Department of Physiology and Pharmacology,
Karolinska Institutet, Stockholm, Sweden

REGULATION OF CARBOHYDRATE METABOLISM IN SKELETAL MUSCLE DURING AND AFTER CONTRACTION

Marie Sandström

Stockholm 2006

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-969-6

I can resist everything but temptation
-Oscar Wilde

ABSTRACT

It is well known that exercise increases glucose transport into skeletal muscles. The regulation of this transport, however, is poorly understood. An increased understanding of the mechanisms underlying glucose transport and glycogen metabolism during exercise will lead to new strategies for treating or preventing increasingly prevalent diseases like type 2 diabetes. The aim of this thesis was to study the regulation of carbohydrate metabolism in skeletal muscle during and after contraction. Three main areas were studied: (A) glucose transport, (B) glycogen synthesis, and (C) glycogen breakdown. (A) The role of endogenously produced reactive oxygen species (ROS) in contraction-mediated glucose transport was investigated in mouse skeletal muscle. An antioxidant (N-acetylcysteine; NAC), added to block the accumulation of ROS during exercise significantly reduced contraction-mediated glucose transport. Furthermore, it was found that the addition of NAC to contracting muscles also inhibited AMPK activity, a key enzyme in contraction-mediated glucose transport. We pharmacologically inhibited cross-bridge force production to assess cross-bridge ATP consumption during contraction. Inhibition of the cross-bridges decreased the initial force by ~95% in fast twitch skeletal muscle. We found that the cross-bridges only account for ~20% of the total ATP production during submaximal contraction and the contraction-mediated glucose transport was only slightly decreased. (B) Glycogen supercompensation (i.e. an increased glycogen concentration above basal) is an established phenomenon but the underlying mechanisms are still unknown. We investigated the insulin independent glycogen supercompensation in skeletal muscle. Muscles were stimulated to deplete glycogen. Glycogen levels were about ~35% greater than basal levels after 6 h of recovery. Glycogen transport was slightly increased whereas glycogen synthase activity was unaffected at the time of supercompensation. Furthermore, glycogen phosphorylase (the rate limiting enzyme of glycogen breakdown) was decreased after stimulation and was still low at the time of supercompensation. (C) We investigated the mechanism behind the increased glycogen breakdown that creatine kinase deficient mice ($CK^{-/-}$) exhibit during contraction. Glycogen phosphorylase, which catalyzes glycogenolysis, is regulated by substrate availability (P_i), phosphorylation/dephosphorylation and allosterically by AMP. The results show that phosphorylase from $CK^{-/-}$muscles has an increased affinity for AMP.
Conclusion: (A) ROS stimulate glucose transport during contraction as well as increasing AMPK activity. Removal of ROS decreases contraction-mediated glucose transport and it is therefore questionable if healthy individuals will benefit from intake of antioxidants. Furthermore, cross-bridges only account for a small part of the total ATP turnover during submaximal contraction and mechanical load does not play a major part in contraction-mediated glucose transport.
(B) Insulin-independent glycogen supercompensation is a result of a decreased glycogen breakdown and increased or constant glycogen synthesis.
(C) $CK^{-/-}$ mice have an increased glycogen breakdown during contraction compared to wild type mice despite the fact that they exhibit low increase in P_i and have a lower phosphorylation of glycogen phosphorylase. These data therefore suggest that allosteric activation of glycogen phosphorylase by AMP could be an important regulatory mechanism for glycogen breakdown.

LIST OF PUBLICATIONS

I. Katz A, Andersson DC, Yu j, Norman B, Sandström ME, Wieringa B, Westerblad H
Contraction-mediated glycenolysis in mouse skeletal muscle lacking creatine kinase: the role of phosphorylase b activation. J Physiol 2003; 553:523-531

II. Sandström ME, Abbate F, Andersson DC, Zhang S-J, Westerblad H, Katz A
Insulin-independent glycogen supercompensation in isolated mouse skeletal muscle: role of phosphorylase inactivation. Pflügers Arch. 2004; 448:533-8

III. Sandström ME, Zhang S-J, Bruton J, Silva JP, Reid MB, Westerblad H, Katz A
Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. J Physiol. 2006; 575:251-62.

IV. Zhang S-J, Andersson DC, Sandström ME, Westerblad H, Katz A
Cross-bridges account for only 20% of total ATP consumption during isometric contraction in mouse fast-twitch skeletal muscle. Am J Physiol Cell Physiol. 2006; 291:147-154

V. Sandström ME, Zhang S-J, Westerblad H, Katz A
Mechanical load plays little role in contraction-mediated glucose transport in mouse skeletal muscle. Submitted

CONTENTS

LIST OF ABBREVIATIONS

ACC	Acetyl CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BTS	N-benzyl-p-toluene sulphonamide
CaMK	Calcium/calmodulin-dependent protein kinase
Cr	Creatine
GLUT	Glucose transport protein
GS	Glycogen synthase
GSH	Glutathione
GSK	Glycogen synthase kinase-3
GSSG	GSH disulfide
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
IRS	Insulin receptor substrate
NAC	N-acetylcysteine
P_i	Inorganic phosphate
PCr	Phosphocreatine
PI3K	Phosphatidylinositol-3-kinase
PP1	Protein phosphatase 1
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SR	Sarcoplasmatic reticulum
TCA	Tricarboxylic acid

INTRODUCTION

Skeletal muscle cells are highly impressive motor cells, giving us the ability to function in everyday life and to communicate through various muscle groups. These muscles are specifically adapted to produce force and can increase their energy turnover ~100 fold during contraction (Sahlin *et al*. 1998). Muscles can account for as much as 90% of the total glucose uptake from blood during insulin stimulation, giving them a central role in whole body glucose homeostasis (DeFronzo *et al*. 1981). Blood glucose concentration increases after ingestion of a meal and it is crucial that the glucose is cleared from the blood into peripheral tissues. An imbalance in blood glucose levels is associated with obesity, diabetes mellitus and cardiovascular disorders (Fonseca, 2005). The glucose that is taken in from the blood stream into the muscles is used as energy to drive all the processes in order for the muscle to contract. Contracting muscles increase glucose clearance from the blood independent of insulin. Exercise therefore plays an important role in whole body glucose homeostasis and is even more important for individuals with decreased insulin sensitivity. There is of course a major interest in new therapeutic agents that can lower the blood glucose concentration, which is needed for people with impaired insulin sensitivity. However, more effort should be invested in the easiest and simplest therapy of them all: exercise.

SKELETAL MUSCLE ACTIVATION

Muscle contraction is initiated through a chain of events, starting with nerve impulses from higher motor centers. Action potentials are transmitted to the muscle fibers, along the surface and down into the transverse tubular system. This tubular system consists of narrow invaginations of the sarcolemma forming a system of branched tubules. The action potential will, in a series of events, open Ca^{2+} channels and induce a Ca^{2+} flow from the sarcoplasmatic reticulum (SR) to the myoplasm, due to a high concentration gradient. The released Ca^{2+} will bind to troponin C, a regulatory protein; this will facilitate the interaction between the two motor proteins myosin and actin. The contraction starts when the myosin heads (also called cross-bridges) interact with actin. During muscle contraction ATP is hydrolyzed to ADP by myosin ATPase to drive the cross-bridges. ATP-driven pumps rapidly pump Ca^{2+} back into the SR and when the impulses from the nerves cease, Ca^{2+} release stops and Ca^{2+} returns to the SR (Fig 1).

Muscle fibers can increase their energy turnover ~100 fold during exercise due to the high ATP consumption mainly by the crossbridges and the ion pumps (Rall, 1982; Baker *et al*. 1994). Intense activation of the muscles leads to muscle fatigue. When muscles fatigue, they display a decreased force, decreased shortening velocity and increased relaxation time. There are several complex mechanisms behind skeletal muscle fatigue (Allen *et al*. 1995). One of the most important is the accumulation of P_i due to its inhibitatory effects on Ca^{2+} release from the SR, cross-

bridge force production, myofibrillar Ca^{2+} sensitivity and re-uptake of Ca^{2+} by the SR (Westerblad & Allen 2002).

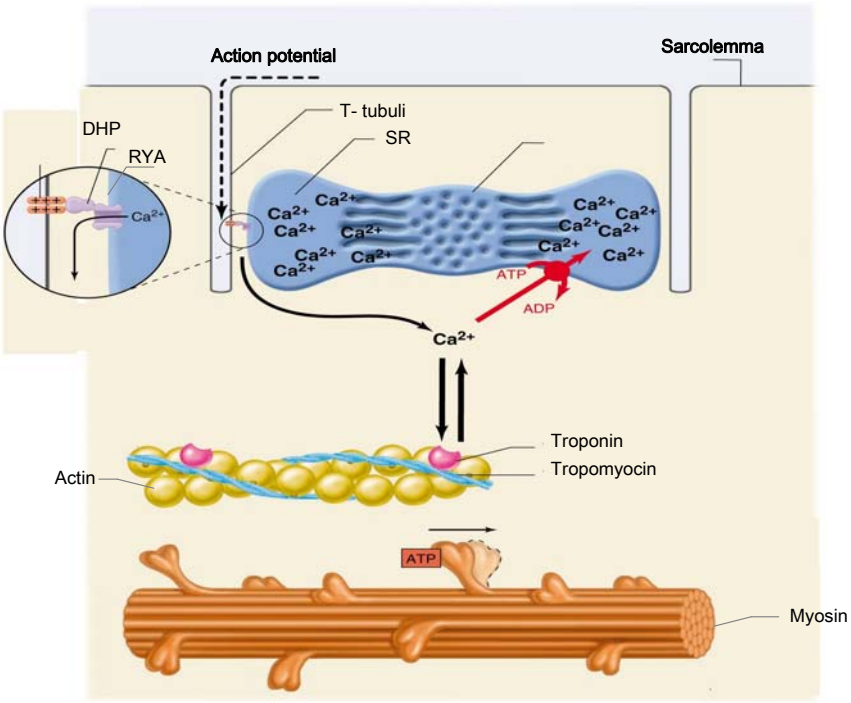

Figure 1. A schematic picture showing skeletal muscle activation. An action potential is transmitted down into the transverse tubuli (T-tubuli). The action potential initiates opening of channels that will release Ca^{2+} from the sarcoplasmatic reticulum (SR) into the myoplasm. Ca^{2+} binds to troponin C, which enables cross-bridge cycling and muscle fiber contraction. ATP driven pumps then pump the Ca^{2+} back into the SR causing a cessation of the contraction.

GLUCOSE TRANSPORT

Skeletal muscle cells are dependent on continuous ATP generation both in the resting state and during contraction. To do this, muscles take up glucose from the blood stream. Glucose, however, cannot penetrate the cell membrane freely and thus is dependent on carrier proteins to facilitate the transport. The two important glucose transporter proteins in skeletal muscle are GLUT 1 and GLUT 4 (Douen *et al*. 1990). GLUT 4 is, in the basal state, stored in intracellular storage sites and is recruited to the membrane by insulin, muscle contraction and hypoxia. Glucose transport stimulated by insulin and contraction complement one another; they activate glucose transport via two distinct pathways (Garetto *et al*. 1984; Brozinick, Jr. *et al*. 1994; Goodyear & Kahn

1998). GLUT 1 is important for glucose transport in the resting state and is constitutively present in the plasma membrane (Mueckler, 1990; Marette *et al*. 1992).

Insulin-mediated glucose transport

To prevent diseases, glucose homeostasis needs to be tightly regulated. When glucose concentration increases after a meal, insulin is secreted from the pancreatic β-cells to stimulate glucose transport by GLUT 4 transporters into the muscle cell. Insulin-mediated glucose transport is stimulated via the insulin receptor in the cell membrane. The insulin receptor is composed of two α- and two β-subunits. Insulin binds onto the extra-cellular α-subunits and this initiates an autophosphorylation of the tyrosine domains on the intra-cellular β-subunits. Upon phosphorylation of the insulin receptor, the insulin receptor substrates (IRS) are also phosphorylated. This subsequently leads to the binding of the IRS to phosphatidylinositol 3'-kinase (PI3K). PI3K is a key enzyme in the insulin-signaling pathway and for the purpose of glucose transport, the catalytic subunit needs to phosphorylate the phosphatidylinositol in the membrane and thereby synthesize phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3, the phosphorylated form of PIP2, binds to phosphatidylinositol -dependent protein kinase which then activates Akt/protein kinase B. To finalize the link between the above-mentioned pathway and glucose uptake, further signaling is needed. A newly found substrate for Akt, AS160, may be important for this stage (Ryder *et al*. 2001). However, the understanding of the insulin-signaling cascade is still incomplete.

Contraction-mediated glucose transport

As mentioned above, insulin is not the only mediator of glucose transport. Contraction stimulates glucose transport via a pathway different from insulin (Holloszy & Hansen 1996). GLUT 4 is translocated to the membrane surface in both pathways. It has however been debated whether contraction and insulin- responsive GLUT 4 “pools” of vesicles exist (Douen *et al*. 1990; Coderre *et al*. 1995). Ca^{2+} has long been recognized as an important signaling molecule for contraction-mediated glucose transport (Holloszy & Narahara 1967). Ca^{2+} is released from SR following an action potential; however, Ca^{2+} is elevated for only a short period after each muscle contraction, whereas glucose transport remains elevated long after muscle contraction ends. Thus, it is likely that Ca^{2+} is not directly regulating glucose transport. There is evidence for the importance of 5´-AMP-activated protein kinase (AMPK) for contraction-mediated glucose transport, which will be discussed below. Another suggested signaling molecules are the Ca^{2+} /calmodulin –dependent protein kinase (CaMK) and protein kinase C (Richter *et al*. 1984; Ojuka *et al*. 2002; Ojuka *et al*. 2003).

It has been debated whether force production *per se* plays a role in the regulation of glucose transport during exercise. An early study by Holloszy and Narahara suggested that mechanical load was not an important component (Holloszy & Narahara 1965). However, more recent studies have found a linear correlation between muscle force

production and glucose transport (Ihlemann *et al*. 2000; Fujii *et al*. 2005). Thus, the importance of force production for glucose transport during exercise is still elusive.

AMP-ACTIVATED PROTEIN KINASE

AMPK is an important signaling molecule for regulation of glucose and fat metabolism in skeletal muscle. It was first discovered as a negative regulator of the rate limiting enzymes of fatty acid and cholesterol synthesis: acetyl CoA carboxylase (ACC) and hydroxymethylglutaryl CoA (Carling *et al*. 1987; Sim & Hardie 1988; Winder *et al*. 1989). AMPK increases fatty acid oxidation through inhibition of ACC (Merrill *et al*. 1997), which will eventually result in the entry of long-chain fatty acyl-CoA into the mitochondria, thus, increasing fatty acid oxidation to yield energy and reduce lipid stores (Kahn *et al*. 2005).

There are now several lines of evidence that AMPK plays an important role in the increased glucose transport during exercise (Sahlin *et al*. 1998; Winder & Hardie 1999; Musi & Goodyear 2003; Jorgensen *et al*. 2006). AMPK is an energy-sensing enzyme and activated during exercise, hypoxia or by supplementation of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which is an AMP analogue (Corton *et al*. 1995; Winder & Hardie 1996; Hardie *et al*. 1998; Wojtaszewski *et al*. 2000). Thus, AMPK is activated to potentiate ATP production and to decrease anabolic processes.

AMPK structure and activation

The enzyme is composed of three subunits, the catalytic α-subunit, and the regulatory β- and γ-subunits, all of which are necessary for full activation (Hardie *et al*. 1998). There are several isoforms of each subunit, thus giving the possibility to form many different heterotrimeric AMPK proteins (Hardie *et al*. 1998). AMPK is activated through phosphorylation of the threonine (Thr172) residue on the α-subunit by upstream kinases, by increased AMP/ATP ratio and by allosteric activation of AMP (Hardie *et al*. 1998). In addition, AMP inhibits dephosphorylation of AMPK while high concentrations of ATP and glycogen inhibit the activation of AMPK (Carling *et al*. 1987; Corton *et al*. 1995; Wojtaszewski *et al*. 2003; Jorgensen *et al*. 2006). Thus, an increased level of AMP has been thought to be the major activator of AMPK. The two known main upstream kinases responsible for phosphorylating AMPK are LKB1 and the Ca^{2+}-dependent CaMK. LKB1 is a tumor suppressor protein and forms a complex with two accessory subunits, STRAD and MO25. LKB1 is constitutively active and neither contraction nor AICAR increase the activity of LKB1 (Sakamoto *et al*. 2004; Hardie & Sakamoto 2006).

Contraction and H_2O_2 – mediated activation of AMPK

AMPK plays a major role in the increased glucose transport during exercise. It has, however, been shown by using transgenic mice that alternative pathways exist (Mu *et al*. 2001). By knocking out the α2-isoform on AMPK Mu *et al* and Jörgensen *et al*

could show that glucose transport during exercise was only partially inhibited, where as knocking out the α1 isoform hade no effect on the contraction-mediated glucose transport (Mu *et al*. 2001; Jorgensen *et al*. 2004b).

AMPK activity and glucose transport are also increased by hydrogen peroxide (H_2O_2), which is formed in contracting muscles (see below). H_2O_2 predominantly activates the α1 isoform on AMPK in an AMP/ATP independent manner (Toyoda *et al*. 2004). By knocking out the upstream kinase LKB1, the H_2O_2 induced AMPK activity is blocked (Woods *et al*. 2003). Recent results have shown that Ca^{2+} also plays an important role in the activation of AMPK via a pathway independent of changes in high-energy phosphates (Ojuka *et al*. 2002; Ojuka *et al*. 2003), probably via a CaMK dependent pathway (Wright *et al*. 2004). Thus, these data indicate that AMPK can be activated by several pathways, which are independent of alterations in the AMP/ATP ratio.

Hypoxia-mediated activation of AMPK

Hypoxia and contraction both increase AMPK activity by a α2 isoform phosphorylation (Mu *et al*. 2001). Some studies have shown that contraction and hypoxia have an additive effect on glucose transport (Derave & Hespel 1999; Fluckey *et al*. 1999) whereas others have found the opposite results (Cartee *et al*. 1991). However, more recent results have shown that it is possible to block the hypoxia-mediated glucose uptake whilst the contraction-mediated uptake remains. Hypoxia-mediated glucose uptake seems to rely on AMPK activation while contraction-mediated transport is partly AMPK independent (Wojtaszewski *et al*. 1998; Mu *et al*. 2001). Thus, hypoxia cannot be used as a model for contraction-mediated glucose transport.

AICAR-mediated AMPK activation

AICAR is a drug that causes phosphorylation of AMPK and increases glucose transport also through an α2 isoform phosphorylation. Upon entering the cell, AICAR is converted to 5-amino-4-imidazolecarboxamide riboside monophosphate (ZMP), which activates AMPK by mimicking the effects of AMP (Merrill *et al*. 1997; Wright *et al*. 2004). The effects of AICAR on glucose transport are completely mediated by AMPK and possibly through the same pathway as hypoxia (Mu *et al*. 2001). AICAR has often been used as a mimetic of contraction-mediated activator of AMPK but the effects of AICAR are, however, non-specific (Long & Zierath 2006).

GLYCOGEN METABOLISM

Of all organs, skeletal muscle contains the largest total depot of glycogen. It is the major energy source for skeletal muscle and it is considered to be a limiting factor for performance during prolonged moderate intensity exercise (Hermansen *et al*. 1967; Robergs *et al*. 1991). Glycogen is built up when there is an excess of energy, as after a meal, and broken down when needed. Glycogen is a branched molecule consisting of glucose residues that are stored in granules. The granules are organized in distinct

subcellular locations within the muscle cell. The granules also contain all enzymes necessary for their own metabolic machinery; in fact, ~70% of the total weight of the granule consists of enzymes (Haschke *et al*. 1970).

When glucose enters the cell it is rapidly phosphorylated to glucose-6-phophate (G6P) by hexokinase. G6P is then further converted to glucose-1-phophate (G1P) by phosphoglucomutase. Next, uridine triphosphate and G1P are combined to form UDP-glucose. The glycogen granule begins to form when glucose residues from UDP-glucose are transferred to the autoglycosylating protein glycogenin (Roach, 2002; Gibbons *et al*. 2002). The transfer from UDP-glucose to the glycogen chain is catalyzed by glycogen synthase (GS). With the help of branching enzymes, the glycogen granule grows around the glycogenin core (Melendez *et al*. 1999).

The rate-controlling enzyme for glycogen breakdown is glycogen phosphorylase. Glycogen is broken down to G1P and then further metabolized in the glycolytic pathway. Glycolysis will eventually give rise to pyruvate, which will be further metabolized either anaerobically or aerobically. Anaerobic glycolysis results in the production of lactate and the production of only 3 ATP whereas the aerobic glycolysis results in the production of 36 ATP (Fig 2).

If glycogen stores are depleted and carbohydrates are ingested following exercise, muscle glycogen levels can exceed basal levels (Bergstrom & Hultman 1967). This phenomenon is called glycogen supercompensation. Many endurance athletes use this method to increase their glycogen stores prior to competing. In humans this can lead to a 2-fold increase in glycogen levels. However, the mechanism behind this phenomenon is still unknown.

Glycogen synthase

GS exists in two isoforms, the D and I form. Both forms are active but the I form is generally regarded as the more active form. GS is regulated by both allosteric and phosphorylation-dephosphorylation mechanisms (Rosell-Perez *et al*. 1962). The phosphorylated D form is dependent on allosteric binding to G6P for its activation whereas the I form is not (Villar-Palasi, 1991). G6P activates GS allosterically as well as making it more susceptible to dephosphorylation mainly by protein phosphatase 1 (PP1) (Villar-Palasi, 1991). The phosphorylation of GS is a fairly complex procedure: there are nine regulatory serine phosphorylation sites on the enzyme: 1a, 1b, 2, 2a, 3a, 3b, 3c, 4, 5. Phosphorylation of sites 2, 2a, 3a and 3b result in a larger decrease of the enzyme activity than phosphorylation of the other sites. However, there is a hierarchical phosphorylation of the enzyme. Phosphorylation of site 5, which is a less potent site, by casein kinase 2 (CK2) is followed by phosphorylation on sites 4, 3c, 3b and 3a by glycogen synthase kinase 3 (GSK3), resulting in a decreased GS activity (Fiol *et al*. 1987). Important kinases that phosphorylate GS are GSK 3, CK 1 and 2, protein kinase

A and AMPK (Roach & Larner 1977; Fiol *et al*. 1987; Jorgensen *et al*. 2004a). PP1 is the main catalyst of the dephosphorylation of GS, thus increasing its activity. Insulin also contributes to the regulation of GS by indirect phosphorylation of serine residues on GSK3. Serine phosphorylation decreases GSK3 activity, which results in less phosphorylation of GS. Insulin and exercise decrease GSK3 to the same extent but not through the same pathway (Markuns *et al*. 1999).

Another important regulator of GS activity is the level of glycogen in the muscle, particularly during and after exercise. There is an inverse relationship between the glycogen concentration and the activity of GS after exercise (Danforth, 1965; Jorgensen *et al*. 2004a). Danforth showed in an early study that GS is activated by electrical stimulation, but that this activity depends on the stimulation program, regarding intensity, duration and the initial level of glycogen (Danforth, 1965). For example, GS activity deceases during short-term intensive exercise (Chasiotis *et al*. 1982; Katz & Raz 1995) wheras prolonged moderate intense exercise increases GS activity (Danforth, 1965; Staneloni & Piras 1969; Bergstrom *et al*. 1972; Brau *et al*. 1997; Wojtaszewski *et al*. 2001).

Glycogen phosphorylase

The rate of glycogenolysis is regulated by the activity of glycogen phosphorylase. Glycogen phosphorylase exists in two molecular forms; phosphorylase **a** and phosphorylase **b**. Glycogen phosphorylase is regulated by substrate availability (mainly P_i, but also glycogen under some conditions), allosterically by AMP and by phosphorylation/dephosphorylation. Phosphorylase **b** is fully dependent on allosteric activation by AMP while phosphorylase **a** is less dependent on AMP (Lowry *et al*. 1964). Classically, the conversion of phosphorylase **b** to phosphorylase **a** has been considered necessary for glycogenolysis. Thus, phosphorylase **a** has been regarded as the active form in vivo. The conversion between the two forms is catalyzed by phosphorylase **b** kinase, which is activated by increased Ca^{2+} levels at the onset of contraction. (Heilmeyer, Jr. *et al*. 1970; Brostrom *et al*. 1971; Browner & Fletterick 1992). This classical view of phosphorylase activation has been questioned throughout the years; Danforth and Lyon showed as early as in 1964 that mice lacking phosphorylase **b** kinase could break down glycogen (Danforth & Lyon 1964). Furthermore, a number of studies questioned the role of interconversion and P_i availability in the activation of glycogenolysis during muscle contraction; for example, ischemic, non-contracting muscle exhibits negligible glycogenolysis despite elevated phosphorylase **a** and P_i (Ren & Hultman 1989; Katz, 1997). It was also shown that the glycolytic rate and P_i levels were not correlated during different stimulation programs in skeletal muscle (Ren & Hultman 1990). Moreover, it was shown that during prolonged continuous stimulation skeletal muscles exhibited a conversion of phosphorylase **b** to **a** during the initial seconds of contraction and thereafter a reversal of phosphorylase **a** back to phosphorylase **b** as the contraction continued, whereas

glycogenolysis continued even after the reversal (Conlee *et al*. 1979). Thus, the regulation of glycogen phosphorylase requires further investigation.

α-Glucosidase

There are two forms of α-glucosidase. One is present in the lysosome and has a pH optimum around 4.5. The other is present in the microsome and has a pH optimum of around 7.0. They both break down glycogen in their respective environment. Lysosomal α-glucosidase defects result in type II glycogen storage disease, in which lysosomal glycogen hyper-accumulates in several tissues, including skeletal muscle. The pathology is severe, leading to disabilities and often to death. The hallmarks of this syndrome are skeletal muscle weakness and hypertrophy of the heart (Roach, 2002; Roth *et al*. 2003)

THE MITOCHONDRIA

The mitochondria function as the power plant of the cells, with the tricarboxylic acid (TCA) cycle as the main engine room. Here glucose, fatty acids and to some extent amino acids, are oxidized to finally give ATP, carbon dioxide and water. The mitochondria exhibit remarkable adaptive capabilities in response to physiological activities and provide the muscle cell with the amount of ATP needed. The mitochondria are also generally believed to be the primary site for reactive oxygen species (ROS) production, a process occurring in all aerobic organisms (Commoner *et al*. 1954; Murrant & Reid 2001).

Function

In skeletal muscle, the number of mitochondria depends on muscle fiber type. Slow twitch fibers like the soleus muscle exhibit more mitochondria than, for example, the fast twitch extensor digitorum longus muscle. The mitochondrion consists of a double membrane, an outer smooth layer and an inner layer, which consists of multiple folds, known as cristae, to increase the surface area. The region enclosed by the outer and inner layer is called the intermembrane space and the space within the inner membrane is called the matrix, which contains most of the enzymes necessary for the TCA cycle. The reactions of the TCA cycle take place inside the matrix. Under aerobic conditions pyruvate is oxidized to acetyl CoA in the TCA cycle. There are eight reactions in the TCA cycle, each catalyzed by a different enzyme. Four of the eight steps are oxidation reactions where an electron is transferred to the intermediate electron acceptors NAD^+ or FAD, which become, together with hydrogen, NADH and $FADH_2$. These molecules will ultimately transfer electrons to the electron transport chain and ultimately to oxygen, which will be reduced to water (Fig 2).

Four closely related enzyme complexes form the electron transport chain. NADH and $FADH_2$ enter the electron transport chain where electrons are transferred from one complex to another in a series of energy-releasing oxidation reactions. Along the

electron transport chain, hydrogen ions from the redox reactions of the enzyme complexes are pumped out into the intermembrane space where a proton gradient is created. When the hydrogen ions flow back into the matrix through the ATPase complex, ATP is formed from ADP. This process is called oxidative phosphorylation. For each glucose molecule about 36 ATP can be produced under aerobic conditions.

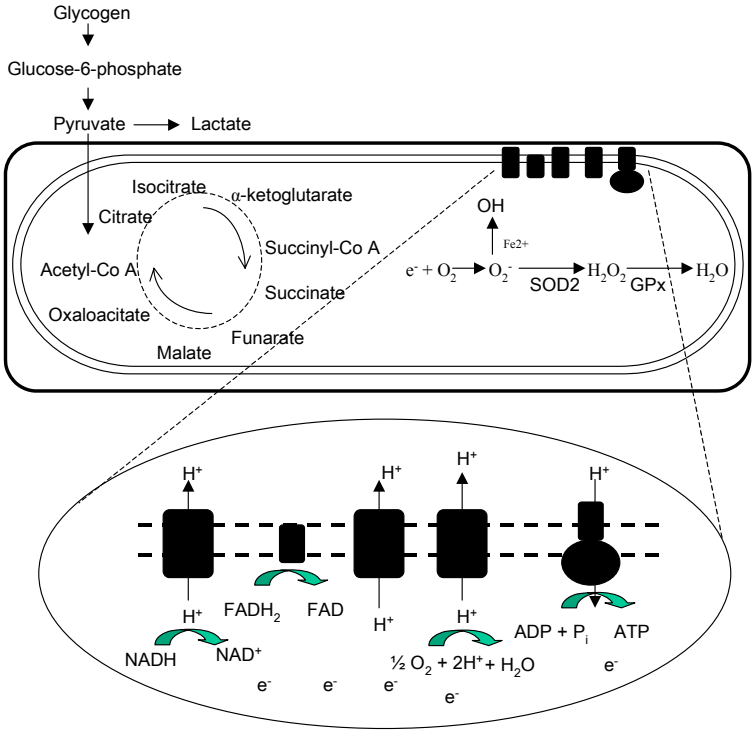

Figure 2. A schematic picture showing glycogenolysis, the production of reactive oxygen species and the production of ATP. Glycogen is broken down to pyruvate, which enters the mitochondrion and the tricarboxylic acid cycle (TCA). Electrons are produced in the TCA cycle, and are subsequently taken up by NAD^+ or FAD. These molecules will transfer the electrons to the electron transport chain where they are ultimately transferred to oxygen. Electrons are, however, lost on the way, which results in the formation of reactive oxygen species. ATP is formed when H^+ ions that have been pumped out to the inter-membrane space flow back through the ATPase complex

Production of reactive oxygen species

There is a constant leakage of electrons from the electron transport chain, which rapidly react to form reactive oxygen species (ROS). About 1-3% of total oxygen consumption result in the formation of ROS (Murrant & Reid 2001). The transport of electrons accelerates in contracting muscles, resulting in an increased formation of ROS. ROS are small molecules containing unpaired electrons (radicals) and are therefore very

reactive. Examples of ROS are the superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Reid *et al*. 1992). $O_2^{\cdot-}$ is very short lived and forms H_2O_2 in the presence of superoxide dismutase (SOD). Although H_2O_2 is not a free radical by definition, it is a potent oxidant. It is more stable than $O_2^{\cdot-}$ and it can freely diffuse through the membrane out of the mitochondria. H_2O_2 can also react with transition metals, like Fe^{2+}, to form the highly reactive hydroxyl radical ($OH^{\cdot}$).

It should be noted that ROS might also be produced by non-mitochondrial sources. For instance, an active NAD(P)H oxidase enzyme complex, which catalyzes the reduction of oxygen to superoxide, has been detected in skeletal muscle (Javesghani et al. 2002). Recent studies have shown that NAD(P)H oxidase is located in association with the t-tubules and can affect SR Ca^{2+} release (Hidalgo *et al*. 2006). Furthermore, the enzyme can be activated by maneuvers that induce SR Ca^{2+} release in cultured rat muscle cells (Espinosa *et al*. 2006). Thus, NAD(P)H oxidase may produce significant amounts of ROS during repeated contractions.

There are several antioxidant systems in the cell to protect against pathological concentrations of ROS. Two of the most important are SOD and glutathione peroxidase. SOD (SOD2 is the isoform in the mitochondria) catalyzes the reaction: $O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$. H_2O_2 is further reduced to water and oxygen in a reaction catalyzed by glutathione peroxidase with glutathione (GSH) as a substrate, which is oxidized to the disulfide, GSSG: $H_2O_2 + 2\ GSH \rightarrow H_2O + GSSG$. Whilst exceedingly high concentrations of ROS can lead to metabolic disturbances and insulin resistance, low/physiological levels appear necessary and beneficial for normal and optimal cell function (Murrant & Reid 2001; Balon & Yerneni 2001; Goldstein *et al*. 2005). It has been shown that exogenously added H_2O_2 stimulates glucose transport in skeletal muscle (Cartee & Holloszy 1990; Toyoda *et al*. 2004). However, the pathway through which ROS work, or the importance of endogenously produced ROS for glucose transport, is poorly understood. Interestingly, it has been shown that muscles adapt to increased ROS production during exercise to keep the right balance of antioxidants. Healthy human muscles therefore seem to control the levels of ROS without ingestion of antioxidants (Ji *et al*. 2006).

AIMS

The overall aim of this thesis is to investigate mechanisms of the regulation of carbohydrate metabolism in skeletal muscle both during and after contraction.
The specific aims that were addressed are as follows:

- To study the regulation of glycogen phosphorylase during contraction using creatine kinase deficient mice, which display little P_i increase during repeated contractions.

- To study the mechanism behind the insulin-independent glycogen supercompensation in skeletal muscle and, specifically, to evaluate the roles of phosphorylase and α-glucosidase activity.

- To investigate the role of endogenously produced ROS in contraction-mediated glucose transport and to study the mechanism whereby ROS activates glucose transport.

- To investigate the role of mechanical load in contraction-mediated glucose transport.

MATERIAL AND METHODS

ANIMALS

All animals were housed at room temperature with a 12 h:12 h light-dark cycle. Food and water were provided *ad libitum*. The following male rodents were used: C57Bl/6 (*study I*), CK-/- mice (Steeghs *et al*. 1997) (*study I*), NMRI mice (*study II-V*), mice over-expressing Mn^{2+} -dependent SOD (strain PAC662D1) and wild type littermates (Silva *et al*. 2005) (*study III*). Mice were killed by rapid cervical dislocation and soleus and/or extensor digitorum longus (EDL) muscles were isolated. Male Wistar rats were used as a control in study *IV*. All procedures were approved by the Stockholm North local ethics committee.

ELECTRICAL STIMULATION

For all contraction studies, stainless steel hooks were tied with a nylon thread to the tendons of the muscles. Muscles were then transferred to a stimulation chamber and mounted between a force transducer and an adjustable holder. The chambers were, at all times, connected to a temperature controlling water-jacket circulation bath. The muscles were bathed in a Tyrode solution with the following composition (in mM): NaCl 121, KCl 5, $CaCl_2$ 1.8, NaH_2PO_4 0.5, $MgCl_2$ 0.4, $NaHCO_3$ 24, EDTA 0.1, glucose 5.5 and 0.1 % fetal calf serum. The solution was gassed continuously with 5 %CO_2/95 %O_2, which results in a pH of 7.4. Muscles were set to the length at which tetanic force was at maximum. Muscles were stimulated with current pulses (0.5 ms duration; ~150% of the current required for maximum force response) delivered via plate electrodes lying parallel to the fibers. The signal from the force transducer was sampled on-line and stored on a desktop computer for subsequent analysis.

Detailed descriptions of stimulation programs are given in the individual papers.

GLUCOSE TRANSPORT

Glucose transport was measured in *study II, III* and *V* as described by Shashkin et al (Shashkin *et al*. 1995) with some modifications. Muscles were transferred to vials containing Tyrode buffer with pyruvate (2 mM). The vials were placed in a temperature controlled shaking bath, shaking at 100 oscillations/min. Radiolabelled 2-deoxy-D-(1,2-^{3}H) glucose (DG) (1 mM; 1mCi/mmol) and inulin (0.2μCi/ml medium) were used in all experiments and added 20 or 30 minutes prior to freezing. Samples were continuously gassed with 5%CO_2/95%O_2, bringing the solution to a pH of 7.4. See the respective papers for specific treatments and incubation times of the muscles. Following incubation, muscles were frozen in liquid N_2. The frozen muscles were added to pre-weighed Eppendorf tubes containing 0.5 ml of 1 N NaOH. The muscles were weighed and then digested at 70 °C for 5 min. The tubes were cooled on ice and

centrifuged at 23,000 g for 5 min. Aliquots of the supernatant were added to scintillation cocktail and counted for ^{14}C and ^{3}H.

ENZYME ASSAYS

Glycogen synthase

Study I and II. Muscles were freeze-dried, dissected free from connective tissue, powered and thoroughly mixed. Muscle powder was homogenized in ice-cold buffer consisting of 10 mM EDTA, 50mM potassium fluoride, 30% (v/v) glycerol, pH 7.0. The homogenate was centrifuged at 23,000 g at 4°C. Aliquots of the supernatant were diluted and assayed with a filter paper technique following the incorporation of [^{14}C]UDP-glucose into glycogen at 30°C. GS activity was measured in the presence of 0.17 or 7.2 mM glucose-6-P as described previously (Jiao *et al*. 1999). GS fractional activity (GSF) is expressed as the ratio of the activity measured in the presence of 0.17 and 7.2 mM G6P.

Glycogen phosphorylase

Study I and II. Muscles were prepared as for GS. Aliquots of the supernatant were diluted and assayed with a filter paper technique following the incorporation of [^{14}C]glucose-1-P into glycogen at 30°C as described elsewhere (Jiao *et al*. 1999). Phosphorylase fractional activity is the ratio of the activity measured in the absence and presence of 3.3 mM AMP (total phosphorylase activity), the latter reflecting the phosphorylated form of the enzyme.

α-Glucosidase

Study II. α-glucosidase activity was assayed with a fluorometric method following the conversion of 4-methylumbelliferyl-α-D-glucopyran (4-MUG, 2 mM) to the fluorescent product 4-methy-lumbelliferone (4-MU) at 35 °C for 60 min as previously described (Salafsky & Nadler 1973). Briefly, muscles were prepared as for GS and the supernatant (4 or 8 µl) was incubated with either 200 µl acetate buffer (0.2 M, pH 4.5) containing KCl (0.2 M) or 200 µl phosphate buffer (0.2 M, pH 7.0) plus substrate. At the end of the incubation 1 ml of 0.5 M glycine, pH 10.4 was added to the incubation mixture to stop the reaction and fluorometric readings were performed. Activity at pH 4.5 is considered to represent lysosomal α-glucosidase, whereas activity at pH 7.0 is considered to reflect microsomal α-glucosidase. Calculations were based on a 4-MU standard curve made in 0.5 M glycine.

AMPK activity

Study III and V. AMPK activity was analyzed based on a method described previously following the incorporation of radiolabelled phosphate from ATP into SAMS peptide (Winder & Hardie 1996). Freeze-dried muscles were homogenized in ice-cold buffer (100 µl/mg dry wt) consisting of (in mM): Tris, 10; sucrose, 250; NaF, 50; EDTA, 1; β-mercaptoethanol, 10; and 1 tablet protease inhibitor cocktail (Roche) per 50 ml of

buffer, pH 7.5. The homogenate was centrifuged at 23,000 g for 30 min at 4°C. The supernatant was diluted with 7 volumes of homogenization buffer and 10 µl of the diluted extract was mixed with 30 µl reaction buffer, resulting in the following final concentrations (in mM): Hepes (pH 7.0), 40; SAMS peptide, 0.2; NaCl, 80; EDTA, 0.8; AMP, 0.2; DTT, 0.8; $MgCl_2$, 5; ATP, 0.2; [γ-^{32}P]ATP, 2 µCi; and glycerol, 8% (v/v);. The assay was performed at 37°C for 10 min. Thereafter, 30 µl of the mixture was spotted onto Whatman P81 discs, washed in 1% phosphoric acid, dried and counted. Blanks consisted of mixtures spotted without incubation.

METABOLITE ASSAYS

Glycogen, PCr, Cr, ATP, Pi, G6P, lactate, IMP, inosine, AMP, ATP, malate

Study I, II, IV and V. For analysis of glycogen, aliquots of powder were digested with hot, 1 N KOH and hydrolyzed enzymatically to free glucose (Jiao *et al*. 1999). Glucose was then analyzed enzymatically with a fluorometric technique (Lowry & Passonneau1972). For analysis of metabolites, ice-cold 0.5 M perchloric acid was added to aliquots of muscle powder. The extract was kept in an ice bath for 15 min while being agitated with a vortex mixer, and then centrifuged. The supernatant was neutralized with 2.2 M $KHCO_3$ and then again centrifuged. The subsequent supernatant was assayed for PCr, Cr, ATP, P_i, G6P, lactate, and malate with enzymatic techniques [changes in NAD(P)H] adapted for fluorometry (Lowry & Passonneau1972). High-performance liquid chromatography was used to analyze the extracts for inosine monophosphate (IMP), inosine, ADP and AMP. To adjust for variability in solid nonmuscle constituents, metabolite values were divided by the sum of PCr + Cr (total Cr) and then multiplied by the mean total Cr content for the whole material.

WESTERN BLOT

Study III and V. Western blots were performed for phosphorylated and total AMPK and ACC. In short, 20 µg (for total and phosphorylated ACC) or 25 µg (for total and phosphorylated AMPK) of supernatant protein (prepared as for AMPK activity, see above) were separated by SDS-PAGE (4-12% Bis-Tris Gels- Invitrogen) and transferred onto polyvinylidine fluoride membranes. Membranes were blocked in 5% (w/v) non-fat milk Tris-buffered saline containing 0.05% Tween 20 followed by incubation with primary antibody, made up in 5% (w/v) bovine serum albumin (all at 1:1000 dilution) over night at 4°C. Membranes were then washed and incubated for 1h at room temperature with secondary antibody (donkey-anti-rabbit at 1:2000 dilution). Immunoreactive bands were visualized using enhanced chemiluminiscence (Super Signal, Pierce).

GLUTHATIONE

Study III and V. A kit was used to analyze the glutathione status (Biooxytech GSH/GSSG-412, Oxis Health Products). Muscles were freeze-dried, dissected free of non-muscle constituents, powdered and thoroughly mixed. The powders were divided

into two aliquots, which were homogenized in ground glass homogenizers containing ice-cold 5% metaphosphoric acid (80 µl/mg dry wt) with and without 1-methyl-2-vinyl-pyridinium trifluoromethane sulphonate (M2VP, 10% v/v), a scavenger of GSH. The homogenates were centrifuged at 23,000 g for 15 min at 4°C. The pellets were digested with 1 M NaOH (60°C) and assayed for protein with the Bio-Rad assay (BIO-RAD). For measurement of total GSH (GSH + GSSG), 4 µl supernatant were mixed with 96µl assay buffer. For GSSG estimation, 5µl supernatant (+M2VP) was mixed with 95µl GSSG assay buffer. For both assays, the samples were mixed with 300 µl of chromogen, glutathione reductase and NADPH. Absorbance (reduction of dithiobis-2-nitrobenzoic acid at 412 nm) was measured after 4.5 min in a spectrophotometer.

DETECTION OF ROS WITH CM-H_2DCFDA

Study III. A fluorometric method was used to detect changes in ROS levels. Small bundles of 5 to 20 fibers were mechanically dissected from the EDL muscle. Each bundle was incubated in 10 µM CM-H_2DCFDA for 20 min at 24 ºC and then washed for 30 minutes with glucose supplemented Tyrode (± 20 mM NAC). A BioRad MRC 1024 confocal unit and a Nikon Diaphot 200 inverted microscope with a 20x objective lens (N.A.) were used. CM-H_2DCFDA was excited with 488 nm light and the emitted light collected through a 522 nm long-pass filter. Confocal images of muscle fibers at rest and after ten minutes of intermittent tetanic contractions were obtained. NAC was added after the 20 minutes loading period and remained in the medium until after the post-contraction scans were performed. The muscles were exposed to 1 mM H_2O_2 for five minutes at the end of the protocol to assess the responsiveness of the indicator.

RESULTS AND DISCUSSION

The questions investigated in this thesis all concern the metabolism of glucose and glycogen during rest and exercise. This is of particular importance in relation to diseases associated with carbohydrate metabolism. Of these, type 2 diabetes mellitus is one of the fastest growing causes of morbidity and mortality to day. It is for this reason that these questions need to be addressed further.

ROS AND GLUCOSE TRANSPORT (*PAPER III*)

It has previously been shown that exogenously added H_2O_2 increases glucose transport in skeletal muscle (Cartee & Holloszy 1990; Toyoda *et al*. 2004). In this study, we investigated whether endogenously produced ROS are involved in contraction-mediated glucose transport and the mechanism whereby ROS activates glucose transport during contraction.

H_2O_2 and glucose transport

We began by investigating the importance of endogenously produced ROS on glucose transport. NAC, which is a general antioxidant, was used to block ROS accumulation in EDL muscles during contraction. We found that the addition of NAC abolished ~50% of contraction-mediated glucose uptake. NAC was also added to resting muscles and muscles treated with insulin, AICAR and hypoxia (Fig 1a, *paper III*) to determine whether NAC was specifically inhibiting contraction-mediated glucose transport or affected other interventions that increase glucose transport. NAC was shown to specifically inhibit contraction-mediated glucose transport, as it did not affect any of the other interventions. Moreover, ebselen, which is a glutathione peroxidase mimetic, and thus removes H_2O_2 in the presence of GSH, was also used to block the ROS accumulation during contraction. Ebselen inhibited 60% of the contraction-mediated glucose transport (Fig 7a, *paper III*), which correspond with the NAC results. However, ebselen also affected basal transport and was therefore not used in subsequent experiments.

H_2O_2 formation

The intracellular changes in GSH and GSSG during contraction reflect the changes in oxidative stress and specifically the formation of H_2O_2. We used two methods to investigate the increase in endogenously produced ROS: the formation of GSSG from GSH and also a ROS sensitive fluorescent indicator, which was loaded into muscle fiber bundles.

Contracting muscles show an increase in GSSG levels and NAC significantly inhibited this GSSG increase (Fig 3, paper III). As a control experiment, we added H_2O_2 to

resting muscles to ensure that we could detect an increased level of GSSG. Accordingly, H_2O_2 increased GSSG and this increase was almost completely abolished by NAC (Fig 2, paper III). This indicates that NAC decreases the oxidative stress that is specifically associated with H_2O_2. EDL fiber bundles were loaded with the ROS sensitive indicator CM-H2DCFDA and stimulated. There was an increase in fluorescence during contraction, which was inhibited by NAC (Fig 4, paper III). Taken together, the NAC inhibition of both contraction-mediated glucose uptake and GSSG increase, indicate that endogenously produced ROS significantly increases glucose transport during contraction.

SOD transgenic mice

Another approach to investigate the importance of ROS was to use mice over-expressing SOD2. SOD catalyzes the reaction $O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$ and we therefore assumed that skeletal muscles from the transgenic mice would exhibit an increased H_2O_2 production due to the increased enzyme activity. This would be expected to increase glucose transport during contraction. This assumption was based on previous studies which showed that these animals had a decreased accumulation of $O_2^{\cdot-}$ during accelerated mitochondrial respiration (Silva *et al*. 2005). Transgenic SOD2 muscles showed normal basal transport as well as a normal increase in insulin-mediated transport and force production (Fig 7c and results, *paper III*). However, glucose transport after contraction was ~25% higher compared to wild type, supporting the idea of an increased H_2O_2 -mediated glucose transport (Fig 7b, *paper III*).

AMPK and ROS

The mechanism whereby ROS activates glucose transport during contraction was investigated by looking at the activity of AMPK. We found that contraction resulted in a 10-fold increase in AMPK activativity and ~50% of this increase was blocked by NAC (Fig 5, *paper III*). We found similar results when measuring AMPK protein phosphorylation (Fig 6a, *paper III*). Thus, NAC inhibited AMPK activation/phosphorylation and glucose transport to the same relative extent. The mechanism whereby exogenous H_2O_2 is activating AMPK is not known but previous studies have shown an AMPK activation by H_2O_2 without alteration in high energy phosphates (Toyoda *et al*. 2004; Quintero *et al*. 2006). Furthermore, a recent study showed that the upstream kinase LK1B is necessary for H_2O_2-mediated activation of AMPK and that LK1B phosphorylation of AMPK is not sensitive to AMP. Thus, it is likely that H_2O_2 enhances the substrate suitability of AMPK for LK1B rather than directly activating LK1B (Woods *et al*. 2003). We therefore suggest that activation of AMPK by endogenously produced ROS is not dependent on alterations in high-energy phosphates.

In conclusion, AMPK seems to be activated through two different pathways during contraction; one that is NAC sensitive and one that is NAC insensitive. The former

would then involve ROS, whereas the latter would depend on some other consequences(s) of repeated contractions.

FORCE AND GLUCOSE TRANSPORT (*PAPER IV AND V)*

There has been contradictory data regarding the importance of force production *per se* on glucose transport. Recent studies have shown a correlation between muscle force production and glucose transport (Ihlemann *et al*. 2000; Fujii *et al*. 2005), whereas earlier results indicate that mechanical load and glucose transport are not related (Holloszy & Narahara 1965). It is, however, a difficult task to specifically inhibit force production without interfering with Ca^{2+} handling or other metabolic events. Previously, two methods have been used to abolish force production; either shorten the length of muscles or to decrease voltage during stimulation. Muscles shortened prior to stimulation, will produce less force owing to inappropriate actomyosin interaction. This method encounters the potential problem of insufficient SR Ca^{2+} release because the action potential may fail to reach the center of the muscle cells (Taylor & Rudel 1970). The other method used was to decrease the voltage during the electrical stimulation to induce different force levels (Goodyear, 2000). Since the action potential generation is based on the 'all or nothing' principle, decreasing the voltage will therefore result in fewer muscle fibers being activated. Inactive muscle cells will of course not show any contraction-mediated glucose uptake.

The effect of BTS on ATP-turnover

To investigate the role of mechanical load on contraction-mediated glucose we used BTS, which blocks the cross-bridges and thus prevents force production. Before BTS could be used for this purpose we had to investigate the specificity and efficiency of BTS. BTS was shown to be a specific inhibitor of myosin II ATPase and it inhibits ~95% of the force production during isometric contractions (Fig 1a, paper *IV*). It was shown earlier that BTS does not interfere with Ca^{2+} release or uptake by SR (Cheung *et al*. 2002; Pinniger *et al*. 2005; Bruton *et al*. 2006). Interestingly, we found that BTS had only a small effect on total ATP-turnover during submaximal isometric contractions (Fig 5, paper *IV*). Since cross-bridges accounted for only ~20% of total ATP consumption this suggests that ion pumps are the major consumers of ATP under the conditions studied. Thus, BTS appeared to provide an appropriate model to study the role of mechanical load in contraction-mediated glucose transport.

The effect of BTS on glucose transport and metabolites

Muscles treated with BTS produced only ~5% of the initial force production compared to control muscles, which is less than what control muscles produced at the end of the stimulation protocol (Fig 1, *paper V*). Despite a marked force depression, BTS had little effect on contraction-mediated glucose transport (Fig 2, *paper V*). Furthermore, metabolites associated with energy consumption were measured. The decrease in glycogen during stimulation in the presence of BTS was ~ 30% less than the decrease

in control muscles. However, the changes in high-energy phosphates and lactate after the series of contraction were similar in both groups. This indicates that ATP turnover was decreased by ~30% during stimulation in the presence of BTS, which is consistent with our findings in *paper IV*. Contraction-mediated glucose transport was slightly decreased in the BTS treated muscles. This difference is probably due to the smaller ATP-turnover in BTS treated muscles. We also found that AMPK was activated and phosphorylated to the same extent in both groups (Fig 3, *paper V*). These findings suggest that rather than mechanical stress, other changes associated with repeated contractions (e.g. increased energy metabolism) play a key role in the activation of glucose transport during contraction. Thus, our results support the early results of Holloszy and Narahara (Holloszy & Narahara 1965). ROS have been shown to be important for contraction-mediated glucose transport (see *paper III*). Muscles treated with BTS showed similar changes in glutathione oxidation status during contraction (Fig 5, *paper V*), which is consistent with the findings that ROS are involved in contraction-mediated glucose transport.

In conclusion, mechanical load plays little role in contraction-mediated glucose transport in mouse fast-twitch muscle. Instead, it is likely that the increased glucose transport during contraction is a consequence of the accelerated Ca^{2+} release form SR, energy metabolism and ROS production.

GLYCOGEN SUPERCOMPENSATION (*PAPER II*)

Glycogen stores can be increased above basal after intense exercise, a phenomenon called glycogen supercompensation. Classically, glycogen supercompensation involves depletion of glycogen stores by exercise followed by rest and ingestion of high carbohydrate diet for several days. Glycogen resynthesis is a product of at least three mechanisms; glucose transport, glycogen synthesis and glycogen breakdown. In healthy humans glycogen supercompensation is always accompanied by insulin release (Maehlum *et al*. 1977). It has been shown that glycogen supercompensation can occur without insulin in skeletal muscle cultures (Mamedova *et al*. 2003) and also in isolated mouse skeletal muscle (Helander *et al*. 2002), however, the underlying mechanism has not been elucidated.

We stimulated the muscles to ~80% depletion of the muscle glycogen stores, as depletion of glycogen is required for glycogen supercompensation to occur (Bergstrom & Hultman 1966). Muscles were then incubated for up to 16 h in the presence of glucose. Glycogen reached maximum levels after 6 h of incubation and was then ~35% greater than the basal levels (Fig 2, *paper II*). Phosphorylase fractional activity (absence/presence of AMP), which was measured to represent the phosphorylated state of the enzyme, decreased right after stimulation and remained low during the first 4 h. It thereafter increased slightly but was still below basal levels after 6 h (Fig 3, *paper II*). Thus, phosphorylase was still lower than basal during the time of supercompensation,

presumably resulting in a low glycogen breakdown. These findings are in line with previous results on primary rat skeletal and cardiac muscle cultures (Mamedova *et al*. 2003; Vigoda *et al*. 2003).

Acid α-glycosidase is a lysosomal enzyme that breaks down glycogen. Lack of acid α-glycosidase results in an accumulation of glycogen in several tissues, including skeletal muscle (Hers , 1963). We measured both the acid and the neutral α-glycosidase and the results show that changes in α-glucosidases activity play no obvious role in glycogen supercompensation (Fig 5, *paper II*).

Furthermore, GS fractional activity (low/high concentrations of G6P) showed a marked increase after stimulation and continued to increase during the initial 30 min of recovery before returning to basal values and was at the time of supercompensation slightly below basal (Fig 4, *paper II*). These results are in agreement with previous studies showing that glycogen synthase activity is not increased at the time of supercompensation (Bergstrom *et al*. 1972; Conlee *et al*. 1978). Glycogen synthase is inhibited in the presence of high concentrations of glycogen, which could explain why the activity decreases when the glycogen levels are increasing (Danforth, 1965; Bergstrom *et al*. 1972).

Glucose transport was still slightly elevated after 4-6 hours of incubation, compared to basal. Thus, all three mechanisms could contribute to the glycogen supercompensation. However, the most important factor for glycogen supercompensation may be the inactivation of phosphorylase, which leads to a decreased rate of glycogen breakdown.

In conclusion, insulin-independent glycogen supercompensation is a result of a decreased glycogen breakdown and increased or constant glycogen synthesis driven by high substrate availability.

GLYCOGENOLYSIS (*PAPER I*)

The regulation of glycogen phosphorylase is still not completely understood as previously discussed. Our aim was to study phosphorylase regulation in mice lacking creatine kinase ($CK^{-/-}$). Mice lacking CK are unable to breakdown PCr. This results in only a negligible increase in P_i during contraction (Dahlstedt *et al*. 2000). Recent studies have shown that $CK^{-/-}$ muscles exhibit a higher rate of glycogenolysis compared to wild type (WT) during contraction despite the fact that they have almost no increase in P_i (van Deursen *et al*. 1994; Dahlstedt *et al*. 2000).

Glycogen phosphorylase is regulated by substrate availability (mainly inorganic phosphate, P_i), allosterically (by AMP) and by phosphorylation/dephosphorylation. It has previously been thought that the Ca^{2+} dependent conversion of phosphorylase **b** to phosphorylase **a** has been necessary for glycogen breakdown (Brostrom *et al*. 1971).

While earlier studies have argued the importance of increased P_i availability for glycogenolysis during contraction (Chasiotis *et al*. 1982), more recent studies have questioned the role of both interconversion and P_i availability (Ren & Hultman 1989; Katz, 1997).

$CK^{-/-}$ and WT muscles were stimulated for 20 seconds to ensure sufficiently large changes in glycogenolytic intermediates, a high and fairly linear glycogenolytic rate and large increases in phosphorylase fractional activity (absence/presence of AMP) (Staneloni & Piras 1969). All experiments were performed in the presence of NaCN to inhibit mitochondrial respiration (Sahlin & Katz 1986). Lactate was measured in the medium and there was no increase in lactate concentration during the 20 sec of stimulation. The increases in glycogenolytic intermediates and lactate in the muscle will therefore reflect the extent of glycogenolysis (Harris *et al*. 1981; Sahlin *et al*. 1989).

$CK^{-/-}$ muscles have a smaller concentration of PCr in the basal state but importantly they show no decrease of PCr during contraction owing to the lack of CK. The P_i concentration was higher in the basal state in $CK^{-/-}$ muscles compared to WT but there was little increase in P_i during stimulation (Table 2, *paper I*). $CK^{-/-}$ muscles have increased accumulation of G6P after contraction, which in addition to the similar lactate production indicates that $CK^{-/-}$ have an increased glycogen break down compared to WT (Table 2, *paper I*). $CK^{-/-}$ muscles fatigue faster and to a greater extent during the high-intensity 20 sec of repeated stimulation (Fig 3, *paper I*). When adjusting glycogenolysis for differences in force production, the glycogenolytic rate/force ratio in $CK^{-/-}$ muscles is more than twofold higher than in WT. This is in line with previous results on $CK^{-/-}$ during more prolonged stimulations (van Deursen *et al*. 1994). Thus, $CK^{-/-}$ muscles exhibit a higher glycogenolytic rate during contraction despite lower P_i availability.

Glycogen phosphorylase fractional activity increased in both $CK^{-/-}$ and WT muscles during exercise but the increase was blunted in the $CK^{-/-}$ muscles (Fig 1, *paper I*). This blunting occurred despite the fact that $CK^{-/-}$ muscles have a similar increase in myoplasmatic Ca^{2+} to WT and should therefore have the same extent of activation of phosphorylase **b** kinase. Moreover, $CK^{-/-}$ muscles had both a lower total phosphorylase activity and phosphorylase **a** activity during contraction compared to WT. Interconversion of the enzyme, therefore, cannot explain the increased glycogenolysis in the $CK^{-/-}$ muscles.

The third mode of phosphorylase activation is allosteric (ie, AMP). We therefore measured phosphorylase activity in the presence of increasing concentrations of AMP. In the presence of low concentrations of AMP (<100μM) a greater proportion of phosphorylase **b** was activated in $CK^{-/-}$ muscles both in the basal state and after

contraction (Fig 2, *paper I*). Thus, phosphorylase in $CK^{-/-}$ muscles has a higher affinity for AMP in both the basal state and after contraction.

In conclusion, all three mechanisms for glycogen phosphorylase activation were investigated in $CK^{-/-}$ muscles and the results indicate that neither interconversion nor substrate availability can be the cause for the increased glycogenolysis in the $CK^{-/-}$ muscles. Rather, we postulate that the AMP-mediated activation of phosphorylase **b** is the mechanism underlying the increased glycogen breakdown in $CK^{-/-}$ muscles.

CONCLUSION

Glucose transport- There are an increasing number of studies supporting the importance of ROS in cell signalling and regulation (Rhee *et al*. 2000; Goldstein *et al*. 2005; Hidalgo *et al*. 2006). We show that the increased production of ROS during contraction has a stimulatory effect on glucose transport and the addition of antioxidants decreases the contraction-mediated glucose transport. Our results also suggest that the reactive oxygen species H_2O_2 is specifically important for the increase in glucose transport. Thus, the traditional role of the "harmful" ROS production should be reconsidered and it is therefore also questionable if the intake of antioxidants is beneficial in healthy humans (Fig 3).

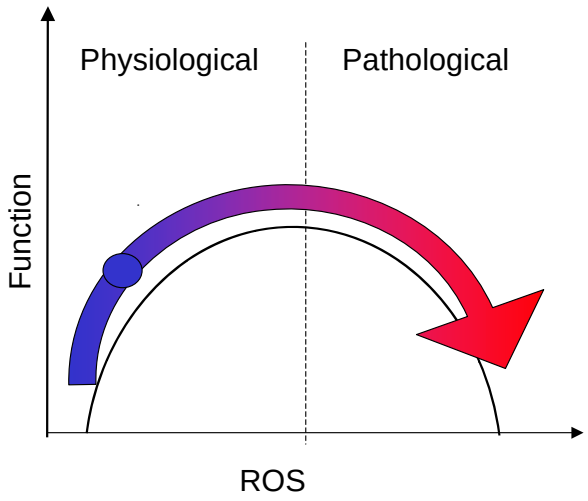

Figure 3. Scheme for the relationship between cellular function and the production of ROS. Blue bulge on left represents resting state (Andrade *et al.* 1998).

Furthermore, our results show that ROS increase AMPK activity in a presumably AMP/ATP independent manner. Another AMP/ATP independent pathway that has been suggested to activate AMPK is the Ca^{2+}-dependent activation of CaMK (Hardie *et al*. 2006). Thus, AMPK activation is much more complex than originally believed. Further studies to clarify the mechanisms involved in activation of AMPK are clearly warranted since AMPK is a potential target for drugs aimed at treating and preventing insulin resistance in human skeletal muscle.

By using a novel compound that specifically inhibits the cross-bridge force production, we show that the cross-bridges account for only a minor part of the energy consumption during submaximal contraction of mouse fast-twitch muscle. Thus, ion pumping, probably by the SR Ca^{2+} pumps, accounted for most of the ATP

consumption. Furthermore, we show that force production *per se* does not play a major role in glucose transport during contraction. Rather, the increased glucose transport during contraction is regulated by multiple signals that originate from increased Ca^{2+} release from the SR, energy turnover and ROS production (Fig 4).

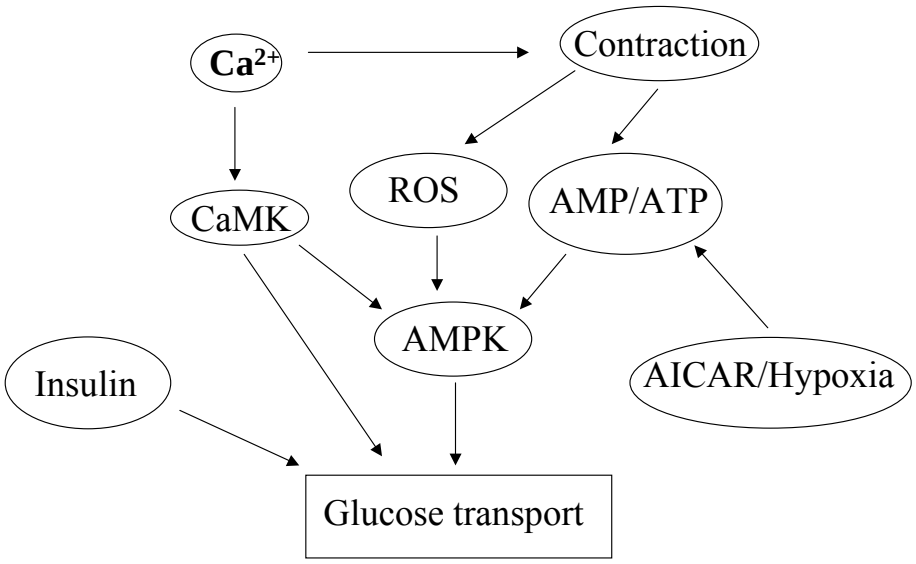

Figure 4. A schematic illustration of the proposed pathways in the regulation of glucose transport in skeletal muscle

Glycogen synthesis- We show that glycogen supercompensation can occur in skeletal muscles in the absence of insulin with the maximal glycogen level being ~35% above basal after 6 h of recovery. Glycogen synthase activity was maximally increased after 30 min of recovery, whereas it had decreased to the basal level at the time of the supercompensation. Although markedly lower than immediately after the series of contractions, glucose transport was still slightly elevated after 6 h of recovery, thus providing more substrate for glycogen resynthesis. Glycogen phosphorylase activity was still suppressed during the period of supercompensation, which suggests that decreased glycogen breakdown was the most important factor behind the supercompensation.

Patients with α-glucosidase deficiencies have an increased glycogen accumulation (Roach, 2002; Roth *et al*. 2003). We therefore investigated the possible link between altered α-glucosidase activity and glycogen supercompensation, but we could, however, not find any involvement of this enzyme.

Glycogenolysis- Animals deficient in creatine kinase have a higher rate of glycogen breakdown than wild type controls during contraction (van Deursen *et al*. 1994). Our

results show this can neither be attributed to the conversion of phosphorylase **b** to phosphorylase **a**, nor to an increase in P_i concentration. Instead we suggest that increased AMP-induced activation of phosphorylase **b** is primarily responsible for the accelerated glycogen breakdown $CK^{-/-}$ muscle. This is a consequence of the combined increases in the concentration of AMP at the enzymatic site during contraction (Lowry *et al*. 1964; Katz & Sahlin 1990) and in the affinity of phosphorylase **b** for AMP. Thus, the present study and a number of earlier studies (Danforth & Lyon 1964; Conlee *et al*. 1979; Katz, 1997) question the classical view of the regulation of glycogen phosphorylase activity; that is, conversion of phosphorylase **b** to **a** is clearly not the sole factor behind the contraction-mediated increase in glycogen breakdown. It is important to understand the regulation of glycogen phosphorylase, as it is a potential target for therapeutic intervention for type 2 diabetes as well as cardiac and cardiovascular diseases (Treadway *et al*. 2001; Oikonomakos, 2002).

CONCLUDING REMARKS

In this thesis, mechanisms of the regulation of glucose and glycogen metabolism were investigated. Despite the fact that this area has been intensively investigated for decades, the exact series of events regulating carbohydrate metabolism during exercise are still elusive. Moreover, thoroughly investigated and supposedly "understood" pathways still need further investigation. In this thesis, for example, we show that the originally established mechanisms for activation of AMPK and glycogen phosphorylase have to be modified. Philosophically one can argue that complete understanding of complex biological processes, such as glucose and glycogen metabolism, may never be obtained. However, this thesis has hopefully provided some additional understanding to the mechanisms underlying the regulation of carbohydrate metabolism during exercise.

To summarize, the major novel findings of this thesis are:

- Endogenously produced ROS play an important role in contraction-mediated glucose transport, probably, through an AMP/ATP independent activation of AMPK.
- Force production *per se* does not play a major role in contraction-mediated glucose transport.
- A major factor in the insulin-independent glycogen supercompensation is a decreased glycogen breakdown.
- Phosphorylase **b** activation by AMP could be an important regulatory mechanism for glycogen breakdown.

ACKNOWLEDGEMENTS

The work for this thesis was carried out at the Department of Physiology and Pharmacology, Karolinska Institute. It was supported by the Swedish National Center for Sports Research, Funds at the Karolinska Institute, Stiftelsen Lars Hiertas Minne, The Swedish Research Council, The Swedish Diabetes Foundation and NIH.

I wish to express my sincere appreciation to all those who have helped me in different ways to complete this thesis:

To my two supervisors: **Abram Katz** and **Håkan Westerblad** for your guidance throughout the work with my thesis, for always seeing positive results in any data and for your never ending discussions. ☺

To all the members of my group; you have all been a part of my work. Thanks to **Shi-Jin Zhang** for all the invaluable help in the lab; **Jan Lännergren** for your technical repair skills every time something 'broke', and for all the entertaining stories; **Joe Bruton** for your expertise and help with the confocal microscope; **Johanna Lanner**, **Jan Aydin** and **Daniel Andersson** for your friendship and for all the help, support and fun in the lab and in the office; and of course to **Maria Ahlsén**, our newest member and a good friend.

The years here at KI could not have been the same without all the wonderful PhD-students. A special thanks to: **Anna Z**, for always being high spirited and out-going, and for being so humble; to the remainder of my colleagues at the **Integrative Physiology** section who made the fourth floor a great place to work; my colleagues at the **Genetic Physiology** section- I loved hearing your laughter, its contagious!; **Lars K** and **Lena N**, "on the other side", for all your advice, the skiing, the partying and taking good care of me; **Stina J**, for great collaboration and friendship; **Sietske S**, **Ellinor K**, **Jessica N, Benita S**, **Oliver S**, **Lydia B, Robert F**, **Pierre R**, **Åsa K**, **Peter L**, **Marco P, Arianne M, Andre F**, & **Kathy Q** for all the parties, entertaining conversations and laughter throughout the years.

Louise B, thank you for your angel like patience, all your help with the teaching; introducing Wednesday and Friday 'fika', and for being such a great person; **Peter W**, for the help with the teaching and for always encouraging me; **Irene L** for all our academic, social, philosophical conversations and for always being so stubborn and positive.

I am also privileged with many wonderful friends outside KI. A special thanks to: **Patrik**, **Sebastian**, **Anders**, **Henrik, Peter** and **Martin**, my oldest friends and also the funniest people I know. You have made me laugh more times than I can count, please sing "Hos frisören" for me again. I also hope our highly philosophical discussions about the world will always continue. Their better halves, who all have wonderful big

hearts: **Frida**, **Kristin**, **Inga-Lill**, **Heidi,** and **Carolina**, who, in particular, has perpetually been teaching me about the concept of time, partying, and lampreys; you have become a great and valuable friend.

Linda, Maria, **Åsa**, **Åsa**, and **Therese**, for all your support, unforgettable trips, wonderful evenings, complaining e-mails and for being yourselves. I am looking forward to our future adventures; **Sara**, for being the most positive person I have ever had the pleasure of meeting; **Julia**, my oldest friend of all! A person I can always be myself with. Take care of Grevgatan for me and of course **Edward**.

Hanna L, through better and worse - through kidney pies and evenings with wine. You are a truly amazing person who has always been by my side, always listening and always giving me good advice. Please take care of yourself as much as you take care of others; **Linda M,** who has been with me through orange juice and laughter.

My family: Mamma **Lena**, Pappa **Göran**, **Fredrik** & **Terese** and the newest member of the family, lilla **Linnéa, Mormor**, **Farmor**, **Mats**, **Ragna**, **Andrea**, **Mathilde, Inger** & **Knud**. All of you - stort stort tack för att ni finns för mig. Thanks for your unconditional love and support. I hope you know how important you are to me and will always be wherever I am in the world.

Shahzad for all the love and support, for making me laugh, cry of happiness and see everything good in life. You have made my life into a treasure hunt! ♥

REFERENCES

Allen DG, Lannergren J & Westerblad H (1995). Muscle cell function during prolonged activity: cellular mechanisms of fatigue. *Exp Physiol* **80**, 497-527.

Andrade FH, Reid MB, Allen DG & Westerblad H (1998). Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol Lond* **509**, 565-575.

Baker AJ, Brandes R, Schendel TM, Trocha SD, Miller RG & Weiner MW (1994). Energy use by contractile and noncontractile processes in skeletal muscle estimated by 31P-NMR. *Am J Physiol* **266**, C825-C831.

Balon TW & Yerneni KK (2001). Redox regulation of skeletal muscle glucose transport. *Med Sci Sports Exerc* **33**, 382-385.

Bergstrom J & Hultman E (1966). Muscle glycogen synthesis after exercise: an enhancing factor localized to the muscle cells in man. *Nature* **210**, 309-310.

Bergstrom J & Hultman E (1967). A study of the glycogen metabolism during exercise in man. *Scand J Clin Lab Invest* **19**, 218-228.

Bergstrom J, Hultman E & Roch-Norlund AE (1972). Muscle glycogen synthetase in normal subjects. Basal values, effect of glycogen depletion by exercise and of a carbohydrate-rich diet following exercise. *Scand J Clin Lab Invest* **29**, 231-236.

Brau L, Ferreira LD, Nikolovski S, Raja G, Palmer TN & Fournier PA (1997). Regulation of glycogen synthase and phosphorylase during recovery from high-intensity exercise in the rat. *Biochem J* **322**, 303-308.

Brostrom CO, Hunkeler FL & Krebs EG (1971). The relation of skeletal muscle phosphorylase kinase by Ca^{2+}. *J Biol Chem* **246**, 1961-1967.

Browner MF & Fletterick RJ (1992). Phosphorylase: a biological transducer. *Trends Biochem Sci* **17**, 66-71.

Brozinick JT, Jr., Etgen GJ, Jr., Yaspelkis BB, III & Ivy JL (1994). The effects of muscle contraction and insulin on glucose-transporter translocation in rat skeletal muscle. *Biochem J* **297**, 539-545.

Bruton J, Pinniger GJ, Lannergren J & Westerblad H (2006). The effects of the myosin-II inhibitor N-benzyl-p-toluene sulphonamide on fatigue in mouse single intact toe muscle fibres. *Acta Physiol* **186**, 59-66.

Carling D, Zammit VA & Hardie DG (1987). A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *FEBS Lett* **223**, 217-222.

Cartee GD, Douen AG, Ramlal T, Klip A & Holloszy JO (1991). Stimulation of glucose transport in skeletal muscle by hypoxia. *J Appl Physiol* **70**, 1593-1600.

Cartee GD & Holloszy JO (1990). Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol* **258**, E390-E393.

Chasiotis D, Sahlin K & Hultman E (1982). Regulation of glycogenolysis in human muscle at rest and during exercise. *J Appl Physiol* **53**, 708-715.

Cheung A, Dantzig JA, Hollingworth S, Baylor SM, Goldman YE, Mitchison TJ & Straight AF (2002). A small-molecule inhibitor of skeletal muscle myosin II. *Nat Cell Biol* **4**, 83-88.

Coderre L, Kandror KV, Vallega G & Pilch PF (1995). Identification and characterization of an exercise-sensitive pool of glucose transporters in skeletal muscle. *J Biol Chem* **270**, 27584-27588.

Commoner B, Townsend J & Pake GE (1954). Free radicals in biological materials. *Nature* **174**, 689-691.

Conlee RK, Hickson RC, Winder WW, Hagberg JM & Holloszy JO (1978). Regulation of glycogen resynthesis in muscles of rats following exercise. *Am J Physiol* **235**, R145-R150.

Conlee RK, McLane JA, Rennie MJ, Winder WW & Holloszy JO (1979). Reversal of phosphorylase activation in muscle despite continued contractile activity. *Am J Physiol* **237**, R291-R296.

Corton JM, Gillespie JG, Hawley SA & Hardie DG (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem* **229**, 558-565.

Dahlstedt AJ, Katz A, Wieringa B & Westerblad H (2000). Is creatine kinase responsible for fatigue? Studies of isolated skeletal muscle deficient in creatine kinase. *FASEB J* **14**, 982-990.

Danforth W (1965). Glycogen synthetase activity in skeletal muscle. Interconversion of two forms and control of glycogen synthesis. *J Biol Chem* **240**, 588-593.

Danforth W & Lyon JB (1964). Glycogenolysis during tetanic contraction of isolated mouse muscle in the presence and absence of phosphorylase a. *J Biol Chem* **239**, 4047-4050.

DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J & Felber JP (1981). The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000-1007.

Derave W & Hespel P (1999). Role of adenosine in regulating glucose uptake during contractions and hypoxia in rat skeletal muscle. *J Physiol* **515**, 255-263.

Douen AG, Ramlal T, Rastogi S, Bilan PJ, Cartee GD, Vranic M, Holloszy JO & Klip A (1990). Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *J Biol Chem* **265**, 13427-13430.

Espinosa A, Leiva A, Pena M, Muller M, Debandi A, Hidalgo C, Carrasco MA & Jaimovich E (2006). Myotube depolarization generates reactive oxygen species through NAD(P)H oxidase; ROS-elicited Ca^{2+} stimulates ERK, CREB, early genes. *J Cell Physiol* **209**, 379-388.

Fiol CJ, Mahrenholz AM, Wang Y, Roeske RW & Roach PJ (1987). Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J Biol Chem* **262**, 14042-14048.

Fluckey JD, Ploug T & Galbo H (1999). Mechanisms associated with hypoxia- and contraction-mediated glucose transport in muscle are fibre-dependent. *Acta Physiol Scand* **167**, 83-87.

Fonseca VA (2005). The metabolic syndrome, hyperlipidemia, and insulin resistance. *Clin Cornerstone* **7**, 61-72.

Fujii N, Hirshman MF, Kane EM, Ho RC, Peter LE, Seifert MM & Goodyear LJ (2005). AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. *J Biol Chem* **280**, 39033-39041.

Garetto LP, Richter EA, Goodman MN & Ruderman NB (1984). Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am J Physiol* **246**, E471-E475.

Gibbons BJ, Roach PJ & Hurley TD (2002). Crystal structure of the autocatalytic initiator of glycogen biosynthesis, glycogenin. *J Mol Biol* **319**, 463-477.

Goldstein BJ, Mahadev K & Wu X (2005). Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets. *Diabetes* **54**, 311-321.

Goodyear LJ (2000). AMP-activated protein kinase: a critical signaling intermediary for exercise-stimulated glucose transport? *Exerc Sport Sci Rev* **28**, 113-116.

Goodyear LJ & Kahn BB (1998). Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* **49**, 235-261.

Hardie DG, Carling D & Carlson M (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* **67**, 821-855.

Hardie DG, Hawley SA & Scott JW (2006). AMP-activated protein kinase--development of the energy sensor concept. *J Physiol* **574**, 7-15.

Hardie DG & Sakamoto K (2006). AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* **21**, 48-60.

Harris RC, Hultman E & Sahlin K (1981). Glycolytic intermediates in human muscle after isometric contraction. *Pflugers Arch* **389**, 277-282.

Haschke RH, Heilmeyer LM, Jr., Meyer F & Fischer EH (1970). Control of phosphorylase activity in a muscle glycogen particle. 3. Regulation of phosphorylase phosphatase. *J Biol Chem* **245**, 6657-6663.

Heilmeyer LM, Jr., Meyer F, Haschke RH & Fischer EH (1970). Control of phosphorylase activity in a muscle glycogen particle. II. Activation by calcium. *J Biol Chem* **245**, 6649-6656.

Helander I, Westerblad H & Katz A (2002). Effects of glucose on contractile function, $[Ca^{2+}]i$, and glycogen in isolated mouse skeletal muscle. *Am J Physiol Cell Physiol* **282**, C1306-C1312.

Hermansen L, Hultman E & Saltin B (1967). Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand* **71**, 129-139.

Hers HG (1963). alpha-Glucosidase deficiency in generalized glycogenstorage disease (Pompe's disease). *Biochem J* **86**, 11-16.

Hidalgo C, Sanchez G, Barrientos G & Aracena-Parks P (2006). A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S -glutathionylation. *J Biol Chem* **281**, 26473-26482.

Holloszy JO & Hansen PA (1996). Regulation of glucose transport into skeletal muscle. *Rev Physiol Biochem Pharmacol* **128**, 99-193.

Holloszy JO & Narahara HT (1965). Studies of tissue permeability. X. Changes in permeability to 3-methylglucose associated with contraction of isolated frog muscle. *J Biol Chem* **240**, 3493-3500.

Holloszy JO & Narahara HT (1967). Enhanced permeability to sugar associated with muscle contraction. Studies of the role of Ca^{2+}. *J Gen Physiol* **50**, 551-562.

Ihlemann J, Ploug T, Hellsten Y & Galbo H (2000). Effect of stimulation frequency on contraction-induced glucose transport in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **279**, E862-E867.

Ji LL, Gomez-Cabrera MC & Vina J (2006). Exercise and hormesis: activation of cellular antioxidant signaling pathway. *Ann N Y Acad Sci* **1067**, 425-435.

Jiao Y, Shashkina E, Shashkin P, Hansson A & Katz A (1999). Manganese sulfate-dependent glycosylation of endogenous glycoproteins in human skeletal muscle is catalyzed by a nonglucose 6-P-dependent glycogen synthase and not glycogenin. *Biochim Biophys Acta* **1427**, 1-12.

Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA & Wojtaszewski JF (2004a). The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53**, 3074-3081.

Jorgensen SB, Richter EA & Wojtaszewski JF (2006). Role of AMPK in Skeletal Muscle Metabolic Regulation and Adaptation In Relation to Exercise. *J Physiol*.

Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA & Wojtaszewski JF (2004b). Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* **279**, 1070-1079.

Kahn BB, Alquier T, Carling D & Hardie DG (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**, 15-25.

Katz A (1997). Differential responses of glycogen synthase to ischaemia and ischaemic contraction in human skeletal muscle. *Exp Physiol* **82**, 203-211.

Katz A & Raz I (1995). Rapid activation of glycogen synthase and protein phosphatase in human skeletal muscle after isometric contraction requires an intact circulation. *Pflugers Arch* **431**, 259-265.

Katz A & Sahlin K (1990). Role of oxygen in regulation of glycolysis and lactate production in human skeletal muscle. *Exerc Sport Sci Rev* **18**, 1-28.

Long YC & Zierath JR (2006). AMP-activated protein kinase signaling in metabolic regulation. *J Clin Invest* **116**, 1776-1783.

Lowry OH & Passonneau JV (1972). *A flexible System Of Enzymatic Analysis* Academic Press, New York.

Lowry OH, SCHULZ DW & Passonneau JV (1964). Effects of adenolytic acid on the kinetic of phosphorylase a. *J Biol Chem* **239**, 1947-1953.

Maehlum S, Hostmark AT & Hermansen L (1977). Synthesis of muscle glycogen during recovery after prolonged severe exercise in diabetic and non-diabetic subjects. *Scand J Clin Lab Invest* **37**, 309-316.

Mamedova LK, Shneyvays V, Katz A & Shainberg A (2003). Mechanism of glycogen supercompensation in rat skeletal muscle cultures. *Mol Cell Biochem* **250**, 11-19.

Marette A, Richardson JM, Ramlal T, Balon TW, Vranic M, Pessin JE & Klip A (1992). Abundance, localization, and insulin-induced translocation of glucose transporters in red and white muscle. *Am J Physiol* **263**, C443-C452.

Markuns JF, Wojtaszewski JF & Goodyear LJ (1999). Insulin and exercise decrease glycogen synthase kinase-3 activity by different mechanisms in rat skeletal muscle. *J Biol Chem* **274**, 24896-24900.

Melendez R, Melendez-Hevia E & Canela EI (1999). The fractal structure of glycogen: A clever solution to optimize cell metabolism. *Biophys J* **77**, 1327-1332.

Merrill GF, Kurth EJ, Hardie DG & Winder WW (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* **273**, E1107-E1112.

Mu J, Brozinick JT, Jr., Valladares O, Bucan M & Birnbaum MJ (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* **7**, 1085-1094.

Mueckler M (1990). Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes* **39**, 6-11.

Murrant CL & Reid MB (2001). Detection of reactive oxygen and reactive nitrogen species in skeletal muscle. *Microsc Res Tech* **55**, 236-248.

Musi N & Goodyear LJ (2003). AMP-activated protein kinase and muscle glucose uptake. *Acta Physiol Scand* **178**, 337-345.

Oikonomakos NG (2002). Glycogen phosphorylase as a molecular target for type 2 diabetes therapy. *Curr Protein Pept Sci* **3**, 561-586.

Ojuka EO, Jones TE, Han DH, Chen M & Holloszy JO (2003). Raising Ca2+ in L6 myotubes mimics effects of exercise on mitochondrial biogenesis in muscle. *FASEB J* **17**, 675-681.

Ojuka EO, Jones TE, Han DH, Chen M, Wamhoff BR, Sturek M & Holloszy JO (2002). Intermittent increases in cytosolic Ca^{2+} stimulate mitochondrial biogenesis in muscle cells. *Am J Physiol Endocrinol Metab* **283**, E1040-E1045.

Pinniger GJ, Bruton JD, Westerblad H & Ranatunga KW (2005). Effects of a myosin-II inhibitor (N-benzyl-p-toluene sulphonamide, BTS) on contractile characteristics of intact fast-twitch mammalian muscle fibres. *J Muscle Res Cell Motil* **26**, 135-141.

Quintero M, Colombo SL, Godfrey A & Moncada S (2006). Mitochondria as signaling organelles in the vascular endothelium. *Proc Natl Acad Sci U S A* **103**, 5379-5384.

Rall JA (1982). Energetics of Ca^{2+} cycling during skeletal muscle contraction. *Fed Proc* **41**, 155-160.

Reid MB, Shoji T, Moody MR & Entman ML (1992). Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol* **73**, 1805-1809.

Ren JM & Hultman E (1989). Regulation of glycogenolysis in human skeletal muscle. *J Appl Physiol* **67**, 2243-2248.

Ren JM & Hultman E (1990). Regulation of phosphorylase a activity in human skeletal muscle. *J Appl Physiol* **69**, 919-923.

Rhee SG, Bae YS, Lee SR & Kwon J (2000). Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci STKE* **2000**, E1.

Richter EA, Garetto LP, Goodman MN & Ruderman NB (1984). Enhanced muscle glucose metabolism after exercise: modulation by local factors. *Am J Physiol* **246**, E476-E482.

Roach PJ (2002). Glycogen and its metabolism. *Curr Mol Med* **2**, 101-120.

Roach RJ & Larner J (1977). Covalent phosphorylation in the regulation glycogen synthase activity. *Mol Cell Biochem* **15**, 179-200.

Robergs RA, Pearson DR, Costill DL, Fink WJ, Pascoe DD, Benedict MA, Lambert CP & Zachweija JJ (1991). Muscle glycogenolysis during differing intensities of weight-resistance exercise. *J Appl Physiol* **70**, 1700-1706.

Rosell-Perez M, Villar-Palasi C & Larner J (1962). Studies on UDPG-glycogen transglucosylase. I. Preparation and differentiation of two activities of UDPG-glycogen transglucosylase from rat skeletal muscle. *Biochemistry* **1**, 763-768.

Roth J, Ziak M & Zuber C (2003). The role of glucosidase II and endomannosidase in glucose trimming of asparagine-linked oligosaccharides. *Biochimie* **85**, 287-294.

Ryder JW, Chibalin AV & Zierath JR (2001). Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. *Acta Physiol Scand* **171**, 249-257.

Sahlin K, Broberg S & Katz A (1989). Glucose formation in human skeletal muscle. Influence of glycogen content. *Biochem J* **258**, 911-913.

Sahlin K & Katz A (1986). The content of NADH in rat skeletal muscle at rest and after cyanide poisoning. *Biochem J* **239**, 245-248.

Sahlin K, Tonkonogi M & Soderlund K (1998). Energy supply and muscle fatigue in humans. *Acta Physiol Scand* **162**, 261-266.

Sakamoto K, Goransson O, Hardie DG & Alessi DR (2004). Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am J Physiol Endocrinol Metab* **287**, E310-E317.

Salafsky IS & Nadler HL (1973). A fluorometric assay of alpha-glucosidase and its application in the study of Pompe's disease. *J Lab Clin Med* **81**, 450-454.

Shashkin P, Koshkin A, Langley D, Ren JM, Westerblad H & Katz A (1995). Effects of CGS 9343B (a putative calmodulin antagonist) on isolated skeletal muscle. Dissociation of signaling pathways for insulin-mediated activation of glycogen synthase and hexose transport. *J Biol Chem* **270**, 25613-25618.

Silva JP, Shabalina IG, Dufour E, Petrovic N, Backlund EC, Hultenby K, Wibom R, Nedergaard J, Cannon B & Larsson NG (2005). SOD2 overexpression: enhanced mitochondrial tolerance but absence of effect on UCP activity. *EMBO J* **24**, 4061-4070.

Sim AT & Hardie DG (1988). The low activity of acetyl-CoA carboxylase in basal and glucagon-stimulated hepatocytes is due to phosphorylation by the AMP-activated protein kinase and not cyclic AMP-dependent protein kinase. *FEBS Lett* **233**, 294-298.

Staneloni R & Piras R (1969). Changes in glycogen synthetase and phosphorylase during muscular contraction. *Biochem Biophys Res Commun* **36**, 1032-1038.

Steeghs K, Benders A, Oerlemans F, de Haan A, Heerschap A, Ruitenbeek W, Jost C, van Deursen J, Perryman B, Pette D, Bruckwilder M, Koudijs J, Jap P, Veerkamp J & Wieringa B (1997). Altered Ca^{2+} responses in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. *Cell* **89**, 93-103.

Taylor SR & Rudel R (1970). Striated muscle fibers: inactivation of contraction induced by shortening. *Science* **167**, 882-884.

Toyoda T, Hayashi T, Miyamoto L, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Inoue G, Otaka A, Sato K, Fushiki T & Nakao K (2004). Possible involvement of the alpha1 isoform of 5'AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* **287**, E166-E173.

Treadway JL, Mendys P & Hoover DJ (2001). Glycogen phosphorylase inhibitors for treatment of type 2 diabetes mellitus. *Expert Opin Investig Drugs* **10**, 439-454.

van Deursen J, Ruitenbeek W, Heerschap A, Jap P, ter Laak H & Wieringa B (1994). Creatine kinase (CK) in skeletal muscle energy metabolism: a study of mouse mutants with graded reduction in muscle CK expression. *Proc Natl Acad Sci U S A* **91**, 9091-9095.

Vigoda A, Mamedova LK, Shneyvays V, Katz A & Shainberg A (2003). Glycogen metabolism in rat heart muscle cultures after hypoxia. *Mol Cell Biochem* **254**, 311-318.

Villar-Palasi C (1991). Substrate specific activation by glucose 6-phosphate of the dephosphorylation of muscle glycogen synthase. *Biochim Biophys Acta* **1095**, 261-267.

Westerblad H & Allen DG (2002). Recent advances in the understanding of skeletal muscle fatigue. *Curr Opin Rheumatol* **14**, 648-652.

Winder WW, Arogyasami J, Barton RJ, Elayan IM & Vehrs PR (1989). Muscle malonyl-CoA decreases during exercise. *J Appl Physiol* **67**, 2230-2233.

Winder WW & Hardie DG (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol* **270**, E299-E304.

Winder WW & Hardie DG (1999). AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* **277**, E1-10.

Wojtaszewski JF, Laustsen JL, Derave W & Richter EA (1998). Hypoxia and contractions do not utilize the same signaling mechanism in stimulating skeletal muscle glucose transport. *Biochim Biophys Acta* **1380**, 396-404.

Wojtaszewski JF, MacDonald C, Nielsen JN, Hellsten Y, Hardie DG, Kemp BE, Kiens B & Richter EA (2003). Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *Am J Physiol Endocrinol Metab* **284**, E813-E822.

Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA & Kiens B (2000). Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* **528**, 221-226.

Wojtaszewski JF, Nielsen P, Kiens B & Richter EA (2001). Regulation of glycogen synthase kinase-3 in human skeletal muscle: effects of food intake and bicycle exercise. *Diabetes* **50**, 265-269.

Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M & Carling D (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**, 2004-2008.

Wright DC, Hucker KA, Holloszy JO & Han DH (2004). Ca^{2+} and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* **53**, 330-335.