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Role of glycogen content on glucose uptake and glycogen synthase in skeletal muscles:

The effect of contraction and insulin

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CONTENTS

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LIST OF PAPERS

This thesis is based on the following papers:

1. Lai YC, Zarrinpashneh E & Jensen J. Additive effect of contraction and insulin on glucose uptake and glycogen synthase in muscles with different glycogen contents. *Manuscript*.

2. Lai YC, Stuenæs JT, Kuo CH & Jensen J (2007). Glycogen content and contraction regulate glycogen synthase phosphorylation and affinity for UDP-glucose in rat skeletal muscle. *Am J Physiol Endocrinol Metab*. 293(6): E1622-E1629.

3. Jensen J & Lai YC (2009). Regulation of muscle glycogen synthase phosphorylation and kinetic properties by insulin, exercise, adrenaline and role in insulin resistance. *Arch Physiol Biochem*. 115(1): 13-21.

4. Lai YC, Lin FC & Jensen J (2009). Glycogen content regulates insulin- but not contraction-mediated glycogen synthase activation in the rat slow-twitch soleus muscles. *Acta Physiol*. 197(2): 139-150.

Note that Paper 1 is the submitted manuscript prior to review and prior to editing by the *Journal of Applied Physiology*. The peer-reviewed and edited version of the article is available at the *Journal of Applied Physiology* online:
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1. INTRODUCTION

Blood glucose concentration fluctuates during a day cycle and it is important to keep blood glucose within normal range. Low levels of blood glucose can result in unconsciousness, lethargy, brain damage, and even death. On the other hand, a persistent elevation in blood glucose can cause diseases and health issues related to diabetes, like limb amputations, blindness, kidney and heart disease (Cohen, 2006). The prevalence of type 2 diabetes is increasing dramatically worldwide and is raising to epidemic proportions (King *et al.*, 1998; Zimmet *et al.*, 2001). This disease not only threatens the quality of human life but also burdens the cost of public health. The rapidly growing number of suffers from type 2 diabetes implies that the current therapeutic approaches are insufficient. In order to improve strategies for preventing and curing type 2 diabetes, a complete understanding of key factors regulating glucose metabolism is warranted.

Blood glucose increases after ingestion of a meal, which stimulates the pancreatic β-cells to secrete insulin to reduce excessive blood glucose. To achieve this goal, insulin suppresses hepatic glucose production and induces glucose uptake in insulin sensitive tissues, including fat, liver and skeletal muscle (Figure 1). Due to its large tissue mass (Janssen *et al.*, 2000), skeletal muscle accounts for most (70-75 %) of postprandial glucose disposal and represents the principal site of insulin-stimulated glucose uptake (DeFronzo, 1988). The metabolic fate of glucose after entering muscle cells is either oxidized to carbon dioxide, converted to lactate, or stored as glycogen. The direct measurement of glycogen synthesis using nuclear magnetic resonance spectroscopy has shown that more than 90 % of glucose taken up by muscle is incorporated into glycogen (Shulman *et al.*, 1990). This result indicates that capacity to store excess glucose as muscle glycogen is critical to maintain optimal blood

glucose homeostasis. Type 2 diabetes is characterized by abnormally high blood glucose and impaired insulin-stimulated glycogen synthesis in skeletal muscle is a major metabolic defect in patients with type 2 diabetes (Shulman *et al.*, 1990).

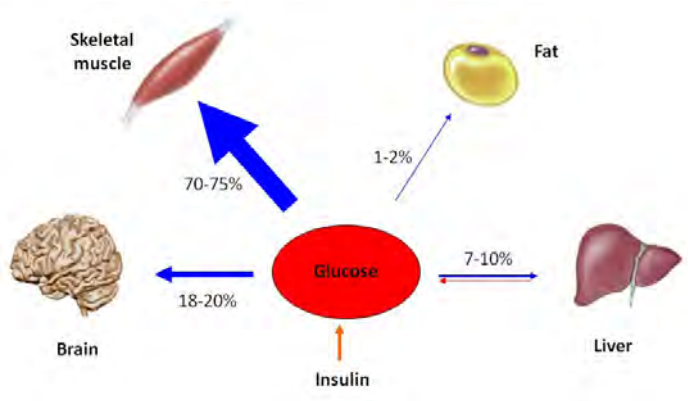

Figure 1. Glucose uptake in different tissues during a hyperinsulinemic euglycemic clamp (DeFronzo, 1988).

Muscle glycogen is one of the major fuel sources providing energy during exercise. The magnitude of glycogen utilization depends on diet, exercise intensity, duration, and oxidative capacity of the working muscle (Sahlin, 1990; Jeukendrup, 2003). At high intensities of prolonged exercise, glycogen is the most important energy substrate and depletion of muscle glycogen store is associated with fatigue (Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967; Sahlin, 1990). The amount of glycogen content in skeletal muscle determines performance, particularly in high intensity and prolonged exercise (Hermansen *et al.*, 1967; Sahlin, 1990). Therefore, repletion of muscle glycogen content during recovery from exercise is an important process. Glycogen content in skeletal muscle is rather stable in resting condition because increased glycogen content can inhibit its own synthesis (Danforth, 1965). However, studies have demonstrated that exhausted exercise followed by a rich

carbohydrate diet can double muscle glycogen content (Bergstrom & Hultman, 1966; Jentjens & Jeukendrup, 2003). This phenomenon is called glycogen supercompensation. Reduction of muscle glycogen, which creates a free space to store excess glucose from the blood stream, is an important characteristic of exercise for improving blood glucose regulation. Thus, decreasing muscle glycogen content is believed to be a key factor for increasing insulin sensitivity.

Glycogen synthesis in skeletal muscle plays an important role in sport performance because rapid replenishment of muscle glycogen is important for next exercise bouts. On the other hand, low muscle glycogen may be beneficial for the regulation of blood glucose homeostasis in type 2 diabetes. The following sections of the introduction will explain the mechanisms for insulin- and contraction-mediated regulation of muscle glucose metabolism and glycogen synthase (GS) activation.

1.1. Muscle glycogen synthesis

Glucose transport

Muscle glycogen synthesis (Figure 2) begins with transport of glucose into muscle cells. This process is mediated by specialized transporters (GLUTs), because glucose is a hydrophilic molecule and needs a facilitated diffusion process to enter cells. It has been identified that there are 14 members of GLUTs in mammals and the principle isoform that mediates glucose transport in skeletal muscle is GLUT4 (Scheepers *et al.*, 2004). In non-stimulated muscle, GLUT4 is sequestered in intracellular storage vesicles. Upon insulin or contraction stimulation, GLUT4 is translocated to the plasma membrane or T-tubules to increase glucose transport (Goodyear *et al.*, 1990; Lund *et al.*, 1995; Ploug *et al.*, 1998). Since GLUT4 has a high affinity for glucose, the number of GLUT4 protein at the cell surface is considered to be the determinant for glucose transport rate. GLUT1 expression is low in skeletal muscle and is located

in the plasma membrane in all conditions (Mueckler, 1994). GLUT1 is thought to mediate basal glucose transport.

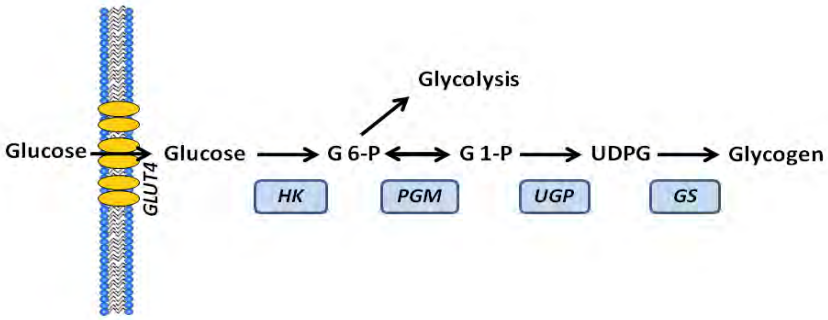

Figure 2. Glycogen synthetic pathway in skeletal muscle. Glucose entry is mainly mediated by GLUT4 and the intracellular glucose is rapidly phosphorylated to G 6-P by HK. Thereafter, glucose can be metabolized through glycolysis or stored as glycogen. In the latter event, GS uses UDPG as a donor to synthesize glycogen. Abbreviations: G 1-P, glucose 1-phosphate; G 6-P, glucose 6-phosphate; GS, glycogen synthase; HK, hexokinase; PGM, phosphoglucomutase; UDPG, uridine diphosphate glucose; UGP, UDP-glucose pyrophosphorylase.

Conversion of glucose into glycogen

Once entering muscle cells, glucose is rapidly phosphorylated by hexokinase to form glucose 6-phosphate. This irreversible reaction traps glucose. Of the two isoforms of hexokinase expressed in skeletal muscle, hexokinase II is the major isoform responsible for glucose phosphorylation (Printz *et al.*, 1993). Glucose 6-phosphate can either turn into glycolysis to produce energy (ATP) for immediate use or turn to glycogen synthesis. In the latter case, glucose 6-phosphate is isomerized by phosphoglucomutase to form glucose 1-phosphate and subsequently converted to UDP-glucose by UDP-glucose pyrophosphorylase. GS uses UDP-glucose as the substrate and adds a glucose molecule to existing glycogen particles via α-1,4 linkage. Glycogen branches are introduced by a branching enzyme. When elongating glycogen chains consist of more than 10 glucose molecules, branching enzyme cleaves the

chain with 7 glucose molecules and re-links it via α-1,6 linkage to form the branches of glycogen (Greenberg *et al.*, 2006). However, an oligosaccharide chain (~10 glucose molecules) has to be formed as a starting point for GS to synthesize glycogen and expand the glycogen granule. Glycogenin is the self-glucosylating protein that attaches the glucose molecule to itself at a tyrosine residue and forms the oligosaccharide chain of ~10 glucose molecules via α-1,4 linkage (Roach, 2002).

Glycogen synthase

GS catalyzes glycogen formation and is a rate-limiting step for glycogen synthesis. A critical role of GS in determining glycogen accumulation in skeletal muscle was evident by following studies. Manchester et al. demonstrated that overexpression of the hyperactive form of GS (both Ser^{7} and Ser^{641} were mutated to Ala) in mouse skeletal muscle was sufficient to increase glycogen content by 5 folds with a normal rate of glucose uptake (Manchester *et al.*, 1996). Conversely, mice lacking functional GS by disruption of *GYS1* gene displayed a lack of muscle glycogen (Pederson *et al.*, 2004). In a more physiological condition, Danforth found that increased glycogen content inhibits its own synthesis through inhibition of GS activity (Danforth, 1965). A number of studies have confirmed these findings (Nielsen *et al.*, 2001; Jensen *et al.*, 2006). Importantly, Jensen et al. (Jensen *et al.*, 2006) demonstrated that high glycogen content decreases insulin-stimulated glycogen synthesis and GS activation without decreasing insulin-stimulated glucose uptake. These findings highlight the important role of GS in the regulation of insulin-stimulated glycogen synthesis. In addition to the finding that glycogen content regulates GS activity, Danforth also found that contraction decreases glycogen and increases GS activity (Danforth, 1965). Contraction-mediated GS activation was also found to be inversely associated with glycogen content (Nielsen *et al.*, 2001). Furthermore, adrenaline stimulates glycogen

breakdown and infusion of adrenaline has been shown to increase GS activity in skeletal muscle (Jensen *et al.*, 2005). These studies not only show the key role of GS in glycogen synthesis but also show that glycogen content is a potent regulator of GS.

1.1.1. Regulation of glycogen synthase

GS was the first example of an enzyme that conserves multiple phosphorylation sites and several kinases are able to phosphorylate these sites (Cohen, 1993). Moreover, GS was the first reported intracellular target protein of insulin (Villar-Palasi & Larner, 1960). GS can also be regulated by muscle contraction and glycogen content (Danforth, 1965). It is believed that insulin and contraction, as well as glycogen content, regulate protein kinases or/and phosphatase activities to modify GS phosphorylation and activity.

GS activity is regulated by phosphorylation of nine serine and by several allosteric effectors, most notably glucose 6-phosphate (Cohen, 1993; Lawrence & Roach, 1997). Phosphorylation of GS decreases enzymatic activity but high concentration of glucose 6-phosphate can restore full activity even in highly phosphorylated GS (Lawrence & Roach, 1997). Conversely, dephosphorylation of GS increases enzymatic activity in the absence of glucose 6-phosphate. Indeed, dephosphorylation of GS increases GS affinities for both glucose 6-phosphate and UDP-glucose (see section about glycogen synthase kinetic properties), which contribute to the enzyme activation (Roach *et al.*, 1976). Site 2 and 2a, corresponding to Ser^{7} and Ser^{10}, are located near the NH_2-terminus of GS. Site 3a, 3b, 3c, 4, 5, 1a, and 1b, corresponding to Ser^{641}, Ser^{645}, Ser^{649}, Ser^{653}, Ser^{657}, Ser^{697}, and Ser^{710}, are located near the COOH-terminus of GS (Figure 3). The nine phosphorylation sites, which theoretically allow 512 combinations of phosphorylation patterns, could cause many different states of GS with different kinetic properties. Indeed, the effect of the

individual phosphorylation sites on GS activity has been tested by site-directed mutagenesis (Ser → Ala which mimics dephosphorylation) on rabbit muscle GS expressed in COS cells (Skurat *et al.*, 1994; Skurat & Roach, 1995). These studies with mutated GS indicate that both NH_2- and COOH-terminal phosphorylation sites are important in regulating GS activity. It is important to note that mutations on GS Ser^{641} and Ser^{645} have the strongest effect on GS activation (Skurat *et al.*, 1994; Skurat & Roach, 1995).

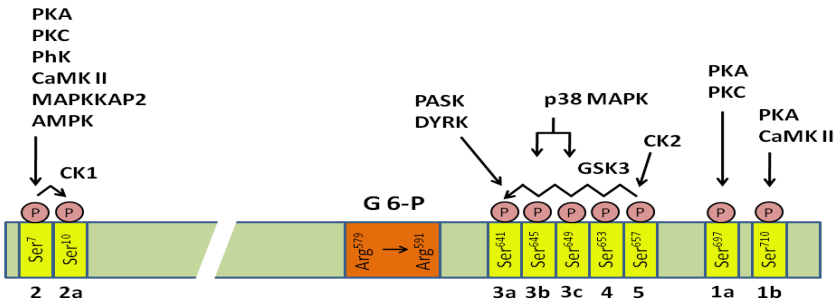

Figure 3. Muscle GS is phosphorylated at nine serine residues by multiple kinases. G 6-P allosterically activates GS through the binding of an argine-rich domain ($Arg^{579\text{-}591}$). Abbreviations: AMPK, AMP-activated protein kinase; CaMK II, calmodulin-dependent kinase II; CK1, casein kinase 1; CK2, casein kinase 2; DYRK, dual-specificity tyrosine phosphorylated and regulated protein kinase; GSK3, glycogen synthase kinase 3; MAPKKAP2, MAPK-activated protein kinase 2; p38 MAPK, p38 mitogen-activated protein kinase; PASK, Per-Arnt-Sim domain kinase; PhK, phosphorylase kinase; PKA, protein kinase A or cyclic AMP-dependent protein kinase; PKC, protein kinase C.

Protein kinases that phosphorylate glycogen synthase

A series of kinases have been reported to phosphorylate various sites of GS (Figure 3). Ser^7 can be phosphorylated by PKA, PKC, CaMK II, MAPKKAP2, AMPK, and PhK (Carling & Hardie, 1989; Roach, 1990; Stokoe *et al.*, 1992; Cohen, 1999). Phosphorylation of Ser^7 acts as a recognition motif for subsequent phosphorylation of Ser^{10} by CK1. This phenomenon has been termed hierarchal phosphorylation (Roach, 1990). GSK3 is the predominant protein kinase of GS phosphorylation and it also

exhibits the hierarchal mechanism (Roach, 1990). CK2 needs to phosphorylate Ser^{657} to create the recognition motif for GSK3 to phosphorylate Ser^{653}, Ser^{649}, Ser^{645}, and Ser^{641} (Figure 3). However, it is possible to phosphorylate GS at Ser^{641}, Ser^{645} and Ser^{649} independently of prior phosphorylation. For example, DYRK and PASK have been reported to directly phosphorylate GS Ser^{641} (Skurat & Dietrich, 2004; Wilson *et al.*, 2005) and p38 MAPK to phosphorylate GS Ser^{645} and Ser^{649} (Kuma *et al.*, 2004).

Protein phosphatases that dephosphorylate glycogen synthase

GS can be dephosphorylated by protein phosphatase 1 (PP1) and protein phosphatase 2A *in vitro* (Ragolia & Begum, 1998). However, PP1 is believed to be the only phosphatase responsible for GS dephosphorylation *in vivo* (Cohen, 1993; Lawrence & Roach, 1997). The current model for the regulation of PP1 activity is through interaction with regulatory subunits (Ragolia & Begum, 1998; Newgard *et al.*, 2000). Some PP1-regulatory subunits target to glycogen. These glycogen targeting subunits serve as molecular scaffolds to attach PP1 to glycogen particles which allows PP1 to dephosphorylate GS (Newgard *et al.*, 2000; Brady & Saltiel, 2001). Five forms of glycogen targeting subunits of PP1, namely muscle-specific glycogen targeting subunit (G_M/R_{GL}; gene *PPP1R3A*), liver-specific glycogen targeting subunit (G_L; gene *PPP1R3B*), protein targeting to glycogen (PTG; gene *PPP1R3C*), the ubiquitous isoform R6 (gene *PPP1R3D*), and R3E (gene *PPP1R3E*), are expressed in skeletal muscles (Cohen, 2002; Montori-grau *et al.*, 2007). G_M/R_{GL} is the primary form expressed in rodent skeletal muscle (Newgard *et al.*, 2000; Brady & Saltiel, 2001). The role of G_M/R_{GL} in the regulation of GS has been demonstrated by two genetic studies. Muscles from G_M/R_{GL} knockout mice display low GS activity and low glycogen content (Suzuki *et al.*, 2001). In contrast, GS is constantly dephosphorylated and activated in muscle from G_M/R_{GL} overexpressing mice, and muscle glycogen

content in these mice is concurrently elevated (Aschenbach *et al.*, 2001).

The fact that so many kinases are capable of mediating GS phosphorylation reflects the crucial role of GS phosphorylation in regulation of enzyme activity under different physiological situations. Regulation of GS activity is far from understood and it is important to illuminate the phosphorylation pattern in response to different physiological stimuli and to characterize how the different phosphorylation statuses influence GS activity.

Allosteric regulation of glycogen synthase

GS is regulated by several metabolic effectors. For example, ATP, ADP, AMP, P_i, and UDP are allosteric inhibitors and Mg^{2+}, UDP-glucose, and glucose 6-phosphate are allosteric activators (Roach & Larner, 1976). However, glucose 6-phosphate is considered the most important allosteric activator (Cohen, 1993; Lawrence & Roach, 1997). Basal concentration of glucose 6-phosphate in muscle is ~0.15 mM (Rothman *et al.*, 1992; Aslesen & Jensen, 1998) so changing this metabolite concentration can easily change GS activity. Insulin and exercise are able to increase intracellular level of glucose 6-phosphate (Rothman *et al.*, 1992; Price *et al.*, 1996) and it is therefore possible that GS is activated by glucose 6-phosphate-mediated allosteric activation. The structure of mammalian GS has not been solved, but mutagenetic studies have suggested that a region (Arg^{579}-Arg^{591}) of GS comprises a binding domain for glucose 6-phosphate (Pederson *et al.*, 2000; Hanashiro & Roach, 2002). However, GS with glucose 6-phosphate binding domain mutated does not respond to phosphorylation modification in the same manner as normal GS (Pederson *et al.*, 2000). The role of glucose 6-phosphate-mediated GS activation has not been clarified in physiological conditions. Covalent modification is also a key mechanism that determines GS activity *in vivo*.

1.1.2. Glycogen synthase kinetic properties

Affinity for glucose 6-phosphate

GS affinity for glucose 6-phosphate is inversely associated with the degree of GS phosphorylation (Roach *et al.*, 1976). GS *Ka* is ~2 mM in rested skeletal muscle (Thorburn *et al.*, 1991; Henry *et al.*, 1996). Dephosphorylated GS (through incubation with purified muscle phospho-protein phosphatase) had a *Ka* of 0.02 mM, whereas highly phosphorylated GS (achieved through incubation with cyclic AMP-independent glycogen synthase kinase 1) increased the *Ka* to 3.2 mM (Ahmad & Huang, 1981). Insulin and exercise increase GS affinity for glucose 6-phosphate (Kochan *et al.*, 1981; Thorburn *et al.*, 1991; Henry *et al.*, 1996), which implies that dephosphorylation activates GS by increasing the affinity for glucose 6-phosphate.

The most accurate way to evaluate GS affinity for glucose 6-phosphate is to measure activity with different concentrations of glucose 6-phosphate (a dose-response curve), but it is laborious to do so. GS activity measured with zero (I form) or low concentrations (fractional activity) of glucose 6-phosphate is dependent on the degree of GS phosphorylation. This means that GS activity measured without and with low concentrations of glucose 6-phosphate are important physiological parameters (Table 1). In contrast, GS activity in the presence of high concentrations of glucose 6-phosphate is independent of phosphorylation state. The concentrations of glucose 6-phosphate required to activate GS to maximal activity can be 5-12 mM, which far exceeds the physiological concentration in skeletal muscles (Rothman *et al.*, 1992; Aslesen & Jensen, 1998). GS activity measured with high concentrations of glucose 6-phosphate is termed total activity. GS I-form and fractional activity are often given a percent of total activity (Table 1). However, fractional activity measured with physiological concentrations (0.1-0.3 mM) of glucose 6-phosphate is proposed to be a more accurate way to describe GS activation than I form (Guinovart *et al.*, 1979).

Table 1. Glycogen synthase activity

Total activity: GS activity at 5-12 mM of G 6-P (phosphorylation independent)
I form (%): $\frac{\text{GS activity at 0 mM of G 6-P (phosphorylation dependent)}}{\text{GS total activity}} \times 100$
Fractional activity (%): $\frac{\text{GS activity at 0.1-0.3 mM of G 6-P (phosphorylation dependent)}}{\text{GS total activity}} \times 100$

Affinity for UDP-glucose

GS affinity for UDP-glucose is also regulated. Dephosphorylation of GS decreases the *Km* for UDP-glucose (Roach *et al.*, 1976). However, this part of regulation on GS has received little attention. The concentration of UDP-glucose is relatively low (0.03-0.06 mM) in skeletal muscle (Piras & Staneloni, 1969; Reynolds *et al.*, 2005), whereas GS *Km* is ~0.4 mM in basal condition (Rossetti & Hu, 1993). Therefore, decreased *Km* can be an important mechanism for increasing GS activity under physiological situations. Furthermore, increased concentration of glucose 6-phosphate in the assay buffer increased the affinity of GS for UDP-glucose (Rossetti & Hu, 1993), which implies that GS affinity for UDP-glucose is regulated by both phosphorylation and glucose 6-phosphate concentration. For many years, it has been known that GS affinity for UDP-glucose is increased by insulin (Rossetti & Hu, 1993). However, it has not been studied whether contraction increases GS affinity for UDP-glucose. Kochan et al. suggested use of a physiological concentration (0.03 mM) of UDP-glucose for measurement of GS fractional activity to study the effect of exercise on skeletal muscle GS activation (Kochan *et al.*, 1981). This suggestion also implies that GS affinity for UDP-glucose is regulated by muscle contraction.

1.2. Insulin-stimulated glucose uptake and glycogen synthesis

Insulin has a wide variety of biological effects in skeletal muscle, including regulation of glycogen synthesis, glucose transport, protein synthesis, and gene expression (Taniguchi *et al.*, 2006). Promotion of glycogen synthesis is one of the most important processes of insulin in skeletal muscle. For insulin-stimulated muscle glycogen synthesis, glucose has to be transported across the cell membrane, phosphorylated to glucose 6-phosphate by hexokinase, and converted to UDP-glucose before GS attaches the glucose molecule to the glycogen particle. Determination of a single process as the rate-limiting step for glycogen synthesis is difficult because these processes are usually well matched. However, it is generally believed that both stimulation of glucose uptake and activation of GS contribute to the control of glycogen synthesis in skeletal muscle (Lawrence & Roach, 1997; Fisher *et al.*, 2002). In the following sections, I will discuss the mechanisms for insulin-stimulated glucose uptake and GS activation

1.2.1. Insulin-stimulated glucose uptake

The intracellular action of insulin is transmitted through its signalling pathways by phosphorylation and protein-protein interactions, which regulate the activation of the key proteins and enzymes. Muscle glucose uptake is a three-step process that includes glucose delivery, transport, and phosphorylation (Richter *et al.*, 2001). However, glucose transport is believed the key regulatory step (Shulman, 2000; Wallberg-Henriksson & Zierath, 2001). Figure 4 presents a current model of the insulin signalling pathway to GLUT4 translocation and glucose transport in skeletal muscle.

Insulin receptor and insulin receptor substrates

The insulin signalling pathway to GLUT4 translocation is initiated by the binding of insulin to its receptor, followed by receptor autophosphorylation. Insulin activates the receptor tyrosine kinase activity which also phosphorylates insulin receptor substrates (IRSs)(Shepherd, 2005). Of the insulin receptor substrate family, IRS1 appears to be the predominant isoform mediating insulin-stimulated glucose uptake in skeletal muscle (Yamauchi *et al.*, 1996).

PI3K

Tyrosine phosphorylation of IRS1 allows IRS1 to bind to class I A phosphatidylinositol 3-kinase (PI3K). Class I A PI3K is a heterodimeric lipid kinase consisting of a regulatory subunit (p85) and a catalytic subunit (p110). The activation of the catalytic subunit of PI3K occurs when the regulatory subunit binds to IRS proteins. Binding p85 to IRS1 will 1) induce a change in the conformation of the enzyme and 2) bring PI3K to its substrates (located at the plasma membrane) and subsequently facilitate the formation of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). Inhibition of PI3K with wortmannin and LY294002 has demonstrated its essential role in insulin-stimulated GLUT4 translocation and glucose uptake in skeletal muscle (Lund *et al.*, 1995; Yeh *et al.*, 1995; Nolte *et al.*, 2003).

PKB

Activated PI3K catalyzes the formation of PIP3, which acts as a platform for recruitment of pleckstrin-homology domain containing proteins, like phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (PKB, also named Akt)(Shepherd, 2005). PKB is activated through phosphorylation at Thr^{308} by PDK1 (Cohen *et al.*, 1997), but full activation of PKB requires additional

phosphorylation at Ser^{473} (Alessi *et al.*, 1996). This site is believed to be phosphorylated by the mammalian target of rapamycin in complex with Rictor, LST8 and SIN1 (mTORC2)(Sarbassov *et al.*, 2005). PKB exists in three isoforms and it is now clear that PKBβ is the major isoform involved in glucose homeostasis. A critical role of PKBβ in insulin-stimulated glucose uptake was appreciated by 1) ablation of PKBβ using siRNA decreases insulin-stimulated glucose uptake (Jiang *et al.*, 2003) and 2) PKBβ knockout mice are glucose intolerant and have decreased insulin-stimulated glucose uptake in skeletal muscle (Cho *et al.*, 2001; McCurdy & Cartee, 2005).

AS160 and TBC1D1

The signalling communication between PKB and GLUT4 traffic is beginning to emerge. AS160 (Akt substrate of 160 kDa; also called TBC1D4) is a Rab GTPase activating protein (GAP) and was identified to be involved in insulin-stimulated glucose transport (Sano *et al.*, 2003). The current idea is that AS160 is unphosphorylated in the basal state and retains GLUT4 vesicles intracellularly. Upon insulin stimulation, AS160 becomes phosphorylated which inhibits its GAP activity and promotes GTP binding to Rabs, thereby allowing translocation of GLUT4 vesicles to plasma membrane (Larance *et al.*, 2005).

AS160 is also called TBC1D4. Recently TBC1D1, a Rab GAP related to AS160, was shown also to regulate GLUT4 translocation (Roach *et al.*, 2007). TBC1D1 knockdown increased GLUT4 translocation in L6 myotubes (Ishikura & Klip, 2008) and studies have reported that TBC1D1 expression was high in skeletal muscle especially with fast-twitch feature (Chavez *et al.*, 2008; Taylor *et al.*, 2008), which suggest that TBC1D1 may be an important component for the regulation of GLUT4 translocation in skeletal muscle. Although the functional significance of

TBC1D1 phosphorylation is currently uncertain, insulin has been shown to phosphorylate TBC1D1 in skeletal muscle (Taylor *et al.*, 2008; Funai & Cartee, 2009) and the insulin-stimulated TBC1D1 phosphorylation was PI3K dependent (Funai & Cartee, 2009).

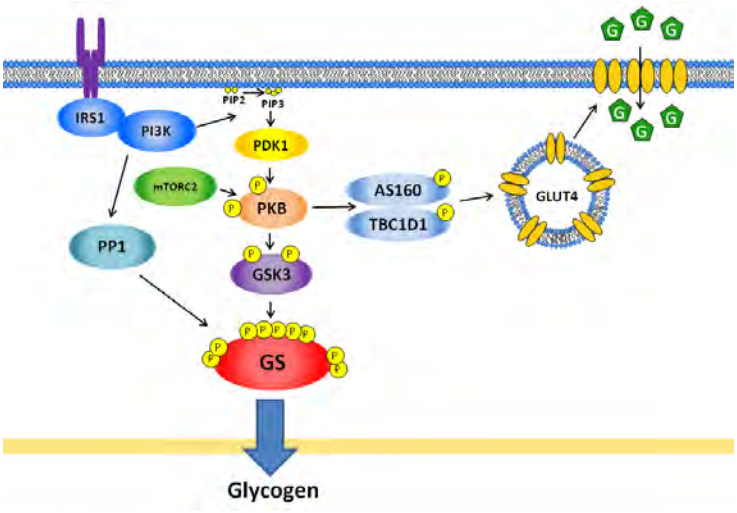

Figure 4. Insulin signalling to increase glucose transport and glycogen synthase activity in skeletal muscle.

1.2.2. Insulin-stimulated glycogen synthase activation

Under insulin stimulation, the majority of glucose uptake occurs in skeletal muscle and is incorporated into glycogen (Shulman *et al.*, 1990). GS catalyzes the final step where UDP-glucose is incorporated into glycogen. Insulin activates GS by dephosphorylation. Dephosphorylation of a protein can be achieved either by decreasing a kinase activity or increasing a protein phosphatase activity.

GSK3

Insulin stimulates GS activation through dephosphorylation of GS Ser^{641}, Ser^{645}, and Ser^{649} (Parker *et al.*, 1983). These sites are phosphorylated by GSK3. The signalling

pathway (Figure 4) for insulin-mediated GS activation has been described to involve the inhibition of GSK3 (Cohen, 1999). Phosphorylation of GSK3α at $Ser^{21}$and GSK3β at Ser^{9} inhibits GSK3 activity; PKB phosphorylates these two sites (Cross *et al.*, 1995). Therefore, insulin-mediated phosphorylation and inhibition of GSK3 will lead to less phosphorylation of GS which increases enzymatic activity (Cross *et al.*, 1995)(Figure 3). The critical role of GSK3 in insulin-mediated GS activation was demonstrated in GSK3α $Ser^{21}$and β Ser^{9} knockin mice (McManus *et al.*, 2005). In GSK3 knockin mice, PKB was unable to phosphorylate and inhibit GSK3 activity; as a result, insulin was unable to dephosphorylate and activate GS (McManus *et al.*, 2005; Bouskila *et al.*, 2008). In the same study, McManus et al. also demonstrated that phosphorylation of GSK3β Ser^{9} is the isoform that leads to GS dephosphorylation and activation in skeletal muscle (McManus *et al.*, 2005).

PP1

All phosphorylation sites of GS are believed to be dephosphorylated by PP1 (Cohen, 1993; Lawrence & Roach, 1997). PP1 is also regulated by insulin (Brady & Saltiel, 2001). It is therefore possible that insulin induces GS dephosphorylation through the activation of PP1. However, some reports have questioned the role of G_M/R_{GL}-regulated PP1 activity in insulin-mediated GS activation (Aschenbach *et al.*, 2001; Suzuki *et al.*, 2001). Results from these reports showed that insulin-stimulated GS activation was the same in muscles from G_M/R_{GL} knockout and wild-type mice (Suzuki *et al.*, 2001), which suggests that G_M/R_{GL}-regulated PP1 activity is not necessary in insulin-mediated GS activation. Other PP1 targeting subunits (ex. G_L, PTG, and *PPP1R6*) may play a role in this event, but no study has demonstrated that insulin-stimulated GS activation in skeletal muscle requires G_L-, PTG-, R6-, or R3E.

1.3. Contraction-stimulated glucose uptake and glycogen synthesis

During exercise muscle glycogen is broken down and glucose uptake is increased to satisfy the energy demand of contracting muscles. After cessation of exercise, muscle glycogen starts to replenish. For re-synthesis of muscle glycogen, both glucose uptake and GS activity have to be increased. The following sections will introduce the potential proteins that have been suggested to mediate contraction-stimulated glucose uptake and GS activation in skeletal muscle (Figure 5).

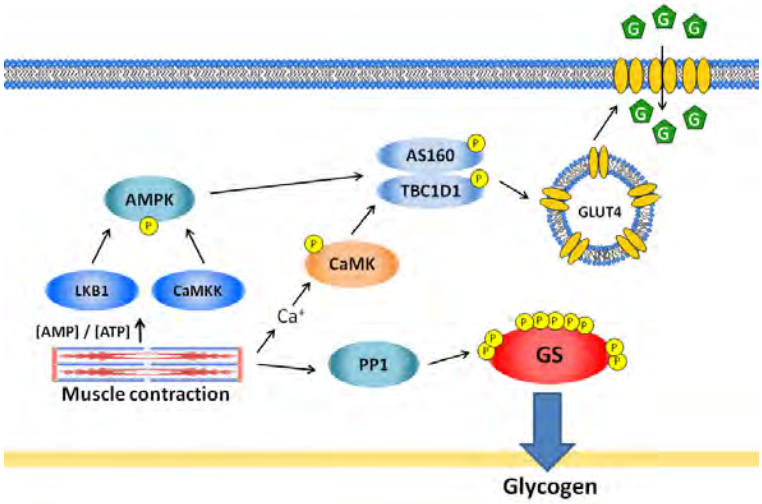

Figure 5. Contraction signalling to increase glucose uptake and glycogen synthase activity in skeletal muscle.

1.3.1. Contraction-stimulated glucose uptake

Contraction increases skeletal muscle glucose uptake by promoting the recruitment of GLUT4 from intracellular compartments to the plasma membrane and t-tubules (Lund *et al.*, 1995; Ploug *et al.*, 1998, Etgen, 1997). Contraction can stimulate glucose uptake in the absence of insulin (Ploug *et al.*, 1984; Aslesen *et al.*, 2001) and early studies have demonstrated that contraction stimulates glucose uptake through distinct signalling mechanisms from insulin. For example, contraction did not increase insulin

receptor and IRS1 tyrosine phosphorylation (Goodyear *et al.*, 1995; Whitehead *et al.*, 2000), and deletion of muscle specific insulin receptor did not reduce exercise-stimulated glucose uptake (Wojtaszewski *et al.*, 1999). Furthermore, inhibition of PI3K with wortmannin, which inhibited insulin-stimulated glucose uptake, did not impair contraction-stimulated glucose uptake (Lund *et al.*, 1995; Whitehead *et al.*, 2000).

The distinction of the initial signalling pathways for insulin- and contraction-stimulated GLUT4 translocation is clinically relevant. Patients with type 2 diabetes, whose insulin action on muscle glucose uptake is impaired, have intact contraction-stimulated glucose uptake (Kennedy *et al.*, 1999). This may suggest that contraction signalling pathways can be used as therapeutic targets to bypass the impaired insulin action in treating type 2 diabetics. However, unlike insulin, the essential signalling pathways that lead to GLUT4 translocation by muscle contraction are largely undefined, but some candidates have been involved in this event.

Ca^{2+} dependent signaling

Increases in intracellular Ca^{2+} level during muscle contraction have for a long time been hypothesized to be involved in the induction of glucose uptake. Caffeine, which causes Ca^{2+} release from the sarcoplasmic reticulum, has been shown to increase glucose uptake without inducing muscle contraction (Youn *et al.*, 1991). In contrast, dantrolene, which blocks Ca^{2+} release, blocks caffeine-induced glucose uptake (Youn *et al.*, 1991). These early studies provide evidence that a rise in cytosolic Ca^{2+} is able to increase muscle glucose uptake.

The signalling mechanisms by which Ca^{2+} signalling mediates glucose uptake are still uncertain, but Ca^{2+}/calmodulin dependent signalling molecules may be involved. For example, inhibition of Ca^{2+}/calmodulin dependent protein kinase

(CaMK) signalling with CaMKs inhibitors (KN62 and KN93) or inhibition of CaMK kinase by STO-609 has been shown to decrease contraction-stimulated muscle glucose uptake (Wright *et al.*, 2004; Wright *et al.*, 2005; Jensen *et al.*, 2007). However, incubation of muscles with KN63 has also been shown to reduce insulin-stimulated glucose transport (Brozinick *et al.*, 1999). Clearly, more studies are needed to verify the link between Ca^{2+}/calmodulin dependent signalling and glucose uptake during muscle contraction.

AMPK signaling

AMPK has received much attention in the regulation of contraction-stimulated glucose uptake. AMPK is a sensor of cellular energy charge (Hardie, 2007). During metabolic stresses and periods of energy demand, such as exercise and muscle contraction, AMPK is activated by the increase in ATP consumption which leads to a rise in AMP/ATP ratio (Towler & Hardie, 2007). Although ADP is the straight product of ATP hydrolysis during contraction, ADP is rapidly converted to AMP through adenylate kinase reaction. AMPK is a heterotrimer comprised of a catalytic α subunit and β and γ regulatory subunits (Hardie, 2007). AMPK is allosterically activated when AMP binds to its γ subunits (Towler & Hardie, 2007). Phosphorylation of Thr^{172} on α subunits is required for high catalytic activity (Hawley *et al.*, 1996). Three AMPK kinases (LKB1, Ca^{2+}/calmodulin dependent protein kinase kinase β, and transforming growth factor-β-activated kinase 1) have been identified. LKB1 appears to be the major AMPK kinase in skeletal muscle and deletion of LKB1 results in a marked decrease in Thr^{172} phosphorylation (Sakamoto *et al.*, 2005). AMPK phosphorylation is also regulated by phosphatase activity. It is widely accepted that binding of AMP activates AMPK because AMPK bound to AMP becomes a poor substrate for protein phosphatase 2C, and thereby increasing AMPK phosphorylation

and activity (Sanders *et al.*, 2007; Richter & Ruderman, 2009).

The hypothesis that AMPK mediates contraction-stimulated glucose uptake came from the following findings 1) contraction-mediated glucose transport coincides with increased AMPK activity (Hayashi *et al.*, 1998), 2) AMPK activity is positively correlated with the level of contraction-stimulated glucose uptake (Ihlemann *et al.*, 1999), and 3) 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a pharmaceutical AMPK activator, increases GLUT4 translocation and glucose uptake independent of insulin signalling pathways (Merrill *et al.*, 1997; Hayashi *et al.*, 1998; Kurth-Kraczek *et al.*, 1999). Genetic modulations with overexpression of dominant negative form of AMPKα2 and knockout of either AMPKα1 or α2 in mice were used to evaluate the role of AMPK in contraction-stimulated glucose uptake. These results showed that inhibition of AMPK partially reduced contraction-stimulated glucose uptake (Mu *et al.*, 2001; Jorgensen *et al.*, 2004b; Fujii *et al.*, 2005), providing the evidence that AMPK is involved in contraction-stimulated glucose uptake. Deletion of LKB1 which prevents AMPK activation in skeletal muscle also partially inhibited contraction-stimulated glucose uptake (Sakamoto *et al.*, 2005). These available data strongly suggest that LKB1-AMPK signalling is involved in contraction-mediated glucose uptake in skeletal muscle.

AS160 and TBC1D1

Recent work has suggested that AS160 and TBC1D1 are downstream targets of AMPK and may mediate contraction-stimulated glucose uptake (Cartee & Wojtaszewski, 2007; Sakamoto & Holman, 2008). Stimulation of AMPK with AICAR or contraction increased glucose uptake and at the same time increased AS160 and TBC1D1 phosphorylation (Bruss *et al.*, 2005; Funai & Cartee, 2008; Taylor *et al.*, 2008). These studies suggest that AMPK may induce GLUT4 translocation by

increasing AS160 and TBC1D1 phosphorylation. Importantly, overexpression of mutant AS160 (four phosphorylation sites mutated) in mouse muscle has been shown to decrease contraction-stimulated glucose uptake (Kramer *et al.*, 2006b), which highlights the role of AS160 in contraction-stimulated glucose uptake. Although direct evidence showing that TBC1D1 is involved in contraction-mediated glucose uptake is unavailable, one recent study has shown that contraction-stimulated TBC1D1 phosphorylation was AMPK dependent, and inhibition of AMPK diminished both contraction-stimulated glucose transport and TBC1D1 phosphorylation (Funai & Cartee, 2009).

Both AS160 and TBC1D1 conserve a calmodulin binding domain (CBD) and because of this, AS160 and TBC1D1 may also be a link between Ca^{2+}/calmodulin dependent signalling and contraction-stimulated GLUT4 translocation (Sakamoto & Holman, 2008). One study has shown that mutation of CBD on AS160 (AS160 cannot bind to calmodulin in the presence of Ca^{2+}) reduced contraction-, but not insulin-, stimulated glucose uptake (Kramer *et al.*, 2007). Taken together, AS160 and TBC1D1 may be a convergent point between AMPK and Ca^{2+} signalling pathways in the regulation of contraction-mediated GLUT4 translocation.

1.3.2. Contraction-stimulated glycogen synthase activation

Glycogen repletion takes place in glycogen depleted muscle after contractile activity. The regulation of glycogen re-synthesis involves the activation of GS (Nielsen & Richter, 2003). Indeed, synthesis and breakdown of glycogen occur at the same time and GS is in fact activated during exercise (Shulman & Rothman, 2001). It was already recognized in the 1960s that skeletal muscle GS is activated in response to contraction (Danforth, 1965), but the mechanisms have not been fully explained.

GSK3

Contraction-stimulated GS activity has been shown to be independent of insulin receptor and PI3K (Wojtaszewski *et al.*, 1999; Sakamoto *et al.*, 2002). These results suggest that contraction activates GS through different signalling mechanisms from insulin. In line with this, GSK3 knockin mice has given conclusive evidence that contraction-stimulated GS activation does not required GSK3α Ser^{21} and β Ser^{9} phosphorylation and inhibition (McManus *et al.*, 2005). Despite this, contraction still appeared to activate GS by dephosphorylation of GS Ser^{641} and Ser^{645} (Sakamoto *et al.*, 2003; McManus *et al.*, 2005; Ruzzin & Jensen, 2005) and these sites are mainly phosphorylated by GSK3. This suggests that contraction-mediated PP1 activation is important for GS dephosphorylation and activation (will be discussed later)

AMPK

AMPK has been proposed as a potential kinase for GS. Carling and Hardie were the first to show in cell free condition (Carling & Hardie, 1989) that AMPK phosphorylates purified GS at Ser^{7}, which reduces GS activity. A recent study showed that treatment with AICAR, which activates AMPK, increased GS Ser^{7} phosphorylation and inactivated GS (Jorgensen *et al.*, 2004a). Furthermore, GS Ser^{7} phosphorylation was reduced in muscles with deletion of AMPKα2. As an energy sensor, increased AMPK activity, which phosphorylates GS, should theoretically decrease glycogen synthesis. However, treatment of rats with AICAR actually increased their muscle glycogen content (Buhl *et al.*, 2001). This discrepancy needs to be clarified. AMPK is activated by muscle contraction (Hayashi *et al.*, 1998) and a question will be whether GS Ser^{7} phosphorylation is increased by muscle contraction.

CaMK II

CaMK II can phosphorylate purified GS at Ser^7 *in vitro* (Roach, 1990). A recent study has reported that GS was phosphorylated by sarcoplasmic reticulum bound CaMK II in fast-twitch muscle of rabbit (Sacchetto *et al.*, 2007). CaMK II is considered a major mediator of Ca^{2+} signalling. A study has shown that contraction can rapidly activate CaMK II by increasing its phosphorylation (Rose *et al.*, 2007). Thus, contraction is possible to increase GS phosphorylation at Ser^7 via CaMK II activation.

Other kinases

It has been suggested that other kinases, like PKA, PKC, and PhK, are able to phosphorylate GS Ser^7 and these kinases may be activated by exercise. PKA is important for adrenaline-mediated GS inactivation, but PKA activity is not increased by contraction. These kinases will not be discussed further as there is no clear evidence showing that they are important for contraction-mediated GS regulation.

PP1

The mechanisms of contraction-stimulated GS activation are most likely through increased phosphatase activity. PP1 activity is controlled by the binding of regulatory subunits and one study has provided evidence that G_M/R_{GL} is required for contraction-stimulated GS activation in skeletal muscle (Aschenbach *et al.*, 2001). In muscle lacking G_M/R_{GL}, contractile activity was not able to activate GS (Aschenbach *et al.*, 2001). These results strongly suggest that G_M/R_{GL}-mediated PP1 activity is involved in the regulation of contraction-mediated GS dephosphorylation and activation. However, the detailed mechanisms by which contraction-mediated PP1 activation activates GS are unknown.

The effect of contraction on GS phosphorylation status in skeletal muscle has

not been carefully studied. Mass spectrometry has previously been used to investigate GS phosphorylation but operating a mass spectrometer is not easy. Now a panel of phospho-specific antibodies against GS has been developed (Table 2). These phospho-specific GS antibodies have made the investigation of GS phosphorylation much easier. Phosphorylation at GS Ser^{641}, Ser^{645}, $Ser^{641,645}$, and $Ser^{645, 649, 653, 657}$ was reported to be reduced in response to muscle contraction (Sakamoto *et al.*, 2003; McManus *et al.*, 2005; Ruzzin & Jensen, 2005). However, GS has nine phosphorylation sites and each site may contribute to its enzymatic activity. Furthermore, increased phosphorylation on one site may influence the phosphorylation of other sites. A detailed understanding of contraction-mediated GS phosphorylation status will be very useful, in order to identify the critical targets that regulate GS activity in response to muscle contraction.

Table 2. A list of phospho-specific antibodies against muscle glycogen synthase

Target	Commercialized
Phospho-GS Ser^{7}	-
Phospho-GS $Ser^{7,10}$	-
Phospho-GS Ser^{641}	+
Phospho-GS Ser^{645}	+
Phospho-GS $Ser^{641,645}$	+
Phospho-GS $Ser^{645,649,653,657}$	+
Phospho-GS Ser^{697}	-
Phospho-GS Ser^{710}	-
GS Total	+

1.4. Effect of contraction on insulin-stimulated glucose uptake and glycogen synthesis

Insulin and contraction are the two most important physiological stimuli for glucose metabolism in skeletal muscle. Combination of insulin and contraction can cause an

additive effect on glucose metabolism. The enhanced effect of contraction on insulin-stimulated glucose uptake can occur during and after contraction. However, the enhanced effect of contraction on insulin-stimulated glycogen synthesis can only occur after contraction. Glycogen re-synthesis after exercise occurs in two phases (Maehlum *et al.*, 1977; Garetto *et al.*, 1984). During the first phase (1-2 h) of glycogen re-synthesis after contraction, glycogen content is restored relatively rapid but incompletely in the absence of insulin (Franch *et al.*, 1999). When insulin is present, glycogen re-synthesis occurs rapidly and the level of glycogen content can be restored to the basal level within a few hours in rat skeletal muscle (Franch *et al.*, 1999). During the following second phase of glycogen re-synthesis, contraction-stimulated glycogen synthesis is low, but insulin-stimulated glycogen synthesis is increased (Franch *et al.*, 1999). This increased insulin action can last for many hours (Richter *et al.*, 1982, 1984). In the following sections, I will discuss the effect of insulin on glucose uptake and GS activity during contraction, immediately after contraction, and several hours after contraction.

1.4.1. Insulin-stimulated glucose uptake and glycogen synthase activation during contraction

Insulin-stimulated glucose uptake during contraction

Contraction performed simultaneously with insulin stimulation causes an additive increase in glucose uptake (Nesher *et al.*, 1985; Lund *et al.*, 1995; Whitehead *et al.*, 2000). GLUT4 content on the cell surface is also additively increased when muscles were stimulated by both contraction and insulin (Lund *et al.*, 1995). This additive increase in glucose uptake is probably because distinct signalling mechanisms are used by contraction and insulin to stimulate GLUT4 translocation (Lund *et al.*, 1995; Holloszy & Hansen, 1996; Jessen & Goodyear, 2005). As described above, insulin

stimulates GLUT4 translocation through a signalling pathway that involves PI3K and PKB. On the other hand, contraction increases GLUT4 translocation possibly through AMPK and Ca^{+} signalling pathways, but PI3K independent. Since both insulin and contraction rely on the GLUT4 translocation to increase glucose uptake, it is possible that two initially distinct mechanisms integrate at a common, distal component. AS160 and TBC1D1 have been suggested as a convergent point between insulin and contraction signalling to GLUT4 translocation (Kramer *et al.*, 2006a; Sakamoto & Holman, 2008; Taylor *et al.*, 2008). Both PKB and AMPK are capable of phosphorylating AS160 and TBC1D1 (Treebak *et al.*, 2006; Sakamoto & Holman, 2008), and the phosphorylation of AS160/TBC1D1 has been shown to be additively increased by insulin and contraction in mouse muscle (Kramer *et al.*, 2006a). Thus, AS160 and TBC1D1 may play a pivotal role regulating both contraction- and insulin-mediated glucose uptake.

Many studies have found an additive increase in glucose uptake when muscles were stimulated by contraction and insulin together (Nesher *et al.*, 1985; Lund *et al.*, 1995; Aslesen & Jensen, 1998; Hayashi *et al.*, 1998; Whitehead *et al.*, 2000; Ruzzin & Jensen, 2005). However, most studies showed that this increased glucose uptake was not fully additive (Lund *et al.*, 1995; Aslesen & Jensen, 1998; Hayashi *et al.*, 1998; Whitehead *et al.*, 2000; Ruzzin & Jensen, 2005). Interestingly, Whitehead et al. reported that inhibition of insulin signalling with wortmannin decreased glucose uptake to the level obtained by contraction alone (Whitehead *et al.*, 2000). Insulin-stimulated IRS1-associated PI3K activity was inhibited during contraction (Whitehead *et al.*, 2000). Therefore, it can be speculated that insulin-mediated glucose uptake is reduced during muscle contraction. On the other hand, insulin-stimulated PKB Ser^{473} phosphorylation was unchanged or slightly reduced during contraction (Whitehead *et al.*, 2000; Ruzzin & Jensen, 2005). And insulin-stimulated GSK3

phosphorylation was reported to be slightly reduced in epitrochlearis but not in soleus muscles during contraction (Ruzzin & Jensen, 2005). Most studies have suggested that these downstream targets of PI3K are slightly reduced or unchanged during contraction in muscles with normal glycogen content. Insulin-stimulated PKB activation and phosphorylation were enhanced in muscles with low glycogen content (Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006), and it would be interesting to know whether insulin-stimulated PKB phosphorylation was reduced in contracting muscle with low glycogen content.

Insulin-stimulated glycogen synthase activation during contraction

From a quantitative view, glycogen content decreases during exercise. However, it has become apparent that contraction increases both glycogen breakdown and synthesis simultaneously (Shulman & Rothman, 2001). GS activity is also increased during contraction (Nielsen *et al.*, 2001) and the increased GS activity is probably the mechanism that assists muscle glycogen re-synthesis after contraction. To the best of my knowledge, no study has investigated the effect of insulin on GS activity during contraction in rodent muscle. However, one human study has showed that GS fractional activity was synergistically increased when exercise was performed in conjunction with insulin infusion (Christ-Roberts *et al.*, 2003). Because two different signalling pathways are used by insulin and contraction to activate GS (Aschenbach *et al.*, 2001; McManus *et al.*, 2005), it is possible that contraction and insulin additively activate GS. Dephosphorylation is a key mechanism to increase GS activity. Insulin or contraction alone has been reported to dephosphorylate GS at Ser^{641} and Ser^{645} in skeletal muscle (Sakamoto *et al.*, 2003; McManus *et al.*, 2005; Ruzzin & Jensen, 2005; Jensen *et al.*, 2006), and it will be interesting to see whether contraction and insulin additively decrease GS phosphorylation and activation. However, it should be noted

that the presence of elevated insulin concentration during exercise does not occur in normal situations; however, this is one of the experimental designs to study the interactions of insulin and muscle contraction.

1.4.2. Insulin-stimulated glucose uptake and GS activation immediately after contraction

Insulin-stimulated glucose uptake immediately after contraction

When muscles are stimulated with insulin immediately after contraction, there is an additive effect on glucose uptake (Garetto *et al.*, 1984; Richter *et al.*, 1984; Wallberg-Henriksson *et al.*, 1988). This is because the contraction effect on glucose uptake has not worn away, as glycogen content remains low and AMPK activity is still elevated (Wallberg-Henriksson *et al.*, 1988; Franch *et al.*, 1999; Musi *et al.*, 2001). After treadmill exercise, insulin-stimulated phosphotyrosine-associated PI3K activity was reported to be enhanced, but IRS1-associated PI3K activity was unchanged (Zhou & Dohm, 1997; Wojtaszewski *et al.*, 1999; Jessen *et al.*, 2003). In contrast, after muscle contraction, insulin-stimulated IRS1-associated PI3K was reported to be reduced (Goodyear *et al.*, 1995).

PKB is downstream of PI3K and a key molecule of insulin signalling for glucose uptake. Some studies have reported that contraction increases PKB phosphorylation and activity in skeletal muscle, even in the absence of insulin (Whitehead *et al.*, 2000; Sakamoto *et al.*, 2002; Sakamoto *et al.*, 2003). Insulin-stimulated PKB activity and phosphorylation at Ser^{473} have been reported to be either no change (Jessen *et al.*, 2003) or enhanced (Wojtaszewski *et al.*, 1999) after treadmill exercise. It is unclear why some studies found elevated activation of insulin signalling pathways after contraction but others did not. Furthermore, PKB has two phosphorylation sites at Ser^{473} and Thr^{308}. These two phosphorylation sites of PKB are

regulated by different mechanisms and their phosphorylation levels are not necessarily parallel. Two recent studies have reported that insulin-stimulated PKB Thr^{308} phosphorylation was enhanced in rat epitrochlearis muscle 3-4 h after a bout of swimming, but no change was found in PKB Ser^{473} phosphorylation (Arias *et al.*, 2007; Koshinaka *et al.*, 2009). It remains to be established whether insulin stimulation of PKB Thr^{308} phosphorylation is elevated at an earlier time point after muscle contraction.

Insulin-stimulated glycogen synthase activation immediately after contraction

During the first few hours after exercise, the enhanced insulin-stimulated glucose uptake in skeletal muscle is preferably incorporated into glycogen (Davis *et al.*, 1986). Studies have demonstrated that insulin-stimulated glycogen synthesis is additively increased during the first few hours after exercise or muscle contraction (Richter *et al.*, 1982; Garetto *et al.*, 1984; Richter *et al.*, 1984; Davis *et al.*, 1986; Franch *et al.*, 1999). The mechanism for this additivity most likely relates to the activation of GS, as insulin-stimulated GS activity is enhanced after contraction (Richter *et al.*, 1984; Richter *et al.*, 2001; Ruzzin & Jensen, 2005). However, this enhanced effect of prior contraction on insulin-stimulated GS activation was not reported in all studies (Franch *et al.*, 1999; Wojtaszewski *et al.*, 1999). These contradictory findings are difficult to explain. GS activity is regulated by phosphorylation, but insulin-mediated GS phosphorylation has not been carefully studied directly after contraction.

1.4.3. Insulin-stimulated glucose uptake and glycogen synthase activation several hours after contraction

Several hours (2-48 h) after exercise, insulin-stimulated glucose uptake, GS activity, and glycogen synthesis are still enhanced in stimulated muscles (Richter *et al.*, 1982;

Garetto *et al.*, 1984; Richter *et al.*, 1984). This increase in skeletal muscle insulin sensitivity contributes to the enhancement of whole body insulin sensitivity after exercise (Dela *et al.*, 1995). The benefits of exercise have been stressed by a longitudinal study showing that regular exercise is more effective than medical intervention in preventing the development of type 2 diabetes (Knowler *et al.*, 2002), suggesting that exercise represents the best way to combat this disease. However the mechanisms related to these have not been studied in the present thesis and will not be discussed further.

1.5. Effect of glycogen content on glucose uptake

It has been suggested that glycogen breakdown participates in the regulation of contraction-stimulated glucose uptake (Aslesen *et al.*, 2001). Studies have shown that insulin-stimulated glucose transport was elevated from 18 h to 48 h after exercise when muscle glycogen was kept low by fat feeding or fasting, whereas insulin-stimulated glucose transport returned to normal when rats were fed with a high carbohydrate diet (Young *et al.*, 1983; Cartee *et al.*, 1989). These results led us to speculate that glycogen content directly regulates glucose uptake and that the elevated glucose uptake after exercise occurs to restore muscle glycogen.

Different protocols have been applied to manipulate muscle glycogen content to test the hypothesis that glycogen regulates glucose uptake. For example, in our lab we use fasting and fasting-refeeding to manipulate muscle glycogen content (Jensen *et al.*, 1997; Jensen *et al.*, 2006) and other groups use a combination of exercise followed by a diet manipulation (Hespel & Richter, 1990; Derave *et al.*, 1999; Kawanaka *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000). The results from these studies are clear: both insulin- and contraction-stimulated glucose uptake were higher in muscle with low glycogen content than in muscle with high glycogen

content. Some studies also demonstrated that the amount of GLUT4 protein on cell surface was inversely correlated with muscle glycogen content (Derave *et al.*, 1999; Kawanaka *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000), which indicates that insulin- or contraction-induced GLUT4 translocation is regulated by muscle glycogen content.

It has been hypothesized that glycogen particles are structurally attached to GLUT4 storage vesicles, and when glycogen is broken down GLUT4 is translocated to the cell membrane (Coderre *et al.*, 1995). However, this hypothesis has not been confirmed. Reduction of glycogen content can change many enzyme activities and gene/protein expression (Blomstrand & Saltin, 1999; Jensen *et al.*, 2006; Parker *et al.*, 2006; Churchley *et al.*, 2007; Mascher *et al.*, 2007), and it is possible that glycogen regulates GLUT4 translocation and glucose transport by changing cellular signals that regulate these processes.

Glycogen content does not influence insulin receptor tyrosine kinase activity, insulin receptor tyrosine phosphorylation, and PI3K activity (Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006). Interestingly, insulin-stimulated PKB activity and phosphorylation were enhanced in muscle with low glycogen content (Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006), suggesting that increased PKB activation by insulin may contribute to the increased glucose uptake in muscles with low glycogen content. Contraction-stimulated glucose uptake was also enhanced when muscle glycogen content was low (Hespel & Richter, 1990; Derave *et al.*, 1999; Kawanaka *et al.*, 2000). AMPK seems to play a regulatory role as contraction- or AICAR-mediated AMPK activity was inversely related to glycogen content (Derave *et al.*, 2000a; Kawanaka *et al.*, 2000; Wojtaszewski *et al.*, 2002). However, evidence for the links between insulin-stimulated PKB or contraction-stimulated AMPK and GLUT4 translocation has not been provided in

muscles with different glycogen contents. Since AS160 and TBC1D1 have emerged as candidates beyond PKB and AMPK in mediation of GLUT4 translocation, it would be interesting to see if glycogen content can affect contraction- or insulin-stimulated AS160 and TBC1D1 phosphorylation.

Combination of insulin and contraction usually leads to an additive increase in glucose uptake (Henriksen *et al.*, 1990; Hansen *et al.*, 1994; Aslesen & Jensen, 1998; Hayashi *et al.*, 1998; Whitehead *et al.*, 2000; Ruzzin & Jensen, 2005). Interestingly, Zorzano et al. reported that exercise enhanced insulin-stimulated glucose uptake in muscle with normal glycogen content, but not in muscle with low glycogen content (Zorzano *et al.*, 1986), which implies that muscle glycogen content may influence the additivity of contraction and insulin on glucose uptake. Many studies have shown that insulin- and contraction-stimulated glucose uptake are enhanced in muscle with low glycogen (Derave *et al.*, 1999; Kawanaka *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006), and it will be interesting to know whether the additive effect of contraction and insulin on glucose uptake is influenced by glycogen content.

1.6. Effect of glycogen content on glycogen synthase activity

It is well documented that glycogen influences its own breakdown and synthesis. Danforth showed that decreased muscle glycogen content increased GS activity, whereas increased glycogen content decreased GS activity (Danforth, 1965). Subsequent studies have confirmed these findings in the absence or presence of insulin (Nielsen *et al.*, 2001; Jensen *et al.*, 2006). Furthermore, insulin was unable to activate GS in skeletal muscle with high glycogen content, suggesting that glycogen is a much potent regulator of GS than insulin (Nielsen *et al.*, 2001; Jensen *et al.*, 2006). Nielsen et al. (Nielsen *et al.*, 2001) showed that contraction-stimulated GS activation

is inversely correlated with glycogen content. In the same study, Nielsen et al. also showed that the correlations between glycogen content and GS fractional activity followed in the same correlation line in both rested and contracted muscles. This suggested that contraction-increased GS activity is solely a result of the decrease in muscle glycogen (Nielsen *et al.*, 2001). In their study, GS fractional activity was measured with 1.6 mM of UDP-glucose, which far exceeds its physiological concentration in muscle. However, Kochan et al. (Kochan *et al.*, 1981) have suggested to use 0.03 mM (a physiological concentration) of UDP-glucose for measurement of GS when studying the effect of exercise in skeletal muscle.

Although GS has been known to be regulated by glycogen for a long time, the underlying mechanisms are largely unknown. Work from our lab has previously shown that phosphorylation of GS at $Ser^{645,649,653,657}$ was low in muscle with low glycogen content, which complements the findings of increased GS fractional activity (Jensen *et al.*, 2006). Surprisingly, GS $Ser^{645,649,653,657}$ phsophorylation was also reduced in muscle with high glycogen content where GS fractional activity was low. Jorgensen et al (Jorgensen *et al.*, 2004a) have reported that GS Ser^{7} and $Ser^{7,10}$ phosphorylation was higher in muscle with high glycogen content than in muscle with low glycogen content, but phosphorylation of GS $Ser^{641,645}$ was found equal in muscles with low and high glycogen contents. Furthermore, phosphorylation of GSK3, a dominant kinase of GS, was reported to be unchanged by glycogen content (Jensen *et al.*, 2006), whereas PP1 activity was reported to be higher in muscle with low glycogen content than in muscle with high glycogen content (Villar-Palasi, 1969). The relationship between GS phosphorylation and activity in muscles with various glycogen contents is still not completely understood.

1.7. Muscle fiber types and glycogen

Muscle fiber types have been classified as slow-twitch (type I) and fast-twitch (type II) fibers based on the expression of myosin heavy chain isoforms and the distinct contractile activities. Fast-twitch fibers are further subcategorized into fast-twitch oxidative (type IIa) and fast-twitch glycolytic (type IIb) fibers (Barnard *et al.*, 1971; Pette & Staron, 1990). Slow-twitch fibers contain high oxidative enzyme activities and mitochondria density, and preferably use fat oxidation for ATP production. In contrast, fast-twitch fibers have higher glycolytic enzyme activities and lower mitochondrial density and more rely on glycolysis for energy supply. Thus different fiber-type compositions will lead to different metabolic properties and energy preferences in skeletal muscles.

We have previously used epitrochlearis muscle to study the effect of glycogen content on glucose metabolism. Epitrochlearis is a fast-twitch muscle composed of 65 % fast-twitch glycolytic fibers, 20 % fast-twitch oxidative fibers, and 15 % slow-twitch fibers (Nesher *et al.*, 1980). We have seen that low glycogen content in epitrochlearis muscle increases insulin-stimulated glucose uptake, GS fractional activity, and glycogen synthesis (Jensen *et al.*, 1997; Jensen *et al.*, 2006). Soleus muscle of rats is commonly used as a slow-twitch muscle. Soleus muscle is composed of 84 % slow-twitch fibers and 16 % fast-twitch oxidative fibers (Ariano *et al.*, 1973). Compared to muscles with higher percentage of fast-twitch fibers, soleus muscle displays higher expressions of key molecules in insulin signalling cascades, like PI3K, PKB, GLUT4, GSK3, and GS (Henriksen *et al.*, 1990; Jensen *et al.*, 1999; Song *et al.*, 1999) and has higher insulin-stimulated glucose uptake and glycogen synthesis. But soleus muscle actually contains lower glycogen content than fast twitch muscles (James & Kraegen, 1984; Brau *et al.*, 1997; Aslesen & Jensen, 1998).

The role of glycogen content on glucose metabolism in soleus muscle is

controversial. Derave et al. reported that insulin-stimulated glucose uptake is not influenced by glycogen content in soleus muscle (Derave *et al.*, 2000b). However, insulin-stimulated GS activity was reported to be inversely correlated with glycogen content (Henriksen *et al.*, 1986; Henriksen & Tischler, 1988; Henriksen *et al.*, 1989) and insulin-stimulated glycogen synthesis was reduced in soleus muscle with high glycogen content (Hoy *et al.*, 2007). According to these reports, GS seems to play a role in the regulation of insulin-stimulated glycogen synthesis in soleus muscle. In another study, Derave et al. also reported that glycogen content did not affect contraction-stimulated glucose uptake in soleus muscle (Derave *et al.*, 1999). Whether glycogen content regulates GS activity and influences glycogen re-synthesis in soleus after contraction are unknown. Therefore, the present thesis also aimed to investigate the effect of glycogen content on GS activity and glycogen synthesis in soleus muscle and compare these results with the data obtained from epitrochlearis muscle.

2. AIMS

Glycogen is an effective modulator for glucose metabolism in skeletal muscle. The primary aim of the present thesis was to investigate the effect of glycogen content on glucose metabolism in skeletal muscle. In particular, the effects of contraction and/or insulin on glucose uptake, GS activity and GS phosphorylation were investigated in epitrochlearis muscles with different glycogen contents. Furthermore, I asked questions as to whether it was possible to manipulate glycogen content in slow-twitch soleus muscle by fasting and refeeding protocols, and whether glycogen content has the same interactions in the regulation of GS activity in slow-twitch soleus and fast-twitch epitrochlearis muscles.

The specific aims of the present thesis were to investigate:

1) Whether contraction and insulin have an additive effect on glucose uptake in muscles with different glycogen contents.
2) The effect of contraction and insulin on PKB and GSK3 phosphorylation in muscles with different glycogen contents.
3) The effect of contraction and insulin on AMPK activity and ACC phosphorylation in muscles with different glycogen contents.
4) The effect of contraction and insulin on AS160/TBC1D1 phosphorylation in muscles with different glycogen contents
5) Whether glycogen content influences GS affinity for UDP-glucose in skeletal muscle.
6) The effect of contraction on GS affinity for UDP-glucose in muscles with different glycogen contents.

7) The effect of insulin on GS fractional activity in muscles with different glycogen contents.
8) The effect of contraction on GS fractional activity in muscles with different glycogen contents.
9) Whether contraction and insulin have an additive effect on GS fractional activity in muscles with different glycogen contents.
10) Whether glycogen content regulates GS phosphorylation in skeletal muscle.
11) The effect of contraction on GS phosphorylation in muscles with different glycogen contents.
12) The additive effect of contraction and insulin on GS phosphorylation in muscles with different glycogen contents.
13) The relationship between GS fractional activity and phosphorylation when GS is modulated by insulin, contraction, and glycogen alone or in combination.

3. MATERIALS AND METHODS

3.1. Animals

The protocols for the animal experiments were approved by National Animal Research Authority and conducted in accordance with the guidelines for live animals in Norway and the European Convention for the Protection of Vertebrate Animals Used in Experimental and Other Scientific Purposes.

Male Wistar rats weighing 85-95 g were purchased from B & K Universal AS, Nittedal, Norway. Rats were acclimatized for 6 or 7 days with free access to standard laboratory chow and tap water in the animal facility of National Institute of Occupational Health. The animal room was maintained at 21 °C and a humidity of ≥ 55% with a 12:12 h light-dark cycle (light from 6:00 $_{AM}$ to 6:00 $_{PM}$). All experiments were performed during the light cycle (between 10:00 $_{AM}$ and 2:00 $_{PM}$).

3.2. Experimental procedures

For paper 1, 2, and 4, the rats (125 ± 0.5 g) were randomly assigned to fasted, normal, and fasted-refed groups to manipulate muscle glycogen content to low, normal, and high levels. The rats with normal glycogen content in muscles (NG) were maintained on their normal diet until the experiments. The rats with low glycogen content in muscles (LG) were fasted from 10:00 $_{AM}$ the day before experiments. The rats with high glycogen content in muscles (HG) were fasted from 10:00 $_{AM}$ two days before to 10:00 $_{AM}$ the day before experiments followed by a normal diet for the last 24 hours prior to the experiments. Figure 6 shows the changes of the rat body weights in response to the fasting and/or refeeding periods. The rats fed with normal diet *ad libitum* increased their body weight by 7.4 ± 0.2 g per day. Fasting for 24 hours decreased rat body weight by 16.4 ± 0.3 g. The fasted rats refed with normal rat chow

for 24 hours increased their body weight by 18.5 ± 0.3 g. The rat body weights on the experimental days were 119.1 ± 1.1, 139.9 ± 0.8, and 130.3 ± 0.9 g in fasted (LG), normal (NG), and refed (HG) rats, respectively.

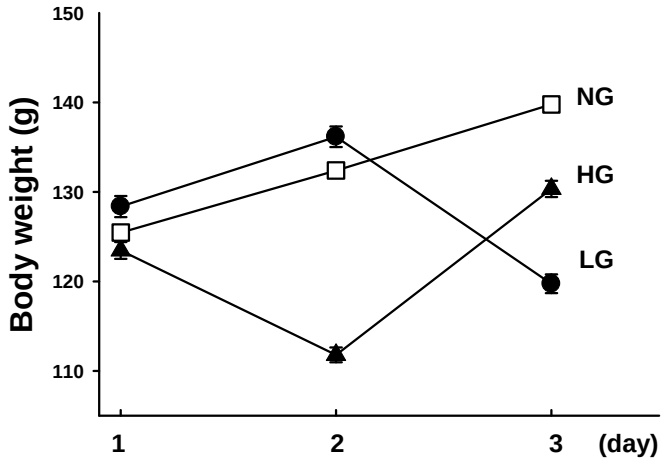

Figure 6. Effect of 24 hour fasting and refeeding on rat body weight. NG: Rats were given a normal diet before the experiments; LG: Rats were fasted for 24 hours before the experiments; HG: Rats were fasted for 24 hours followed by a normal diet for 24 hours before the experiments. n = 52, 80, and 60 in LG, NG, and HG, respectively.

In paper 3, the rats had free access to laboratory chow and tap water in our animal facility. We used only muscles with normal glycogen content and the rats weighed ~140 g on experimental days.

3.3. Muscle harvests and incubations

On the days of the experiments, rats were anaesthetized with an intraperitoneal injection of ~10 mg pentobarbital (50 mg/ml). The muscles were dissected from the rats and rapidly mounted on the contraction apparatus in their resting lengths.

Epitrochlearis muscle (paper 1, 2, and 3) was studied intact, whereas soleus muscle (paper 4) was split into two strips. This is because the thickness of intact soleus from rats above 100 g (Crettaz *et al.*, 1980) can cause that oxygen and substrates cannot reach the deep part of this muscle at a sufficient rate.

Once mounted on the contraction apparatuses, all muscles were placed into glass tubes and pre-incubated for 30-50 min in a pre-warmed (30 °C) Krebs Henseleit buffer containing 5.5 mM glucose, 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2 mM sodium pyruvate, and 0.1 % bovine serum albumin, pH 7.4 before interventions of stimuli. All incubations were executed at 30 °C using a temperature controlled water bath and gas (95 % O_2 – 5 % CO_2) was bubbled continuously through the buffer. After incubation procedures, muscles were rapidly removed from the apparatuses, blotted on filter papers, frozen in liquid nitrogen, and stored at -70 °C until analysis.

The merits of pre-incubation are 1) recovery from the pulling and twitching that occurred while the muscles were dissected out of the rats, and 2) removing such individual variables as plasma hormones, that occurred when rats were under fasting or refeeding conditions. Thus, this *in vitro* incubation system allows muscle metabolisms to be studied in a simple and well controlled environment.

In paper 1, the pre-incubated epitrochlearis muscles were stimulated with 10 mU/ml insulin, contraction, insulin + contraction, or kept rested for 30 min. Muscle contraction was stimulated electrically with impulse trains of 200 ms (100 Hz, pulses duration of 0.2 ms, 10 V) delivered every 2 seconds. Glucose uptake was measured during stimulation, whereas glycogen content, GS activity and protein phosphorylation measurements were taken from muscle frozen immediately after stimulations (Figure 7).

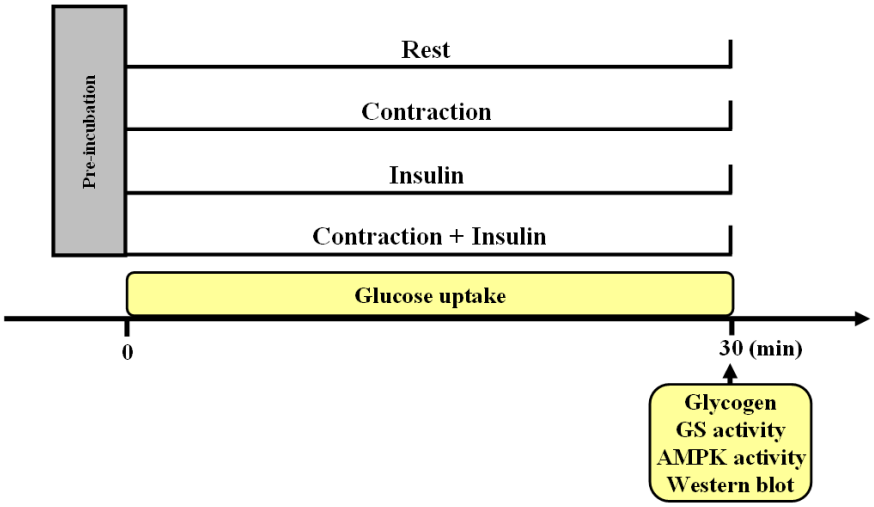

Figure 7. Schematic illustration of incubation protocols in paper 1. Epitrochlearis muscles with different glycogen contents were kept rested or stimulated with contraction, insulin, or contraction + insulin for 30 min. Glucose uptake was measured during the incubation, whereas glycogen content, GS activity, AMPKα2 activity, and western blot were measured in muscle frozen immediately after incubation. The upward arrow indicates the stop point of incubations.

In paper 2, the pre-incubated epitrochlearis muscles were kept rested or contracted electrically with 200 ms trains (100 Hz, square wave pulses of 0.2 ms duration, 10 V) delivered every 2 seconds for 30 min. Muscles were then incubated for an additional 30 min for analysis of glycogen content, GS activity and Western blot. Other muscles were incubated 60 min for measurement of glycogen synthesis (Figure 8).

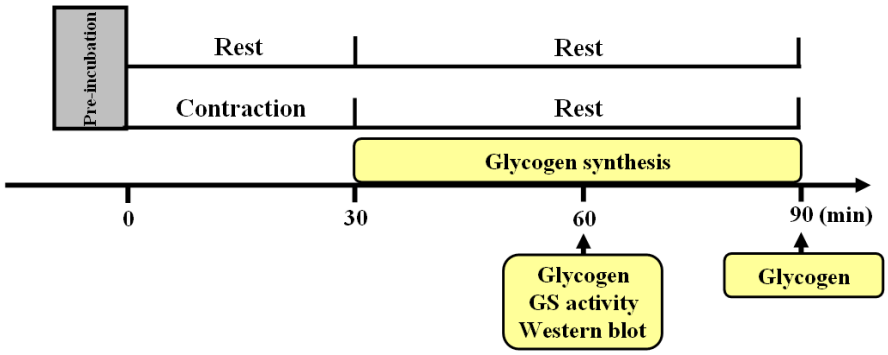

Figure 8. Schematic illustration of incubation protocols in paper 2. Epitrochlearis muscles with different glycogen contents were rested or contracted for 30 min followed by a 60 min incubation period for measurement of glycogen synthesis. Other muscles were incubated for additional 30 min after contraction for analysis of GS activity and western blot. The upward arrows indicate the stop point of incubations.

In paper 3, some epitrochlearis muscles were frozen with liquid nitrogen immediately after pre-incubation for use as a control rested group, others were stimulated to contract with 200 ms trains (100 Hz, square wave pulses of 0.2 ms duration, 10 V) delivered every 2 seconds for 1, 2, 5, 10, or 30 min before being frozen in liquid nitrogen (Figure 9).

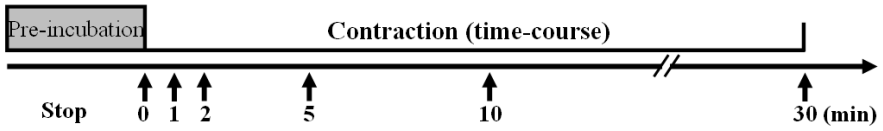

Figure 9. Schematic illustration of incubation protocols in paper 3. After pre-incubation, epitrochlearis muscles were stimulated to contract for 1, 2, 5, 10, or 30 min or frozen immediately (0 min). Glycogen content, GS activity, and western blot were measured in these muscles.

In paper 4, we studied soleus muscles using the same protocol as in paper 2, but two additional groups with insulin stimulation were included. Stripped soleus

muscles were kept rested or stimulated to contract with 200 ms trains (100 Hz, square-wave pulses of 0.2 ms, 10 V) delivered every 2 seconds for 30 min followed by a 60 min incubation period with or without insulin (10 mU/ml) for measurement of glycogen synthesis. Other muscle strips, for measurement of glycogen content, GS activity, and protein phosphorylation, were incubated for 30 min with or without insulin after contraction (Figure 10).

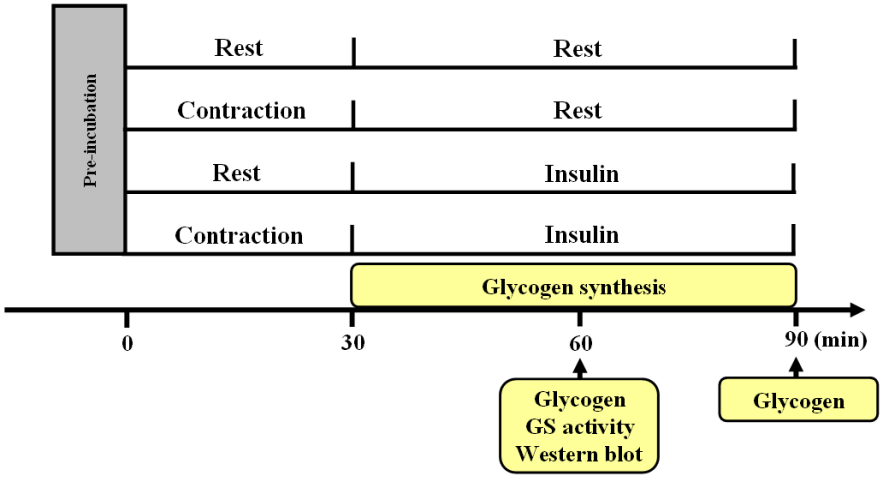

Figure 10. Schematic illustration of incubation protocols in paper 4. Soleus muscles with different glycogen contents were kept rested or contracted for 30 min followed by a 60 min incubation period with or without insulin for measurement of glycogen synthesis. For measurement of glycogen content, GS activity, and western blot, muscles were incubated for 30 min after the contraction bout. The upward arrows indicate the stop point of incubations.

3.4. Materials and methods for paper 3

The materials and methods in papers 1, 2, and 4 are referred to their sections of "materials and methods". The materials and methods for paper 3 are described below because paper 3 is an opinion paper with some original data presented.

Glycogen content

Muscles were hydrolyzed with 1 ml of 1 M HCl for 2.5 hours at 100 °C and the digests were then centrifuged at 3000 g for 10 min at 4 °C. Glycogen content was determined as glucose units analyzed fluorometrically with appropriate standard curves (Passonneau & Lowry, 1993).

Glycogen synthase activity

GS fractional activity was measured as described in paper 1.

Western blot analysis

Immunoblotting was performed as described in paper 1. The signals were detected and quantified by an LAS-4000mini image analysis system (Fujifilm, Tokyo, Japan).

Statistics

Data are presented as means ± SEM. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 13.0 for Windows. One-way ANOVA was conducted to evaluate whether there were differences between time-points. When the ANOVA indicated significant differences, further analysis was performed using LSD *post hoc* test.

4. RESULTS

The main results of included papers are given in the following sections.

4.1. Paper 1: Additive effect of contraction and insulin on glucose uptake and glycogen synthase in muscles with different glycogen contents.

The aims of this study were to investigate whether contraction and insulin have an additive effect on glucose uptake and GS activation in muscles with different glycogen contents, and to investigate the effect of glycogen content on phosphorylation of AMPK, PKB, AS160/TBC1D1 and GSK3 in muscles exposed to contraction and insulin alone or in combination. We also aimed to investigate the relationship between GS phosphorylation and fractional activity when muscles with different glycogen contents were exposed to contraction and insulin alone or in combination.

Contraction and insulin additively increased glucose uptake and GS fractional activity in muscles with NG and HG, but not in LG. Nevertheless, glucose uptake and GS fractional activity were highest in LG during contraction, insulin stimulation alone or when these two stimuli were combined. In LG, contraction-stimulated AMPK activity and insulin-stimulated PKB phosphorylation were higher than in NG and HG, but we found that AS160/TBC1D1, as well as GSK3, phosphorylation was not elevated correspondingly.

The Group mean of GS fractional activity was inversely correlated with GS phosphorylation at Ser^{641} ($R = -0.94$, $P < 0.001$) and at $Ser^{645,649,653,657}$ ($R = -0.75$, $P < 0.006$). GS fractional activity was not correlated with GS Ser^{7}phosphorylation ($R = -0.29$, $P = 0.360$). Phosphorylation of GS Ser^{7} was increased during contraction and not regulated by insulin.

4.2. Paper 2: Glycogen content and contraction regulate glycogen synthase phosphorylation and affinity for UDP-glucose in rat skeletal muscles.

The aims of this study were to investigate the effects of glycogen content and contraction on GS fractional activity and GS affinity for UDP-glucose. Furthermore, GS Ser^{7} and Ser^{641} phosphorylation was also investigated.

GS affinity for UDP-glucose was enhanced in muscle with low glycogen content and reduced with high glycogen content. GS fractional activity measured with a physiological concentration of UDP-glucose (0.03 mM) was higher in contracted HG than in rested NG, despite higher glycogen content in contracted HG than in rested NG. These results show that contraction has additional mechanisms other than glycogen reduction for activating GS. However, when GS fractional activity was measured with 1.67 mM of UDP-glucose, the independent effect of glycogen content on contraction-mediated GS activation disappeared. GS affinity for UDP-glucose was increased after contraction and the effect of contraction on GS affinity for UDP-glucose was increased more than expected from contraction-mediated glycogen depletion.

GS phosphorylation at Ser^{7} and Ser^{641} was increased in HG and decreased in LG, compared to NG. Phosphorylation of GSK3β Ser^{9} and AMPKα Thr^{172} was not regulated by glycogen content in rested muscles. Contraction decreased GS Ser^{641} phosphorylation, but increased GS Ser^{7} phosphorylation despite increased GS activity.

4.3. Paper 3: Regulation of muscle glycogen synthase phosphorylation and kinetic properties by insulin, exercise, adrenaline and role in insulin resistance.

In paper 3, we conducted a time-course study to explore GS fractional activity and

phosphorylation status during muscle contraction.

GS fractional activity started to increase after 5 min of contraction and reached a plateau after 10 min of contraction; GS fractional activity remained at the same high level until 30 min of contraction. Contraction decreased GS Ser^{641} phosphorylation after 5 min of contraction ($P < 0.02$) and decreased GS Ser^{641} phosphorylation further after 10 min of contraction ($P < 0.001$ vs. 0 min; $P < 0.005$ vs. 5 min); GS Ser^{641} phosphorylation remained at the same low level until 30 min of contraction. Interestingly, GS Ser^{7} phosphorylation was increased after 1 min of contraction and remained at a high level during the 30 min of contraction ($P < 0.05$). Muscle glycogen content was decreased after 2 min of contraction ($P < 0.05$) and decreased continuously through the 30 min of contraction.

4.4. Paper 4: Glycogen content regulates insulin- but not contraction- mediated glycogen synthase activation in the rat slow-twitch soleus muscles.

The primary aim of paper 4 was to study whether glycogen content regulates GS activation in slow-twitch soleus muscles during insulin stimulation, after contraction, or when these two stimuli were combined. Furthermore, GS phosphorylation at Ser^{7}, Ser^{641}, and $Ser^{645,649,653,657}$ were assessed and related to GS fractional activity.

The glycogen content was lower in soleus muscle, compared to epitrochlearis muscle. Fasting for 24 h reduced glycogen content by 40 % in soleus muscle. 24 h fasting followed by a period of 24 h refeeding increased glycogen content by 40 % in soleus muscle. Insulin-stimulated GS fractional activity and glycogen synthesis were inversely correlated with glycogen content ($R = -0.95$, $P < 0.001$, $n = 24$ and $R = -0.55$, $P < 0.002$, $n = 36$, respectively). In contrast, GS fractional activity after contraction was increased to similar levels in all groups despite different levels of glycogen content. Rate of glycogen synthesis after contraction was equal in all groups.

Contraction enhanced insulin-stimulated GS fractional activity to similar levels in all groups.

The group mean of GS fractional activity was inversely correlated with GS phosphorylation at Ser^{641} ($R = -0.94$, $P < 0.001$) and at $Ser^{645,649,653,657}$ ($R = -0.80$, $P < 0.003$). GS fractional activity was not correlated with GS Ser^{7} phosphorylation ($R = -0.49$, $P = 0.106$) in soleus muscles.

5. DISCUSSION

It has been known for many years that glycogen content, contraction, and insulin regulate GS activity in skeletal muscle (Villar-Palasi & Larner, 1960; Danforth, 1965). However, the cellular mechanisms by which glycogen content, contraction, and insulin regulate GS activity are still not completely understood. Glycogen content also regulates glucose uptake in skeletal muscle (Richter *et al.*, 2001) and influences the effect of contraction and insulin on glucose uptake (Hespel & Richter, 1990; Jensen *et al.*, 1997; Derave *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006), but whether glycogen content influences the additive effect of insulin and contraction on glucose uptake has not been systematically studied. In the present thesis, I have investigated the effect of contraction and insulin on glucose uptake and GS activation in muscles with different glycogen contents. In order to understand the molecular mechanisms by which insulin and contraction increase glucose uptake, the phosphorylation of the key signalling molecules, like AMPK, PKB, and AS160/TBC1D1, was investigated. To understand the regulation of GS activity better, I have investigated the effect of glycogen content and contraction on GS affinity for UDP-glucose. Three phospho-specific antibodies raised against GS (Ser^{7}, Ser^{641}, and $Ser^{645,649,653,657}$) were used to assess the phosphorylation status when muscles were modulated by insulin, contraction, and glycogen content alone or in combination. The phosphorylation states were then related to GS fractional activity. Finally, the effect of glycogen content on GS fractional activity and phosphorylation was investigated in soleus muscles exposed to insulin and/or contraction, and these results were compared with those obtained from epitrochlearis muscles. In the following sections, I will discuss the specific questions raised in the aims.

5.1. Additive effect of insulin and contraction on glucose uptake in muscles with different glycogen contents

In paper 1, our focus was to investigate whether glycogen content influences the additive effect of insulin and contraction on glucose uptake in epitrochlearis muscle. Before we studied the additive effect of contraction and insulin, the individual effect of insulin and contraction on glucose uptake was established. Insulin-stimulated glucose uptake was higher in LG than in NG and HG, which agrees with our and other previous studies (Jensen *et al.*, 1997; Kawanaka *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006). This is the first study to investigate the effect of glycogen content on contraction-stimulated glucose uptake when muscle glycogen content was manipulated by fasting or fasting-refeeding, and our data showed that contraction-stimulated glucose uptake was inversely correlated with glycogen content. These results agree with the finding of other studies where muscle glycogen content was manipulated by a combination of exercise and diet (Hespel & Richter, 1990; Derave *et al.*, 1999; Kawanaka *et al.*, 2000).

In paper 1, we found that insulin and contraction additively increased glucose uptake in NG and HG. An additive effect of contraction and insulin on glucose uptake in NG has been reported many times (Nesher *et al.*, 1985; Henriksen *et al.*, 1990; Hansen *et al.*, 1994; Holloszy & Hansen, 1996; Aslesen & Jensen, 1998). The additive increase in glucose uptake is probably because distinctive signalling pathways are used by insulin and contraction to increase GLUT4 translocation (Lund *et al.*, 1995; Whitehead *et al.*, 2000). Another possibility for the additive increase in glucose uptake is because there are two different pools of GLUT4, one is recruited by insulin and the other recruited by contraction (Ploug *et al.*, 1998). Although these fundamental findings may explain the additive effect of contraction and insulin on glucose uptake, it is clear that the additivity was not full. Studies have demonstrated

that GLUT4 translocation was partially additive when insulin and contraction were combined (Lund *et al.*, 1995; Ploug *et al.*, 1998), which agrees with our data and most previous studies (Brozinick *et al.*, 1994; Aslesen & Jensen, 1998; Hayashi *et al.*, 1998; Whitehead *et al.*, 2000; Ruzzin & Jensen, 2005). However, some studies have reported a fully additive effect of contraction and insulin on glucose uptake (Nesher *et al.*, 1985; Henriksen *et al.*, 1990).

No additive effect on glucose uptake was found in LG when insulin and contraction were combined, but glucose uptake was higher in LG than in NG and HG. In agreement with our findings, Zorzano et al. previously reported that contraction additively increased insulin-stimulated glucose uptake in muscles from rats on a normal diet, but not in muscles from fasted rats (Zorzano *et al.*, 1986). Furthermore, we found that insulin-stimulated glucose uptake was higher than contraction-stimulated glucose uptake in LG. Insulin-stimulated glucose uptake appears to be higher than contraction-stimulated glucose uptake in soleus muscle (Henriksen *et al.*, 1990; Brozinick *et al.*, 1994; Aslesen & Jensen, 1998; Ruzzin & Jensen, 2005), and several studies did not find an additive effect of insulin and contraction on glucose uptake in soleus muscle (Brozinick *et al.*, 1994; Aslesen & Jensen, 1998; Ruzzin & Jensen, 2005). The absence of additivity in epitrochlearis muscle with low glycogen content, which is similar to soleus muscle from normal rats, is probably because low levels of glycogen content increase glucose uptake. Fasting for 24 h increased GLUT4 expression by 50 % and the enhanced glucose uptake in LG can be attributed to this increased GLUT4 expression (Henriksen *et al.*, 1990; Jensen *et al.*, 2006). GLUT4 content on the cell surface was shown to be higher in LG than in HG either during contraction or insulin stimulation (Derave *et al.*, 1999; Kawanaka *et al.*, 1999; Derave *et al.*, 2000b; Kawanaka *et al.*, 2000). However, when contraction and insulin were combined, we found no further increase in glucose

uptake, which suggests that the GLUT4 content on the cell surface was not additively increased in LG.

Overall, our data suggest that low glycogen content prevented the additive effect of contraction and insulin on glucose uptake in epitrochlearis muscle, but glucose uptake was highest in muscles with low glycogen content when insulin and contraction were combined.

5.2. Effect of insulin and contraction on PKB and GSK3 phosphorylation in muscles with different glycogen contents

In epitrochlearis muscle, insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation was higher in LG than in NG and HG (paper 1). These results agree with previous studies (Derave *et al.*, 2000a; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006) and suggest that the enhanced PKB phosphorylation may contribute to the enhanced glucose uptake in epitrochlearis muscle with low glycogen content. For the first time, insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation was found to be enhanced in soleus muscle with low glycogen content (paper 4). Insulin-stimulated GS fractional activity was higher in soleus muscle with low glycogen content than in soleus muscle with normal glycogen content, but glycogen synthesis was similar in these two groups. These data may suggest that glucose uptake is not elevated in soleus muscle with low glycogen content. Derave et al. also reported that decreased glycogen content did not enhance insulin-stimulated glucose uptake in soleus muscle (Derave *et al.*, 2000b). In line with this, GLUT4 content was not increased in soleus muscle with low glycogen content (paper 4), as occurred in epitrochlearis muscle with low glycogen content (paper 1)(Jensen *et al.*, 2006). These results therefore suggest that the enhanced PKB phosphorylation in soleus muscle with low glycogen content does not enhance insulin-stimulated glucose uptake.

GSK3 is a downstream target of PKB. Insulin-stimulated GSK3β Ser^{9} phosphorylation did not parallel with PKB phosphorylation in epitrochlearis muscle, which agrees with a previous study (Jensen *et al.*, 2006). In paper 4, we found that insulin-stimulated GSK3β Ser^{9} phosphorylation did not parallel with PKB phosphorylation in soleus muscles. Although GSK3β phosphorylation was similar in all groups, GS phosphorylation at Ser^{641} and $Ser^{645,649,653,657}$ was reduced in muscles with low glycogen content. These results indicate that GSK3β activity does not regulate glycogen-mediated GS phosphorylation at the sites phosphorylated by GSK3 in both soleus and epitrochlearis muscles.

PKB phosphorylation was not increased after contraction in both soleus and epitrochlearis muscles, which agrees with most previous studies (Brozinick & Birnbaum, 1998; Lund *et al.*, 1998; Ruzzin & Jensen, 2005). However, some studies reported that contraction increased PKB phosphorylation in fast-twitch muscles (Whitehead *et al.*, 2000; Sakamoto *et al.*, 2003). The results of the phospho-Akt substrate (PAS) antibody showed that contraction increased phosphorylation on several PKB substrate proteins (paper 1), which suggests that PKB activity is increased by contraction. However, it is apparent that the contraction-mediated PKB activation is not involved in contraction-stimulated glucose uptake, since wortmannin completely inhibited contraction-activated PKB without interfering in contraction-stimulated glucose uptake (Whitehead *et al.*, 2000). Sakamoto et al. also reported that deletion of PKBβ did not affect exercise-stimulated glucose uptake in mouse skeletal muscles (Sakamoto *et al.*, 2006).

GSK3β Ser^{9} phosphorylation was increased by contraction in epitrochlearis muscle (paper 1 and 2). By contrast, GSK3β Ser^{9} phosphorylation was not increased in soleus muscle after contraction (paper 4), which agrees with the findings of a previous study (Ruzzin & Jensen, 2005). These findings suggest that contraction

induces GSK3 phosphorylation in a fiber-type dependent manner. Even though contraction increases GSK3 phosphorylation in epitrochlearis but not in soleus muscles, dephosphorylation of GS Ser^{641} and $Ser^{645,649,653,657}$ occurred in both muscles. GSK3 is not required in contraction-mediated GS activation in gastrocnemius and extensor digitorum longus muscles (McManus *et al.*, 2005), and since contraction did not phosphorylate GSK3 in soleus, contraction also activates GS independent of GSK3 in soleus muscle.

We found that insulin-stimulated PKB and GSK3β phosphorylation was reduced during contraction in epitrochlearis muscles with low and normal glycogen contents (paper 1). The reduction of insulin-stimulated PKB and GSK3 phosphorylation in contracting epitrochlearis muscle with normal glycogen content was limited (~20 %) and has been reported in one previous study (Ruzzin & Jensen, 2005), but not in the other (Whitehead *et al.*, 2000) from the laboratory. At low glycogen content, we found that the reduction of PKB and GSK3 phosphorylation was more obvious. It is known that insulin-stimulated PKB and GSK3 phosphorylation are dependent on PI3K activation (Shepherd, 2005). Contraction has been reported to inhibit insulin-stimulated PI3K activation (Whitehead *et al.*, 2000), which supports that the activation of insulin signalling is reduced during muscle contraction. In addition, evidence appears that treatment with AICAR, which activates AMPK, decreases insulin-stimulated PKB and GSK3 phosphorylation in skeletal muscle (Fediuc *et al.*, 2006). Conversely, deletion of LKB1, which blunts AMPK activation, enhances insulin-stimulated PKB phosphorylation (Koh *et al.*, 2006). In paper 1, we found that contraction decreased insulin-stimulated PKB and GSK3 phosphorylation in LG and NG, where AMPK activities were high. Therefore, the reduced PKB and GSK3 phosphorylation may occur because AMPK was activated.

In soleus muscle, the combined effect of contraction and insulin on PKB and

GSK3β phosphorylation was studied 30 min after contraction; therefore, these data cannot be compared directly with the data from epitrochlearis muscles. In soleus muscles, insulin-stimulated PKB Ser^{473} and GSK3β Ser^{9} phosphorylation was not changed in any of the groups after contraction. PKB activity is regulated by two phosphorylation sites and each site is regulated by different mechanisms (Alessi *et al.*, 1996). Interestingly, insulin-stimulated PKB Thr^{308} phosphorylation was found to be enhanced in all soleus muscles after contraction, whereas PKB Ser^{473} phosphorylation was unchanged. Two recent studies reported similarly that insulin-stimulated PKB Thr^{308} phosphorylation was increased in epitrochlearis muscles 4 h after swimming exercise, without increasing insulin-stimulated PKB Ser^{473} phosphorylation (Arias *et al.*, 2007; Koshinaka *et al.*, 2009). Our data provides evidence that the elevation of PKB Thr^{308} phosphorylation also occurs in soleus muscle after contraction. It is therefore possible that the increased PKB Thr^{308} phosphorylation plays a role in improving insulin sensitivity after muscle contraction.

In conclusion, insulin-stimulated PKB phosphorylation was enhanced in both soleus and epitrochlearis muscles with low glycogen content, whereas insulin-stimulated GSK3β Ser^{9} phosphorylation was independent of glycogen content. Although PKB phosphorylation was not increased by contraction, phosphorylation of GSK3 and other PKB substrates was increased by contraction in epitrochlearis muscles. Insulin-stimulated PKB and GSK3 phosphorylation was reduced during contraction in epitrochlearis muscles with low and normal, but not with high, glycogen contents. In soleus muscle, insulin-stimulated PKB Thr^{308} phosphorylation was enhanced after contraction, whereas PKB Ser^{473} phosphorylation was unchanged.

5.3. Effect of contraction and insulin on AMPK activity and ACC phosphorylation in muscles with different glycogen contents

Contraction-stimulated AMPKα2 activity and AMPKα Thr^{172} phosphorylation were decreased as glycogen content increased in epitrochlearis muscle (paper 1). These data are consistent with the data of contraction-stimulated glucose uptake. There is little doubt that contraction-stimulated AMPK activation contributes to stimulation of glucose uptake in fast-twitch muscle (Derave *et al.*, 2000a; Wright *et al.*, 2004). However, we can only provide correlative data and a direct link between AMPK activation and glucose uptake is currently lacking. Furthermore, previous studies have reported that contraction-stimulated AMPK activity was higher in LG than in HG (Derave *et al.*, 2000a; Kawanaka *et al.*, 2000), and we now show that contraction-stimulated AMPK activity in NG is intermediate. In contrast to that of fast-twitch muscles, the activation of AMPK does not seem to participate in contraction-stimulated glucose uptake in slow-twitch soleus muscle (Derave *et al.*, 2000a; Wright *et al.*, 2004). However, we still found that AMPK phosphorylation was increased 30 min after contraction in soleus muscles with low and normal, but not with high, glycogen contents (paper 4), which agrees with other studies (Derave *et al.*, 2000a; Ai *et al.*, 2002).

The mechanism for inhibition of AMPK activity by glycogen content in skeletal muscle is not well understood. AMPK β-subunit has a glycogen binding domain (McBride *et al.*, 2009) and a recent study has demonstrated that purified glycogen and oligosaccharides with α-1,6 branch points inhibit AMPK activity *in vitro* (McBride *et al.*, 2009). Therefore, it is possible that contraction-stimulated AMPK activity is inhibited through its direct binding to glycogen. This may explain the finding that contraction-stimulated AMPK activation is higher in LG and lower in HG, compared to NG.

Insulin did not change AMPK activity and phosphorylation in rested muscles, but insulin slightly reduced contraction-stimulated AMPK activation in epitrochlearis muscle with normal glycogen content. Insulin has been reported to inhibit AMPK activity in heart and hepatoma cells (Witters & Kemp, 1992; Horman *et al.*, 2006). In paper 1, we found that insulin decreased contraction-stimulated AMPK activation and phosphorylation in skeletal muscle, but we do not know why this insulin-mediated reduction of AMPK activation occurred only in NG but not in LG and HG. Insulin was reported to increase ACCβ activity in hepatoma cells (Witters & Kemp, 1992), but we found that insulin did not affect contraction-stimulated ACC phosphorylation in any of the groups despite reduced AMPK activity in NG (paper 1). Furthermore, although contraction-stimulated AMPK activity was inversely regulated by glycogen content, contraction-stimulated ACC phosphorylation was independent of glycogen content. A contraction time-course study has also reported that AMPK and ACC phosphorylation are not correspondingly increased (Miranda *et al.*, 2008). ACC is a well defined substrate of AMPK (Winder *et al.*, 1997) and it has been reported that a small increase in AMPK activity is sufficient to fully phosphorylate ACC (Miranda *et al.*, 2008), which may explain why contraction-stimulated ACC phosphorylation does not parallel with AMPK activity. However, it is also possible that ACC is phosphorylated by a kinase other than AMPK that is also activated by contraction.

In conclusion, contraction-stimulated activation and phosphorylation of AMPK was inversely regulated by glycogen content, which may contribute to the magnitude of contraction-stimulated glucose uptake in epitrochlearis muscle.

5.4. Effect of contraction and insulin on AS160/TBC1D1 phosphorylation in muscles with different glycogen content

Insulin and contraction are the two most important physiological stimuli for glucose

uptake. Earlier studies have demonstrated that these two stimuli use different signalling mechanisms to induce GLUT4 translocation (Lund *et al.*, 1995; Whitehead *et al.*, 2000). However, recent studies have suggested that the mechanisms for insulin- and contraction-stimulated glucose uptake are different at the early stages, but converge on AS160 and TBC1D1 (Kramer *et al.*, 2006a; Cartee & Wojtaszewski, 2007; Sakamoto & Holman, 2008). AS160 and TBC1D1 have similar phosphorylation motifs (Roach *et al.*, 2007) and studies have reported that the PAS antibody can recognize both AS160 and TBC1D1 phosphorylation at the expected band of 160 kDa (Chavez *et al.*, 2008; Funai & Cartee, 2008). Indeed, when the electrophoresis was run for a longer period of time, we could vaguely see two bands near 160 kDa; AS160 has a slightly higher molecule weight (160 kDa) than TBC1D1 (150 kDa)(Funai & Cartee, 2008). However, we were not able to separate these two bands clearly and our data most likely includes both AS160 and TBC1D1 phosphorylation (paper 1).

Insulin and contraction additively increased AS160/TBC1D1 phosphorylation in epitrochlearis muscles with normal and high glycogen contents, which agrees with the data from mouse muscles that had a normal glycogen content (Kramer *et al.*, 2006a). Interestingly, the additive effect of AS160/TBC1D1 phosphorylation supports the findings of an additive increase in glucose uptake in NG and HG, but not in LG. This shows that the phosphorylation of AS160/TBC1D1 reflects the additive effect of contraction and insulin on glucose uptake. However, we also noticed that the levels of AS160/TBC1D1 phosphorylation did not parallel with the extent of glucose uptake in muscles stimulated by insulin and contraction either alone or in combination. Particularly, in LG, contraction-mediated AS160/TBC1D1 phosphorylation was low despite the highest AMPK activation and glucose uptake. Several experiments have provided evidence that contraction can increase glucose uptake without a detectable increase in AS160/TBC1D1 phosphorylation in skeletal muscle (Treebak *et al.*, 2006;

Jensen *et al.*, 2008). A time-course study did not find a correlation between contraction-stimulated glucose transport and AS160/TBC1D1 phosphorylation (Funai & Cartee, 2008), which supports that AS160/TBC1D1 phosphorylation does not determine the extent of glucose uptake. However, our data does not preclude the importance of AS160/TBC1D1 phosphorylation for stimulation of glucose uptake because their phosphorylation could have been higher at earlier time points during contraction (Funai & Cartee, 2008). Furthermore, the PAS antibody has been reported to interact preferentially with Thr^{642} phosphorylation on AS160 and with Thr^{596} on TBC1D1, and these two proteins can be regulated by other phosphorylation sites (Kane & Lienhard, 2005; Roach *et al.*, 2007).

Taken together, AS160/TBC1D1 phosphorylation reflected the additivity of glucose uptake when muscle was stimulated by insulin and contraction simultaneously. However, AS160/TBC1D1 phosphorylation does not determine the extent of contraction- and/or insulin-mediated glucose uptake in skeletal muscle.

5.5. Effect of glycogen content on GS affinity for UDP-glucose in skeletal muscle

An important finding in paper 2 is that decreased glycogen content in skeletal muscle increased GS affinity for UDP-glucose, and increased muscle glycogen content decreased GS affinity for UDP-glucose (paper 2). Danforth described long ago that GS I form (%) is strongly regulated by glycogen content (Danforth, 1965) and it has been shown many times that GS fractional activity is reduced in muscles with high glycogen content (Nielsen *et al.*, 2001; Jensen *et al.*, 2006). However, GS fractional activity only explains the affinity for glucose 6-phosphate. GS affinity for UDP-glucose is also regulated and it is surprising to me that no study has investigated the effect of glycogen content on GS affinity for UDP-glucose. We now provide evidence that GS affinity for UDP-glucose is also inversely regulated by glycogen

content. Importantly, high glycogen content increased GS *Km*-$_{0.17}$ to 1.11 mM, which is far above the physiological contraction (0.03-0.06 mM) of UDP-glucose in skeletal muscle (Piras & Staneloni, 1969; Reynolds *et al.*, 2005). Therefore, increased GS *Km* may have a strong effect on the limitation of glycogen synthesis. In contrast, GS *Km*-$_{0.17}$ decreased to a low level in LG, which suggests that decreased GS *Km* may assist glycogen accumulation. Previous studies have reported that GS *Km* was decreased when GS activity was measured with a high concentration of glucose 6-phosphate (Roach *et al.*, 1976; Rossetti & Hu, 1993). We also found that GS *Km* measured with 12 mM of glucose 6-phosphate was decreased at all glycogen contents; however, GS *Km*$_{-12}$ was still regulated by glycogen content (paper 2).

In conclusion, GS affinity for UDP-glucose was regulated by muscle glycogen content, which may be a physiological mechanism for regulation of glycogen synthesis in skeletal muscle.

5.6. Effect of contraction on GS affinity for UDP-glucose in muscles with different glycogen contents

To the best of my knowledge, the effect of contraction on GS affinity for UDP-glucose has not been determined. In paper 2, we reported that GS affinity for UDP-glucose was increased by muscle contraction. GS *Km*-$_{0.17}$ decreased to low levels at all glycogen contents after contraction. Notably, GS *Km*-$_{0.17}$ was reduced from 1.11 to 0.33 mM in HG after contraction and this reduction of GS *Km*-$_{0.17}$ is remarkable. This is the first report showing the data of GS affinity for UDP-glucose in contracted muscle, and we hypothesized that increased GS affinity for UDP-glucose is an important mechanism for glycogen re-synthesis after contraction.

To determine the effect of contraction on GS affinity for UDP-glucose independently of glycogen content, GS *Km*-$_{0.17}$ values were plotted against individual

glycogen content (paper 2). GS *Km*-$_{0.17}$ was decreased dramatically after contraction and contraction decreased GS *Km*-$_{0.17}$ independently of glycogen content. However, at a high concentration (12 mM) of glucose 6-phosphate, contraction did not increase GS affinity for UDP-glucose independently of glycogen content. These data suggest that a physiological concentration of glucose 6-phosphate is necessary for detecting the changes of GS *Km* independently of glycogen content in contracted muscles. GS total activity is measured through high concentrations of glucose 6-phosphate in assay buffers. We found that contraction was unable to decrease GS *Km* in HG and only had a minor effect in LG and NG at 12 mM of glucose 6-phosphate. It is interesting to see that a high concentration of glucose 6-phosphate decreased GS *Km* to similar levels in both contracted and rested muscles. These data indicate that high concentrations of glucose 6-phosphate not only maximally activate GS, but also powerfully increase GS affinity for UDP-glucose. Therefore, a physiological concentration of glucose 6-phosphate is required to evaluate the physiological state of GS activity.

In conclusion, contraction increased GS affinity for UDP-glucose in skeletal muscle. Furthermore, contraction increased GS affinity for UDP-glucose independently of glycogen content, but a physiological concentration of glucose 6-phosphate for analysis of GS *Km* is required to see the effect of contraction independent of glycogen content.

5.7. Effect of insulin on GS fractional activity in muscles with different glycogen contents

Basal and insulin-stimulated GS fractional activities were inversely correlated with glycogen content in epitrochlearis muscles (paper 1). These results agree with the findings of other studies in fast-twitch muscles (Nielsen *et al.*, 2001; Jensen *et al.*, 2006). In soleus muscle (paper 4), we also found that basal and insulin-stimulated GS

fractional activities were inversely correlated with glycogen content, which agrees with other studies using different experimental settings (Henriksen *et al.*, 1986; Henriksen & Tischler, 1988; Henriksen *et al.*, 1989). To compare fiber-type differences, similar glycogen contents are needed in both epitrochlearis and soleus muscles. If we exclude the group of epitrochlearis muscle with high glycogen content, we then have glycogen contents between 70-250 mmol kg dw^{-1} in both soleus and epitrochlearis muscles. Interestingly, the relationship between glycogen content and GS fractional activity was similar in soleus and epitrochlearis muscles (Figure 11), which suggests that the feedback mechanisms of glycogen on insulin-stimulated GS activation in soleus muscle are the same as in epitrochlearis muscle.

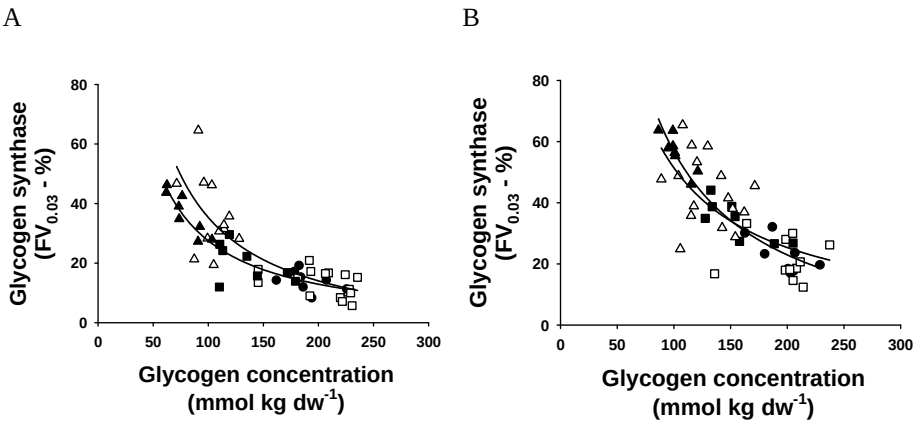

Figure 11. Correlations between GS fractional activity and glycogen content in muscles with glycogen contents between 70-250 mmol kg dw^{-1} (epitrochlearis muscles with LG and NG; soleus muscles with LG, NG, and HG). A: GS fractional activity plotted against glycogen content in rested muscles. B: GS fractional activity plotted against glycogen content in muscles during insulin stimulation. Open symbols: epitrochlearis muscles; filled symbols: soleus muscles; circles: muscles with high glycogen content; squares: muscles with normal glycogen content; triangles: muscles with low glycogen content.

Insulin-stimulated glycogen synthesis was also inversely correlated with glycogen content in soleus muscle (paper 4), as in epitrochlearis muscle (Jensen *et al.*, 2006).

Our results therefore suggest that high glycogen content inhibits insulin-stimulated glycogen synthesis through similar feedback mechanisms in both epitrochlearis and soleus muscles.

Although the feedback mechanism of glycogen on insulin-stimulated GS activation seems similar in epitrochlearis and soleus muscles, we have noticed that glycogen content in soleus muscle from the refed rats was less than half of that in epitrochlearis muscle (paper 4). Why soleus muscle accumulated less glycogen is not understood. The exposure to endogenous insulin should be similar in both soleus and epitrochlearis muscles after refeeding. Furthermore, soleus muscle has higher expressions of the key insulin signalling molecules for glucose storage, such as PI3K, PKB, GLUT4, GSK3, and GS (Henriksen *et al.*, 1990; Song *et al.*, 1999)(Lai YC and Jensen J, unpublished data) and has higher insulin-stimulated glucose uptake and glycogen synthesis than epitrochlearis muscle (Henriksen *et al.*, 1990; Ruzzin & Jensen, 2005). The extent of prior glycogen reduction is related to subsequent glycogen restoration (Price *et al.*, 2000). In the period of 24 h fasting of the rats, glycogen content decreased less in soleus muscle than in epitrochlearis muscle (paper 4) and this may explain why soleus muscle supercompensated less glycogen. In fact, a previous study has shown that glycogen content reached the highest levels at ~8 h after refeeding and after that glycogen content began to decrease in soleus muscle, but remained high in fast-twitch muscles (Holness *et al.*, 1988). This is probably because soleus muscle is more active than fast-twitch muscles during free moving conditions. Other protocols to increase muscle glycogen content, like 2 h of swimming followed by a high carbohydrate diet (Derave *et al.*, 2000b) and unloading or/and reloading to the rats (Henriksen *et al.*, 1986; Henriksen & Tischler, 1988), were not able to increase glycogen content above 200 mmol kg dw^{-1} in soleus muscle. Even infusion of the rat with a high concentration of glucose (Hoy *et al.*, 2007) or perfusion of

isolated hindlimbs with high concentrations of insulin and glucose (Richter *et al.*, 1988) did not increase glycogen content in soleus muscle to a similar level of that in fast-twitch muscles, which suggests that soleus muscle has lower capacity to store glycogen than fast-twitch muscles.

In conclusion, insulin-stimulated GS fractional activity was inversely regulated by glycogen content in both soleus and epitrochlearis muscles.

5.8. Effect of contraction on GS fractional activity in muscles with different glycogen contents

A number of studies have shown that contraction increases GS activity, but some of these studies reported only GS I form (%) or fractional activity when measured with high concentrations of UDP-glucose (Danforth, 1965; Nielsen *et al.*, 2001). Kochan et al. have previously suggested that a physiological concentration (0.03 mM) of UDP-glucose is more sensitive for studying the effect of exercise on GS fractional activity (Kochan *et al.*, 1981). GS fractional activity measured with 0.03 mM ($FV_{0.03}$) of UDP-glucose was increased by contraction (paper 1 and 2). We found that contraction-stimulated GS $FV_{0.03}$ was inversely correlated with glycogen content in epitrochlearis muscle, which agrees with a earlier study on other fast-twitch muscles (Nielsen *et al.*, 2001). Interestingly, plotting GS $FV_{0.03}$ against glycogen content showed that GS $FV_{0.03}$ in contracted epitrochlearis muscles was clustered above the level of that for rested muscles (paper 2), suggesting that contraction increases GS $FV_{0.03}$ independently of glycogen content. In paper 3, we reported that glycogen content decreased continuously during the contraction time-course, but GS $FV_{0.03}$ increased between 5-10 min of contraction and remained at the same high level until the 30 min of contraction, which shows that glycogen content is not closely related to contraction-mediated GS fractional activity.

In paper 2, we also showed that using different conditions for measuring GS fractional activity can influence the physiological interpretation of the data. When GS fractional activity was measured with 1.67 mM of UDP-glucose (GS $FV_{1.67}$), contraction did not increase GS $FV_{1.67}$ independently of glycogen content (paper 2). This result is similar to the result observed by Nielsen et al (Nielsen *et al.*, 2001), but is different from the result when GS fractional activity was measured with 0.03 mM of UDP-glucose. Our results therefore indicate that a physiological concentration of UDP-glucose is required to detect the effect of contraction on GS fractional activity independent of glycogen content.

In soleus muscle, GS $FV_{0.03}$ was increased to a similar level in all groups after contraction (paper 4), which suggests that contraction increases GS $FV_{0.03}$ independently of glycogen content in soleus muscle. Glycogen synthesis was also increased to a similar level in all soleus muscles after contraction (paper 4). These data seem to contrast with those in epitrochlearis (paper 2) and other fast-twitch muscles (Davis *et al.*, 1986; Nielsen *et al.*, 2001). If we were to investigate fiber-type differences, we would need to compare muscles with similar glycogen content. Then, if we exclude the epitrochlearis muscles with high glycogen content, we will have glycogen contents between 20-180 mmol kg dw^{-1} in both contracted soleus and epitrochlearis muscles. Interestingly, GS $FV_{0.03}$ was increased to similar levels after contraction (Figure 12) and no correlation between glycogen content and GS $FV_{0.03}$ was observed in both muscles with comparable glycogen contents (Figure 12). These results raise the possibility that high glycogen content (above 180 mmol kg dw^{-1}) may be important to achieve the inhibitory effect of glycogen on contraction-mediated GS fractional activity. However, soleus muscle has lower capacity to store glycogen content than epitrochlearis. Therefore, it is difficult to verify that glycogen content did not regulate contraction-meditated GS fractional activity in soleus muscle.

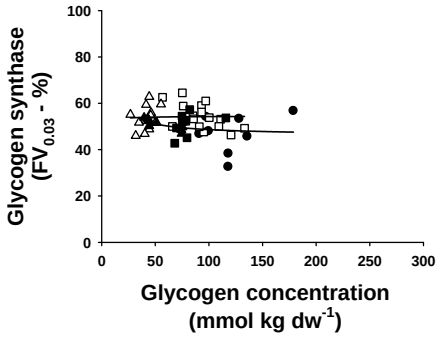

Figure 12. Correlation between GS fractional activity and glycogen content in contracted muscles with glycogen content between 20-180 mmol kg dw^{-1} (contracted epitrochlearis muscles with LG and NG; contracted soleus muscles with LG, NG, and HG). Open symbols: epitrochlearis muscles; filled symbols: soleus muscles; circles: muscles with high glycogen content; squares: muscles with normal glycogen content; triangles: muscles with low glycogen content.

In paper 3, we investigated the effect of contraction on GS fractional activity measured at different concentrations of UDP-glucose. We found that contraction increased GS $FV_{1.67}$ only 50 %; whereas contraction increased GS $FV_{0.03}$ 3-4 fold (paper 2 and 3). These data indicate that using a physiological concentration of UDP-glucose to measure GS fractional activity is more relevant to physiological conditions, as contraction increased the rate of glycogen synthesis several fold in skeletal muscle. Furthermore, plotting GS $FV_{0.03}$ against the Km-$_{0.17}$ values from the same samples, we found that these two parameters were not changed simultaneously (paper 3). When muscles have high glycogen contents, contraction led to a higher increase in GS affinity for UDP-glucose than in GS $FV_{0.03}$. On the other hand, contraction did not increase GS affinity for UDP-glucose and only increased GS $FV_{0.03}$ in muscles with low glycogen content (paper 2 and 3). These results highlight the fact that GS affinities for both UDP-glucose and glucose 6-phosphate have to be considered together when GS activity is evaluated.

Taken together, contraction-stimulated GS fractional activity was inversely regulated by glycogen content in epitrochlearis muscle, but the reduction of glycogen content is not the only factor that promotes GS activation. Furthermore, a physiological concentration of UDP-glucose for analyzing GS fractional activity is essential to find the effect of contraction independent of glycogen content. Contraction-mediated GS fractional activity was not regulated by glycogen content in soleus muscle, but this may be because soleus muscle has too low glycogen content to cause an inhibitory effect on GS fractional activity after contraction.

5.9. Additive effect of contraction and insulin on GS fractional activity in muscles with different glycogen contents

In paper 1, we addressed the question as to whether there is an additive increase in GS fractional activity when epitrochlearis muscles with different glycogen contents were stimulated by contraction and insulin simultaneously. In epitrochlearis muscle, our data showed that contraction and insulin additively increased GS fractional activity in NG and HG, but not in LG. Nevertheless, insulin, contraction, and combination of the two stimuli increased GS fractional activity to the highest level in LG. This pattern is rather similar to the data of glucose uptake. The combined effect of contraction and insulin on glycogen synthesis was not measured in paper 1, but our data on glucose uptake and GS fractional activity may suggest that the additive effect of contraction and insulin on glycogen synthesis occurs in NG and HG, but not in LG.

Several studies have reported that insulin-stimulated glucose uptake, GS activation, and glycogen synthesis were enhanced after contraction in soleus muscle with normal glycogen content (Leighton *et al.*, 1989; Etgen *et al.*, 1996; Richter *et al.*, 2001; Ruzzin & Jensen, 2005). In paper 4, we also demonstrated that contraction prior to insulin stimulation enhanced GS fractional activity and glycogen synthesis in

soleus muscles with low and high glycogen contents. However, we found that insulin-stimulated GS fractional activity was similar in all contracted soleus muscles despite different levels of glycogen content. Since contraction alone increased GS fractional activity to a similar level, insulin action of GS fractional activity was also similar in contracted soleus muscles with different glycogen contents, which contrasts with the results in rested soleus muscle where insulin action was higher in LG (paper 4). Insulin-stimulated glycogen synthesis after contraction in soleus was similar in LG and NG, but slightly lower in HG than in NG. These results may suggest that glucose uptake is the rate-limiting step for insulin-stimulated glycogen synthesis in soleus muscle after contraction.

Overall, when epitrochlearis muscles were stimulated by contraction and insulin simultaneously, GS fractional activity was additively increased in NG and HG but not in LG. In soleus muscles, prior contraction increased insulin-stimulated GS fractional activity to a similar level at all glycogen contents.

5.10.Effect of glycogen content on GS phosphorylation in skeletal muscle

A significant finding in the present thesis is that in rested epitrochlearis muscle GS Ser^{641} and Ser^{7} phosphorylation was higher in HG and lower in LG, compared to NG, which agrees with the data of GS fractional activity and GS affinity for UDP-glucose (paper 1 and 2). These findings support the view that phosphorylation/dephosphorylation regulates GS activity. The consistence between GS activity and GS phosphorylation at Ser^{641} and Ser^{7} may also suggest that GS Ser^{641} and Ser^{7} phosphorylation are involved in glycogen-mediated regulation of GS activity. GS Ser^{7} phosphorylation has previously been reported to be higher in HG than in LG (Jorgensen *et al.*, 2004a), and we now report that GS Ser^{7} phosphorylation in NG is intermediate. For the first time, GS Ser^{641} phosphorylation was shown to be paralleled

with glycogen content and phosphorylation of this site has a strong effect on GS activity (discussed later). In soleus muscle, GS Ser^{641} and Ser^{7} phosphorylation was also reduced in LG, compared to NG (paper 4), which agrees with the increased GS fractional activity in LG. However, GS Ser^{641} and Ser^{7} phosphorylation was similar in NG and HG, despite the fact that GS fractional activity was reduced in HG. Glycogen content in soleus muscle from the refed rats was much lower than in epitrochlearis muscle, which may explain why the increased GS Ser^{641} and Ser^{7} phosphorylation was not observed in soleus muscle with high glycogen content.

In both epitrochlearis and soleus muscles, GS $Ser^{645,649,653,657}$ phosphorylation was higher in NG than in LG and HG, whereas GS activity was lower in HG than in NG. Jensen et al. has previously reported similar discrepancies in epitrochlearis muscle (Jensen *et al.*, 2006) and it is unclear why this antibody showed low binding to GS in HG. GS $Ser^{641,645}$ phosphorylation has been reported to be similar in HG and LG (Jorgensen *et al.*, 2004a). These data may suggest that GS Ser^{641} phosphorylation is increased in HG whereas GS Ser^{645} phosphorylation is not. A problem in interpreting these results is that we do not know whether these multiple phospho-site antibodies interact with GS when all these sites are phosphorylated or if they interact with GS when only one of these sites is phosphorylated. Therefore, the data obtained with antibodies against multiple phosphorylation sites must be interpreted with caution. However, electrophoretic mobility of GS indicate that this protein was highly phosphorylated in epitrochlearis muscle with high glycogen content (paper 1 and 2).

In agreement with previous studies (Derave *et al.*, 2000a; Kawanaka *et al.*, 2000; Jensen *et al.*, 2006), GSK3 phosphorylation and AMPK activity were not influenced by altering glycogen contents in rested soleus and epitrochlearis muscles (paper 1, 2 and 4). These results suggest that GSK3 and AMPK do not regulate glycogen-modulated GS phosphorylation. Other kinases can also phosphorylate GS,

but we do not know if any of these kinase activities is regulated by glycogen content. On the other hand, high glycogen content has reportedly decreased PP1 activity (Villar-Palasi, 1969) and deletion of G_M/R_{GL} decreased muscle glycogen content (Aschenbach *et al.*, 2001). These reports indicate that PP1 is the key effector in mediating glycogen-modulated GS phosphorylation and activation, which determines glycogen accumulation.

In conclusion, the degree of GS Ser^{641} and Ser^{7} phosphorylation was positively correlated with the level of glycogen content; therefore, GS Ser^{641} and Ser^{7} phosphorylation may be involved in glycogen-mediated regulation of GS activity.

5.11. Effect of contraction on GS phosphorylation in muscles with different glycogen contents

Contraction decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation at all glycogen contents in epitrochlearis muscle (paper 1). These findings agree with the increased GS fractional activity and GS affinity for UDP-glucose (paper 1 and 2). Dephosphorylation of these sites with contraction stimulus has been reported in rat and mouse muscles containing normal glycogen content (McManus *et al.*, 2005; Ruzzin & Jensen, 2005). In paper 1 and 2, we found that GS phosphorylation was higher in HG than in NG after contraction, which shows that high glycogen content can reduce contraction-meditated GS dephosphorylation and activation.

GS $Ser^{645,649,653,657}$ phosphorylation was reduced immediately after contraction (paper 1), but not 30 min after contraction (paper 2), in epitrochlearis muscles with high glycogen content. These data suggest that the lower glycogen re-synthesis in HG after contraction is because high glycogen content 1) reduces contraction-meditated GS dephosphorylation and 2) accelerates the restoration of GS phosphorylation. The mechanisms by which contraction increases GS activity have been reported to be

independent of GSK3 (McManus *et al.*, 2005), whereas contraction was not able to dephosphorylate and activate GS in muscle from G_M/R_{GL} knockout mice (Aschenbach *et al.*, 2001). These studies indicate that G_M/R_{GL}-mediated PP1 activity is essential for contraction-stimulated GS dephosphorylation and activation.

An interesting finding in the present thesis is that GS Ser^7 phosphorylation was increased by contraction in epitrochlearis muscle. Contraction-stimulated GS Ser^7 phosphorylation occurred at all glycogen contents in epitrochlearis muscles (paper 1 and 2). The increased GS Ser^7 phosphorylation was surprising because GS activity was increased by contraction at all glycogen contents. A study has reported that AMPK phosphorylated purified GS at Ser^7, which decreased GS activity (Carling & Hardie, 1989). Jorgensen et al. have also shown that inactivation of GS was associated with increased GS Ser^7 phosphorylation during AICAR stimulation (Jorgensen *et al.*, 2004a). In AMPKα2 knockout muscle, AICAR did not increase GS Ser^7 phosphorylation and GS fractional activity was higher in AMPKα2 knockout muscle than in wild-type muscle (Jorgensen *et al.*, 2004a). Our data does not exclude that increased GS Ser^7 phosphorylation decreases enzymatic activity. However, it does show that increased GS Ser^7 phosphorylation does not prevent contraction-mediated GS activation.

Our study was not aimed at determining the mechanism for contraction-stimulated GS Ser^7 phosphorylation, but it is surprising that contraction increased GS Ser^7 phosphorylation and simultaneously decreased Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation. PP1 is the known phosphatase that dephosphorylates GS. It is therefore possible that PP1 preferentially acts on specific sites. But it is also possible that a contraction-induced kinase activity overcomes the activity of PP1 on GS Ser^7. Furthermore, our data suggest that AMPK is not the major kinase responsible for contraction-stimulated GS Ser^7 phosphorylation. First,

contraction-stimulated GS Ser^{7} phosphorylation was low in LG where AMPK activity was highest (paper 1 and 2). Second, GS Ser^{7} phosphorylation was rapidly increased during 30 min of contraction (paper 3), but contraction-stimulated AMPK activation appeared to increase gradually (Miranda *et al.*, 2008). It is therefore possible that contraction-stimulated GS Ser^{7} phosphorylation was mediated by other contraction-activated protein kinases. CaMK II has been reported as a good GS Ser^{7} kinase in fast-twitch muscle of rabbit (Roach, 1990; Sacchetto *et al.*, 2007). Rose et al. (Rose *et al.*, 2007) also reported that contraction-mediated CaMK II activation occurred rapidly and in a pattern similar to GS Ser^{7} phosphorylation during contraction (paper 3). These results lead me to suggest that CaMK II is the kinase responsible for contraction-stimulated GS Ser^{7} phosphorylation in epitrochlearis muscle.

In paper 4, we found that GS Ser^{7} phosphorylation was not increased 30 min after contraction in soleus muscle, which implies that contraction-stimulated GS Ser^{7} phosphorylation is fiber-type specific. Even though GS Ser^{7} phosphorylation was unchanged in all soleus muscles after contraction, CaMK II phosphorylation has been reported to be increased by contraction in soleus muscle (Canto *et al.*, 2006), which may suggest that contraction-stimulated CaMK II activation does not phosphorylate GS Ser^{7} in soleus muscle. This interpretation also raises the possibility that contraction-activated protein kinases may have different responsibilities in soleus compared with epitrochlearis muscles, like contraction-stimulated activation of AMPK regulates glucose uptake only in fast-twitch, but not in soleus, muscles (Derave *et al.*, 2000a; Wright *et al.*, 2004). Many kinases, as well as phosphatases, have been identified to be regulated by contraction, but little is known about whether they regulate cellular functions in a fiber-type specific manner.

In conclusion, contraction decreased GS phosphorylation at Ser^{641} and $Ser^{645,649,653,657}$. High glycogen content diminished contraction-stimulated GS dephosphorylation and accelerated the restoration of contraction-mediated GS phosphorylation. On the other hand, contraction increased GS Ser^{7} phosphorylation in epitrochlearis muscle despite increased GS activity

5.12.Additive effect of contraction and insulin on GS phosphorylation in muscles with different glycogen contents

Contraction and insulin alone decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation, which is accompanied by the increased GS activity. In epitrochlearis muscle, we found that the combination of contraction and insulin additively dephosphorylated GS Ser^{641} and $Ser^{645,649,653,657}$ only in HG, but not in LG and NG (paper 1). In HG, we also saw an additive increase in GS fractional activity when insulin and contraction were combined. However, contraction alone decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation to a low level in NG, and no further decrease was found when insulin and contraction were combined.

In soleus muscle, insulin-stimulated dephosphorylation at GS Ser^{641} and $Ser^{645,649,653,657}$ was enhanced after contraction in all groups (paper 4). Interestingly, prior contraction decreased insulin-mediated GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation to a similar level in all soleus muscles, which agrees with the GS fractional activity data (paper 4). Insulin dephosphorylates and activates GS through phosphorylation and inactivation of GSK3β (McManus *et al.*, 2005). On the other hand, G_M/R_{GL}-mediated PP1 activation is required for contraction-stimulated GS activation (Aschenbach *et al.*, 2001). Despite the two distinctive mechanisms, our results showed that both stimuli dephosphorylate GS Ser^{641} and $Ser^{645,649,653,657}$, which contribute to GS activation.

In conclusion, contraction and insulin additively decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation in epitrochlearis muscle with high glycogen content, but not in epitrochlearis muscles with low and normal glycogen contents. In soleus muscle, prior contraction decreased insulin-mediated GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation to a similar level despite different levels of glycogen content.

5.13.The relationship between GS fractional activity and phosphorylation in skeletal muscles modulated by contraction, insulin, and glycogen content

GS activity is regulated by insulin, contraction and glycogen content. In paper 1 and 4, we investigated the correlation between GS fractional activity and GS phosphorylation when muscle GS activation was modulated by insulin, contraction, and glycogen content alone or in combination. GS phosphorylation and GS fractional activity were measured in different muscles; therefore, we can only make a correlation between the group means. We found that even though contraction-mediated GS fractional activity and phosphorylation was measured at different time points in epitrochlearis and in soleus muscles, the correlation results were similar. Interestingly, GS fractional activity was found to be inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation. Mutagenetic studies have reported that dephosphorylation of GS Ser^{641} and Ser^{645} is important for increasing GS activity (Skurat *et al.*, 1994; Skurat & Roach, 1995), and the most recent study also found that GS $Ser^{641,645}$ phosphorylation was inversely correlated with GS fractional activity in human muscles modulated by exercise and diet (Prats *et al.*, 2009). Our findings therefore support that GS Ser^{641} and Ser^{645} phosphorylation are physiologically important for regulating GS activity. Importantly, GS Ser^{641} phosphorylation appeared to correlate most strongly with GS fractional activity, which suggests that GS Ser^{641} phosphorylation is the most important regulator for GS activity under various

physiological conditions. Furthermore, the results from the time-course study (paper 3) also showed that contraction-mediated GS activation occurred at the same time as GS Ser^{641} phosphorylation started to decrease, which shows that the degree of GS Ser^{641} phosphorylation can be easily translated to GS activity.

GS Ser^{7} phosphorylation did not correlate with GS fractional activity, suggesting that this phosphorylation site poorly reflects the activation of GS. However, if we exclude the data of contracted muscles, GS fractional activity was correlated with GS Ser^{7} phosphorylation in both rested epitrochlearis and soleus muscles. In epitrochlearis muscle, contraction and insulin activate GS without decreasing GS Ser^{7} phosphorylation (paper 1), which agrees with the observation in rabbit skeletal muscle (Parker *et al.*, 1983). However, GS Ser^{7} phosphorylation was decreased by insulin in soleus muscles with low and normal glycogen contents (paper 4). Our results cannot verify that the dephosphorylation of GS Ser^{7} contributes to GS activation, but they do suggest that insulin-mediated GS Ser^{7} dephosphorylation may be fiber-type specific.

In conclusion, GS fractional activity was inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation, supporting that Ser^{641} and Ser^{645} are important sites for regulating GS activity under physiological conditions. Nevertheless, GS Ser^{641} phosphorylation is the most sensitive site in describing GS activity. However, GS fractional activity did not correlate with GS Ser^{7} phosphorylation, suggesting that GS Ser^{7} phosphorylation poorly describes physiological GS activity.

6. CONCLUSION

1) Contraction and insulin additively increased glucose uptake in epitrochlearis muscles with normal and high glycogen contents, but not in muscle with low glycogen content. Despite this, the highest rate of glucose uptake occurred in muscle with low glycogen content when insulin and contraction were combined.
2) Insulin-stimulated PKB phosphorylation was enhanced in both epitrochlearis and soleus muscles with low glycogen content, whereas insulin-stimulated GSK3β phosphorylation was not influenced by glycogen content. Insulin-stimulated PKB and GSK3β phosphorylation was reduced in contracting epitrochlearis muscles with low and normal, but not with high, glycogen contents. In soleus muscle, insulin-stimulated PKB Thr^{308} phosphorylation was enhanced at all glycogen contents after contraction, whereas insulin-stimulated PKB Ser^{473} and GSK3β phosphorylation was not changed after contraction.
3) AMPKα2 activity and ACC phosphorylation were not influenced by glycogen content in rested epitrochlearis muscle. Contraction-stimulated AMPK activation was inversely regulated by glycogen content, but contraction-stimulated ACC phosphorylation was not regulated by glycogen content.
4) Contraction and insulin additively increased AS160/TBC1D1 phosphorylation in epitrochlearis muscles with normal and high glycogen contents, but not in muscles with low glycogen content. However, AS160/TBC1D1 phosphorylation did not correlate with the magnitude of glucose uptake.
5) Low glycogen content increased GS affinity for UDP-glucose. Conversely, high glycogen content decreased GS affinity for UDP-glucose in skeletal muscle.
6) GS affinity for UDP-glucose was increased in epitrochlearis muscle after contraction. Contraction increased GS affinity for UDP-glucose independently of

glycogen content, but a physiological concentration of glucose 6-phosphate for analyzing GS *Km* is required to see this effect.

7) Insulin-stimulated GS fractional activity was inversely regulated by glycogen content in both soleus and epitrochlearis muscles.
8) Contraction-stimulated GS fractional activity was inversely correlated with glycogen content in epitrochlearis muscle. Contraction increased GS fractional activity independently of glycogen content, but a physiological concentration of UDP-glucose is essential to obtain this outcome when analyzing GS fractional activity. In soleus muscle, contraction increased GS fractional activity to a similar level in all groups despite different levels of glycogen content. This lack of correlation between GS fractional activity and glycogen content occurred because it is difficult to increase glycogen content in soleus muscle to a high enough level.
9) Contraction and insulin additively increased GS fractional activity in epitrochlearis muscles with normal and high glycogen contents, but not in muscle with low glycogen content. Nevertheless, GS fractional activity was highest in epitrochlearis muscle with low glycogen content. In soleus muscle, prior contraction increased insulin-stimulated GS fractional activity to a similar level independently of glycogen content.
10) GS Ser^{641} and Ser^{7} phosphorylation was decreased in rested muscle with low glycogen content and increased in rested epitrochlearis muscle with high glycogen content. GS $Ser^{645,649,653,657}$ phosphorylation was higher in muscle with normal glycogen content than in muscles with low and high glycogen contents.
11) Contraction decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation. High glycogen content diminished contraction-stimulated GS dephosphorylation at Ser^{641} and $Ser^{645,649,653,657}$. GS Ser^{7} phosphorylation was increased by contraction

in epitrochlearis muscle, but contraction-stimulated GS Ser^{7} phosphorylation did not prevent contraction-stimulated GS activation.

12) Contraction and insulin additively decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation only in epitrochlearis muscle with high glycogen content. However, in soleus muscle, prior contraction decreased insulin-mediated GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation to a similar level irrespective of glycogen content.

13) GS fractional activity was inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation when GS was modulated by contraction, insulin, and glycogen content in both epitrochlearis and soleus muscles. In contrast, GS fractional activity did not correlate with GS Ser^{7} phosphorylation.

7. SUMMARY

The present thesis is based on 4 papers which have investigated contraction- and insulin-mediated regulations of glucose uptake and GS activation in skeletal muscles with different glycogen contents. The level of glycogen content in skeletal muscle has a strong effect on the regulation of glucose metabolism. In the present thesis, we have shown that low glycogen content strongly increased glucose uptake in epitrochlearis muscle either under insulin and contraction stimulation alone or in combination. GLUT4 content was increased in epitrochlearis muscle with low glycogen content which can contribute the increased glucose uptake. Although insulin-stimulated PKB phosphorylation and contraction-stimulated AMPK activation were highest in muscle with low glycogen content, AS160/TBC1D1 phosphorylation was not elevated correspondingly, suggesting that these key signalling molecules do not determine the rate of glucose uptake. Furthermore, low glycogen content prevents the additivity of glucose uptake when insulin and contraction were combined.

Increased glycogen content can limit its own synthesis through an inhibition of GS activity. We have demonstrated that increased glycogen content decreased not only GS fractional activity but also GS affinity for UDP-glucose, which can be an important mechanism for determining muscle glycogen accumulation. Contraction increased both GS fractional activity and GS affinity for UDP-glucose. Although contraction-stimulated GS fractional activity and GS affinity for UDP-glucose were inversely regulated by glycogen content, our data revealed that contraction can activate GS through mechanisms independently of glycogen content. Furthermore, we have provided evidence that GS assay conditions will influence the physiological interpretation of GS data. Low glycogen content prevented the additive increase in GS fractional activity when epitrochlearis muscles were stimulated by insulin and

contraction simultaneously. Despite this, GS fractional activity was highest in epitrochlearis muscle with low glycogen content during insulin and/or contraction stimulations. In soleus muscle, however, prior contraction increased insulin-stimulated GS fractional activity to a similar level irrespective of glycogen content.

Phosphorylation is the key mechanism for regulating GS activity. We found that phosphorylation of GS Ser^{7} and Ser^{641} was associated with glycogen content in rested muscle, which suggests that glycogen regulates GS activity through phosphorylation probably at these two sites. Contraction decreased GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation in epitrochlearis muscle at all glycogen contents, which was associated with the increased GS fractional activity and affinity for UDP-glucose. On the other hand, contraction increased GS Ser^{7} phosphorylation in epitrochlearis muscle which did not prevent contraction-stimulated GS activation. When muscles with different glycogen contents were exposed to insulin and contraction alone or in combination, GS Ser^{641} phosphorylation had the strongest correlation with GS fractional activity. This result shows that insulin, contraction and glycogen content all regulate GS Ser^{641} phosphorylation in concert with physiological GS activity.

8. PERSPECTIVES

Optimal regulation of glycogen metabolism in skeletal muscle is important not only in sport performance but also in controlling glucose homeostasis of the body. Type 2 diabetes is approaching epidemic proportions globally. While the prevalence of diabetes is on the increase, it has been recognized that physical activity can prevent, and in some case treat, this disease. Impaired insulin-stimulated glucose uptake and glycogen synthesis are the hallmark of type 2 diabetes and exercise has proven to improve these metabolic processes. Glycogen is the most important fuel for providing energy to allow muscle to contract during exercise, and decreased muscle glycogen content has a profound effect on insulin-stimulated glucose uptake and GS activity. It was thought that contraction increases muscle GS activity solely by decreasing glycogen content, but data from this thesis show that contraction can activate GS through mechanisms other than decreasing glycogen content. It will be important to fully clarify these mechanisms.

The present study highlights the importance of using a sensitive assay to determine GS activation in response to muscle contraction. For future research, I suggest that GS affinity for both glucose 6-phosphate and UDP-glucose should be studied at the same time to obtain a more comprehensive estimation of GS activity. Many studies only reported GS I form or fractional activity for estimating GS activation, but this assay is unable to completely detect the physiological state of the enzyme. Furthermore, most studies measured GS fractional activity with a high concentration of UDP-glucose, which is not sensitive enough to describe the physiological activation of GS after an exercise or contraction challenge. I suggest that GS fractional activity should be measured with a physiological concentration of UDP-glucose and GS affinity for UDP-glucose with a physiological concentration of

glucose 6-phosphate.

The illumination of the complete picture on contraction-mediated GS phosphorylation should focus on the underlying mechanisms of GS activation. This work will be useful for identifying the upstream targets that are involved in contraction-mediated GS dephosphorylation. The activation of PP1 has emerged as a potential mediator for regulating contraction- and glycogen-mediated GS phosphorylation and activation. Future work should also develop a reliable method to investigate PP1 activity and clarify the mechanisms for the regulation of PP1 activity on GS.

In the present thesis, I have not discussed intracellular GS distribution, which may also play a role in the regulation of GS activity. Nielsen et al. have reported that GS was primarily located in a glycogen-rich membrane fraction in resting muscle, whereas in glycogen depleted muscle GS translocated to a cytoskeleton fraction (Nielsen *et al.*, 2001). It has been reported that phosphorylation may regulate the translocation of GS in skeletal muscle (Prats *et al.*, 2005; Prats *et al.*, 2009). In the present thesis, GS Ser^{7} phosphorylation was increased by contraction in epitrochlearis muscles, and I hypothesize that phosphorylation at GS Ser^{7} may regulate GS translocation. Based on their intracellular locations in skeletal muscle, glycogen particles have been classified into three different pools, namely subsarcolemmal, intramyofibrillar, and intermyofibrillar glycogen. Since GS is bound to glycogen particles, one could expect that at least three distinctive pools of GS are present in skeletal muscle. It will be interesting to know if GS phosphorylation regulates GS localization and the physiological role of GS translocation. Another important question to solve is the crystal structure of GS. Data on the structure of GS with different phosphorylation statuses should provide important information about the mechanisms for the regulation of GS activity.

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Glycogen content regulates insulin- but not contraction-mediated glycogen synthase activation in the rat slow-twitch soleus muscles

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Abstract

Aim: The aim of this study was to investigate the effect of glycogen content on glycogen synthase (GS) activation and phosphorylation in the slow-twitch soleus muscles after contraction, during insulin stimulation and when these two stimuli were combined.

Methods: Glycogen content was manipulated *in vivo* with 24 h fasting and fasting followed by 24 h refeeding. Soleus strips were electrically stimulated for 30 min *in vitro*, and GS activation and phosphorylation were investigated after an additional 30 min incubation with or without insulin.

Results: Fasting reduced glycogen content in soleus muscle by 40% and refeeding enhanced by 40%, compared to rats with free access to chow. Insulin-stimulated GS fractional activity was inversely correlated with glycogen content ($R = -0.95$, $P < 0.001$, $n = 24$) and rate of glycogen synthesis was also inversely correlated with glycogen content ($R = -0.70$, $P < 0.001$, $n = 36$). After contraction, GS fractional activity was increased to similar levels in muscles with low, normal and high glycogen content; rate of glycogen synthesis after contraction was also similar. After contraction, insulin additively increased GS activation at all glycogen contents. Group means of GS fractional activity was inversely correlated with GS Ser^{641} ($R = -0.93$, $P < 0.001$) and $Ser^{645,649,653,657}$ ($R = -0.85$, $P < 0.001$) phosphorylation, but not with Ser^{7} phosphorylation.

Conclusion: Glycogen content regulates insulin- but not contraction-stimulated GS activation and glycogen synthesis in soleus muscles. Furthermore, phosphorylation of GS Ser^{641} and $Ser^{645,649,653,657}$ seems to regulate GS activity in soleus.

Keywords Akt, fibre types, glycogen synthesis, GSK3, phosphorylation, PKB.

Skeletal muscles are composed of different muscle fibre types with different metabolic characteristics and energy preferences (Pette & Staron 1990, Zierath & Hawley 2004). According to their expressions of myosin heavy chain isoforms and the distinctive contractile activities, muscle fibre types are classified as slow-twitch (type I) and fast-twitch (type II) fibres (Barnard *et al.* 1971, Pette & Staron 1990). Slow-twitch fibres contain high oxidative enzyme activities and mitochondrial density, and have high capacity for fat oxidation (Buchthal & Schmalbruch 1980, Zierath & Hawley 2004). In contrast, fast-twitch fibres have high glycolytic enzyme activities and lower mitochondrial density.

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In addition to the metabolic properties of the muscle fibres, glycogen content also regulates glucose metabolism. Insulin- and contraction-stimulated glucose uptake, glycogen synthase (GS) and glycogen synthesis are enhanced in muscles with low glycogen content and reduced with high glycogen content (Davis *et al.* 1986, Jensen *et al.* 1997, 2006, Derave *et al.* 1999, 2000b, Kawanaka *et al.* 2000, Nielsen *et al.* 2001, Lai *et al.* 2007). Protein kinase B (PKB), which mediates insulin-stimulated glucose uptake (Jiang *et al.* 2003), also showed higher activation and phosphorylation during insulin stimulation in muscles with low glycogen content and elevated glucose uptake (Derave *et al.* 2000b, Jensen *et al.* 2006). GS incorporates the glucose molecules into the glycogen particle and GS represents a potential rate-limiting step during insulin-stimulated glycogen synthesis and during glycogen re-synthesis after exercise (Cohen 1993, Lawrence & Roach 1997, Wojtaszewski *et al.* 2002, Nielsen & Richter 2003). The activity of GS is regulated by phosphorylation and particularly dephosphorylation of Ser^{641} and Ser^{645} increases activity (Ahmad & Huang 1981, Skurat & Roach 1995). Insulin, contraction and low glycogen content reduce GS phosphorylation of these sites and increase GS activity (Sakamoto *et al.* 2003, Jorgensen *et al.* 2004, McManus *et al.* 2005, Lai *et al.* 2007).

Most of the above-mentioned studies have been performed in rat muscles comprising a high percentage of fast-twitch fibres (Davis *et al.* 1986, Jensen *et al.* 1997, 2006, Derave *et al.* 1999, 2000b, Kawanaka *et al.* 1999, 2000, Nielsen *et al.* 2001, Lai *et al.* 2007). Soleus is a slow-twitch muscle comprising ~80% type I fibres (Ariano *et al.* 1973). Soleus muscles contain lower glycogen content than fast-twitch muscles (James & Kraegen 1984, Brau *et al.* 1997, Aslesen & Jensen 1998), and glycogen content does not seem to affect contraction- or insulin-stimulated glucose uptake (Henriksen & Ritter 1993, Derave *et al.* 1999, 2000b). However, Hoy *et al.* (2007) found that insulin-stimulated glycogen synthesis was reduced in soleus muscle with high glycogen content, and GS activity was inversely correlated with glycogen content in soleus during unloading and reloading (Henriksen *et al.* 1986, 1989, Henriksen & Tischler 1988). Furthermore, we recently reported that GS fractional activity and phosphorylation is inversely correlated with glycogen content in fast-twitch epitrochlearis muscles after contraction (Danforth 1965, Nielsen *et al.* 2001, Lai *et al.* 2007), but this question has not been studied in soleus muscles.

We previously used a fasting and fasting–refeeding protocols to manipulate glycogen content in fast-twitch epitrochlearis muscle of young rats and obtained a fourfold variation in glycogen content (Jensen *et al.* 1997, 2006, Lai *et al.* 2007). We hypothesized that this protocol would also regulate glycogen content in soleus muscles and modulate GS activation and phosphorylation. The first aim of the present study was to investigate the variation of glycogen content in soleus muscles after fasting and fasting–refeeding. Furthermore, the effect of contraction and insulin on GS activation was studied after manipulation of glycogen content. Finally, GS phosphorylation at Ser^{7}, Ser^{641} and $Ser^{645,649,653,657}$ after contraction and during insulin stimulation was investigated in muscles with different glycogen contents and related to GS activity.

Materials and methods

Chemicals and antibodies

Insulin (Actrapid) was from Novo Nordisk (Bagsværd, Denmark). Amyloglucosidase was from Boehringer Mannheim (Indianapolis, IN, USA). D-[U-^{14}C] glucose (303 mCi mmol^{-1}) and D-[^{14}C] uridine diphosphate glucose (302 mCi mmol^{-1}) were from Perkin Elmer Life and Analytical Sciences (Waltham, MA, USA). Anti-GLUT4 was a gift from David E. James (Sydney, Australia). Anti-GS was a gift from Oluf Pedersen (Copenhagen, Denmark). Anti-phospho-GS Ser^{7} was a gift from Graham Hardie and described previously (Jorgensen *et al.* 2004). Anti-phospho-GS $Ser^{645,649,653,657}$ was from Oncogene (San Diego, CA, USA). Anti-phospho-GS Ser^{641}, anti-phospho-GS kinase3 (GSK3) α/β Ser^{21}/Ser^{9}, anti-phospho-PKB Ser^{473}, anti-phospho-PKB Thr^{308}, anti-phospho-5′-AMP-activated protein kinase α (AMPKα) Thr^{172} and anti-rabbit HRP-linked antibodies were from Cell Signaling (Beverly, MA, USA). Anti-GSK3, anti-mouse HRP-conjugate, and anti-sheep HRP-conjugate antibodies were from Upstate (Lake Placid, NY, USA). ECL was from Millipore (Billerica, MA, USA). Other chemicals were standard analytical grades from Merck (Darmstadt, Germany) or Sigma (St Louis, MO, USA).

Animals

Male Wistar rats were obtained from B&K Universal (Nittedal, Norway) and acclimatized in our laboratory animal facility for 6 or 7 days with free access to food and tap water. The animal room was maintained at 21 °C, humidity ≥55% and a 12 : 12 h light : dark cycle (lights on from 6:00 AM to 6:00 PM). The experiments were performed between 10:00 AM and 2:00 PM. Muscle glycogen content was manipulated *in vivo* to acquire muscles with low (LG), normal (NG) and high (HG) glycogen levels as described previously (Jensen *et al.* 1997). In brief, the rats with NG were kept on their normal diet until the experiments. The rats with LG were fasted for 24 h before the experiments. The

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rats with HG were fasted for 24 h and then fed on normal chow for 24 h preceding the experiments. On the day of the experiment the weights of the rats were 120–150 g. All experimental procedures were approved by National Animal Research Authority and conducted in conformity with the guidelines for live animals in Norway and the European Convention for the Protection of Vertebrate Animals Used in Experimental and Other Scientific Purposes.

Muscle preparation and incubation

The rats were anaesthetized with an intraperitoneal injection of ~10 mg pentobarbital (50 mg mL^{-1}). Soleus muscles were split longitudinally into two, dissected out and suspended on contraction apparatuses at their approximate resting length. Muscles were first pre-incubated for 30–50 min in 3.5 mL modified Krebs-Henseleit buffer, containing 116 mM NaCl, 4.6 mM KCl, 1.16 mM $MgSO_4$, 1.16 mM KH_2PO_4, 25.4 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5.5 mM glucose, 2 mM sodium pyruvate, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) and 0.1% bovine serum albumin, pH 7.4. After pre-incubation, muscles were kept rested or contracted electrically with 200 ms trains (100 Hz, square wave pulses of 0.2 ms duration, 10 V) delivered every 2 s for 30 min as described previously (Aslesen & Jensen 1998). After the stimulation bout, muscles were incubated 60 min with or without insulin (10 mU mL^{-1}) for measurements of glycogen synthesis. Other muscles, for measurements of GS activity and Western blot were incubated 30 min with or without insulin after the contraction bout. All incubations were executed at 30 °C and gas (95% O_2 / 5% CO_2) was bubbled continuously through the buffer. After incubations, muscles were removed from the apparatuses, blotted on filter papers, frozen in liquid nitrogen and stored at −70 °C until analysis.

Glycogen synthesis

For measurement of glycogen synthesis, 0.2 μCi mL^{-1} D-[U-^{14}C] glucose was added to the buffer and the incorporation of radio-labelled glucose into glycogen was measured as described previously (Franch *et al.* 1999).

Glycogen synthase activity

Glycogen synthase activity was measured by a modified method of Thomas *et al.* (1968) as described previously (Jensen *et al.* 2005). Briefly, freeze-dried muscles were homogenized with a Polytron (Kinematica AG, Littau-Luzern, Switzerland) in 1 mg : 400 μL (dry weight : volume) of buffer containing 50 mM Tris/HCl (pH 7.8), 100 mM NaF, 10 mM EDTA. The homogenates were centrifuged at 3000 ***g*** for 30 min at 4 °C. For analysis, 20 μL of the supernatant was added to 40 μL of assay buffer containing 25 mM Tris/HCl (pH 7.8), 50 mM NaF, 5 mM EDTA, glycogen (10 mg mL^{-1}), 0.5 μCi mL^{-1} D-[^{14}C] uridine diphosphate glucose. Activity was measured at 37 °C with 0.03 mM uridine diphosphate glucose and 0.17 or 12 mM glucose 6-phosphate for 8 min. After reaction, 50 μL of the supernatant/assay buffer was spotted on a filter paper (Whatman ET-31, 1 cm × 2.5 cm) and immediately dropped into ice-cold 66% ethanol and washed to remove free D-[^{14}C] uridine diphosphate glucose. The fractional activity of GS was calculated as per cent of the activity at 0.17 mM glucose 6-phosphate in the activity at 12 mM glucose 6-phosphate.

Glycogen content

In muscles where the rate of glycogen synthesis was measured, 100 μL of the KOH digest was neutralized and hydrolysed with amyloglucosidase. In muscles where GS activity was measured, glycogen was hydrolysed with 1 M HCl (2.5 h at 100 °C). Glycogen content was determined as glucose units analysed fluorometrically with appropriate standard curves (Passonneau & Lowry 1993).

Western blot

Muscles were weighed and homogenized (1 mg wet wt: 25 μL) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 30 mM NaF, 1 mM Na_3VO_4, 10 mM EDTA, 2.5 mM benzamidine and 2 μL mL^{-1} protease inhibitor cocktail (P-8340; Sigma), pH 7.4. The homogenates were rotated with 1% Triton X-100 at 4 °C for 1 h and centrifuged (11 500 ***g***) at 4 °C for 10 min. Protein concentration in lysates was determined (D_C Protein Assay; Bio-Rad, Hercules, CA, USA), and lysates were diluted to 2.5 μg μL^{-1} and prepared with Laemmli buffer. Proteins (~30 μg) were separated by electrophoresis in 10% SDS-PAGE. Proteins were transferred from the gel into a polyvinylidene difluoride (PVDF) membrane at 250 mA for 1 h. The membranes were washed (3 × 10 min) in phosphate-buffered saline (PBS)-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20) and blocked in PBS-T containing 5% dry milk for 2 h at room temperature. After blocking, the membranes were washed shortly in PBS-T, and incubated at 4 °C overnight with appropriate primary antibodies. After washing with PBS-T (6 × 10 min), the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature. The membranes were washed again (6 × 10 min) and the antibody binding was

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detected with the enhanced chemiluminescence (ECL) kit and quantified according to densitometry with Scion Image software (Scion, Frederick, MD, USA).

Statistics

Data are presented as means ± SEM. One-way analysis of variance (ANOVA) was performed to determine the differences between groups. When ANOVA revealed significant differences, further analysis was performed using least significant difference (LSD) as *post hoc* tests. Pearson's test was used for correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Glycogen content

Glycogen content in normal rested soleus (NG) was 118.4 ± 6.3 mmol kg dw^{-1} (Fig. 1a). Fasting for 24 h (LG) reduced glycogen content by ∼40% ($P < 0.002$). When 24 h fasted rats were given access to normal chow for another 24 h, the glycogen content was increased by ∼40% ($P < 0.001$; Fig. 1a). Contraction decreased glycogen content by ∼40% in NG and HG ($P < 0.003$) and by ∼30% in LG ($P = 0.084$; Fig. 1a). After contraction, glycogen content was similar in LG and NG, and higher in HG than in LG and NG. In Table 1, we report glycogen content in soleus and epitrochlearis muscles from rats exposed to fasting and fasting–refeeding. The glycogen content in epitrochlearis from rats on normal diet was ∼70% higher than in soleus muscles ($P < 0.001$; Table 1). Fasting reduced glycogen to rather similar levels in both soleus and epitrochlearis. After refeeding, glycogen content was more than twice as high in epitrochlearis muscles than in soleus muscles ($P < 0.001$; Table 1).

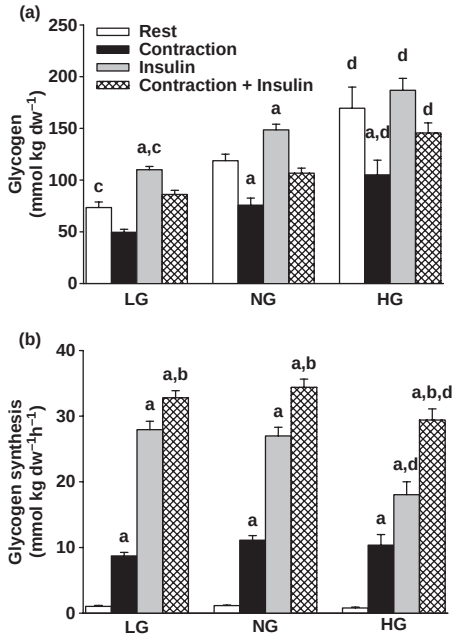

Figure 1 Glycogen content and rate of glycogen synthesis in soleus muscles with different glycogen contents. Muscles were contracted or kept rested for 30 min followed by 60 min incubation with or without insulin (10 mU mL^{-1}). (a) Glycogen content. (b) Rate of glycogen synthesis. Data are means ± SEM; n = 8–12. [a]Significantly different from rested muscles. [b]Significantly higher than contracted and insulin-stimulated muscles. [c]Significantly different from NG and HG. [d]Significantly different from LG and NG.

Glycogen synthesis

Glycogen synthesis was increased to a similar level in all groups during the first hour after contraction (Fig.1b). Insulin increased glycogen synthesis in all groups (Fig. 1b), but the rate of insulin-stimulated glycogen synthesis was ∼30% lower in HG than in NG and LG. The rate of insulin-stimulated glycogen synthesis was inversely correlated with glycogen content ($R = -0.70$, $P < 0.001$, $n = 36$). In addition, insulin increased glycogen synthesis more than contraction in all groups (Fig. 1b). In contracted muscles, insulin-stimulated glycogen synthesis was higher than in rested muscles independent of glycogen content, but the rate of glycogen synthesis was slightly lower in HG than in LG and NG (Fig. 1b). Basal glycogen synthesis was not influenced by glycogen content.

Glycogen synthase fractional activity

The fractional activity of GS in rested muscles decreased gradually as glycogen content increased (LG > NG > HG, $P < 0.05$; Fig. 2a). Pearson's correlation between GS fractional activity and glycogen content was $R = -0.88$ ($P < 0.001$, $n = 24$; Fig. 2b). Insulin increased GS fractional activity in all groups (Fig. 2a). Insulin-stimulated GS fractional activity decreased gradually as glycogen content increased (LG > NG > HG, $R = -0.95$, $P < 0.001$, $n = 24$; Fig. 2). After contraction, GS fractional activity was elevated to similar levels in all groups (Fig. 2a). Therefore, the increase in GS fractional activity 30 min after contraction was independent of glycogen content (Fig. 2). In contracted muscles, insulin-stimulated GS fractional activity was higher than in rested muscles and independent of glycogen content (Fig. 2a).

Table 1 Comparison of glycogen content (mmol kg dw^{-1}) in epitrochlearis and soleus from fasted and re-fed rats

	Fasted	Normal	Re-fed
Epitrochlearis	103.1 ± 4.9 (20)†	204.8 ± 8.2 (20)	449.3 ± 26.0 (20)‡
Soleus	78.7 ± 2.9 (20)†	120.5 ± 4.3 (20)*	169.1 ± 11.0 (20)*‡

Data are means ± SEM. Number of muscles is given in parentheses. Epitrochlearis and soleus muscles were from the same rats. Data from eight of the epitrochlearis muscles in each group are from figure 1B of Lai *et al.* (2007).
*Significantly different from epitrochlearis.
†Significantly different from normal and re-fed rats.
‡Significantly different from normal and fasted rats.

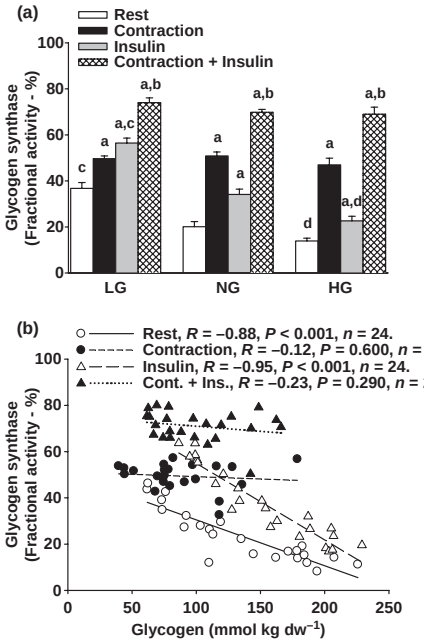

Figure 2 Effect of contraction and insulin on glycogen synthase fractional activity in soleus muscles with different glycogen contents. Muscles were contracted or kept rested for 30 min followed by 30 min incubation with or without insulin (10 mU mL^{-1}). (a) Glycogen synthase fractional activity. Data are means ± SEM; n = 8 in each group. [a]Significantly different from rested muscles. [b]Significantly higher than contracted and insulin-stimulated muscles. [c]Significantly different from NG and HG. [d]Significantly different from LG and NG. (b) Relationships between glycogen content and glycogen synthase fractional activity.

Glycogen synthase phosphorylation

Phosphorylation of GS at Ser^{641}, Ser^{645,649,653,657} and Ser^{7} is shown in Figures 3 and 4. Phosphorylation of GS at Ser^{641}, Ser^{645,649,653,657} and Ser^{7} was lower in rested LG than in NG. In HG, phosphorylation of GS at Ser^{641} and Ser^{7} was not significantly different from NG, but GS Ser^{645,649,653,657} phosphorylation was lower compared with NG. Insulin decreased GS Ser^{641} phosphorylation in all groups, whereas insulin decreased GS Ser^{645,649,653,657} phosphorylation only in LG and NG (Fig. 4). Insulin reduced GS Ser^{7} phosphorylation in NG and nearly significantly in LG (P < 0.08), but not in HG (Fig. 4c). GS Ser^{641}, Ser^{645,649,653,657} and Ser^{7} phosphorylation, when insulin was present, was lower in LG than in NG and HG (Fig. 4). After contraction, GS Ser^{641} phosphorylation was reduced in all groups (Fig. 4). GS Ser^{645,649,653,657} phosphorylation was reduced only in NG and LG. GS Ser^{641}, Ser^{645,649,653,657} and Ser^{7} phosphorylation was lower in LG than in NG 30 min after contraction (Fig. 4). GS Ser^{7} phosphorylation was unchanged 30 min after contraction in all groups of soleus muscles. In contracted muscles, insulin decreased GS Ser^{641} and Ser^{645,649,653,657} phosphorylation to similar levels in all groups (Fig. 4).

In the present study 12 groups were included. Figure 4 shows the relationships between group means of GS fractional activity and group means of GS phosphorylation. GS Ser^{641} phosphorylation was inversely correlated with GS fractional activity (R = −0.93, P < 0.001; Fig. 4d). GS Ser^{645,649,653,657} phosphorylation was also inversely correlated with GS fractional activity (R = −0.85, P < 0.001; Fig. 4e). In contrast, GS Ser^{7} phosphorylation did not correlate with GS fractional activity (Fig. 4f).

PKB and GSK3β phosphorylation

Protein kinase B Ser^{473} and Thr^{308} phosphorylation was undetectable 30 min after contraction and non-contracted muscles when insulin was absent (Fig. 5a,b). Insulin increased PKB Ser^{473} and Thr^{308} phosphorylation in all groups and insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation was higher in LG than in NG and HG (Fig. 5a,b). In contracted muscles, insulin increased PKB Ser^{473} phosphorylation to similar level as in non-contracted muscles (Fig. 5a), but insulin-stimulated PKB

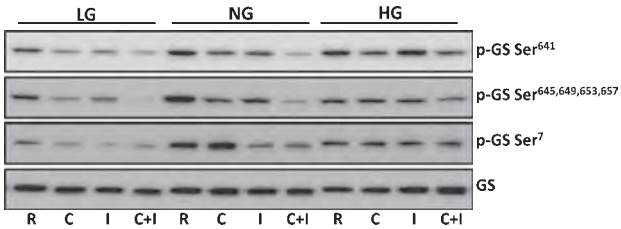

Figure 3 Representative immunoblots showing expression and phosphorylation of glycogen synthase (GS). Soleus muscles with low (LG), normal (NG) and high (HG) glycogen contents were kept rested for 30 min followed by 30 min incubation without (R) or with 10 mU mL^{-1} insulin (I), or contracted for 30 min followed by 30 min incubation without (C) or with 10 mU mL^{-1} insulin (C+I). From top: blots showing p-GS Ser^{641}, p-GS $Ser^{645,649,653,657}$, p-GS Ser^{7} and total GS.

Thr^{308} phosphorylation was slightly higher after contraction in all groups (Fig. 5b).

GSK3β Ser^{9} phosphorylation was low 30 min after contraction and in non-contracted muscles in LG, NG and HG (Fig. 5c). Insulin increased GSK3β Ser^{9} phosphorylation to similar extents in all groups (Fig. 5c). Insulin-stimulated GSK3β Ser^{9} phosphorylation was not altered 30 min after contraction in any of the groups.

AMPKα phosphorylation

AMPKα Thr^{172} phosphorylation was increased in LG and NG 30 min after contraction, but there was no difference between contracted LG and NG (Fig. 5d). Phosphorylation of AMPKα Thr^{172} was not significantly increased 30 min after contraction in HG. Insulin did not alter AMPKα Thr^{172} phosphorylation in any of the groups (Fig. 5d).

GLUT4 content

Total GLUT4 protein content in LG, NG and HG was 101.8 ± 7.6, 100.0 ± 6.9 and 85.9 ± 7.1 respectively ($n = 8$ in each group). There were no significant differences between groups.

Discussion

Skeletal muscles are composed of fibre types with different metabolic properties and energy utilizations. In the present study we report that glycogen content did not increase to the same level in soleus after refeeding compared with the fast-twitch epitrochlearis muscles. Nevertheless, glycogen content differed twofold between soleus muscles from fasted and fasted–refed rats, and insulin-stimulated glycogen synthesis and GS activation were inversely correlated with glycogen content in soleus muscles. On the other hand, the rate of glycogen synthesis after contraction was similar in muscles with different glycogen contents agreeing with similar GS fractional activity. Moreover, GS fractional activity was inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation in soleus muscles.

Soleus muscles in young rats kept on their normal diet had a glycogen content of ~120 mmol kg dw^{-1} which is ~60% of that in the fast-twitch epitrochlearis muscles, agreeing with other studies (James & Kraegen 1984, Brau *et al.* 1997, Aslesen & Jensen 1998, Aslesen *et al.* 2001, Ruzzin & Jensen 2005). Moreover, whereas 24 h fasting reduced glycogen content to similar levels in soleus and epitrochlearis (Table 1), glycogen content in soleus muscles after 24 h refeeding was less than the half of that in epitrochlearis muscles (Jensen *et al.* 1997, 2006, Lai *et al.* 2007). The reason for the lower glycogen content in soleus, compared with epitrochlearis, is not obvious as soleus muscles have higher expressions of GLUT4 and insulin signalling molecules like phosphatidylinositol 3-kinase (PI3-kinase), PKB, GSK3 and GS (Henriksen *et al.* 1990, Jensen *et al.* 1999, Song *et al.* 1999), and have higher insulin-stimulated glucose uptake and glycogen synthesis than epitrochlearis muscles (Henriksen *et al.* 1990, Ruzzin & Jensen 2005). Indeed, it has been reported that glycogen content in soleus muscles peaks 8 h after refeeding after which it decreases (Holness *et al.* 1988, James *et al.* 1998). Glycogen content in fast-twitch muscles also reaches the maximal level at an earlier time point but remains at a higher level for a longer period of time (Holness *et al.* 1988). The glycogen content achieved in soleus muscles after glucose infusion in conscious rats was also 30% lower than in the fast-twitch red quadriceps muscles (Hoy *et al.* 2007), and manipulation of glycogen content with other protocols like combinations of 2 h swimming and diets (Derave *et al.* 2000b) and unloading or reloading (Henriksen *et al.* 1986, Henriksen & Tischler 1988) also increase glycogen content to only about 200 mmol kg dw^{-1}. Even perfusion of hind limbs with glucose and high insulin concentration was unable to increase glycogen content in soleus to the same level as in fast-twitch muscles (Richter *et al.* 1988), which suggests that soleus muscles have lower capacity to store glycogen.

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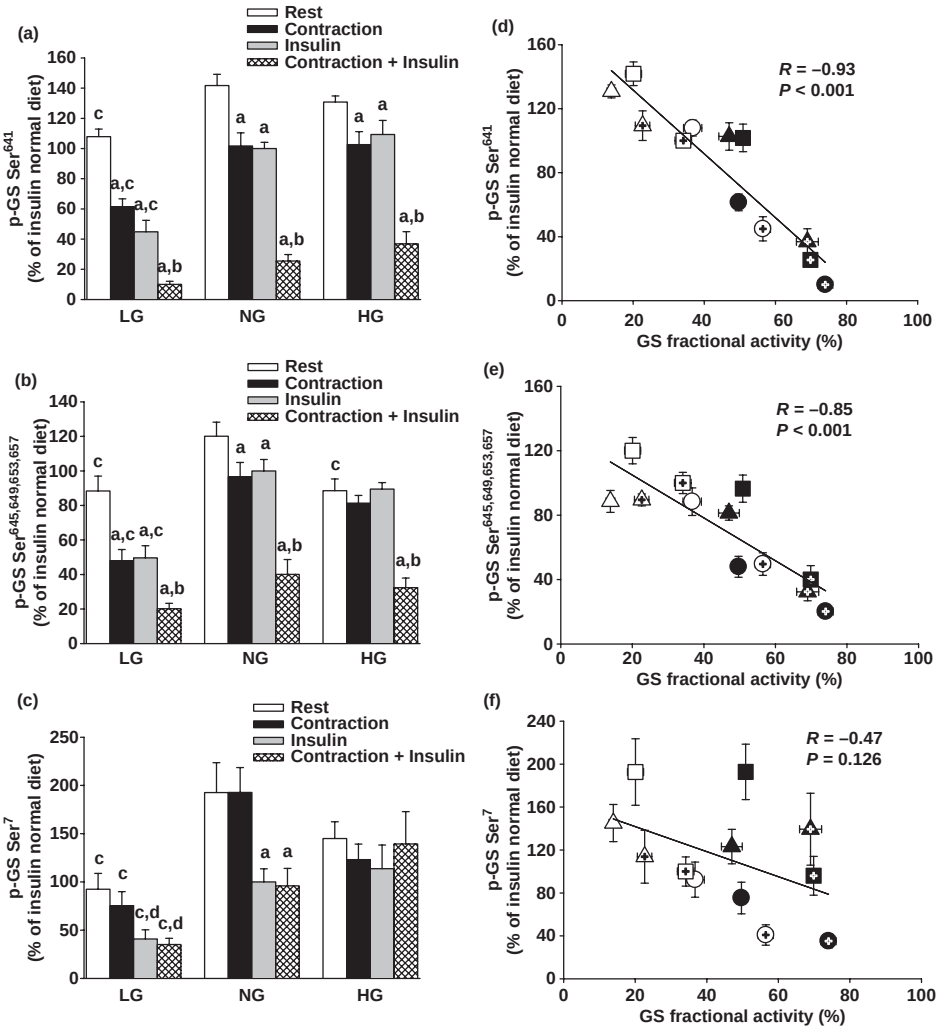

Figure 4 Effect of contraction and insulin on glycogen synthase (GS) phosphorylation in soleus muscles with different glycogen contents. Muscles were contracted or kept rested for 30 min followed by 30 min incubation with or without insulin (10 mU mL^{-1}). (a) p-GS Ser^{641} phosphorylation. (b) p-GS $Ser^{645,649,653,657}$ phosphorylation. (c) p-GS Ser^{7} phosphorylation. Data are means ± SEM; $n = 8$ in each group. [a]Significantly different from rested muscles. [b]Significantly lower than contracted and insulin-stimulated muscles. [c]Significantly different from NG. [d]$P < 0.08$ vs. rested muscles. (d–f) Correlations between GS fractional activity and GS phosphorylation at p-GS Ser^{641} (d), p-GS $Ser^{645,649,653,657}$ (e) and p-GS Ser^{7} (f). Open symbols: rested muscles; filled symbols: contracted muscles; open symbols with black cross: rested muscles incubated with insulin; filled symbols with white cross: contracted muscles incubated with insulin; circles: muscles with low glycogen content; squares: muscles with normal glycogen content; triangles: muscles with high glycogen content.

Although glycogen content only varied twofold in soleus muscles, we still observed that insulin-stimulated GS fractional activity was inversely correlated with glycogen content. However, the rate of glycogen synthesis was similar in LG and NG which suggests that glucose transport may be the rate-limiting step. Indeed, insulin-stimulated glucose uptake has been reported to be similar in soleus muscles with low and high glycogen contents (Derave *et al.* 2000b). Hoy *et al.* (2007) also found that glycogen content did not influence insulin-stimulated glucose transport, whereas insulin-stimulated glycogen synthesis was reduced in

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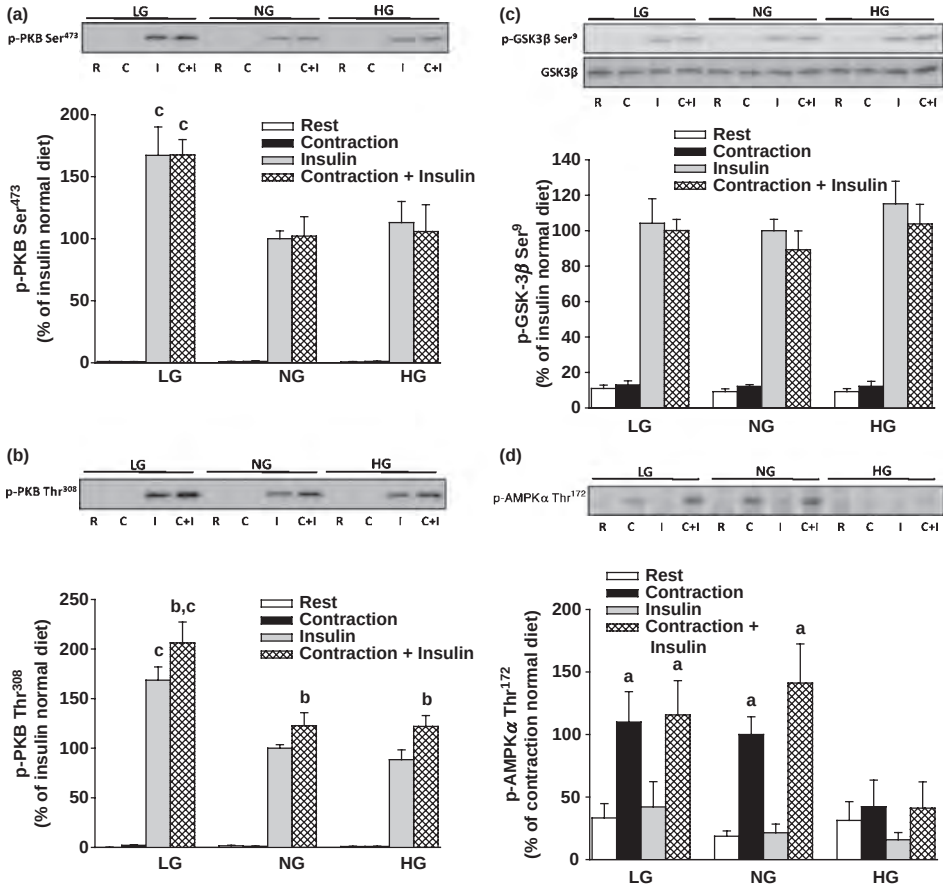

Figure 5 Effect of contraction and insulin on PKB, GSK3β and AMPKα phosphorylation in soleus muscles with different glycogen contents. Muscles with low (LG), normal (NG) and high (HG) glycogen contents were kept rested for 30 min followed by 30 min incubation without (R) or with 10 mU mL^{-1} insulin (I), or contracted for 30 min followed by 30 min incubation without (C) or with 10 mU mL^{-1} insulin (C+I). (a) p-PKB Ser^{473} phosphorylation. (b) p-PKB Thr^{308} phosphorylation. (c) p-GSK3β Ser^{9} phosphorylation. (d) p-AMPKα Thr^{172} phosphorylation. Data are means ± SEM; n = 8 in each group. [a]Significantly different from rested muscles. [b]Significantly higher than insulin-stimulated muscles. [c]Significantly different from NG and HG.

soleus muscles with high glycogen content. We did not measure glucose transport in the present study, but GS only becomes the rate-limiting step for glycogen synthesis in soleus with high glycogen content (Hoy *et al.* 2007) as occurs in epitrochlearis (Jensen *et al.* 2006). Moreover, insulin-stimulated GS fractional activity in soleus was inversely correlated with glycogen content with a similar trend as in epitrochlearis containing comparable glycogen contents (Jensen *et al.* 2006), which suggests that the mechanisms for glycogen-mediated feedback inhibition on GS activity may be similar in these two muscles.

The rate of glycogen synthesis after contraction was increased to a similar level in all groups. Moreover, GS fractional activity in soleus muscles was also increased to a similar level at all glycogen levels and therefore agrees with glycogen synthesis. These data contrast those reported in epitrochlearis (Lai *et al.* 2007) and other fast-twitch muscles (Davis *et al.* 1986, Brau *et al.* 1997, Nielsen *et al.* 2001). However, the epitrochlearis muscles with high glycogen content are important to achieve this correlation between glycogen content and GS fractional activity (Lai *et al.* 2007), and such high glycogen content was not obtained in soleus muscles.

It is also worth noting that GS fractional activity in soleus was much higher 30 min after contraction than after insulin stimulation, whereas the rate of glycogen synthesis was higher during insulin stimulation (Figs 1

and 2a). Glucose transport seems therefore to be the rate-limiting step for glycogen re-synthesis in soleus after contraction. Consistent with this interpretation, glucose transport determines glycogen re-synthesis in rat soleus muscle during reloading (Henriksen *et al.* 1996). Glucose transport returns much faster to the basal level after contraction in soleus than in fast-twitch muscles (Ploug *et al.* 1987), and glucose transport therefore becomes the rate-limiting step for glycogen re-synthesis in soleus. In a physiological perspective, these findings suggest that insulin is more important for replenishing glycogen in soleus muscles than in fast-twitch muscles.

In the present study, insulin-stimulated GS fractional activity and rate of glycogen synthesis were higher after contraction compared with rested muscles independent of glycogen content. Contraction prior to insulin stimulation enhances glucose uptake, glycogen synthesis and GS activation in soleus with normal glycogen content (Leighton *et al.* 1989, Etgen *et al.* 1996, Richter *et al.* 2001, Ruzzin & Jensen 2005). Interestingly, insulin-stimulated GS fractional activity increased to similar levels after contraction independent of glycogen content. However, the rate of glycogen synthesis remained lower in HG than in NG, and our data therefore suggest that glucose transport is the rate-limiting step for insulin-stimulated glycogen re-synthesis after exercise.

This is the first study to investigate the combined effects of glycogen content, contraction and insulin on GS phosphorylation in soleus muscles. Unfortunately, GS phosphorylation and fractional activity were not studied in the same muscles, and we can therefore only make correlations between group means. GS phosphorylation and fractional activity were investigated in 12 groups, and GS fractional activity was inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation. Insulin dephosphorylates and activates GS via GSK3β Ser^{9} phosphorylation (McManus *et al.* 2005) whereas protein phosphatase 1 is required for contraction-mediated GS activation (Aschenbach *et al.* 2001), but our data show that both stimuli dephosphorylate GS Ser^{641} and $Ser^{645,649,653,657}$ and activate GS. Previously, mutational studies have reported that GS phosphorylation at Ser^{641} and Ser^{645} are strong regulators of activity (Skurat & Roach 1995), and our data suggest that these phosphorylation sites are of physiological importance in soleus muscle.

GS Ser^{7} phosphorylation did not correlate with GS fractional activity in soleus muscles. GS Ser^{7} phosphorylation has been reported to decrease GS fractional activity (Carling & Hardie 1989, Jorgensen *et al.* 2004), but we recently found that contraction increased GS Ser^{7} phosphorylation in epitrochlearis when GS fractional activity was high (Lai *et al.* 2007). However, GS Ser^{7} phosphorylation was not increased 30 min after contraction in soleus as reported in epitrochlearis (Lai *et al.* 2007). The reason for this is not clear, but contraction-mediated activation of signalling pathways may be a key feature of fibre type differences, and contraction e.g. increases phosphorylation of GSK3 and p38 MAP-kinase in fast-twitch muscles but not in soleus muscles (Wretman *et al.* 2000, Ruzzin & Jensen 2005, Lai *et al.* 2007).

Insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation was higher in LG than in NG and HG. This finding agrees with previous findings in fast-twitch muscles (Derave *et al.* 2000b, Kawanaka *et al.* 2000, Jensen *et al.* 2006). The mechanisms for the higher PKB activation in muscles with low glycogen content have not been understood, but the higher insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation agrees with the higher insulin-stimulated glycogen synthesis in LG compared with HG. This interpretation can seem too simple as insulin-stimulated GSK3β Ser^{9} phosphorylation was not higher in LG. However, it is still possible that the magnitude of PKB Ser^{473} and Thr^{308} phosphorylation plays a role in the regulation of insulin-stimulated glucose uptake.

In humans, PKB phosphorylation has been reported to be elevated after exercise when insulin sensitivity is improved (Howlett *et al.* 2006, Wilson *et al.* 2006). In the present study, insulin-stimulated PKB Thr^{308} phosphorylation was slightly elevated 30 min after contraction. These data agree with two recent studies reporting increased insulin-stimulated PKB Thr^{308} phosphorylation in epitrochlearis after exercise without elevated Ser^{473} phosphorylation (Arias *et al.* 2007, Koshinaka *et al.* 2009), which may suggest that contraction specifically increases PKB Thr^{308} phosphorylation. PKB phosphorylation in the absence of insulin was not elevated in soleus 30 min after contraction, which agrees with other studies (Brozinick & Birnbaum 1998, Lund *et al.* 1998).

In the present study, AMPK phosphorylation was elevated 30 min after exercise in soleus with LG and NG, but not in HG. High glycogen content has previously been reported to prevent AMPK activation immediately after contraction in soleus (Derave *et al.* 2000a) and fast-twitch muscles (Derave *et al.* 2000a, Lai *et al.* 2007). The mechanism for this lack of activation of AMPK in HG is unclear, but the AMPK β-subunit has a glycogen-binding domain. Purified glycogen and oligosaccharides with α-1,6 branch points inhibit AMPK activity *in vitro* (McBride & Hardie 2009, McBride *et al.* 2009). Therefore, it is possible that contraction does not reduce glycogen content sufficient to allow AMPK activation in HG.

In conclusion, glycogen content does not increase to the same level in soleus muscles after refeeding as seen

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in fast-twitch epitrochlearis muscles; nevertheless, insulin-stimulated glycogen synthesis and GS activation inversely correlated with glycogen content in soleus muscles. On the other hand, rate of glycogen synthesis and GS activation after contraction were similar in muscles with different glycogen contents. GS fractional activity was inversely correlated with GS Ser^{641} and $Ser^{645,649,653,657}$ phosphorylation which suggests that glycogen content, insulin and contraction regulate GS activity via modulation of GS Ser^{641} and Ser^{645} phosphorylation.

Conflict of interest

The authors have no conflict of interest for this study.

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