during contraction since white light, which breaks S-nitrosylation bonds (Borutaite *et al.*, 2000; Benhar *et al.*, 2009), did not affect increases in glucose uptake during contraction (Figure 4.6). Although protein S-nitrosylation was not measured in the present study, S-glutathionylation of a protein band at ~270 kDa was prevented by white light providing some evidence that white light affects signalling in skeletal muscle (Figure 4.7A). However, further work is needed to ensure S-nitrosylation is not involved in regulating skeletal muscle glucose uptake during contraction. This is of particular importance since S-nitrosylation can act as an intermediate for ROS signalling via S-glutathionylation; the nitrosothiol formed by S-nitrosylation of a thiol represents an active protein cysteine thiol which can then react with GSH and produce S-glutathionylation (Martinez-Ruiz & Lamas, 2007). Thus S-glutathionylation may act as a convergence point between ROS and NO signalling.

In agreement with previous studies (Reid *et al.*, 1992a; Sandstrom *et al.*, 2006) and Chapter 3 (Figure 3.8), we found that contraction increases skeletal muscle oxidant levels, and that this increase can be prevented by NAC treatment (Figure 4.3). As discussed in Chapter 2 (*2.1.5 Oxidant levels assay*) DCF fluorescence was measured as a marker of muscle ROS levels. Contraction-stimulated increases in DCF fluorescence were prevented by NAC (Figure 4.3B), suggesting that although DCFH can be oxidised to DCF by reactive nitrogen species (Murrant *et al.*, 1999) as well as by ROS (Murrant & Reid, 2001), in our hands contraction-stimulated increases in DCFH oxidation primarily resulted from an increase in intracellular ROS levels. This is supported by the finding that contraction-stimulated muscle S-glutathionylation, which is promoted by oxidative stress (Dalle-Donne *et al.*, 2009), of protein bands at ~270 kDa and ~37 kDa was prevented by NAC (Figure 4.7A). In addition to attenuating contraction-stimulated increases in oxidative stress, NAC also attenuated increases in muscle glucose

uptake (Figure 4.4 and 4.6). This is in agreement with Chapter 3 (Figure 3.11) and others who have shown that NAC attenuates stretch (Chambers *et al.*, 2009) and contraction-stimulated increases in skeletal muscle glucose uptake (Sandstrom *et al.*, 2006). Interestingly, the effect of NOS inhibition and NAC treatment on the attenuation of increases in contraction-stimulated muscle glucose uptake was not additive (Figure 4.4). This suggests that there is some degree of overlap between the pathways though which NO and ROS regulate skeletal muscle glucose uptake during *ex vivo* contractions.

The ROS superoxide ($O_2^{\bullet-}$) and NO interact to produce $ONOO^-$ (Sawa *et al.*, 2000), and each of $O_2^{\bullet-}$, NO and $ONOO^-$ can promote protein S-glutathionylation; the reversible formation of mixed disulfides between protein sulfide groups and glutathione (Dalle-Donne *et al.*, 2009) which is becoming increasingly recognised as the redox equivalent of phosphorylation in regulating protein signalling (Martinez-Ruiz & Lamas, 2007; Dalle-Donne *et al.*, 2009). Therefore, the $ONOO^-$ scavenger urate (Reiter *et al.*, 2000) and thiol reducing agent DTT (which reduces thiols, therefore S-glutathionylated bonds) (Dalle-Donne *et al.*, 2005) were used to investigate the role of $ONOO^-$ and S-glutathionylation in regulating skeletal muscle glucose uptake. Like NAC, DTT prevented contraction stimulated increases in DCF fluorescence. This is not surprising since DTT reduces glutathione-protein-mixed disulfides (Lou *et al.*, 1987) and oxidised glutathione (GSSG), elevating cellular levels of the antioxidant reduced glutathione (GSH) (Rothwarf & Scheraga, 1992). Interestingly, urate, which scavenges $ONOO^-$, tended to attenuate contraction-stimulated increases in oxidant levels suggesting that during contraction $ONOO^-$ is produced. In support of this tyrosine nitration, which is caused by $ONOO^-$ (Pacher *et al.*, 2007), of a protein band at ~37 kDa (Figure 4.7B) was increased with contraction and this increase was prevented by all antioxidants (NAC, DTT and urate) and NOS inhibition. In support, $ONOO^-$ has been shown previously to be produced by contracting skeletal muscle cells (Pattwell *et al.*, 2004). Similarly, all antioxidants and NOS inhibition prevented increases in S-glutathionylation at ~37 kDa during contraction (Figure 4.7A). Importantly, like L-NMMA and NAC, DTT and urate also attenuated increases in skeletal muscle glucose uptake during contraction (Figure 4.4 and 4.6). Whether the effect of DTT was a result of its thiol reducing properties, or the resulting antioxidant effects is difficult to discern and requires further investigation.

Taken together, these data suggest that during contraction ROS and NO interact to alter protein S-glutathionylation and tyrosine nitration, and this may play a role in the regulation of

skeletal muscle glucose uptake during contraction. However, tyrosine nitration is promoted by high $ONOO^-$ concentrations, and normally associated with irreversible pathophysiological inhibition of protein phosphorylation (Pacher *et al.*, 2007). To reconcile these two views of $ONOO^-$, it is possible that during intense *ex vivo* contractions higher than normal physiological levels of $ONOO^-$ may be formed causing substantial tyrosine nitration. On the other hand, it is likely that during more physiological exercise lower concentrations of $ONOO^-$, that do not cause tyrosine nitration, are produced, and cause transient and reversible phosphotyrosine signalling which can alter protein activities (Mallozzi *et al.*, 1997; Pacher *et al.*, 2007). It is, therefore, important that future studies identify whether relationships exist between protein tyrosine nitration, $ONOO^-$ signalling, S-glutathionylation and glucose uptake during contraction. Interestingly, both S-glutathionylation and tyrosine nitration of a protein band at ~37 kDa was increased with contraction and this increase was attenuated by NOS inhibition and all antioxidant treatments (Figure 4.7). This protein band was identified using mass spectrometry to have a 67% convergence with GAPDH. The primary function of GAPDH is to catalyse the breakdown of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during glycolysis (Sirover, 1996), however, it also has roles in the facilitation of transcription (Zheng *et al.*, 2003), apoptosis (Berry & Boulton, 2000), and protein vesicular transportation (Tisdale, 2001). Indeed, both S-glutathionylation (Lind *et al.*, 1998) and $ONOO^-$ (Souza & Radi, 1998), potentially via tyrosine nitration (Mohr *et al.*, 1994), have been shown to inhibit the activity and alter the function of GAPDH (Mohr *et al.*, 1994; Souza & Radi, 1998; Mohr *et al.*, 1999). Whether GAPDH has the potential to signal skeletal muscle glucose uptake, and whether the regulation of GAPDH through S-glutathionylation or peroxynitrite signalling during muscle contraction is also involved in facilitating glucose uptake should now be investigated.

It is notable that the incubation of skeletal muscle with urate, an $ONOO^-$ scavenger (Reiter *et al.*, 2000; Sawa *et al.*, 2000), reduced initial contraction force and inhibited muscle signalling (ACCβ and p38) during contraction, independent of NOS inhibition (Figure 4.8B and 4.10B). This indicates that urate is having effects on skeletal muscle independent of $ONOO^-$ scavenging. Furthermore, urate stimulated an increase in resting muscle passive tension (Figure 4.11B) which is likely to have caused the increase in resting muscle AMPK phosphorylation and glucose uptake (Figure 4.12A and 4.11A) (Ihlemann *et al.*, 1999b). Although skeletal muscle glucose uptake during contraction may not be dependent on force production *per se* (Sandstrom *et al.*, 2007), the various affects of urate on the muscle likely

confounds interpretation of $ONOO^-$ effects on skeletal muscle glucose uptake during contraction. Interestingly, however, the increase in resting muscle glucose uptake with urate was similar to that seen after the combination of urate and contraction (Figure 4.4 and 4.11A); suggesting that urate prevented any further increase in skeletal muscle glucose uptake during contraction. Therefore, investigating the role of $ONOO^-$ in regulating skeletal muscle glucose uptake during contraction using more specific $ONOO^-$ scavengers is important.

Although $ONOO^-$ produced endogenously during contraction may play a role in glucose regulation, surprisingly, exogenous $ONOO^-$ did not affect resting skeletal muscle glucose uptake. The possible different effects of $ONOO^-$ produced endogenously during contraction, and that added exogenously, on glucose uptake may relate to differences in intracellular conditions. During contraction muscle energy balance is alter and numerous signalling pathways are upregulated, and therefore, it may be that during such conditions $ONOO^-$ is able to stimulate pathways associated with glucose uptake. Furthermore, the addition of exogenous $ONOO^-$ to the cell surface is likely to activate different signalling pathways than that produced from internal sources (Chambers *et al.*, 2009). Alternatively, or concurrently, the higher than physiological level of $ONOO^-$ employed (500 μM) may have a different affect to the lower concentration normally observed endogenously (10-200 μM (Pacher *et al.*, 2007)). Indeed, at low concentrations $ONOO^-$ has been shown to promote tyrosine phosphorylation, activating proteins, whereas at higher pathophysiologcal levels (>500 μl) $ONOO^-$ inhibits protein phosphorylation through tyrosine nitration (Mallozzi *et al.*, 1997). Five hundred μM $ONOO^-$ was used in the current study because similar concentrations have been shown previously in cells to activate PI3K and AMPK pathways (Zou *et al.*, 2002; Zou *et al.*, 2003), and indeed 500 μM of exogenous $ONOO^-$ increased AMPK phosphorylation in muscle (Figure 4.12A). However, $ONOO^-$-stimulated increases in resting AMPK phosphorylation did not result in an increase in glucose uptake. Since increases in AMPK phosphorylation in resting muscle increase glucose uptake (Figure 3.12) it suggests that this concentration of $ONOO^-$ may be inhibiting glucose uptake pathways, and future studies should test the effect of lower exogenous $ONOO^-$ concentrations, and $ONOO^-$-donors on skeletal muscle glucose uptake.

While the mechanisms through which NO regulates glucose uptake during contraction have not been examined previously, ROS have been suggested to increase glucose uptake during contraction through the activation of AMPK (Sandstrom *et al.*, 2006), and Chambers *et al.*

(2009) have provided some evidence that ROS may regulate stretch-stimulated skeletal muscle glucose uptake via p38 MAPK (p38). In contrast, we found that the antioxidants NAC and DTT attenuated contraction-stimulated glucose uptake independent of p38 phosphorylation, and neither NAC, DTT nor urate affected AMPK phosphorylation during contraction (Figure 4.8A and 4.10B). This supports our finding from Chapter 3 (Figure 3.11) that ROS regulate glucose uptake during contraction via an AMPK independent pathway, and also provides evidence to suggest that ROS may not regulate glucose uptake during contraction via p38 MAPK phosphorylation. This suggests that stretch and contraction stimulate glucose uptake through separate pathways. However, it must it must be acknowledged that p38 MAPK phosphorylation may not fully represent *in vivo* p38 MAPK activity, therefore future investigations should assess the effect of antioxidants on p38 MAPK activity. The discrepancy between Sandstrom *et al.* (2006) and our finding in regards to the effects of NAC on AMPK is more difficult to discern and, as discussed in Chapter 3, may relate to Sandstrom *et al.* (2006) using a longer pre-contraction NAC incubation (30 vs 60 min), cooler incubation temperatures (25 vs 30°C) or a more strenuous contraction protocol than was employed in the present study (see Chapter 3, *3.4 Discussion*).

In support of findings in Chapter 3 (Figure 3.7) neither contraction nor any other treatment affected PAS-160 phosphorylation (Figure 4.9). As discussed in detail in Chapter 3, it is possible that longer that 10 min of contraction is required to increase PAS-160 phosphorylation. Regardless, this suggests that PAS-160 phosphorylation may not be required for normal increases in glucose uptake during contraction of mouse skeletal muscle *ex vivo*, but does not rule out a possible role for AS160 or TBC1D1 in regulating ROS and/or NO-stimulated skeletal muscle glucose uptake during contraction (discussed in detail in Chapter 3, *3.4 Discussion*).

Previously, it has been shown that exogenous ROS increase basal (resting) muscle glucose uptake through a PI3K/Akt dependent pathway (Higaki *et al.*, 2008; see Chapter3, 3.4 Discussion for details). Therefore, in an attempt to determine the mechanism by which NAC reduced basal glucose uptake (Figure 4.11A), basal Akt Ser^{473} was examined. NAC did not affect basal Akt Ser^{473} (Figure 4.12B), or AMPK Thr^{172} phosphorylation (Chapter 3, Figure 3.12B), suggesting that NAC was not affecting basal AMPK and Akt regulation of glucose uptake. It is plausible, however, that the high NAC concentration (20 mM), required to attenuate increases in ROS during contraction had a direct affect on basal glucose uptake,

possibly through changing the osmolality of the incubation media. A 20 mM sodium chloride control may be advisable for future investigations that use 20 mM NAC. Importantly, the effect of NAC on reducing basal glucose uptake was considerably less than that of NAC reducing contraction-stimulated muscle glucose uptake (Figure 4.11A), suggesting that effect of NAC on contraction stimulated glucose uptake was not merely the result of a reduced basal glucose uptake.

In addition to contraction increasing S-glutathionylation of a protein band at ~37 kDa, S-glutathionylation at ~270 kDa was also increased with contraction, and this increase was prevented by NAC, white light and DTT (Figure 4.7). Since neither L-NMMA nor urate affected the contraction-stimulated increase in S-glutathionylation at ~270 kDa, but reduced glucose uptake (Figure 4.4 and 4.6) it is unlikely that this protein band is involved in the regulation of contraction-stimulated glucose uptake. This protein band was identified to contain Myh4 and SERCA1. SERCA1 is a 110 kDa protein and Myh4 is a 223 kDa protein, this suggests that during contraction SERCA1 may be S-glutathionylated to Myh4 or another protein to be present at ~270 kDa. Indeed, in Chapter 6 (Figure 6.8), SERCA1 is confirmed to be present at ~270 kDa in contracted skeletal muscle. SERCA is found in the sarcoplasmic reticulum (SR) of skeletal muscles and is associated with Ca^{2+} transport and S-glutathionylation of SERCA in response to oxidative stress increases its activity (Adachi *et al.*, 2004; Ying *et al.*, 2007). However, we provide evidence for the first time that skeletal muscle SERCA is S-glutathionylated during contraction, and this can be prevented with antioxidants. Whether S-glutathionylation of SERCA during muscle contraction is important in the regulation of Ca^{2+} uptake and normal contraction force development, is worthy of further experimental investigation.

Interestingly, NAC and other antioxidants have been shown to reduce fatigue during exhaustive exercise and intense muscle contractions (Reid *et al.*, 1992a; Reid *et al.*, 1992b; Sen *et al.*, 1994; Medved *et al.*, 2004b). Whether at high contraction intensities the S-glutathionylation of SERCA acts to disrupt calcium homeostasis, a common factor associated with muscular fatigue (Bruton *et al.*, 1998; Reid, 2001), and whether antioxidant treatment may reduce this is an interesting hypothesis for future consideration, but beyond the scope of this thesis.

In conclusion, this study suggests that during *ex vivo* contractions NO regulates skeletal muscle glucose uptake through a cGMP-PKG independent pathway that does not involve p38 MAPK or AMPK phosphorylation. Furthermore, we provide evidence that NO and ROS interact during contraction to regulate skeletal muscle glucose uptake, potentially through peroxynitrite and S-glutathionylation signalling. Interestingly, we provide some evidence that GAPDH, Myh4 and SERCA1 are targets of S-glutathionylation during skeletal muscle contractions.

CHAPTER FIVE

LOCAL HINDLIMB ANTIOXIDANT INFUSION DOES NOT AFFECT MUSCLE GLUCOSE UPTAKE DURING IN SITU CONTRACTIONS IN RAT

5.1 INTRODUCTION

In agreement with Sandstrom *et al.* (2006), evidence is provided in Chapter 3 and 4 that ROS are involved in the regulation of skeletal muscle glucose uptake during *ex vivo* contractions. However, it remains to be determined whether ROS are involved in regulating contraction-stimulated skeletal muscle glucose uptake using intact and more physiological models. This is important because *ex vivo* models rely on diffusion gradients for substrate delivery and clearance (Allen *et al.*, 2008), unlike *in vivo* exercise where muscle blood flow is integral to skeletal muscle glucose uptake (Wheatley *et al.*, 2004; Rattigan *et al.*, 2005). Furthermore, unlike *in vivo*, *ex vivo* models of muscle contraction generally involve non-uniform delivery of oxygen to muscle fibres, an absence of antioxidant systems surrounding muscle (such as that supplied by blood flow) and supra-maximal highly fatiguing stimulation protocols (Allen *et al.*, 2008) which are all likely to artificially inflate oxidative stress and alter ROS signalling (Reid, 2008). As such, it may be that during these highly fatiguing *ex vivo* contraction conditions ROS are produced at a greater rate than during *in vivo* exercise and preferentially regulate skeletal muscle glucose uptake.

Reactive oxygen species have been suggested to regulate stretch- and contraction-stimulated glucose uptake through p38 MAPK and AMPK (Sandstrom *et al.*, 2006; Chambers *et al.*, 2009), respectively (see Chapter 3 and 4 for detailed discussion). However, evidence is provided in Chapter 3 and 4 that the antioxidant NAC attenuates contraction-stimulated glucose uptake independent of p38 MAPK and AMPK during contractions *ex vivo* (Figures 3.5 and 4.8). Interestingly, the antioxidant allopurinol has been shown to prevent contraction-stimulated increases in p38 MAPK phosphorylation during exhaustive exercise *in vivo* in rats (Gomez-Cabrera *et al.*, 2005). Whether ROS are involved in the regulation of skeletal muscle

glucose uptake through p38 MAPK and AMPK signalling in intact preparations has not yet been investigated. Importantly, however, NAC has been shown to reduce contraction-induced lowering of muscle GSH content and attenuate the rate of muscle fatigue development *in situ* (Shindoh *et al.*, 1990; Supinski *et al.*, 1995) and *in vivo* (Medved *et al.*, 2004b). This suggests that NAC is effective in attenuating increases in muscle ROS levels during *in situ* and *in vivo* contraction.

Therefore, this study determined whether local infusion of NAC attenuates the increase in hindlimb skeletal muscle glucose uptake, AMPK and p38 MAPK phosphorylation during physiologically relevant low-moderate intensity contractions *in situ* in rats. We hypothesised that low-moderate intensity contractions *in situ* would increase skeletal muscle glucose uptake and that NAC infusion, like *ex vivo* contractions, would attenuate this increase without affecting AMPK or p38 MAPK phosphorylation. An *in situ* preparation was utilised here since it allows delivery of NAC locally to the hindlimb muscles, but unlike *ex vivo* preparations blood supply is intact. A low-moderate intensity contraction protocol, which substantially increases muscle glucose uptake and metabolic signalling without being highly fatiguing (Ross *et al.*, 2007), was employed because is more applicable to submaximal endurance exercise than maximal intensity tetanic contractions commonly used in contractions *ex vivo* (Hayashi *et al.*, 1998; Higaki *et al.*, 2001; Sandstrom *et al.*, 2006).

5.2 METHODS

5.2.1 Experimental procedure

As described in detail in Chapter 2 (*2.3.1 Experimental model*) an *in situ* model using male hooded Wistar rats at ~8 weeks of age, weighing 238 ± 2g was employed. The experimental protocol is outlined in Figure 5.1. Briefly, isotonic saline (154 mmol $^{-1}$ NaCl) containing N-acetylcysteine (NAC; 125 mg·kg·hr^{-1}) or isotonic saline alone was infused locally into one hindlimb (contracted leg) via the epigastric artery. Sham surgery was performed on the contralateral leg (rest). The systemic infusion of NAC at 125 mg·kg·hr^{-1} has been shown previously to elevate muscle NAC, cysteine and total GSH during exercise in humans (Medved *et al.*, 2004b). After a 15 min pre-infusion, the contracted leg was electrically stimulated to contract (0.1 ms impulse at 2 Hz and 35 V) (Ross *et al.*, 2007) for 15 min while NAC/saline infusion continued. This contraction protocol was chosen because our group have previously shown it to substantially increase muscle glucose uptake and metabolic signalling without being highly fatiguing (Ross *et al.*, 2007). The knee was secured by the tibiopatellar ligament and the Achilles tendon was attached to a Harvard Apparatus (MA, USA) isometric transducer thereby allowing measurement of tension development from the gastrocnemius-plantaris-soleus muscle group during contraction. Blood pressure, heart rate, and femoral blood flow were monitored throughout the experiment (see *2.3.1 Experimental model*). Following contraction hindlimb muscle (which consisted of soleus, plantaris, gastrocnemius red and gastrocnemius white muscle) from the contracted and contralateral (rest) leg were rapidly dissected and freeze clamped using liquid nitrogen-cooled thongs (~ 60 sec after last contraction).

5.2.2 Analytical techniques

Muscle 2-deoxy-D-glucose (2-DG) glucose uptake was measured during the final 10 min of contraction as described in Chapter 2 (*2.3.2 2-Deoxy-D-glucose uptake*). Hindlimb muscles were analysed for AMPKα Thr^{172} phosphorylation, ACCβ Ser^{222} phosphorylation, PAS-160 phosphorylation, p38 MAPK Thr^{180}/Tyr^{182} phosphorylation, S-glutathionylation, 3-nitrotyrosine and NAC and thiol content. Blood samples were analysed for glucose and lactate concentration as well as plasma NAC and cysteine content. The methodology used for these analytical procedures is described in detail in Chapter 2. The thiols measured were cysteine and glutathione as markers antioxidant defences and oxidative state (see Chapter 2 for details).

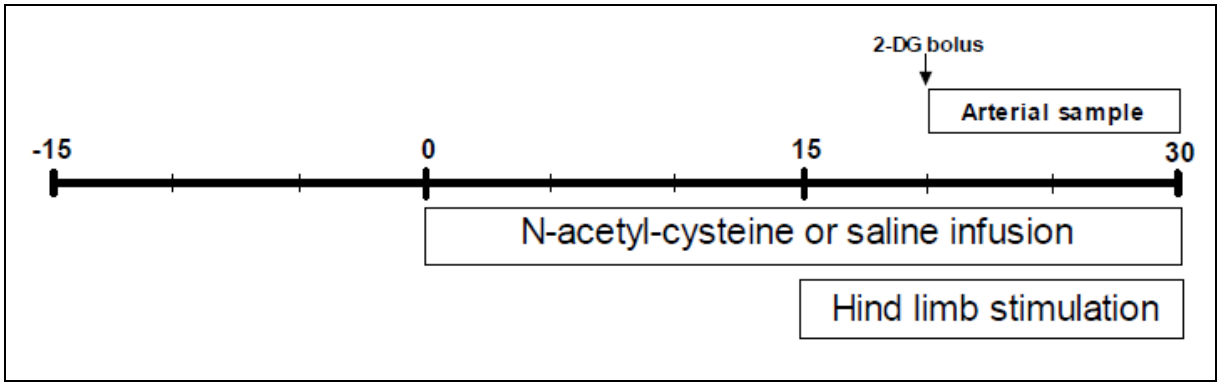

Figure 5.1 Experimental protocol. Saline containing N-acetylcysteine (NAC; 125 mg·kg·hr^{-1}) or saline only was infused into the epigastric artery of the contracted leg from *t* = 0 min for 15 min prior to stimulation and continued during stimulation. Electrical stimulation (0.1-ms impulse at 2Hz and 35 V) of the contracted leg commenced at *t* = 15 min and continued for 15 min. A bolus of radioactive 2-deoxyglucose (2-DG) was injected at *t* = 20 min and a continuous arterial sample was drawn at 50 µl·min^{-1} from the carotid artery for 10 min. At *t* = 30 min contracted and contralateral rest leg hindlimb muscles were rapidly dissected and freeze clamped.

5.2.3 Statistical Analysis

All data are expressed as means ± SEM. Results were analysed by SPSS statistical package using two-factor ANOVA as well as two-factor repeated measures ANOVA (for contraction force). Since NAC infusion started pre-contraction, the repeated measure ANOVA's were partitioned to assess the effect of NAC at rest (0 to 15 min) and during exercise (15 to 30 min). If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

5.3 RESULTS

5.3.1 Force development

Peak contraction force decreased by ~30% by the end of the 15 min contraction period ($P<0.001$; Figure 5.2). Local NAC infusion did not affect initial peak contraction force ($P>0.05$) or the rate of fatigue ($P>0.05$, Figure 5.2).

5.3.2 Plasma and muscle NAC

Local NAC infusion resulted in plasma NAC and reduced NAC concentration of 19.1 ± 2.6 and 3.1 ± 1.1 μM, respectively (Figure 5.3A). Local NAC infusion increased muscle NAC in the contracted leg to a greater extent than the rest leg ($P<0.05$, Figure 5.3B). Similar concentrations of reduced NAC were found in the muscle of the rest and contracted legs ($P>0.05$; Figure 5.3B). NAC was not detected in the plasma or muscle during saline infusion.

5.3.3 Blood glucose and lactate

Hindlimb contraction increased blood glucose and lactate and this increase was not affected by local NAC infusion (Figure 5.4A and B)

5.3.4 Heart rate and blood pressure

Local NAC infusion did not affect resting heart rate (Figure 5.5A). Heart rate was only measured during the first 5 min of contraction, and was not increased significantly from rest at this point (Figure 5.5A). Resting mean arterial pressure (MAP) was not affected by local NAC infusion (Figure 5.5B), however local NAC infusion attenuated the contraction induced increase in MAP by ~12 mmHg at t=20 min ($P<0.05$, Figure 5.5B) suggesting some systemic affects of NAC infusion. HR and blood pressure were not measured during the last 10 min of contraction due to the withdrawal of blood from the arterial line which was required to measure 2-DG uptake as well as BP and HR.

5.3.5 Leg blood flow and vascular resistance

Pre-contraction vascular resistance was reduced in NAC rest leg compared with saline infused rest leg ($P=0.02$, Figure 5.5C), however resting vascular resistance was not affected by local NAC infusion in contracted leg ($P>0.05$, Figure 5.5C). During contraction, vascular resistance was increased in the rest leg and reduced in the contracted leg ($P<0.05$, Figure 5.5C), due to

sympathetic outflow contributing to blood flow redistribution (Thomas & Segal, 2004). Although NAC infusion appeared to attenuate (by ~60% at *t*=20 min) vascular resistance in the resting leg during contraction, NAC did not significantly alter vascular resistance during contraction (P>0.05 for interaction). Leg blood flow remained unchanged during the pre-contraction infusion period (Figure 5.5D). Contraction increased femoral blood flow to the contracted leg by ~400% (P<0.001; Figure 5.5D). Local NAC infusion did not affect the contraction-induced increase in leg blood flow (P.0.05; Figure 5.5D).

5.3.7 Muscle and plasma thiols

Figure 5.6A, C and E show that muscle GSH, GSSG, and GSSG/GSH ratio were not significantly affected by contraction and/or local NAC infusion. Local NAC infusion increased muscle cysteine similarly by 50% in the rest and contracted leg (P=0.001, Figure 5.6). There was a tendency for local NAC infusion to increase muscle cystine (P=0.07), and contraction tended to increase this affect (P=0.08; Figure 5.6D). However, contraction alone did not affect muscle cysteine or cystine concentrations (Figure 5.6B and D). Local NAC infusion increased (P<0.05) plasma cysteine and cystine 360- and 1.4-fold, respectively (Figure 5.6F).

5.3.8 Muscle S-glutathionylation and tyrosine nitration

Contraction significantly (P<0.05) increased muscle S-glutathionylation of protein bands at ~250 kDa and ~150 kDa in the contracted saline infused leg ~1.7-fold and NAC infusion prevented these increases (P<0.05; Figure 5.7A). Neither contraction nor NAC affected protein S-glutathionylation of any other visible protein bands. Muscle tyrosine nitration was not significantly affected by contraction or NAC infusion (Figure 5.7B).

5.3.9 Muscle glucose uptake

The contracted leg had a muscle glucose uptake ~20-fold greater than the rest leg (P<0.001; Figure 5.8). Local NAC infusion did not affect muscle glucose uptake in the rest or in the contracted leg (P>0.05, Figure 5.8).

5.3.10 AMPK*α*, ACCβ, p38 MAPK and PAS-160 phosphorylation

Contraction increased (P<0.05) phosphorylation of AMPK (6-fold) and ACCβ (10-fold) and this increase was not affected by local NAC infusion (Figure 5.9A and B). Similarly, contraction increased p38 MAPK phosphorylation (by ~60-fold; P=0.002) and this increase

was not affected by local NAC infusion ($P>0.05$; Figure 5.9C). PAS-160 phosphorylation was not affected by either contraction or NAC infusion ($P>0.05$; Figure 5.9D).

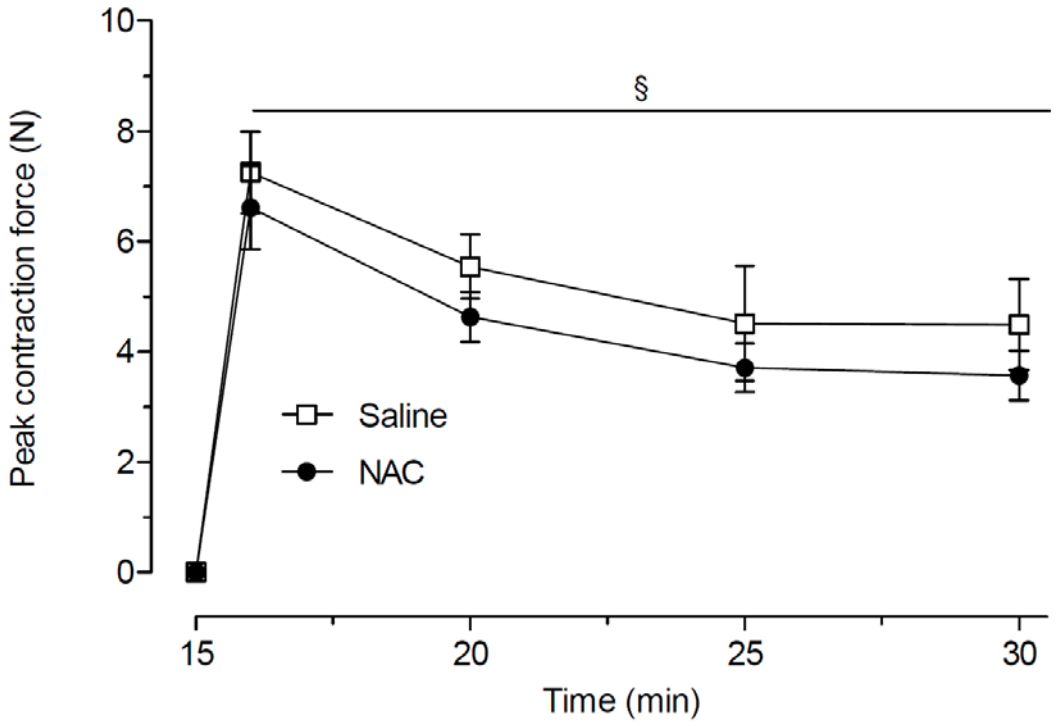

Figure 5.2 Force production of the hindlimb of rats when locally infused with N-acetylcysteine (NAC) or saline during 15 min of in situ contractions. Data are means ± SEM, n=4 per group, §P<0.05 for time.

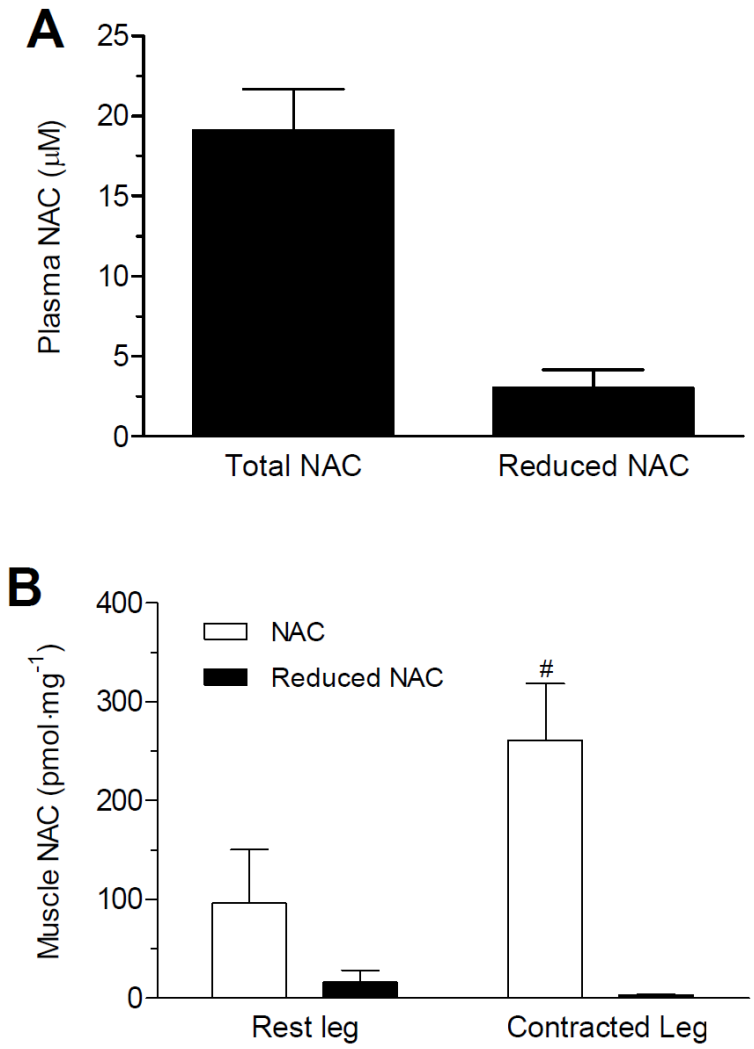

Figure 5.3 Plasma (A) and, rest and contracted leg muscle (B) N-acetylcysteine (NAC) content during in situ hindlimb contractions in rats receiving local NAC infusion into the contracted leg. Data are means ± SEM, n=6-8 per group, #P<0.05 vs rest leg.

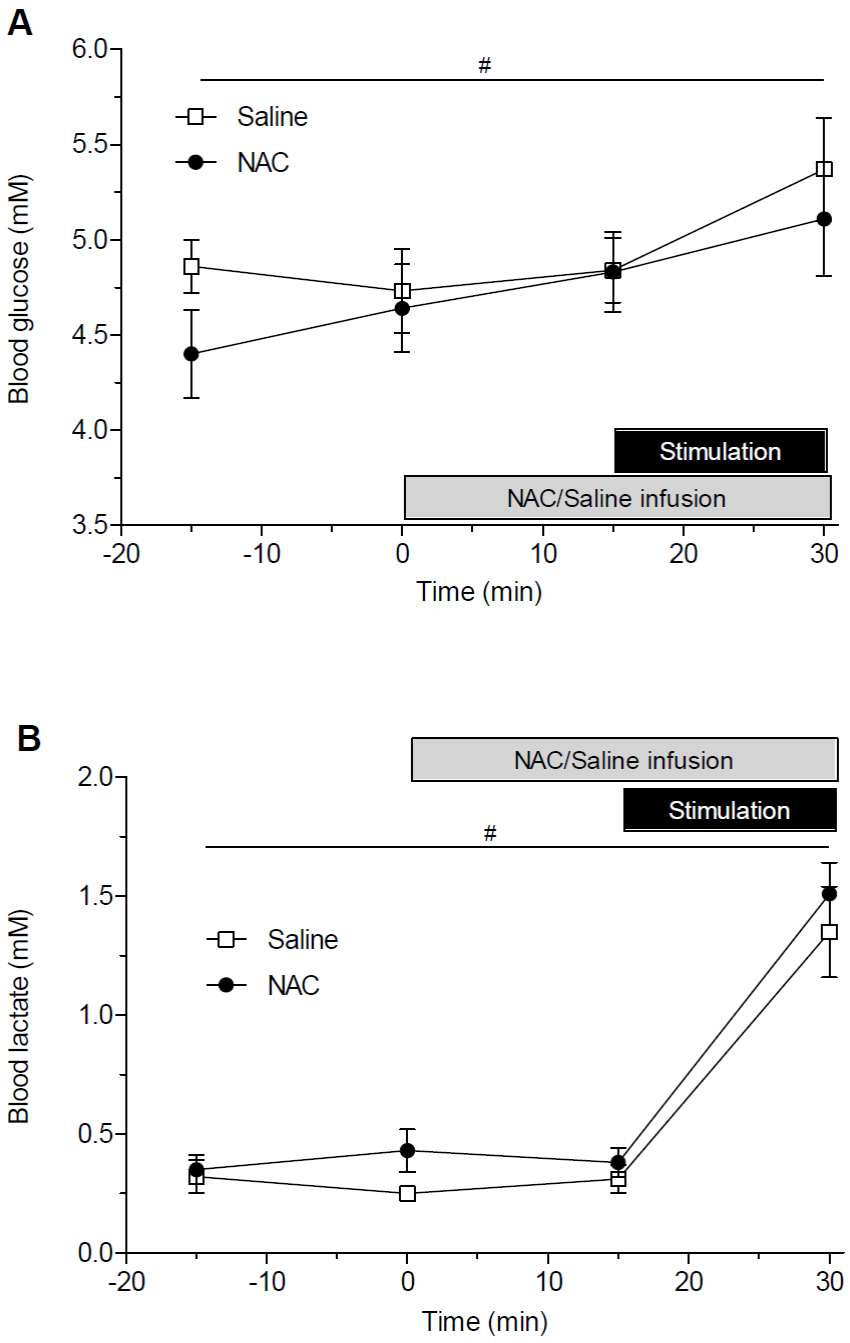

Figure 5.4 Blood glucose (A) and blood lactate (B) content at rest and during in situ hindlimb contractions in rats receiving either local N-acetylcysteine (NAC) or saline infusion into the contracted leg. Data are means ± SEM, n=6-8 per group, #P<0.05 for time.

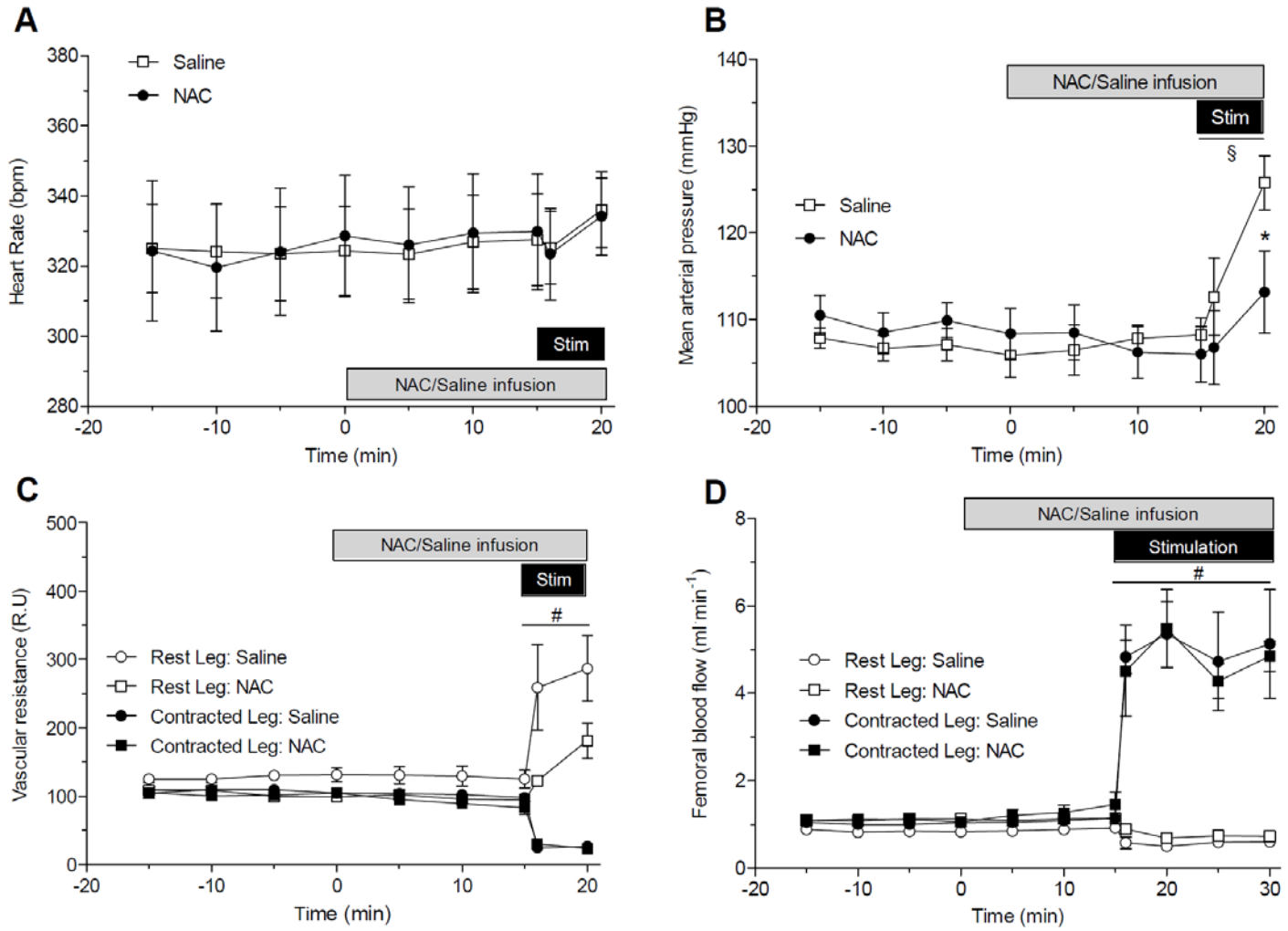

Figure 5.5 Effect of local N-acetylcysteine (NAC) or saline infusion on heart rate (A) mean arterial pressure (B), and rest and contracted leg femoral blood flow (C), and vascular resistance (D) at rest and during in situ hindlimb contractions in rats. Data are means ± SEM, n=6-8 per group, §P<0.05 for time, #P<0.05 for condition (rest vs contraction), *P<0.05 vs saline.

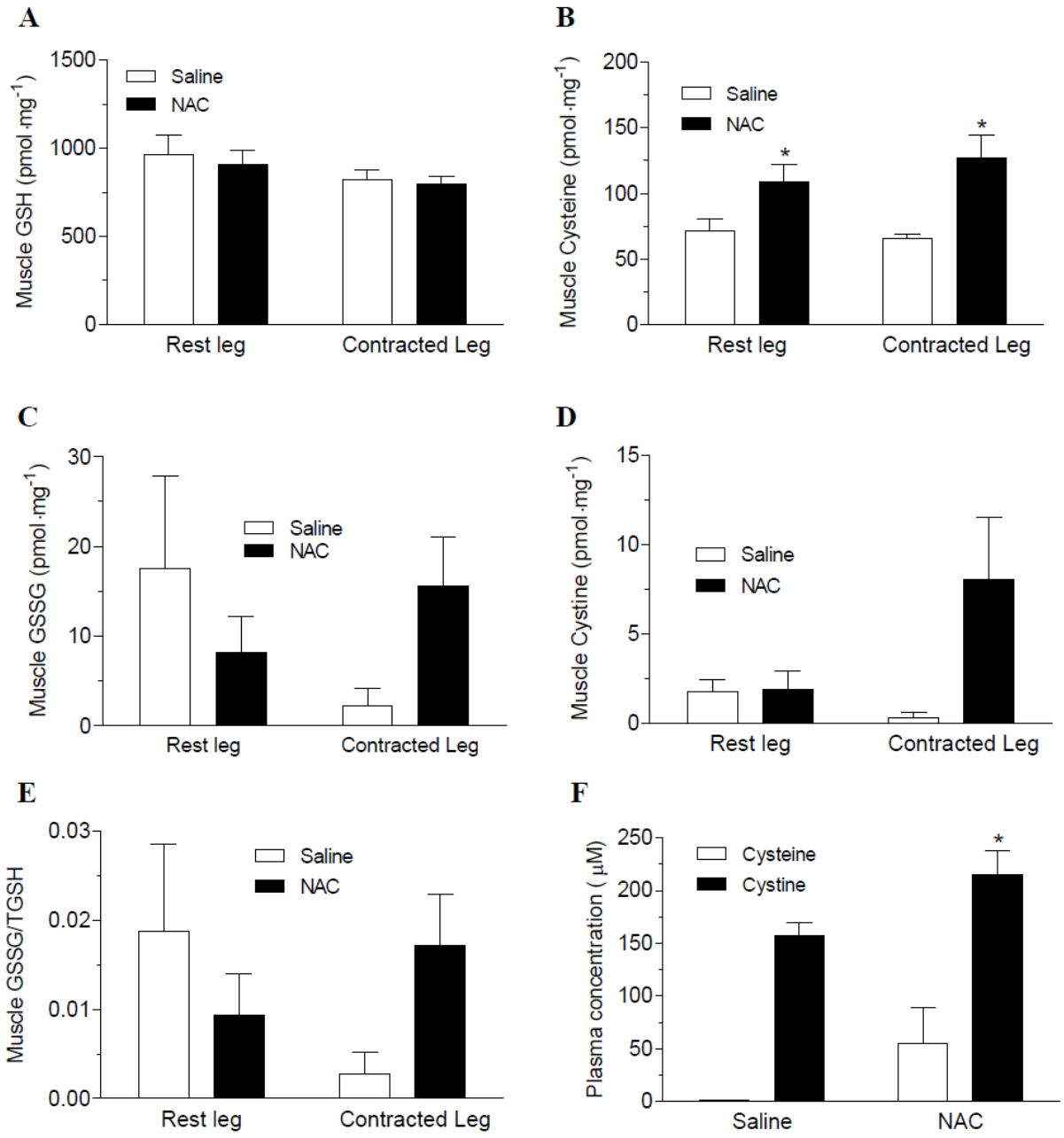

Figure 5.6 Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg muscle reduced glutathione (GSH) (A), cysteine (B), oxidised glutathione (GSSG) (C), cystine (D) and GSSG/GSH ratio (E) following 15 min of in situ hindlimb contractions in the contacted leg of rats, and plasma cysteine and cystine levels during contraction (at t=30 min) (F). Data are means ± SEM, n=6-8 per group, *P<0.05 for treatment (NAC vs saline).

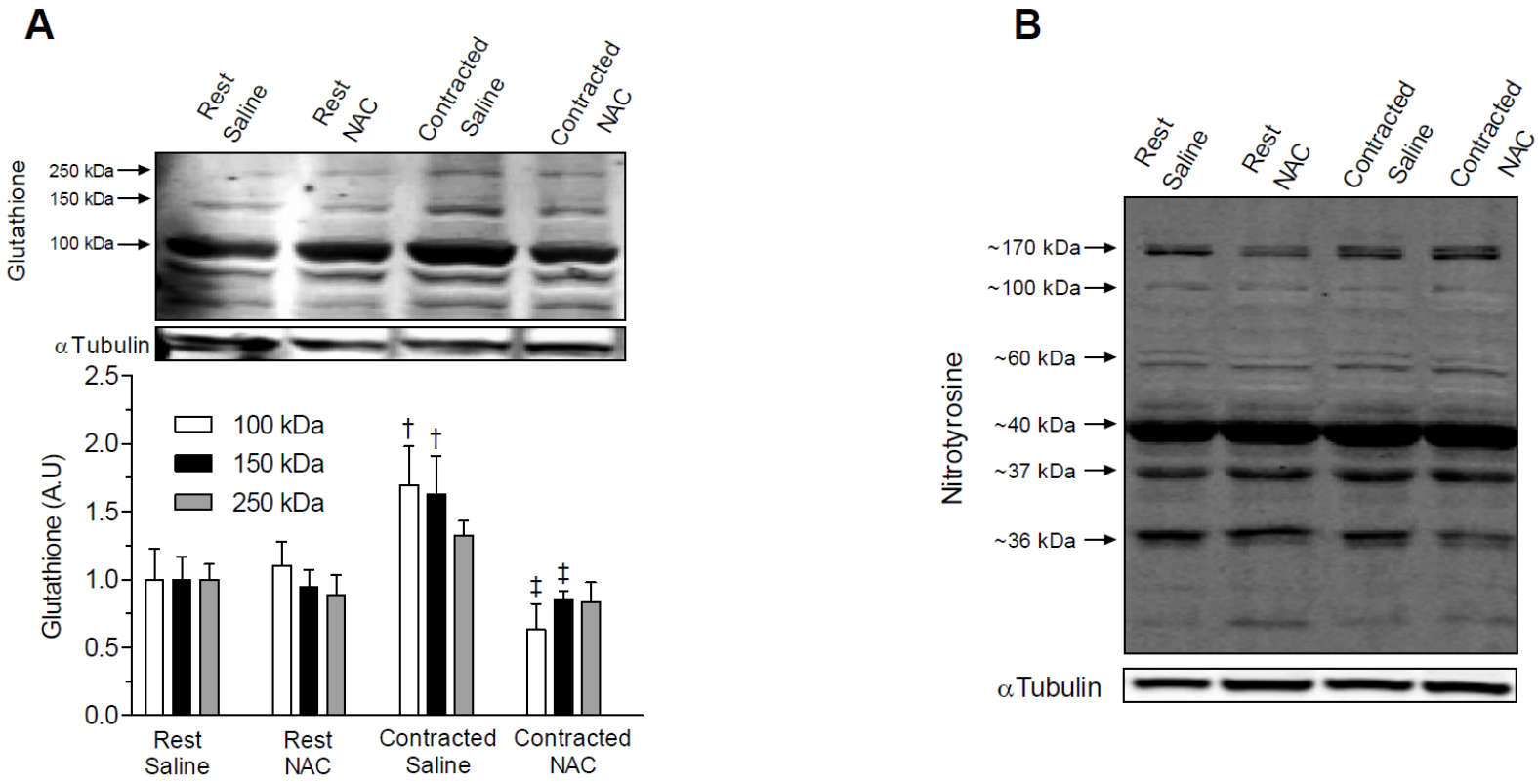

Figure 5.7 Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg muscle S-glutathionylation (A) and tyrosine nitration (B) following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SEM, n=6 per group, ‡P<0.05 vs saline of same condition, †P<0.05 vs rest of same treatment.

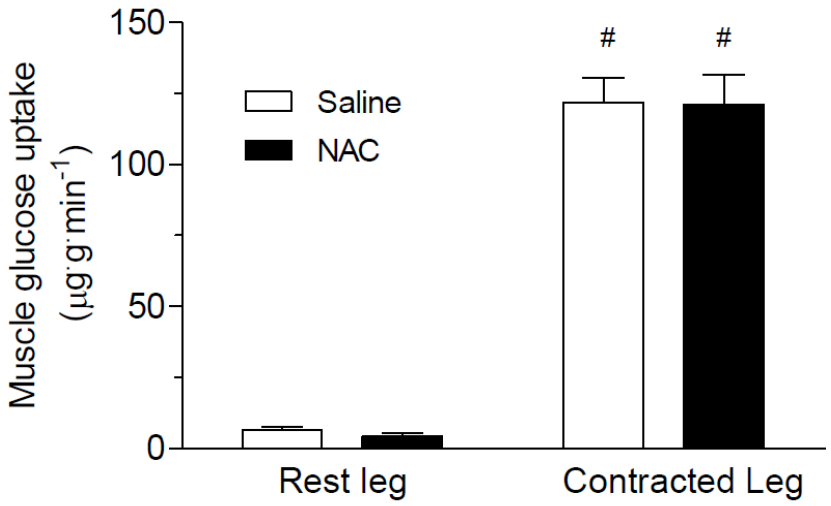

Figure 5.8 Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg muscle glucose uptake following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SEM, n=6 per group, #P<0.05 for condition (rest vs contraction).

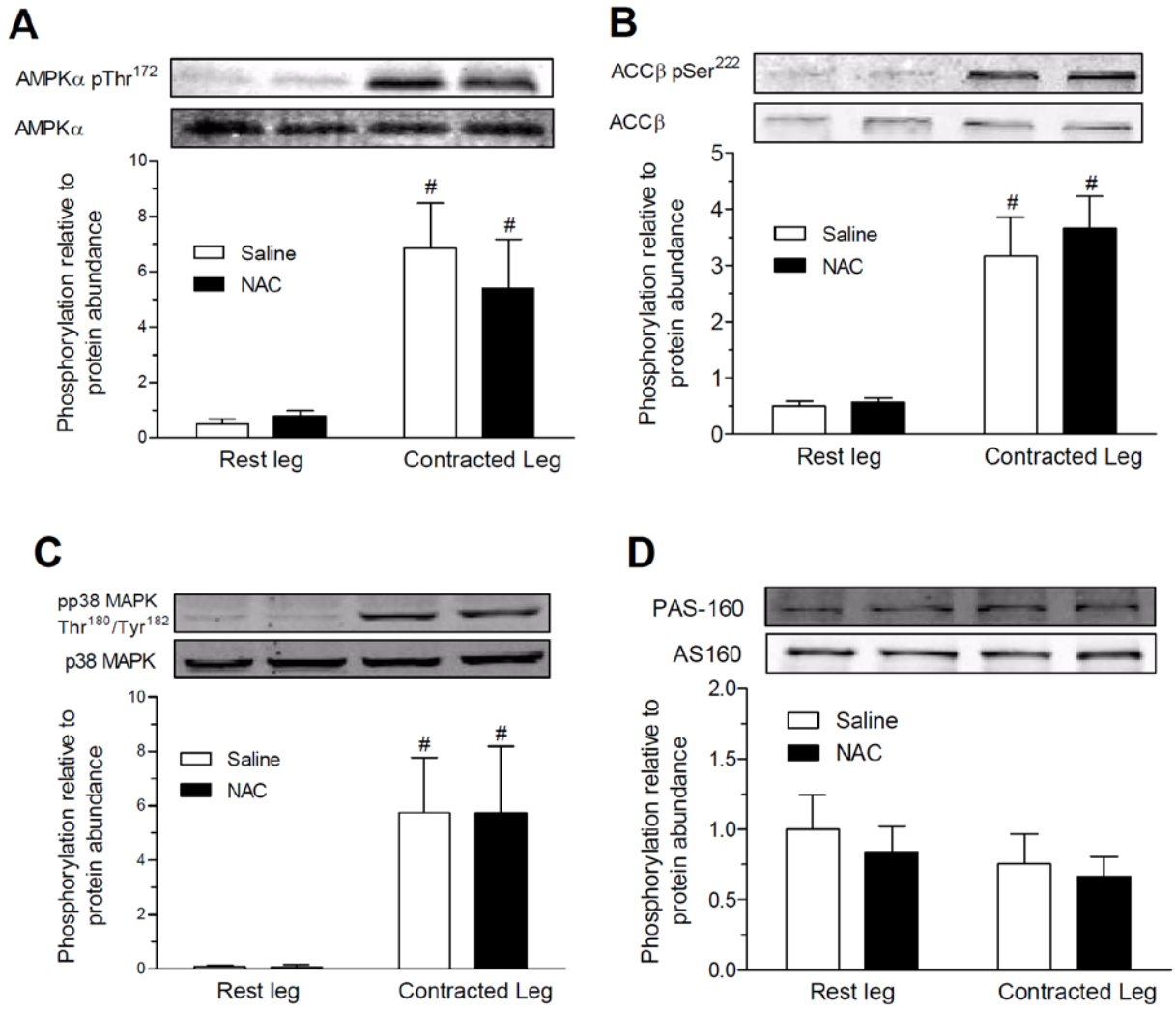

Figure 5.9 Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg AMPK Thr^{172} (A), ACCβ Ser^{222} (B) p38 MAPK Thr^{180}/Tyr^{182} (C) and PAS-160 (D) phosphorylation following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SEM, n=6 per group, #P<0.05 for condition (rest vs contraction).

5.4 DISCUSSION

The major finding of this study was that local infusion of the antioxidant N-acetylcysteine (NAC) does not attenuate the increase in rat hindlimb skeletal muscle glucose uptake (Figure 5.8), AMPK signalling or p38 MAPK phosphorylation (Figure 5.9) during *in situ* contractions. Although the low-moderate stimulation protocol did not affect muscle tyrosine nitration, reduced glutathione (GSH) or oxidised glutathione (GSSG) content (Figure 5.6), it significantly increased S-glutathionylation of protein bands at ~250 kDa and ~150 kDa and these increases in S-glutathionylation were prevented by NAC infusion (Figure 5.7). Therefore, because our low-moderate contraction protocol also substantially increased skeletal muscle glucose uptake and metabolic signalling (AMPK and p38 MAPK phosphorylation), and resulted in a physiological degree of fatigue, this study provides evidence to suggest that unlike during *ex vivo* contractions ROS are not essential for the regulation of skeletal muscle glucose uptake, AMPK signalling or p38 MAPK phosphorylation during low-moderate intensity contractions *in situ*.

The deacylation of NAC to produce cysteine (Deneke, 2000), both extra- and intra-cellularly, combined with the ability of cysteine to be transported into the cell (Bannai & Tateishi, 1986), resulted in an elevation of plasma and muscle cysteine during NAC infusion (Figure 5.6). Surprisingly, and in contrast to several studies in humans and rats (Sen *et al.*, 1994; Medved *et al.*, 2004b; Gomez-Cabrera *et al.*, 2005) a change in muscle GSH or GSSG following contraction was unable to be detected. Interestingly, however, similar to mouse muscle in Chapter 3 and 4 (Figures 3.9 and 4.7), S-glutathionylation of protein bands at ~250 and ~150 kDa was increased during contraction. This suggests that ROS production increased during the contraction protocol causing a small oxidative shift in cell redox that was not large enough to substantially deplete antioxidant defences and therefore alter glutathione levels, but sufficient to increase S-glutathionylation, which NAC prevented. The S-glutathionylated protein bands at ~150 kDa have not yet been identified, but the band at ~250 kDa is likely to be similar to the protein band S-glutathionylated during contraction at ~270 kDa in mouse (Chapter 4, *4.3.4 S-glutathionylation and tyrosine nitration*) and human muscle (Chapter 6, Figure 6.8) which appear to contain SERCA and Myh4 (for detailed discussion see Chapter 4, *4.4 Discussion*).

It is likely that no measurable changes in muscle glutathione status were seen in this study because of the low-moderate intensity (0.1 ms impulse at 2 Hz and 35 V), but physiological, short duration (15 min) stimulation protocol employed. In support, previously Sahlin *et al.* (1992) has shown that muscle glutathione levels are not affected during submaximal short duration (20 min) dynamic exercise in humans. Like glutathione status, muscle tyrosine nitration was also not affected by this contraction protocol (Figure 5B). Nitric oxide and superoxide interact to form peroxynitrite, which at high concentrations promotes protein tyrosine nitration (Halliwell, 1989). Tyrosine nitration is generally associated with deleterious inhibitory affects on muscle signalling (Pacher *et al.*, 2007), and therefore it is not surprising that if any peroxynitrite was produced during contraction it was not sufficient to increase tyrosine nitration. It is, however, important to acknowledge that the stimulation intensity employed is physiologically comparable to submaximal exercise in humans as it substantially increased muscle glucose uptake (Figure 5.8) and metabolic signalling (AMPK, ACCβ and p38 MAPK phosphorylation; Figure 5.9) without being highly fatiguing (Figure 5.1). Conversely, the majority of those studies reporting a reduction in muscle glutathione or an increase in GSSG/GSH ratio following contraction either employ exercise to exhaustion (Sen *et al.*, 1994; Gomez-Cabrera *et al.*, 2005) or prolonged high intensity exercise (>70% VO_{2peak}) (Svensson *et al.*, 2002; Medved *et al.*, 2004b; Zhang *et al.*, 2007) which results in greater increases in ROS production and depletion of antioxidant defences (O'Neill *et al.*, 1996; Ji, 1999; Reid, 2001). It is during such exercise that NAC can attenuate muscle GSH depletion (Supinski *et al.*, 1995; Medved *et al.*, 2004b) by promoting its resynthesis (Sen *et al.*, 1992; Cotgreave, 1997). Therefore, because our contraction protocol did not deplete GSH, there may have been little requirement for increased GSH resynthesis and thus NAC did not affect muscle GSH/GSSG content.

Unlike NAC treatment of mouse skeletal muscle during contractions *ex vivo* (Chapter 3, 4 and Sandstrom *et al.*, 2006), local NAC infusion during *in situ* hindlimb contractions in rats did not affect the increase in skeletal muscle glucose uptake (Figure 5.8). Similarly, NAC infusion did not affect blood glucose levels or anaerobic metabolism as indicated by similar increases in blood lactate in NAC and saline conditions (Figure 5.4). In support of findings during contractions *ex vivo* in Chapters 3 (Figure 3.6) and 4 (Figure 4.8), NAC did not affect AMPK phosphorylation, or the phosphorylation of the major downstream target of AMPK, ACCβ during *in situ* contractions (Figure 5.9). Thus, because contraction increased skeletal muscle glucose uptake and AMPK phosphorylation to a similar extent during saline and NAC

infusion (Figure 5.8 and 5.9), it is likely that the low levels of oxidative stress associated with physiological contraction intensities is not involved in the regulation of skeletal muscle glucose uptake or AMPK signalling. Although NAC infusion prevented increases in S-glutathionylation of protein bands at ~150 and ~250 kDa during contraction, suggesting that NAC attenuated some ROS signalling (Dalle-Donne *et al.*, 2009), it does not exclude the possibility that the concentration of NAC in the tissue may have been to low to prevent *all* ROS signalling events. Therefore, it may be that NAC-derived increases in antioxidant defences in the muscle were not sufficient to prevent ROS signalling of glucose uptake. Indeed, very high (20 mM) concentrations of NAC (which presumably results in even greater antioxidant reserves) are used to attenuate skeletal muscle contraction-stimulated glucose uptake *ex vivo* (Figures 3.11 and 4.4 and Sandstrom *et al.*, 2006). Future investigations should utilise different antioxidants, and multiple concentrations of antioxidants to further investigate the role of ROS in regulating glucose uptake in intact preparations.

Alternatively, or concurrently, it is possible that under contraction conditions which, unlike low-moderate intensity contractions, greatly elevate oxidative stress, ROS may play a greater role in the regulation of skeletal muscle glucose uptake than during lower intensities. Such conditions may include exhaustive endurance exercise, or *ex vivo* contraction conditions of supra-maximal stimulation, non-uniform oxygen delivery (due to oxygen diffusion limitations), and reduced antioxidant capacity (in the absence of antioxidant systems found in blood) (Reid, 2001). This may explain the discrepancy between NAC affects on skeletal muscle contraction-stimulated glucose uptake *ex vivo* (Chapter 3 and 4) and *in situ*. Therefore, experimental investigation is required to assess the role of ROS and oxidative stress in signalling glucose uptake during high intensity exercise in intact preparation with blood flow. However, the contraction intensity required to increase skeletal muscle ROS levels to a large enough extent to increase glucose uptake as seen in *ex vivo* preparations (Sandstrom *et al.*, 2006), is not likely to be physiologically realistic to humans. Therefore, the hypothesis that ROS regulate skeletal muscle glucose uptake during contraction may be an artefact of non-physiological *ex vivo* contraction conditions, and our results suggest that mechanisms other than ROS regulate skeletal muscle glucose uptake during normal submaximal muscle contractions.

It has been proposed that muscle glucose uptake during contraction is differentially regulated in muscles containing predominantly oxidative compared with muscles containing

predominantly glycolytic type fibres (Wright *et al.*, 2005). The hindlimb muscle sampled was estimated to contain only a small proportion of oxidative type fibres (~14%) (Laughlin & Armstrong, 1983). It is likely that ROS would play a greater signaling role in glycolytic fibres because they have lower levels of endogenous antioxidant enzymes (Laughlin *et al.*, 1990). However, since ROS regulated contraction-stimulated glucose uptake in muscle containing both predominantly glycolytic and oxidative fibres *ex vivo* (Chapter 3, Figure 3.11), the finding that NAC infusion did not affect hindlimb muscle glucose uptake during contraction is unlikely to be a result of fibre type composition of the sampled muscle.

Exogenous ROS have been shown to increase skeletal muscle p38 MAPK phosphorylation (Kefaloyianni *et al.*, 2006) and the attenuation of exercise-induced oxidative stress by the xanthine oxidase inhibitor, allopurinol, attenuates increases in p38 MAPK phosphorylation during exhaustive exercise (Gomez-Cabrera *et al.*, 2005). Indeed, there is some evidence, as discussed in detail in Chapter 4 (*4.4 Discussion*), that the inhibition of p38 MAPK attenuates glucose uptake during contraction (Somwar *et al.*, 2000), and ROS signalling via p38 MAPK may be involved in regulating skeletal muscle stretch-induced glucose uptake (Chambers *et al.*, 2009). Interestingly, we show here that *in situ* contractions can increase skeletal muscle p38 MAPK phosphorylation without significant alterations in the GSSG/GSH ratio, and that local NAC infusion, which prevented S-glutathionylation, does not affect p38 MAPK phosphorylation during contraction. This is in agreement with findings from Chapter 4 (Figure 4.10) that suggest that ROS may not be essential for increases in p38 MAPK phosphorylation during *ex vivo* contractions. The inconsistency between the results of Gomez-Cabrera *et al.* (2005) and ours may be related to the use of a xanthine oxidase specific inhibitor, rather than the use of a general antioxidant such as NAC and requires further investigation.

Similar to observations in mouse muscle contracted *ex vivo* (Figure 3.7 and 4.9), rat hindlimb muscle PAS-160 phosphorylation was not affected by contractions *in situ* or local NAC infusion during contraction. As discussed in detail in Chapter 3, this is likely the result of contraction duration and intensity. At moderate (67% VO_{2peak}) exercise intensities *in vivo* it appears that PAS-160 phosphorylation does not increase until 60 min of exercise (Treebak *et al.*, 2006). Therefore, given that our contraction protocol resulted in minimal fatigue and was only 15 min in duration, it is not surprising that contraction did not increase PAS-160 phosphorylation. However, it does suggest that because glucose uptake was considerably

increased (~20-fold; Figure 5.8) with this contraction protocol, PAS-160 phosphorylation is not essential for increases in skeletal muscle glucose uptake during *in situ* contraction.

A small oxidative shift in cell redox is required for optimal skeletal muscle contraction force, however, high levels of oxidative stress appear to contribute to the development of muscular fatigue (Reid, 2001). NAC treatment has been shown to attenuate fatigue during longer duration and/or more strenuous exercise/muscle contractions (Khawli & Reid, 1994; Reid *et al.*, 1994) than used in the current study by attenuating the exercise induced increases in oxidative stress (Supinski *et al.*, 1995; Medved *et al.*, 2004b). Since our contraction protocol did not alter skeletal muscle glutathione levels this suggests that the level of oxidative stress was not sufficient to negatively impact on muscle function, and this may therefore explain why NAC did not affect the rate of fatigue during contraction (Figure 5.2).

Although NAC was locally infused into the contracted hindlimb only, increased levels of NAC in the rest leg and plasma indicate NAC entered the systemic circulation. Interestingly, NAC had a small but significant affect on blood pressure during contraction (Figure 5.5). This suggests that NAC or ROS may have been exerting some systemic affects during contraction, which is worthy of further investigation. However, it is unlikely that these small systemic effects can account for the finding that ROS are not involved in the regulation of skeletal muscle glucose uptake during low-moderate intensity contractions *in situ*.

In conclusion, this study shows for the first time that local infusion of the antioxidant NAC during *in situ* hindlimb contractions in rats does not attenuate increases in skeletal muscle glucose uptake. Although the low-moderate intensity *in situ* muscle contraction protocol, which is more relevant to normal submaximal exercise than tetanic contractions associated with most *ex vivo* studies, did not alter muscle GSH/GSSG levels or tyrosine nitration, it did increase protein S-glutathionylation indicating a small increases in muscle oxidative stress. NAC prevented the increases in S-glutathionylation of protein bands at ~250 kDa and ~150 kDa during contraction, but did not affect the large increases in skeletal muscle glucose uptake (20-fold) or phosphorylation of AMPK and p38 MAPK. These results suggest that, unlike during highly fatiguing *ex vivo* contraction conditions in mice, ROS do not regulate skeletal muscle glucose uptake or metabolic signalling during physiologically relevant, low-fatiguing, skeletal muscle contractions *in situ* in rat.

CHAPTER SIX

N-ACETYLCYSTEINE INFUSION HAS NO EFFECT ON GLUCOSE DISPOSAL DURING PROLONGED MODERATE INTENSITY EXERCISE IN HUMANS

6.1 INTRODUCTION

In Chapters 3 (Figures 3.8 and 3.11) and 4 (Figures 4.3 and 4.4) the antioxidant N-acetylcysteine (NAC) was shown to attenuate increases in ROS production and glucose uptake during *ex vivo* contraction of skeletal muscle. These studies and Sandstrom *et al.* (2006) provide evidence that ROS signalling during contraction may, in part, mediate skeletal muscle glucose uptake. In contrast, however, it was reported in Chapter 5 that the local infusion of NAC into the hindlimb of rats during *in situ* contractions did not affect skeletal muscle glucose uptake (Figure 5.8). This provides evidence that, in contrast to *ex vivo* models, where there is no blood flow, ROS may not be involved in regulating skeletal muscle glucose uptake during *in situ* contraction in intact preparations. *Ex vivo* models of skeletal muscle contraction rely on diffusion gradients for substrate delivery and clearance, do not have uniform delivery of oxygen to all muscle fibres (Allen *et al.*, 2008), and generally involve supra-maximal highly fatiguing stimulation protocols (Allen *et al.*, 2008). These conditions may alter ROS production (Reid, 2008) and therefore the contribution of ROS signalling during contraction. Chapter 5 is the first study to have examined the involvement of ROS in regulating contraction-stimulated skeletal muscle glucose uptake in an intact physiological preparation, by employing an anaesthetised rat model (*in situ* contractions). Therefore, to further extend these studies to *in vivo* and physiologically realistic exercise conditions, investigations are required to determine the involvement of ROS in regulating skeletal muscle glucose uptake during *in vivo* exercise, particularly in humans.

ROS have been suggested to contribute to the regulation of skeletal muscle AMPK activity (Sandstrom *et al.*, 2006) and p38 MAPK phosphorylation (Gomez-Cabrera *et al.*, 2005) during contraction (see Chapter 3-5 for detailed discussion). As discussed in Chapters 3-5,

AMPK (Mu *et al.*, 2001; Sandstrom *et al.*, 2006; Lefort *et al.*, 2008) and p38 MAPK (Somwar *et al.*, 2000; Chambers *et al.*, 2009) have been implicated in the regulation of stretch- and contraction-stimulated skeletal muscle glucose uptake. In contrast, we found that ROS regulate *ex vivo* contraction stimulated skeletal muscle glucose uptake independent of AMPK (Figures 3.11) and p38 MAPK (Figure 4.4, 4.6 and 4.10), and ROS do not appear to be essential for contraction stimulated increases in p38 MAPK (Figures 4.10 and 5.9) or AMPK (Figures 3.5, 4.8 and 5.9) phosphorylation *ex vivo* or *in situ*. However, the involvement of ROS in regulating AMPK and p38 MAPK phosphorylation during exercise in humans has not previously been examined, and may be important for understanding the regulation of skeletal muscle contraction-stimulated glucose uptake.

As discussed in Chapters 3-5, we have found that there is S-glutathionylation of a protein band at ~270 kDa during contraction of mouse muscle *ex vivo* (Figures 3.9 and 4.7) and at ~250 kDa in rat muscle contracted *in situ* (Figure 5.7). In addition, we showed that this increase in S-glutathionylation can be attenuated by NAC (Figures 3.9, 4.7 and 5.7). The protein band at ~270 kDa in mouse muscle appears to contain SERCA1 and Myh4 (Chapter 4). It has also been shown that total S-glutathionylation of skeletal muscle increases during treadmill running to exhaustion in rats and this is blocked by the xanthine oxidase inhibitor allopurinol (Gomez-Cabrera *et al.*, 2005). No study has examined whether S-glutathionylation increases in skeletal muscle of humans during exercise and if so, if this is blocked by NAC. Determining whether ROS regulate S-glutathionylation during exercise in humans, and identifying any targets of S-glutathionylation may improve the understanding of the role of ROS signalling during exercise.

In a series of studies, Medved *et al.* (2003; 2004a; 2004b) demonstrated that NAC can be safely infused intravenously into humans during prolonged exercise. Moreover, the cysteine donating properties of NAC promoted the resynthesis of muscle reduced glutathione (GSH) during exercise indicating an attenuation of exercise-induced increases in skeletal muscle oxidative stress (Medved *et al.*, 2004b). Therefore, in the current study NAC was infused intravenously in humans during prolonged cycle ergometry to determine the role of ROS in the regulation of glucose disposal, metabolic signalling (via and AMPK and p38 MAPK phosphorylation) and S-glutathionylation during exercise. It was hypothesised that NAC infusion would attenuate the increases in glucose disposal and S-glutathionylation during exercise in humans without affecting AMPK or p38 MAPK phosphorylation.

6.2 METHODS

6.2.1 Participants

Nine healthy recreationally active ($\dot{V}O_{2\ peak}$: 51.7 ± 2.3 $ml \cdot kg^{-1} \cdot min^{-1}$) adult males volunteered. The participants' age, weight and height (mean ± SEM) were 23 ± 2 yr, 79.7 ± 3.4 kg, 179 ± 3 cm, respectively. As mentioned in Chapter 2, this study was approved by The University of Melbourne Human Ethics Committee and conducted in accordance with the Declaration of Helsinki.

6.2.2 Experimental design

As outlined in detail in Chapter 2 (*2.4.1 VO_2 Peak test and familiarisation*) on two separate occasions at least 1-2 weeks prior to experimental trial the participants completed a $\dot{V}O_{2\ peak}$ test and familiarisation trial. The experimental trials involved a double-blind randomised crossover design, with counterbalanced testing order. Experimental trials were conducted at the same time of day and separated by at least two weeks. A 24 h food diary was completed prior to the first trial, and this was photocopied and returned to the participant who was asked to follow the same diet prior to the second trial.

Participants fasted overnight and reported to the laboratory at 6.30 am on trial days, having abstained from exercise, caffeine and alcohol for the preceding 24 h. A 22-gauge cannula was inserted into an antecubital forearm vein for the infusion of stable isotope glucose tracer (6,6-^{2}H-glucose; Cambridge Isotope Laboratories, MA, USA) and NAC (Parvolex, Faulding Pharmaceuticals, Salisbury, SA, Australia), and another cannula was inserted into the contralateral forearm for blood sampling. An initial blood sample was obtained and then the glucose tracer was administered as described in Chapter 2 (*2.3.4 Glucose kinetics*). As described previously by Medved *et al.* (2003), an initial loading dose of either NAC (125 $mg \cdot kg^{-1} \cdot h^{-1}$ in 0.9% saline) or 0.9% saline alone (control; CON) was co-infused intravenously 35 min prior to exercise for 15 min, followed by a constant infusion (25 $mg \cdot kg^{-1} \cdot h^{-1}$ NAC) that continued until the end of exercise. As outlined in Figure 6.1, following 35 min of NAC/CON infusion at rest, participants cycled for 80 min

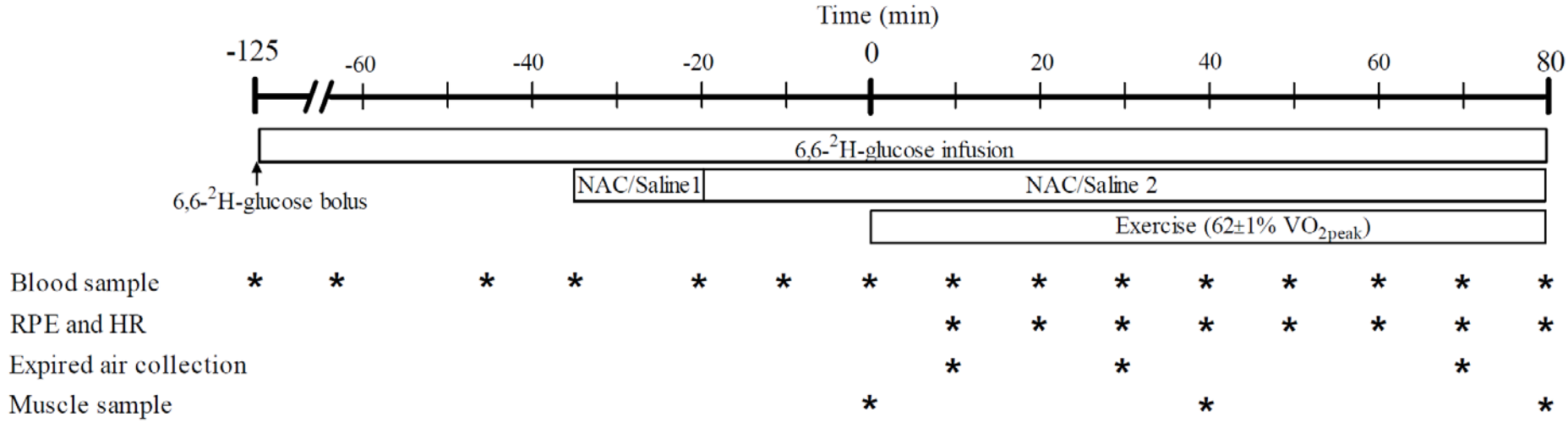

Figure 6.1 Outline of the experimental protocol. RPE= Rating of perceived exertion, HR= Heart rate, NAC/CON 1= Infusion of N-acetylcysteine (NAC; 125 $mg \cdot kg^{-1} \cdot h^{-1}$) or saline (CON), NAC/CON 2= Infusion of N-acetylcysteine at lower rate (25 $mg \cdot kg^{-1} \cdot h^{-1}$) or saline (CON).

at 62±1% $\dot{V}O_{2\ peak}$ in standard laboratory conditions (~20°C) with a fan on high setting positioned ~1 m directly in front of the handlebars. Water was consumed *ad libitum*.

6.2.3 Experimental trial sample collection and measurements

Heart rate was measured using a heart rate monitor (Polar Favor, Oulu, Finland) and recorded at 10 min intervals during exercise. Expired respiratory gases were sampled for 3 min at *t*=10, 30 and 70 min, and volume expired, O_2 and CO_2 content were measured as described in Chapter 2 (*2.4.1 VO_2 Peak test and familiarisation*). Rating of perceived exertion (Borg, 1975) was obtained at 10 min intervals during exercise, and any adverse reactions to the infusions were recorded during the trial as described by Medved *et al.* (2003; 2004b) (see Appendix A) Venous blood samples were obtained at *t*= -120, -65, -45, -35 and -20 min (for glucose kinetics), and then every 10 min until the end of exercise, and vastus lateralis muscle samples were obtained at *t*= 0, 40 and 80 min (Figure 6.1). See Chapter 2 (*2.4.2 Blood and muscle sampling*) for details.

6.2.4 Plasma analysis

Plasma lactate, glucose, non-esterified fatty acids (NEFA) and insulin levels were determined, as well as plasma NAC and thiol content (see Chapter 2 for plasma collection and storage details). The thiols measured were cysteine and glutathione as markers of antioxidant defences and oxidative state (see Chapter 2 for details). Plasma was also analysed for the % enrichment of 6,6-^{2}H-glucose and glucose appearance rate (glucose R_a), glucose disappearance rate (glucose R_d) and glucose clearance rate (glucose CR) were estimated from the changes in % enrichment and plasma glucose concentration. The methodology used for these analytical procedures is described in detail in Chapter 2.

6.2.5 Muscle analysis

Muscle lactate, glycogen, ATP, creatine and creatine phosphate were determined (see Chapter 2 for details), and free ADP and free AMP were calculated as described previously (Chen *et al.*, 2000). Muscles samples were also analysed for AMPKα Thr^{172} phosphorylation, ACCβ Ser^{221} phosphorylation, PAS-160 phosphorylation, p38 MAPK Thr^{180}/Tyr^{182} phosphorylation, S-glutathionylation, tyrosine nitration, and sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase 1 (SERCA1) protein, NAC and thiol content. The methodologies used for these analytical procedures and calculations are described in detail in Chapter 2.

6.2.6 Statistical analysis

All data are expressed as means ± SEM. Results were analysed by SPSS statistical package using two-factor (condition x time) repeated measures ANOVA. Since NAC infusion started pre-exercise. To assess any effects at rest, the ANOVA was partitioned to assess the effect of NAC during rest (-30 to -10 min) and during exercise (0 to 80 min). If the ANOVA revealed a significant treatment by time interaction, specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

6.3 RESULTS

6.3.1 Respiratory measures, heart rate and rating of perceived exertion

Participants exercised at the same workload (130 ± 22 watts) and as would be expected there was no sign diff in $\dot{V}O_2$ between the two trials, (62 ± 1% $\dot{V}O_{2\ peak}$). During exercise there were also no significant differences in respiratory exchange ratio (RER), heart rate (HR) or rating of perceived exertion (RPE) between the treatments (Table 6.1).

6.3.2 Muscle and plasma NAC and adverse reactions

No adverse reactions to either NAC or CON infusions were observed. By the onset of exercise NAC infusion increased ($P<0.05$) plasma NAC and plasma reduced NAC concentration to 148.2 ± 16.1 μmol and 79.8 ± 18.4 μmol, respectively, and these concentrations were maintained throughout exercise (Figure 6.2A). NAC infusion increased the levels of resting muscle NAC and resting muscle reduced NAC to 74.2 ± 22.5 and 46.2 ± 16.4 pmol·mg^{-1} wet wt, respectively, and these levels remained essentially unchanged during exercise (Figure 6.2B). NAC was not detected in the plasma or muscle during saline infusion.

6.3.3 Plasma thiols

NAC infusion elevated plasma cysteine ($P<0.01$), and tended to increase plasma cystine ($P=0.07$) (Figure 6.3). Plasma cysteine concentration was increased by exercise during NAC ($P<0.05$), but not CON infusion (Figure 6.3A). Exercise did not affect plasma cystine (Figure 6.3).

6.3.4 Plasma lactate, NEFA and insulin

During exercise plasma insulin concentration decreased ($P<0.01$) and plasma NEFA and lactate concentration increased ($P<0.05$) to a similar extent in the two trials (Figure 6.4).

6.3.5 Glucose kinetics

Plasma glucose concentration was not affected by NAC infusion or exercise (Figure 6.5A; $P<0.05$). The increase in glucose appearance rate (glucose R_a,) glucose disappearance rate (glucose R_d), and glucose clearance rate (glucose CR) during resting pre-exercise infusion period ($P<0.05$) was not affected by NAC infusion (Figure 6.5). Glucose R_a, R_d, and CR

increased with exercise (P<0.05) and this increase was not affected by NAC infusion (Figure 6.5).

6.3.6 Muscle thiols

NAC infusion increased muscle cysteine at rest and during exercise (P<0.05), but cystine was only increased during exercise (P<0.05; Figure 6.6B and D). Muscle cysteine was increased with exercise at 40 min and cystine at 40 and 80 min but only during NAC infusion (P<0.05; Figure 6.6B and D). Muscle GSH, GSSG or GSSG/GSH ratio was not affected by exercise or NAC infusion (Figure 6.6A, C and E).

6.3.7 S-glutathionylation and tyrosine nitration

Exercise increased (P<0.05) muscle S-glutathionylation of a protein band at ~270 kDa (Figure 6.7A) by ~3-fold and this was prevented by NAC infusion. Exercise did not affect S-glutathionylation of any other protein bands. Muscle tyrosine nitration was not significantly affected by exercise or NAC infusion (Figure 6.7B).

6.3.8 SERCA1 protein

In Chapter 4 evidence is provided that a protein band S-glutathionylated at ~270 kDa in mouse muscle contains SERCA1. Since SERCA1 has a molecular weight of 110 kDa, whether SERCA1 was present at ~270 kDa was investigated by probing S-glutathionylation extracted samples (i.e. non-reducing extraction) with a SERCA1 antibody. Figure 6.8 shows that SERCA appears to be present as ~270, ~135 as well as ~110 kDa.

6.3.9 Muscle metabolites

NAC infusion had no affect on resting muscle metabolite concentrations (Table 2). Exercise did not affect muscle ATP concentration (P>0.05), but resulted in an increase in muscle lactate, creatine content and calculated free ADP, free AMP and free AMP/ATP ratio (P<0.05), and a reduction in muscle PCr and glycogen content. There were no significant differences between treatments (Table 6.2).

6.3.10 AMPK*α*, ACC*β*, PAS-160 and p38 MAPK phosphorylation

Exercise increased skeletal muscle AMPKα Thr^{172} and ACCβ Ser^{221} phosphorylation by ~3- and ~6-fold respectively, and these increases were not affected by NAC infusion (Figure 6.9A and B). PAS-160 phosphorylation was not affected by exercise or NAC infusion (Figure

6.10A). Exercise transiently increased p38 MAPK Thr^{180}/Tyr^{182} phosphorylation in CON infusion, with a significant elevation being evident at 40 but not 80 min (Figure 6.10B). NAC infusion prevented the increase in p38 MAPK Thr^{180}/Tyr^{182} phosphorylation at 40 min, but increased phosphorylation above that of CON at 80 min (Figure 6.10B).

Table 6.1. Mean physiological responses to exercise at 62±1% $\dot{V}O_{2\,peak}$ during saline (CON) or saline + N-acetylcysteine (NAC) infusion.

	CON	**NAC**
$\dot{V}O_2$ ($L \cdot min^{-1}$)	2.6 ± 0.1	2.5 ± 0.2
RER	0.90 ± 0.02	0.91 ± 0.02
HR (BPM)	154 ± 2	154 ± 4
RPE	13 ± 1	14 ± 2

N=9, $\dot{V}O_2$ = oxygen consumption, RER= respiratory exchange ratio, HR= heart rate, BPM= beats per minute, RPE= rating of perceived exertion

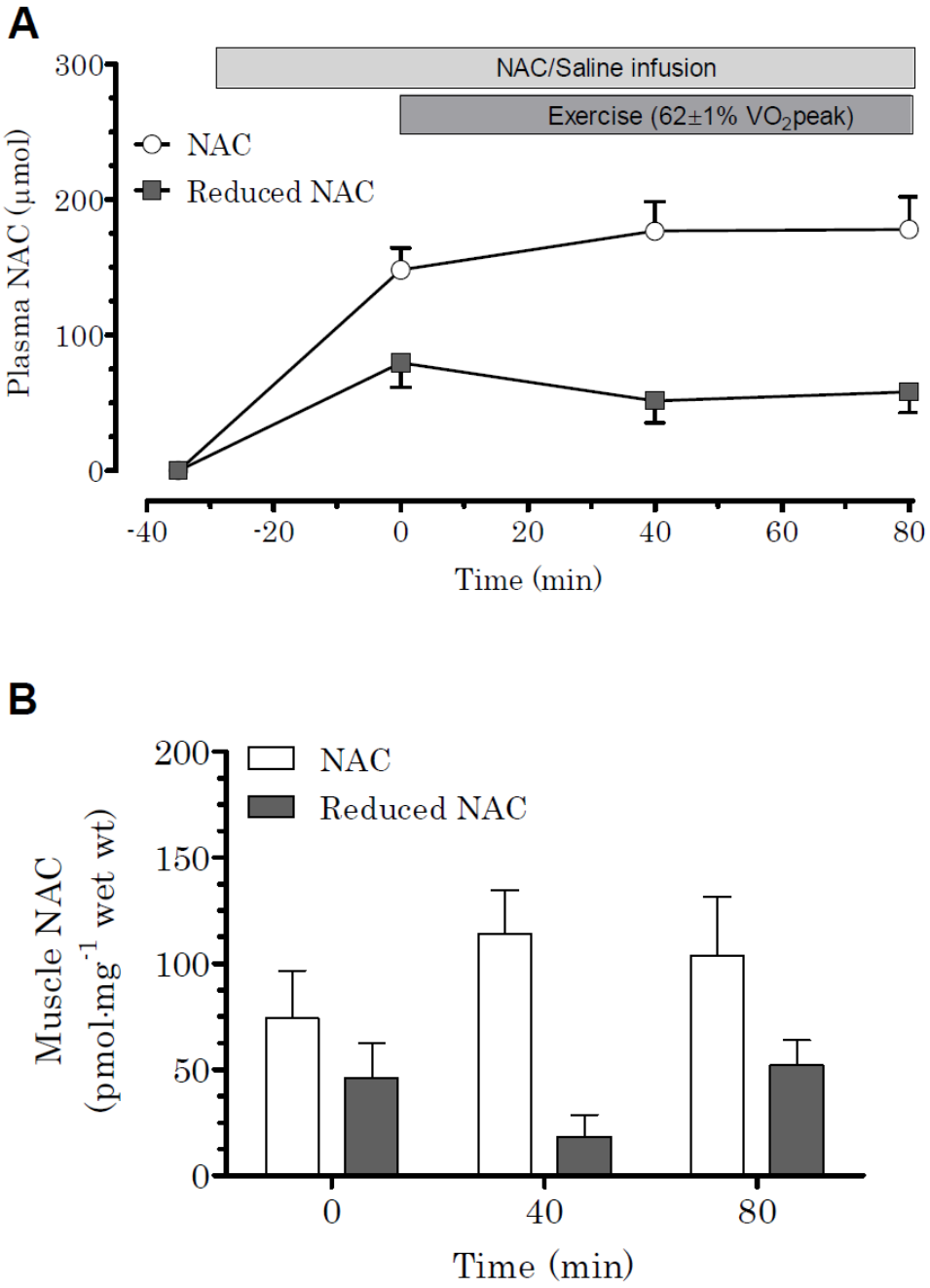

Figure 6.2 Plasma (A) and muscle (B) N-acetylcysteine (NAC) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\,peak}$ while receiving NAC infusion. Data are means ± SEM, n=8.

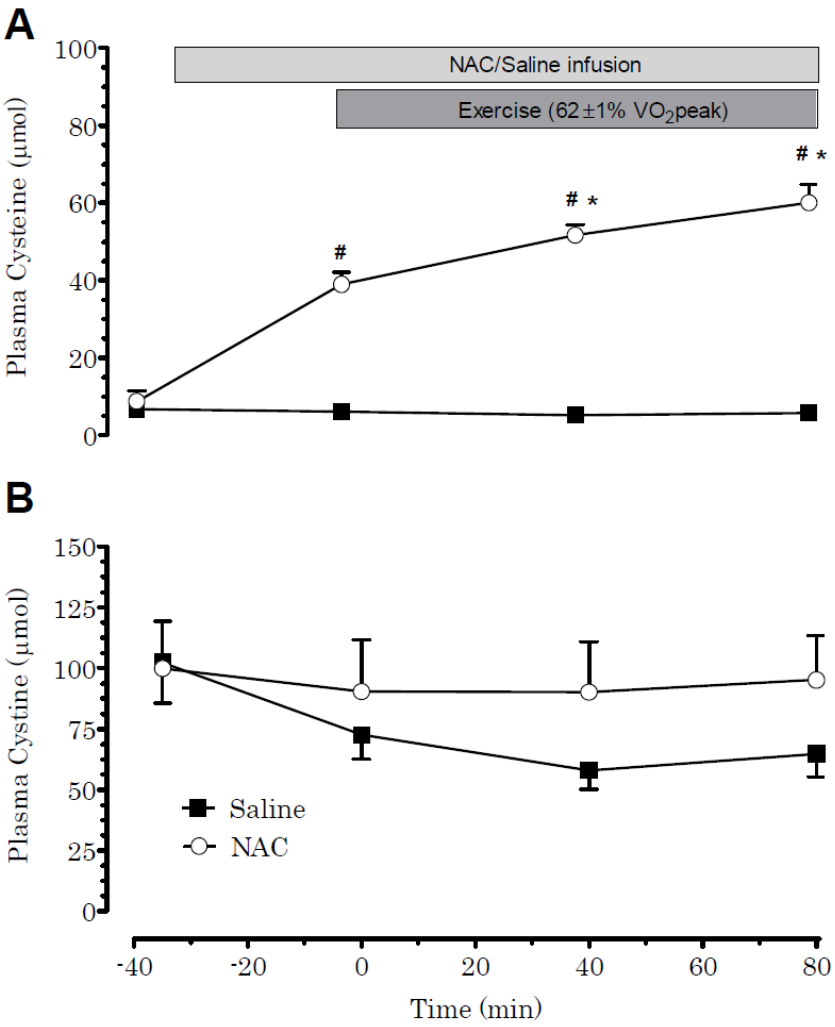

Figure 6.3 Plasma cysteine (A) and cystine (B) and oxidised glutathione (GSSG; C) concentration at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Data are means ± SEM, n=8, #P<0.05 vs saline (CON), *P<0.05 vs. *t*=0 of same treatment.

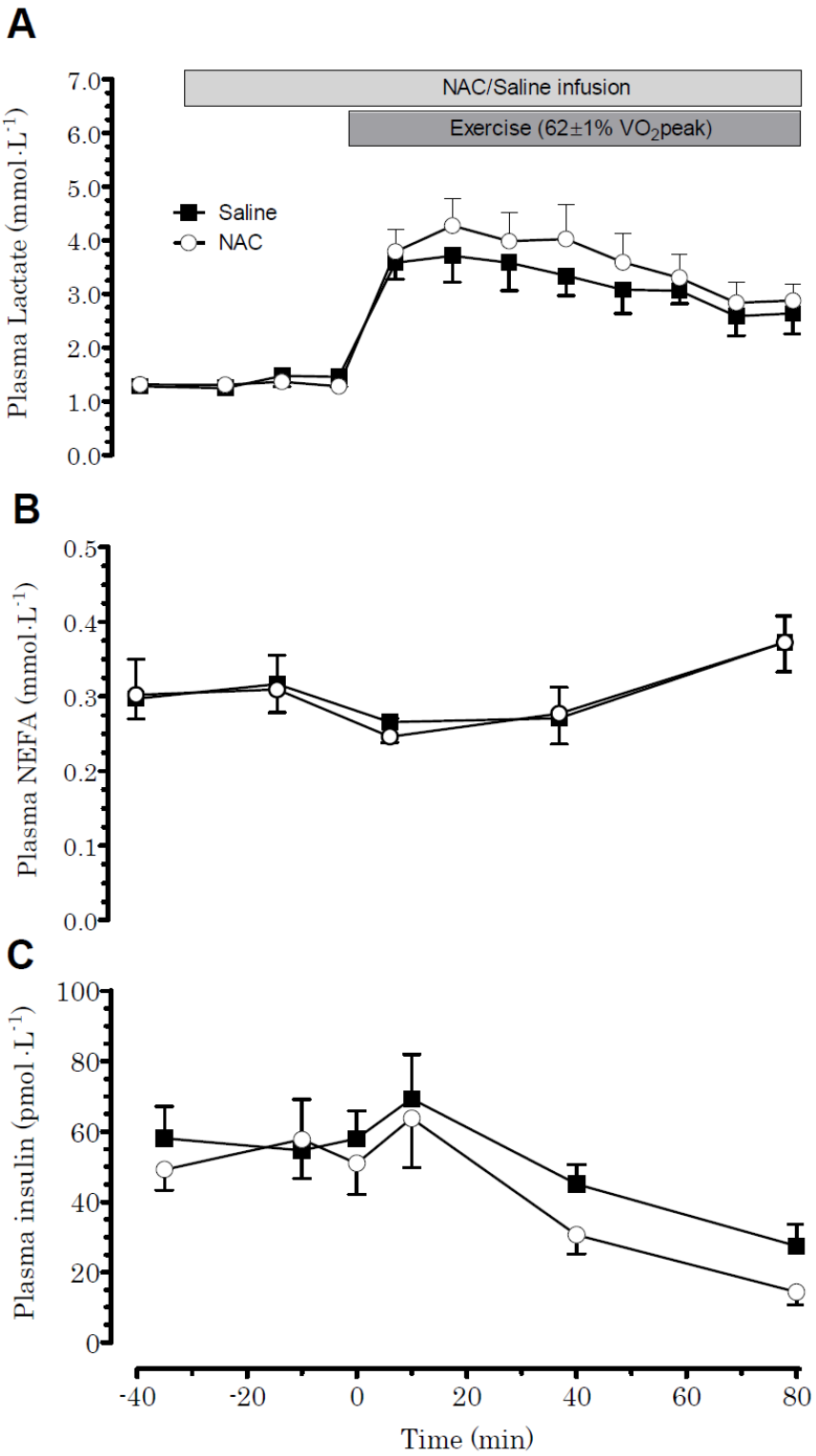

Figure 6.4 Plasma lactate (A), non-esterified free fatty acids (NEFA; B) and insulin (C) concentration at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Data are means ± SEM, n=9.

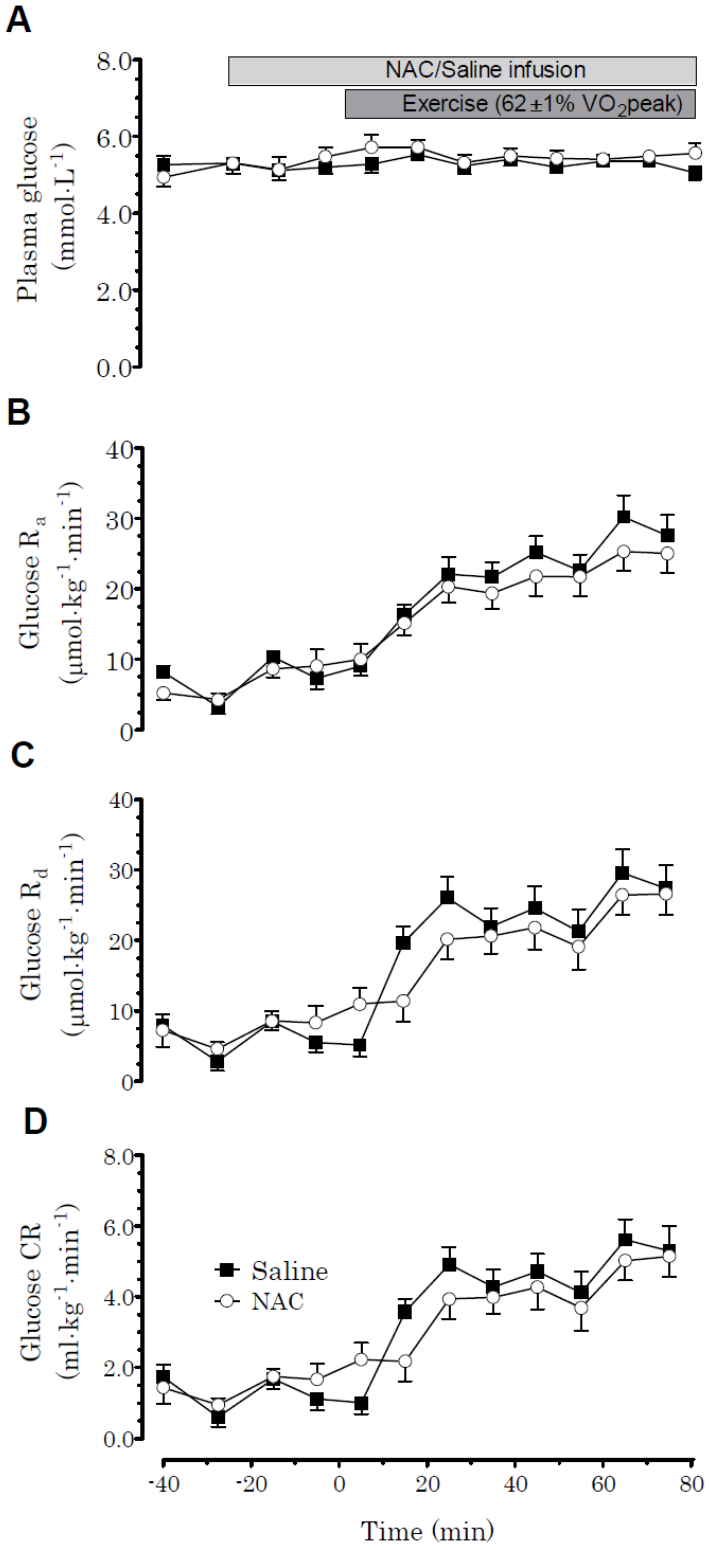

Figure 6.5 Plasma glucose (A), rate of glucose appearance (glucose Ra; B), rate of glucose disappearance (glucose Rd; C) and glucose clearance rate (glucose CR; D) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\,peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Data are means ± SEM, n=9.

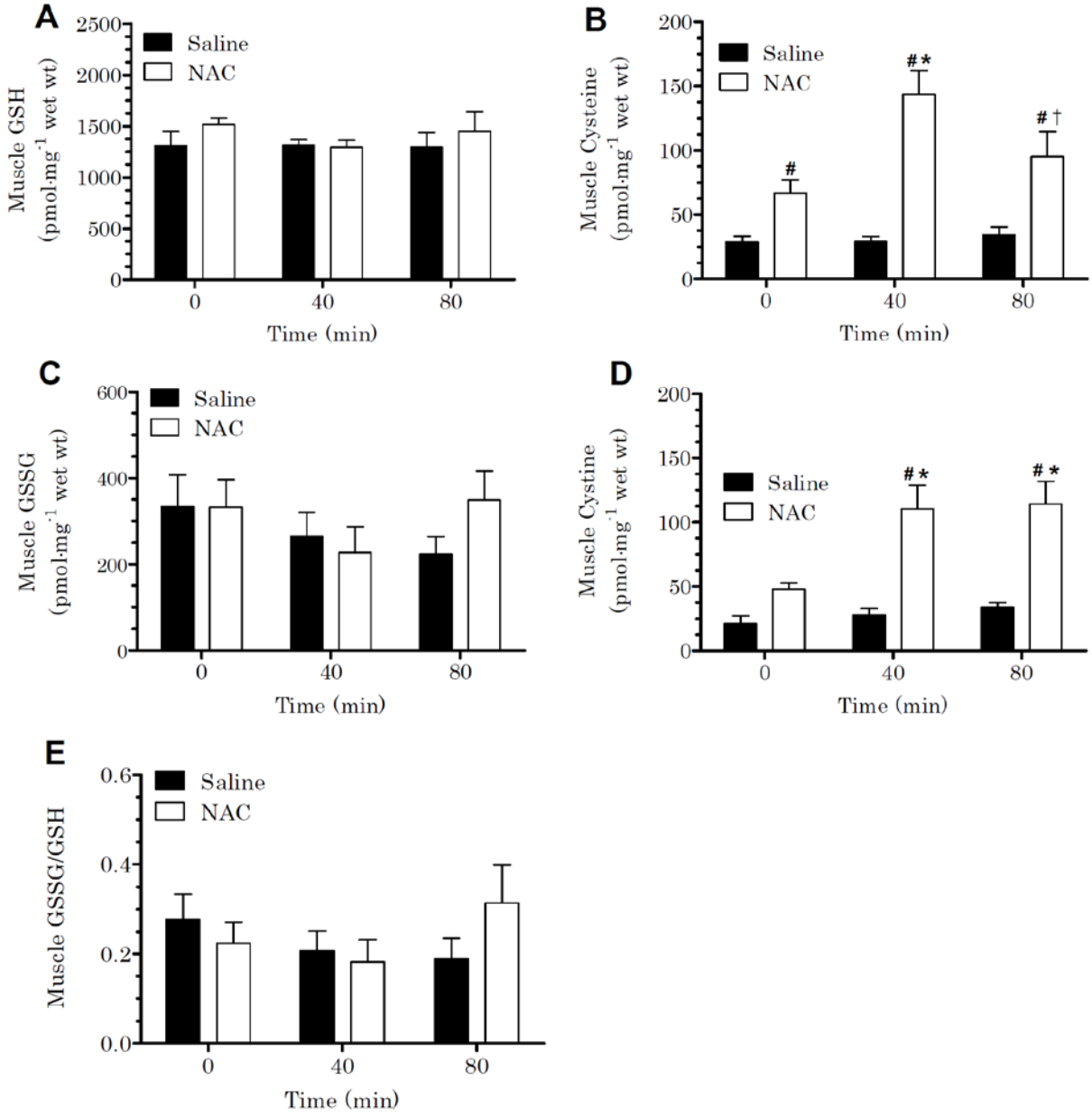

Figure 6.6 Muscle reduced glutathione (GSH; A), cysteine (B), oxidised glutathione (GSSG; C), cystine (D) and GSSG/GSH ratio (E) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Data are means ± SEM, n=9, #P<0.05 vs. saline (CON), *P<0.05 vs *t*=0 min of same treatment, †P<0.05 vs. *t*=40 min of same treatment.

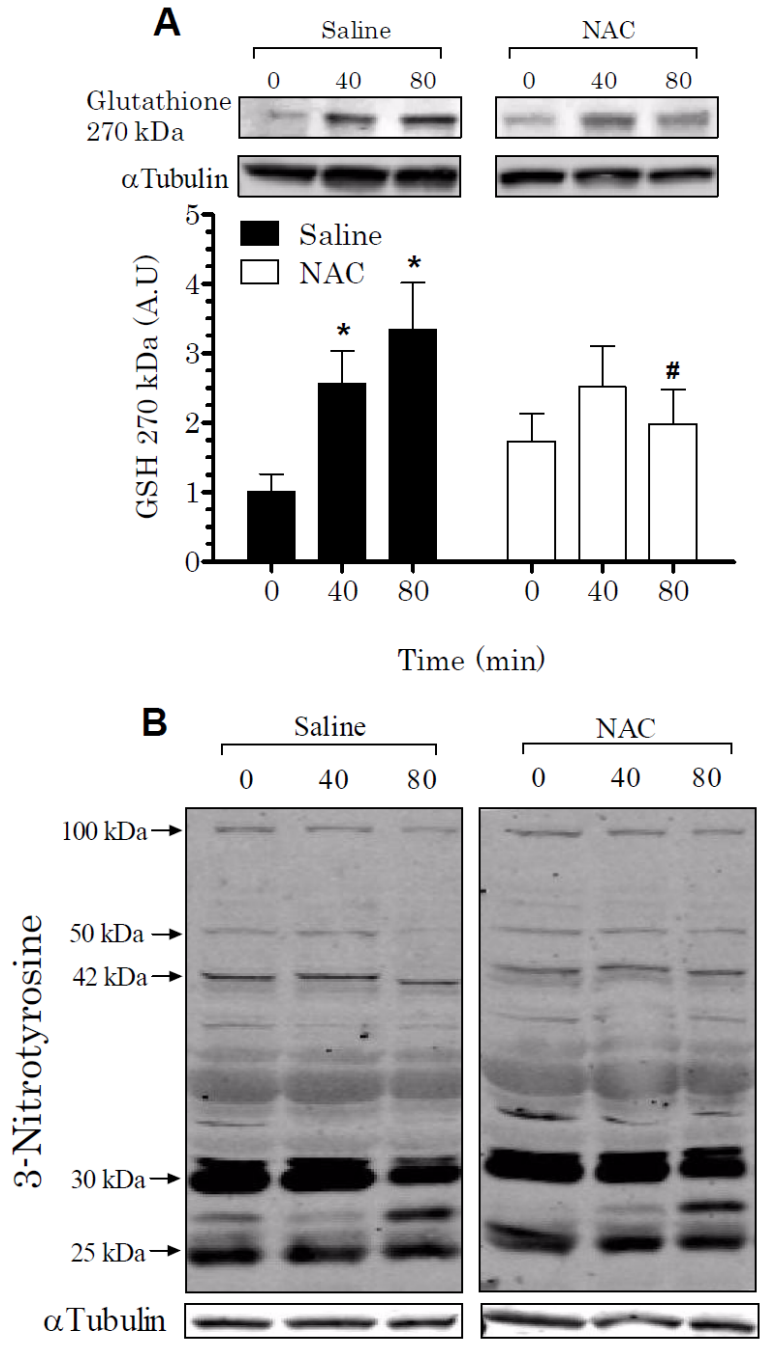

Figure 6.7 Muscle protein S-glutathionylation (A) and tyrosine nitration (B) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ saline (CON) or N-acetylcysteine (NAC) infusion. Data are means ± SEM, n=9, #P<0.05 vs. saline (CON), *P<0.05 vs. *t*=0 min of same treatment.

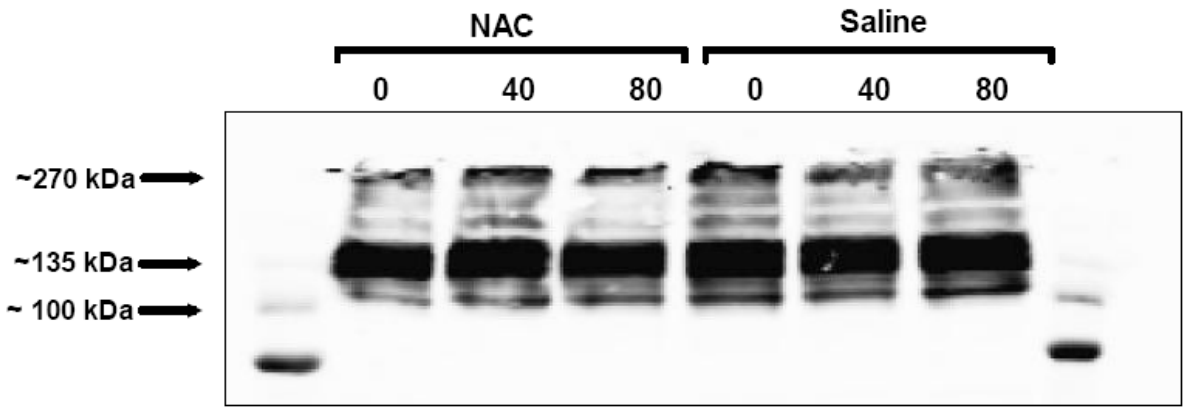

Figure 6.8 Muscle extracted using S-glutathionylation extraction method (non-reducing) and probed with SERCA1 antibody at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\,peak}$ saline (CON) or N-acetylcysteine (NAC) infusion.

Table 6.2 Muscle metabolites at rest and during exercise at 62±1% $\dot{V}O_{2\,peak}$ while receiving saline (CON) or N-acetylcysteine (NAC) infusion.

Metabolite	Trial	0 min	40 min	80 min
Muscle lactate	CON §	3.4 ± 0.7	21.7 ± 4.5	19.0 ± 6.1
(mmol·kg^{-1} d·m)	NAC §	4.2 ± 0.5	12.8 ± 2.9	18.2 ± 3.8
PCr	CON §	94.8 ± 3.8	63.4 ± 5.5	67.2 ± 4.8
(mmol·kg^{-1} d·m)	NAC §	95.6 ± 4.2	72.2 ± 4.5	67.4 ± 7.6
Cr	CON §	46.6 ± 1.7	78.0 ± 3.6	74.3 ± 4.2
(mmol·kg^{-1} d·m)	NAC §	45.8 ± 2.6	69.2 ± 7.1	74.1 ± 6.4
ATP	CON	25.9 ± 0.3	25.2 ± 1.0	25.2 ± 0.8
(mmol·kg^{-1} d·m)	NAC	26.2 ± 0.7	25.6 ± 1.1	23.7 ± 1.4
AMPfree	CON §	0.6 ± 0.1	3.2 ± 0.6	2.5 ± 0.4
(mmol·kg^{-1} d·m)	NAC §	0.6 ± 0.1	2.5 ± 0.9	2.5 ± 0.9
ADPfree	CON §	120.8 ± 5.2	266.6 ± 25.1	239.9 ± 21.6
(µmol·kg^{-1} d·m)	NAC §	115.0 ± 9.2	219.5 ± 34.2	218.1 ± 42.0
AMPfree:ATP	CON §	0.02 ± 0.00	0.13 ± 0.03	0.09 ± 0.01
	NAC §	0.02 ± 0.00	0.11 ± 0.05	0.11 ± 0.03
Muscle glycogen	CON §	373.6 ± 18.7	216.9 ± 16.9	177.8 ± 26.5
(mmol·kg^{-1} d.m)	NAC §	348.5 ± 24.1	266.1 ± 23.6	206.8 ± 33.7

§P<0.05 for time effect, n=9, PCr= creatine phosphate, Cr= creatine, ATP= Adenosine 5'-triphosphate, ADP= Adenosine 5'-diphosphate, AMP= adenosine-5'-monophosphate.

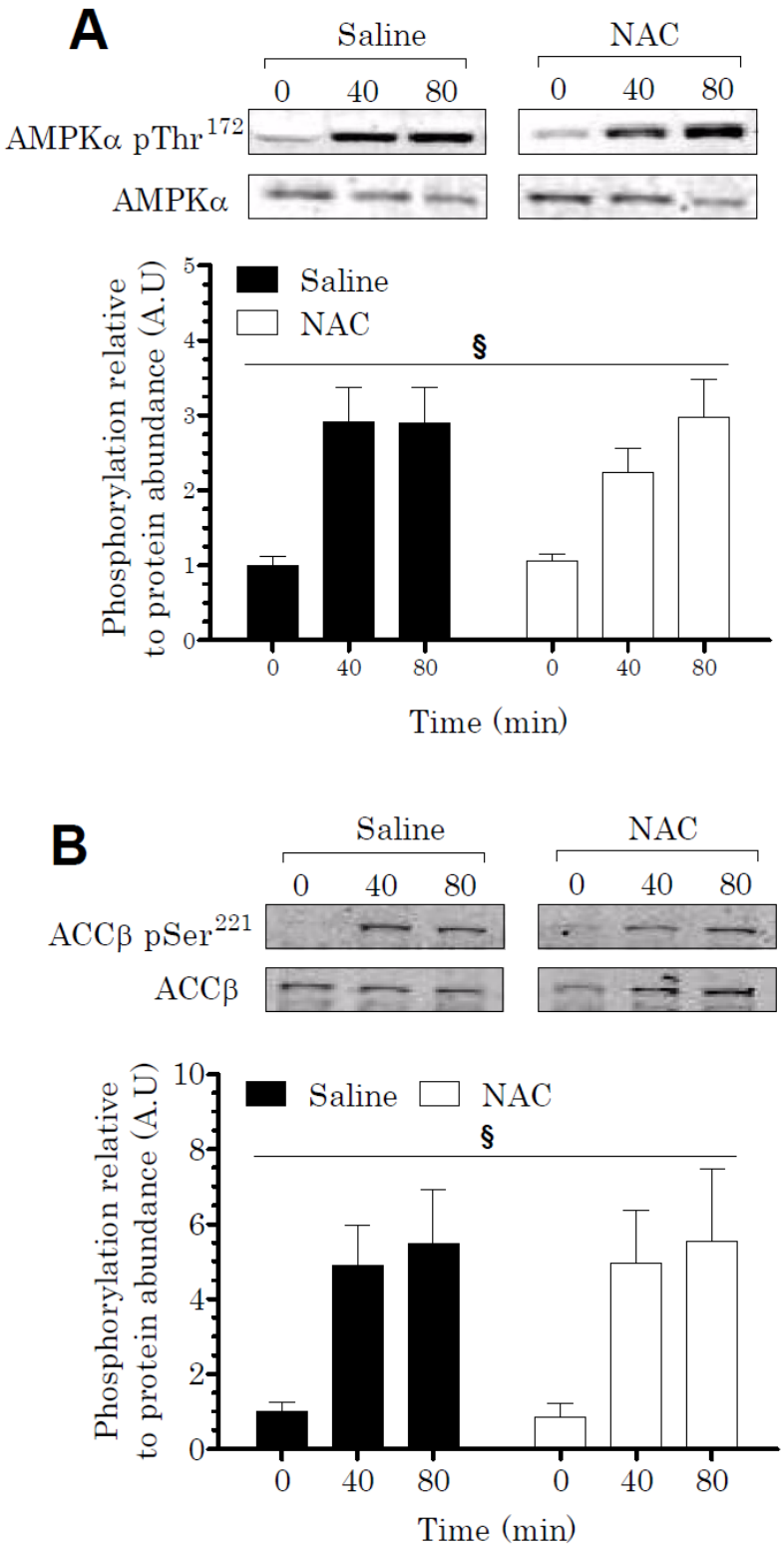

Figure 6.9 Muscle AMPK Thr^{172} phosphorylation (A) and ACCβ Ser^{221} phosphorylation (B) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Western blots are representative of one participant. Data are means ± SEM, n=9, §P<0.05 for time effect.

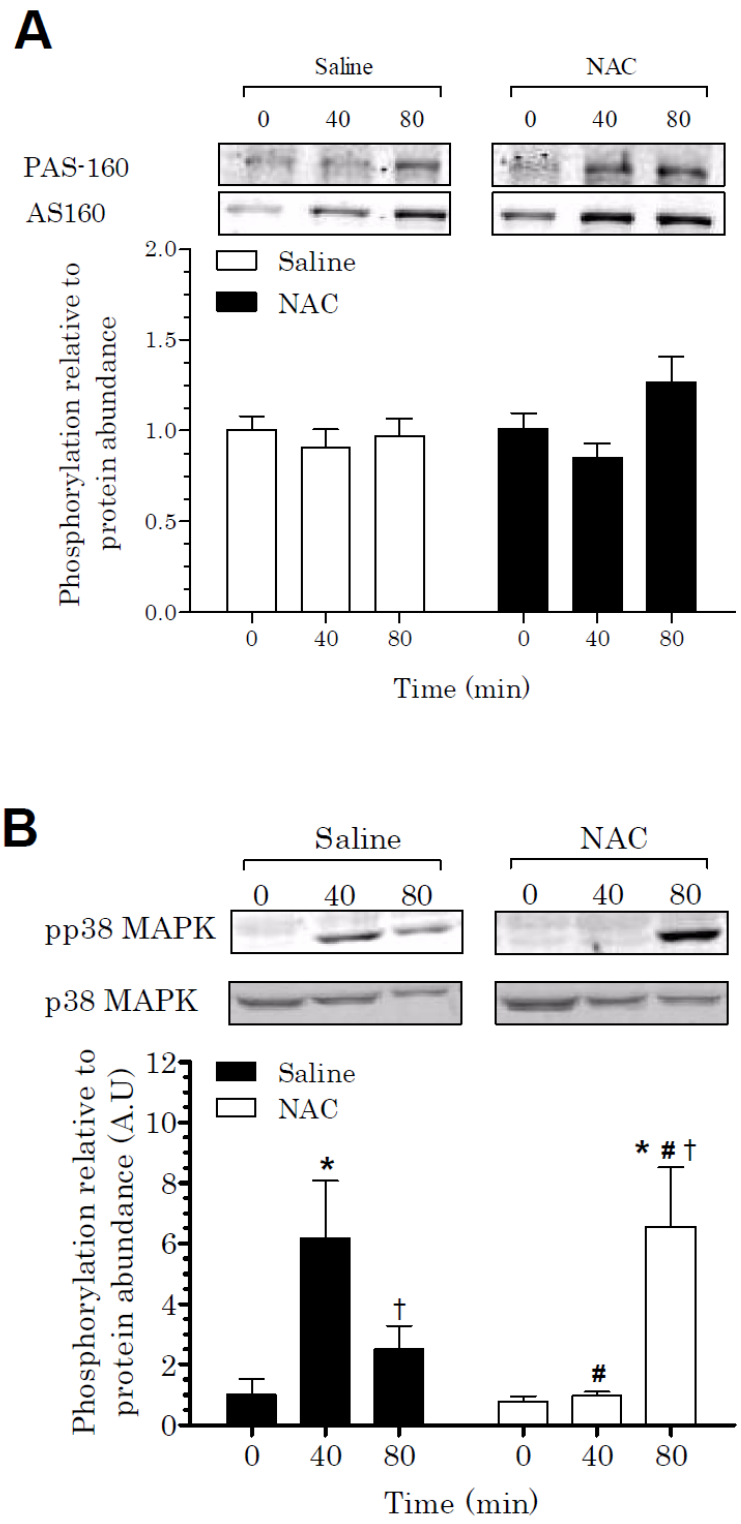

Figure 6.10 Muscle PAS-160 phosphorylation (A) and p38 MAPK Thr^{180}/Tyr^{182} phosphorylation (B) at rest and during 80 min of steady state exercise at 62±1% $\dot{V}O_{2\ peak}$ while receiving either saline (CON) or N-acetylcysteine (NAC) infusion. Western blots are representative of one participant. Data are means ± SEM, n=9, *P<0.05 vs. rest (0 min) of same condition, †P<0.05 vs. 40 min of same conditions, #P<0.05 vs. saline at same time point.

6.4 DISCUSSION

The major finding of this study was that the systemic infusion of NAC in humans did not affect glucose disposal during prolonged exercise. Secondly, we show that skeletal muscle AMPK signalling during exercise is unaffected by NAC infusion, although some influence on p38 MAPK phosphorylation was observed. Interestingly, as we found in rodent muscle contracted *ex vivo* and *in situ*, skeletal muscle S-glutathionylation of a protein band at ~270 kDa was increased during moderate intensity exercise, despite no significant changes in muscle GSH and GSSG, and NAC infusion prevented this increase in S-glutathionylation. Furthermore, as was the case in rodent muscle, the protein band S-glutathionylated at ~270 kDa appeared to contain SERCA1.

The antioxidant NAC has been shown to attenuate skeletal muscle glucose uptake during contractions *ex vivo* (Sandstrom *et al.*, 2006; Figures 3.11 and 4.6), but not to affect skeletal muscle glucose uptake during *in situ* contractions (Figure 5.8). In agreement with findings from Chapter 5 (Figure 5.8), NAC infusion does not affect glucose disposal during exercise in humans (Figure 6.5). Furthermore, other measures of substrate utilisation during exercise such as respiratory exchange ratio (RER), muscle glycogen use and plasma NEFA concentration were not affected by NAC infusion (Table 1, 2 and Figure 4). Importantly, NAC infusion also did not affect other possible mediators of glucose uptake such as plasma insulin or skeletal muscle metabolic stress as indicated by similar exercise-induced changes in muscle metabolites, oxygen consumption and plasma lactate during CON and NAC infusion trials (Table 1, 2 and Figure 6.4). Since skeletal muscle is responsible for >95% of glucose disposal during exercise (Jeukendrup *et al.*, 1999), this suggests that ROS signalling may not be essential for the regulation of skeletal muscle glucose uptake during moderate intensity exercise in humans. However, although the NAC dose infused was sufficient to prevent exercise-stimulated increases in S-glutathionylation at ~270 kDa, it may not have increased muscle NAC or cysteine levels enough to prevent all ROS signalling events during exercise. Indeed, a much higher NAC concentrations (20 mM) is used to attenuate skeletal muscle glucose uptake during *ex vivo* contractions (Sandstrom *et al.*, 2006; Chapters 3 and 4). Therefore, based on our findings ROS cannot be excluded from playing a role in the regulation of skeletal muscle glucose uptake during exercise in humans.

Sandstrom *et al*. (2006) has previously reported that NAC similarly attenuates contraction-stimulated glucose uptake and AMPK activity to a similar extent during *ex vivo* skeletal muscle contraction in mouse muscle. In contrast, as was the case *ex vivo* (Figures 3.5, 3.6 and 4.8) and *in situ* (Figure 5.9), we found that NAC infusion did not affect AMPK signalling (AMPK and ACCβ phosphorylation) during exercise in humans (Figure 6.9). High concentrations of exogenous ROS lower cell energy levels (AMP/ATP and Creatine/PCr ratio) (Leon *et al.*, 2004), activate AMPK and increase skeletal muscle glucose uptake (Toyoda *et al.*, 2004). However, NAC infusion did not affect moderate intensity exercise-induced lowering of cell energy in the present study, suggesting that the increase in ROS levels during moderate intensity exercise was not sufficient to alter cell energy status (Table 2). As discussed in detail in Chapter 3, it is possible that the highly fatiguing *ex vivo* muscle contraction protocol utilised by Sandstrom *et al*. (2006) may have resulted in higher than physiological ROS levels in skeletal muscle (Reid, 2001) which then altered cell energy balance and subsequently activated AMPK (Higaki *et al.*, 2008). Therefore, it may be that during high intensity exhaustive exercise in humans, when ROS production is greatly elevated and substantially increases skeletal muscle oxidative stress (Reid & Li, 2001; Medved *et al.*, 2004b; Zhang *et al.*, 2007), ROS may be involved in the regulation of skeletal muscle glucose uptake potentially via AMPK.

Antioxidant treatment (allopurinol) has previously been shown to abolish p38 MAPK phosphorylation following exhaustive exercise in rats (Gomez-Cabrera *et al.*, 2005). However, in the current thesis NAC treatment during contractions of mouse muscle *ex vivo* (Figure 4.10) and rat muscle *in situ* (Figure 5.9) do not affect contraction-stimulated increases in p38 MAPK phosphorylation. Interestingly, in this study NAC infusion attenuated p38 MAPK phosphorylation at 40 min (Figure 6.10A). However, because the exercise-induced increase in p38 MAPK phosphorylation during CON infusion was transient, being lower at 80 min than 40 min, further research is required to assess the role of ROS in activating p38 MAPK during moderate intensity exercise in humans. As discussed in detail in Chapter 4, the inhibition of p38 MAPK has been implicated in the regulation of H_2O_2-, contraction- and stretch-stimulated skeletal muscle glucose uptake (Somwar *et al.*, 2000; Kim *et al.*, 2006; Chambers *et al.*, 2009). In the current study glucose disposal was consistently elevated throughout exercise despite the transient increase in p38 MAPK phosphorylation, making it unlikely that p38 MAPK plays a substantial role in regulating skeletal muscle glucose uptake during exercise in humans. In support, the overexpression of the major p38 MAPK isoform

expressed in skeletal muscle, p38 MAPKγ, does not affect *ex vivo* contraction-stimulated glucose uptake in mouse muscle (Ho *et al.*, 2004). Furthermore, the antioxidants NAC and DTT, and NOS inhibitor L-NMMA attenuate contraction-stimulated glucose uptake *ex vivo* without affecting p38 MAPK phosphorylation (Figue 4.10).

PAS-160 phosphorylation (as a marker of AS160 and/or TBC1D1 phosphorylation; see Chapter 2 for details) has been suggested as a convergent point between insulin and contraction-stimulated glucose uptake regulation (Sakamoto & Holman, 2008). Neither exercise nor NAC infusion affected PAS-160 phosphorylation during moderate intensity exercise in humans (Figure 6.10). This is agreement with observations in mouse muscle contracted *ex vivo* (Figures 3.7 and 4.9) and rat hindlimb muscle contracted *in situ* (Figure 5.9), but in contrast to Treebak *et al.* (2007) who reported that PAS-160 phosphorylation increased at 60 min during moderately-high (67% $VO_{2\ peak}$) intensity exercise in humans. As discussed in detail in Chapter 3, this may be the result of exercise intensity being too low (62% $VO_{2\ peak}$) to stimulate PAS-160 phosphorylation by 80 min in the current study. However, this finding supports *ex vivo* and *in situ* results in this thesis (Figures 3.7, 4.9 and 5.9) that suggest PAS-160 phosphorylation is not essential for increases in skeletal muscle glucose uptake during contraction/exercise.

In support of findings from Chapters 3-5 muscle S-glutathionylation of a protein band of ~270 kDa (Figure 6.7A) was increased with moderate intensity exercise and this increase was prevented by NAC infusion. This provides evidence that moderate intensity exercise caused an increase in muscle oxidative stress and this increase was prevented by the infusion of NAC. As discussed in Chapter 5, it is only during moderately-high exercise *in vivo* (Sen *et al.*, 1994; Svensson *et al.*, 2002; Medved *et al.*, 2004b; Gomez-Cabrera *et al.*, 2005; Zhang *et al.*, 2007) ROS levels increase enough to cause detectable depletion of muscle GSH and/or increases in GSSG. Therefore, not surprisingly, and as reported previously (Ji *et al.*, 1992; Chapter 5; Sahlin *et al.*, 1992) muscle GSH and GSSG levels were not affected by moderate intensity exercise. This is supported by our finding that report tyrosine nitration was not increased during exercise or influenced by NAC (Figure 6.7B). As reported previously (Medved *et al.*, 2004b), systemic infusion of NAC into humans during exercise elevated skeletal muscle NAC content and increases plasma and muscle cysteine concentration (Figure 6.3 and 6.6). However, NAC infusion did not affect skeletal muscle GSH or GSSG/GSH ratio. As discussed in Chapter 5, NAC generally only increases muscle GSH availability

under conditions where muscle GSH oxidation is elevated (Medved *et al.*, 2004b; Sandstrom *et al.*, 2006). Therefore, it is likely NAC did not affect muscle GSH or GSSG concentration in the current study because GSH was not depleted by moderate intensity exercise.

In Chapter 4 the protein band S-glutathionylated at ~270 kDa in mouse muscle was identified to contain SERCA1 and Myh4. Indeed, SERCA1 appeared to be present at ~270 kDa in human muscle extracted for S-glutathionylation analysis (non-reducing conditions) (Figure 6.8). Interestingly, since SERCA1 has a molecular weight of 110 kDa these results suggest that SERCA1 may be forming a dimer, possibly via S-glutathionylation, with Myh4 or another protein to be present at ~270 kDa. However, further experimental investigation is required to confirm that the main protein at ~270 kDa being S-glutahionylated is SERCA1. This could include immunoprecipitating SERCA1 and then confirming the presence of glutathione on SERCA1 with a western blot. For discussion of the potential functional significance of the S-glutathionylation of SERCA1 during exercise see Chapter 4 (*4.4 Discussion*). Notably, in addition to SERCA1 being present at ~270 and ~110 kDa, a strong band was apparent at ~135 kDa (Figure 6.8). Whether this protein band is indeed SERCA1, and not the result of non-specific binding, is also worthy of further investigation but is beyond the scope of this thesis.

In conclusion, although skeletal muscle glutathione balance was not affected by moderate intensity exercise, S-glutathionylation of a protein band of ~270 kDa, which appears to contain SERCA1, was increased and this increase was prevented by NAC infusion. Since glucose disposal and AMPK signalling during exercise were not attenuated by NAC, this study provides evidence that small to moderate increases in ROS levels that occur during moderate intensity exercise in humans are not involved in the regulation of skeletal muscle glucose disposal or AMPK signalling. This suggests factors in addition to ROS regulate skeletal muscle glucose uptake during moderate intensity exercise, and also suggests that previous results obtained using intense *ex vivo* contractions in mouse muscle may not be relevant to normal prolonged exercise.

CHAPTER SEVEN

DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

This chapter will discuss the contribution that the main findings of this thesis make to the understanding of how contraction regulates skeletal muscle glucose uptake. In addition, suggestions will be made, based on the findings of this thesis, for future investigations regarding the role of ROS and NO in the regulation of skeletal muscle glucose uptake during contraction/exercise, and conclusions will be drawn.

There are numerous signalling intermediates within skeletal muscle which have been implicated in the regulation of GLUT4 translocation and glucose uptake during contraction (i.e. calcium (Ca^{2+}) (Holloszy & Hansen, 1996), CaMK's (Wright *et al.*, 2004), PKC (Wojtaszewski *et al.*, 1998), and AMP-activated protein kinase (AMPK) (Mu *et al.*, 2001; Jensen *et al.*, 2007b; Lefort *et al.*, 2008), however this thesis focused on the contribution of ROS and NO, and to some extent their interactions with AMPK. The major findings of this thesis were that during skeletal muscle contractions *ex vivo*, ROS and NO regulate skeletal muscle glucose uptake through a similar pathway that is independent of AMPK and cGMP-PKG but may involve signalling through $ONOO^-$ and/or S-glutathionylation. In the introduction of this thesis (Chapter 1) signalling pathways were proposed through which NO (Figure 1.2) and ROS (Figure 1.5) may regulate glucose uptake during contraction. Based on findings from this thesis the proposed pathways through which ROS and NO regulate glucose uptake during contractions *ex vivo* have been modified (Figure 7.1). Interestingly, however, and in contrast to *ex vivo* contractions, ROS do not appear to play an integral role in the regulation of skeletal muscle glucose uptake during low-moderate intensity hindlimb contractions in rats *in situ*, or during moderate intensity exercise in humans. This suggests that highly fatiguing *ex vivo* contraction conditions may artificially promote ROS signalling of skeletal muscle glucose uptake.

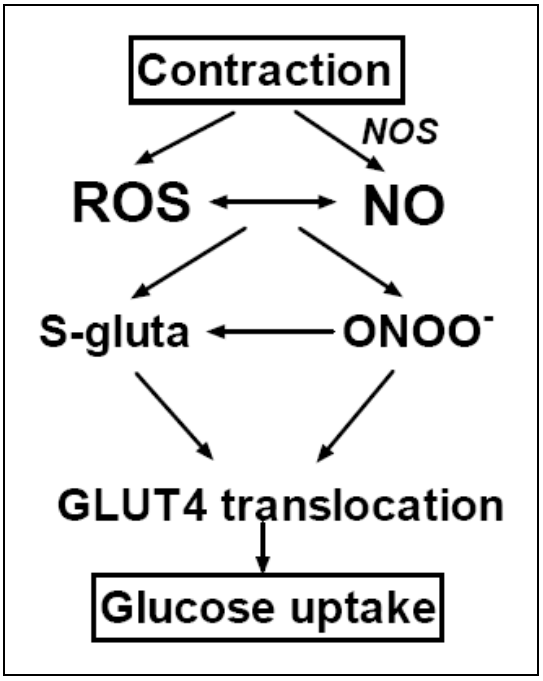

Figure 7.1 Proposed pathways through which reactive oxygen species (ROS) and nitric oxide (NO) may regulate skeletal muscle glucose uptake during contractions *ex vivo*. S-gluta= S-glutathionylation, $ONOO^-$= peroxynitrite.

7.1 AMPK AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

Through the use of the pharmacological activator of AMPK, AICAR, it has become universally recognised that AMPK can increase skeletal muscle glucose uptake independent of insulin (Merrill *et al.*, 1997; Hayashi *et al.*, 1998; Kurth-Kraczek *et al.*, 1999; Koistinen *et al.*, 2003). Since AMPK is also activated during skeletal muscle contraction, AMPK has been implicated in the regulation of insulin-independent skeletal muscle glucose uptake during contraction (Hayashi *et al.*, 1998; Musi *et al.*, 2001b; Wojtaszewski *et al.*, 2003). However, disassociations between contraction-stimulated skeletal muscle glucose uptake and AMPK activity during contraction (Derave *et al.*, 2000; Fujii *et al.*, 2000; Wojtaszewski *et al.*, 2000; Jorgensen *et al.*, 2004; Fujii *et al.*, 2005; McConell *et al.*, 2005; Wadley *et al.*, 2006; Fujii *et al.*, 2007; Maarbjerg *et al.*, 2009) suggest that AMPK may not be required for normal increases in glucose uptake during contraction. Indeed, studies have reported that the knocking out either AMPKα1 or AMPKα2 isoforms (Jorgensen *et al.*, 2004), or the overexpression of a kinase dead form of AMPKα2 (AMPK KD) does not affect skeletal

muscle glucose uptake during contractions *ex vivo* (Fujii *et al.*, 2005; Fujii *et al.*, 2007) or *in vivo* exercise (Maarbjerg *et al.*, 2009).

In agreement with Fuji et al. (2005), it was found that in the absence of any increases in AMPKα1 or AMPKα2 activity (Figure 3.4) or impairment of force production (Figures 3.1 and 3.2), muscles from AMPK KD mice had normal increases in glucose uptake during contractions *ex vivo* (Figure 3.11). Previously, studies using muscles from AMPK KD mice to investigate the role of AMPK in regulating glucose uptake during contraction have used higher intensity stimulation protocols (longer and more frequent stimulation trains), and therefore less physiological, than used in Chapter 3 (Mu *et al.*, 2001; Jensen *et al.*, 2007b; Lefort *et al.*, 2008). The lower intensity contraction protocol employed in Chapter 3 may account for why, in contrast to some (Mu *et al.*, 2001; Jensen *et al.*, 2007b; Lefort *et al.*, 2008), it was found that AMPK KD mice have normal glucose uptake during *ex vivo* contractions. Indeed, higher intensity skeletal muscle contractions result in greater AMPK activation and glucose uptake (Ihlemann *et al.*, 1999b; Chen *et al.*, 2003). This may suggest that it is only during high intensity contractions that AMPK play a significant role in regulating contraction-stimulated skeletal muscle glucose uptake. In support, during *in vivo* exercise (which generally involves much lower intensity contractions than used *ex vivo*) AMPK appears to play little role in the regulation of skeletal muscle glucose uptake (Lee-Young *et al.*, 2009; Maarbjerg *et al.*, 2009). Furthermore, it was found that the NOS inhibitor, L-NMMA, and the antioxidants, NAC, DTT and urate (Figure 4.4 and 4.6), attenuated contraction-stimulated glucose uptake *ex vivo* without affecting AMPK phosphorylation (Figure 4.8). This further supports the notion that skeletal muscle glucose uptake during contraction is regulated through pathways that are independent of AMPK.

7.2 NO AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

NO has been implicated in the regulation of skeletal muscle glucose uptake during contraction (Balon & Nadler, 1997; Roberts *et al.*, 1997; Bradley *et al.*, 1999; Kingwell *et al.*, 2002; Ross *et al.*, 2007), however few studies have investigated the mechanisms through which NO acts.

7.2.1 Is AMPK involved?

High doses (>10 mM SNP) of NO-donors increase resting skeletal muscle glucose uptake and AMPK activity (Higaki *et al.*, 2001; Lira *et al.*, 2007), suggesting that NO regulates glucose uptake via AMPK. Alternatively, AMPK may act upstream of NO, increasing glucose uptake through the activation of NOS and indeed, NOS inhibition has been shown to attenuate AICAR-stimulated glucose uptake in skeletal muscle (Fryer *et al.*, 2000). Therefore, it is possible that NO and AMPK interact to increase skeletal muscle glucose uptake during contraction, however this has not previously been investigated.

The inhibition of NOS in EDL muscles from AMPK KD mice (Figure 3.11), which show no increases in AMPK activity with contraction (Figure 3.4), attenuated contraction-stimulated increases in glucose uptake (Figure 3.11). Furthermore, NOS inhibition attenuated the increase in glucose uptake during contraction of EDL muscle from C57Bl/6 mice (Figure 4.4) without affecting phosphorylation of AMPK or ACCβ (Figure 4.8), the primary downstream target of AMPK. This indicates that NO regulates EDL muscle glucose uptake during contractions *ex vivo* independent of AMPK. This is supported by the previous finding that NOS inhibition attenuates glucose uptake during *in situ* hindlimb contractions in rats without affecting AMPK signalling (Ross *et al.*, 2007), and the finding that L-NMMA does not affect AICAR-stimulated glucose up in mouse (Figure 3.12) or rat skeletal muscle (Stephens *et al.*, 2004). However, in soleus muscles L-NMMA did not attenuate contraction-stimulated glucose uptake (Figure 3.11). Thus it appears that NO is more important in regulating glucose uptake in glycolytic than in oxidative muscles. This is likely the result of nNOS being more highly expressed in the glycolytic muscles compared with oxidative muscles (Figure 3.10B).

7.2.1 Is cGMP or S-nitrosylation involved?

There is strong evidence to suggest that NO-donors increase basal skeletal muscle glucose uptake through a cGMP-PKG dependent pathway (Young *et al.*, 1997; Young & Leighton, 1998a, b). However, NO can act as a signalling molecule through cGMP-PKG independent mechanisms such as S-nitrosylation (Stamler & Meissner, 2001). Therefore, it was important to establish whether NO requires cGMP-PKG signalling to increase skeletal muscle glucose uptake during contraction. It was found that the inhibition of cGMP and PKG did not affect skeletal muscle glucose uptake during *ex vivo* contractions (Figure 4.5), however, the inhibition of cGMP prevented NO donor-stimulated skeletal muscle glucose uptake (Figure 4.5). This suggests that, unlike NO donor-stimulated skeletal muscle glucose uptake, NO

regulates glucose uptake during contraction via a cGMP-PKG independent pathway. Furthermore, it provides evidence that exogenous substances which promote insulin-independent increases in basal glucose uptake, such as the activation of AMPK by AICAR (Jorgensen *et al.*, 2004), may not necessarily utilise the same pathway as that involved during contraction. This is likely the result of high, and often extremely high (Higaki *et al.*, 2001; Toyoda *et al.*, 2004), concentrations of exogenous substrates (such as AICAR, NO-donors, H_2O_2) applied to the cell surface activating pathways that differ from that of signalling intermediates produced at low concentrations endogenously during contraction (Chambers *et al.*, 2009). Therefore care must be taken when extrapolating resting muscle glucose uptake findings to that of contraction.

NO is proposed to increase glucose uptake into adipocytes through the induction of protein S-nitrosylation (Kaddai *et al.*, 2008). During contraction, muscles were treated with white light to break any S-nitrosyl bonds that may have been formed (Borutaite *et al.*, 2000; Benhar *et al.*, 2009). This did not affect glucose uptake (Figure 4.6), suggesting that NO stimulates glucose uptake during contraction through an alternative mechanism. However, the effect of contraction and white light on S-nitrosylation of critical proteins needs to be determined to confirm this finding. Other potential mechanisms though which NO may regulate glucose uptake during contraction include S-glutathionylation and $ONOO^-$ signalling, both of which require the presence of ROS (Stamler & Meissner, 2001; Zou *et al.*, 2002; Dalle-Donne *et al.*, 2009) and are therefore discussed in section 7.4 *ROS and NO interaction and contraction-stimulated skeletal muscle glucose uptake*.

7.3 ROS AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

There has only been one previous study which has investigated the involvement of ROS in the regulation of skeletal muscle glucose uptake during contraction (Sandstrom *et al.*, 2006). Sandstrom *et al.* (2006) reported that the antioxidant NAC attenuated EDL muscle glucose uptake and AMPK activity during contractions *ex vivo*. This lead to the hypothesis that the increase in ROS production during contraction contributes to the activation of AMPK, and AMPK, in part, mediates skeletal muscle glucose uptake during contraction (Sandstrom *et al.*, 2006; Katz, 2007). However, as discussed above (7.1 *AMPK and contraction-stimulated skeletal muscle glucose uptake*), AMPK may not be essential for normal increases in skeletal

muscle glucose uptake during contraction. Furthermore, recent evidence suggests that although exogenous ROS can activate AMPK, AMPK activation is not required for exogenous ROS-stimulated skeletal muscle glucose uptake (Higaki *et al.*, 2008; Jensen *et al.*, 2008). Whether ROS regulate skeletal muscle glucose uptake during contraction through an AMPK-dependent pathway had not previously been directly investigated.

EDL and soleus muscles from AMPK KD mice, were contracted *ex vivo* and it was found that the antioxidant NAC attenuated contraction-stimulated glucose uptake in muscles from AMPK KD mice to a similar extent as in muscles from WT mice (Figure 3.11). This was despite no increases in AMPK activity during contraction (Figure 3.4). This suggests that ROS are involved in the regulation of skeletal muscle glucose uptake during contraction in muscles containing both predominantly glycolytic (EDL) and predominantly oxidative (soleus) type fibres independent of AMPK. In support, it was also found that the antioxidants NAC, DTT and urate attenuated contraction-stimulated skeletal muscle glucose without affecting AMPK phosphorylation (Figure 4.6). It is likely that the lower intensity, but more physiological, stimulation protocol utilised accounts for why, in contrast to Sandstrom *et al.* (2006), it was found that NAC did not attenuate AMPK phosphorylation during contraction. At high concentrations exogenous ROS reduce cellular energy balance (Leon *et al.*, 2004) which activates AMPK (Toyoda *et al.*, 2004), whereas at lower concentrations exogenous ROS increase skeletal muscle glucose uptake independent of AMPK (Higaki *et al.*, 2008; Jensen *et al.*, 2008). Therefore, at high contraction intensities where ROS production is maximised (Reid, 2008), ROS levels may be high enough to disrupt cellular energy status, activating AMPK, and this may contribute to the increase in skeletal muscle glucose uptake during contraction. However, it is likely that during less intense *ex vivo* contraction protocols ROS regulate glucose uptake through an AMPK independent pathway. Indeed, at low concentrations exogenous ROS increase basal skeletal muscle glucose uptake through the activation of PI3K and Akt (Higaki *et al.*, 2008). However, it is well established that PI3K and Akt signalling is not involved in the regulation of skeletal muscle glucose uptake during contraction (Lee *et al.*, 1995; Yeh *et al.*, 1995). Alternative mechanisms through which ROS may regulate skeletal muscle glucose uptake during contractions *ex vivo* are discussed in section 7.4 *ROS and NO interaction and contraction-stimulated skeletal muscle glucose uptake*.

Although ROS appear to regulate skeletal muscle glucose uptake during contractions *ex vivo*, the role of ROS in regulating skeletal muscle glucose uptake during contraction in more physiological models (intact preparations with blood flow) was previously unknown. Therefore, NAC was infused IA in the hindlimb of rats during *in situ* contractions (Chapter 5), and IV in humans during exercise (Chapter 6) to examine whether ROS are involved in the regulation of glucose uptake during skeletal muscle contractions in intact preparations. This was important because, unlike *ex vivo* preparations, during *in situ* contractions and *in vivo* exercise, blood flow is intact and therefore delivery of metabolic substrates (Clifford & Hellsten, 2004; Rattigan *et al.*, 2005) and antioxidant systems (i.e. glutathione and cysteine) (Powers *et al.*, 1999) evenly to all muscle fibres, and removes potentially deleterious products of metabolism from the muscle (Holloszy & Booth, 1976). Furthermore, *ex vivo* muscle contraction protocols generally involve frequent, and prolonged tetanic (maximal) stimulations (Hayashi *et al.*, 1998; Higaki *et al.*, 2001; Mu *et al.*, 2001; Sandstrom *et al.*, 2006) which are substantially more intense than what is physiological achievable *in vivo* (Allen *et al.*, 2008). As such, these conditions may artificially increase ROS production and potentially influence ROS signalling mechanisms (Reid, 2001; Allen *et al.*, 2008; Reid, 2008) and increase the relative importance of ROS in the regulation of skeletal muscle glucose uptake during contraction.

Indeed, it was found that NAC infusion did not affect contraction-stimulated increases in glucose uptake in rat hindlimb muscles (Figure 5.9) or during exercise in humans (Figure 6.5). This was despite NAC infusion increasing muscle NAC (Figures 5.3 and 6.2) and cysteine content (Figures 5.6 and 6.6), as well as preventing contraction-stimulated increases in S-glutathionylation of a protein band at ~250 kDa in rats and ~270 kDa in humans (Figures 5.7 and 6.7). Importantly, the *in situ* stimulation protocol in rats (0.1 ms impulse at 2 Hz and 35 V) and exercise intensity in humans (62±1% $\dot{V}O_{2\ peak}$) were sufficient to substantially increase glucose uptake (Figures 5.8 and 6.5), and metabolic signalling (AMPK, ACCβ and p38 MAPK phosphorylation; Figures 5.9, 6.9 and 6.10), as well as reducing muscle energy status (Table 6.2) without being highly fatiguing (Figure 5.2 and Table 6.2). Although these exercise/contraction protocols did not significantly increase muscle oxidative stress as indicated by GSH/GSSG ratio (Figures 5.6 and 6.6), they did induce an increase in muscle S-glutathionylation which was prevented with NAC infusion (Figures 5.7 and 6.7). This suggests that the ROS scavenging properties of NAC attenuated any increases in ROS that may have occured during muscle contraction, but were not great enough to increase GSH

oxidation. Therefore, Chapters 5 and 6 provide evidence to suggest that during low-moderate intensity exercise/contractions ROS are not essential for the regulation of skeletal muscle glucose uptake. This is in contrast to the *ex vivo* findings of Chapters 3 and 4 and Sandstrom *et al.* (2006), suggesting that results gained from *ex vivo* models of skeletal muscle contraction may not always be relevant to normal exercise conditions.

It is important to acknowledge that although NAC infusion increased muscle NAC and cysteine levels (Figures 5.6 and 6.6), and prevented contraction-stimulated increases in S-glutathionylation (at ~250 and 270 kDa; (Figures 5.7 and 6.7), this does not prove that NAC infusion prevented all increases in ROS during exercise/contraction. It may be that the NAC infusion-derived increases in muscle NAC and cysteine were to low to prevent ROS signalling of glucose uptake. Therefore, future investigations should employ higher concentrations of antioxidants (where possible, i.e. the maximum dose of NAC that can be infused into humans safely without serious side effects was used (Medved *et al.*, 2003)) and alternative means of preventing increases in ROS, such as the inhibition of xanthine oxidase with allopurinol (Gomez-Cabrera *et al.*, 2005), or ROS scavenging with ascorbic acid (Ashton *et al.*, 1999). Alternatively, or concurrently, it may be that the modest elevation in muscle oxidative stress induced by low-moderate intensity exercise and *in situ* contractions was insufficient to activate ROS-stimulated signalling of skeletal muscle glucose uptake. Therefore, it is now important to determine whether ROS play a greater role in regulating skeletal muscle glucose uptake during higher intensity exercise that substantially increases muscle oxidative stress (Svensson *et al.*, 2002; Medved *et al.*, 2004b; Zhang *et al.*, 2007). It also must be noted, however, that *ex vivo* studies which provide evidence that ROS are involved in the regulation of skeletal muscle glucose uptake (Chapters 3 and 4 and (Sandstrom *et al.*, 2006)) during contraction have all used mouse muscle. Although unlikely, it may be that ROS play a greater role in regulating glucose uptake during contraction in mouse muscle than in either human or rat muscle.

7.4 ROS AND NO INTERACTIONS AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

Since ROS and NO are highly integrated molecules (Brown & Borutaite, 2006), is not surprising that the co-treatment of skeletal muscle with L-NMMA and NAC did not have an

additive affect on the attenuation of glucose uptake during contractions *ex vivo* (Figure 4.4). This suggests that ROS and NO regulate skeletal muscle glucose uptake during contractions *ex vivo* through a common pathway. The possible involvement of interactions between NO and ROS in the regulation of skeletal muscle glucose uptake during contraction has not previously been examined. Interactions between NO and $O_2^{\bullet-}$ can produce $ONOO^-$, which at low concentrations acts as a signalling molecule (Pacher *et al.*, 2007), and NO, $O_2^{\bullet-}$ and $ONOO^-$ can all promote S-glutathionylation (Martinez-Ruiz & Lamas, 2007; Dalle-Donne *et al.*, 2009).

7.4.1 Ex vivo

The effect of an $ONOO^-$ scavenger (urate) and a reducing agent (DTT; which prevents S-glutathionylation) on contraction-stimulated glucose uptake was examined. It was found that urate attenuated contraction-stimulated glucose uptake to a similar extent as NAC and L-NMMA, and that DTT prevented any increase in skeletal muscle glucose uptake during contraction (Figure 4.6). Furthermore, like NAC and L-NMMA, DTT and urate also attenuated contraction-stimulated increases in tyrosine nitration (a marker of $ONOO^-$) of a protein band at ~37 kDa (Figure 4.7), and S-glutathionylation of a protein band at ~37 kDa (Figures 3.9 and 4.7; S-glutathionylation at 250/270 kDa is addressed below). This suggests that NO and ROS may regulate glucose uptake during contraction through a similar pathway that involves signalling through S-glutathionylation and/or $ONOO^-$. However, further experimental investigation using alternative methods to alter $ONOO^-$ production and S-glutathionylation is required to confirm this relationship. This is of particular importance since urate increased skeletal muscle basal glucose uptake, reduced initial contraction force and affected some contraction-stimulated muscle signalling (p38 MAPK and ACCβ phosphorylation) independent of NOS inhibition and therefore presumably $ONOO^-$. This indicates that urate may have been having some effects beyond $ONOO^-$ scavenging.

To further investigate the potential for $ONOO^-$ in stimulating glucose uptake, skeletal muscle was treated with 500 μM of exogenous $ONOO^-$. Surprisingly, this did not affect basal glucose uptake (Figure 4.11), questioning whether $ONOO^-$ is involved in the regulation of contraction-stimulated increases in skeletal muscle glucose uptake. Alternatively, however, this result may highlight the inherent differences in signalling targets of $ONOO^-$ produced endogenously during contraction and that applied exogenously to the cell surface of resting muscle (Chambers *et al.*, 2009). Furthermore, it may be that at the high concentration used

(500 μM) (Zou *et al.*, 2003), $ONOO^-$ inhibited (through tyrosine nitration) insulin-independent glucose uptake signalling, rather than activating it via tyrosine phosphorylation (which is promoted by lower, 10-200 μM, concentrations of $ONOO^-$) (Mallozzi *et al.*, 1997; Pacher *et al.*, 2007). Previously $ONOO^-$ has been reported to increase AMPK phosphorylation in cells (Zou *et al.*, 2003). Interestingly, in the current study, $ONOO^-$ increased AMPK phosphorylation (Figure 4.12), however the increase in basal glucose uptake normally associated with AMPK activation (Figure 3.12) was not seen (Figure 4.11). This supports the notion that high concentrations of $ONOO^-$ may inhibit insulin-independent glucose uptake. Therefore, future investigations should identify whether $ONOO^-$-mediated tyrosine phosphorylation is increased during exercise, the proteins affected, and whether this, rather than tyrosine nitration, is involved in the regulation of skeletal muscle glucose uptake.

S-glutathionylation (Figures 3.9 and 4.7) and tyrosine nitration (Figures 4.7) of a protein band at ~37 kDa was increased with contraction and this increase, like glucose uptake (Figures 3.11, 4.4 and 4.6) was attenuated by NOS inhibition (L-NMMA) and antioxidant treatment (NAC, DTT and urate). In Chapter 4, this protein band was identified to contain the glycolysis enzyme, GAPDH. This suggests a role for GAPDH in regulating NO and ROS-mediated skeletal muscle glucose uptake during contraction. There is currently no evidence to suggest that GAPDH is involved in the regulation of insulin-independent glucose uptake, however, both S-glutathionylation and $ONOO^-$ signalling have been shown to inhibit the activity of GAPDH under conditions of oxidative stress (Mohr *et al.*, 1994; Souza & Radi, 1998; Mohr *et al.*, 1999). Therefore, future investigations should determine whether GAPDH has the potential to regulate contraction-stimulated skeletal muscle glucose uptake, and whether its activity is altered by S-glutathionylation and/or $ONOO^-$ signalling during contraction.

7.4.2 In vivo

In contrast to the infusion of the antioxidant NAC *in situ* and *in vivo*, our group and others have previously shown that inhibition of NOS attenuates increases in skeletal muscle glucose uptake during *in situ* hindlimb contractions in rats (Ross *et al.*, 2007) and during *in vivo* exercise in rats (Roberts *et al.*, 1997) and humans (Bradley *et al.*, 1999; Kingwell *et al.*, 2002). This suggests that since NO, but not ROS, appear to be important in the regulation of glucose uptake during moderate intensity contractions in intact preparations, NO and ROS may not be required to interact to increase glucose uptake. However, it is possible that during *ex vivo* contraction conditions, high enough levels of ROS are produced to also activate the pathway

through which NO-stimulates glucose uptake, and the levels of ROS required to activate this pathways are not produced during moderate intensity exercise in intact preparations.

In contrast to mouse muscle contracted *ex vivo*, *in situ* hindlimb contractions in rats and exercise in humans did not increase S-glutathionylation of a protein band at ~37 kDa. This suggests that S-glutathionylation at ~37 kDa is not involved in the regulation of skeletal muscle glucose uptake during normal exercise. However, this may also suggest that it is only during conditions of high oxidative stress, such as high intensity contractions *ex vivo*, that S-glutathionylation of GAPDH (as a result of increased ROS) is involved in the regulation of glucose uptake during skeletal muscle contraction. This supports the idea that ROS regulation of skeletal muscle glucose uptake during contraction may be a largely redundant mechanism which is only engaged underconditions of very high oxidative stress. It is possible that when greater levels of glucose transport into the muscle than can be efficiently supplied through alternative signalling of insulin-independent GLUT4 translocation (i.e. NO and CaMK signalling) that this mechanisms is required. Indeed, conditions that require maximal insulin-independent glucose uptake, like high intensity exercise, are often associated with higher than normal ROS levels.

7.5 PAS-160 AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

AS160 and TBC1D1 have been implicated in the activation of GLUT4 translocation to increase glucose uptake (Stone *et al.*, 2006; Sakamoto & Holman, 2008), and hypothesised to be a convergent point in the signalling of insulin-dependent and insulin-independent skeletal muscle glucose uptake (Sakamoto & Holman, 2008). Several Akt phosphorylation sites of AS160 and TBC1D1 are recognised by the PAS antibody at 150-160 kDa (Taylor *et al.*, 2008). In this thesis PAS phosphorylation at ~160 kDa (PAS-160) was measured as a marker of AS160 and TBC1D1 phosphorylation. Skeletal muscle PAS-160 phosphorylation was not increased during contractions *ex vivo* (Figures 3.7 and 4.9), *in situ* (Figure 5.1) or during *in vivo* exercise (Figure 6.1). This is in contrast to some *ex vivo* and *in situ* studies that report PAS-160 phosphorylation to be increased with skeletal muscle contraction (Bruss *et al.*, 2005; Kramer *et al.*, 2006a; Funai & Cartee, 2008), but in support of others (Treebak *et al.*, 2007; Jensen *et al.*, 2008) that have found PAS-160 phosphorylation is not always increased with skeletal muscle contraction. This discrepancy is likely a result of contraction intensity, with

most studies reporting increases in PAS phosphorylation using more intense skeletal muscle contraction protocols (Bruss *et al.*, 2005; Kramer *et al.*, 2006a; Funai & Cartee, 2008) than the lower, and more physiological, intensity protocols used in this thesis. Furthermore, it appears that PAS-160 phosphorylation may not increase until some time after the onset of skeletal muscle contraction, ranging from 10 min to 60 min of continous contraction (Bruss *et al.*, 2005; Treebak *et al.*, 2007). Therefore, it is also possible that the sampling time points used in this thesis did not coincide with increases in PAS-160 phosphorylation. Regardless, since skeletal muscle glucose uptake was increased independent of PAS-160 phosphorylation during contractions in all models, it suggests that PAS-160 phosphorylation is not required for normal increases in skeletal muscle glucose uptake during contraction. Importantly, however, since PAS-160 collectively recognises several Akt phosphorylation sites, non-Akt phosphorylation sites and/or specific Akt phosphorylation sites may be more important in regulating the activity of AS160 and TBC1D1 during contraction. Indeed, insulin-stimulation at rest and following exercise results in similar increases in PAS-160 phosphorylation, however several specific AS160 phosphorylation sites (Ser-318, Ser-341, Ser-588 and Ser-751) are differentitally phosphorylated in rest and contracted muscle stimulated with insulin (Treebak *et al.*, 2009a). Therefore, the role of AS160 and TBC1D1 in regulating skeletal muscle glucose uptake during contraction, and possible interactions with ROS and NO to this end requires further investigation.

7.6 p38 MAPK AND CONTRACTION-STIMULATED SKELETAL MUSCLE GLUCOSE UPTAKE

There is evidence to suggest that during exhaustive exercise ROS regulate p38 MAPK phosphorylation (Gomez-Cabrera *et al.*, 2005) and that p38 MAPK is involved in the regulation of stretch-stimulated and contraction-stimulated skeletal muscle glucose uptake (Somwar *et al.*, 2000; Chambers *et al.*, 2009). To further examine this relationship, p38 MAPK phosphorylation was measured in contracted muscles exposed to antioxidants. It was found that, despite attenuating *ex vivo* contraction-stimulated skeletal muscle glucose uptake, NAC and DTT did not affect increases in p38 MAPK phosphorylation (Figure 4.10). Furthermore, the increase in p38 MAPK phosphorylation during exercise in humans was transient (Figure 6.10), and NAC infusion did not affect the increase in p38 MAPK phosphorylation during *in situ* rat hindlimb contractions (Figure 5.9). This questions whether ROS are involved in regulating p38 MAPK phosphorylation during contraction, and the

disassociations between skeletal muscle glucose uptake and p38 MAPK phosphorylation suggest that p38 MAPK phosphorylation may not be required for normal increases in glucose uptake during contraction. In support, Ho *et al.* (2004) showed that the over expression of p38 MAPKγ in mouse skeletal muscle does not affect contraction-stimulated glucose uptake. To reconcile discrepancies between the findings that ROS do not contribute to the regulation of p38 MAPK phosphorylation during contractions, and that of others (Gomez-Cabrera *et al.*, 2005), future research should investigate whether the level of exercise intensity and the particular antioxidant(s) employed alters the relationship between ROS and p38 MAPK phosphorylation during exercise.

7.7 S-GLUTATHIONYLATION OF A PROTEIN BAND AT ~270 KDA DURING CONTRACTION

In mouse skeletal muscle the protein band S-glutathionylated at ~270 kDa during contraction was identified to contain both SERCA1 and Myh4 (Chapter 4, *4.3.4 S-glutathionylation and tyrosine nitration*). Indeed, under non-reducing conditions SERCA1 was also present at ~270 kDa in human skeletal muscle (Figure 6.8). Since S-glutathionylation has already been recognised to increase SERCA activity under conditions of oxidative stress (Adachi *et al.*, 2004; Ying *et al.*, 2007), SERCA1 is most likely to be the protein being S-glutathionylated at ~270 kDa in skeletal muscle during contraction. However, SERCA1 has a molecular weight of 110 kDa. This suggests that for SERCA1 to be present at ~270 kDa, under conditions of oxidative stress a heterodimer is formed between SERCA and another protein, possibly the contractile protein Myh4, and possibly via S-glutathionylation. SERCA facilitates sarcoplasmic reticulum (SR) Ca^{2+} transport and is associated with skeletal muscle relaxation (Berchtold *et al.*, 2000), and therefore is unlikely to be directly involved in the regulation of skeletal muscle glucose uptake during contraction. Indeed, in Chapter 4 it is shown that S-gulathionylation of 270 kDa profile does not match that of contraction stimulated glucose uptake (Figures 4.3, 4.6 and 4.7) However, Ca^{2+} transport disruption (Bruton *et al.*, 1998; Allen *et al.*, 2008) and ROS (Reid, 2008) have long been associated with the development of skeletal muscle fatigue during exercise. Therefore, understanding the function of skeletal muscle SERCA S-glutathionylation during contraction may prove to be important for future research into factors contributing to fatigue during exercise.

7.8 FUTURE DIRECTIONS

This section summarises some of the directions in which research regarding the role of ROS and NO regulating skeletal muscle glucose uptake during contraction may take, and is based on the findings from this thesis.

In Chapter 4, evidence is provided that S-nitrosylation is not involved in the regulation of skeletal muscle glucose uptake during contraction by using white light to break S-nitrosyl bonds (Borutaite *et al.*, 2000; Benhar *et al.*, 2009) that may have been formed during contraction. However, S-nitrosylation during contraction was not measured. It is important to determine whether S-nitrosylation is increased during skeletal muscle contraction. Furthermore, it is important to confirm that white light prevented S-nitrosylation during skeletal muscle contraction to verify that S-nitrosylation is not playing a role in the regulation of glucose uptake. This may be achieved by measuring S-nitrosylation using an S-nitrosocysteine antibody (Sun *et al.*, 2001) or a biotin switch assay (Jaffrey *et al.*, 2001; Jaffrey & Snyder, 2001). This is of particular importance given that S-nitrosylation can facilitate protein S-glutathionylation (Martinez-Ruiz & Lamas, 2007) which is suggested above (7.4 *ROS and NO interactions and contraction-stimulated skeletal muscle glucose uptake*) to be involved in the regulation of skeletal muscle glucose uptake during contractions *ex vivo*.

Some evidence is provided, through the observations that contraction increased tyrosine nitration (Figure 4.7) and an $ONOO^-$ scavenger (urate) reduced muscle ROS levels during contraction (Figure 4.3), that $ONOO^-$ is produced during contraction. This should now be confirmed by measuring $ONOO^-$ levels in muscles following contraction. This is important because urate also attenuated increases in contraction-stimulated skeletal muscle glucose uptake, suggesting a role for $ONOO^-$ in regulating glucose uptake during contraction (Figure 4.6). However, urate appeared to be having $ONOO^-$ scavenging–independent effects on muscle (see *4.4 Discussion* for details), therefore alternative $ONOO^-$ scavengers, such as 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) (Kalinowski *et al.*, 2004; Di Marco *et al.*, 2005), should be utilised to establish whether $ONOO^-$ is involved in the regulation of skeletal muscle contraction-stimulated glucose uptake in both *ex vivo* and *in vivo* models. In addition, since tyrosine nitration is generally associated with the

inhibition of signalling pathways induced by high levels of $ONOO^-$ (Pacher *et al.*, 2007), identifying whether or not $ONOO^-$-stimulated tyrosine phosphorylation is increased during contraction may provide insight into how NO regulates skeletal muscle glucose uptake during contraction.

S-glutathionylation of GAPDH was shown to increase in response to contractions *ex vivo* and it was suggested that GAPDH may play a role in regulating contraction stimulated glucose uptake (Figures 3.9 and 4.7; Chapter 4). However, this requires further investingation. It is suggested that firstly, the role of GAPDH in regulating skeletal muscle glucose uptake during contraction should be investigated using one or more of the many GAPDH inhibitors (Leitao *et al.*, 2004) available or through si-RNA depletion of GAPDH protein (Hara *et al.*, 2005) in muscle. However, it is acknowledged that the effect of any inhibition or deletion of GAPDH on glucose uptake would be difficult to interpret due to the role of GAPDH in regulating flux through glycolysis. The role of S-glutathionylation in regulating insulin-independent glucose uptake can be further explored by using S-glutathionylation promoters such as S-nitrosoglutathione (GSNO) (although care muscle be taken when using GSNO as it is also a NO donor), and its involvement in regulating glucose uptake during contraction through the use of transgenic mouse models where increases in S-glutathionylation during contraction would be attenuated, such as glutathione peroxidise KO mice (Crack *et al.*, 2001). Providing such experiments give further evidence that GAPDH and S-glutathionylation are involved in the regulation of skeletal muscle glucose uptake, then a relationship between them should be considered by investigating the effect of S-glutathionylation promoters on glucose uptake in muscle from mice that have GAPDH down regulated.

As discussed above (7.3 *ROS and contraction-stimulated skeletal muscle glucose uptake*), in contrast to *ex vivo* studies (Figure 3.11 and 4.4), NAC infusion did not affect glucose uptake during low-moderate intensity hindlimb *in situ* contractions in rat (Figure 5.9) or during moderate intensity exercise in humans (Figure 6.5). Furthermore, the contraction protocols employed did not significantly alter muscle glutathione status (Figures 5.7 and 6.6). This suggests that the increase in ROS production was not sufficient to signal ROS-stimulated increases in glucose uptake. To further investigate the role of ROS in regulating glucose uptake during contraction in *in vivo* and *in situ* preparations these experiments should now be repeated using higher intensity exercise protocols, such as exercise to exhaustion, which alter muscle glutathione status (Medved *et al.*, 2004b; Gomez-Cabrera *et al.*, 2005). Additionally,

to further reconcile the inconsistency between results from *ex vivo* vs. intact preparations, the effect of antioxidants on glucose uptake during low intensity/twitch contractions *ex vivo* should be investigated.

It is possible the concentration of NAC infused during muscle contractions in rats (Chapter 5) and humans (Chapter 6) was not sufficient to prevent all ROS signalling in the muscle. Therefore, is important now that investigations employ higher concentrations of antioxidants and alternative means of preventing increases in ROS, such as the inhibition of xanthine oxidase with allopurinol, or the over expression of antioxidants such as superoxide dismutase or GPx in mice, to further investigate the role of ROS in regulating glucose uptake during contractions in intact preparations. It must be acknowledged, however, that in this thesis the maximum dose of NAC that can be infused into humans safely without serious side effects was used (Medved *et al.*, 2003).

7.9 CONCLUSION

In summary, the major conclusions of this thesis are:

1. ROS and NO are involved in the regulation of skeletal muscle glucose uptake during contractions *ex vivo*.

2. ROS and NO regulate skeletal muscle glucose uptake during *ex vivo* contractions through an AMPK-independent pathway, furthermore, AMPK does not appear to be essential for normal increases in skeletal muscle contraction-stimulated glucose uptake.

3. During contractions *ex vivo*, ROS and NO regulate skeletal muscle glucose uptake through a similar pathway that is independent of cGMP-PKG signalling.

4. ROS and NO promote protein S-glutathionylation and tyrosine nitration during skeletal muscle contractions *ex vivo*, and signalling through S-glutathionylation and peroxynitrite may be involved in the regulation of *ex vivo* contraction-stimulated glucose uptake.

5. In contrast to skeletal muscle contractions *ex vivo*, ROS are not likely to be involved in the regulation of skeletal muscle glucose uptake during low-moderate intensity hindlimb contractions *in situ* in rats, or during moderate intensity exercise in humans. This suggests that results implicating ROS in the regulation of contraction stimulated skeletal muscle glucose uptake obtained *ex vivo*, using high intensity stimulation to contract muscle, may not be relevant to normal exercise.

6. ROS promote skeletal muscle S-glutathionylation of a protein band at ~270 kDa during contraction, and this protein band contains SERCA1. Furthermore, as protein band S-glutathionylated at ~37 kDa during *ex vivo* contractions was shown to contain GAPDH and Mhy4.

CHAPTER EIGHT

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APPENDIX A

Questionaires and forms related to Chapter 7 (NAC infusion in humans study) including:

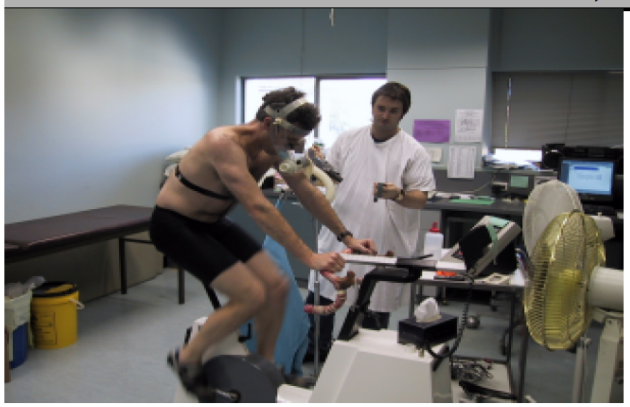
Participant recruitment advertisement
Plain language statement
Consent form
Medical questionnaire
Adverse event reporting form

Participant recruitment advertisement

April 2008

MALE SUBJECTS WANTED FOR EXERCISE RESEARCH

DEPARTMENT OF PHYSIOLOGY, THE UNIVERSITY OF MELBOURNE



Benefits of participation:

- VO_2max (maximal cycling test; normally valued ~$200)
- Remuneration for travel expenses/inconvenience ($120)
- Contribute to research which may help diabetics

The factors regulating skeletal muscle glucose use during exercise are unclear, a better understanding of these factors may help the development of drugs that improve glucose control in diabetics. Reactive oxygen species (ROS) are produced by the body and the inhibition of ROS in rodents reduces glucose uptake into contracting skeletal muscle.

Aim: To examine whether ROS influence glucose regulation in exercising humans.

Participants required: Active (exercise >2 times wk) males aged 18-35 years

Approximately 13.5 hours in total is involved in this study over 4 visits. This study involves muscle sampling (biopsy) and the use of catheters placed in the forearm. Catheters will be used for blood sampling and infusion of a glucose tracer and a ROS preventing drug (an antioxidant) commonly used in the treatment of paracetamol overdose (N-acetylcysteine).

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Plain language statement

THE UNIVERSITY OF MELBOURNE

Does an antioxidant reduce muscle glucose uptake during exercise?

Thank you for your interest in the study. This study will be conducted within the Department of Physiology, at The University of Melbourne, and has been approved by The University of Melbourne Human Research Ethics Committee, Ethics Application number 0715356.2. The Chief Investigator is Dr Glenn McConell and this study will contribute to the PhD thesis of Troy Merry.

Aims of Project

We are interested in the relationship between type 2 diabetes and exercise. In diabetes, blood sugar levels are high because organs such as the muscles don't absorb glucose normally. However, when diabetics exercise their muscles have a normal ability to absorb glucose and blood sugar levels fall. We would like to know why and how this occurs, as it might provide new means of improving blood sugar levels in diabetics.

We are focusing on molecules called reactive oxygen species (ROS) because animal studies suggest that they might explain the benefits of exercise in diabetes. However, the situation in humans is unknown. We can test whether ROS are important by inhibiting their action with an antioxidant compound called N-acetylcysteine (NAC). We can study muscle glucose uptake and muscle metabolism during exercise with and without NAC to see whether ROS are involved. For these studies we need healthy volunteers and we are grateful for your interest.

Specifically, we are seeking healthy male athlete's who undertake endurance exercise training 4-5 times a week for at least 1 hr and are prepared to participate in the following experimental procedure which involves blood sampling, muscle biopsies and intravenous (into vein) infusions.

If you agree to take part and are eligible for this study you will be required to visit the lab on a total of 4 occasions (as described in detail below). The first visit is a screening visit and will take approximately 1 hour. This visit will identify those volunteers who fit the criteria to proceed in the study and you will then return 2-3 days later for about 20 min. The subsequent 2 visits will take approximately 6 hours each and will be separated by at least 2 weeks. Therefore, the total time involved is approximately 13.5 hours and would be spread over 4 weeks.

Your Involvement

Visit 1: Medical questionnaire and fitness test.
The first visit involves a medical and training questionnaire to determine if you are suitable for participation in the study. If for whatever reason the questionnaires reveal reasons why you could not be included in the study we shall thank you for your time and return your medical information.

If you go on, we shall ask you to perform a fitness test and determine your maximal exercise capacity on an exercise bicycle that involves continuous, incremental cycling until exhaustion (12 to 20 minutes). The test may be terminated earlier if you wish.

Visit 2: Familiarisation ride
At the second visit you will exercise for approximately 20 min at a workload calculated to be 70% of your maximum capacity (determined at the first visit) to ensure that the exercise workload for the experimental trials is correct.

Visit 3: First experimental trial
The third visit is for an exercise trial and will take place approximately 1 week after the familiarisation ride.

We'll ask you to abstain from alcohol, caffeine and exercise for 24 hrs and to fast overnight before this trial. We'd also like you to record what you eat during the 24 hours prior to this trial.

When you come to the laboratory a small plastic tube (catheter) will be inserted and secured into a vein of each forearm using a needle (the needle is then removed and the catheter remains in the vein). One catheter will be used for regular blood sampling (saving the need for repeated needles). A total of about 90 ml of blood (a third of a cup) will be withdrawn during the trial. The other catheter will be used for infusion of a solution containing a small amount of "heavy" glucose (a non-radioactive tracer). In one experimental trial you will also receive an infusion of the antioxidant NAC and during the other saline (water with some salt in it) will be infused. You won't be aware of which trial you receive the NAC

Prior to the commencement of exercise, the first muscle biopsy will be obtained from the side of the thigh about 15 cm above the knee. Antiseptic methods will be used at all times and a qualified medical practitioner will conduct the muscle biopsies.

The local hair will be shaved and you will be given a local anaesthetic, which can sting slightly before the area goes numb. A small cut (approximately 7mm) will then be made into the skin so that the biopsy needle can be inserted into the muscle. The biopsy sample is about the size of two grains of rice. The time taken for muscle removal is 4-6 seconds. Participants usually describe the biopsy procedure as uncomfortable but not painful.

You will then perform the exercise trial. The exercise bout will consist of cycling continuously for 80 min at a workload equivalent to 70% of your maximum capacity.

During exercise we will monitor your heart rate and rating of perceived exertion at regular intervals, and collect expired gas air samples.

Two further muscle biopsies will be performed, the first after 40 min of exercise (this will require a short stop in exercise bout) and the second immediately following the conclusion of exercise. Participants will be required to remain in the laboratory for 2hrs following exercise trial for observation.

Visits 4: Second experimental trial
Approximately two weeks later you will complete a second trial that will be very similar to the first except that subjects who were given NAC in the first trial will not receive it on this occasion and vice versa.

You will be required to consume the same diet that you did in the 24 hours previous to the first experimental trial, and abstain from alcohol and caffeine for twenty-four hours and exercise for 24 hours and to fast overnight. The catheter placement, labelled glucose infusion, blood sampling and muscle biopsies will all be performed in an identical fashion to the first trial. The exercise will also be the same as outlined above.

Explanation of Risks
There are slight risks associated with invasive procedures in normal volunteers. These are minimised by having all procedures undertaken by a qualified and experienced medical practitioner using accepted antiseptic technique. There is a small chance of minor bruising as a result of insertion of the catheters. Very occasionally, however, there can be infections or clot formation due to insertion of catheters. We consider the risk extremely low given the aseptic/barrier techniques used in inserting the catheter and the relatively short (~4 hours) period of time that it is in place.

Some discomfort will be experienced following the muscle biopsy procedures similar to a corked thigh. However, normal daily activities can typically continue with minimal discomfort, and most participants are able to undertake vigorous exercise within 24-48 hours. In rare cases, the procedure may be associated with bruising in the muscle and / or aching for several days, which may require you to take oral analgesics (e.g. panadol). You will be given a post-biopsy information sheet and the home phone number of the chief investigator in the event of excessive bruising following the biopsy or any other unforeseen incident arising from the study. You will be given an elastic tubular bandage to wear until the evening to prevent swelling. If the bandage feels too tight, or uncomfortable, remove it. In addition the chief investigator will ring you on the evening of a trial and four days following the trial to check on your comfort.

NAC is a drug that is used in clinical practice (eg. for Panadol overdose) in higher doses than we are using in this study. When NAC is infused at high doses into healthy human volunteers, adverse reactions have been reported and include nausea, diarrhoea, vomiting, rash, altered moods, sleepiness, dizziness, coughing and in rare cases anaphylaxis (severe allergic reaction which, if left untreated, often results in death). However, when given in smaller doses to healthy volunteers (as in the current project) very few side effects have been reported.

Although unlikely, there is a possibility that you may experience a mild acute reaction to NAC that might cause one or more of the above symptoms. In the extremely unlikely event that you experience a serious adverse reaction, feel faint or unwell as a result of the cycling task or infusion the trial and infusion will be immediately terminated and you will be instructed to lie down and rest until you feel better while being monitored closely by a medical practitioner who will present throughout each experimental trial. In most cases reactions usually respond quickly to withdrawal of the infusion. In the very unlikely event that the cession of infusion does not improve symptoms a full resuscitation kit including defibrillator, antihistamines, steroids, adrenaline and IV fluids will be available for the attending medical practitioner to treat the reaction.

Our collaborators from Victoria University have conducted several studies using the same infusion of NAC that we will be using in the current study. Overall very few side-effects that could be attributed to NAC were reported and none that would be viewed as severe or require intervention or stopping of infusion.

Summary

In summary, you will be required to attend the laboratory on 4 occasions. These will all be between the hours of 7:00 am and 5:00 pm. You will be required to spend approximately 13.5 hours in the laboratory. You will receive results from your maximal cycling test which is the most accurate measure of aerobic fitness. In addition, following the completion of the study, you will be mailed a document outlining your personal results as well as group means. The name of any other subject will not be disclosed to any participant.

Confidentiality

No findings that could identify any individual participant will be published. The anonymity of your participation is assured by our procedure, in which your name will not be revealed in any results. Access to data is restricted to only the staff members directly involved in the study. The researchers may wish to contact you after the conclusion of the study to ask permission for other researchers to use your de-identified data (as indicated in the Consent Form). Coded data are stored for five years, as prescribed by University regulations.

Voluntary Participation

Participation in this research is entirely voluntary, and if you agree to participate, you may withdraw your consent at any time without being penalised or disadvantaged in any way. You may also decline to participate in any section of the procedure, by expressing your desire that you do not wish to undertake the task to the experimenters. You will receive $120 for participation in the trial to reimburse you for your transport expenses and the inconvenience of attending the laboratory. Should you withdraw from the study you will receive an appropriate payment based on a pro-rata basis.

If you have any queries or would like to be informed of the aggregate research findings, please contact me on: telephone (03) 8344 3672 mobile: 0404511171

Should you require any further information, or have any concerns, please do not hesitate to contact: Troy Merry: 8344 3672 or 0404511171, or Dr Glenn McConell: 8344 5844. Should you have any concerns about the conduct of the project, you are welcome to contact the Executive Officer, Human Research Ethics, The University of Melbourne, on ph: 8344 2073; fax 9347 6739

Thank you for taking the time to read this document.

Troy Merry (Ph.D student)

Consent form

THE UNIVERSITY OF MELBOURNE

Consent form for persons participating in research projects

Does the antioxidant N-acetylcysteine attenuate increases in muscle glucose uptake during exercise in humans?

Name of participant:

Name of investigator(s): Troy Merry BPhEd (Hons) Dr. Glenn McConell, PhD

1. I consent to participate in the project named above, the particulars of which - including details of a maximal exercise aerobic capacity test (VO_2 max test); glucose tracer infusion ; muscle biopsies; blood sampling; N-acetylcysteine infusion - have all been explained to me. A written copy of the information has been given to me to keep.

2. I authorise the researcher or his or her assistant to **perform** the testing and experimental procedures referred to under (1) above.

3. I acknowledge that:

   (a) The possible effects of the testing and experimental procedures have been explained to me to my satisfaction.

   (b) I have been informed, and I understand that I am free to withdraw from the project at any time without explanation or prejudice and to withdraw any unprocessed data previously supplied.

   (c) The project is for the purpose of research and not for treatment.

   (d) I have been informed that the confidentiality of the information I provide will be safeguarded subject to any legal requirements.

Please tick the box if appropriate

- ❑ The information I provide can be used by other researchers **as long as my permission is obtained** and my name and contact information is removed before it is given to them

Signature Date

(Participant)

Independent witness to participant's voluntary and informed consent

I believe that …….. understands the above project and gives her/his consent voluntarily

Name:

.....(please print)

Signature Date

(Witness to consent)

Medical questionnaire

THE UNIVERSITY OF MELBOURNE

MEDICAL QUESTIONNAIRE

Does the antioxidant N-acetylcysteine attenuate increases in muscle glucose uptake during exercise in humans?

NAME:_____ **AGE:** _____

WEIGHT: _____ **HEIGHT (cm):** _____ **SEX:** _____

HEALTH STATUS:

1) Do you have high blood pressure? YES NO DON'T KNOW

If yes, please elaborate: _____

2) Do you smoke? YES NO

3) Do you have or a family member have diabetes? YES NO DON'T KNOW

4) Do you, or a member of your family,
suffer from a bleeding disorder? YES NO DON'T KNOW

5) Is there a family history of heart disease? YES NO DON'T KNOW

If yes, please elaborate: _____

6) Are you overweight? YES NO DON'T KNOW

7) Do you have a heart murmur? YES NO DON'T KNOW

8) Are you asthmatic? YES NO DON'T KNOW

9) Do you have a current injury which may effect
your ability to take part in the study? YES NO DON'T KNOW

If yes, please elaborate: _____

10) Do you have allergies (including to medications)? YES NO DON'T KNOW

If yes, please elaborate: _____

11) Are you currently on any medication? YES NO

If yes, what is the medication?:_____

If you answer "yes" to any one of questions one to four, it is necessary for you to be excluded from the study. If you answer "don't know" to question one, it will be necessary for us to test your blood pressure before a decision is made concerning your involvement in the study. If you answer "yes" to any of questions five to eleven or "don't know" to questions three or four we will need to ask you for more details and then make a medical decision based on your specific circumstances.

I believe that the information I have provided is correct. I have not used anabolic steroids or any other growth-enhancing agent.

Name of Subject: _____ Signature of Subject: _____

Adverse event reporting form

Participant: Date: Time:

Dose: Rout:

Attending Medical Doctor:

Information regarding the frequency and severity of any adverse reactions found to NAC and saline (called placebo below) in this research project will be reported to the human ethics committee in the below tables. Any unforeseen adverse reaction observed or reported that is additional to those listed in the below tables will be included in our report. In the case of any moderate to severe reaction to NAC (as listed in table 1), an adverse event other than a reaction to NAC/saline, or an adverse event/reaction that can not be adequately summarised in the below tables a full description of the reaction, treatment procedures and outcome of treatment will also be provided (see page 2: Detailed adverse event reporting form).

Table 1. Adverse reactions to NAC

	***N*-ACETYL CYSTEINE**				**SALINE**			
	NO	MILD	MOD	SEV	NO	MILD	MOD	SEV
Asthma/Bronchospasm								
Breathing difficulties								
Tachycardia								
Hypotension								
Conjunctival irritation								
Vein redness								
Swelling								
Pain								
Flushing								
Rash								
Coughing								
Altered moods								
Metallic taste								
Sleepiness								
Itchy Skin								
Asthma								
Nausea								
Vomiting								
Other								

CODE:

No: no adverse effects were reported and/or observed

Mild: observed but not causing discomfort, allowing protocol to be completed

Mod (moderate): causing discomfort to the participant and interruption of protocol, but no active treatment after stopping infusion

Sev (severe): severe effects that require interruption of protocol and active treatment after stopping infusion

Detailed adverse event reporting form

Adverse event/reaction description:_____
_____
_____
_____
_____
_____
_____

When did adverse event take place:_____
_____

Treatment/course of action:_____
_____
_____
_____
_____
_____
_____
_____
_____

Outcome:
_____
_____
_____
_____
_____
_____
_____
_____
_____

Could this event have been prevent? How?
_____
_____
_____

Other comments: _____
_____
_____
_____

Sign:_____ Name:_____ Date:__/__/__

APPENDIX B

Merry, T.L., and McConell, G.K. (2009) The regulation of skeletal muscle glucose uptake during exercise: a focus on reactive oxygen species and nitric oxide signalling. *IUBMB life*, 61(5):479-484. IF 2.36, Citations: 1

IUBMB *Life*, 61(5): 479–484, May 2009

Critical Review

Skeletal Muscle Glucose Uptake During Exercise: A Focus on Reactive Oxygen Species and Nitric Oxide Signaling

Troy L. Merry and Glenn K. McConell
Department of Physiology, The University of Melbourne, Parkville, Victoria, Australia

Summary

Like insulin, muscle contraction (*in vitro* or *in situ*) and exercise increase glucose uptake into skeletal muscle. However, the contraction/exercise pathway of glucose uptake in skeletal muscle is an independent pathway to that of insulin. Indeed, skeletal muscle glucose uptake is normal during exercise in those who suffer from insulin resistance and diabetes. Thus, the pathway of contraction-mediated glucose uptake into skeletal muscle provides an attractive potential target for pharmaceutical treatment and prevention of such conditions, especially as skeletal muscle is the major site of impaired glucose disposal in insulin resistance. The mechanisms regulating skeletal muscle glucose uptake during contraction have not been fully elucidated. Potential regulators include Ca^{2+} (via CaMK's and/or CaMKK), AMPK, ROS, and NO signaling, with some redundancy likely to be evident within the system. In this review, we attempt to briefly synthesize current evidence regarding the potential mechanisms involved in regulating skeletal muscle glucose uptake during contraction, focusing on ROS and NO signaling. While reading this review, it will become clear that this is an evolving field of research and that much more work is required to elucidate the mechanism(s) regulating skeletal muscle glucose uptake during contraction.
IUBMB *Life*, 61(5): 479–484, 2009

Keywords AMPK; contraction; CaMK; ROS; NO; metabolism.

INTRODUCTION

Glucose is an essential substrate for the metabolism and homeostasis of all eukaryotic cells, with skeletal muscle being critical for glucose disposable and blood glucose regulation. Glucose can not passively diffuse into a cell and must be transported through the cell membrane by glucose transporters (GLUTS). GLUT4 is the major glucose transporter isoform expressed in skeletal muscle, and has a large capacity to increase glucose transport across the cell membrane through facilitative diffusion. GLUT4 is located intracellularly; therefore, its translocation to the cell surface to facilitate glucose transport into the cell is essential for the maintenance of whole-body glucose homeostasis in response to acute perturbations in blood glucose (*1*). Although insulin and contraction both facilitate glucose uptake into skeletal muscle by increasing GLUT4 translocation to the cell membrane, the mechanism(s) through which they signal GLUT4 translocation and glucose uptake, although not yet fully elucidated, are known to be independent (*2*) (Fig. 1).

Skeletal muscle glucose uptake during dynamic exercise can increase as much 50-fold (*3*), and is regulated through three important steps; delivery to the muscle cell, transport through the cell membrane, and flux through intracellular metabolism (Fig. 1). Under normal submaximal exercise conditions, and providing that adequate extracellular glucose is available, skeletal muscle glucose uptake appears to be limited by glucose transport though the cell membrane (*4*). The mechanism(s) through which contraction/exercise stimulates GLUT4 translocation and glucose uptake appear to arise from local factors within skeletal muscle such as calcium (Ca^{2+}), CaMK, reactive oxygen species (ROS), nitric oxide (NO), and AMP-activated protein kinase (AMPK) (Fig. 1). This review will attempt to synthesize current literature concerning the pathway(s) though which contraction signals glucose transport, focusing on the role of reactive oxygen species and nitric oxide.

POTENTIAL REGULATION OF GLUCOSE UPTAKE DURING EXERCISE

Muscle Glycogen Concentration

Skeletal muscle glucose uptake in exercising humans has been reported to be either enhanced (*5*) or unaffected (*6*) by

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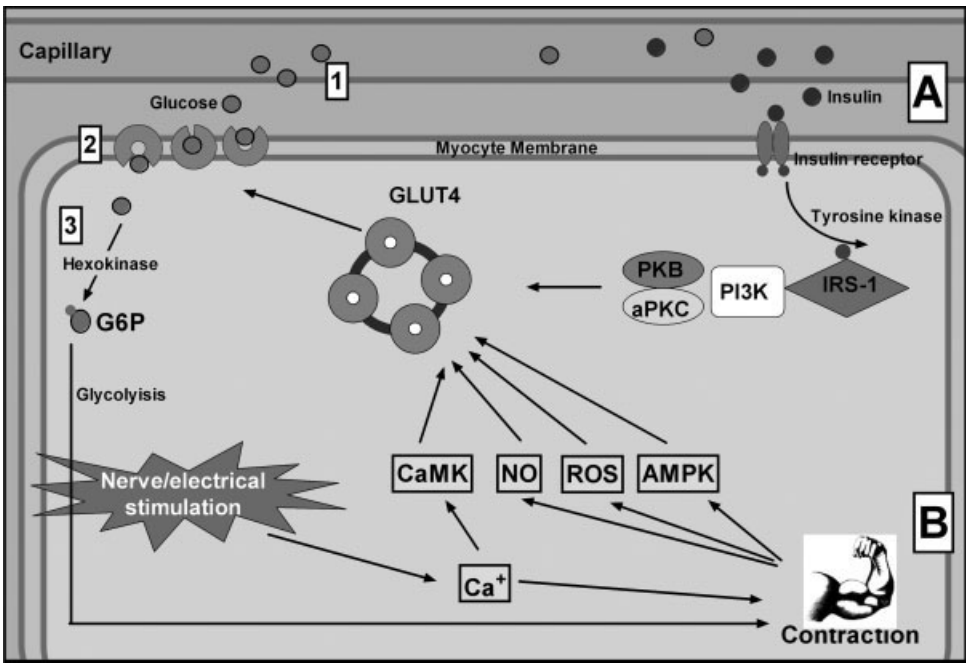

Figure 1. Mechanisms of glucose uptake into skeletal muscle. (A) insulin-stimulated glucose uptake, (B) Potential mechanisms involved in contraction-stimulated glucose uptake. 1) Glucose delivery to the muscle cell, 2) glucose transport through the membrane and 3) glucose phosphorylation and therefore 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, phosphoinositide-3 kinase; IRS-1, insulin receptor substrate 1.

low muscle glycogen levels. *In vitro* studies have shown that glucose transport is elevated in response to low muscle glycogen content in fast-twitch (glycolytic), but not slow-twitch (oxidative) muscle fibers (*7*). Because GLUT4 is hypothesized to be structurally bound to glycogen particles, it has been speculated that depleting glycogen releases GLUT4 to facilitate glucose transport (*4*). However, this process and the overall regulatory role of muscle glycogen content on glucose transport are yet to be comprehensively defined. If muscle glycogen has some influence on contraction mediated glucose uptake, it is likely to be via one or more of the signaling pathways discussed below.

AMP-Activated Protein Kinase

AMP-activated protein kinase (AMPK) is an intracellular fuel sensor that is activated by a variety of pathological, pharmacological as well as physiological induced stresses, which generally deplete or interfere with cellular ATP production (*8*). Indeed, exercise/contraction activates AMPK, and the level of activation is dependent on the metabolic demand on the muscle (*9–12*). The α2-containing complex of AMPK is the primary catalytic isoform activated during exercise in rodents (*13*), and in humans (*12*), whereas both AMPKα1 and AMPKα2 are activated during *in vitro* contractions of rodent muscles (*13, 14*).

Correlations between rates of glucose uptake and AMPK activity have been reported during contraction/exercise, suggesting a relationship between AMPK activation and glucose uptake during exercise. Although the pharmacological activator of AMPK, adenosine analogue 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR), increases AMPK activity and glucose uptake in rodent muscle *in vitro* (*15*) and *in vivo* (*16*) to a similar nonadditive extent as contraction, apparent disassociations between AMPK activation and skeletal muscle glucose uptake during exercise has brought into question the importance of AMPK in contraction-stimulated glucose transport (*7, 11*). Indeed, mice that overexpress a dominant negative inhibitory catalytic sub unit of AMPK α2 (AMPK DN) have abolished AICAR stimulated glucose uptake (*17*), but when matched for contraction force, their contraction-stimulated glucose uptake is similar to wild type litter mate controls (*18, 19*). Furthermore, neither AMPKα1 nor -α2 knockout appears to affect glucose uptake during contraction (*20*), although this may depend on contraction parameters and muscle fiber type (*14*).

Thus, it appears that skeletal muscle AMPK activation has the capacity to increase glucose uptake independent of insulin, but is not essential for contraction-stimulated glucose uptake. Indeed, AMPK has been shown to phosphorylate protein kinase B (PKB/Akt) substrate of 160 kDa (AS160), a protein that may act as one site of convergence between insulin- and contraction-

stimulated glucose uptake by promoting the docking of GLUT4 into the cell membrane (for comprehensive review see (*21*)). AS160 total phosphorylation is increased by insulin, AICAR and contraction stimulation, thus providing an attractive target for further investigation of its potential role in regulating glucose uptake.

Interestingly, mice with deficient skeletal muscle expression of a known upstream kinase of AMPK, LKB1 demonstrate an abolished AMPKα2 activation and a severely blunted increase in glucose uptake during contraction (*22*). LKB1 is not only an upstream kinase for AMPK but also regulates numerous other AMPK-related kinases that may be necessary for normal contraction-stimulated glucose transport independent of AMPK, thus warranting further investigation.

Calcium and CAMK

Early studies using caffeine to induce nondepolarization release of Ca^{2+} from the SR of isolated frog sartorious showed that raising skeletal muscle intracellular concentrations of Ca^{2+} increased *in vitro* glucose uptake (*23*), leading to the proposal that Ca^{2+} activates downstream kinases which then facilitate glucose uptake. One such kinase is calcium-calmodulin dependent protein kinase (CaMK), as its inhibition has been shown to block caffeine-stimulated glucose uptake (*24*). Furthermore, the inhibition of CaMK and CaMK kinase (CaMKK) reduces contraction stimulated glucose uptake in rodent muscles (*19, 24*). However, this relationship is not universal and seems to depend on contraction duration and/or intensity, as well as muscle fiber composition (*19, 24, 25*). There is evidence that the Ca^{2+}/CaMKK/CaMK pathway interacts to some extent with AMPK, but AMPK activation is not required for Ca^{2+}-stimulated glucose uptake during contraction (*19, 24*).

Nitric Oxide

The mu neural isoform of NOS (nNOSμ) is the primary isoform expressed in skeletal muscle. nNOSμ is constitutively active and its activity, and therefore $NO^{\bullet}$ production, increases by 1.5–2-fold with contraction (*26, 27*). The $NO^{\bullet}$ donor sodium nitroprusside (SNP) increases $NO^{\bullet}$ production in isolated skeletal muscle and enhances glucose transport independently of insulin (*26, 28, 29*), but whether $NO^{\bullet}$ simulates muscle glucose transport through the same pathway as contraction is controversial. NOS inhibition has been shown by some to substantially attenuate or abolish the increases in skeletal muscle contraction-stimulated glucose uptake in rodents *in vitro* and *in vivo* (*27, 28, 30*) while others have reported no affect (*29, 31, 32*). Although it is difficult to explain these conflicting findings (*31*), they are likely due to methodological inconsistencies. In particular, a number of studies that have found NOS inhibition not to affect glucose uptake, have measured glucose uptake >20 min following contraction/exercise and/or did not inhibit NOS until after contraction (*28, 29*).

Few studies have investigated the effect of NOS inhibition on glucose uptake during, or immediately following contraction. The two studies that have used NOS inhibitor (N^G-nitro-L-arginine methyl ester; L-NAME) ingestion to assess the effect of $NO^{\bullet}$ on glucose uptake following treadmill running in rats have yielded conflicting results of abolished (*30*) and unaffected (*32*) muscle glucose uptake. However, these studies are limited by the deleterious effects of L-NAME ingestion on homeostasis and only modest exercise induced increases in glucose uptake. In a recent study from our group, local infusion of L-NAME into the epigastric artery of the contracting hindlimb of male Hooded Wister rats attenuated the increase in muscle glucose uptake by ~35% compared with saline infusion without affecting muscle capillary blood flow, blood pressure or heart rate (*27*). Importantly, our group has shown in humans that infusion of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) during supine cycling attenuates the normal increase in leg glucose disposal during exercise in healthy males (*33, 34*), and to a greater extent, in diabetics (*33*) without altering leg blood flow. These results not only indicate that healthy humans require $NO^{\bullet}$ for normal glucose uptake during exercise (*33, 34*) but also that people with type 2 diabetes have a greater reliance on the NO-mediated glucose transport pathway during exercise (*33*).

To summarize, $NO^{\bullet}$ donors increase glucose uptake in skeletal muscle. NOS is expressed in skeletal muscle, and its activity and resulting production of $NO^{\bullet}$ increases dramatically with exercise/contraction. There is building evidence that $NO^{\bullet}$ is important for the regulation of glucose uptake into skeletal muscle of rodents and humans *during* contraction/exercise independent of blood flow, and likely to be associated with signaling GLUT4 translocation.

Downstream Mechanisms of Nitric Oxide-Mediated Glucose Uptake. Currently, there have been only a handful of studies that have investigated the possible downstream mechanism(s) of $NO^{\bullet}$ stimulated glucose uptake, with none conducted during contraction. The $NO^{\bullet}$ donor, SNP appears to increase glucose uptake in muscle through a cGMP-PKG (cGMP-dependent protein kinase) dependent pathway. Young and Leighton showed that the cGMP analogue, 8-bromo-cGMP, and the cGMP phosphodiesterase inhibitor, zaprinast, increased glucose uptake, and that by inhibiting soluble guanylate cyclase (and therefore preventing the $NO^{\bullet}$ induced increase in cGMP formation) using LY-83583, dramatically attenuates the increase in glucose uptake stimulated by the $NO^{\bullet}$ donor SNP in isolated rat soleus and EDL muscles (*35, 36*). In addition, SNP increased PKG activity and LY-83583 abolished this increase (*36*). Taken together, these studies suggest that, in resting muscle at least, $NO^{\bullet}$ stimulates glucose uptake through a cGMP-dependent pathway that may involve PKG activation. Similar to $NO^{\bullet}$ donors, contraction increases cGMP formation in skeletal muscle (*37*) and it is likely, therefore that $NO^{\bullet}$ increases glucose uptake during contraction via a cGMP-dependent pathway. However, this

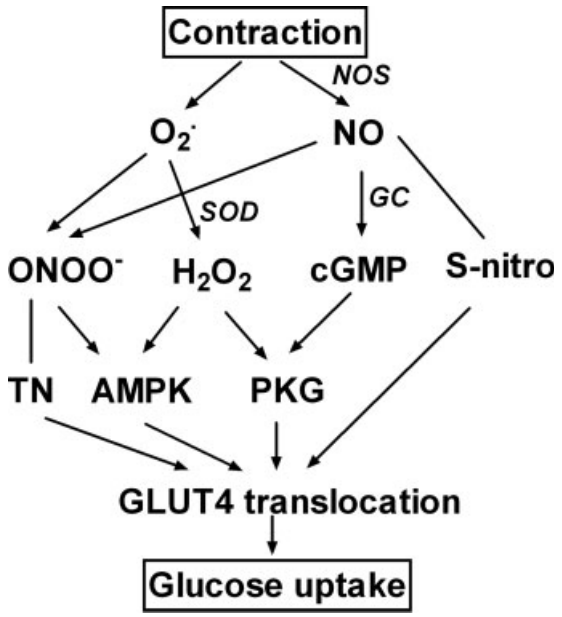

Figure 2. Potential mechanisms involved in ROS and NO-mediated glucose uptake during contraction. O_2, superoxide; NO•, nitric oxide; SOD, superoxide dismutase; sGC, soluble granulate cyclase; cGMP, cyclic guanosine monophosphate; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; S-nitro, S-nitrosylation; TN, tyrosine nitration; AMPK, AMP-activated protein kinase; PKG, cGMP-dependent protein kinase.

is yet to be determined. This is an important area of investigation given that NO• is known to also exert its affects via cGMP-independent signaling such as through the formation of peroxynitrite ($ONOO^-$) from superoxide ($O_2^{\bullet -}$) and NO•, through s-nitrosylation of proteins, and through interactions with AMPK (see Fig. 2, (*29, 38*). Indeed, $ONOO^-$ can tyrosine nitrosylate proteins associated with glucose transport such as AMPK and PI3-Kinase (*39*), and in adipocytes NO• increases GLUT4 translocation and glucose uptake independent of the cGMP/PKG pathway and AMPK activation, with the s-nitrosylation of proteins appearing to be responsible (*40*). Thus, there is building evidence that NO• plays a critical role in signaling glucose uptake during contraction; however, further investigation is needed to identify the pathway(s) though which NO• acts during contraction.

Reactive Oxygen Species

There is a basal generation of ROS in skeletal muscle, and this generation is increased substantially with contraction (*41*). ROS generation is typically associated with various disease states and deleterious muscle function and fatigue during exercise (*41*) but recent evidence, suggests that at physiological levels ROS may also be important signaling molecules regulating various cellular processes, metabolism and gene expression (*42–44*). The seemingly contrasting dynamics of ROS is likely to be a product of duration and extent of exposure, with chronic low level oxidative stress or acute large increases in ROS generation having deleterious effects on muscle cells and acute modest changes initiating signaling cascades.

Early observations that exogenous hydrogen peroxide can increase basal glucose uptake in isolated rat epitrochlearis muscle (*45*) led to more recent studies implicating ROS in the regulation of glucose uptake during contraction (*46*). Sandström et al. (*46*) showed that the antioxidants NAC and ebselen reduce the contraction-induced elevation in ROS and attenuated contraction-stimulated glucose uptake in isolated mouse EDL muscles by ~50%. Moreover, EDL muscles from mice that overexpress Mn^{2+}-dependent superoxide dismutase (SOD), and therefore presumably have increased intracellular H_2O_2 production, were shown to have greater contraction-induced glucose uptake compared with wild type controls (*46*). In support of H_2O_2 being the primary ROS associated with the regulation of glucose uptake, the anti-oxidant catalase (which reduces H_2O_2 to H_2O) but not SOD, inhibits the increase in rat EDL muscle glucose uptake in response to the superoxide generating system hypoxanthine+xanthine oxidase (*47*).

While there is preliminary evidence that ROS may be involved in signaling glucose uptake during contraction, studies examining the mechanism(s) involved have largely focused on exogenous H_2O_2-stimulated glucose transport rather than ROS produced endogenously during contraction. The increase in both glucose uptake and total AMPK activity in contracting (*46*), or AMPKα1 activity in H_2O_2 treated (*48*) muscles is diminished by NAC. This observation has lead to the hypothesis that ROS signal glucose uptake via an AMPK-dependent pathway. In contrast, Jensen et al. (*14*) found the treatment of AMPK DN, AMPK-α1 and -α2 knock out mice soleus with 3 mM H_2O_2 stimulates glucose uptake to a similar extent as in wild types. Similarly, treatment of rat EDL muscles with 600 μM of H_2O_2 increases glucose uptake without affecting AMPK α1 or α2 activity (*47*). Thus it appears that high concentrations of H_2O_2 activate AMPK, but this is not necessarily the mechanism by which ROS stimulates glucose uptake.

Interestingly, H_2O_2 treatment increases skeletal muscle PKB/Akt phosphorylation (*14, 47*). Furthermore, the PI3K inhibitor wortmannin can abolish H_2O_2 simulated glucose uptake without affecting IRS-1 phosphorylation, but combination of insulin and H_2O_2 was partially additive (*47*). Furthermore, the nonspecific antioxidant NAC does not affect insulin-stimulated glucose uptake in mouse EDL muscles (*46*). This suggests that ROS and insulin pathways of glucose uptake interact to some extent but one may not be completely reliant on the other. It is unlikely that ROS are regulating glucose uptake during contraction via a PI3K-PKB/Akt dependent pathway because PI3K and PKB/Akt phosphorylation is not required for glucose uptake during contraction (*2*). However, it is plausible that during contraction ROS may act below PI3K and PKB/Akt in the insulin pathway to simulate glucose uptake, or via a completely independent mechanism. Indeed, exposure of skeletal muscle to

exogenous H_2O_2 increases Ca^{2+} release, activates PKG and increases peroxynitrite, all of which have been implicated in signaling glucose uptake independent of insulin (see above and Fig. 2). Therefore there are numerous potential pathways through which ROS may act as intermediate signaling molecules to increase glucose uptake during contraction, making it an important area for future study.

SUMMARY AND CONCLUDING THOUGHTS

Both insulin and contraction increase skeletal muscle GLUT-4 translocation to the cell membrane and increase glucose uptake, but it is clear that independent pathways are involved. The mechanisms by which contraction/exercise increases glucose uptake into skeletal muscle are not fully elucidated, but may involve Ca^{2+} (via CaMK's and/or CaMKK), AMPK, ROS, and $NO^{\bullet}$ signaling. It is likely that more than one pathway is involved in signaling GLUT4 translocation and glucose uptake during contraction and that overlapping of pathways and redundancy may be occurring; such that if one pathway is inadequate or prevented another pathway may be upregulated.

Exciting new, yet limited, evidence suggests that $NO^{\bullet}$ and ROS (in particular H_2O_2) may play an essential role in regulating glucose uptake during exercise/contraction. Further research is required to establish the extent of free radical involvement (ROS and $NO^{\bullet}$) in signaling glucose transport during contraction/exercise and identifying the mechanisms involved. Given that ROS and $NO^{\bullet}$ are highly interrelated, it is also important to establish any possible signaling interaction and/or convergences between these molecules that may be involved in glucose uptake signaling during contraction/exercise.

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APPENDIX C

Merry, T.L., Steinberg G.R., Lynch, G.S., McConell, G.K. (2010) Nitric oxide and ROS regulate skeletal muscle glucose uptake during contraction independent of AMPK. *Am J Physiol Endocrinol Metab*, 298(3):577-85. IF= 3.89, Citations= 1

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Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK

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Merry TL, Steinberg GR, Lynch GS, McConell GK. Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK. *Am J Physiol Endocrinol Metab* 298: E577–E585, 2010. First published December 15, 2009; doi:10.1152/ajpendo.00239.2009.—Reactive oxygen species (ROS) and nitric oxide (NO) have been implicated in the regulation of skeletal muscle glucose uptake during contraction, and there is evidence that they do so via interaction with AMP-activated protein kinase (AMPK). In this study, we tested the hypothesis that ROS and NO regulate skeletal muscle glucose uptake during contraction via an AMPK-independent mechanism. Isolated extensor digitorum longus (EDL) and soleus muscles from mice that expressed a muscle-specific kinase dead AMPKα2 isoform (AMPK-KD) and wild-type litter mates (WT) were stimulated to contract, and glucose uptake was measured in the presence or absence of the antioxidant *N*-acetyl-L-cysteine (NAC) or the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA). Contraction increased AMPKα2 activity in WT but not AMPK-KD EDL muscles. However, contraction increased glucose uptake in the EDL and soleus muscles of AMPK-KD and WT mice to a similar extent. In EDL muscles, NAC and L-NMMA prevented contraction-stimulated increases in oxidant levels (dichloroflourescein fluorescence) and NOS activity, respectively, and attenuated contraction-stimulated glucose uptake in both genotypes to a similar extent. In soleus muscles of AMPK-KD and WT mice, NAC prevented contraction-stimulated glucose uptake and L-NMMA had no effect. This is likely attributed to the relative lack of neuronal NOS in the soleus muscles compared with EDL muscles. Contraction increased AMPKα Thr^{172} phosphorylation in EDL and soleus muscles of WT but not AMPK-KD mice, and this was not affected by NAC or L-NMMA treatment. In conclusion, ROS and NO are involved in regulating skeletal muscle glucose uptake during contraction via an AMPK-independent mechanism.

nitric oxide; reactive oxygen species; exercise; metabolism; contraction; glucose uptake

EXERCISE AND CONTRACTION INCREASE translocation of GLUT4 to the cell surface, facilitating glucose transport into skeletal muscle (55). Although the mechanism(s) by which contraction increases GLUT4 translocation and glucose uptake into skeletal muscle are not yet fully defined, there is consensus that the signals regulating this pathway are independent of the insulin-signaling pathway (31, 39). Numerous mechanisms have been implicated in regulating contraction-stimulated glucose uptake (43), and one of the most attractive and well-studied candidates is AMP-activated protein kinase (AMPK). While initial studies demonstrated that the AMPK activator 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) increased glucose uptake (17) and that increased AMPK activity during contraction correlates with glucose uptake (20, 38), disassociations between glucose uptake and contraction have also been reported (9, 34). Importantly, mice that overexpress a kinase dead (KD) AMPKα2 isoform, the major isoform activated with contraction (13, 52), or have a genetic deletion of AMPKα1 or α2 isoforms, have normal increases in glucose uptake during contraction (15, 25). However, this is not a universal finding since attenuation of glucose uptake during contraction in mice with deficient AMPK signaling has also been reported (14, 23, 29, 35), and these discrepancies have been attributed to differences in the contraction protocols employed (29).

Recent studies have focused on free radical signaling of glucose uptake in skeletal muscle, with donors of nitric oxide (NO) and reactive oxygen species (ROS) shown to increase glucose uptake in resting skeletal muscle (18, 19, 24). Indeed, acute physiological increases in ROS, nitric oxide synthase (NOS) activity, and NO production occur during contraction (22, 40, 44, 46) and antioxidant treatment (46) and NOS inhibition, in some (1, 2, 26, 42, 44) but not all studies (11, 18, 45), attenuates skeletal muscle glucose uptake during contraction. Because ROS increase AMPK activity (19, 24) and antioxidant treatment during contraction attenuates increases in AMPK activity (46), it has been proposed that ROS, particularly hydrogen peroxide (H_2O_2), may regulate skeletal muscle glucose uptake during contraction via an AMPK-dependent mechanism (46). However, more recent studies (19) have shown that at low concentrations (600 μM) H_2O_2 treatment of rat EDL muscles can increase glucose uptake without affecting AMPK activity. Thus it appears that although ROS can increase AMPK activity, AMPK activation is not required for ROS-stimulated glucose uptake in noncontracted muscles. Similarly, NO has been proposed to act both upstream and downstream of AMPK (30) in regulating contraction and NO-mediated glucose uptake (12, 18, 50). However, it has been shown that NOS inhibition during contraction attenuates glucose uptake without affecting AMPK signaling (44).

Based on these equivocal findings, the aim of the present study was to determine whether NO and ROS regulate glucose uptake during contraction via an AMPK-dependent pathway by contracting isolated skeletal muscle from AMPK-KD and wild-type mice in the presence of a NOS inhibitor [N^G-monomethyl-L-arginine (L-NMMA)] or an antioxidant [*N*-acetyl-L-cysteine (NAC)]. We tested the hypothesis that ROS and NO regulate glucose uptake during contraction via an AMPK-independent mechanism.

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RESEARCH DESIGN AND METHODS

Animals. This study used male C57Bl/6 mice aged 12–14 wk, and male and female mice with muscle-specific overexpression of a kinase dead form of the AMPKα2 isoform (AMPK-KD), which have been described in detail previously (10, 35). AMPK-KD and littermate controls were used for experiments at 22–24 wk of age (Table 1), and since no differences were found between male and female mice for muscle glucose uptake during contraction, the results were pooled. Mice were maintained in an environmentally controlled room at 21°C with a 12-h light-dark cycle (light 0630–1830). Mice were given ad libitum access to standard rodent chow and water. The experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, as described by the National Health and Medical Research Council (Australia).

Materials and antibodies. NAC and L-NMMA were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). 2-Deoxy-D-[1,2-^{3}H]glucose and D-[^{14}C]mannitol were purchased from Amersham Biosciences (Piscataway, NJ), and 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR) was purchased from Toronto Chemicals (Toronto, Canada). Primary antibodies for AMPKα and α-tubulin were purchased from Cell Signaling Technology (Hartsfordshire, England), and anti-phospho-ACCβ Ser^{221} and anti-phospho-AMPKα Thr^{172} were from Upstate Biotechnology (New York, NY), neuronal NOS (nNOS) was from BD Transduction Laboratories (Sydney, New South Wales, Australia), and glutathione was from Abcam (Cambridge, England). AMPKα1 and α2 antibodies used for immunoprecipitation were as previously described (5). IRDye 800-labeled streptavidin and secondary antibodies IRDye 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA).

Muscle dissection and incubation. Mice were anesthetized with pentobarbital sodium (60 mg/kg ip Nembutal; Rhone Merieux, Pinkenba, Queensland, Australia) and the proximal and distal tendons of the extensor digitorum longus (EDL) and soleus muscles of both hindlimbs were tied with 5/0-silk suture. Muscles were carefully excised tendon-to-tendon, with the proximal tendon tied to a force transducer (PanLab), and the muscles were suspended in incubation chambers (Radnoti, Monrovia, CA) filled with Ringer solution (in mM: 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, and 2.5 $CaCl_2$ pH 7.4) containing 0.01% BSA, 8 mM mannitol, and 2 mM sodium pyruvate and in the presence or absence of a AMPK activator AICAR (2 mM), NOS inhibitor L-NMMA (100 μM; Ref. 18), nonspecific antioxidant NAC (20 mM; Ref. 46), AICAR + NAC, or AICAR + L-NMMA. Chambers were oxygenated with 95% O_2-5% CO_2 gas (Carbogen; BOC Gases, Preston, Victoria, Australia) and maintained at 30°C. After all the muscles had been excised, the mice were killed by cervical dislocation while still anesthetized deeply.

Muscle contraction protocol. In contraction experiments, muscles were stimulated by square wave electrical pulses generated by a Grass S48 stimulator (model DC-300A Series II; Crown International, MA), amplified (EP500B power amplifier; Audio Assemblies, Cambellfield, Victoria, Australia), and delivered via two platinum plate electrodes that flanked but did not touch the muscle preparation. The stimulation was sufficient to recruit all motor units within the contracting muscle and elicit maximum force (32), which was recorded using a Power Lab running Chart 5.0 software (AD Instruments, Castle Hill, New South Wales, Australia). Muscles were first stimulated with a series of isometric twitch contractions to determine the muscle's optimum length (L_o), as described previously (48). Following a 30-min incubation at rest in the presence or absence of L-NMMA or NAC, muscles were stimulated to contract for 10 min [pulse durations: 350 ms (EDL) and 600 ms (soleus) at a frequency of 60 Hz, 12 contractions/min]. This contraction protocol was designed to maximize glucose uptake yet maintain muscle viability and minimize fatigue. Noncontracted muscles were treated the same as contracted muscles except that they were not stimulated during the contraction period.

Table 1. *Morphologic characteristics of AMPK-KD and WT mice*

	WT	AMPK-KD
Age, wk	23 ± 0.2	23 ± 0.2
Body mass, g	24.5 ± 0.6	24.1 ± 0.4
EDL muscle mass, mg	10.8 ± 0.5	10.1 ± 0.5
Soleus muscle mass, mg	8.8 ± 0.4	8.7 ± 0.4

Values are means ± SE; n = 28–30 per group; AMPK-KD, kinase dead AMPKα2 isoform; WT, wild type; EDL, extensor digitorum longus.

Glucose uptake measurements. 2-Deoxy-D-glucose glucose uptake was measured during the final 5 min of contraction and for 5 min of recovery in contracted muscles (51) and during the final 10 min of incubation in noncontracted muscles. This involved exchanging the incubation buffer for a buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H]glucose (0.128 μCi/ml) and 8 mM D-[^{14}C]mannitol (0.083 μCi/ml). Following incubation in this buffer, muscles were washed in ice-cold Ringer solution, blotted on filter paper, and then snap frozen in liquid nitrogen. Whole (intact) muscles were digested in 125 μl of 1 M NaOH for 10 min at 80°C and then neutralized by the addition of 125 μl of 1 M HCl, vortexed, and spun at 13,000 rpm for 2 min. The supernatant (175 μl) was recovered and added to 4.5 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA). Radioactivity of both tracers was measured by a β-scintillation counter (Packard TriCarb 2900TR; PerkinElmer), and glucose uptake was calculated (50). Separate muscles were used for immunoblotting and glucose uptake. Muscles used for immunoblotting were frozen immediately after contraction.

Immunoblotting and AMPK activity. Frozen muscles were homogenized in ice-cold lysis buffer (20 μl/mg tissue; 50 mM Tris·HCl at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM PMSF, and 5 μl/ml protease inhibitor cocktail), incubated for 20 min on ice and then centrifuged at 13,000 *g* for 20 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard. Total protein was diluted 1:3 in sample buffer (1.5 M Tris·HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, and 0.0012% bromophenol blue), heated for 10 min at 100°C, and stored at −20°C before 80 μg of total protein were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked in PBS containing 5% nonfat milk for 1 h at room temperature. For analysis of protein *S*-glutathionylation (the addition of glutathione to the cysteine sulfhydryls on proteins; a commonly used marker of oxidative stress; Ref. 8), muscle was extracted under nonreducing conditions with lysis and sample buffer containing 5 and 10 mM of *N*-ethylmaleimide, respectively, in the absence of DTT. After blocked membranes were incubated overnight at 4°C with phosphorylation-specific antibodies for ACCβ Ser^{221}, or with primary antibodies for nNOS or glutathione, binding was detected with anti-mouse or rabbit IgG secondary antibody. Direct fluorescence was detected and quantified using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NB). Membranes were then stripped [2% SDS (wt/vol) in 25 mM glycine pH 2.0] and reprobed with primary antibodies for ACCβ (streptavidin), nNOS, and tubulin to determine total protein levels. However, for AMPKα Thr^{172} phosphorylation, membranes were first probed with a AMPKα primary antibody before being stripped and reprobed with a AMPKα Thr^{172} phosporylation-specific antibody, as we find that AMPKα Thr^{172} phosphorylation cannot be effectively stripped. Protein phosphorylation was expressed relative to the total protein abundance of the protein of interest or tubulin as indicated.

For AMPKα1 and AMPKα2 activity measurements, muscles were homogenized as described above, and homogenates were incubated with AMPKα1 or AMPKα2 antibody bound protein A beads for 2 h at 4°C.

Immunocomplexs were washed with PBS and suspended in 50 mM Tris·HCl buffer (pH 7.4) for AMPK activity assay (7). In the presence of 200 μM of AMP, activities were calculated as picomoles of phosphate incorporated into the SAMS peptide [acetyl-CoA carboxylase (ACC)α (73-87)A^{77}] per minute per milligrams of total protein subjected to immunoprecepitation.

Oxidant levels and NOS activity. Oxidant levels were measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate

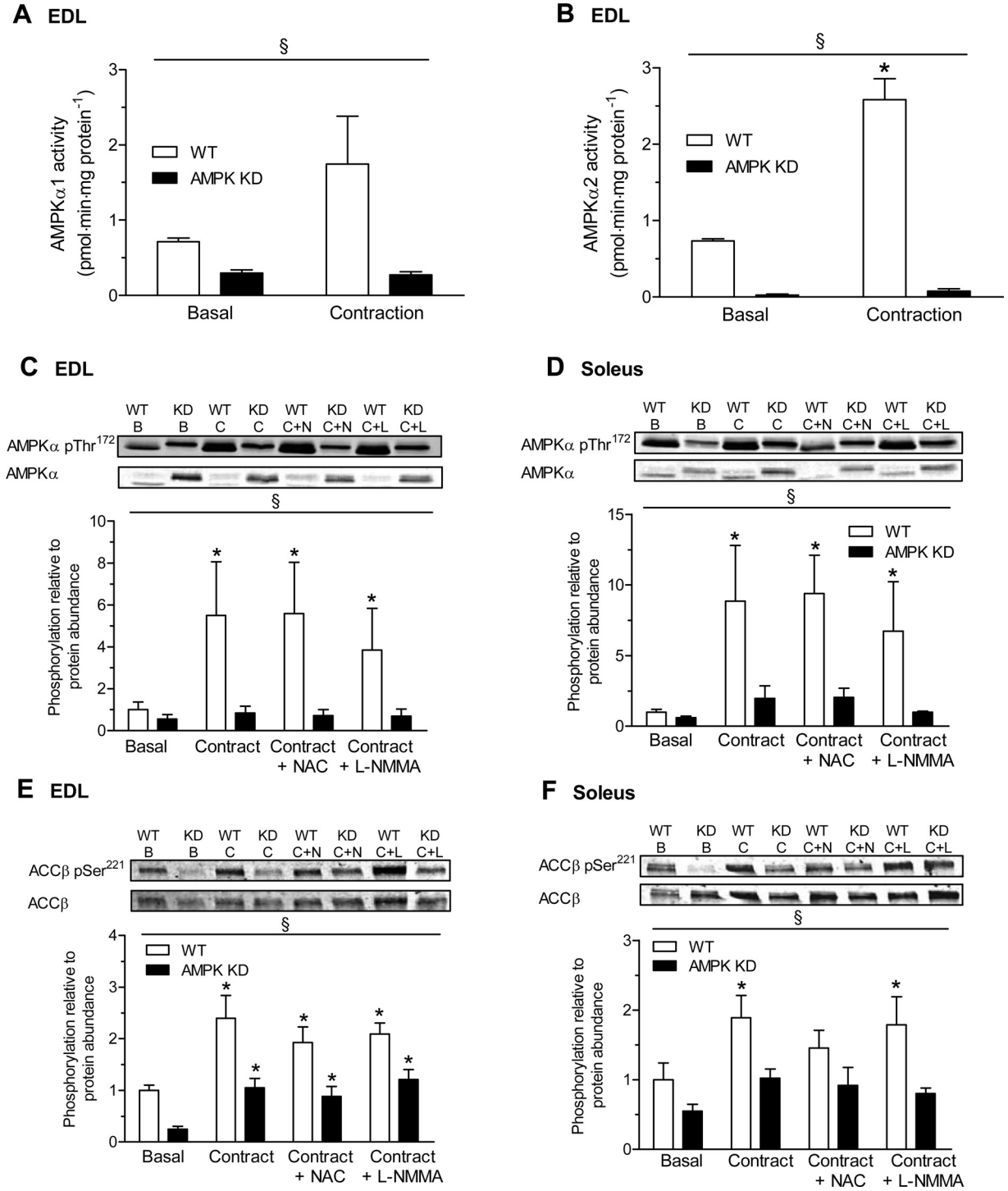

Fig. 1. AMPKα1 (*A*) and AMPKα2 (*B*) activity in the extensor digitorum longus (EDL) muscles of wild-type (WT) and AMPK kinase dead (KD) mice with either basal incubation or when stimulated to contact for 10 min. AMPKα Thr^{172} phosphorylation relative to total AMPKα protein abundance in EDL (*C*) and soleus (*D*) muscles of WT and AMPK-KD mice, and ACCβ Ser^{221} phosphorylation relative to total ACCβ (streptavidin) protein abundance in EDL (*E*) and soleus (*F*) muscles of WT and AMPK-KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of N^G-monomethyl-L-arginine (L-NMMA) and *N*-acetyl-L-cysteine (NAC). Data are means ± SE; $n = 3–4$ per group for AMPK activity and 6–7 per group for all others measures. *$P < 0.05$ vs. basal group of same genotype. §$P < 0.05$ for genotype effect. For representative blots: KD, AMPK-KD mice; B, basal; C, contraction; N, NAC; L, L-NMMA.

(DCFH-DA; Molecular Probes, Eugene, OR; Ref. 37) in muscle cross sections. Following incubation muscles were mounted in OCT and frozen in isopentane cooled in liquid nitrogen. Muscles were sectioned and treated with DCFH-DA (5 μM) and allowed to dry overnight at room temperature. The oxidized derivative of DCFH-DA, dichlorofluorescein (DCF), was measured using confocal microscopy (480 nm excitation, 520 nm emissions; Zeiss LSM-510 Meta confocal microscope, Carl Zeiss Microimaging, Thornwood, NY), and emission intensity was determined with Zeiss LSM-510 accusation and analysis software. These procedures were carried out in low light conditions to avoid photo-oxidation. NOS activity was determined in separate muscles by measuring the conversion of labeled L-arginine to labeled L-citrulline (NOS activity assay; Cayman Chemicals, Ann Arbor, MI).

Statistical analysis. All data are expressed as means ± SE. Results were analyzed by SPSS statistical package using one (condition)-, two (genotype and condition)-, and three (genotype, condition and time)-factor ANOVA. If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher's least significance difference test. Morphological properties were compared using an unpaired *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

Morphological properties. Body mass, EDL, or soleus mass was not different between age and sex matched AMPK-KD and WT mice (Table 1).

AMPK signaling. EDL muscles from AMPK-KD mice showed lower ($P < 0.01$) basal AMPKα1 and AMPKα2 activity than EDL muscles from WT mice (Fig. 1, *A* and *B*). AMPKα1 activity in EDL muscles from WT and AMPK-KD mice were not affected by contraction (Fig. 1*A*). Contraction increased ($P = 0.001$) AMPKα2 activity in EDL muscles from WT mice by 3.5-fold but did not increase AMPKα2 activity in EDL muscles from AMPK-KD mice (Fig. 1*B*).

Muscles from AMPK-KD mice showed a twofold greater expression of AMPKα than muscles from WT mice (data not shown), but WT mice had greater AMPKα Thr^{172} phosphorylation relative to AMPKα expression than AMPK-KD mice for both EDL and soleus muscles ($P < 0.05$; Fig. 1, *C* and *D*). Contraction increased ($P < 0.05$) AMPKα Thr^{172} phosphorylation four- and sevenfold above basal in EDL and soleus muscles of WT mice, respectively, but did not affect AMPKα Thr^{172} phosphorylation in EDL or soleus muscles of AMPK-KD mice ($P > 0.05$). AMPKα Thr^{172} phosphorylation in EDL and soleus muscles of both genotypes was not affected by NAC or L-NMMA (Fig. 1, *C* and *D*).

ACCβ Ser^{221} phosphorylation in EDL and soleus muscles was greater in WT than in AMPK-KD mice ($P < 0.005$; Fig. 1, *E* and *F*). Contraction increased ACCβ Ser^{221} phosphorylation in EDL and soleus muscles of WT mice ($P < 0.05$) and in EDL ($P = 0.05$) but not soleus ($P = 0.17$) muscles of AMPK-KD mice (Fig. 1, *E* and *F*). NAC treatment prevented the contraction-induced increase in ACCβ Ser^{221} phosphorylation in soleus ($P = 0.17$) but not EDL muscles of WT mice

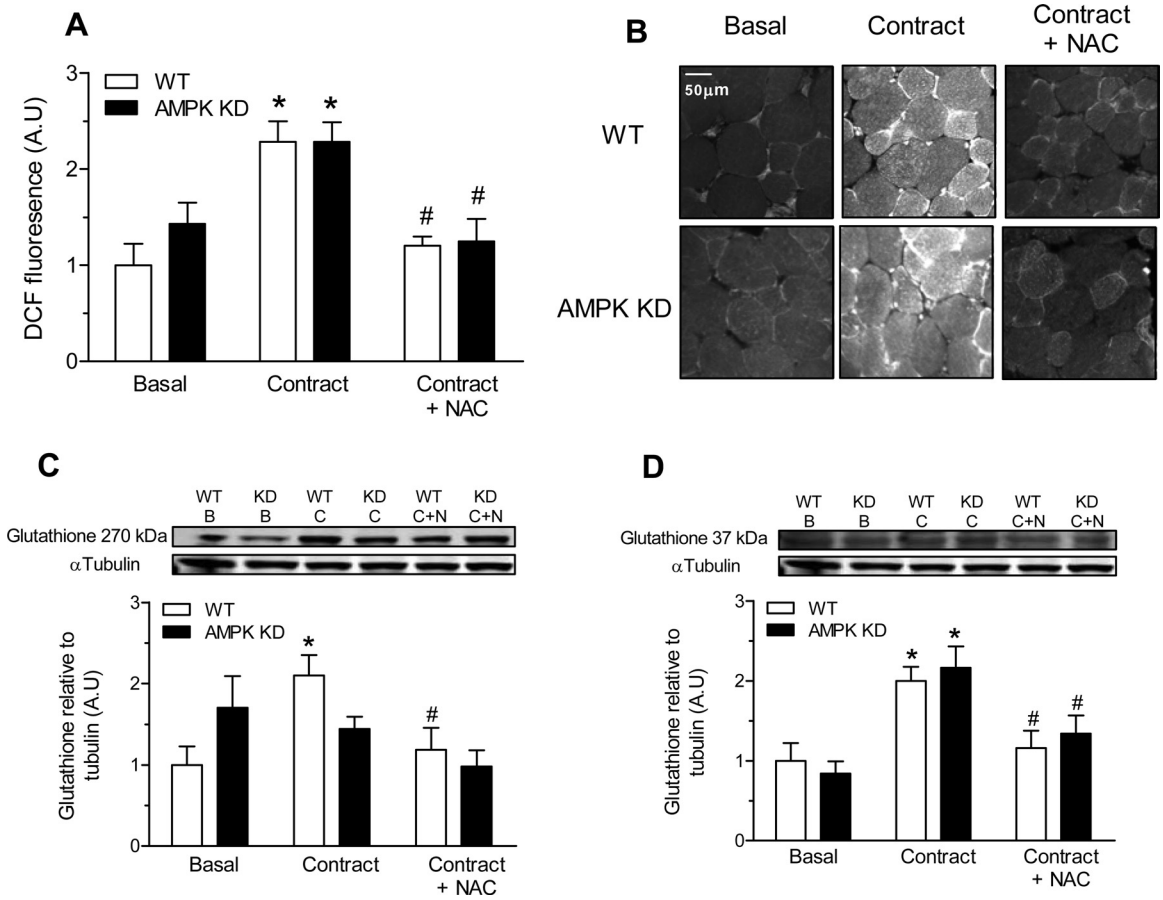

Fig. 2. Representative images (*A*) and emission intensity (*B*) of dichloroflourescein (DCF) fluorescence and *S*-glutathionylation of a protein band at ~270 kDa (*C*) and 37 kDa (*D*) in EDL muscles of WT and AMPK-KD mice with either basal incubation or when stimulated to contract for 10 min in the presence or absence of NAC. Data are means ± SE; $n = 4$–7 per group. A.U., arbitrary units. $^{*}P < 0.05$ vs. basal group of same genotype. $^{\#}P < 0.05$ vs. contraction group of same genotype.

and did not affect ACCβ Ser^{221} phosphorylation in the EDL or soleus muscles of AMPK-KD mice (Fig. 1, *E* and *F*). L-NMMA treatment did not affect ACCβ Ser^{221} phosphorylation during contraction in either EDL or soleus muscles of either genotype (Fig. 1, *E* and *F*).

Oxidant levels and S-glutathionylation. Contraction increased ($P < 0.05$) oxidant levels by ~50% in the EDL muscles of both AMPK-KD and WT mice, and this increase was prevented by NAC (Fig. 2, *A* and *B*). *S*-glutathionylation is a redox signaling event and therefore was measured as a marker of oxidative stress (8). Contraction also increased ($P < 0.05$) *S*-glutathionylation of protein bands at ~270 and ~37 kDa by approximately twofold in EDL muscles of WT mice, and these increases were prevented by NAC. In EDL muscles from AMPK-KD mice contraction did not increase *S*-glutathionylation at ~270 kDa (Fig. 2*C*) but increased ($P < 0.05$) *S*-glutathionylation at ~37 kDa (Fig. 2*C*) by ~2.3-fold ($P < 0.05$), and this increase was prevented by NAC.

NOS activity and nNOS expression. EDL muscles from AMPK-KD mice showed a ~30% lower NOS activity than muscles from WT mice ($P = 0.046$; Fig. 3*A*). Contraction increased ($P < 0.05$) NOS activity approximately twofold in the EDL muscles of both WT and AMPK-KD mice, and this increase was prevented with L-NMMA (Fig. 3*A*).

As in previous studies (27), nNOS protein was threefold more abundant in EDL than in soleus muscles ($P < 0.001$), and there was no difference in nNOS protein expression in the EDL and soleus muscles of WT and AMPK-KD mice (Fig. 3*B*).

Glucose uptake. Despite inhibition of AMPK signaling in AMPK-KD mice, contraction increased ($P < 0.05$) glucose uptake in the EDL (2.3-fold) and soleus (1.6-fold) muscles from both WT and AMPK-KD mice to a similar extent (Fig. 4). NAC reduced resting C57Bl/6 muscle EDL muscle glucose uptake from 1.1 ± 0.1 to 0.7 ± 0.1 $\mu mol \cdot g \cdot^{-1} \cdot h^{-1}$ ($P = 0.02$). In EDL muscles from WT mice, NAC attenuated the increase in glucose uptake during contraction by ~50% ($P = 0.01$) and prevented increases in muscle glucose uptake in EDL muscles of AMPK-KD mice during contraction (Fig. 4*A*). In the soleus muscles of both WT and AMPK-KD mice, NAC prevented the increase in glucose uptake during contraction (Fig. 4*B*).

l-NMMA did not affect resting glucose uptake in EDL muscles of C57Bl/6 mice (data not shown). However, L-NMMA attenuated the increases muscle glucose uptake during contraction in EDL muscles of WT ($P = 0.03$) and AMPK-KD mice ($P = 0.05$; ~40%), but L-NMMA did not affect glucose uptake during contraction in the soleus muscles of either WT ($P = 0.34$) or AMPK-KD mice ($P = 0.51$; Fig. 4, *A* and *B*).

Muscle contraction. Neither treatment nor genotype affected peak force (normalized to muscle mass) during contraction of either the EDL or soleus muscles (Fig. 5, *A* and *B*). Peak force in both muscles was obtained within the first minute of the 10-min contraction protocol and declined thereafter ($P < 0.001$). Similarly, force produced by EDL and soleus muscles was not affected by NAC or L-NMMA treatment in AMPK-KD or WT mice ($P > 0.05$).

AICAR-stimulated glucose uptake, AMPK phosphorylation and oxidant levels. AICAR increased glucose uptake 2.6-fold ($P < 0.01$) in the EDL muscles of C57Bl/6 mice, and this increase was prevented by NAC (Fig. 6*A*). L-NMMA had no effect on AICAR-stimulated glucose uptake in EDL muscles

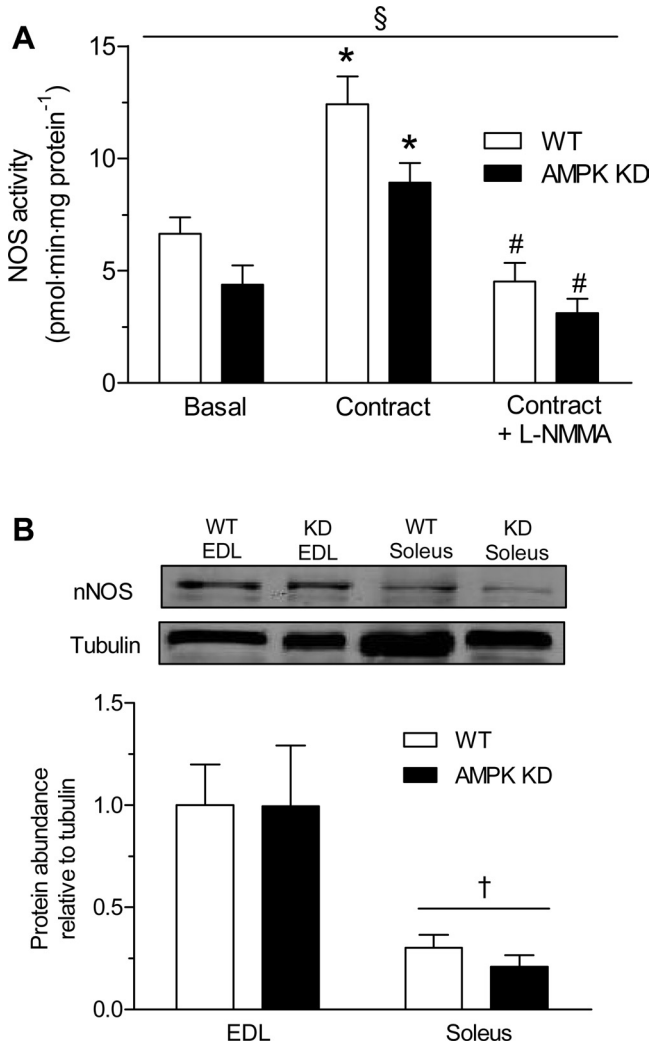

Fig. 3. Nitric oxide synthase (NOS) activity in EDL muscles of WT and AMPK-KD mice with basal incubation or when stimulated to contract for 10 min in the presence or absence of L-NMMA (*A*) and neuronal NOS (nNOS; *B*) expression relative to tubulin in EDL and soleus muscles of WT and AMPK-KD mice. Data are means ± SE; $n = 6–8$ per group. $^{*}P < 0.05$ vs. basal group of same genotype. $^{\#}P < 0.05$ vs. contraction of same genotype. $^{\dagger}P < 0.05$ vs. EDL. $^{\S}P < 0.05$ for genotype effect.

(Fig. 6*A*). NAC had no effect on basal AMPK phosphorylation in EDL muscles, but it prevented the 2.3-fold increase in AMPKα Thr^{172} phosphorylation in response to AICAR ($P = 0.01$; Fig. 6*B*). AICAR did not affect oxidant levels in EDL muscles, but the addition of NAC to AICAR tended to reduce oxidant levels in EDL muscles ($P = 0.09$; Fig. 6, *C* and *D*).

DISCUSSION

The most important finding of this study was that ROS and NO are involved in regulating glucose uptake during contraction via a mechanism independent of AMPK. We also provided further evidence that AMPK activity is not required for normal increases in glucose uptake during contraction of mouse skeletal muscles in vitro.

As reported previously (10, 29), AMPK-KD mice have reduced AMPKα1 and α2 activity in EDL muscles, and unlike WT mice, AMPK-KD mice show no increases in AMPKα2

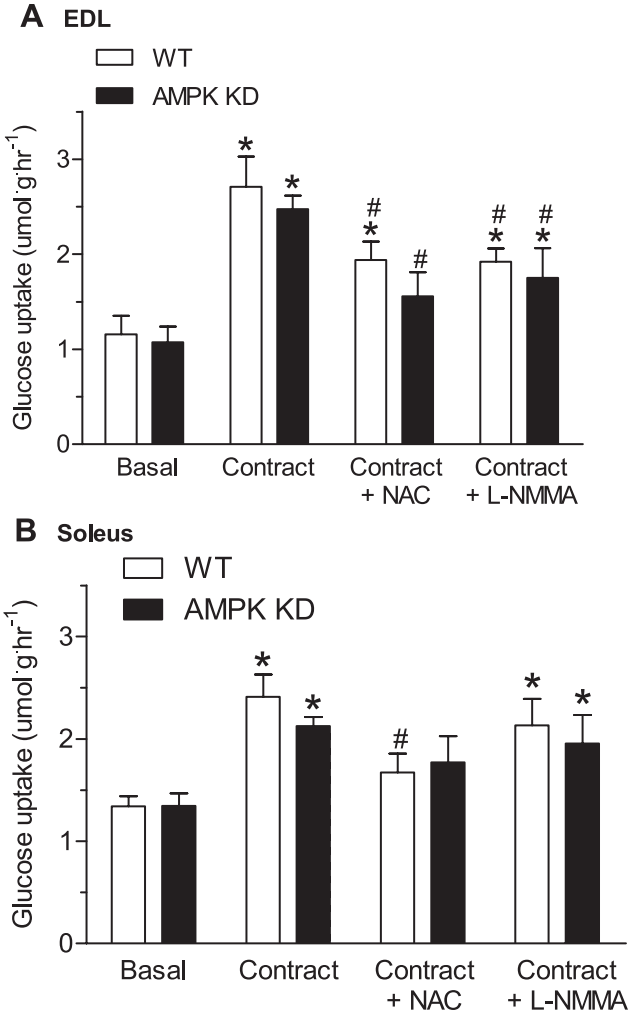

Fig. 4. 2-Deoxy-D-glucose uptake in EDL (*A*) and soleus (*B*) muscles from WT and AMPK-KD mice with either basal incubation or when stimulated to contact for 10 min in the presence or absence of L-NMMA and NAC. Data are means ± SE; $n = 6–12$ per group. $^{*}P < 0.05$ vs. basal group of same genotype. $^{\#}P < 0.05$ vs. contraction of same genotype.

activity in EDL muscles during contraction (Fig. 1). Despite this, glucose uptake increased to a similar extent with contraction in the EDL and soleus muscles of WT and AMPK-KD mice (Fig. 4). Previously, isolated muscles from AMPK-KD mice have been reported to have a slight but significant attenuation of the increase in glucose uptake during contraction (14, 23, 35). However, when the force produced by muscles from WT mice, which is greater than that in AMPK-KD mice at high stimulation frequencies (≥75 Hz; Ref. 29), is matched to the force of muscles from AMPK-KD mice by reducing the stimulation voltage, muscles from AMPK-KD mice have similar increases in glucose uptake during contraction as muscles from WT mice (14). Despite a similar workload, reducing stimulation voltage in muscles of WT mice may alter muscle fiber recruitment and signaling, thus potentially reducing glucose uptake (29, 47). Furthermore, recent evidence (47) suggests that force production is not necessarily synonymous with glucose uptake but rather metabolic demand. In the present study, we used a more physiologically relevant contraction protocol than used previously (18, 25, 46), specifically by using lower stimulation frequencies, train durations, and contraction rates (54). Similar to the findings of Lefort et al. (29), we found no difference in force production between muscles of AMPK-KD and WT mice (Fig. 5) using a low (50 Hz) frequency stimulation. In contrast, Lefort et al. (29) reported that contraction-stimulated glucose uptake was reduced by ~50% in muscles from AMPK-KD mice. It is difficult to discern the reason for the discrepancy between our findings and those of Lefort et al. (29), but it may be attributed to differences in the contraction protocols. Lefort et al. (29) stimulated muscles at a higher intensity for a shorter duration at one train per second for 2 min, compared with our protocol of 12 contractions per minute for 10 min. This is consistent with the fact that AMPK activation is higher during more intense exercise (6, 21) and thus may be playing a greater role in regulating glucose uptake during exercise at higher intensities.

The nonspecific antioxidant NAC attenuated the increase in glucose uptake during contraction (Fig. 4) of glycolytic (EDL) muscles from WT mice to a similar extent as reported previously (Fig. 4; Ref. 46). However, Sandstrom et al. (46) also reported that NAC attenuated the increases in AMPK activity and AMPK phosphorylation during contraction, suggestive of a causative relationship between ROS, AMPK, and glucose

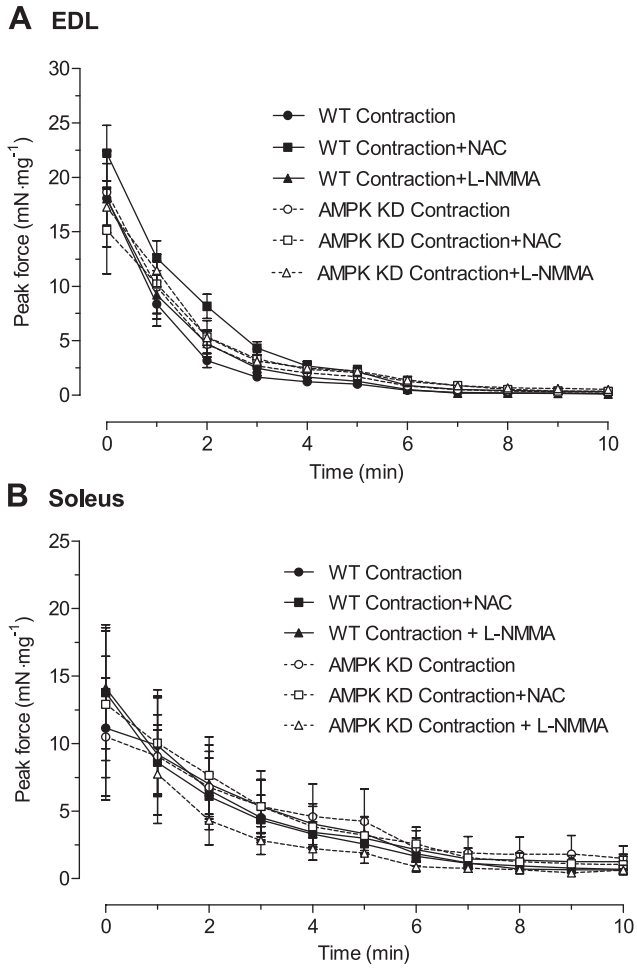

Fig. 5. Force production of isolated EDL and soleus muscles of WT and AMPK-KD mice at the beginning of each minute of the 10-min contraction protocol (see RESEARCH DESIGN AND METHODS for details). Data are means ± SE; $n = 6–12$ per group.

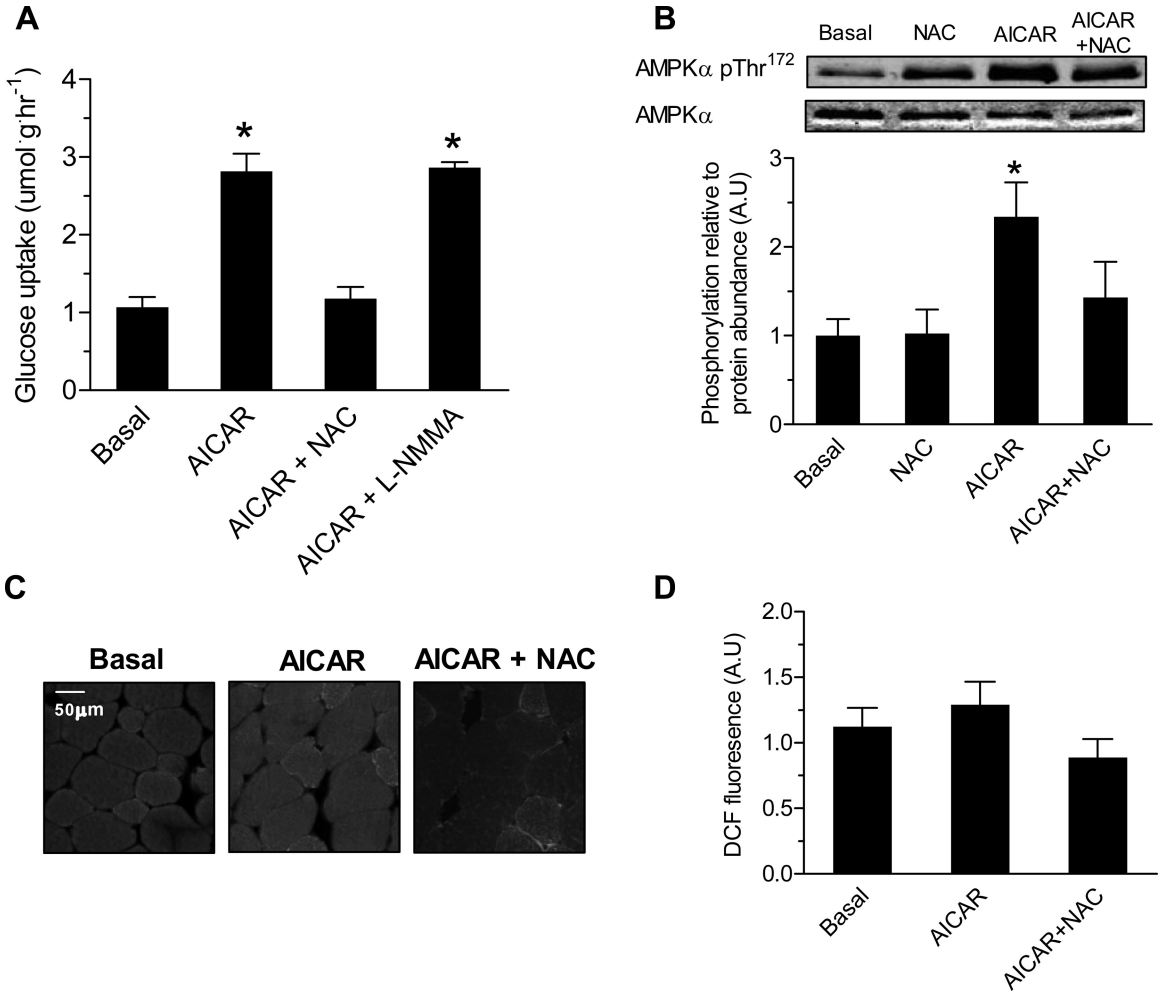

Fig. 6. 2-Deoxy- D-glucose uptake (*A*), AMPKα Thr^{172} phosphorylation relative to total AMPKα protein abundance (*B*), representative images (*C*), and emission intensity (*D*) of DCF fluorescence in EDL muscles of C57Bl/6 mice when incubated in the presence and absence of NAC, 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR), NAC + AICAR, or AICAR + L-NMMA. Data are means ± SE; $n = 4–6$ per group. *$P < 0.05$ vs. basal.

uptake during contraction. In the present study, we showed that in addition to NAC attenuating increases in glucose uptake during contraction in glycolytic muscles, NAC also prevented increases in glucose uptake during contraction in oxidative (soleus) muscles (Fig. 4). Furthermore, we found that NAC attenuated increases in skeletal muscle glucose uptake during contraction even in the absence of any increases in AMPKα1 or α2 activity during contraction (Fig. 1). In support of previous findings, we report that skeletal muscle oxidant levels are increased during contraction (41) and that this increase is prevented by NAC (46). In the present study, we measured DCF fluorescence as a marker of oxidant levels in cross-sections of EDL muscles. Although DCFH can be oxidized to DCF by both reactive nitrogen species (36) and ROS (37), because NAC prevented contraction-induced increases in DCF, we are confident of this method as a marker of muscle ROS levels. Furthermore, oxidative stress promotes the addition of glutathione to protein residuals (*S*-glutathionylation; Ref. 8). Since contraction-stimulated increases in *S*-glutathionylation of protein bands at ~37 kDa in AMPK-KD and ~270 and ~37 kDa in EDL muscles of WT mice and since this increase was prevented by NAC (Fig. 2), this provides further evidence that contraction increased muscle ROS levels. Therefore, our findings suggest that ROS are involved in regulating glucose uptake in both glycolytic and oxidative muscles independent of AMPK.

In contrast to Sandstrom et al. (46), we found that NAC did not affect AMPK Thr^{172} phosphorylation during contraction in EDL muscles. The reason for this discrepancy is also difficult to discern, but it could be attributed to minor differences in methodology such as the incubation temperature (25°C in Ref. 46 vs. 30°C used in the present study), longer NAC preincubation period (60 min in Ref. 46 vs. 30 min used in the present study), and differences in contraction protocol as discussed above. Regardless, our finding that NAC did not affect muscle AMPK Thr^{172} phosphorylation during contraction in WT mice supports the involvement of ROS in regulating skeletal muscle contraction-stimulated glucose uptake independently of AMPK. In agreement, recent studies (19, 24) have reported that exogenous ROS can increase glucose uptake in isolated glycolytic and oxidative skeletal muscles independent of AMPK. Higaki et al. (19) showed that the phosphatidylinositol 3-kinase inhibitor wortmannin prevented exogenous H_2O_2-stimulated skeletal muscle glucose uptake. However, contraction increases skeletal muscle glucose uptake via a phosphatidylinositol 3-kinase-independent pathway (31, 39). Therefore, it is likely that

during contraction ROS regulate skeletal muscle glucose uptake through a pathway that differs from that of exogenous H_2O_2. Interestingly, ROS appear to regulate p38 MAPK (p38) phosphorylation during exercise (16), and p38 has been implicated in the regulation of skeletal muscle contraction-stimulated glucose uptake (49). Furthermore, ROS have been proposed to increase stretch-stimulated skeletal muscle glucose uptake via p38 (4). Given the numerous pathways beginning to be recognized as being redox sensitive (22), it is important to investigate whether ROS regulate skeletal muscle glucose uptake during contraction via p38 or alternative pathways. Indeed, ROS have already been implicated in the regulation of pathways involved in glucose uptake signaling including $ONOO^-$ signaling (56) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase activity (3, 53).

Interestingly, AICAR-stimulated AMPK phosphorylation and glucose uptake in EDL muscle were prevented by NAC. Although AICAR stimulation did not increase basal oxidant levels in EDL muscles, NAC tended to reduce AICAR oxidant levels. This suggests that basal ROS levels may be required for AICAR-stimulated AMPK phosphorylation and glucose uptake. However, it is important to acknowledge that the mechanisms through which AICAR activates AMPK and stimulates glucose uptake are considerably different from those of contraction (25) and the effect of NAC on AICAR-stimulated glucose uptake appears to be depend on treatment order (46).

The role of NO in the regulation of skeletal muscle glucose uptake during contraction is controversial, with some studies reporting that inhibition of NOS during contraction attenuates the increases in glucose uptake (1, 2, 26, 42, 44), while others have reported no effect (11, 18, 45). These differences are also likely attributed to inconsistencies in methodology (33). In the present study, L-NMMA treatment attenuated the increase in glucose uptake in EDL but not soleus muscles of AMPK-KD and WT mice during contraction (Fig. 4). Since NOS activity and glucose uptake in EDL muscles of WT and AMPK-KD mice were increased with contraction and L-NMMA prevented these increases (Fig. 3), this suggests that NO is involved in the regulation of glucose uptake during contraction independent of AMPK in muscles with a higher proportion of glycolytic fibers. This was supported by the finding that L-NMMA also did not affect contraction-stimulated AMPK Thr^{172} or ACCβ Ser^{221} phosphorylation in EDL muscles (Fig. 1). However, as reported previously (28), EDL muscles from AMPK-KD mice showed ~30% lower NOS activity than in WT mice. This supports evidence that AMPK phosphorylates NOS (7) and that this phosphorylation increases NOS activity. Interestingly, the lower absolute NOS activity in EDL muscles of AMPK-KD mice did not affect glucose uptake during contraction. This suggests that because muscles from AMPK-KD and WT mice showed similar increases in NOS activity and glucose uptake during contraction (Fig. 4), it may be that the contraction-stimulated increase in NO production from basal, rather than the absolute NO concentration, is more important in regulating glucose uptake during contraction. As discussed above, it has been reported previously that during intense stimulation protocols ex vivo that the muscles of AMPK-KD mice have attenuated increases in glucose uptake during contraction compared with muscles from WT mice. Therefore, whether this lower glucose uptake is a result of lower AMPK or lower NOS activity requires further examination. Interestingly, L-NMMA did not affect glucose uptake during contraction in the soleus muscles of WT or AMPK-KD mice, suggesting that NO plays a greater role in regulating glucose uptake during contraction in glycolytic vs. oxidative muscles. Indeed, nNOS protein abundance was similar between genotypes, and lower in the soleus than in EDL muscles (Fig. 3) consistent with reports that soleus muscles produce less NO than EDL muscles (27).

In conclusion, this study provides evidence that ROS and NO are involved in regulating skeletal muscle glucose uptake during contraction independently of AMPK.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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APPENDIX D

Merry, T.L., Dywer, R.M. Bradley, E.A., Rattigan, S., McConell, G.K., Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat. *J Appl Physiol*. 108(5):1275-83 IF= 3.6. Citations= N/A.

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Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat

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Merry TL, Dywer RM, Bradley EA, Rattigan S, McConell GK. Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat. *J Appl Physiol* 108: 1275–1283, 2010. First published March 4, 2010; doi:10.1152/japplphysiol.01335.2009.—There is evidence that reactive oxygen species (ROS) contribute to the regulation of skeletal muscle glucose uptake during highly fatiguing ex vivo contraction conditions via AMP-activated protein kinase (AMPK). In this study we investigated the role of ROS in the regulation of glucose uptake and AMPK signaling during low-moderate intensity in situ hindlimb muscle contractions in rats, which is a more physiological protocol and preparation. Male hooded Wistar rats were anesthetized, and then *N*-acetylcysteine (NAC) was infused into the epigastric artery (125 mg·kg^{-1}·h^{-1}) of one hindlimb (contracted leg) for 15 min before this leg was electrically stimulated (0.1-ms impulse at 2 Hz and 35 V) to contract at a low-moderate intensity for 15 min. The contralateral leg did not receive stimulation or local NAC infusion (rest leg). NAC infusion increased ($P < 0.05$) plasma cysteine and cystine (by ~360- and 1.4-fold, respectively) and muscle cysteine (by 1.5-fold, $P = 0.001$). Although contraction did not significantly alter muscle tyrosine nitration, reduced (GSH) or oxidized glutathione (GSSG) content, S-glutathionylation of protein bands at ~250 and 150 kDa was increased ($P < 0.05$) ~1.7-fold by contraction, and this increase was prevented by NAC. Contraction increased ($P < 0.05$) skeletal muscle glucose uptake 20-fold, AMPK phosphorylation 6-fold, ACCβ phosphorylation 10-fold, and p38 MAPK phosphorylation 60-fold, and the muscle fatigued by ~30% during contraction and NAC infusion had no significant effect on any of these responses. This was despite NAC preventing increases in *S*-glutathionylation with contraction. In conclusion, unlike during highly fatiguing ex vivo contractions, local NAC infusion during in situ low-moderate intensity hindlimb contractions in rats, a more physiological preparation, does not attenuate increases in skeletal muscle glucose uptake or AMPK signaling.

exercise; metabolism; *S*-glutathionylation; reactive oxygen species; AMP-activated protein kinase

WHOLE BODY GLUCOSE HOMEOSTASIS is largely dependent on the transport of glucose into skeletal muscle cells (5). Insulin and contraction both regulate skeletal muscle glucose uptake by signaling the translocation of the glucose transporter protein GLUT4 to the cell surface, which facilitates the transport of glucose through the cell membrane into the cell (5). However, insulin and contraction increase skeletal muscle glucose uptake through different signaling pathways (13, 49, 51). The pathway through which contraction signals glucose uptake is not yet fully elucidated (25) but may include discrete or integrated signaling via calcium/calmodulin-dependent protein kinase (CaMK) (48), AMP-activated protein kinase (AMPK) (13), and nitric oxide (NO) (4, 37).

Although chronic elevation in oxidative stress (a pro-oxidant shift in cell redox status) is associated with the pathophysiology of type 2 diabetes and insulin resistance (21), acute treatment of isolated skeletal muscle with exogenous ROS stimulates insulin-independent glucose uptake (14, 16). Acute increases in skeletal muscle ROS production occur during in vivo exercise (11, 24, 40) and during ex vivo contractions (34, 39). Sandstrom et al. (39) have shown that the treatment of isolated skeletal muscle ex vivo with the antioxidant *N*-acetylcysteine (NAC) attenuates both the contraction-stimulated increase in oxidative stress and increases in glucose uptake. Thus there is evidence that acute increases in ROS are involved in the signaling of skeletal muscle glucose uptake during ex vivo contractions. Interestingly, Sandstrom et al. (39) also reported that, similar to glucose uptake, NAC attenuates the contraction-stimulated increases in skeletal muscle AMPK activity. Furthermore, antioxidant supplementation (allopurinol) has been shown to prevent increases in p38 MAPK phosphorylation during exhaustive exercise in rats (11), and p38 MAPK has been implicated in the regulation of contraction and stretch-stimulated skeletal muscle glucose uptake (6, 42). This suggests that during contraction ROS may regulate skeletal muscle glucose uptake via the activation of AMPK and/or p38 MAPK.

Although ROS appear to be essential for normal increases in skeletal muscle glucose uptake and AMPK signaling during ex vivo contractions (39), it remains to be determined whether ROS are involved in regulating contraction-stimulated glucose uptake and AMPK signaling using intact and more physiological models. This is important because ex vivo models rely on diffusion gradients for substrate delivery and clearance (1), unlike in vivo exercise where muscle and capillary blood flow are involved in the regulation of skeletal muscle glucose uptake (31, 47). Furthermore, unlike in vivo, ex vivo models of muscle contraction generally involve nonuniform delivery of oxygen to all muscle fibers, an absence of antioxidant systems surrounding muscle (such as that supplied by blood flow), and supramaximal highly fatiguing stimulation protocols (1), which are all likely to artificially inflate oxidative stress and alter ROS signaling (32). As such, it may be that during these highly fatiguing ex vivo contraction conditions, ROS preferentially regulate skeletal muscle glucose uptake and AMPK activity.

Therefore, in this study, we determined whether local infusion of the antioxidant NAC attenuates the increase in hindlimb skeletal muscle glucose uptake and AMPK signaling during physiologically relevant low-moderate intensity contractions in situ in rats. We hypothesized that low-moderate intensity contractions in situ would increase skeletal muscle glucose

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uptake and AMPK signaling and that NAC infusion would attenuate these increases.

RESEARCH DESIGN AND METHODS

Animals

Male hooded Wistar rats weighing 238 ± 2 g and ~8 wk of age were maintained in an environmentally controlled room at 21°C with 12:12-h light-dark cycle at the University of Tasmania. Rats were given ad libitum access to standard rodent chow and water. The present study was approved by the University of Tasmania Ethics Committee and conformed to the guidelines for the care and use of experimental animals, as described by the National Health and Medical Research Council (Australia).

Materials and Antibodies

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). 2-Deoxy-D-[1-^{14}C]glucose (2DG; specific activity 1.92 TBq/mmol) was purchased from Amersham life science (NSW, Australia). Primary antibodies for AMPKα, p38 MAPK, α-tubulin, and anti-phospho-p38 MAPK Thr^{180}/Tyr^{182} were purchased from Cell Signaling Technology (Hartsfordshire, UK), and 3-nitrotyrosine from Chemicon. Anti-phospho-ACCβ Ser^{222} and anti-phospho-AMPK Thr^{172} were purchased from Upstate Biotechnology, and anti-glutathione was purchased from Abcam (Cambridge, UK). IRDye 800-labeled streptavidin and secondary anti-bodies IRDye 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA).

Experimental Procedure

An anesthetized rat model was utilized in this study as described previously (30, 37, 47). Briefly, rats were anesthetized using pentobarbital sodium (1.5 μl/g body wt ip), and cannulas were inserted into the carotid artery and jugular veins for arterial sampling and continuous administration of anesthetic, respectively. Isotonic saline (154 mmol/l NaCl) containing NAC (125 mg·kg^{-1}·h^{-1}) or isotonic saline alone was infused locally (at 1:100 of the arterial flow rate) into one hindlimb (contracted leg) via the epigastric artery. Sham surgery was performed on the contralateral leg (rest). NAC is a nonspecific antioxidant that directly scavenge ROS (2) and is deacetylated to cysteine, which promotes the resynthesis of reduced glutathione (GSH) (8, 41). The systemic infusion of NAC at 125 mg·kg^{-1}·h^{-1} has been shown previously to elevate muscle NAC, cysteine, and total GSH during exercise in humans (24). After a 15-min preinfusion, the contracted leg was electrically stimulated to contract (0.1-ms impulse at 2 Hz and 35 V) for 15 min while NAC infusion continued. The knee was secured by the tibiopatellar ligament, and the Achilles tendon was attached to a Harvard Apparatus isometric transducer, thereby allowing measurement of tension development from the gastrocnemius-plantaris-soleus muscle group during contraction. It has previously been shown that during highly fatiguing contractions ex vivo, ROS are involved in the regulation of glucose uptake during contraction (39). Therefore this contraction protocol was chosen because we have previously shown it to substantially increase muscle glucose uptake and metabolic signaling while causing only a physiological level of fatigue (37). At 10 min before the completion of the experiment (from t = 20 to 30 min), a 1.85-MBq bolus of 2DG in isotonic saline was administered via the right jugular vein. Immediately following the 2DG bolus, an arterial blood sample (0.5 ml) was withdrawn by an automated syringe pump at 50 μl/min over 10 min. From this blood sample a plasma sample (25 μl) was collected to determine the average plasma specific radioactivity of 2DG. At t = 30 min the lower leg muscles (soleus, plantaris, gastrocnemius red, and gastrocnemius white) from the contracted and contralateral (rest) leg were rapidly dissected and freeze-clamped using liquid nitrogen-cooled thongs. Throughout the experimental protocol, heart rate (HR), mean arterial pressure (MAP), and femoral blood flow were monitored and recorded as described previously (30, 47).

Muscle Glucose Uptake

The lower leg muscles (soleus, plantaris, gastrocnemius red, and gastrocnemius white) were ground under liquid nitrogen, and 100 mg was homogenized with 1.5 ml water before free and phosphorylated glucose were separated by ion-exchange chromatography using anion-exchange resin (AG1-X8; Bio-Rad, Hercules, CA). Inorganic liquid scintillation cocktail (Amersham Life Science) was added to samples, and radioactivity was measured by a β-scintillation counter (Packard TriCarb 2900TR, Perkin-Elmer, Boston, MA). Based on plasma glucose, muscle, and plasma 2DG concentrations, muscle 2DG glucose uptake (R′g) was calculated as follows:

$$R'g = \frac{\text{muscle}\left[^3\text{H}\right]\text{2DG6-P}\left(\text{dpm/g}\right) \times \text{plasma}\left[\text{glucose}\right]\left(\mu\text{g/ml}\right)}{\text{average plasma}\left[^3\text{H}\right]\text{2DG}\left(\text{dpm/ml}\right) \times 10\left(\text{min}\right)}$$

where 2DG6-P is 2-deoxy-D-glucose-6-phosphate.

NAC and Thiols

Muscle preparation. Thirty milligrams of ground muscle sample was rapidly homogenized on ice in 300 μl of 0.42 M PCA; immediately following homogenization PCA was neutralized with 40 μl of 2.5 M K_2CO_3. Samples were then centrifuged at 13,000 g for 5 min at 4°C and the supernatant was recovered.

HPLC method for plasma and muscle sample. For analysis of reduced thiols (reduced NAC, GSH, and cysteine), 10 μl of distilled H_2O was added to 50 μl of sample, and for the determination of total thiols (TNAC, TGSH, and total cysteine) 10 μl of tributylphosphine solution diluted 1:10 was added to 50 μl of sample to oxidize reduced thiols. All samples were then incubated for 30 min on ice and 25 μl of 4-fluoro-7-sulfamoylbenzofurazan (ADB-F; 5 mg/ml in borate buffer: 0.2 M boric acid, 2 mM sodium EDTA, pH 8.0) was added. Following 10 min incubation at 50°C, 10 μl of 2 M PCA was added and samples were centrifuged at 13,000 g for 5 min. A 40-μl aliquot of the supernatant was then injected onto a reverse-phase HPLC Gemini column (5 μm C_{18} 110 Å, phenomenex) with 0.1 M sodium acetate buffer (pH 4.0) in 10% methanol as a mobile phase, at a flow rate of 1.5 ml/min and detection wavelength of 386 nm excitation and 516 nm emission. All HPLC values obtained for thiols were measured against standards. Oxidized thiols (NAC, GSSG, cystine) were calculated from the difference between the measured reduced and total thiols.

Muscle Signaling

Three-hundred milligrams of ground muscle was homogenized (10 μl/mg tissue) in ice-cold lysis buffer (50 mM Tris·HCl at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM PMSF, and 5 μl/ml Protease Inhibitor Cocktail). Lysates were then incubated for 20 min on ice and centrifuged at 13,000 g for 20 min at 4°C. For analysis of *S*-glutathionylation, a marker of oxidative stress (9), muscle was extracted under nonreducing conditions with lysis and sample buffer (1.5 M Tris·HCl, pH 6.8, 30% glycerol, 10% SDS, 0.6

M DTT, 0.0012% bromophenol blue) containing no DTT, and 5 mM and 10 mM of *N*-ethylmaleimide, respectively, to alkylate free thiol groups. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with BSA as the standard. Total protein was diluted 1:3 in sample buffer, heated for 10 min at 100°C (with the exception of samples used for 3-nitrotyrosine analysis, which were heated at 37°C for 10 min), and stored at −20°C before 80 μg of total protein was separated by SDS-PAGE, transferred to PVDF membrane, and blocked in PBS containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody for glutathione and phosphorylation-specific primary antibodies for ACCβ Ser^{222} and p38 MAPK Thr^{180}/Tyr^{182} before binding was detected with rabbit IgG secondary antibody. Direct fluorescence was detected and quantified using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE). Membranes were then stripped [2% SDS (wt/vol) in 25 mM glycine, pH 2.0] and reprobed with primary antibodies for ACCβ (streptavidin) and p38 MAPK to determine total protein levels. However, for AMPKα Thr^{172} phosphorylation, membranes were first probed with a AMPKα primary antibody before being stripped and reprobed with a AMPKα Thr^{172} phosphorylation-specific antibody as we find that AMPKα Thr^{172} phosphorylation cannot be effectively stripped. Protein phosphorylation was expressed relative to the total protein abundance of the protein of interest or α-tubulin.

Statistical Analysis

All data are expressed as means ± SE. Results were analyzed by SPSS statistical package using two-factor ANOVA as well as two-factor repeated-measures ANOVA. Because NAC infusion started precontraction, the repeated-measures ANOVAs were partitioned to assess the effect of NAC at rest (0–15 min) and during exercise (15–30 min). If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

RESULTS

Force Development

Peak contraction force decreased by ~30% by the end of the 15-min contraction period ($P < 0.001$; Fig. 1). Local NAC infusion did not affect initial peak contraction force ($P = 0.56$) or the rate of fatigue ($P = 0.65$, Fig. 1).

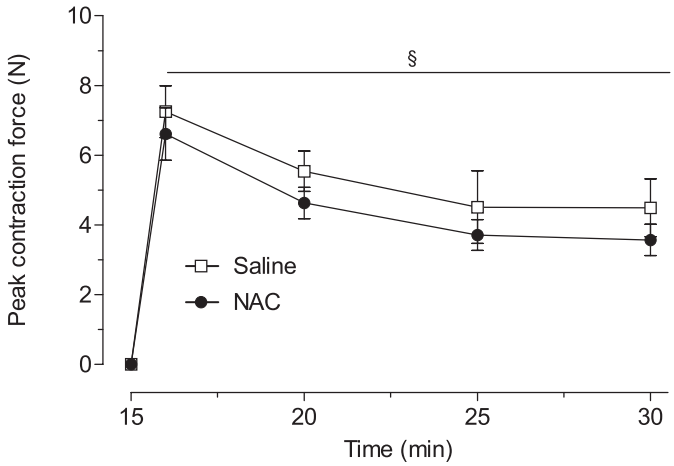

Fig. 1. Force production of the hindlimb of rats when locally infused with *N*-acetyl-L-cysteine (NAC) or saline during 15 min of in situ contractions (see RESEARCH DESIGN AND METHODS for details). Data are means ± SE; $n = 4$ per group. §$P < 0.05$ for time.

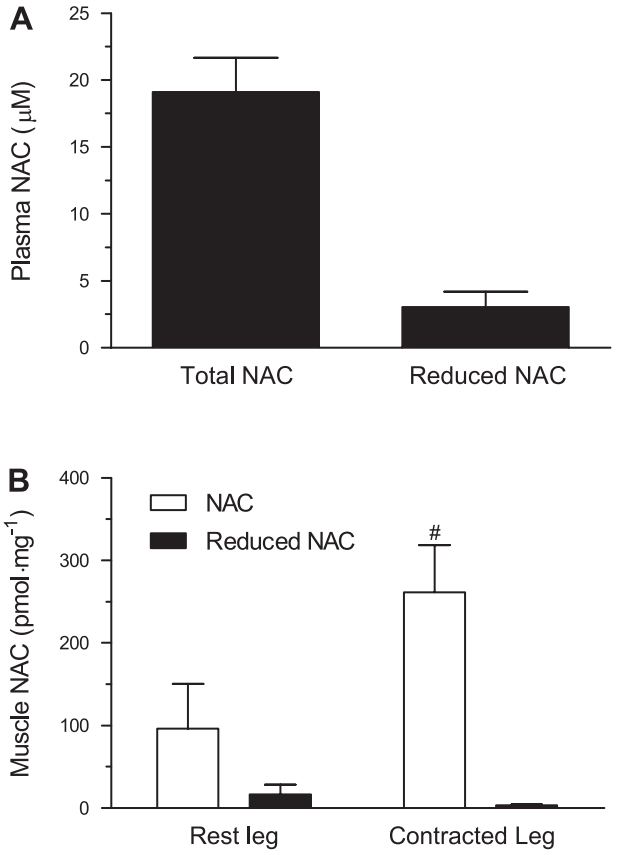

Fig. 2. Plasma (*A*) and rest and contracted leg muscle (*B*) NAC content during in situ hindlimb contractions (taken at $t = 30$ min) in rats receiving local NAC infusion into the contracted leg. Data are means ± SE; $n = 6–8$ per group. #$P < 0.05$ vs. rest leg.

Plasma and Muscle NAC

Local NAC infusion resulted in plasma NAC and reduced NAC concentration of 19.1 ± 2.6 and 3.1 ± 1.1 μM, respectively (Fig. 2*A*). Local NAC infusion increased muscle NAC in the contracted leg to a greater extent than the rest leg ($P = 0.03$, Fig. 2*B*). Similar concentrations of reduced NAC were found in the muscle of the rest and contracted legs ($P = 0.16$; Fig. 2*B*). NAC was not detected in the plasma or muscle during saline infusion (data not shown).

Heart Rate and Blood Pressure

Local NAC infusion did not affect resting heart rate (325 ± 38 beats/min). Heart rate was only measured during the first 5 min of contraction and was not increased significantly from rest at this point (data not shown). Resting MAP was not affected by local NAC infusion (Fig. 3*A*); however, local NAC infusion attenuated the contraction-induced increase in MAP by ~12 mmHg at $t = 20$ min ($P < 0.05$, Fig. 3*A*), suggesting some systemic affects of NAC infusion.

Leg Blood Flow and Vascular Resistance

Leg blood flow remained unchanged during the precontraction infusion period (Fig. 3*B*). Contraction increased femoral

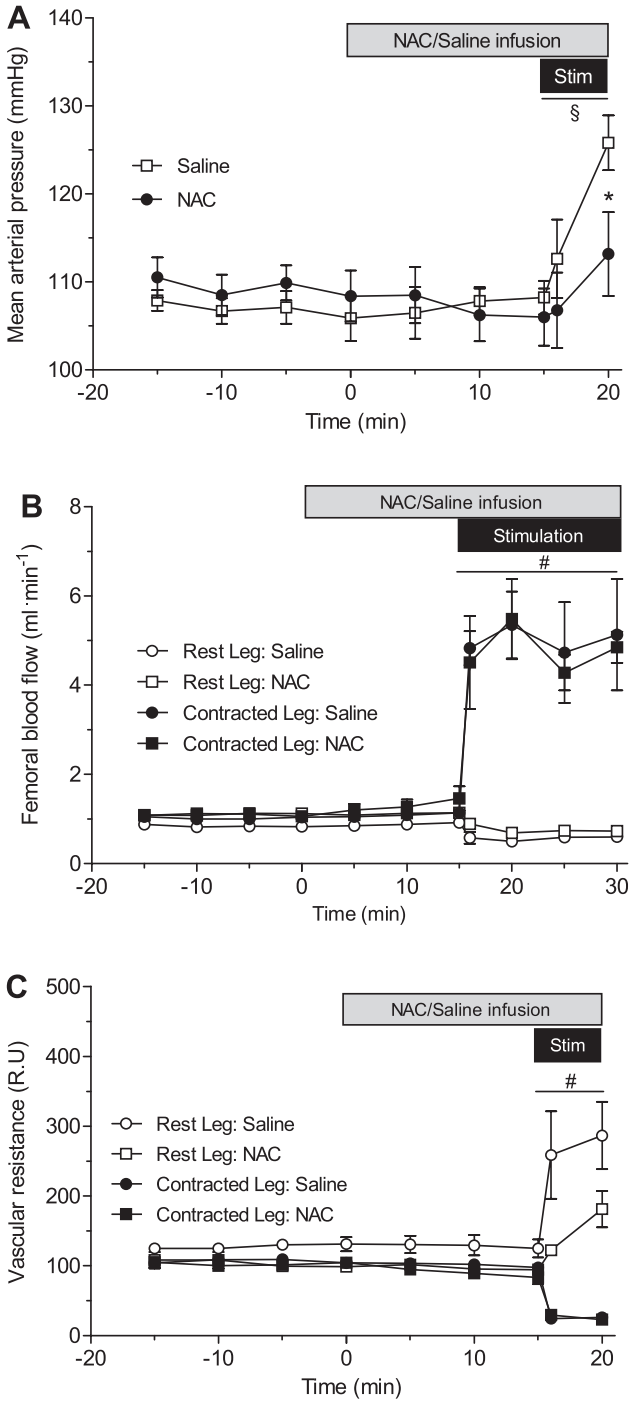

Fig. 3. Effect of NAC or saline infusion on mean arterial pressure (*A*), and rest and contracted leg femoral blood flow (*B*), and vascular resistance (*C*) at rest and during in situ hindlimb contractions in rats. Data are means ± SE; n = 6–8 per group. §$P < 0.05$ for time, #$P < 0.05$ for condition (rest vs. contraction), *$P < 0.05$ vs. saline.

blood flow to the contracted leg by ~400% ($P < 0.001$; Fig. 3*B*). Local NAC infusion did not affect the contraction-induced increase in leg blood flow ($P = 0.78$; Fig. 3*B*). Precontraction vascular resistance was reduced in NAC rest leg compared with saline infused rest leg ($P = 0.02$, Fig. 3*C*); however, resting vascular resistance was not affected by local NAC infusion in contracted leg ($P = 0.19$, Fig. 3*C*). During contraction, vascular resistance was increased in the rest leg, due to sympathetic outflow contributing to blood flow redistribution (45), and reduced in the contracted leg ($P < 0.05$, Fig. 3*C*). Although NAC infusion appeared to attenuate (by ~60% at $t = 20$ min) vascular resistance in the resting leg during contraction, NAC did not significantly alter vascular resistance during contraction ($P = 0.09$ for interaction).

Muscle and Plasma Thiols

Muscle glutathione levels were measured as marker of cellular oxidative state (29) since under conditions of oxidative stress reduced glutathione (GSH) is more rapidly oxidized to oxidized glutathione (GSSG). Figure 4, *A, C,* and *E*, shows that muscle GSH, GSSG, and GSSG/GSH ratio were not significantly affected by contraction or local NAC infusion. NAC is rapidly deacylated to produce cysteine (10), and like NAC, cysteine can directly scavenge ROS (2, 8). Therefore, cysteine levels were measured as a marker of NAC-enhanced antioxidant defenses. Local NAC infusion increased muscle cysteine similarly by 50% in the rest and contracted leg ($P = 0.001$, Fig. 4*B*). There was a tendency for local NAC infusion to increase muscle cystine ($P = 0.07$), and NAC infusion tended to increase the affect of contraction on muscle cystine ($P = 0.08$; Fig. 4*D*). Contraction did not affect muscle cysteine or cystine concentrations (Fig. 4, *B* and *D*). Local NAC infusion increased ($P < 0.05$) plasma cysteine and cystine 360- and 1.4-fold, respectively (Fig. 4*F*).

Muscle Glucose Uptake

The contracted leg had a muscle glucose uptake ~20-fold greater than the rest leg ($P < 0.001$; Fig. 5*A*). Local NAC infusion did not affect muscle glucose uptake in the rest or in the contracted leg ($P > 0.05$, Fig. 5*A*).

Muscle S-Glutathionylation and Tyrosine Nitration

Contraction significantly ($P < 0.05$) increased muscle *S*-glutathionylation of protein bands at ~250 and 150 kDa in the contracted saline infused leg ~1.7-fold, and NAC infusion prevented this increase ($P < 0.05$; Fig. 5*B*). Neither contraction nor NAC affected protein *S*-glutathionylation of any other visible protein bands. Muscle tyrosine nitration was not significantly affected by contraction or NAC infusion (Fig. 5*C*).

AMPKα, ACCβ, and p38 MAPK Phosphorylation

Contraction increased ($P < 0.05$) phosphorylation of AMPK (6-fold) and ACCβ (10-fold) (Fig. 6, *A* and *B*), and this increase was not affected by local NAC infusion (Fig. 6, *A* and *B*). Similarly, contraction increased ($P = 0.002$) p38 MAPK phosphorylation (by ~60-fold), and this increase was not affected by local NAC infusion ($P > 0.05$; Fig. 6*C*).

DISCUSSION

The major finding of this study was that local infusion of the antioxidant NAC does not attenuate the increase in hindlimb skeletal muscle glucose uptake or AMPK signaling during in situ contractions in rats. Although our low-moderate stimulation protocol did not affect muscle tyrosine nitration, GSH, or GSSG contents, it significantly increased *S*-glutathionylation of protein bands at ~250 and 150 kDa, and these increases in

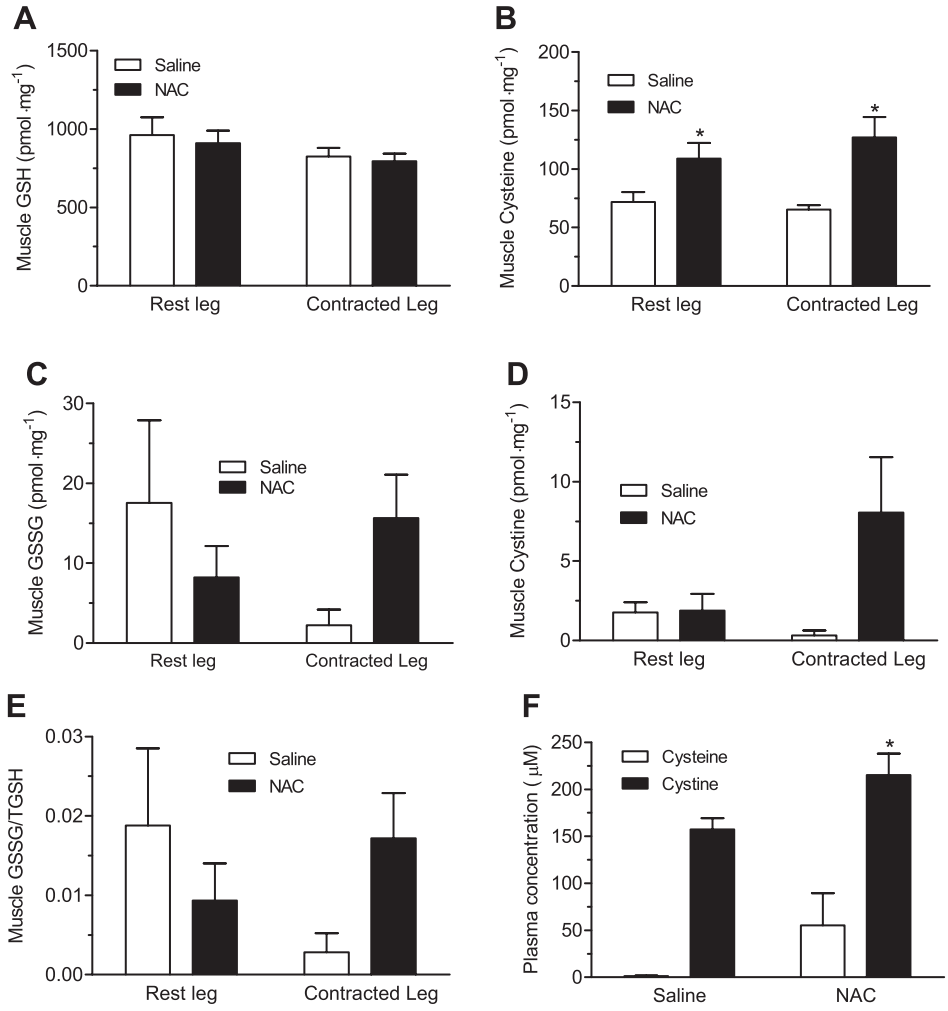

Fig. 4. Effect of local NAC or saline infusion on rest and contracted leg muscle reduced glutathione (GSH) (*A*), cysteine (*B*), oxidized glutathione (GSSG) (*C*), cystine (*D*), and GSSG/GSH ratio (*E*) following 15 min of in situ hindlimb contractions in the contacted leg of rats, and plasma cysteine and cystine levels during contraction (at $t = 30$ min) (*F*). Data are means ± SE; $n = 6–8$ per group. *$P < 0.05$ for treatment (NAC vs. saline).

S-glutathionylation were prevented by NAC infusion. Therefore, because our low-moderate contraction protocol also substantially increased skeletal muscle glucose uptake and metabolic signaling, and resulted in a physiological degree of fatigue, this study provides evidence to suggest ROS are not essential for the regulation of skeletal muscle glucose uptake or AMPK signaling during low-moderate intensity contractions in situ.

Surprisingly, and in contrast to several studies in humans and rats (11, 24, 40) we were unable to detect a change in muscle GSH or GSSG following contraction. Interestingly, however, we found that *S*-glutathionylation of protein bands at ~250 and 150 kDa was increased during contraction. We are currently conducting experiments to determine the nature of these proteins. *S*-glutathionylation is the addition of glutathione to protein cysteine residues, which is enhanced during times of nitrosative and oxidative stress (9). Although contraction increases nitrosative stress (3, 37), the increase in *S*-glutathionylation during contraction was likely the result of oxidative stress because NAC infusion abolished these increases. This suggests that ROS production increased during our contraction protocol causing a small oxidative shift in cell redox that was not large enough to substantially deplete antioxidant defenses and therefore alter glutathione levels, but sufficient to increase *S*-glutathionylation, which NAC prevented.

It is likely that no measurable changes in muscle glutathione status were seen in this study because of the low-moderate intensity (0.1-ms impulse at 2 Hz and 35 V), but physiologically relevant, short-duration (15 min) stimulation protocol we employed. In support, previously Sahlin et al. (38) has shown that muscle glutathione levels are not affected by submaximal short-duration (20 min) dynamic exercise in humans. Like glutathione status, muscle tyrosine nitration was also not affected by the low-moderate intensity contraction protocol (Fig. 5*B*). Nitric oxide and superoxide interact to form peroxynitrite, which at high concentrations promotes protein tyrosine nitration (12). Tyrosine nitration is generally associated with deleterious inhibitory effects on muscle signaling (28), and therefore it is not surprising that if any peroxynitrite was produced during contraction it did not increase tyrosine nitration. It is, however, important to acknowledge that the stimulation intensity employed is physiologically comparable to submaximal exercise in humans as it substantially increases muscle glucose uptake (Fig. 5) and metabolic signaling (Fig. 6) without being highly fatiguing (Fig. 1). Conversely, the majority of those studies reporting a reduction in muscle GSH or an increase in GSSG/GSH ratio following contraction either employ exercise

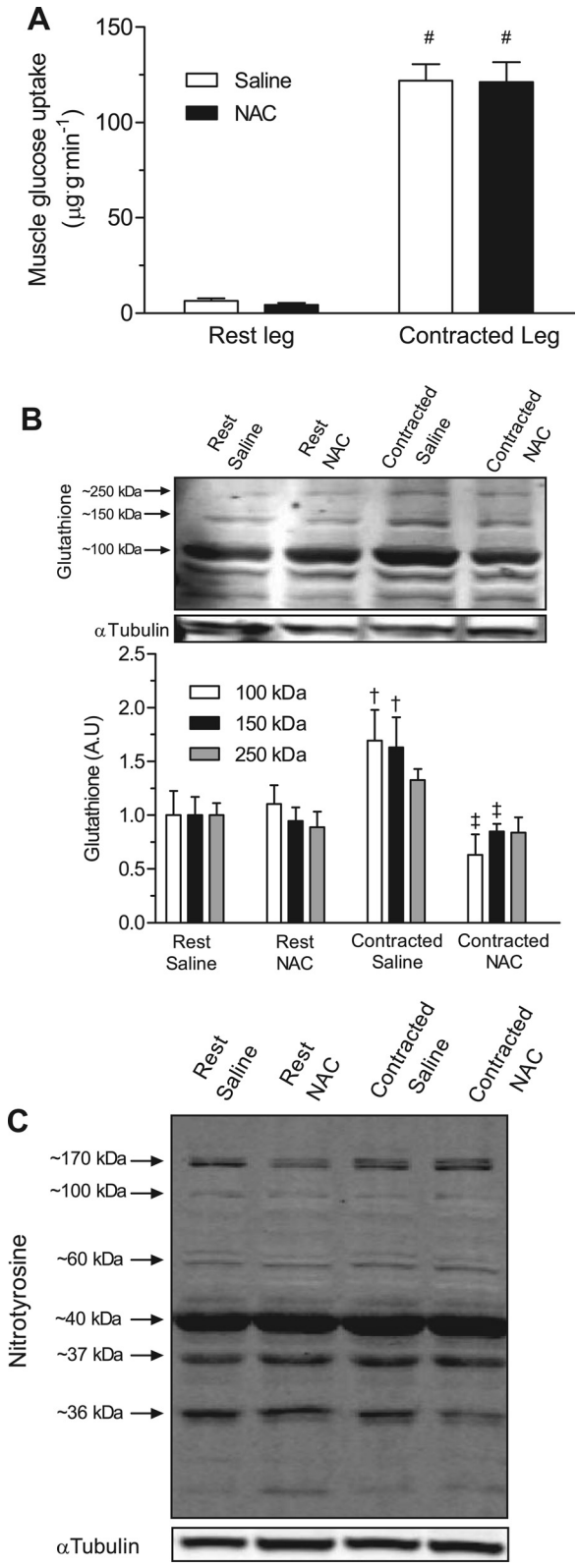

Fig. 5. Effect of local NAC or saline infusion on rest and contracted leg muscle glucose uptake (*A*), *S*-glutathionylation (*B*), and tyrosine nitration (*C*) following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SE; $n = 6$ per group. #$P < 0.05$ for condition (rest vs. contraction). ‡$P < 0.05$ vs. saline of same condition. †$P < 0.05$ vs. rest, of same treatment.

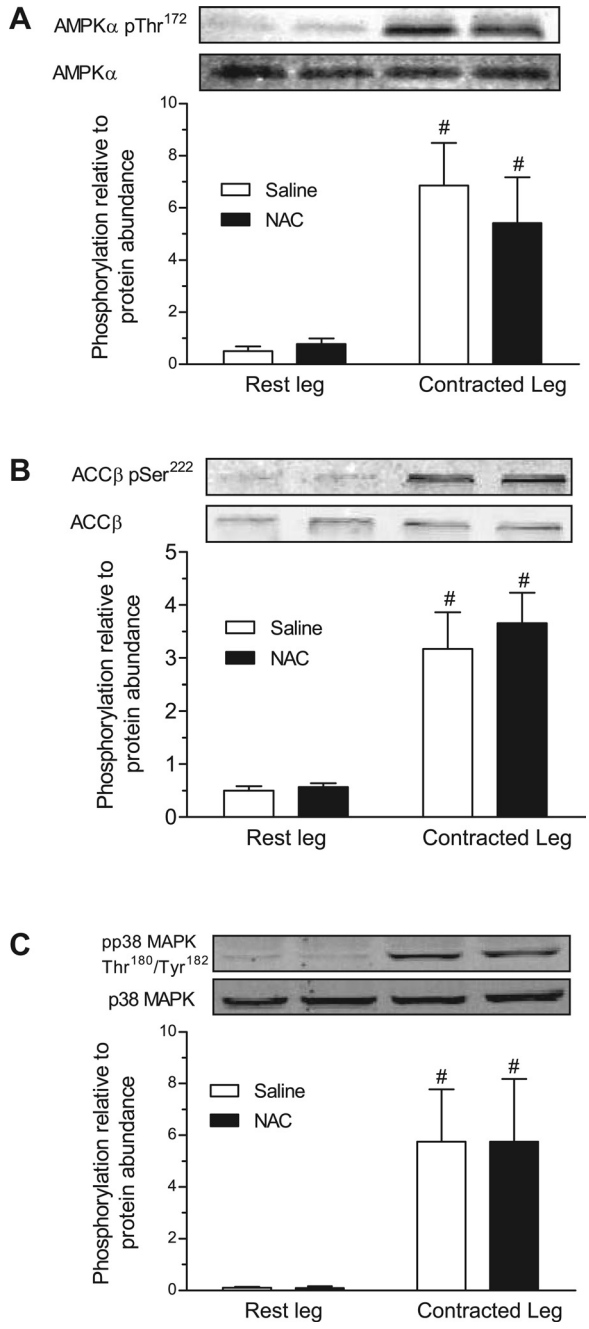

Fig. 6. Effect of local NAC or saline infusion on rest and contracted leg AMPK Thr^{172} (*A*), ACCβ Ser^{222} (*B*), and p38 MAPK Thr^{180}/Tyr^{182} (*C*) phosphorylation following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SE; $n = 6$ per group. #$P < 0.05$ for condition (rest vs. contraction).

to exhaustion (11, 40) or prolonged high to strenuous exercise (>70% peak oxygen consumption) (24, 44, 52), which results in greater increases in ROS production and depletion of antioxidant defenses (17, 27, 33). It is during such exercise that NAC can attenuate muscle GSH depletion (24, 43) by promoting its resynthesis (8, 41). Therefore, because our contraction protocol did not deplete GSH, there was no requirement for increased GSH resynthesis and thus NAC did not affect muscle GSH/GSSG content. However, NAC prevented exercise-stimu-

lated muscle *S*-glutathionylation, providing evidence that it was having GSH-independent antioxidant effects in the muscle (2, 8).

It has been previously reported that the treatment of isolated mouse extensor digitorum longus (EDL) muscles with NAC attenuates increases in ex vivo contraction-induced oxidative stress and glucose uptake (39). The authors propose that because NAC also inhibited AMPK phosphorylation and activity, ROS activate AMPK during contraction and this contributes to the activation of skeletal muscle glucose uptake during ex vivo contractions (18, 39). Indeed, we have also found that NAC attenuates increases in glucose uptake during ex vivo contractions in mouse EDL and soleus muscles (26). However, here we report that local NAC infusion did not affect the increase in skeletal muscle glucose uptake, AMPK phosphorylation, or the phosphorylation of the major downstream target of AMPK, ACCβ during in situ contractions (Fig. 5 and 6). This suggests that the low levels of oxidative stress associated with physiologically relevant contraction intensities is not involved in the regulation of skeletal muscle glucose uptake or AMPK signaling.

It has been proposed that muscle glucose uptake during contraction is differentially regulated in muscles containing predominantly oxidative and predominantly glycolytic type fibers (50). The hindlimb muscle sampled was estimated to contain only a small proportion of oxidative type fibers (~14%) (22). It is likely that ROS would play a greater signaling role in glycolytic fibers because they have lower levels of endogenous antioxidant enzymes (23). Furthermore, ROS are involved in the regulating contraction-stimulated glucose uptake in muscle containing predominantly glycolytic and oxidative fibers ex vivo (26, 39). Therefore, the finding that NAC infusion did not affect hindlimb muscle glucose uptake during contraction is unlikely to be a result of fiber-type composition of the sampled muscle. Alternatively, however, it is possible that the NAC-derived increases in muscle antioxidant defenses [NAC and cysteine content (2, 8)] were insufficient to prevent all ROS signaling, and therefore the signaling of glucose uptake during contraction.

Interestingly, NAC had a small but significant affect on blood pressure during contraction. This suggests that NAC or ROS may have been exerting some systemic affects during contraction, which is worthy of further investigation. However, it is unlikely that these small systemic effects can account for the finding that ROS are not involved in the regulation of skeletal muscle glucose uptake during low-moderate intensity in situ contractions. It is possible, however, that during exhaustive endurance exercise, or under the normal ex vivo conditions of supramaximal stimulation, nonuniform oxygen delivery (due to oxygen diffusion limitations), and reduced antioxidant capacity (in the absence of antioxidant systems found in blood), oxidative stress would be greatly elevated (33) and play a role in the regulation of skeletal muscle glucose uptake via the activation of AMPK. Indeed it is during high-intensity exercise, which substantially elevates ROS production (32), when glucose uptake (36) and AMPK activation (7) are greatest, and it is only at high concentrations that exogenous ROS (H_2O_2; 3 mM) activate AMPK (14, 16, 46). Therefore, experimental investigation is required to assess the role of ROS and oxidative stress in signaling glucose uptake and AMPK activity during high-intensity exercise in intact preparations with blood flow. However, the contraction intensity required to increase skeletal muscle ROS levels to a large enough extent to activate AMPK and increase glucose uptake as seen in ex vivo preparations (39) is not likely to be physiologically realistic to humans. Therefore, the hypothesis that ROS regulate skeletal muscle glucose uptake during contraction may be an artifact of nonphysiological ex vivo contraction conditions, and our results suggest that mechanisms other than ROS regulate skeletal muscle glucose uptake during normal submaximal muscle contractions.

It has been shown that the phosphorylation of p38 MAPK is increased by exogenous ROS (19) and that the attenuation of exercise-induced oxidative stress by the xanthine oxidase inhibitor, allopurinol, attenuates increases in p38 MAPK phosphorylation during exhaustive exercise (11). Indeed, there is some evidence that the inhibition of p38 MAPK attenuates glucose uptake during contraction (42), and ROS signaling via p38 MAPK may be involved in regulating skeletal muscle stretch-induced glucose uptake (6). However, we show here that contraction can increase skeletal muscle p38 MAPK phosphorylation without significant alterations in the GSSG/GSH ratio, and that local NAC infusion, which prevented *S*-glutathionylation, does not affect p38 MAPK phosphorylation during contraction. This suggests that during low-moderate intensity contractions, ROS are not involved in the regulation of p38 MAPK signaling. The inconsistency between the results of Gomez-Cabrera et al. (11) and ours may be related to the use of a xanthine oxidase-specific inhibition, rather than the use of a general antioxidant such as NAC, and requires further investigation. Furthermore, the role of p38 MAPK in regulating skeletal muscle glucose uptake during contraction, and potential interactions with ROS at high exercise intensities, is worthy of further investigation since some isoforms of p38 MAPK appear to play a greater role in regulating glucose up than others (15, 42).

A small oxidative shift in cell redox is required for optimal skeletal muscle contraction force; however, high levels of oxidative stress appear to contribute to the development of muscular fatigue (33). NAC treatment has been shown to attenuate fatigue during longer duration and/or more strenuous exercise/muscle contractions (20, 35) than used in the present study by attenuating the exercise-induced increase in oxidative stress (24, 43). Since our contraction protocol did not alter skeletal muscle glutathione levels, this suggests that the level of oxidative stress was not sufficient to negatively impact on muscle function and therefore may explain why NAC did not affect the rate of fatigue during contraction (Fig. 1).

In conclusion, this study shows for the first time that local infusion of the antioxidant NAC during in situ hindlimb contractions in rats does not attenuate increases in skeletal muscle glucose uptake or AMPK signaling. Although our low-moderate intensity in situ muscle contraction protocol, which is more relevant to normal submaximal exercise than tetanic contractions associated with ex vivo studies, did not alter muscle GSH/GSSG levels or tyrosine nitration, it did increase protein *S*-glutathionylation, indicating small increases in muscle oxidative stress. NAC prevented the increases in *S*-glutathionylation during contraction but did not affect the large increases in skeletal muscle glucose uptake (20-fold) or phosphorylation of AMPK and p38 MAPK. These results suggest that, unlike during highly fatiguing ex vivo contraction conditions, ROS do not regulate skeletal muscle glucose uptake or metabolic sig-

naling during physiologically relevant, low-fatiguing, skeletal muscle contractions in situ in rat.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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APPENDIX E

Merry, TL., Wadley, G.D., Stathis, C.G., Garnham, A.P., Rattigan, S., Hargreaves, M., McConell, G.K. N-acetylcysteine infusion does not affect glucose disposal during prolonged moderate intensity exercise in humans. *J. Physiol.*, 588:1623-34 IF= 4.3. Citations= 0

N-Acetylcysteine infusion does not affect glucose disposal during prolonged moderate-intensity exercise in humans

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There is evidence that reactive oxygen species (ROS) signalling is required for normal increases in glucose uptake during contraction of isolated mouse skeletal muscle, and that AMP-activated protein kinase (AMPK) is involved. The aim of this study was to determine whether ROS signalling is involved in the regulation of glucose disposal and AMPK activation during moderate-intensity exercise in humans. Nine healthy males completed 80 min of cycle ergometry at 62 ± 1% of peak oxygen consumption ($\dot{V}_{O_2,peak}$). A 6,6-^{2}H-glucose tracer was infused at rest and during exercise, and in a double-blind randomised cross-over design, *N*-acetylcysteine (NAC) or saline (CON) was co-infused. NAC was infused at 125 mg kg^{-1} h^{-1} for 15 min and then at 25 mg kg^{-1} h^{-1} for 20 min before and throughout exercise. NAC infusion elevated plasma NAC and cysteine, and muscle NAC and cysteine concentrations during exercise. Although neither NAC infusion nor exercise significantly affected muscle reduced or oxidised glutathione (GSH or GSSG) concentration ($P > 0.05$), *S*-glutathionylation (an indicator of oxidative stress) of a protein band of ∼270 kDa was increased ∼3-fold with contraction and this increase was prevented by NAC infusion. Despite this, exercised-induced increases in tracer determined glucose disposal, plasma lactate, plasma non-esterified fatty acids (NEFAs), and decreases in plasma insulin were not affected by NAC infusion. In addition, skeletal muscle AMPK*α* and acetyl-CoA carboxylase-*β* (ACC*β*) phosphorylation increased during exercise by ∼3- and ∼6-fold ($P < 0.05$), respectively, and this was not affected by NAC infusion. Unlike findings in mouse muscle *ex vivo*, NAC does not attenuate skeletal muscle glucose disposal or AMPK activation during moderate-intensity exercise in humans.

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Abbreviations ACC*β*, acetyl-CoA carboxylase-*β*; AICAR, 5-amino-imidazole 4-carboxamide ribonucleoside; CaMK, calcium/calmodulin-dependent kinase; GSH, reduced glutathione; GSSG, oxidised glutathione; NAC, *N*-acetylcysteine; NEFA, non-esterified fatty acids; PVDF, polyvinylidene difluoride membrane; R_a, appearance rate; R_d, disappearance rate; ROS, reactive oxygen species.

Introduction

Exercise stimulates skeletal muscle glucose uptake by increasing GLUT-4 translocation from intracellular vesicles to the cell membrane (Kennedy *et al.* 1999) through a mechanism(s) that differs from insulin-stimulated GLUT-4 translocation and glucose uptake (Zisman *et al.* 2000). However, the pathway(s) through which contraction stimulates muscle glucose uptake is unclear (Rose & Richter, 2005; Merry & McConell, 2009) with evidence for separate and collective contribution of several signalling intermediates including AMP-activated protein kinase (AMPK) (Hayashi *et al.* 1998), nitric oxide (NO) (Bradley *et al.* 1999; Ross *et al.* 2007), calcium–calmodulin-dependent kinase (CaMK) (Witczak *et al.* 2007), and Akt substrate of 160 kDa and 150 kDa (AS160 and TBC1D1) (Funai & Cartee, 2008, 2009). Interestingly, recent *ex vivo* evidence suggests that reactive oxygen species (ROS) may also play a role in signalling skeletal muscle contraction-mediated glucose uptake (Sandstrom *et al.* 2006).

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Exposure of isolated skeletal muscle to exogenous ROS increases glucose uptake (Toyoda *et al.* 2004; Higaki *et al.* 2008; Jensen *et al.* 2008). Similarly, the rate of ROS production in skeletal muscle increases with intense contraction *ex vivo* (Reid *et al.* 1992*a*,*b*) and *in vivo* (Sen *et al.* 1994; Medved *et al.* 2004*b*), and causes an acute oxidative shift in cell redox status. By attenuating the increase in ROS production and oxidative stress during contraction of isolated extensor digitorum longus (EDL) muscle with the antioxidant *N*-acetylcysteine (NAC) Sandstrom *et al.* (2006) provided the first evidence that ROS signalling during contraction may, in part, mediate glucose uptake via the activation of AMPK.

The AMPK activator 5-amino-imidazole 4-carboxamide ribonucleoside (AICAR) increases skeletal muscle glucose uptake (Merrill *et al.* 1997), and during contraction the increase in AMPK activity correlates with glucose uptake (Musi *et al.* 2001; Chen *et al.* 2003), thus implicating AMPK in the regulation of glucose uptake during contraction. Interestingly, high levels of exogenous ROS increase skeletal muscle AMPK activity (Toyoda *et al.* 2004; Jensen *et al.* 2008) and the attenuation of contraction-mediated increases in ROS levels attenuate increases in contraction-mediated AMPK activity (Sandstrom *et al.* 2006). This suggests that an increase in ROS levels during skeletal muscle contraction may be partially responsible for activating AMPK and thus regulating glucose uptake (Sandstrom *et al.* 2006). However, dissociations between skeletal muscle AMPK activity and contraction-stimulated glucose uptake have been frequently reported (Derave *et al.* 2000; Jorgensen *et al.* 2004; Wadley *et al.* 2006; McConell *et al.* 2008). Indeed, AMPK activation does not appear to be required for normal increases in skeletal muscle glucose uptake following short-term exercise training (McConell *et al.* 2005) in humans and during *in vivo* exercise in mice (Lee-Young *et al.* 2009; Maarbjerg *et al.* 2009). Importantly, at present the role of ROS in the regulation of contraction-stimulated muscle glucose uptake has only been examined in isolated muscle models. In the absence of blood flow, such models depend on diffusion gradients for substrate delivery and clearance, and result in non-uniform delivery of oxygen to all muscle fibres (Allen *et al.* 2008). Furthermore, *ex vivo* muscle preparations generally involve supra-maximal highly fatiguing stimulation protocols. These factors may alter contraction-induced ROS production (Allen *et al.* 2008; Reid, 2008) and signalling of glucose uptake. Therefore, it is important to investigate the role of ROS signalling in the regulation of skeletal muscle glucose uptake during exercise *in vivo*.

In a series of studies, Medved *et al.* (2003, 2004*a*,*b*) demonstrated that the non-specific anti-oxidant NAC can be safely infused intravenously into humans during prolonged exercise. Moreover, the cysteine-donating properties of NAC promoted the resynthesis of muscle reduced glutathione (GSH) during exercise indicating an attenuation of exercise-induced increases in skeletal muscle oxidative stress (Medved *et al.* 2004*b*). Therefore, in the current study, we infused NAC intravenously in humans during prolonged cycle ergometry to determine the role of ROS in the regulation of glucose disposal and AMPK signalling during exercise. We hypothesised that NAC infusion would attenuate the increases in glucose disposal and skeletal muscle AMPK signalling during exercise in humans.

Methods

Participants

Nine healthy recreationally active ($\dot{V}_{O_2,peak}$: 51.7 ± 2.3 ml kg^{-1} min^{-1}) adult males volunteered. The participants' age, weight and height (mean ± S.E.M.) were 23 ± 2 years, 79.7 ± 3.4 kg and 179 ± 3 cm, respectively. This study was approved by the University of Melbourne Human Ethics Committee, and was conducted in accordance with the *Declaration of Helsinki* and *The Journal of Physiology* standards (Drummond, 2009). Participants were informed of the experimental procedures and provided written consent. All participants were non-smokers, were not taking any medication and had no history of cardiovascular, cerebrovascular or respiratory disease.

Preliminary procedures

$\dot{V}_{O_2,peak}$ was measured in normal ambient laboratory conditions (∼20°C) using a graded cycle ergometer (electronically braked ergometer; Lode, Groningen, the Netherlands) protocol to voluntary exhaustion with respiratory gas analysis for volume expired (Air flow meter; Vacuumed, Ventura, CA, USA), O_2 and CO_2 content (S-3A O_2 and AMETEK CO_2 analyser; Applied Electrochemistry, Sunnyvale, CA, USA). On a separate day, 1–2 weeks before the first experimental trial, participants completed a familiarisation session where they cycled for 20 min at ∼60% of $\dot{V}_{O_2,peak}$.

Experimental trials

The study involved a double-blind randomised cross-over design, with counterbalanced testing order. Experimental trials were conducted at the same time of day and separated by at least 2 weeks. A 24 h food diary was completed prior to the first trial, and this was photocopied and returned to the participants who were asked to follow the same diet prior to the second trial.

Participants fasted overnight and reported to the laboratory at 6.30 am on trial days, having abstained from exercise, caffeine and alcohol for the preceding 24 h. A 22-gauge cannula was inserted into an antecubital forearm vein for the infusion of stable isotope glucose tracer (6,6-^{2}H-glucose; Cambridge Isotope Labratories, MA, USA) and NAC, and another cannula was inserted into the contralateral forearm for blood sampling. An initial blood sample was obtained and then a bolus of 40.2 μmol kg^{-1} of tracer was administered followed by a 2 h pre-exercise continuous constant infusion (0.39 μmol kg^{-1} min^{-1}), which was continued through to the end of exercise (McConell *et al.* 2006). As described previously by Medved *et al.* (2003), an initial loading dose of either NAC (Parvolex, Faulding Pharmaceuticals; 125 mg kg^{-1} h^{-1} in 0.9% saline) or 0.9% saline alone (control, CON) was co-infused intravenously 35 min prior to exercise for 15 min, followed by a constant infusion of saline or NAC (25 mg kg^{-1} h^{-1}) that continued until the end of exercise. As outlined in Fig. 1, following 35 min of NAC/CON infusion at rest, participants cycled for 80 min at 62 ± 1% $\dot{V}_{O_2,peak}$ in standard laboratory conditions (∼20°C) with a fan on high setting positioned ∼1 m directly in front of the handlebars. Water was consumed *ad libitum*.

Experimental trial sample collection and measurements

Heart rate was measured using a heart rate monitor (Polar Favor, Oulu, Finland) and recorded at 10 min intervals during exercise. Expired respiratory gases were sampled for 3 min at $t = 10$, 30 and 70 min, and volume expired, O_2 and CO_2 content were measured. Rating of perceived exertion (Borg, 1974) was obtained at 10 min intervals during exercise, and any adverse reactions to the infusions were recorded during the trial as described by Medved *et al.* (2003, 2004*b*).

Venous blood samples were obtained at $t = -120$, −65, −45, −35 and −20 min, and then every 10 min until the end of exercise (Fig. 1). Blood for glucose, per cent enrichment of [6,6-^{2}H]glucose, lactate, insulin and thiol analysis was transferred immediately to tubes containing lithium–heparin. Blood for non-esterified fatty acid (NEFA) analysis was transferred immediately to tubes containing EDTA. All blood tubes were placed on ice until the end of the trial then spun at 3000 *g* for 20 min and plasma stored at −80°C for later analysis.

For the sampling of muscle, during the pre-exercise infusion period three separate ∼1 cm incisions were made under local anaesthesia above the vastus lateralis of one leg. Muscle samples were then obtained at $t = 0$, 40 and 80 min (Fig. 1) using the percutaneous needle biopsy technique (distal-to-promimal order, at least 1 cm apart). Muscle samples were frozen in liquid nitrogen while still in the biopsy needle within 6–12 s following the cessation of exercise. Muscle samples were stored in liquid nitrogen for later analysis and were obtained from the contralateral leg during the second trial.

Blood analysis

Plasma lactate was measured using an automated L-lactate oxidase method (YSI 2300 Stat, Yellow Springs, OH, USA) and plasma glucose was determined using an enzymatic fluorometric assay involving NADPH production (Lowry & Passonneau, 1972). Plasma NEFA was measured by an enzymatic colourimetric procedure (NEFA-C test; Wako, Osaka, Japan) and plasma insulin using a human radioimmunoassay kit (Linco Research, St Charles, MO, USA). As described previously (McConell *et al.* 1994), glucose kinetics were measured using a modified one-pool, non-steady-state model (Steele *et al.* 1956; Radziuk *et al.* 1978). We assumed 0.65 as the rapidly mixing portion of the glucose pool and estimated the apparent glucose space as 25% of body weight. Plasma glucose appearance (R_a) and disappearance (R_d) rates were determined from changes in the per cent enrichment of 6,6-^{2}H-glucose and the plasma glucose concentration. Over 95% of tracer-determined R_d is oxidised at power outputs requiring ∼60% $\dot{V}_{O_2,peak}$ (Jeukendrup *et al.* 1999).

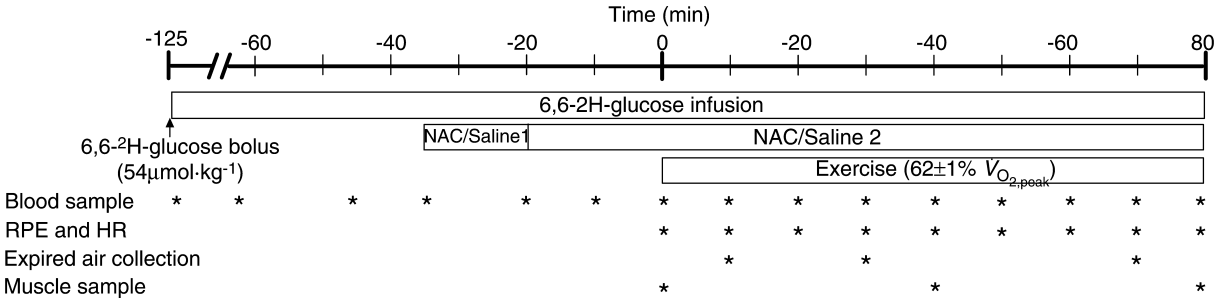

Figure 1. Experimental protocol
See Methods for details. RPE, rating of perceived exertion; HR, heart rate; NAC/Saline 1, infusion of *N*-acetylcysteine (NAC; 125 mg kg^{-1} h^{-1}) or saline; NAC/Saline 2, infusion of *N*-acetylcysteine at lower rate (25 mg kg^{-1} h^{-1}) or saline.

For the analysis of plasma total thiols and NAC, 10 μl of 1:10 tributylphosphine (Sigma-Aldrich Chemicals, St Louis, MO, USA) was added to 50 μl of plasma, and following a 30 min incubation on ice, 25 μl of 4-fluoro-7-sulfamoylbenzofurazan (Sigma-Aldrich Chemicals; ADB-F; 5 mg ml^{-1} in borate buffer: 0.2 M boric acid, 2 mM sodium EDTA, pH 8.0) was added. Samples were then incubated at 50°C for 10 min, 10 μl of 2 M perchloric acid (PCA) was added and they were spun at 13,000 *g* for 5 min. The supernatant was recovered and 40 μl was injected into a reverse-phase HPLC Gemini column (5 μm C18 110Å, phenomenex) with 0.1 M sodium acetate buffer (pH 4.0) in 10% methanol as a mobile phase, at a flow rate of 1.5 ml min^{-1} and detection wavelength of 386 nm excitation and 516 nm emission. For the determination of plasma reduced thiols and NAC, tributylphosphine was replaced with H_2O and the protocol was repeated. Oxidised thiols were calculated from the difference between total and reduced thiols.

Muscle analysis

Approximately 25 mg of muscle were freeze-dried and ground to a powder. For the determination of muscle glycogen, ~1 mg of freeze-dried muscle was incubated at 95°C for 2 h in 250 μl of 2 M HCl and then neutralised with 750 μl of 0.67 M NaOH. The extracts were then analysed for glucosyl units using an enzymatic fluorometric assay (Passonneau & Lauderdale, 1974). Muscle metabolites (ATP, creatine, creatine phosphate (PCr) and lactate) were determined by extracting ~2 mg of freeze-dried muscle in 250 μl of 0.5 M PCA and 1 mM EDTA, and the supernatant analysed using enzymatic fluorometric assays (Lowry & Passonneau, 1972). To account for any non-muscle contamination, muscle metabolites were corrected to the highest muscle total creatine content for each participant and free AMP and ADP were calculated as described previously (Chen *et al.* 2000).

Extraction for NAC and thiols analysis involved the homogenization of 30 mg of frozen muscle in 300 μl of ice-cold 0.42 M PCA; 40 μl of 2.5 M K_2CO_3 was then added to neutralize samples before spinning at 13,000 *g* for 5 min at 4°C and recovering supernatant. The protocol described above for the determination of plasma thiols was then repeated with the muscle supernatant.

For Western blot analysis, 50 mg of frozen muscle were homogenized in ice-cold lysis buffer (10 μl mg^{-1} tissue; 50 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% v/v glycerol, 1% v/v Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mm phenylmethylsulphonyl floride (PMSF)

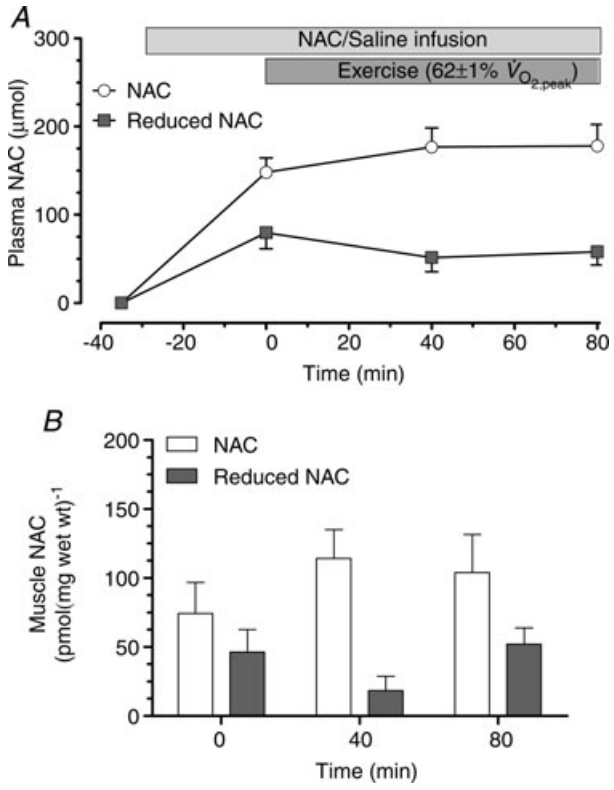

Figure 2. Plasma and muscle NAC levels
Plasma (*A*) and muscle (*B*) *N*-acetylcysteine (NAC) during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving *N*-acetylcysteine infusion. $N = 8$.

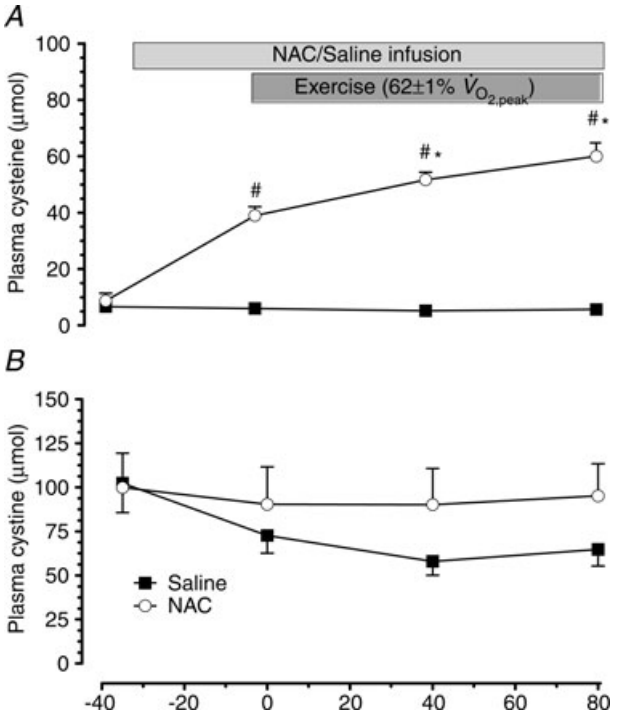

Figure 3. Plasma cysteine and cystine levels
Plasma cysteine (*A*) and cystine (*B*) concentration at rest and during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving either saline (CON) or saline + *N*-acetylcysteine (NAC) infusion. $N = 8$, #$P < 0.05$ *vs.* CON, *$P < 0.05$ *vs.* $t = 0$ of same treatment.

and 5 μl ml^{-1} Protease Inhibitor Cocktail (Sigma-Aldrich Chemicals, St Louis, MO, USA), incubated for 20 min on ice and spun at 16,000 *g* for 20 min at 4°C. The supernatant was recovered and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with BSA as the standard. The remaining supernatant was solubilised in Laemmli sample buffer (1.5 M Tris-HCl, pH 6.8, 30% glycerole, 10% SDS, 0.6 M DTT, 0.0012% bromophenol blue), heated for 10 min at 100°C and stored at −20°C. For analysis of protein *S*-glutathionylation, muscle was extracted under non-reducing conditions with lysis and Laemmli sample buffer containing 5 mM and 10 mM of *N*-ethylmaleimide, respectively, in the absence of DTT. SDS-PAGE was used to separate 80 μg of total protein before transferring to PVDF membrane and blocking in 5% non-fat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies for 3-nitrotyrosine (Chemicon; Temecula, CA, USA), glutathione (Abcam, Cambridge, UK) and phosphorylation-specific antibodies for ACCβ Ser^{221} and AMPK Thr^{172} (Upstate Biotechnology, NY, USA) before binding was detected with rabbit IgG and mouse IgG secondary fluorescent antibodies (Rockland, Gilbertsville, PA, USA). Direct fluorescence was detected and quantified using the Odyssey infrared imaging system

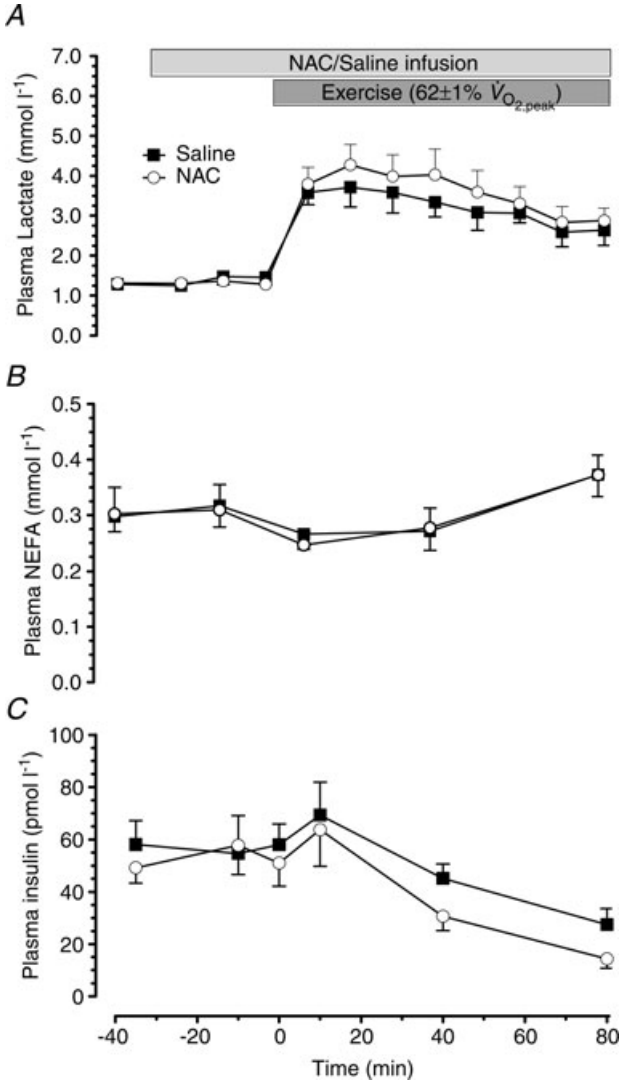

Figure 4. Plasma lactate, non-esterified free fatty acids and insulin concentration
Plasma lactate (*A*), non-esterified free fatty acids (NEFA; *B*) and insulin (*C*) concentration at rest and during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving either saline or saline + *N*-acetylcysteine (NAC) infusion. $N = 9$.

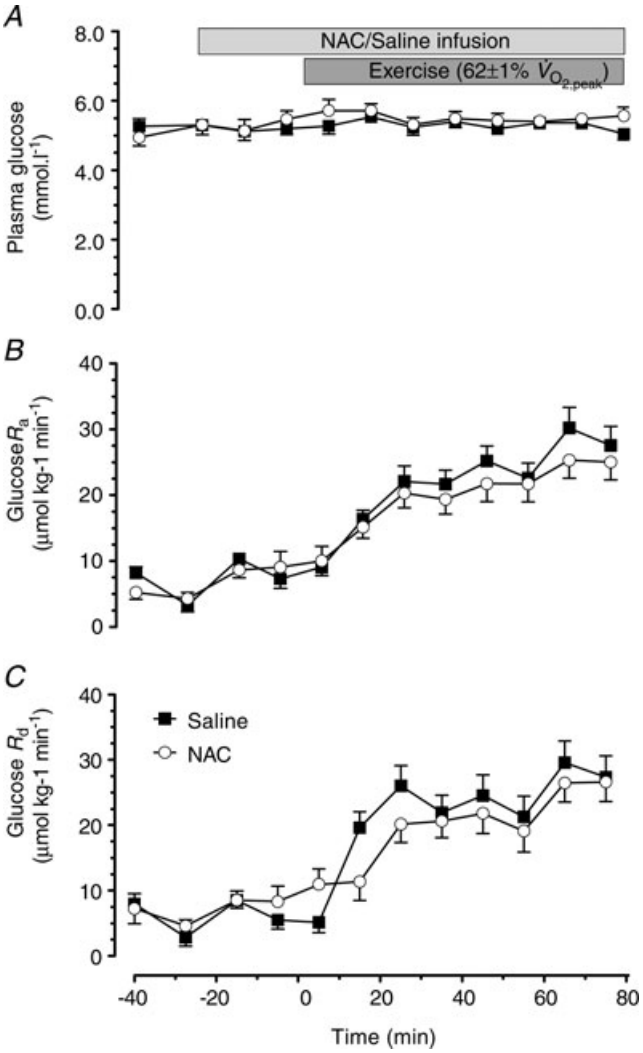

Figure 5. Plasma glucose, rate of glucose appearance and rate of glucose disappearance
Plasma glucose (*A*), rate of glucose appearance (Glucose R_a; *B*) and rate of glucose disappearance (Glucose R_d; *C*) at rest and during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving either saline or saline + *N*-acetylcysteine (NAC) infusion. $N = 9$.

(LICOR Biosciences, Lincoln, NB, USA). Membranes were then stripped (2% SDS (w/v) in 25 mM glycine, pH 2.0) and re-probed with primary antibodies for ACCβ (streptavidin) (Rockland), AMPKα and α-tubulin (Cell Signaling Technology, Hertfordshire, UK) to determine total protein levels. Protein phosphorylation was expressed relative to the total protein abundance of the protein of interest.

Statistical analysis

All data are expressed as means ± S.E.M. Results were analysed by SPSS statistical package using two-factor repeated measures ANOVA. To assess the resting effects and because NAC infusion started pre-exercise, the ANOVA was partitioned to assess the effect of NAC during rest (−30 to −10 min) and during exercise (0 to 80 min). If the ANOVA revealed a significant treatment by time interaction, specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

Results

Respiratory measures, heart rate and rating of perceived exertion

Participants exercised at 62 ± 1% $\dot{V}_{O_2,peak}$ during both trials. Oxygen consumption (2.6 ± 0.1 *vs.* 2.5 ± 0.2 l min^{-1}), respiratory exchange ratio (RER; 0.90 ± 0.02 *vs.* 0.91 ± 0.02), heart rate (HR; 154 ± 2 *vs.* 154 ± 4 beats min^{-1}) and rating of perceived exertion

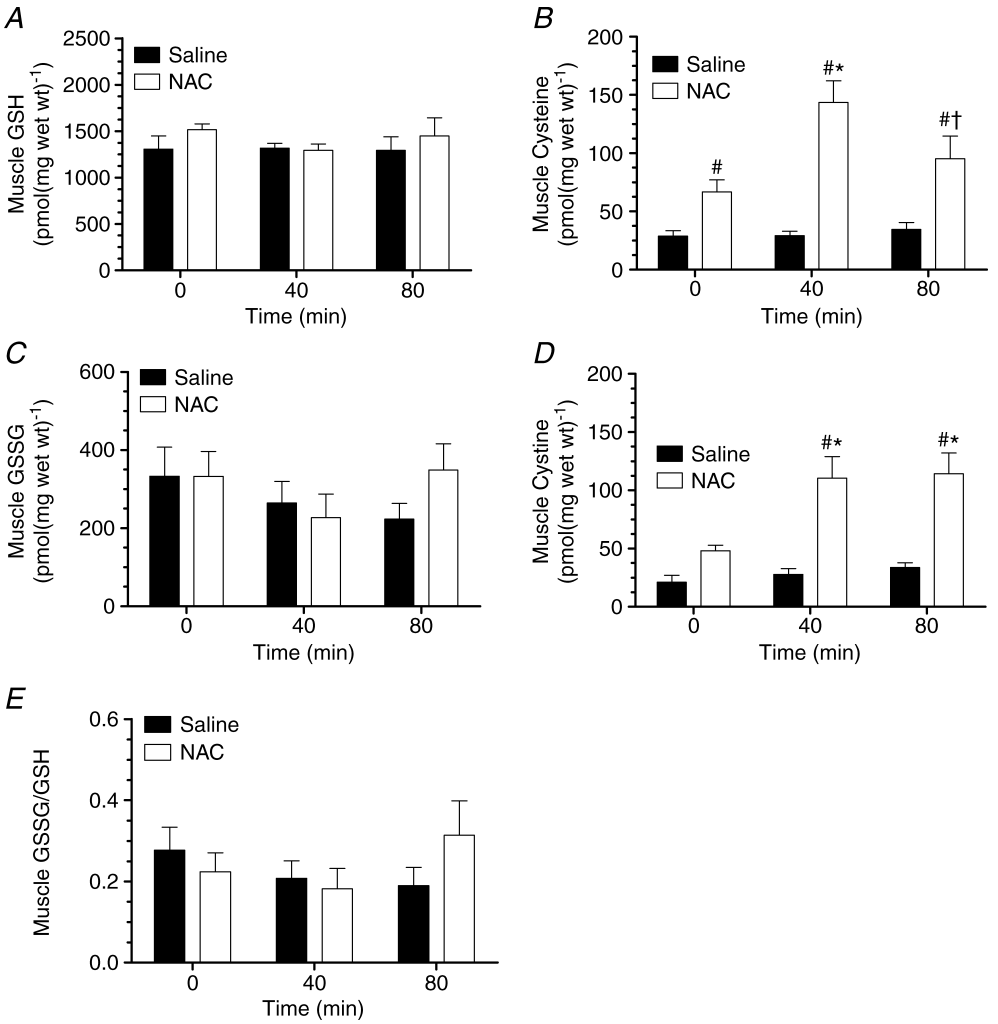

Figure 6. Muscle reduced glutathione, cysteine, oxidised glutathione, cystine and GSSG/GSH ratio
Muscle reduced glutathione (GSH; *A*), cysteine (*B*), oxidised glutathione (GSSG; *C*), cystine (*D*) and GSSG/GSH ratio (*E*) at rest and during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving either saline (CON) or saline + *N*-acetylcysteine (NAC) infusion. $N = 9$, #$P < 0.05$ *vs.* CON, *$P < 0.05$ *vs.* $t = 0$ min of same treatment, †$P < 0.05$ *vs.* $t = 40$ min of same treatment.

(RPE; 13 ± 1 *vs.* 14 ± 2) were similar ($P > 0.05$) in saline (control) *vs.* NAC trials, respectively.

Muscle and plasma NAC and adverse reactions

No adverse reactions to either NAC or control infusions were observed. No NAC was detectable in muscle or plasma of control infusion. By the onset of exercise, NAC infusion increased ($P < 0.05$) plasma NAC and reduced NAC content to 148.2 ± 16.1 μmol and 79.8 ± 18.4 μmol, respectively, and this content was maintained throughout exercise (Fig. 2*A*). NAC infusion increased the levels of resting muscle NAC and resting muscle reduced NAC to 74.2 ± 22.5 and 46.2 ± 16.4 pmol (mg wet wt)$^{-1}$, respectively, and these levels remained essentially unchanged during exercise (Fig. 2*B*).

Plasma cysteine

NAC infusion elevated plasma cysteine ($P < 0.01$), and tended to increase plasma cystine ($P = 0.07$; Fig. 3). Plasma cysteine concentration was increased by exercise during NAC ($P < 0.05$), but not saline infusion (Fig. 3*A*). Exercise did not affect plasma cystine (Fig. 3*B*).

Plasma lactate, NEFA and insulin

During exercise plasma insulin concentration decreased ($P < 0.01$) and plasma NEFA and lactate concentration increased ($P < 0.05$) to a similar extent in the two trials (Fig. 4).

Glucose kinetics

Plasma glucose concentration was not affected by NAC infusion or exercise (Fig. 5*A*; $P < 0.05$). The increase in glucose appearance (R_a) and glucose disappearance (R_d) were not affected by NAC infusion (Fig. 5). The pattern of the glucose clearance rate (CR) was very similar to the glucose R_d results and therefore has not been presented. This is not surprising given the similarity of the plasma glucose concentrations in the two trials. Glucose R_a, and R_d increased with exercise ($P < 0.05$) and this increase was not influenced by NAC infusion (Fig. 5).

Muscle thiols

NAC infusion increased muscle cysteine at rest and during exercise ($P < 0.05$), but cystine was only increased during exercise ($P < 0.05$; Fig. 6*B* and *D*). Muscle cysteine was increased with exercise at 40 min and cystine at 40 and 80 min but only during NAC infusion ($P < 0.05$; Fig. 6*B* and *D*). Muscle GSH, GSSG or GSSG/GSH ratio were not affected by exercise or NAC infusion (Fig. 6*A*, *C* and *E*).

S-Glutathionylation and tyrosine nitration

Exercise increased muscle *S*-glutathionylation of a protein band of approximately 270 kDa (Fig. 7*A*) by ~3-fold ($P < 0.05$). NAC infusion prevented the exercise-induced increase in *S*-glutathionylation of this protein band. The nature of this protein band is currently being investigated. Muscle tyrosine nitration was not significantly affected by exercise or NAC infusion (Fig. 7*B*).

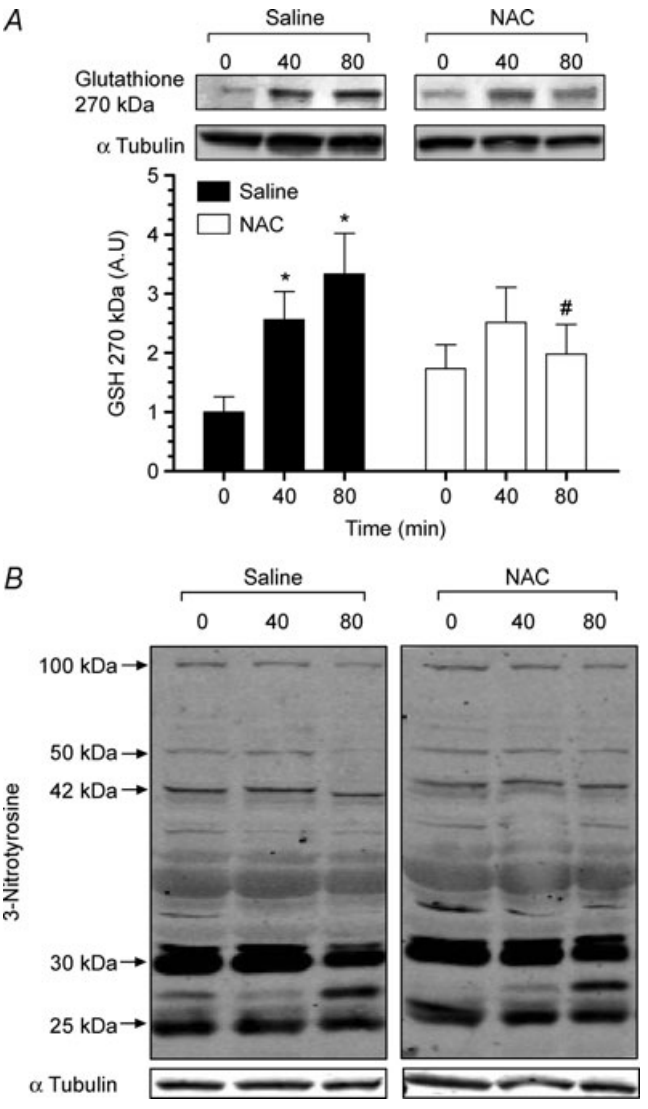

Figure 7. Muscle protein *S*-glutathionylation and tyrosine nitration
Muscle protein *S*-glutathionylation (*A*) and tyrosine nitration (*B*) at rest and during 80 min of steady-state exercise at $62 \pm 1\%$ $\dot{V}_{O_2,peak}$ saline (CON) or saline + *N*-acetylcysteine (NAC) infusion. Western blots are representative for one participant from each trial at each timepoint. $N = 9$, #$P < 0.05$ *vs.* CON, *$P < 0.05$ *vs.* $t = 0$ min of same treatment.

Table 1. Muscle metabolites at rest and during exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving saline (CON) or saline + *N*-acetylcysteine (NAC) infusion

Metabolite	Trial	0 min	40 min	80 min
Muscle lactate (mmol (kg dry wt)$^{-1}$)	CON §	3.4 ± 0.7	21.7 ± 4.5	19.0 ± 6.1
	NAC §	4.2 ± 0.5	12.8 ± 2.9	18.2 ± 3.8
PCr (mmol (kg dry wt)$^{-1}$)	CON §	94.8 ± 3.8	63.4 ± 5.5	67.2 ± 4.8
	NAC §	95.6 ± 4.2	72.2 ± 4.5	67.4 ± 7.6
Cr (mmol (kg dry wt)$^{-1}$)	CON §	46.6 ± 1.7	78.0 ± 3.6	74.3 ± 4.2
	NAC §	45.8 ± 2.6	69.2 ± 7.1	74.1 ± 6.4
ATP (mmol (kg dry wt)$^{-1}$)	CON	25.9 ± 0.3	25.2 ± 1.0	25.2 ± 0.8
	NAC	26.2 ± 0.7	25.6 ± 1.1	23.7 ± 1.4
Free AMP (mmol (kg dry wt)$^{-1}$)	CON §	0.6 ± 0.1	3.2 ± 0.6	2.5 ± 0.4
	NAC §	0.6 ± 0.1	2.5 ± 0.9	2.5 ± 0.9
Free ADP (μmol (kg dry wt)$^{-1}$)	CON §	120.8 ± 5.2	266.6 ± 25.1	239.9 ± 21.6
	NAC §	115.0 ± 9.2	219.5 ± 34.2	218.1 ± 42.0
Free AMP:ATP	CON §	0.02 ± 0.00	0.13 ± 0.03	0.09 ± 0.01
	NAC §	0.02 ± 0.00	0.11 ± 0.05	0.11 ± 0.03
Muscle glycogen (mmol (kg dry wt)$^{-1}$)	CON §	373.6 ± 18.7	216.9 ± 16.9	177.8 ± 26.5
	NAC §	348.5 ± 24.1	266.1 ± 23.6	206.8 ± 33.7

§$P < 0.05$ for time effect, $N = 9$, PCr, creatine phosphate; Cr, creatine.

Muscle metabolites

NAC infusion had no affect on resting muscle metabolite concentrations (Table 1). Exercise did not affect muscle ATP concentration ($P = 0.20$), but resulted in an increase in muscle lactate, creatine content and calculated free ADP, free AMP and free AMP/ATP ratio ($P < 0.05$), and a reduction in muscle PCr and glycogen content with no significant differences between treatments (Table 1).

AMPKα and ACCβ phosphorylation

Exercise increased skeletal muscle AMPKα Thr^{172} and ACCβ Ser^{221} phosphorylation by ∼3- and ∼6-fold, respectively, and these increases were not affected by NAC infusion (Fig. 8*A* and *B*).

Discussion

The major finding of this study was that the systemic infusion of the antioxidant *N*-acetylcysteine (NAC) in humans did not affect glucose disposal during prolonged moderate-intensity exercise. In addition we show that skeletal muscle AMPK signalling during exercise is also unaffected by NAC infusion. Interestingly, we provide evidence in humans that skeletal muscle *S*-glutathionylation is increased during moderate-intensity exercise, despite no significant changes in muscle GSH and GSSG, and that NAC infusion prevented the increase in *S*-glutathionylation of a protein band at ∼270 kDa during exercise.

The antioxidant NAC has been shown previously to attenuate glucose uptake in isolated skeletal muscles contracted *ex vivo* (Sandstrom *et al.* 2006; Merry *et al.* 2009). In contrast, we show in this study that NAC infusion does not affect glucose disposal during exercise in humans (Fig. 5), despite preventing exercise-induced increases in muscle *S*-glutathionylation (the post-translational addition of glutathione to a specific cysteine residual of a protein which is promoted by oxidative stress (Dalle-Donne *et al.* 2009)) (Fig. 7). Furthermore, other measures of substrate utilisation during exercise such as respiratory exchange ratio (RER), muscle glycogen use, muscle metabolites, plasma lactate and plasma NEFA concentration were also not affected by NAC infusion (Table 1 and Fig. 4). This suggests that ROS signalling may not be essential for the regulation of skeletal muscle glucose uptake during moderate-intensity exercise in humans. However, although NAC infusion prevented *S*-glutathionylation, it does not exclude the possibility that the concentration of NAC in the muscle was insufficient to prevent all ROS signalling events. Therefore, it may have been the case that muscle NAC-enhanced cysteine levels were too low to prevent ROS effects on glucose uptake. Indeed, very high (20 mM) concentrations of NAC are used to attenuate skeletal muscle contraction-stimulated glucose uptake *ex vivo* (Sandstrom *et al.* 2006).

Our finding that NAC treatment also did not affect AMPK signalling during exercise in humans is in contrast to the previously reported finding that NAC similarly attenuates contraction-stimulated glucose uptake and AMPK activity during *ex vivo* skeletal muscle contraction (Sandstrom *et al.* 2006). High concentrations of exogenous ROS lower cell energy levels (AMP/ATP and creatine/PCr ratio) (Leon *et al.* 2004), activate AMPK and increase skeletal muscle glucose uptake (Toyoda *et al.* 2004). However, NAC infusion did not affect moderate-intensity exercise-induced lowering of cell energy in the present

study, suggesting that the increase in ROS levels during moderate-intensity exercise was not sufficient to alter cell energy status (Table 1). It is possible that *ex vivo* muscle incubation conditions of non-uniform oxygen delivery, and highly fatiguing contraction protocols, promote higher than physiological ROS levels in skeletal muscle (Reid, 2001) which may alter cell energy balance and therefore activate AMPK. Therefore, it may be that during high-intensity exhaustive exercise/contractions when ROS production is greatly elevated and substantially increases skeletal muscle oxidative stress (Reid, 2001), ROS signalling may be involved in regulating skeletal muscle glucose uptake via AMPK.

A novel finding of this study was that muscle *S*-glutathionylation of a protein band at ∼270 kDa (Fig. 7*A*) was increased with moderate intensity exercise, and this increase was prevented by NAC infusion. Protein *S*-glutathionylation has been shown to be involved in redox-related regulation of cellular processes from protein folding (Demasi *et al.* 2008) to energy metabolism (Cotgreave *et al.* 2002); however, targets of *S*-glutathionylation during skeletal muscle contraction are yet to be investigated. The nature of the protein band at ∼270 kDa has not yet been conclusively identified. Regardless, these data provide some evidence that moderate-intensity exercise caused an increase in muscle oxidative stress and this increase was prevented by the infusion of the antioxidant NAC. Since oxidative stress causes the rapid oxidation of GSH to GSSG, the GSSG/GSH ratio is commonly used as a marker of oxidative stress (Powers & Jackson, 2008). However, it is only during moderately high (Medved *et al.* 2004*b*) (>70% $\dot{V}_{O_2,peak}$) and strenuous (Svensson *et al.* 2002; Medved *et al.* 2004*b*; Zhang *et al.* 2007) (>80% $\dot{V}_{O_2,peak}$) exercise in humans, and exercise to exhaustion in rats (Sen *et al.* 1994; Gomez-Cabrera *et al.* 2005) that ROS levels increase enough to cause detectable depletion of muscle GSH and/or increases in GSSG. Therefore, not surprisingly, and as reported previously (Ji *et al.* 1992; Sahlin *et al.* 1992), we found that muscle GSH and GSSG levels were not affected by moderate-intensity exercise. In support, skeletal muscle nitric oxide production is increased during contraction (Balon & Nadler, 1994) and NO can interact with superoxide to form peroxynitrite ($ONOO^-$; Halliwell, 1989), which at high concentrations promotes tyrosine nitration (Halliwell, 1989). Here we report that tyrosine nitration was not increased during exercise or influenced by NAC (Fig. 7). This supports our finding of only a modest oxidative shift in muscle redox during moderate-intensity exercise.

Although NAC can directly scavenge ROS (Aruoma *et al.* 1989), the primary antioxidant properties of NAC are derived from its rapid deacetylation to cysteine (Deneke, 2000), a precursor to GSH synthesis (Sen *et al.* 1992). The increased bioavailability of cysteine enhances antioxidant defences by promoting the regeneration of GSH (Sen *et al.* 1992) and reduces ROS by direct scavenging (Cotgreave, 1997). As reported previously (Medved *et al.* 2004*b*), systemic infusion of NAC into humans during exercise elevates skeletal muscle NAC content and increases plasma and muscle cysteine concentration (Fig. 2). However, NAC infusion did not affect skeletal muscle GSH or GSSG/GSH ratio. In agreement, NAC generally only increases muscle GSH availability under conditions where muscle GSH oxidation is elevated (Sandstrom *et al.* 2006), such as during strenuous exercise/muscle contraction (Medved

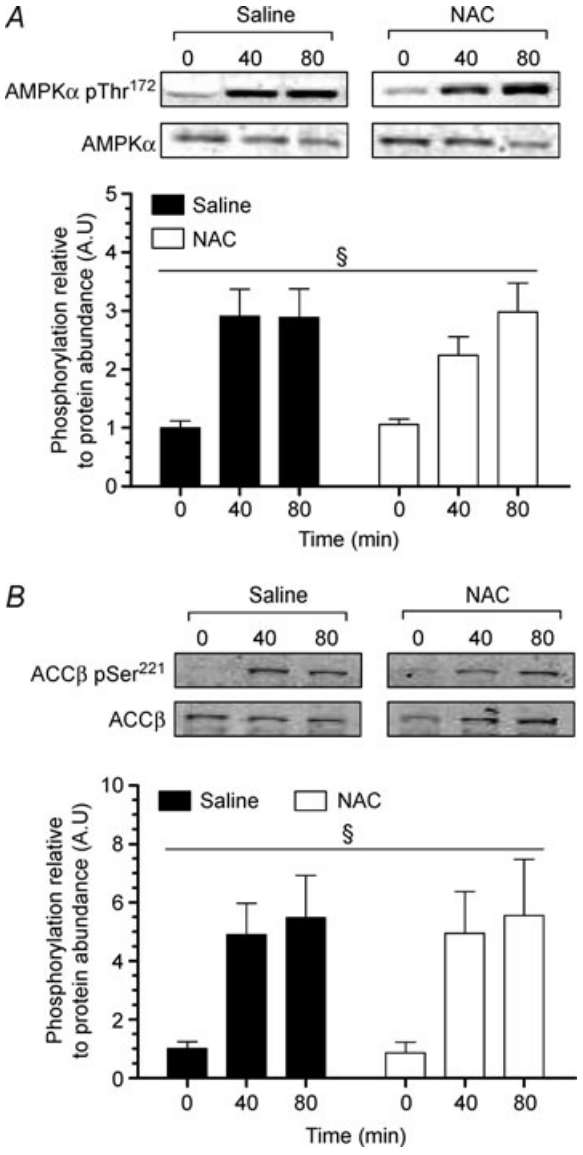

Figure 8. Muscle AMPK Thr^{172} and ACCβ Ser^{221} phosphorylation
Muscle AMPK Thr^{172} phosphorylation (*A*) and ACCβ Ser^{221} phosphorylation (*B*) at rest and during 80 min of steady-state exercise at 62 ± 1% $\dot{V}_{O_2,peak}$ while receiving either saline or saline + *N*-acetylcysteine (NAC) infusion. Western blots are representative for one participant from each trial at each timepoint. $N = 9$, §$P < 0.05$ for time effect.

et al. 2004*b*; Sandstrom *et al.* 2006). Therefore, it is likely that NAC did not affect muscle GSH or GSSG concentration in the current study because GSH was not depleted by moderate-intensity exercise.

In conclusion, although skeletal muscle glutathione balance was not affected by moderate-intensity exercise, *S*-glutathionylation of a protein band of ∼270 kDa was increased and this increase was prevented by NAC infusion. Since glucose disposal during exercise was not attenuated by NAC, this study provides evidence to suggest that small to moderate increases in ROS levels during moderate-intensity exercise in humans may not be involved in the regulation of skeletal muscle glucose disposal or AMPK signalling. This provides evidence to suggest previous results obtained using intense *ex vivo* contractions may not always be relevant to normal prolonged exercise.

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T.L.M., M.H. and G.K.M. contributed to the conception and design of experiments. T.L.M., G.D.W., C.G.S., A.P.G., S.R., M.H. and G.K.M. contributed to the execution, analysis and interpretation of experiments. T.L.M. wrote the initial draft of the manuscript and G.D.W., M.H. and G.M. contributed to writing and revising the manuscript. All authors approved the final version of the manuscript.

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