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**THE EFFECT OF THE K_{ATP} CHANNEL ON ENERGY
METABOLISM IN SKELETAL MUSCLE DURING
FATIGUE**

By

Maria Benkhalti

A thesis submitted to the Faculty of Graduate and Post-Doctoral Studies

of the University of Ottawa

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ABSTRACT

K_{ATP} channels are found on the sarcolemma where they affect muscle contractility and thus, energy demand. They are also found in the mitochondria where they affect ATP production. The overall objective was to determine how the K_{ATP} channel affects skeletal muscle metabolism during fatigue, elicited with one tetanic contraction every sec for 3 min. Exposing Kir6.2^{-/-} flexor digitorum brevis (FDB) muscles, which have no sarcolemmal K_{ATP} channels, to the K_{ATP} channel blocker, glibenclamide, or the opener, pinacidil, did not affect lactate production during the three min fatigue. However, DMSO, used to dissolve glibenclamide and pinacidil caused a decrease in lactate content during the last two min of fatigue, an effect not observed in its absence of DMSO. It is therefore possible that DMSO also influence how glibenclamide and pinacidil affect lactate content during the last two minutes of fatigue. Further studies were then restricted to a comparison between wild type and Kir6.2^{-/-} FDB. The amount of glucosyl units entering glycolysis, from glycogen breakdown and glucose uptake, was the same between wild type and Kir6.2^{-/-} FDB, while lactate production was much less in Kir6.2^{-/-} FDB following fatigue. It is suggested that the absence of K_{ATP} channel activity in Kir6.2^{-/-} FDB results in an increased oxidative capacity in which more pyruvate enters the Krebs cycle than in wild type FDB. This suggestion is supported by the fact that the oxygen uptake is greater in Kir6.2^{-/-} than wild type mice during a 24 hour period.

ABBREVIATIONS

ABS: ATP-binding site

ADP: adenosine diphosphate

AMP: adenosine monophosphate

AMPK: 5'-AMP-activated protein kinase

ANOVA: Analysis of variance

AP: action potential

ATP: adenosine-5'-triphosphate

CaMK: Ca²⁺-calmodulin-dependent protein kinase

CoA: coenzyme-A

DHPR: Dihydropyridine receptor

DMSO: dimethyl sulfoxide

EC coupling: excitation-contraction coupling

EDL: extensor digitorum longus

ETC: electron transport chain

FADH: flavin adenine dinucleotide

FDB: flexor digitorum brevis

FFA: free fatty acids

G-1-P: glucose-1-phosphate

G-6-P: glucose-6-phosphate

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GLUT: glucose transporter

IMP: inosine monophosphate
IMTG: intramyocellular triacylglycerol
 K_{ATP} channel: ATP-sensitive potassium channel
Kir: potassium inward rectifier
ml: milliliter
mM: millimolar
mV: millivolt
n: number of samples
NADH: nicotinamide adenine dinucleotide
NBD: nucleotide-binding domain
NBF: nucleotide-binding fold
°C: degrees Celsius
PCr: phosphocreatine
PGK: phosphoglycerate kinase
 pH_i : intracellular pH
 P_i : inorganic phosphate
PIP₂: phosphatidylinositol-4, 5-bisphosphate
RER: respiratory exchange ratio
RQ: respiratory quotient
RYR: Ryanodine receptor
S.E.: standard error
SR: sarcoplasmic reticulum
SUR: sulfonylurea receptor

TMD: transmembrane domain

v/v: volume/volume

Vo: maximal shortening velocity

VO₂: volume of oxygen inhaled

VCO₂: volume of carbon dioxide exhaled

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CHAPTER 1

INTRODUCTION

Skeletal muscles possess different pathways to ensure energy production matches energy demand during muscle contraction. However, during any exercise, energy demand eventually becomes greater than energy production. This could potentially lead to deleterious energy depletion, which then jeopardizes muscle integrity and function. Consequently, muscles have developed different protective mechanisms to prevent this from occurring. One of these is the ATP-sensitive potassium (K_{ATP}) channel. The channel got its name from the fact that the binding of ATP to the channel closes its pore.

It has been shown that a lack of K_{ATP} channel activity leads to muscle fiber damage both *in vivo* following swimming and treadmill running and during fatigue *in vitro* in single fibre (Kane et al, 2004; Thabet et al, 2005; Bourassa, 2006). The K_{ATP} channel prevents fibre damage via two different mechanisms. The first involves protecting against Ca^{2+} overload by preventing excessive membrane depolarization (Cifelli et al., 2008). This in turn prevents activation of Ca^{2+} -dependent proteases and increase in reactive oxygen species, two conditions known to result in fiber damage (Nethery et al., 2000).

The second mechanism is suggested to protect against deleterious energy depletion whereby the activation of the K_{ATP} channel results in decreased action potential amplitude, which in turn results in decreased Ca^{2+} release from the sarcoplasmic reticulum leading to decreased force production. These events decrease ATP utilization and thus decrease its depletion. One study found that the absence of K_{ATP} channel activity results in faster decrease but not greater extent of ATP depletion during 1 hour metabolic

inhibition (Gramolini & Renaud, 1997). Another study found no change in ATP levels in extensor digitorum brevis muscle in the presence or absence of K_{ATP} channel activity following fatigue, whereas in soleus the absence of K_{ATP} channel activity led to greater ATP depletion following fatigue (Matar et al., 2000). In a third study, Li (2007) obtained interesting results in that the genetic modulation, which eliminates sarcolemmal K_{ATP} channel activity, resulted in lower ATP and lactate whereas the pharmacological modulation, which has been found to modulate both sarcolemmal and mitochondrial K_{ATP} channels, resulted in higher ATP and lactate when compared to their respective controls. This could be indicative of the involvement of the mitochondrial K_{ATP} channel in metabolic changes. However, it is also possible that the solvent used to dissolve the pharmacological modulators, dimethyl sulfoxide (DMSO) is also responsible for the difference in outcome. Lastly, because the genetically modulated muscles had lower lactate levels than those pharmacologically modulated when compared to control, it is possible that a chronic lack of K_{ATP} channel activity results in increased oxidative capacity. Thus, the three objectives of this thesis were to determine how, during fatigue i) mitochondrial K_{ATP} channels affect lactate levels, ii) DMSO affects lactate levels, and iii) chronic lack of K_{ATP} channel activity leads to increase oxidative capacity.

MUSCLE CONTRACTION

MUSCLE CONTRACTION AND FIBRE TYPES

Muscle contractions are initiated when a motor neuron triggers an action potential (AP) at the neuromuscular junction. An AP is an electrochemical signal which consists of a depolarization phase generated by an influx of sodium (Na^+) through voltage-sensitive Na^+ channels (NaV) followed by a repolarization phase caused by the efflux of potassium

(K⁺) through voltage-sensitive K⁺ channels. The AP is then transmitted along the membrane and down the transverse tubular invagination (t-tubule), which is adjacent to the sarcoplasmic reticulum (SR) system where Ca²⁺ is stored (Bezanilla et al., 1972). The t-tubules contain voltage sensitive L-type Ca²⁺ channels (also named dihydropyridine receptors, DHPR, which are activated by AP. The DHPR are in turn associated with ryanodine receptors (RYR) on the sarcoplasmic reticulum (SR), involving a protein-protein interaction. The activation of the DHPR results in the activation of the ryanodine receptors (RYR) and the release of Ca²⁺ (Tanabe et al., 1990). At the sarcomere, which encompasses the contractile components actin and myosin, Ca²⁺ activates the contraction process allowing an interaction between myosin and actin where the chemical energy of ATP is transformed into mechanical energy, i.e. force and muscle shortening. At the end of the AP, Ca²⁺ is pumped back into the SR by Ca²⁺-ATPase pumps allowing for muscle relaxation (Allen et al., 2007). The speed at which these events occur correlates with the type of fibres composing each muscle.

Fibres were originally characterized as either slow (type I) or fast twitch (type II) according to their speed of contraction (Hamalainen et Pette, 1995). With the advent of new technologies, different isoforms were reported for many of the contractile components, including troponin, tropomyosin, myosin, and myosin light chain (Schachat, 1985; Moore, 1987). However, the isoform most commonly used to describe different fibre types is the myosin. Thus, skeletal muscles are said to be composed of fibre types I, IIA, IIX, and/or IIB according to the myosin isoform they contain (Schiaffino and Reggiani, 1996). The different fibre types correlate with different maximal shortening velocities (Vo) in the following order: I<IIA<IIX<IIB (Johnson et al., 1994; Pellegrino et

al, 2003). The different shortening velocities correlate with the maximal myosin ATPase activity. Different fibre types also possess different twitch durations whereby type II fibres have shorter twitches than type I. This duration is reflective of the faster myosin ATPase activity and greater capacity of Ca^{2+} ATPase pump to pump Ca^{2+} back into the SR (Essen et al, 1975). The slower type I and IIA fibres are most used in longer, endurance exercises, such as marathon running, or to maintain posture. On the other hand, the faster type IIX and IIB fibres can contract faster and are thus used in more intense and powerful exercise, such as weight lifting (Hamalainen and Pette, 1995).

ENERGY METABOLISM DURING MUSCULAR ACTIVITY

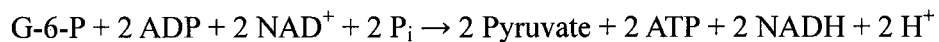
During contractile activity, energy is necessitated at three major sites. Firstly, the Na^+ - K^+ -ATPase restores Na^+ and K^+ gradients across the membrane by pumping three Na^+ molecules out and two K^+ molecules in. This is necessary for the generation of AP because the increase in intracellular Na^+ and extracellular K^+ , which occurs when several APs are generated, results in decreases in AP amplitude (Clausen, 1996). Secondly, the energy utilized by myosin ATPase converts chemical energy into mechanical work. Lastly, the Ca^{2+} -ATPase pump reuptakes Ca^{2+} back into the SR, which allows for relaxation following a contraction (Gibbs, 1987).

All ATPases create energy by hydrolyzing ATP to ADP and Pi. Furthermore, different ATPases consume different amounts of ATP. For instance, it is generally accepted that myosin ATPase consumes the most energy with values ranging from 50% to 80% of the total ATP used during muscular activity (Baker et al, 1994; Szentesi, 2001). Nonetheless, during intense exercise, ATP utilization can increase up to 100 fold (Fitts, 1994). Thus, muscles must have mechanisms that increase ATP production in response to increases in

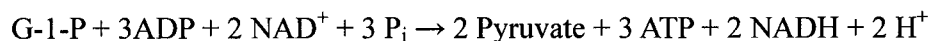
energy demand.

ENERGY SOURCES IN SKELETAL MUSCLE

There are several pathways used by myocytes to generate energy. First, phosphocreatine (PCr) is hydrolyzed and transfers its high energy phosphate to ADP to create ATP and creatine (Cr). Because this reaction only consists of one step, it can be used immediately upon the onset of exercise. However, this source is rather limited and only lasts up to 30 sec (de Feo, 2003; Widmaier et al, 2004). The second pathway is glycolysis, which has two possible substrates, extracellular glucose or glycogen. Extracellular glucose is uptaken into the myocyte by glucose transporters. While there are 12 different isoforms of glucose transporters, the two found in skeletal muscle are GLUT1 and GLUT4 (Gulve et al, 1994). GLUT4 is the transporter responsible for glucose uptake during exercise (Miki et al., 2002). At the onset of exercise, GLUT4 is translocated to the cell membrane allowing for glucose uptake (Wright et al., 2004). Once in the cytosol, glucose is rapidly phosphorylated to glucose-6-phosphate (G-6-P) and enters the glycolytic pathway. The glycolytic pathway converts glucose into pyruvate and produces ATP as per the following:



Glycogen is another source of glucose for glycolysis. It is broken down via a process called glycogenolysis, which utilizes the enzyme glycogen phosphorylase. The end-product, glucose-1-phosphate (G-1-P), then enters glycolysis to follow the following reaction:



Thus, aside from ATP, glycolysis yields two other products, NADH and pyruvate. Under

anaerobic conditions, pyruvate is converted to lactate. This produces more NAD^+ and ensures the driving forward of glycolysis. In the presence of oxygen, pyruvate enters the Krebs's cycle in the mitochondrial matrix where the third pathway begins. There, it combines with co-enzyme A to produce acetyl CoA. Acetyl CoA then enters the Krebs's cycle allowing for more ATP and NADH synthesis. At this point, all the NADH molecules from glycolysis and Krebs's cycle reduce the complexes of the electron transport chain located in the mitochondrial inner membrane. This results in a proton gradient in the mitochondrial inter-membrane space which will eventually lead to the production of more ATP molecules; this process is called oxidative phosphorylation. Under aerobic conditions, 36 moles of ATP per glucose molecule or 38 moles of ATP per glucosyl molecule (from glycogen) are produced (Widmaier et al, 2004). It is clear that ATP production is far greater under aerobic conditions than under anaerobic conditions.

Another energy production pathway utilizes free fatty acids. After CoA molecule is linked to a fatty acid chain, acetyl CoA is cleaved off; this process is termed β -oxidation. Acetyl CoA will then enter Krebs's cycle in the mitochondria and undergo oxidative phosphorylation, while another coenzyme-A molecule will attach onto the remaining fatty acid chain and repeat the cycle. Furthermore, the cleavage of acetyl CoA also generates one molecule of NADH and one molecule of FADH_2 , which will also enter the mitochondria and reduce the electron transport chain. Thus, fatty acids produce energy through the following equation:



where n represents the numbers of carbon in the FFA divided by 2.

This pathway is capable of generating much more energy compared to that from

carbohydrates. For instance, the catabolism of an 18-carbon fatty acid results in the production of 146 ATP molecules whereas the catabolism of a glucose molecule yields up to 36. Thus, when taking the molecular weight into account, one gram of fat would produce approximately 2.5 times the amount of ATP produced by one gram of glucose or glycogen (Widmaier et al, 2004).

Lastly, myocytes possess a way to produce ATP when its consumption exceeds its production, leading to increased ADP. Here, adenylate kinase uses two molecules of ADP and converts them into one ATP and one AMP molecule. AMP deaminase then further deaminates AMP, which results in inosine monophosphate (IMP). This drives adenylate kinase towards the production of ATP (Sahlin et al, 1990).

METABOLIC CHANGES DURING EXERCISE

Since ATP is the source of energy for myocytes, its levels would be expected to decrease during muscular activity. Furthermore, this decrease should become greater with increased exercise intensity. In fact, while it is generally accepted that resting ATP levels in skeletal muscle range between 27 and 30 mmol/kg dry weight (Spriet et al, 1995), changes during exercise vary according to the fibre types that make up the muscle. Indeed, muscles containing mainly type I and IIA fibres are slower but are used for longer periods of time. Therefore, they must also be able to produce energy for longer. That is why they are most reliant on aerobic energy metabolism and contain higher mitochondrial and myoglobin content. This allows for large generation of ATP. Consequently, the majority of studies show that these fibres experience slower decrease of ATP levels during exercise. For instance, Meyer et al. (1979) found that soleus stimulated at 120 trains/min of 250 ms each, ATP levels only decreased by 2 mmol/kg dry

weight. In contrast, type IIX and IIB fibres are faster and sustain muscle contraction for much shorter periods of time. This is because they have lower mitochondrial content and furthermore, the powerful exercises in which they are used partially block blood flow creating anaerobic conditions, which can produce much less ATP than aerobic conditions. Consequently, ATP decreases at a much greater rate. Indeed, Meyer et al. (1979) found that in gastrocnemius stimulated at only 60 trains/min at 100 ms each, ATP decreased by 12 mmol/kg dry weight. More recent studies follow the same trend with relatively small or no changes at all in slower fibres and faster decreases in faster ones (Matar et al., 2000; Szentesi et al., 2001).

Accordingly, ADP levels also increase, albeit much lower than expected. Indeed, Tullson et al. (1991) reported a 10-fold increase in ADP. However, changes in ADP are only in micromoles while the decrease in ATP falls in the millimoles range. The discrepancy in the changes in ATP versus ADP is explained by the fluctuations in AMP and IMP levels. In fact, while AMP levels do not change greatly, IMP content increases as such that it accounts for the decreases in total adenosine nucleotides (Chasiotic et al, 1987; Sahlin and Broberg, 1990).

Muscular activity also results in the accumulation of lactate. Once again, there are differences in accumulation depending on muscle type. Resting levels are always approximately 10 mmol/kg dry weight (Carvalho et al, 1996). Following exercise, lactate content increases by 3 to 4 fold in slow oxidative muscles (Meyer et al, 1979). In contrast, fast glycolytic muscles have an increase of as much as 25 fold (Meyer et al, 1979). Along with lactate increases, H^+ levels also rise; this results in a decrease in intracellular pH from 7.0 to 6.3 (Cady et al, 1989).

In addition to changes in metabolic by-products, changes in energy sources also occur. As previously mentioned, since PCr is the first energetic source, it is also the first one to be depleted. Tesch et al (1989) found resting fast twitch muscles to contain 82.7 mmol/kg dry weight. This represents 13% higher levels than slow twitch muscles, which had 73.1 mmol/kg dry weight. Following exercise, fast twitch muscles had a 69% decrease in PCr content whereas in slow twitch muscles it was only of 59%. In anticipation of PCr's rapid decrease, changes start occurring at the onset of muscular activity to ensure optimal glycolytic response. Thus, GLUT4-containing vesicles are translocated to the cell membrane, which increases glucose uptake into the cell (Wright et al, 2004; Kramer et al, 2007). *In vivo* experiments have found that following muscle contractions, glucose uptake increases 2 to 4 fold (Wright et al, 2004).

Glycogen is the main way by which glucose-derived energy is stored in skeletal myocytes. Resting levels range between 200 and 300 mmol/kg dry weight. Decreases in both slow and fast muscles are similar approaching 100 mmol/kg dry weight (Carvalho et al, 1996). Instead, it is the time to decrease which varies between muscles whereby fast glycolytic muscles have faster decreases in glycogen due to their increased dependence on this pathway as previously explained (Allen et al., 2008). Furthermore, decreases in glycogen are linear with the time to fatigue (Sahlin et al, 1998).

In conclusion, although exercise increases energy consumption, energy sources are used to maintain ATP levels. However, when energy demand becomes greater than energy production, a protective mechanism must exist against large ATP depletion. This mechanism is believed to be the "raison d'être" of muscle fatigue (McKenna & Renaud, 2008), which is observed when the capacity of a muscle to generate force or to do work is

diminished (Vollestad & Sejersted, 1988).

MECHANISMS FOR THE DECREASE IN FORCE DURING EXERCISE

Originally, decreases in force during exercise were thought to be due to metabolite changes acting at the level of the sarcomere. One of the most thorough studies was undertaken by Godt and Nosek (1989) and found that at 25°C, of all changes in metabolites occurring during exercise, the only ones that could explain the decrease in force were changes in pH and P_i . Increases in both metabolites caused decreases in Ca^{2+} sensitivity of the sarcomere and maximal force production in skeletal and cardiac muscles. However, these decreases could only account for a third of the decrease in force observed during fatigue. Moreover, when experiments were undertaken at physiological temperatures, changes in pH and P_i could no longer account for a decrease in force (Wiseman et al, 1996; Debold et al, 2006). Together, these studies and others show that the major causes for the decrease in force during fatigue must be upstream of the sarcomere.

It is now well accepted that fatigue is related to a decrease in Ca^{2+} release resulting in sub maximal levels for force production at the level of the sarcomere (Allen & Westerblad, 2001; Allen et al., 2002). However, the mechanisms leading to these events remain largely unknown. Studies at the present time are looking at the possibility of a decrease in membrane excitability as a cause for the lower Ca^{2+} release.

Changes in membrane excitability are mostly associated with changes in Na^+ and K^+ gradients. Repeated action potentials result in a net Na^+ influx and K^+ efflux. The Na^+/K^+ pump transports Na^+ out of the cell and K^+ back in to allow the maintenance of ionic gradients as well as ensure the maintenance of the membrane potential between -75 and -

85 mV. However, as exercise intensifies, the pump is not always able to maintain ionic gradients and the membrane potential may decrease to range between -65 and -70 mV (Balog and Fitts, 1996). Consequently, this leads to reduced membrane excitability. This in turn decreases AP amplitude resulting in decreased Ca^{2+} release, which translates into a failure to produce the expected force (Cairns et al, 2003). Mitochondrial function was also found to affect membrane excitability. When ATP production in mitochondria was inhibited while maintaining constant ATP levels, twitch force, elicited by electrical stimulation of the t-tubules, was depressed by up to 90%. The authors suggested that the decrease in force was related to a decrease in membrane excitability via a signaling pathway originating from the mitochondria (Ortenblad & Stephenson, 2002).

Furthermore, glucose and glycogen can also affect decreases in force. For instance, muscles that were allowed to recover from a first fatigue bout in the presence of glucose, had up to 20% slower decrease in force during the 2nd bout of fatigue. Conversely, muscles that recovered in the absence of glucose had a 40% faster decrease in force. Thus, the presence of glucose delays the decrease in tetanic force during fatigue (Helander et al., 2002). Chin & Allen (1997) also found that a decrease of glycogen to 27% of control resting levels resulted in faster decreases in Ca^{2+}_i and force. Thus, muscle fibres are capable of altering intracellular events and depress force to conserve energy. This means that there has to be some cellular component that can first sense a change in energy levels and then act to preserve them. The cell possesses several such components, one of which is the K_{ATP} channel.

ATP-SENSITIVE POTASSIUM CHANNEL

The K_{ATP} channel is a ligand-sensitive, voltage-insensitive potassium channel. It was

initially found that ATP inhibits the channel's activity via allosteric interaction, which is how the channel retained its name (Noma, 1983). Although it was originally discovered in cardiomyocytes, it is today known to be present in several different tissues throughout the body including skeletal and smooth muscle, pancreatic cells, neurons, kidneys, and mitochondria (Ashcroft, 1988).

MOLECULAR STRUCTURE OF THE K_{ATP} CHANNEL

The K_{ATP} channel is a hetero-octamer which consists of the pore-forming Kir subunits and regulatory sulfonylurea receptor (SUR) subunits (Seino, 1999, Fig. 1-1) in a 4:4 stoichiometric ratio (Clement et al, 1997).

Kir subunit

The Kir subunits of the K_{ATP} channel are members of the superfamily of inward rectifier potassium channel, comprising seven subfamilies, Kir1-7. The K_{ATP} channel is constituted of the Kir6.x subfamily. Three Kir genes have been cloned and sequenced to date, Kir6.1 and 6.2 in humans, rats, and mice (Inagaki, 1995; Ashcroft, 1988; Inagaki ^a, 1995), and Kir6.3 in zebra fish (Zhang et al, 2005). As opposed to the voltage-sensitive K^+ channel superfamily (Kv), which contains six putative transmembrane domains, Kir subunits consist of only two putative transmembrane domains, M1 and M2 (Figure 1-1, A). While most Kir channels possess a G-Y-G motif on the H5 external pore loop, which confers K^+ selectivity location, Kir 6.x contains a G-F-G motif. Kir 6.x subunits also possess an R-K-R motif on the C-terminal. This motif acts as a SR retention signal and prevents the trafficking of the Kir subunit in the absence of the SUR subunit (Zerangue et al, 2001). Kir 6.x only shares 40-50% amino acid identity with other Kir channels, while

Kir6.1 is mapped onto chromosome 12p11.23 whereas Kir6.2 is mapped onto chromosome 11p15.1. Consequently, Kir6.1 and Kir6.2 only share 71% amino acid identity (Seino, 1999).

SUR subunit

The second subunit completing the octamer is the sulfonylurea receptor (SUR). Its name was derived from the fact that anti-diabetic sulfonylurea drugs, such as tolbutamide and glibenclamide, bind to SUR and inhibit the channel (Sturgess et al., 1985). SUR belongs to the ATP-binding cassette (ABC) superfamily (Higgins, 1992), which also includes a variety of transmembrane transporters. As per the molecular structure of the ABC superfamily, the subunit consists of three putative transmembrane domains TMD0, TMD1, and TMD2. While TMD0 has five membrane spanning regions, TMD1 and TMD2 each contain six. Furthermore, two intracellular folds termed nucleotide binding folds, NBF1 and NBF2 (also called nucleotide binding domains (NBD), NBF1 being located between TMD1 and TMD2 and NBF2 on the C-terminal (Figure 1-1, A). Each NBF contains Walker A and B motifs, which are involved in the binding of ATP and ADP (Dean et al, 2001).

Two genes have been sequenced and encode for SUR1 and SUR 2. SUR1 is known to have high affinity for sulfonylureas whereas SUR2 has low affinity. In fact, SUR2 has up to 500 times lower affinity than SUR1 (Miki et al, 1999). Furthermore, there are several splice variants for SUR2, of which the most studied are SUR2A and SUR2B isoforms (Seino, 1999). The different isoforms of each subunit come together in different fashion across tissues. This enables them to adopt widely different electrophysiological properties leading to different roles and pharmacological responses in each tissue. The pancreatic β -

cell K_{ATP} channel contains SUR1 and Kir6.2 subunits (Inagaki et al, 1996), while in vascular smooth muscle it is composed of SUR2B and Kir6.1 and in non-vascular smooth muscle the SUR2B-Kir6.2 combination is formed (Yamada et al, 1997). In skeletal and cardiac muscles, the sarcolemmal K_{ATP} channel consists of 4 SUR2A and 4 Kir6.2 subunits (Inagaki et al, 1996).

It is more and more accepted that mitochondria also contain a K_{ATP} channel. However, they have not yet been genetically sequenced and their molecular nature remains controversial. For instance, Seharaseyon et al. (2000) found that both Kir6.1 and 6.2 were expressed in ventricular myocytes but could not determine the role it would play in the mitochondria. It was later found that PKC ϵ promotes the transport of Kir6.2 into the mitochondria under ischemic conditions. Furthermore, it was found that SUR2A may be present as well (Garg and Hu, 2007). Yet another study demonstrated that the mitochondrial K_{ATP} channel associates with succinate dehydrogenase forming a macromolecular complex (Ardehali et al, 2004). These are only a few examples to illustrate the complexity of this debate.

REGULATION OF THE SARCOLEMAL K_{ATP} CHANNEL ACTIVITY

As stated earlier, the K_{ATP} channel is ligand sensitive and is regulated by different metabolic changes. ATP was the initial metabolite found to regulate the channel (Noma, 1983). However, several other metabolites are now known to regulate the channel's activity. The regulation sites involve both the Kir and SUR subunit.

Regulation through the Kir subunit

It was found that the K_{ATP} channel can be regulated through its pore-forming Kir subunit

Figure 1-1

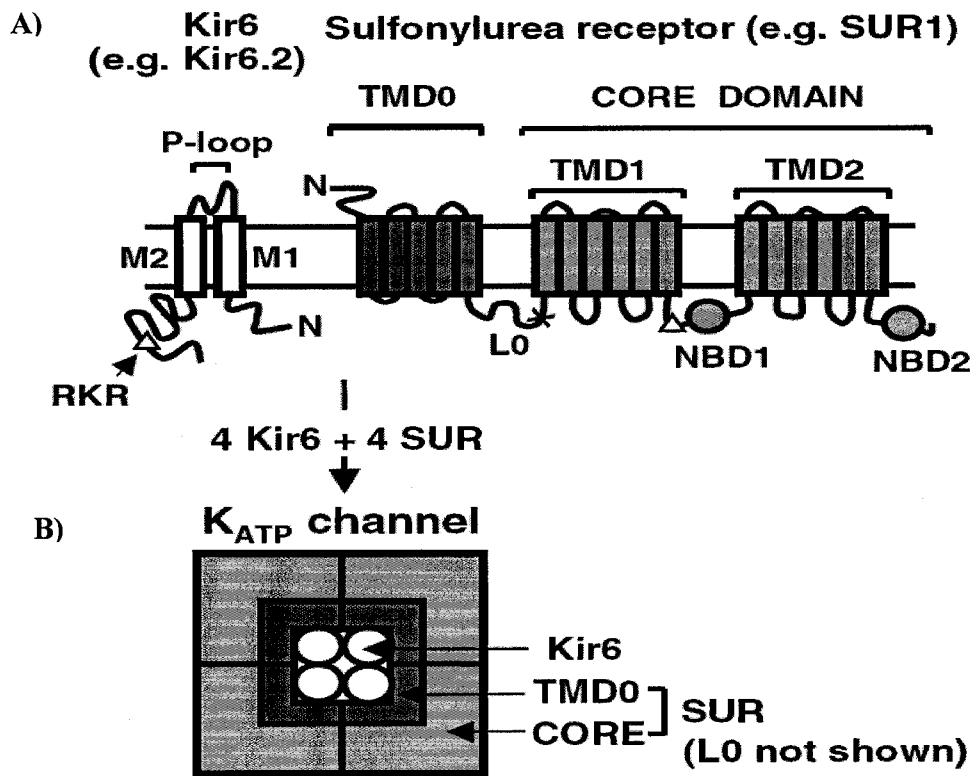


Figure 1-1 Molecular structure of the K_{ATP} channel. (a) Membrane topology of Kir and SUR subunits. Kir6.x consists of two transmembrane domains, M1 and M2 on each side of the selectivity filter formed by the P-loop. SUR consists of TMD0, which associated with the Kir subunit, and the core domain, which contains TMD1, TMD2 and both nucleotide binding domains. (b) Schematic representation of the K_{ATP} channel assembly. It is a hetero-octamer comprised of 4 pore-forming Kir subunits and 4 regulatory SUR subunits. (Fang et al, 2006)

when Tucker et al. (1997) were able to form a K_{ATP} channel in the absence of the SUR subunits following the truncation of the last 36 amino acids of the C-terminus. ATP allosterically inhibits the channel whereby the ATP binding site on Kir6.2 involves two subunits. The ATP phosphate tail interacts with two residues on the C terminus, Arg201 and Lys185, of one subunit and a third residue, Arg50, on the N terminal of a second subunit; the N6 atom of the adenine ring also interacts with the N-terminal of the second subunit. Thus, one ATP molecule can interact with each Kir subunit or 4 per K_{ATP} channel (Antcliff et al, 2005). Magnesium-free ADP is also capable of inhibiting K^+ flux through the channel but with much lower affinity (Markworth et al, 2000). Although ATP can regulate a Kir6.x subunit alone, the presence of a SUR subunit increases ATP affinity by nearly 10 fold (Tucker et al, 1997).

Other activating mechanisms exist which compete with the inhibitory effects of ATP. Phosphatidylinositol-4,5-bisphosphate (PIP_2) is capable of opening the channel through the Kir subunit. The anionic heads of PIP_2 interact with the positively charged amino acid residues Arg 54, Arg 176, Arg 177, and Arg 206 , which are located near the residues with which ATP interacts (Zhuo et al, 2004; Nichols, 2006). Furthermore, decreases in pH levels have been found to activate the K_{ATP} channel. When decreases in pH were mimicked either through the use of NH_3Cl or CO_2 the channel is activated (Davies et al, 1992; Xu et al, 2001). Not only does this occur with relatively high levels of ATP, but the presence of ATP is in fact necessary for H^+ to open the channel (Davies et al, 1992). Three amino acids have so far been shown to contribute to pH sensitivity, Thr-71 on the N terminus, and Cys-166 within the M2 region and His-175 on the C terminus (Piao et al, 2001). This is important because it demonstrates how the K_{ATP} channel can be activated

during exercise despite relatively small changes in ATP levels.

Regulation through the SUR subunit

Regulation through the SUR subunit involves Mg^{2+} nucleotides that lead to channel activation. This activation results from the binding of nucleotides on the NBF's. Furthermore, these NBF's contain two ATP-binding sites (Walker A and B motifs) onto which the binding of Mg^{2+} ADP is thought to be the preferential activation mechanism. In fact, it is thought that Mg^{2+} ATP is hydrolyzed to Mg^{2+} ADP by NBF2 which thus provides the energy necessary to overcome the inhibitory effect of ATP on the Kir subunit (Nichols, 2006). Mg^{2+} ADP can also directly bind to NBF2 without being hydrolyzed. Thus, a decrease in the ATP/ADP ratio results in the activation of the K_{ATP} channel. Conversely, an increase in the ratio will lead to channel closure (Ueda et al, 1999).

However, changes in ATP/ADP ratios to regulate K_{ATP} channel activity were mainly found under patch-clamp conditions. The relevance of this ratio under physiological conditions is somewhat different. As previously explained, even under stress, intramyocellular ADP levels remain rather low since it is eventually deaminated to IMP. Consequently, it cannot be expected that ADP levels be the sole mechanism of activation through the SUR subunit. It is also known that lactate levels between 20 and 40 mM can activate the channel at physiological conditions during fatigue. Furthermore, this effect is observed with ATP levels between 2 and 5 mM (Keung et al., 1991). However, it is not clear whether this effect occurs via the Kir or SUR subunits.

In conclusion, the K_{ATP} channel is activated following decreases in energy levels which are detected either by the decrease of ATP or by the increase of metabolic end-products including ADP, H^+ , and lactate. Keeping in mind that this ion channel can alter membrane

excitability, it can thus be said that the K_{ATP} channel links cellular energy state to membrane electrical activity.

PHYSIOLOGICAL ROLES OF THE SARCOLEMMA K_{ATP} CHANNEL

The K_{ATP} channel is expressed in many tissues with different functions which can, for the most part, be grouped into two main categories: glucose homeostasis and cytoprotection.

Glucose homeostasis

In the pancreas, K_{ATP} channel activity influences insulin secretion by β -cells and glucagon secretion by α -cells (Bokvist et al, 1999). At low plasma glucose levels, β -cell K_{ATP} channels are open (Miki et al, 1998). Following an increase in plasma glucose, glucose is transported into pancreatic β -cells by GLUT2 transporters. This occurs because the ATP/ADP ratio affects K_{ATP} channel activity in SUR1-containing channels present in pancreatic α - and β -cells and not in SUR2-containing channels present in skeletal muscles. As it is metabolized, the ATP to ADP ratio increases resulting in the closure of K_{ATP} channels. The subsequent decrease in K^+ efflux causes membrane depolarization, which in turn activates the L-type Ca^{2+} channels (Gopel et al, 1999). The influx of Ca^{2+} increases intracellular Ca^{2+} concentration which prompts the exocytosis of insulin-filled vesicles (Ashcroft et al, 2006). The role of the K_{ATP} channel was further confirmed in null mice for the Kir6.2 gene (Kir6.2^{-/-} mice), which fail to release insulin when exposed to elevated glucose concentrations (Miki et al., 1998). In pancreatic α -cells, K_{ATP} channels have been found to be electrically active at low glucose concentrations (Yoshimoto et al, 1999). However, their exact involvement in glucagon secretion remains to be elucidated. The K_{ATP} channel is also involved in glucose-sensing neurons. Located in the lateral,

arcuate, and ventromedial region of the hypothalamus, these neurons respond to extracellular fluctuations in glucose by altering their firing rate (Burdakov et al, 2005). There are two types of glucose-sensing neurons, those whose firing is inhibited by glucose and those whose firing is increased by glucose. The K_{ATP} channel is thought to play a role in the latter where an increase in firing rate results in an inhibition of glucagon from the pancreatic α -cells (Miki et al., 2001).

Lastly, the K_{ATP} channel indirectly modulates glucose uptake in skeletal muscles. When Chutkow et al. (2001) measured glucose uptake on soleus muscles from $Sur2^{-/-}$ mice in vitro, there was no difference at rest when compared to wild type muscles. However, $Sur2^{-/-}$ soleus had significantly higher uptake in the presence of 2000 μ U/ml of insulin. Similarly, Miki et al. (2001) also found increased glucose uptake in $Kir6.2^{-/-}$ soleus muscles albeit at a much lower insulin concentration of 100 μ U/ml. As well, $Kir6.2^{-/-}$ extensor digitorum longus (EDL) muscles also had increased glucose uptake although at much higher concentrations of 32 mM. Thus, although different experimental conditions lead to differences in results, it is clear that a lack of K_{ATP} channel results in increased glucose uptake via an unknown mechanism.

Myoprotection

Cytoprotection refers to the general prevention of cell damage and function impairment. In neurons, it is referred to as neuroprotection, whereas in muscles it is referred to as myoprotection. It has been shown that the K_{ATP} channel is important in myoprotection in both cardiac and skeletal muscle. For instance, Zingman et al. (2002) found that $Kir6.2^{-/-}$ mice have decreased exercise tolerance compared to wild type mice, which was due to cardiac arrhythmia. In skeletal muscles, Thabet et al. (2005) found that following

treadmill running, Kir6.2^{-/-} hind limb and diaphragm muscles had increased necrosis and collagen infiltration, which is indicative of fibre damage. They also found significantly more fibres with internal nuclei, which is indicative of fibre regeneration. These studies show that the K_{ATP} channel is crucial in preventing fibre damage.

Protection via vasodilation

The K_{ATP} channel is indirectly involved in myoprotection through its action within vascular smooth muscle. Its activation, resulting in vasodilation, allows for increased transport of oxygen, fatty acids, and glucose to exercising muscles. This phenomenon, known as reactive hyperaemia, ensures the capability of muscles to continue exercise without being energy depleted (Peter et al, 1996). However, this is not always possible. For instance, during short and intense exercise bouts, such as weight lifting, large muscle contractions obstruct optimal blood flow and thus prevent the transport of oxygen and energy sources. Furthermore, under certain circumstances, even increased blood flow is not sufficient. This can occur during prolonged exercise when the energy sources available and transported are not sufficient. In such instances, the K_{ATP} channels within striated muscles themselves are required to avoid damage. It is thought that this is mainly achieved by protecting against Ca²⁺ overload and against deleterious energy depletion.

Protection against Ca²⁺ overload

Ca²⁺ overload represents any uncontrolled increase in intracellular Ca²⁺ resulting from an imbalance between Ca²⁺ influx and Ca²⁺ pumping into the SR. Ca²⁺ overload leads to cellular damage because of the activation of Ca²⁺-dependent proteases and increase in reactive oxygen species (ROS) (Nethery et al, 2000). There is evidence that the absence

of K_{ATP} channel activity leads to increased membrane depolarization either during metabolic inhibition or fatigue (Gramolini & Renaud, 1997; Matar et al., 2000). It has also been found that a lack of K_{ATP} channel activity leads to increased intracellular Ca^{2+} and contractile dysfunction such as the development of unstimulated force, which can be eliminated by exposure of the muscle to the L-type Ca^{2+} channel blocker, verapamil (Cifelli et al., 2007; Cifelli et al., 2008). Thus, the K_{ATP} channel was suggested to protect against Ca^{2+} overload. This suggestion was reinforced when Cifelli et al. (2008) observed that decreasing extracellular Ca^{2+} in such muscles also decreased contractile dysfunctions and fibre damage.

Protection against energy depletion

Since decreases in ATP levels lead to the activation of the K_{ATP} channel, it was suggested that the channel protects energy metabolism (Noma, 1983). This occurs since activation of the channel decreases action potential duration in cardiac muscle and amplitude in skeletal muscle. This in turn leads to decreased Ca^{2+} release from the SR during contraction resulting in decreased force. It has been suggested these events are important because, less ATP is needed by myosin ATPase for cross-bridge cycle repetition and by the Ca^{2+} -ATPase pump for Ca^{2+} reuptake into the SR.

When cardiomyocytes were exposed to the K_{ATP} channel opener pinacidil following ischemia, ATP and PCr were preserved. Furthermore, the channel blocker, glibenclamide, not only eliminated the effects of pinacidil but also resulted in increased depletion of ATP (McPherson et al., 1993). In skeletal muscle, decreases in ATP and PCr following metabolic inhibition were faster in the presence of glibenclamide in frog sartorius muscle (Gramolini & Renaud, 1997). In mammalian muscle, Matar et al. (2000) found that

following fatigue, glibenclamide resulted in 8.1 $\mu\text{moles/ g}$ dry weight greater decrease in ATP levels in soleus but not in EDL muscles. Similarly, pinacidil only affected the soleus muscle whereby ATP increased by 1.6 $\mu\text{moles/ g}$ dry weight following fatigue whereas it decreased by 3.8 $\mu\text{moles/ g}$ dry weight in control muscle. The reason for the discrepancy between both muscles may be because, as observed by Gramolini & Renaud (1997), the K_{ATP} channel may affect the rate rather than the final extent of ATP and PCr in EDL muscle. Furthermore, Matar et al. suggested that the differences in ATP levels between EDL and soleus could be due to i) the difference in mitochondrial content and ii) the fact that pinacidil and glibenclamide were found to affect both sarcolemmal and mitochondrial K_{ATP} channels under certain conditions (Gribble & Reimann, 2001; Ardehali & O'Rourke, 2005).

Consequently, Li (2007) undertook to determine how PCr, ATP and lactate levels change over time during fatigue while K_{ATP} channel activity was modulated using both a pharmacological approach, using glibenclamide and pinacidil, and a genetic approach, using the flexor digitorum brevis (FDB) from $\text{Kir6.2}^{-/-}$ mice. $\text{Kir6.2}^{-/-}$ FDB had faster rates but similar final extent of decrease in ATP as previously observed in frog sartorius and EDL. However, glibenclamide did not affect ATP levels at any time during fatigue in wild type FDB. Muscles exposed to pinacidil had a slower initial rate of decrease of ATP during the first 60 sec but a subsequent faster rate, which resulted in similar final ATP levels. Lactate accumulation was much less in $\text{Kir6.2}^{-/-}$ than in wild type FDB at all times of fatigue. However, the presence of glibenclamide had no effect on lactate content during the first 60 sec of fatigue and resulted in higher levels during the last 120 sec of fatigue. This was surprising since it was the first time that glibenclamide wild type and

Kir6.2^{-/-} muscles did not result in the same outcome. The difference may be linked to the fact that while Kir6.2^{-/-} FDB only lacks functional sarcolemmal K_{ATP} channels, glibenclamide and pinacidil modulate both sarcolemmal and mitochondrial K_{ATP} channels (Ardehali & O'Rourke, 2005). It is therefore important to study whether pinacidil and glibenclamide affect metabolite levels via the mitochondrial K_{ATP} channel.

Another possibility could be an effect of dimethyl sulfoxide (DMSO), the solvent used to dissolve pinacidil and glibenclamide. Normally, DMSO is included in all control conditions, and at 0.1% it has no effect on force kinetics. However, while Li used 0.1% DMSO in her pharmacological modulation of K_{ATP} channel activity in wild type muscles, she did not include the solvent when she compared wild type and Kir6.2^{-/-} muscles. She then observed that FDB lactate level increases during the last two min of fatigue in the absence of DMSO while it decreases in the presence of DMSO (Li, 2007). At higher concentrations (0.3-10%), DMSO has been shown to affect force production and action potential duration in ventricular myocytes (Ogura et al, 1995; Ogura et al., 1996). It is thus possible that although at 0.1% DMSO does not affect contractile properties, it may affect how metabolites change during fatigue. Therefore, it is possible that the effect of glibenclamide and pinacidil are in fact the result of the effect of DMSO.

OBJECTIVES AND HYPOTHESES

The objective of this study was to test the following three hypotheses:

1. Pinacidil and glibenclamide affect lactate production by modulating mitochondrial K_{ATP} channel activity. To test this hypothesis, lactate was measured during fatigue in Kir6.2^{-/-} FDB, which has no sarcolemmal K_{ATP} channel activity, in the presence of either pinacidil or glibenclamide.

2. DMSO does not affect lactate content during fatigue. This was tested by fatiguing wild type FDB in the absence and presence of DMSO.
3. Kir6.2^{-/-} FDB has more glucosyl entering glycolysis than wild type FDB. This was tested by measuring the amount of glucose uptake and glycogen content during fatigue.

CHAPTER 2

METHODS AND MATERIALS

ANIMALS AND MUSCLE PREPARATIONS

Two to three months old C57-B16 and Kir6.2^{-/-} mice were used for all experiments. The C57-B16 mice were used as wild type mice while Kir6.2^{-/-} mice were null mice for the Kir6.2 gene. Kir6.2^{-/-} muscles have no K_{ATP} channel activity in the cell membrane of skeletal muscle (Miki et al., 1998). The genetic background between the two mice was mixed over 4 generations. All mice were fed ad libitum and housed according to the guidelines of the Canadian Council for Animal Care. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa.

Mice were anaesthetized with a single intraperitoneal injection of 2.5 mg ketamine, 0.5 mg xylazine, and 0.25 mg acepromazine per 10 g of body weight. The fibres controlling the 4th digit of the flexor digitorum brevis (FDB) muscles were separated by cutting along the fascia between the 3rd and 4th digit as described by Cifelli (2007).

SOLUTIONS

FDB bundles were immersed in acrylic cuvettes measuring 1 X 1 X 4.5 cm (width, length, height) filled with 2 mL of physiological saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 CaCl₂, 3.1 MgCl₂, 25 Na₂HCO₃, 2 NaH₂PO₄ and 5.5 D-glucose. The solution was constantly bubbled with 95% O₂ - 5% CO₂ and had a pH of 7.4. All experiments were carried out at 37°C.

Solutions containing either 100 µM pinacidil or 10 µM glibenclamide were prepared by first dissolving the drugs in DMSO before it was added to the physiological saline

solution with a DMSO concentration of 0.1% (v/v). Control solutions also contained DMSO only in experiments in which the effects of glibenclamide and pinacidil were studied.

FORCE MEASUREMENT

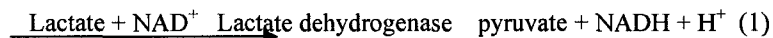
Muscles were attached with silk (Black Braided Silk Suture, 6-0, USA) at one end to a force transducer (Grass Instrument, Model FT03C, USA), and the other end to a fixed hook. The transducer was connected to a Grass Physiograph (Grass Instrument, Model 79E, USA). Force measurements were divided into three types: peak tetanic force, unstimulated force, and peak total force (Figure 3-1). Peak tetanic force was defined as the force generated upon stimulation. Unstimulated force occurred when muscles failed to relax after a contraction and was defined as the difference in force between two contractions during fatigue and the resting force before fatigue.

STIMULATION AND FATIGUE PROTOCOL

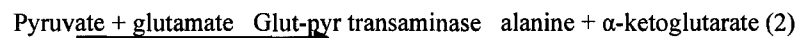
Muscles were stimulated by passing a current between parallel platinum wires located on both sides of the muscle. Stimulations were generated using a Grass S88 stimulator (USA) and Grass isolation unit (SIU5, USA). Muscles were first set at optimal length for maximum tetanic force. They were then allowed to equilibrate for 30 min in control conditions or in the presence of glibenclamide or pinacidil. During that time muscles were elicited to contract every 100 s with 200 ms train of 0.3 ms, 8 V pulses at 200 Hz. Fatigue was elicited by increasing the frequency of contractions to one every second for 3 min. Muscles were then freeze-clamped in liquid nitrogen at different times during fatigue and stored at -80°C , until used for metabolite determinations.

LACTATE MEASUREMENT

Muscles were lyophilized with a freeze drier (Freezemobile, 6, Virtis, USA). Dried muscle fibres were separated from tendons at room temperature and weighed on an analytical balance (Mettler Toledo, XS105, USA). Lactate was extracted by adding 500 μ l of ice cold 6% perchloric acid per 0.1-0.2 mg of dry muscles. After sonification (sonic Dismembrator, model 100, Fisher Scientific) at maximum power for 15 s, solutions were centrifuged during 30 min at 20,000 g and 4°C. Supernatants were neutralized with ice cold 3 M K_2CO_3 and the K^+ salt was centrifuged (International equipment company, IEC, Micromax 3590F2282, USA) 15 min at 10,000 g and 4°C. Lactate concentration was determined enzymatically as described by Passoneau and Lowry (1983)., Briefly, lactate was converted into pyruvate as follows:



Pyruvate was then converted to alanine as follows:



NADH is a fluorescent compound and the amount produced was measured using a Perkin Elmer fluorometer (Model LS50B, USA) and lactate content was determined using a standard curve

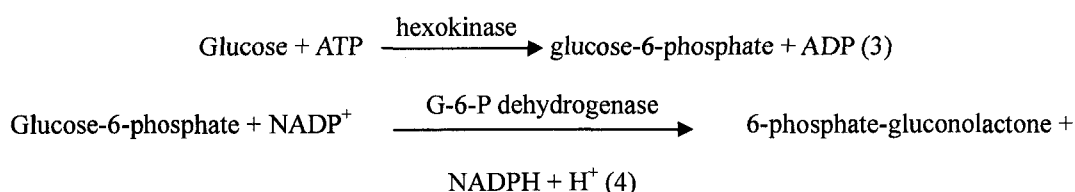
GLUCOSE UPTAKE MEASUREMENT

Physiological saline solution containing 2 μ Ci/mL ^3H - 2-deoxyglucose (^3H -2DG) (glucose analog), in the presence of either 5.5 mM 2DG or endogenous glucose, and 0.9 μ Ci/mL ^{14}C -sucrose (extracellular marker) was added before equilibrium. In some experiments, 2DG and sucrose were washed-out in glucose-free 4°C physiological solution every min for 20 or 60 min. In other experiments, muscles were quickly covered

in Kim wipe and freeze-clamped without a wash-out. In all experiments, ^3H -2DG and ^{14}C -sucrose were extracted from muscles using the same protocol as for lactate except that PCA supernatants were not neutralized with K_2CO_3 . 500 μl of the PCA supernatant was added to 10 mL of Biodegradable Counting Scintillant to count the ^3H and ^{14}C using a Wallac (Fisher Scientific, model 1414) scintillation counter.

GLYCOGEN CONTENT MEASUREMENT

Muscles were lyophilized and powdered and connective tissue was removed as described above. Muscle glucose was degraded by adding 1 N NaOH (v/v) and incubated at 80°C for 15 min. Three times more H_2O was then added along with 0.25 mol/L HCl and 0.15 mol/L Na acetate buffer. Glycogen was hydrolyzed to glucose by adding 1.5 g/L amyloglucosidase and incubating at 30°C for 4 hours. Glucose content was then determined enzymatically as described by Passoneau and Lowry (1983). Briefly, glucose was first converted to glucose-6-phosphate using hexokinase and the latter was converted to 6-phosphate-gluconolactone using glucose-6-phosphate dehydrogenase as follows:



Changes in NADPH fluorescence were then measured using a Perkin Elmer fluorometer (Model LS50B, USA) and the glucose content was obtained from a standard curve.

INDIRECT CALORIMETRY MEASUREMENTS

Whole-body oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) were measured for a 24 hour period using a four chamber Oxymax v5.11 system (Columbus

Instruments, Ohio); each chamber having a volume of 2.5 L and an airflow of 0.5 L/min. The temperature was maintained at 24°C and light was present from 7:00 to 19:00. Mice were fed ad libitum and the system had a measurement time of 1 min, a settle time of 2 min.

STATISTICAL ANALYSIS

Values are given as Mean \pm standard error (S.E) with the number of samples (n). Split plot Analysis of Variance (ANOVA) designs were used to determine significant differences; the treatment being i) drug or mice treatment in the whole plot because muscles were from different mice and ii) time treatment was in the split plot since all measurements were from the same muscle. ANOVA calculations were made using the Version 9.0 GLM (General Linear Model) procedures of the Statistical Analysis Software (SAS Institute Inc., Cary, NC USA). When a main effect or an interaction was significant, the least square difference (L.S.D) was used to locate significant differences (Steel and Torrie, 1980). The word “significant” refers only to a statistical difference ($P < 0.05$).

Chapter 3

RESULTS

EFFECT OF PINACIDIL AND GLIBENCLAMIDE ON MITO K_{ATP} CHANNEL

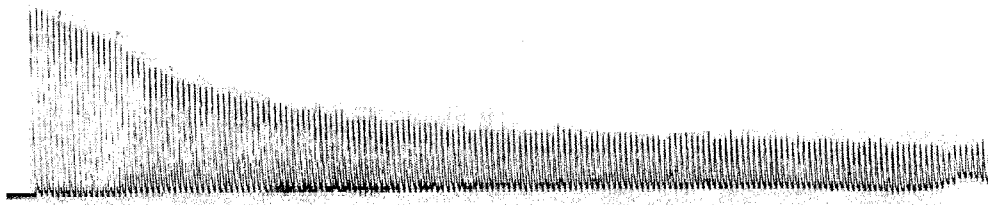
The first aim of the study was to determine whether the sarcolemmal K_{ATP} (sarco K_{ATP}) channel activator, pinacidil, and blocker, glibenclamide, alter lactate levels during fatigue by also modulating mitochondrial K_{ATP} (mito K_{ATP}) channel activity. This was tested by exposing Kir6.2^{-/-} FDB, which lack any functional sarco K_{ATP} channel, to glibenclamide and pinacidil and measure lactate content. An effect of pinacidil or glibenclamide would then indicate a possible modulation of the mito K_{ATP} channels on lactate production.

FATIGUE KINETICS

Figure 3-1B depicts an example of the force trace generated from Kir6.2^{-/-} FDB during fatigue, which was elicited by one contraction every second for 3 min. Figure 3-2 A shows, on an average basis, that peak tetanic force in Kir6.2^{-/-} control muscles decreased to 60% of pre-fatigue force during the first 20 sec of fatigue. By 40 sec, force had declined 30%, reaching 22% by 60. Thereafter, it decreased very little being 20% by 180 sec. When muscles were exposed to either glibenclamide or pinacidil, peak tetanic force decreased in similar fashion with no statistically significant difference between all three conditions. One major feature of the fatigue kinetics in Kir6.2^{-/-} FDB is the large increase in unstimulated force (Figure 3-1B), which is due to excessive Ca^{2+} influx via L-type Ca^{2+} channels (Cifelli et al., 2008). Under control conditions, unstimulated force, reached a mean peak of 25% at 30

Figure 3-1

A) Wild type



B) Kir 6.2^{-/-}

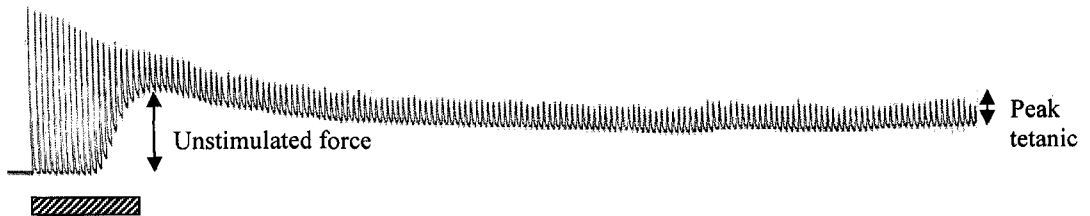


Figure 3-1 Recordings of tetanic contractions from A) wild type and B) Kir6.2^{-/-} FDB muscle bundles. Fatigue was elicited with contractions every second during 3 minutes. Measurements of peak tetanic force and unstimulated force are shown in B. Horizontal hatched bar represents 20 seconds.

Figure 3-2

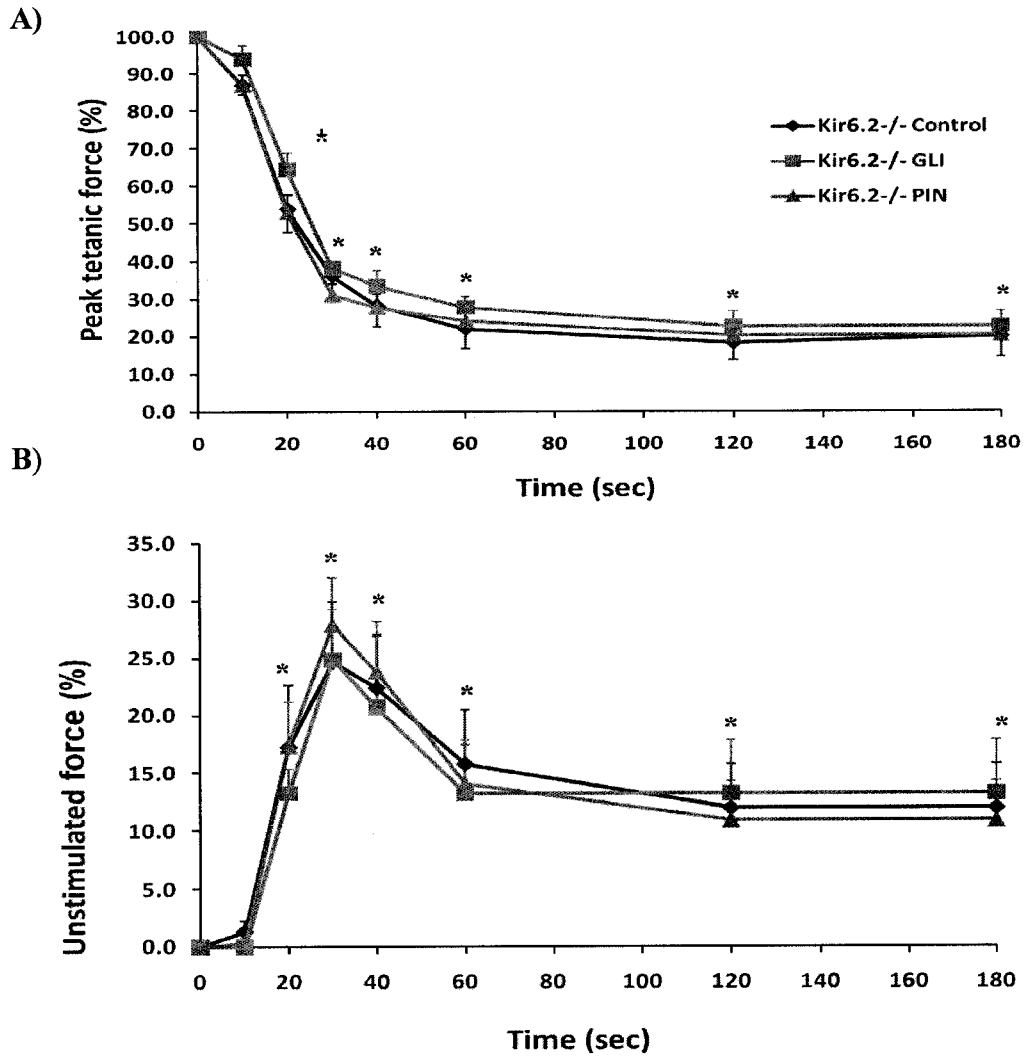


Figure 3-2 Glibenclamide and pinacidil had no effect on A) peak tetanic force and B) unstimulated force during fatigue in Kir6.2^{-/-} FDB. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was of 37°C. Vertical bars represent S.E. for 6 muscles.

*Significantly different from time 0, ANOVA and L.S.D., P<0.05.

sec and then decreased to 13% by 60 sec remaining constant thereafter (Figure 3-2B). Glibenclamide and pinacidil had no effect on unstimulated force.

LACTATE CONCENTRATION

Before fatigue, control Kir6.2^{-/-} FDB muscles contained 4 μmoles/g of dry weight of lactate (Figure 3-3). Lactate levels increased steadily during fatigue peaking at 59 μmoles/g of dry weight by 60 sec. During the last 120 sec of fatigue, lactate levels decreased reaching 26 μmoles/g of dry weight by 180 sec. When muscles were exposed to glibenclamide, lactate levels started higher than control at 24 μmoles/g of dry weight; however, the difference was not statistically significant. By 60 sec, levels were comparable to those of control conditions and remained so during the rest of fatigue. When exposed to pinacidil, muscles contained similar amounts of lactate as control conditions at all time points.

EFFECT OF DMSO ON WILD TYPE MUSCLES

The second aim of this study was to determine whether the solvent DMSO has an effect on muscle contractility and lactate production in wild type FDB.

FATIGUE KINETICS

A typical fatigue trace is shown on Figure 3-1A and the average changes in peak tetanic force and unstimulated force are shown in Figure 3-4A. Peak tetanic force, under control conditions, decreased to 43% of pre-fatigue levels after only 30 sec. By 60 sec, peak tetanic force had reached 27% and continued to decrease slightly to 22% by the end of fatigue. The difference between the two conditions was the highest between 30 and 60

Figure 3-3

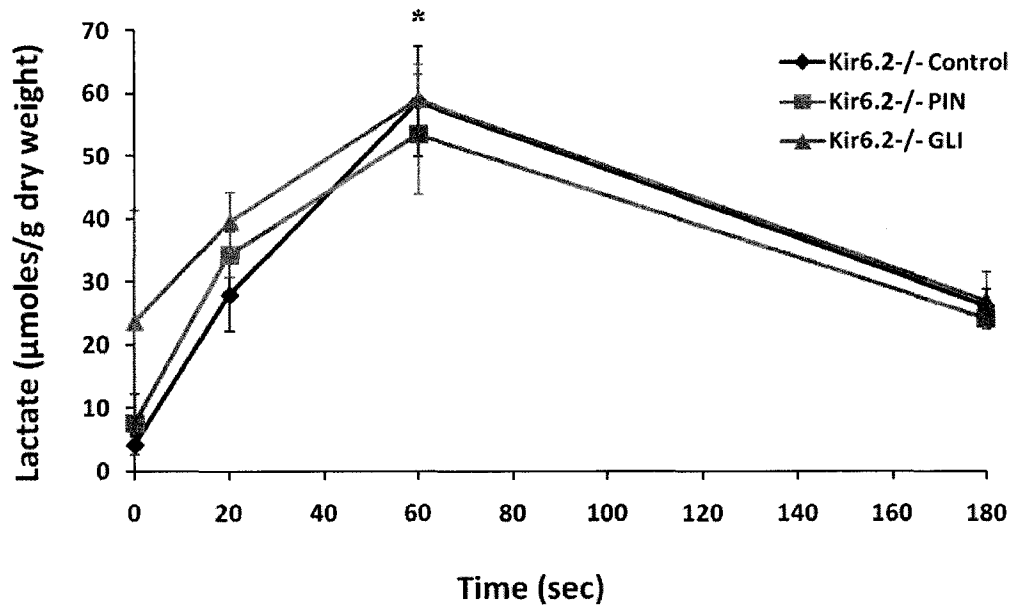


Figure 3-3. Glibenclamide and pinacidil had no effect on lactate content in Kir6.2^{-/-} FDB muscle during fatigue. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was of 37°C. Vertical bars represent S.E. for 5 muscles.

*Significantly different from time 0, ANOVA and L.S.D., P<0.05.

Figure 3-4

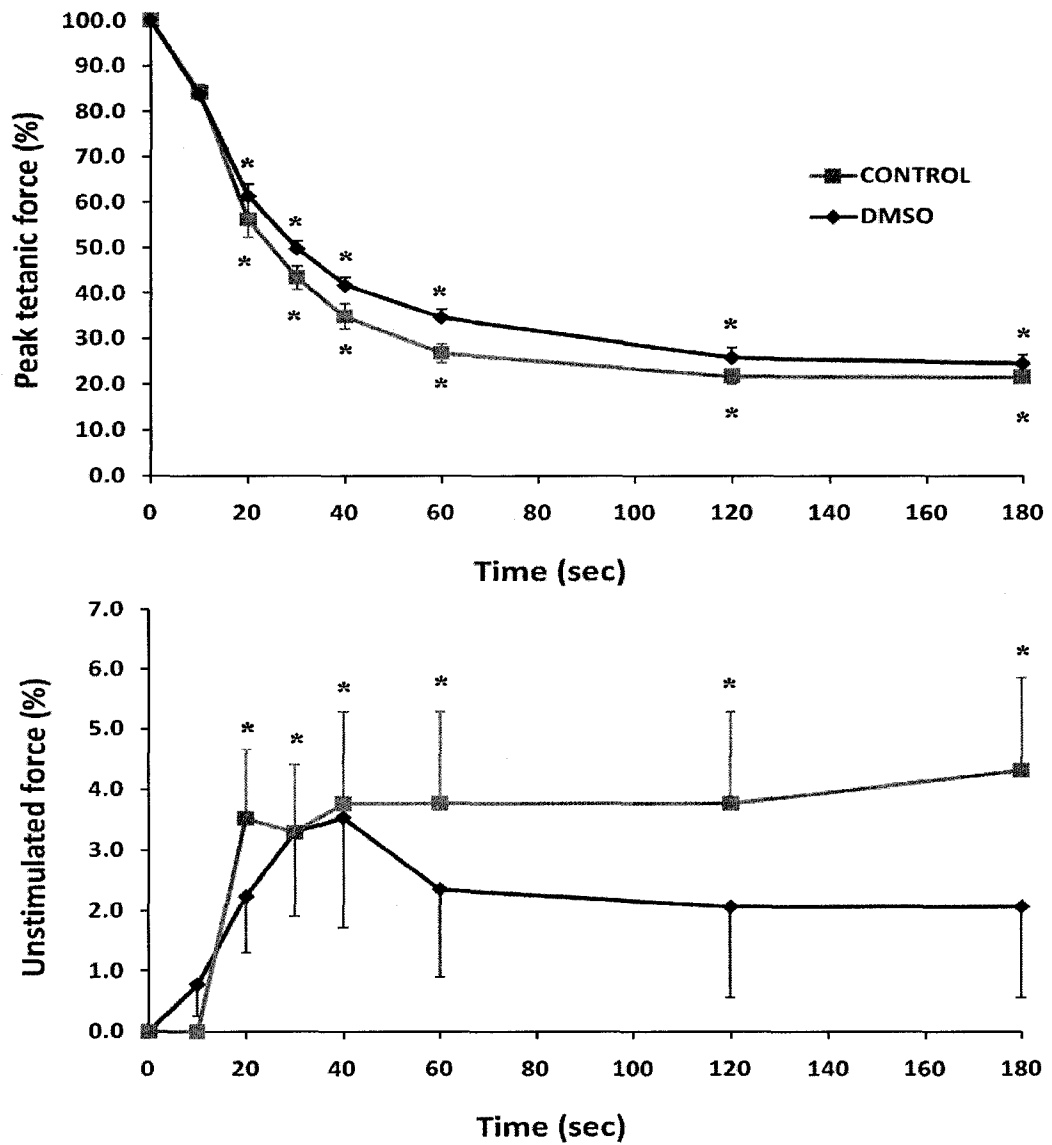


Figure 3-4. DMSO had no significant effect on A) peak tetanic force and B) unstimulated force on wild type muscles during fatigue. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was of 37°C. Vertical bars represent S.E. for 6 muscles.

*Significantly different from time 0, ANOVA and L.S.D., P<0.05.

sec (7-8%) and decreased to 2 % by 180 sec. None of these differences were statistically significant. Unstimulated force was slightly greater in control muscles being two times greater after 40 sec mainly because it remained constant for the rest of the fatigue period in the absence of DMSO and decreased slightly in the presence of DMSO. However, these differences were also insignificant (Figure 3-4B).

LACTATE CONCENTRATION

Pre-fatigue lactate levels were similar in control and DMSO-treated muscles being 5 and 3 $\mu\text{moles/g}$ of dry weight, respectively (Figure 3-5). Under control conditions, lactate levels during fatigue increased to 33 $\mu\text{moles/g}$ of dry weight by 20 sec and to 49 $\mu\text{moles/g}$ of dry weight by 60 sec. Thereafter, lactate content increased at a slower rate, reaching 58 $\mu\text{moles/g}$ of dry weight by 180 sec. The increase in lactate content during the first 40 sec was very similar in the absence and presence of DMSO. However, contrary to control muscles, lactate levels decreased back to 39 $\mu\text{moles/g}$ of dry weight. Although the difference at 180 sec was not statistically significant, the presence of DMSO constantly resulted in a decreased lactate content from 60 to 180 sec of fatigue in both wild type (Figure 3-5; Li, 2007) and Kir6.2^{-/-} (Figure 3-3), while the decrease was never observed in the absence of DMSO (Figure 3-5, Li, 2007). Furthermore, a DMSO concentration as low as 0.02%, while being enough to dissolve 10 μM glibenclamide and 100 μM pinacidil, still results in lower lactate levels than in the absence of DMSO (Appendix 1). Consequently, in the absence of a proper inert solvent for pinacidil and glibenclamide, all further experiments were limited to wild type and Kir6.2^{-/-} FDB exposed to control solution with no added DMSO.

Figure 3-5

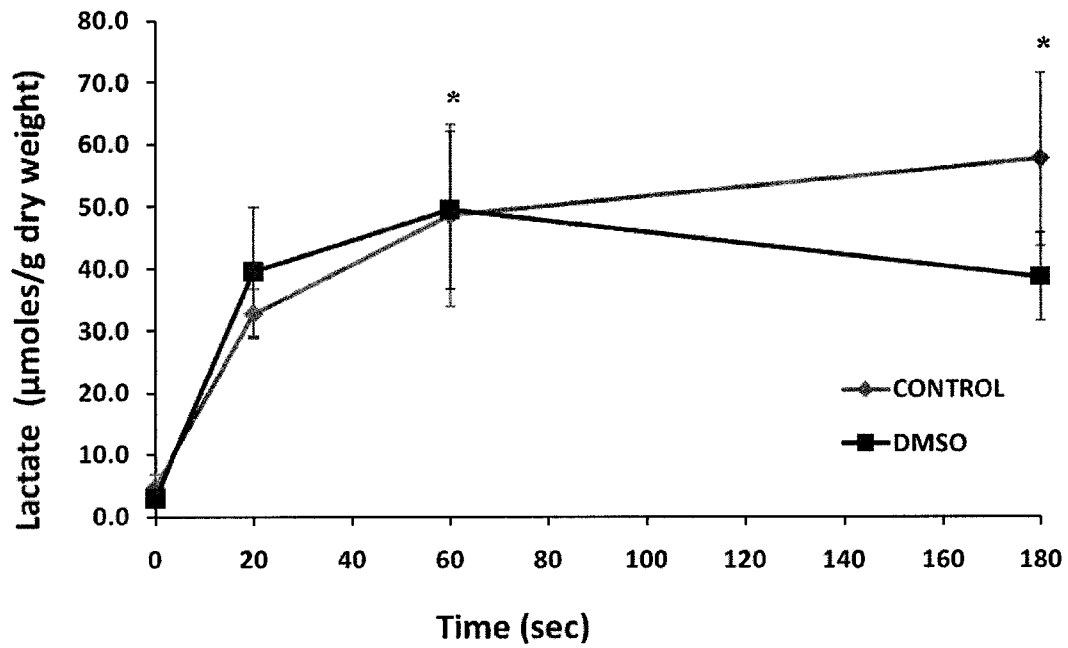


Figure 3-5. Lactate content decreased between 60 and 180 sec of fatigue in the presence of DMSO, while it increased under control conditions. There was no significant difference in lactate content. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was of 37°C. Vertical bars represent S.E. for 5 muscles.

**Significantly different from time 0, ANOVA and L.S.D., P<0.05*

EFFECT OF K_{ATP} CHANNEL ABSENCE ON GLUCOSE METABOLISM

The final aim of the study was to determine whether a chronic lack of K_{ATP} channel activity leads to increased oxidative capacity during fatigue.

FATIGUE KINETICS

As observed in Figure 3-6A, most of the decrease in peak tetanic force occurred in the first 60 sec, being slightly faster in Kir6.2^{-/-} compared to wild type muscles. This difference was not statistically significant. Wild type muscles developed little unstimulated force during fatigue, reaching 5% at 30 sec (Figure 3-6B). On the other hand, Kir6.2^{-/-} muscles developed significantly higher unstimulated force, reaching 20% by 30 sec, followed by a decrease to 12%, which was sustained during the last 2 minutes of fatigue.

GLUCOSE UPTAKE

Wash-out of extracellular glucose

One method for glucose uptake measurement involves using the radioactive non-metabolized glucose analog, ³H-2-deoxyglucose (³H-2DG) in conjunction with ¹⁴C-sucrose as an extracellular volume marker (Hansen et al, 1994). This method assumes i) that the diffusion rate of both sucrose and 2DG into the interstitial space are similar and ii) that diffusion rates are not limiting to 2DG for transport; i.e. that the 2DG concentration in t-tubules and interstitial space is always in equilibrium with that in the bulk solution. However, while the second assumption holds at resting levels, the same may not be true during intense exercise. The increased demand for energy would increase 2DG transport rate and the diffusion rate of 2DG into the interstitial space may become

Figure 3-6

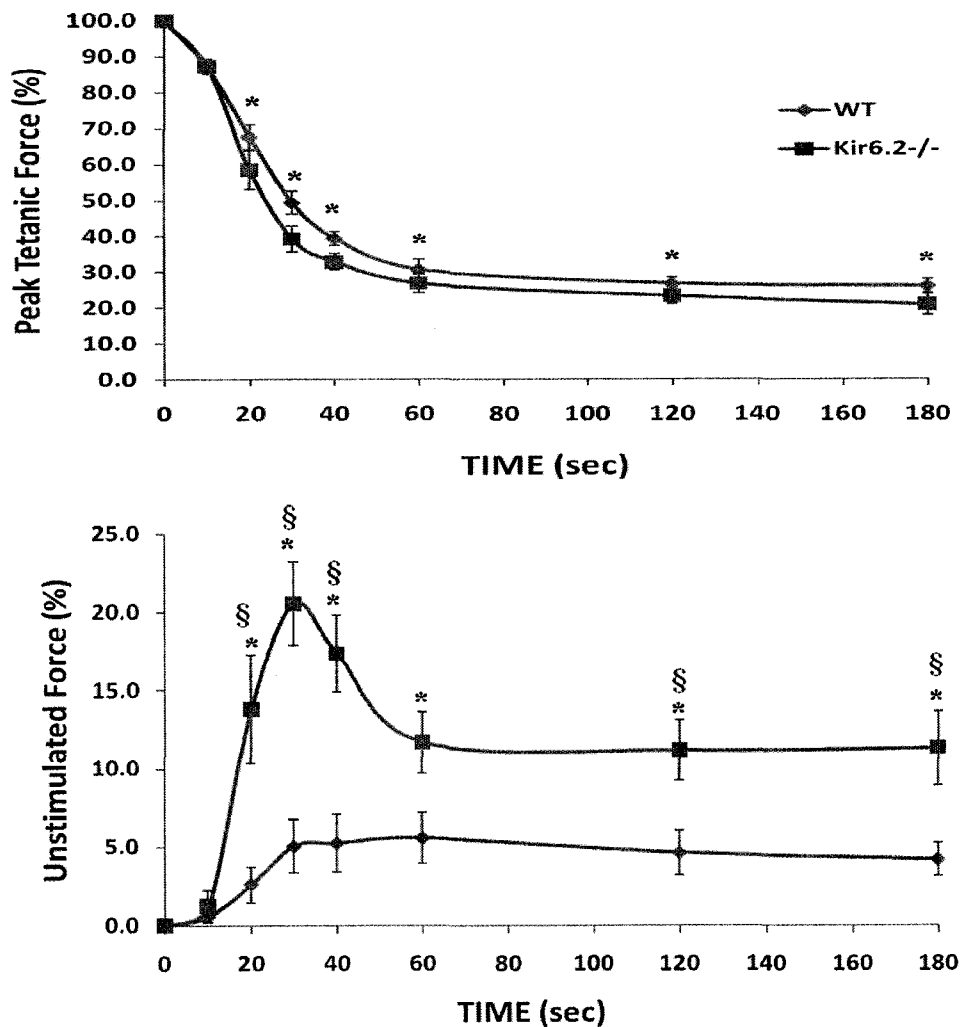


Figure 3-6. A) The decrease in peak tetanic force during fatigue was slightly slower in wild type than in Kir6.2^{-/-} FDB muscles, while B) Kir6.2^{-/-} FDB muscles developed significantly more unstimulated force. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent S.E. for 15 muscles.

* Significantly different from time 0, ANOVA and L.S.D., P<0.05.

§Significantly different from wild type FDB, ANOVA and L.S.D., P<0.05.

the limiting step. Consequently, the interstitial concentration would become lower than the bulk concentration resulting in an overestimation of the calculated extracellular glucose and an underestimation of glucose uptake.

An experiment was therefore designed to determine 2DG uptake during fatigue using the method described above concomitantly with an estimation of the uptake following a series of washout. Here, after the 30 min uptake period, which ended with the 3 min fatigue, muscles were transferred to a series of glucose-free solutions at 4°C. After counting the ³H-2DG and ¹⁴C-sucrose content in each fraction and muscle, the ³H-2DG and ¹⁴C-sucrose muscle content was calculated and plotted over time. ³H-2DG and ¹⁴C-sucrose content both had a rapid loss during the first 3 min, which most likely reflects the wash-out of the interstitial space (Figure 3-7).

From 3 to 12 min, the decrease was slower. The ¹⁴C-sucrose content reached 0 by 12 min, which indicates the complete wash-out of all extracellular radioactive compounds. Consequently, the decrease in ³H-2DG thereafter must be from a loss of intracellular 2DG, which persisted even after a 60 min wash-out (data not shown). Extrapolating to 0 min the relationship between log (³H-2DG DPM) with time will then give the intracellular 2DG content at the start of the experiment (Figure 3-8). The average DPM calculated using this method was 4498 whereas the average DPM calculated using ¹⁴C-sucrose as an extracellular marker to calculate extra- and intracellular 2DG was 4759. The difference was not statistically significant. Therefore, for the remainder of the study, glucose uptake was determined as in other studies, i.e. using ¹⁴C-sucrose as an extracellular marker.

Figure 3-7

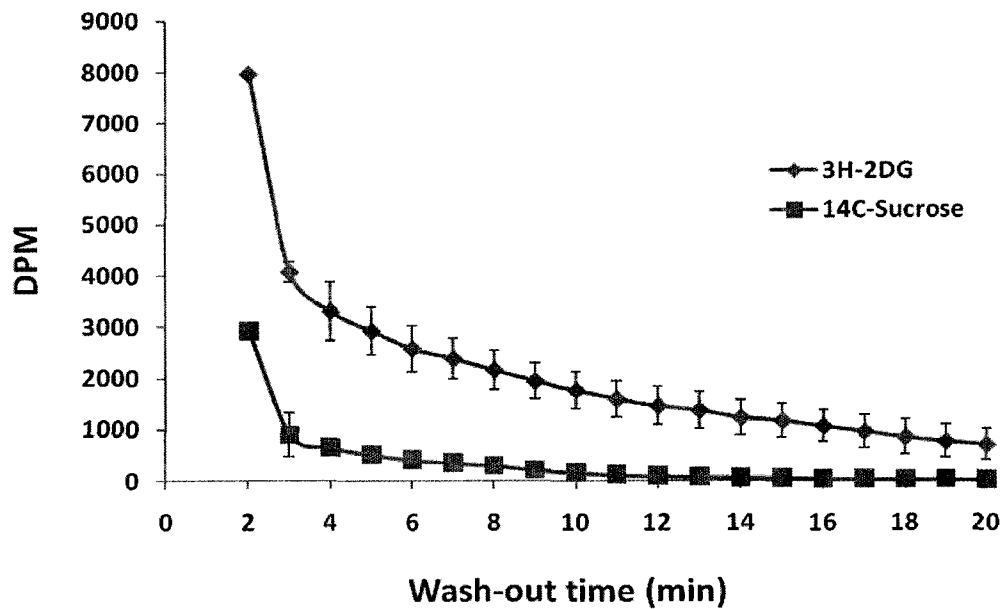


Figure 3-7. ^3H -2DG and ^{14}C -sucrose wash-out. FDB was exposed to $2\ \mu\text{Ci}/\text{mL}$ ^3H -2DG and $0.9\ \mu\text{Ci}/\text{mL}$ ^{14}C -sucrose for 30 min. Fatigue was elicited at 27 min with one contraction every sec for 3 min. Muscles were then transferred to ^3H -2DG and ^{14}C -sucrose free solution 20 times during 1 min each time. ^3H -2DG and ^{14}C -sucrose were measured in all wash-out solutions and in the muscle. ^3H -2DG and ^{14}C -sucrose contents at each time point were then calculated. Experimental temperature was 37°C during incubation and fatigue and of 4°C during wash-out. Vertical bars represent S.E. for 5 muscles.

Figure 3-8

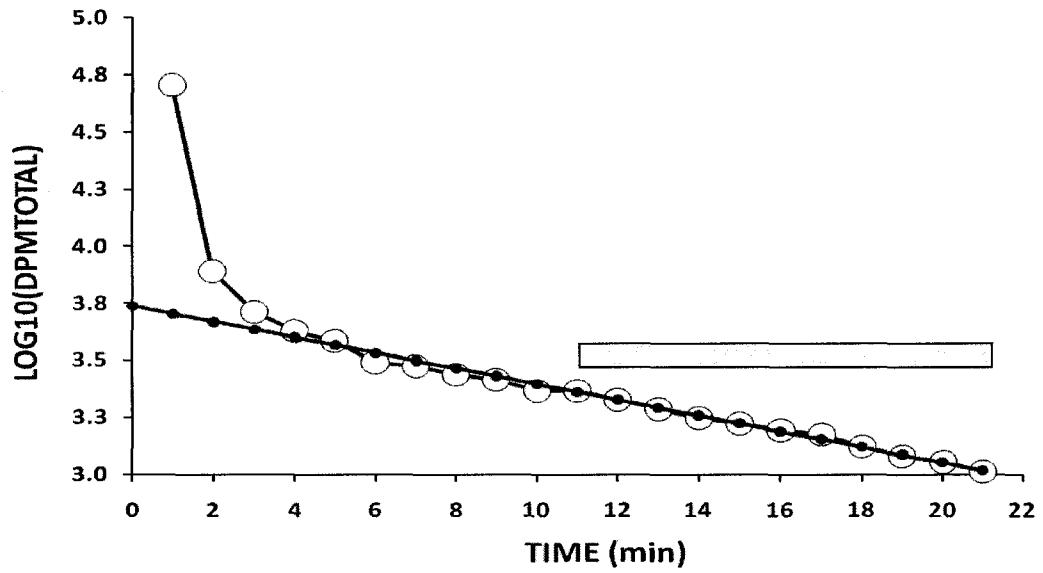


Figure 3-8. Example of the calculation of the log of ^3H -2DG and illustrating the extrapolation to the Y-intercept which indicates the intracellular ^3H -2DG loss.

Difference in ³H-2DG uptake in the presence of cold glucose and cold 2DG

When 2DG uptake is measured, the physiological solution contains ³H-2DG and cold 2DG instead of glucose. However, for this study, 2DG uptake must be measured during intense exercise requiring large amounts of energy. Since 2DG cannot be metabolized, removing glucose would be similar to inhibiting glycolysis. It is also important to measure the real glucose uptake, but the use of ¹⁴C-glucose is technically difficult as some of the ¹⁴C will be lost via lactate efflux and CO² production. After a literature search, no previous studies were found which determined whether the GLUT1 and 4 have similar affinity for glucose. Thus, we determined whether the uptake of ³H-2DG was the same in the presence of cold 2DG or glucose at rest in the absence of insulin (at low uptake rate) and in the presence of insulin (at high uptake rate, which is associated with increased GLUT4 incorporation into the cell membrane as it occurs during exercise).

In the absence of insulin, 2DG uptake was 10.2 μmoles/g dry weight in the presence of glucose and 8.7 μmoles/g dry weight in the presence of cold 2DG (Figure 3-9). In the presence of 1000 μU/mL insulin, 2DG uptake increased to 49.0 μmoles/g dry weight in the presence of glucose and to 54.4 μmoles/g dry weight in the presence of cold 2DG. None of the differences in ³H-2DG between glucose and 2DG conditions were significant. Thus, it is possible to determine glucose uptake using a solution containing ³H-2DG and 5mM cold glucose.

Glucose uptake during fatigue

Preceding fatigue, wild type and Kir6.2^{-/-} muscles transported 42 and 35 μmoles/g dry weight of glucose, respectively (Figure 3-10). This difference was not statistically significant. Following fatigue, wild type muscles had transported 75 μmoles glucose/g

Figure 3-9

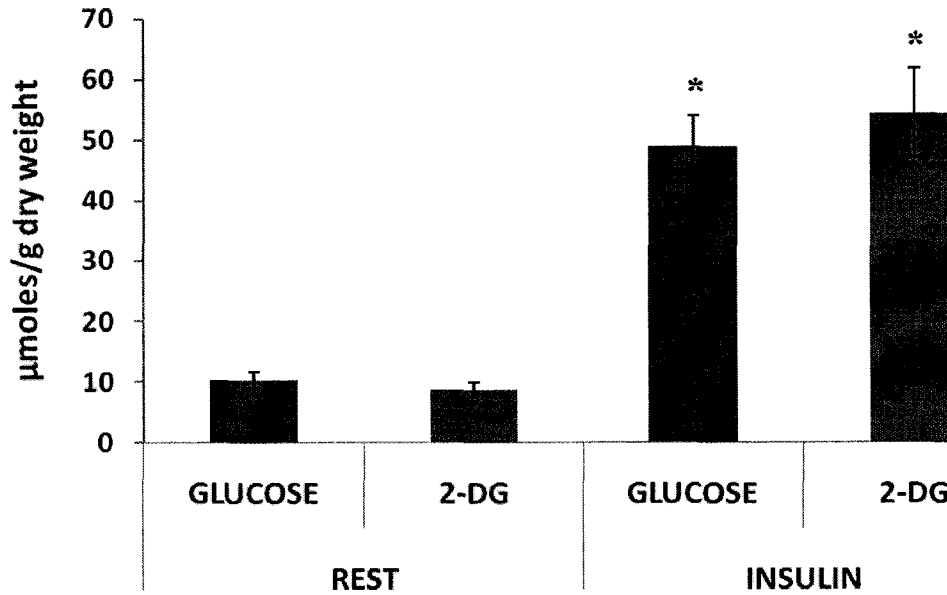


Figure 3-9. ³H-2DG uptake was the same in the presence of 5 mM cold glucose or 2DG both at rest and after exposure to insulin. Muscles were incubated in 1000 μU/mL of insulin for a total of one hour and 2 μCi/mL ³H-2DG and 0.9 μCi/mL ¹⁴C-sucrose during 30 min.

Experimental temperature was 37°C. Vertical bars represent S.E. for 6 muscles.

* Significantly different from rest, ANOVA and L.S.D., P<0.05.

Figure 3-10

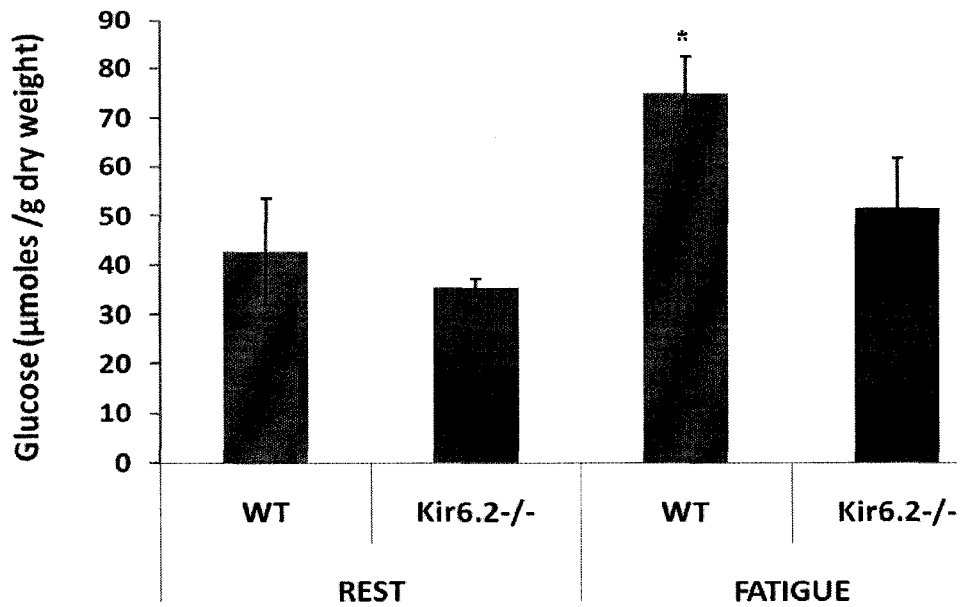


Figure 3-10. Glucose uptake during fatigue was less in Kir6.2^{-/-} than in wild type FDB, although there was no statistically significant difference. Muscles were incubated in 2 µCi/mL ³H-2DG and 0.9 µCi/mL ¹⁴C-sucrose during 30 min before the onset of fatigue. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent S.E. for 5 muscles.
* Significantly different from rest, ANOVA and L.S.D., P<0.05.

dry weight compared to 51 $\mu\text{moles/g}$ dry weight for Kir6.2^{-/-} FDB. Thus, the net glucose uptake during the 3 min fatigue was 33 $\mu\text{moles/g}$ dry weight in wild type and 16 $\mu\text{moles/g}$ dry weight in Kir6.2^{-/-} muscles.

GLYCOGEN CONTENT DURING FATIGUE

Figure 3-11 shows that wild type muscles contained 163 $\mu\text{moles glycogen/g}$ dry weight at rest. Following fatigue, levels decreased significantly to 87 $\mu\text{moles/g}$ dry weight. Kir6.2^{-/-} muscles had slightly lower resting glycogen content compared to wild type being of 143 $\mu\text{moles/g}$ dry weight at rest and of 54 $\mu\text{moles/g}$ dry weight following fatigue. Thus, during fatigue 76 μmoles of glucosyl units were generated in wild type compared to 89 μmoles in Kir6.2^{-/-} FDB.

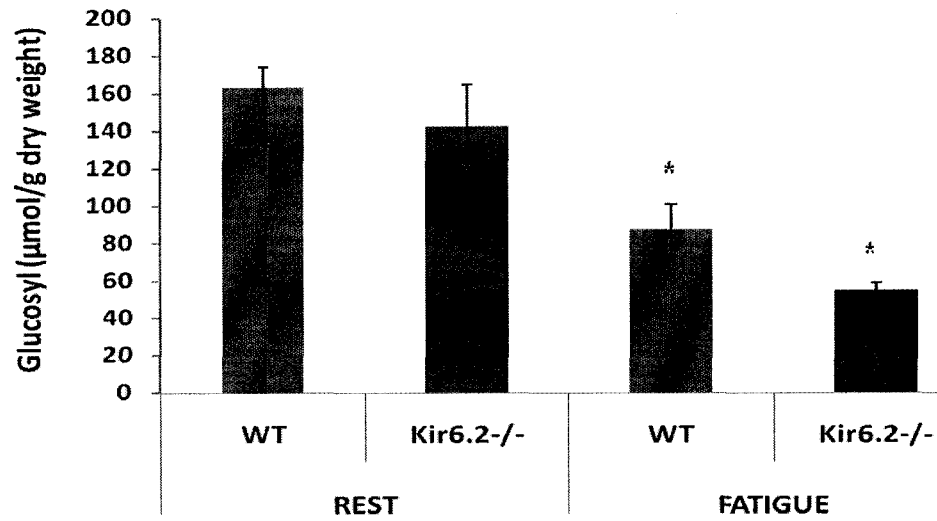
LACTATE CONTENT DURING FATIGUE

At rest, lactate levels in wild type FDB were 4.7 $\mu\text{moles/g}$ dry weight and increased significantly to 65.6 $\mu\text{moles/g}$ dry weight after fatigue (Figure 3-12). Kir6.2^{-/-} FDB had higher resting lactate levels of 16.9 $\mu\text{moles/g}$ dry weight. Following fatigue, lactate levels were lower in Kir6.2^{-/-} muscles being 37.3 $\mu\text{moles/g}$ dry weight compared to 65.6 $\mu\text{moles/g}$ dry weight in wild type. Thus, the net increase in lactate content in wild type FDB was 60.9 $\mu\text{moles/g}$ dry weight and significantly less in Kir6.2^{-/-} FDB, being only 20.4 $\mu\text{moles/g}$ dry weight.

EFFECT OF K_{ATP} CHANNEL ABSENCE ON INDIRECT CALORIMETRY *IN VIVO*

As discussed in the Introduction, the lower lactate production may be due to greater

Figure 3-11



Fatigue 3-11. Glycogen breakdown was greater in Kir6.2^{-/-} than in wild type FDB, although there was no statistically significant difference. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent S.E. for 5 muscles.

* Significantly different from rest, ANOVA and L.S.D., P<0.05.

Figure 3-12

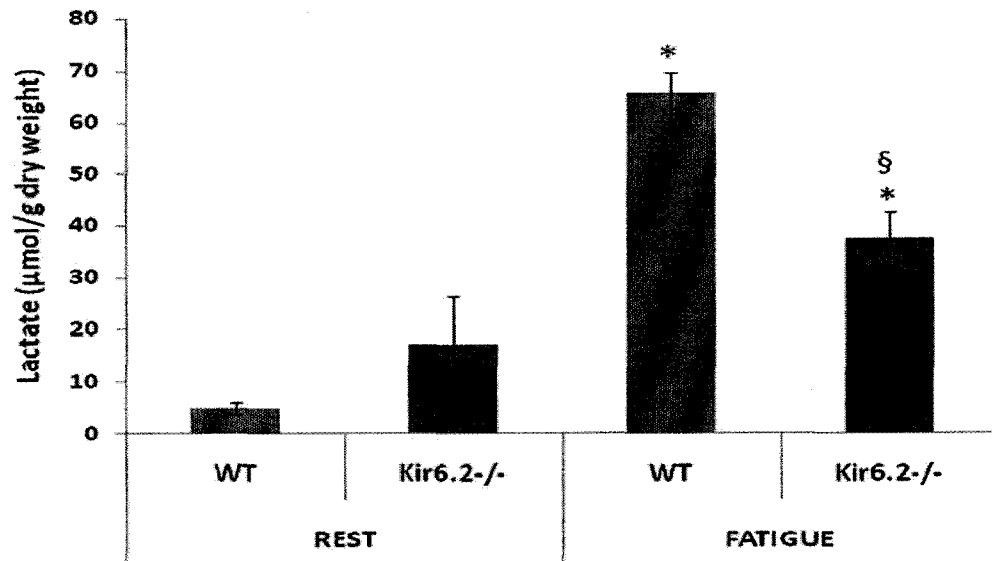


Figure 3-12. After fatigue, Kir6.2-/- muscles contained significantly less lactate compared to wild type muscles. Fatigue was elicited with one contraction every sec for 3 min. Experimental temperature was 37°C. Vertical bars represent S.E. for 5 muscles.

* Significantly different from time 0, ANOVA and L.S.D., $P < 0.05$.

§ Significantly different from wild type mouse, ANOVA and L.S.D., $P < 0.05$.

oxidative capacities of Kir6.2^{-/-} muscles compared to wild type muscles. Considering that skeletal muscles constitute a major portion of the body mass, oxygen consumption (VO₂) and respiratory exchange ratio (RER) were measured in wild type and Kir6.2^{-/-} mice over a 24 hour period.

OXYGEN UPTAKE MEASUREMENTS

Over a 24 hour period, the mean VO₂ was 1.29 O₂/min in wild type mice compared to a significantly higher VO₂ of 1.51 O₂/min in Kir6.2^{-/-} mice (Figure 3-13). This higher VO₂ was also reflected by a shift to the right of 20% of the percent cumulative frequency (PRCF) curve, which was calculated as explained by Riachi et al. (2004). Figure 3-14 shows that Kir6.2^{-/-} mice tended to have higher VO₂ at most times of the day. However, the difference was only statistically significant during more active hours of the day, which is at hours 24 and 1.

RESPIRATORY EXCHANGE RATIO MEASUREMENTS

RER is defined as the volume of carbon dioxide exhaled divided by the volume of oxygen inhaled (VCO₂/VO₂). When measuring the RER, the point of inflection of the PRCF curves of both wild type and Kir6.2^{-/-} mice were similar (Figure 3-15). This indicates that there was no significant difference between the mean RER of wild type and Kir6.2^{-/-}. Indeed, they both equaled 0.93. However, Kir6.2^{-/-} mice had a significantly wider range of RER, ranging from 0.83 to 1 compared to that of wild type mice which was from 0.86 to 0.98. When plotting RER at different hours of the day, Kir6.2^{-/-} mice had higher RER values between 17 and 2 hours, which is the time period when mice are most active. Conversely, Kir6.2^{-/-} mice had lower RER values between 4 and 16 hours

(Figure 3-16).

Figure 3-13

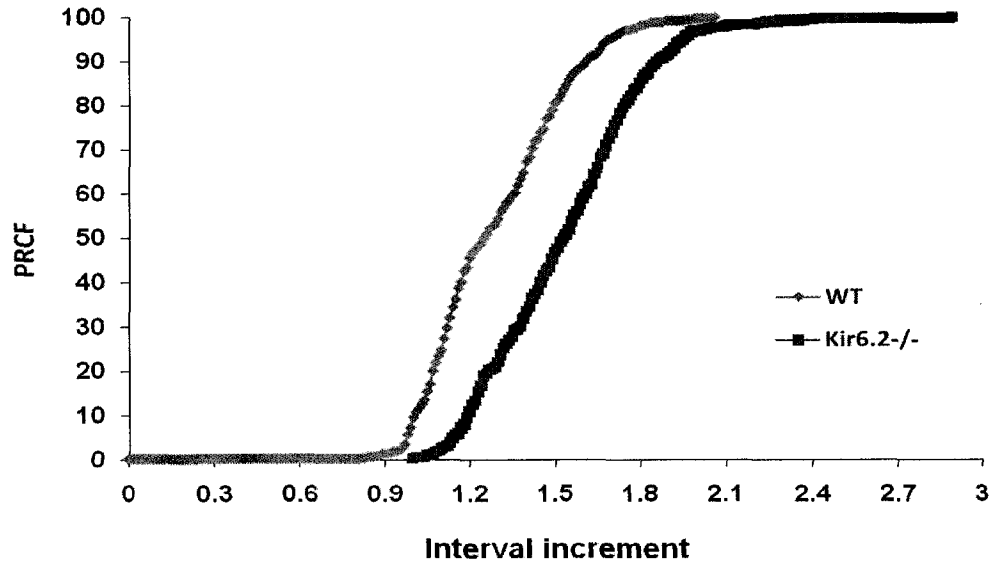


Figure 3-13. Kir6.2^{-/-} mice had PRCF VO₂ curve shifted to the right compared to wild type mice over a 24 hour period. The temperature was maintained at 24°C and light was present from 7:00 to 19:00 and mice were fed ad libitum. N=5.

Figure 3-14

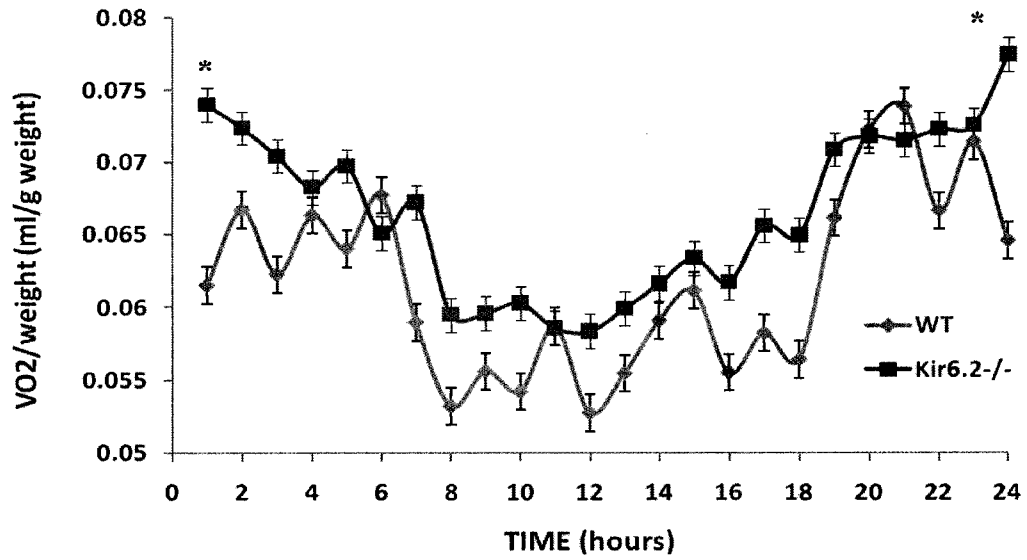


Figure 3-14. Kir6.2^{-/-} mice had higher VO₂ when compared to wild type mice.

Experimental temperature was 24°C. Vertical bars represent S.E. for 5 muscles.

* Significantly different from wild type, ANOVA and L.S.D., P<0.05.

Figure 3-15

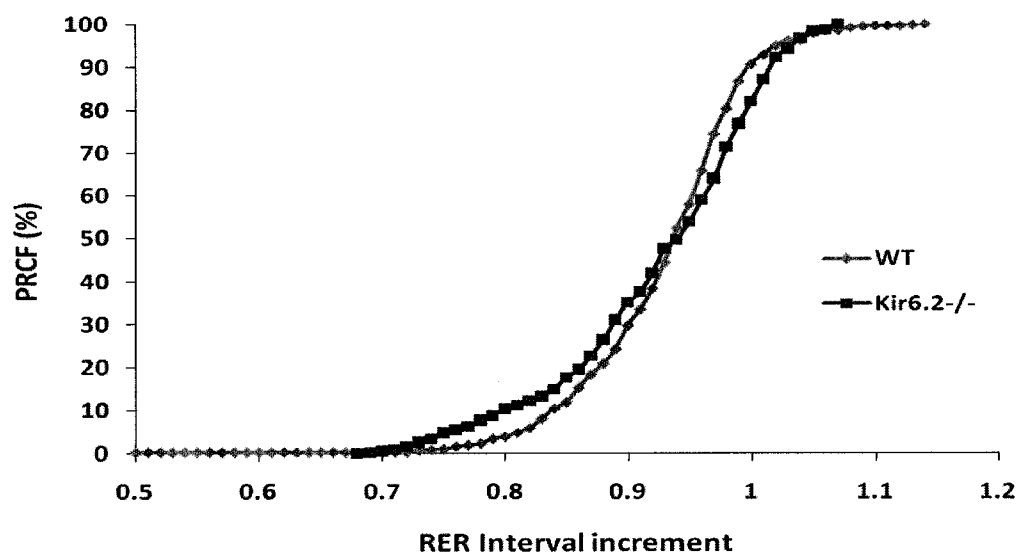


Figure 3-15. Kir6.2^{-/-} mice had the same point of inflection but wider range of PRCF RER curve compared to wild type mice over a 24 hour period.

The temperature was maintained at 24°C and light was present from 7:00 to 19:00 and mice were fed ad libitum. N=5.

Figure 3-16

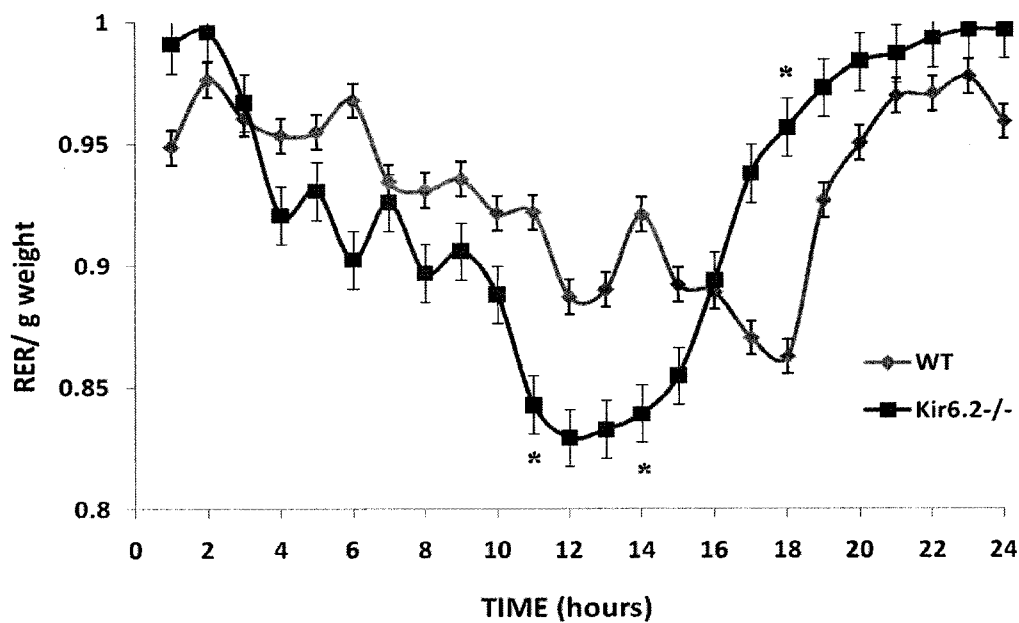


Figure 3-16. Kir6.2^{-/-} mice had significantly lower RER at hours 11 and 14 but significantly higher RER at hour 18 when compared to wild type mice.

Experimental temperature was 24°C. Vertical bars represent S.E. for 5 muscles.

* Significantly different from wild type, ANOVA and L.S.D., P<0.05.

Chapter 4

DISCUSSION

This study has found that the K_{ATP} channel modulators, pinacidil and glibenclamide, did not affect peak tetanic force, unstimulated force, nor lactate content in Kir6.2^{-/-} FDB during fatigue. Secondly, the presence of 0.1% DMSO had no effect on peak tetanic force and unstimulated force but decreased lactate content during the last 2 min of fatigue in wild type FDB. Furthermore, Kir6.2^{-/-} FDB transported less glucose and utilized more glycogen than wild type during 3 min of fatigue, which amounted to similar total glucosyl units. However, Kir6.2^{-/-} FDB had a significantly lower increase in lactate content. This study also found that Kir6.2^{-/-} mice possess higher levels of oxygen consumption especially during the night when they are most active. Lastly, it has been found that Kir6.2^{-/-} mice have a wider range of respiratory exchange ratio whereby rates were higher than wild type during more active times of day and lower than wild type during less active times of day.

PINACIDIL AND GLIBENCLAMIDE DO NOT AFFECT LACTATE CONTENT IN KIR6.2^{-/-} FDB DURING FATIGUE

Li (2007) found different effects on metabolite content during fatigue when the K_{ATP} channel activity was abolished pharmacologically, using glibenclamide, compared to using a genetic approach, i.e. Kir6.2^{-/-} FDB. Specifically, glibenclamide-exposed wild type FDB had higher lactate levels during the last 2 min of fatigue than in control. Kir6.2^{-/-} FDB, on the other hand, had lower lactate levels than wild type through-out fatigue. Pinacidil and glibenclamide are known to modulate both sarcolemmal and mitochondrial K_{ATP} channels (mito K_{ATP}) (Ardehali & O'Rourke, 2005). So far, pinacidil and

glibenclamide did not affect force production and fatigue kinetics in Kir6.2^{-/-} FDB (Gong et al, 2003; Cifelli et al., 2007). However, the mitochondrial K_{ATP} channel activity is thought to affect mitochondrial function by increasing reactive oxygen species (ROS) production (Krenz et al. 2002). Furthermore, as a result of increased osmolarity, it increases mitochondrial volume, which increases ATP production (Jouaville et al., 1999). It was therefore hypothesized that pinacidil and glibenclamide affect the pyruvate flux through Kreb's cycle and thereby affect lactate content.

Consequently, the first approach was to determine whether mitoK_{ATP} channel modulation with pinacidil and glibenclamide on Kir6.2^{-/-} FDB affect lactate content during fatigue because these muscles only lack sarcolemmal K_{ATP} channel activity. The fatigue kinetic measurements obtained in this study showed no effect of pinacidil and glibenclamide on peak tetanic force and unstimulated force as previously reported for pinacidil (Gong et al., 2003) and glibenclamide (Cifelli et al., 2007). More importantly, no difference was found between the three conditions in lactate content either. These results would then suggest that a modulation of mitoK_{ATP} channel activity by glibenclamide and pinacidil does not affect lactate production in FDB and is not responsible for the complex effect of these modulators reported by Li.

However, recent evidence has emerged suggesting the presence of the Kir6.2 subunit in mitoK_{ATP} channel (Garg & Hu, 2007). If this is the case, then Kir6.2^{-/-} FDB also lacks mitoK_{ATP} in addition to sarcoK_{ATP} channel. Thus, future studies will be necessary to determine if pinacidil and glibenclamide affect mitochondrial activity before we can clearly establish whether mitoK_{ATP} channel activity affects lactate content. Such studies could include exposing glibenclamide and pinacidil to isolated mitochondria and

determining differences in mitochondrial activity via the measurement of flavoprotein oxidation.

DMSO DECREASES LACTATE CONTENT IN FDB DURING FATIGUE

When Li (2007) compared how the metabolite levels change during fatigue between Kir6.2^{-/-} and wild type FDB, she did not use DMSO since no drugs were used in these experiments. In both wild type and Kir6.2^{-/-} FDB, lactate content increased during the last 2 min of fatigue. When she tested the effects of glibenclamide and pinacidil in wild type FDB, her control solution also contained 0.1% DMSO and lactate content decreased during the last 2 min of fatigue. However, Li's results were obtained from two separate sets of experiments. Thus, lactate content in the presence or absence of DMSO was measured in this study using paired muscles in order to avoid differences between animals and over time. The presence of 0.1% DMSO resulted in a 22% decrease in lactate content but a 16% increase in the absence of DMSO during the last 2 min of fatigue. Furthermore, a decrease in lactate was also observed in Kir6.2^{-/-} FDB when exposed to DMSO. Additionally, when reducing the DMSO concentration as low as 0.02% concentration to dissolve glibenclamide, lactate levels were still lower than with no DMSO (Appendix 1). It can therefore be concluded that DMSO lowers lactate content in FDB especially during the last 2 min of fatigue.

The first possible mechanism by which DMSO decreases lactate content is by decreasing force production and therefore decreasing energy demand. Ogura et al. (1996) determined

that DMSO depresses force in rabbit and guinea-pig papillary muscles. Furthermore, they found that this effect was due to a decrease in Ca^{2+} overload. DMSO also decreases intracellular Ca^{2+} levels in human erythroleukemia cells (Michel, 1998) and promyelocytic leukemia cells (Choi et al, 1999). However, these studies were performed at high DMSO concentrations of 3-10%. In the present study, the DMSO concentration was of 0.1% and DMSO had no effect on peak tetanic force or on unstimulated force. Therefore, it is suggested that the decrease in lactate in the presence of DMSO is not due to a decrease in energy demand from contractile activity.

It is not possible from the results of this thesis to determine the mechanism by which DMSO decreases lactate content during fatigue. One possibility could be due to an effect of DMSO on mitochondria by increasing the amount of pyruvate going through the Krebs's cycle. In fact, in 1982, Shlafer et al. found that 70 mM DMSO prevented mitochondrial changes resulting from cardiac ischemia and reperfusion by improving respiratory rates and oxidative phosphorylation rates. It is possible that at extremely high exercise intensity leading to fatigue, this effect is exacerbated and thus noticeable even at low DMSO concentrations. Another possibility is an effect of DMSO on glycolytic enzymes, thus affecting the rate of lactate production. Finally, if DMSO alters lactate production, it may also modulate the effect of pinacidil and glibenclamide. Therefore, experiments using glibenclamide and pinacidil will need to be redone with a more inert solvent.

EFFECT OF K_{ATP} CHANNEL ABSENCE ON GLUCOSE METABOLISM DURING FATIGUE IN FDB

GLUCOSE UPTAKE METHODOLOGY

It was first necessary to determine whether, during high energy demands associated with

exercise, the diffusion rate of 2DG into the interstitial space from a bulk solution does not become a limiting step, resulting in an underestimation of glucose uptake. Therefore, an experiment was designed whereby the wash-out of extracellular radioactive compounds would allow the direct measurement of intracellular ^3H -2DG. During the wash-out, the time at which the extracellular marker, ^{14}C -sucrose, reaches 0 is when all radioactive compounds have been washed-out from the extracellular space, i.e. ^3H -2DG and ^{14}C -sucrose. Knowing that once 2DG enters the cell, it is phosphorylated and becomes “trapped” in the cell (Nordlie, 1971; Jenkins et al., 1986), ^3H -2DG levels were then expected to plateau once ^{14}C -sucrose reaches 0 and the value at that time would be equivalent to the intracellular ^3H -2DG levels. However, such plateau was not observed. Instead, ^3H -2DG levels continued to decrease at an average rate of 100 DPM/min, which is indicative of a loss of intracellular 2DG. Nonetheless, by extrapolating ^3H -2DG values to time 0, it was possible to determine the total 2DG uptake. The results showed that the uptake was the same as observed in the conventional method of measuring 2DG uptake using ^{14}C -sucrose as an extracellular marker to subtract extracellular ^3H -2DG. It thus appears that the diffusion of 2DG from the bulk solution to the interstitial space does not become the limiting step for uptake during fatigue.

Measurement of glucose uptake is normally done using ^3H -2DG and cold 2DG. However, 2DG cannot be metabolized to produce ATP. Consequently, if a similar method was used, an important source of energy would be lacking, seriously altering the fatigue kinetics. The use of ^{14}C -glucose would be complicated since it can be metabolized and it would therefore be necessary to also measure the $^{14}\text{CO}_2$ produced. So, the best approach is to use ^3H -2DG with 5 mM cold glucose.

If GLUT 1 and 4 have different affinities for 2DG and glucose, then measuring glucose uptake with ^3H -2DG would not estimate the real glucose uptake. For instance, if 2DG has higher affinity for GLUT transporters, glucose uptake will be overestimated. Conversely, if 2DG has a lower affinity than glucose, glucose uptake will be underestimated. No studies were found in the literature comparing affinities of glucose and 2DG for GLUT1 and 4. To test if such a problem exists, the uptake of ^3H -2DG was done in paired muscles where one muscle was exposed to cold 2DG and the other to cold glucose. At rest, when a large uptake of glucose is via GLUT1, there was no significant difference in ^3H -2DG uptake in the presence of cold 2DG or cold glucose. When exposed to 1000 $\mu\text{U}/\text{mL}$ of insulin, which increases the cell membrane GLUT4 content, there was also no difference in uptake. Accordingly, glucose uptake can be properly measured using cold glucose with ^3H -2DG.

GLUCOSE UPTAKE, GLYCOGEN AND LACTATE CONTENT

In order to compare energy metabolism during fatigue, it was necessary to first compare fatigue kinetics between Kir6.2^{-/-} and wild type muscles. Although there was no significant difference in peak tetanic force, Kir6.2^{-/-} FDB developed five times more unstimulated force. Furthermore, Cifelli (2007) also reported an increase in unstimulated intracellular Ca²⁺ concentration. Together, these differences should lead to increased ATP utilization by myosin ATPase and Ca²⁺-ATPase pump. In support of this, Li (2007) showed that Kir6.2^{-/-} FDB had lower ATP levels during fatigue compared to wild type. This decrease in ATP is then expected to result in greater ATP production. Surprisingly, glucose uptake was 17 $\mu\text{moles}/\text{g}$ dry weight lower in Kir6.2^{-/-} than in wild type FDB during fatigue. Not only is this finding surprising but it is different from previous studies

showing increased glucose uptake observed in the absence of K_{ATP} channel activity. Miki et al. (2002) found that $Kir6.2^{-/-}$ muscles had higher glucose uptake at rest in EDL when 2DG concentration exceeded 32 mM and in presence of insulin in soleus. Additionally, Chutkow et al. (2001) also observed that in the presence of insulin, $SUR2^{-/-}$ soleus had 1.5 fold greater glucose uptake than wild type. However, insulin and contraction-induced glucose uptake result from different intracellular signaling pathways in which the exact involvement of the K_{ATP} channel remains unclear. Furthermore, the experiments in the following study were performed on FDB muscles, which is mainly composed of fibre types IIA and IIX as opposed to soleus and EDL, which mainly contain types I, IIA and IIB, IIX, respectively (Banas, unpublished data). So, this study suggests that while at rest and upon insulin stimulation glucose uptake is greater in $Kir6.2^{-/-}$ muscle, the reverse occurs during exercise.

While $Kir6.2^{-/-}$ muscles transported less glucose, they utilized more glycogen than wild type muscles. The decrease in glycogen content following fatigue was 12 μ moles of glucosyl/g dry weight greater in $Kir6.2^{-/-}$ than in wild type FDB. Cifelli et al. (2007) have shown that $Kir6.2^{-/-}$ FDB have higher intracellular Ca^{2+} following fatigue. Furthermore, increased Ca^{2+} results in increased glycogenolysis (Jakob et al., 1980). This could explain the higher glycogen breakdown in $Kir6.2^{-/-}$ FDB. Furthermore, it has been shown that increased glycogenolysis decreases glucose uptake (Fell et al., 1982). Thus, this could also explain the lower glucose uptake observed. When adding total glucosyl used from glucose uptake and glycogen breakdown, it is found that $Kir6.2^{-/-}$ and wild type muscles have similar values with 104 and 109 μ moles/ g dry weight, respectively.

During glycolysis, glucosyl is transformed into pyruvate, which can either enter the

Kreb's cycle or be transformed into lactate. Wild type muscles generated 61 μ moles of lactate/ g dry weight during fatigue compared to 20 μ moles/ g dry weight generated by Kir6.2^{-/-}. Thus, 79 μ moles of glucose are unaccounted for in wild type muscles compared to 94 μ moles in Kir6.2^{-/-} muscles. If all the pyruvate molecules generated by these glucose molecules went through the Kreb's cycle, it can be deduced that an extra 518 ATP molecules were produced in Kir6.2^{-/-} muscles (Widmaier et al, 2004), representing a 18% greater ATP production in Kir6.2^{-/-} FDB compared to wild type FDB.

However, it is important to keep in mind that not all glucosyl molecules measured do end up in pyruvate or lactate and not all pyruvate molecules entering the Kreb's cycle result in CO₂ production. Indeed, there are many glycolytic and mitochondrial intermediates and it is unknown whether the rate at which these are produced is similar for Kir6.2^{-/-} and wild type muscles. Future studies would therefore need to determine the levels of different glycolytic intermediates during fatigue as well as mitochondrial function and ¹⁴CO₂ production.

Nonetheless, if there is increased pyruvate flux into the Kreb's cycle, there should be increased oxygen consumption by the mouse. Considering that nearly 80% of the mouse total body mass is muscle mass of the limbs (Proctor et al., 1999), *in vivo* calorimetric analysis of oxygen consumption (VO₂) and respiratory exchange ratio (RER) measurements were deemed appropriate as preliminary testing. This allowed us to determine whether the K_{ATP} channel affects oxidative capacity and energy consumption patterns in the whole mouse. The PRCF curve of Kir6.2^{-/-} mice was shifted to the right by 20% which indicates that Kir6.2^{-/-} mice possess an overall higher VO₂. When standardizing VO₂ for weight at different hours of the day, Kir6.2^{-/-} mice retain a higher

VO₂. The difference was accentuated and statistically significant between 12 and 1 am, which is the most active period of the day. These results support the concept that Kir6.2^{-/-} mice have a higher oxidative capacity and are in agreement with the *in vitro* findings that Kir6.2^{-/-} muscles have increased flow of pyruvate to the Krebs's cycle.

The RER, defined as VCO₂/VO₂, expresses the type of energy source upon which an organism relies on the most at a certain point in time. An RER closer to 1 indicates a metabolism highly reliant on carbohydrates whereas an RER closer to 0.7 indicates a metabolism highly reliant on fatty acids. The RER PRCF curves indicated that Kir6.2^{-/-} mice had a greater variability in fuel sources compared to wild type. In other words, Kir6.2^{-/-} mice tend to use either energy source more exclusively and thus possess a higher metabolic flexibility whereas wild type mice utilize a mix of energy sources. This was better illustrated when RER values were plotted at each hour of the day. Indeed, between hours 18 and 3, the RER of Kir6.2^{-/-} remained between 0.95 and even reached 1 during 4 hours. This indicates a metabolism relying purely on carbohydrates. On the other hand, the highest RER value reached by wild type mice was of 0.98 indicating a metabolism which is heavily reliant on carbohydrates. Since these hours are when mice are most active, it can be suggested that Kir6.2^{-/-} mice rely more heavily on carbohydrates during physical activity when compared to wild type. Conversely, during least active times of the day, Kir6.2^{-/-} mice have significantly lower RER values compared to wild type reflecting increased dependence on fatty acids. Interestingly, Kir6.2^{-/-} RER begins to increase while wild type RER is still decreasing. This could either indicate a difference in the rate of metabolic response or a difference in daily activity patterns. Furthermore, when calculating metabolic energy expenditure following the equation from Jay et al. (2007),

Kir6.2^{-/-} mice had higher energy expenditure than wild type mice at most times of the day and significantly higher between 12 and 1 am (Figure 4-1).

Figure 4-1

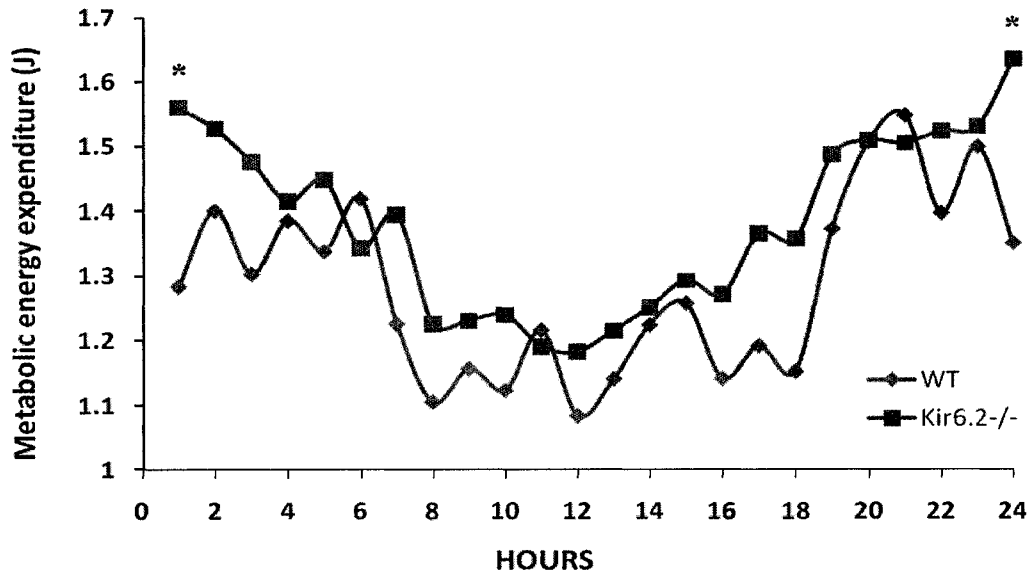


Figure 4-1 Metabolic energy expenditure was higher in Kir6.2^{-/-} compared to wild type mice. Experimental temperature was 24°C.

* Significantly different from wild type, ANOVA and L.S.D., P<0.05.

It is impossible to apply the following results to the in vitro experiments previously discussed since Kir6.2-containing K_{ATP} channels are present in tissues other than skeletal muscle, which could also influence energy use in unknown manners, notably the pancreas, which in Kir6.2^{-/-} mice does not possess normal insulin responses. Furthermore, the RER is an estimation of energy metabolism since the measurement of O_2 and CO_2 exchange is performed from the mouth. In order to precisely determine fuel metabolism at the level of the muscle, it is necessary to calculate the respiratory quotient (RQ), which is the VCO_2/VO_2 measured directly from the muscle. Although in theory, RER and RQ should be equal under steady state conditions, this assumption cannot be made since it is unknown how eliminating Kir6.2-containing K_{ATP} channels affects ventilation. If indeed, they are found to hyperventilate, RER would not be reflective of the RQ value. Thus, it would be necessary for future studies to determine the RQ of Kir6.2^{-/-} muscles. It would also be interesting to perform calorimetric measurements during intense exercise such as treadmill running not only to verify changes in VO_2 but also to determine how fluctuations in RER will be affected.

Chapter 5

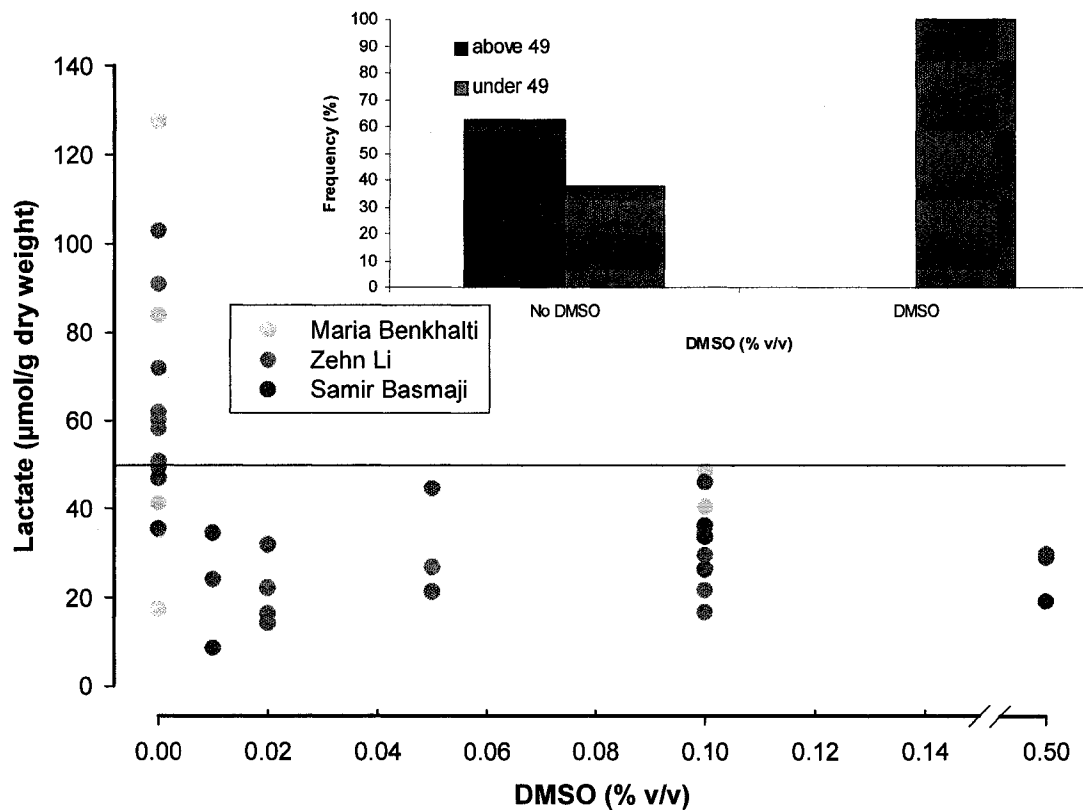
CONCLUSION

In conclusion, it was found that pinacidil and glibenclamide do not affect fatigue kinetics and lactate levels in Kir6.2^{-/-} FDB during fatigue. Furthermore, 0.1% DMSO results in a decrease in lactate levels during the last 2 minutes of fatigue. Lastly, although Kir6.2^{-/-} muscles had similar glucosyl utilization, they had decreased lactate levels. This suggests that more pyruvate enters the Krebs's cycle, thus generating more ATP than in wild type muscles. This increase in mitochondrial activity also suggests an increase in oxygen consumption. This was reflected in vivo where Kir6.2^{-/-} mice had higher VO₂. Furthermore, Kir6.2^{-/-} mice rely more exclusively on either carbohydrates or fatty acids unlike wild type mice that use a mixture of fuel sources. Thus, Kir6.2^{-/-} muscles possess increased oxidative capacity, which results in increased ATP production. These findings are significant to understanding diabetes and for the potential development of more effective treatment, which would optimize glucose uptake and utilization by skeletal muscles. An understanding of the specific mechanisms by which Kir6.2^{-/-} muscles have an altered glucose uptake and glycogen utilization would enable to develop a treatment which could specifically target the upregulation of glucose uptake during exercise.

These findings are also significant to enhancing weight management programs. Indeed, understanding the mechanism by which Kir6.2^{-/-} mice have developed a manner of producing increased ATP with a similar amount of glucosyl would be key in aiding overweight individuals reduce their caloric intake while still maintaining appropriate energetic levels. Thus, it is crucial that the mechanisms by which Kir6.2^{-/-} mice have altered energy metabolism is better understood for they could have a drastic impact on

the treatment of two of the most prominent health issues of our society today.

APPENDIX 1



Appendix 1 from (Basmaji, 2008). Distribution of lactate production after 180 s of fatigue in the absence and presence of DMSO. Individual data points from 3 different researchers performing the same experiment to measure lactate levels in FDB skeletal muscles after 180s of fatigue is shown. A horizontal bar is traced at 49 $\mu\text{mol/g dry weight}$ to delimit the highest value of lactate production obtained in the presence of DMSO at any concentration from 0.01 to 0.5% (v/v). The inset figure shows the frequency of data contained above and below 49 $\mu\text{mol/g dry weight}$ in control solution and control solution containing DMSO at any concentration (Benkhalti, 2009; Li, 2007).

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