Adrenaline action in normal and insulin-resistant Zucker rat skeletal muscles

Effect on insulin-stimulated glucose uptake and glycogen breakdown

Nader I. M. Alhaj

Master thesis in pharmacology for the degree Master of Pharmacy

Department of Pharmaceutical Biosciences,
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This thesis was conducted between August.2009 and September 2010 at the National Institute of Occupational Health (STAMI) and the Norwegian School of Sport Sciences

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Preface

My best thanks and regards to my external advisor who helped me through this job, followed me step by step, learned me exactly how to WORK SMARTER NOT HARDER, my special thanks and appreciations to **Jørgen Jensen**.

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A warm gratitude and thanks to my family members for their support and positive directions.

My great love to my wife and son, I thank them for being the best family and friends for me during my work in this project.

At the end, I dedicate this simple and modest job to every Palestinian student who can't reach his or her school because of the occupation, I hope they can get their schools and graduate at the scheduled time, and hope all Palestinian children can go to school in peace and without occupational borders.

My best wishes and regards.

Nader Alhaj

11.09.2010

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Abbreviations

ANOVA	Analysis Of Variance
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CVA	cerebrovascular accident
dw	Dry weight (freeze-dried)
EDL	Extensor Digitorium Longus
GS	Glycogen synthase
PI3-Kinase	Phosphatidylinositol 3-kinase
PKA	cAMP dependent protein kinase
PKB	Protein kinase B (AKT)
rEDL	red Extensor Digitorium Longus
wEDL	white Extensor Digitorium Longus
BW	Body weight

Abstract

Carbohydrates are the major source of energy for most humans and account for about 60% of the energy consumed. In the human body carbohydrates are stored as glycogen in the liver and in the skeletal muscles. Regulation of glycogen synthesis is of great importance in the process of blood glucose homeostasis as glucose is stored as glycogen. Insulin and adrenaline are the most important hormones which regulate glycogen breakdown and glucose uptake. Adrenaline normally contrasts the effect of insulin in metabolic regulation. Insulin stimulates glucose uptake and synthesis of glycogen and lipids leading to energy accumulation whereas adrenaline decreases insulin-stimulated glucose uptake, increases energy expenditure and stimulates breakdown of glycogen and lipids.

The aim of this study was to investigate the effect of adrenaline on insulin-stimulated glucose uptake and glycogen breakdown in soleus and EDL muscles from lean and insulin resistant obese Zucker rat.

Experiments were done in vitro, as muscles were surgically removed from rats and incubated with insulin, adrenaline or insulin and adrenaline together. Other muscles were stimulated electrically. Glucose uptake was calculated from the intracellular accumulation of ^{3}H-deoxy-D-glucose during incubation while glycogen content was measured through hydrolyzing of glycogen with amyloglucosidase. The formed glucose measured indirectly by measuring the amount of hexokinase and glucose-6-phosphate dehydrogenase fluorometrically.

In this study my results showed that adrenaline decreased insulin-stimulated glucose uptake in soleus and EDL muscles from lean and obese Zucker rats. Adrenaline was without effect on basal glucose uptake in soleus muscles from lean and obese Zucker rats. We found also that, glycogen content was higher in soleus muscles from obese Zucker rats than in soleus muscles from lean littermates in control rested state and after exposure to adrenaline. Adrenaline stimulated glycogen breakdown in insulin resistant EDL muscles but not in normal muscles.

In conclusion, adrenaline decreased insulin-stimulated glucose uptake in both normal and insulin resistant muscles. Furthermore adrenaline stimulated glycogen breakdown in EDL but not soleus muscles from obese Zucker rats. This suggests that β-adrenergic signaling is not impaired in insulin resistant muscles.

1. Background

1.1 Diabetes

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin (type I), or when the body cannot effectively respond to insulin (Type II). In a generation, diabetes has had a six-fold increase. In 1985 there were an estimated 30 million people with diabetes. Today diabetes affects more than 230 million people, almost 6% of the world's adult population (WHO, World diabetes foundation). The most important type is type II as it accounts for 90% of diabetes cases in the world. Type II diabetes can really managed or even prevented by a healthy diet and regular exercise.

The human body has the ability to manage the blood glucose level within 4-8 mmol/l despite food intake. The first step in the metabolism of glucose in muscles is the stimulation of cells by insulin. Insulin stimulates muscle cells to take up glucose and to store it as glycogen. This storage is crucial to maintain blood glucose within narrow limits despite continuous utilization of glucose and supply of carbohydrates in larger meals. Despite that carbohydrates are essential, the body contains limited amount of it. The total human body content of carbohydrates is nearly 500 g distributed as (4 g blood glucose, 80 g liver glycogen and 400 g muscle glycogen). Skeletal muscles managing about 80%of the insulin-stimulated glucose uptake in the human body (DeFronzo et al., 1981). And 70-90% of the glucose in the human body converts to glycogen in the skeletal muscles (Shulman et al., 1990).

One of the major causes of the type II diabetes is the defect or decrease in the insulin sensitivity in the cell. The major part of the body, which is of special importance in the study of the insulin sensitivity is skeletal muscle as some studies have reported that the chronic insulin resistance is in large part due to the skeletal muscle insulin resistance (Ivy et al., 1986).

Insulin resistance is generally defined as a decreased ability of insulin to stimulate usage and storage of glucose, precedes the development of diabetes and is detectable prior to the development of hyperglycaemia. This reduction in insulin ability to exert its action stimulates insulin production by pancreas, which results in hyperinsulinemia which is the most important symptom of insulin resistance. Insulin resistance results in decrease of glucose uptake into the muscle cells leading to increase glucose level in the blood. There are clear interactions between adrenergic and insulinergic systems that affect the net result of glucose uptake. Adrenaline stimulates glycogenolysis in skeletal muscles and accumulates glucose 6-phosphate, which inhibits hexokinase and phosphorylation of glucose and limit

glucose uptake process. As well, previous studies have reported that adrenaline decreases insulin-stimulated glucose transport but only when insulin is within a low to moderate physiological range. This supports the fact that adrenaline inhibits glucose uptake in skeletal muscles but not in the absence of insulin, which may indicate a different effect of adrenaline in normal skeletal muscles and insulin-resistant skeletal muscles on glucose uptake.

Adrenaline could inhibit the effect of insulin by the insulin receptor substrate-1(IRS-1)-associated phosphatidylinositol 3-kinase (PI3-kinase) in skeletal muscle (Hunt & Ivy 2002). Activation of PI3-kinase is recognized as an essential step in the activation of muscle glucose transport by insulin. However Hunt & Ivy (Hunt & Ivy, 2002) hypothesized that inhibition of glucose uptake by adrenaline in the presence of moderate physiological insulin concentration may be due to the inhibition of glucose transport rather than to a step distal to transport (Hunt & Ivy, 2002). The ability of adrenaline to attenuate insulin-stimulated glucose uptake by inhibiting transport appears to be limited to insulin concentrations in the low to moderate physiological range, as Hunt & Ivy found in 2002.

Type II diabetes can be managed or prevented by healthy diet and exercise, so obesity and physical inactivity in addition to genetical factors are the most important causes of this disease. Insulin resistant obese individuals often have abdominal obesity and high levels of LDL-cholesterol and triglycerides, and low level HDL-cholesterol. This will lead to accumulation of cholesterol in the blood and results in a high risk of heart attack and cerebrovascular accident (CVA). It is important to mention that people with type II diabetes have the same risk of heart attack and CVA as the people without diabetes who have already had one (World Diabetes Foundation).

In the treatment of diabetes, increasing physical activity and managing food intake toward the healthy sort, will give much better life with diabetes. Oral hypoglycemic medications will help to improve the disease and give much better control over blood glucose, but with the progression of the disease and after 5-10 years, many type II diabetic individuals must use insulin injection. Depending on the symptoms of diabetes, medical researches continue to find new therapies for this disease. The most promising researches are those applied on diabetic animals.

1.2 Animal model (Zucker rat)

Many different rodent models have been used to study the characteristics and causes of muscle insulin resistance, but probably the most popular model to date is the obese Zucker rat. This rat type was discovered in 1961 by Lois and Theodore Zucker (Strobl et al., 1993). This model has since been well characterized as a model of obesity, showing commonly published metabolic symptoms including insulin resistance and hyperlipidemia. The obese Zucker rat is hyperphagic and demonstrates a number of metabolic characteristics in common with human obese subjects.

Obese Zucker rat is the preferred rat model of insulin resistance and a combination of reduced insulin sensitivity and responsiveness, suggesting that its muscle insulin resistance resulted from both receptor and postreceptor defects. These defects included a reduced insulin receptor number and a defective glucose transport system. Some studies have reported that, the insulin resistance in the obese Zucker rats is associated with a decreased insulin binding (Crettaz et al., 1980), rate of glycogen synthesis (Ivy et al., 1986), and rate of glycolysis (Creattaz et al., 1983) when compared with lean littermates. Another study characterized the insulin receptor signaling system in skeletal muscle of the obese Zucker rat and found extreme insulin resistance consistent with the decrease in insulin-stimulated glucose uptake into skeletal muscle (Sherman et al., 1988).

The obese Zucker rats have hyperinsulinaemia, circulating triglycerides and skeletal muscle triacylglycerols are higher than lean littermates (Berthiaume & Zinker, 2002). Moreover circulating non-esterified fatty acids are increased in obese rats. This indicates that lipid utilization by muscle of obese rats could be increased and thereby alter glucose metabolism by the mechanism of the glucose-fatty acid cycle (Crettaz et al., 1980).

1.3 Carbohydrates

Carbohydrates are the major source of energy for most humans and account for about 60% of the energy consumed (Cryer, 2002). Most of the carbohydrates found in nature occur in the form of high molecular weight polymers called polysaccharides. The monomeric building blocks used to generate polysaccharides can be varied, however, the predominant monosaccharide found in polysaccharides is D-glucose. In blood, glucose is the main monosaccharide molecule. Although the utilization of energy varies greatly at rest and exercise, the concentration of glucose in the blood is highly regulated around 5 mM. A reduced concentration of blood glucose will cause fatigue, and concentration below 2 mM will cause seizure and even death (Cryer, 2002). The high blood glucose concentration is also unhealthy which leads to the diabetic complications. Endocrine system is very important in the regulation of blood glucose concentration. Insulin and adrenaline are the most important hormones to regulate blood glucose concentration.

1.4 Glycogen

The storage form of carbohydrates in humans is glycogen, this storage is crucial to maintain blood-glucose within narrow limits despite continuous utilization of glucose and supply of carbohydrates in larger meals. Despite that carbohydrates are essential, the body contains limited amount of it. Muscle glycogen is an important energy substrate during exercise, and the stores of glycogen limits performance in many types of endurance exercises (Coyle, 2000).

1.5 Synthesis of glycogen

The synthesis of glycogen from glucose is carried out by the enzyme glycogen synthase, this enzyme utilizes UDP-glucose as substrate. Glycogen synthase exists as iso-forms, the muscle iso-form GYS1 and the liver iso-form GYS2.

The activation of glucose to be used for glycogen synthesis is carried out by the enzyme UDP-glucose pyrophosphorylase. This enzyme exchanges the phosphate on C-1 of glucose-1-phosphate for UDP. The energy of the phospho-glycosyl bond of UDP-glucose is utilized by glycogen synthase to catalyze the incorporation of glucose into glycogen. UDP is subsequently released from the enzyme. The α-1,6 branches in glucose are produced by amylo-(1,4–1,6)-transglycosylase, also termed the branching

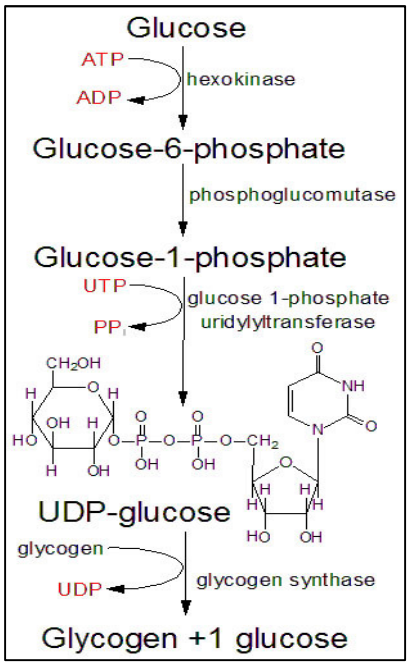

Figure A: Glycogen synthesis. The first step in glycogen synthesis is the formation of glucose 6-phosphate mediated by hexokinase, then and catalysed by phosphoglucomutase, glucose 6-phosphate converts to glucose 1-phosphate which transformed to UDP-glucose and under the action of glycogen synthase glycogen become the end result of this biochemical reaction (reproduced from themedicalbiochemistrypage.org) with a special permission from Dr. Michael W. King).

enzyme. This enzyme transfers a terminal fragment of 6-7 glucose residues (from a polymer at least 11 glucose residues long) to an internal glucose residue at the C-6 hydroxyl position.

1.6 Regulation of glycogen synthesis

Regulation of glycogen synthesis occurs mainly through the regulation of glycogen synthase activity. Glycogen synthase activity in vivo is regulated by phosphorylation as well as the concentration of glucose 6-phosphate. Most likely, both mechanisms participate in regulation of glycogen synthesis, but their contributions in physiological conditions are still uncertain. There are large numbers of regulators for the glycogen synthase that reflects its crucial role in metabolic regulation. As mentioned before, the glycogen is the only storage form of glucose in the human body, and the tight regulation of glycogen synthase and phosphorylase allows blood glucose level to be maintained within narrow ranges. External stimuli like insulin, adrenaline and exercise regulating glycogen synthase activity via changing the state of phosphorylation and the concentration of allosteric activators.

The activity of glycogen synthase is regulated by phosphorylation of serine residues. Phosphorylation of glycogen synthase reduces its activity towards UDP-glucose. In the non-phosphorylated state, glycogen synthase does not require glucose-6-phosphate as an allosteric activator; when phosphorylated it does (figure B).

Furthermore, glycogen synthase activity in the absence of glucose 6-phosphate (I-form) or with low (physiological) concentration of glucose 6-phosphate (fractional activity) is regulated by insulin, adrenaline, glycogen content and muscle contraction (Jensen et al. 1999).

Adrenaline decreases insulin-stimulated glycogen synthesis, however it does not inhibit glycogen synthesis after contraction (Franch et al., 1999). Glycogen synthesis needs the glucose transported through the cell membrane and phosphorylated to glucose 6-phosphate. During insulin stimulation and contraction activity, adrenaline has no effect in the transport of glucose through the cell membrane. (Lee et al., 1997; Aslesen & Jensen, 1998).

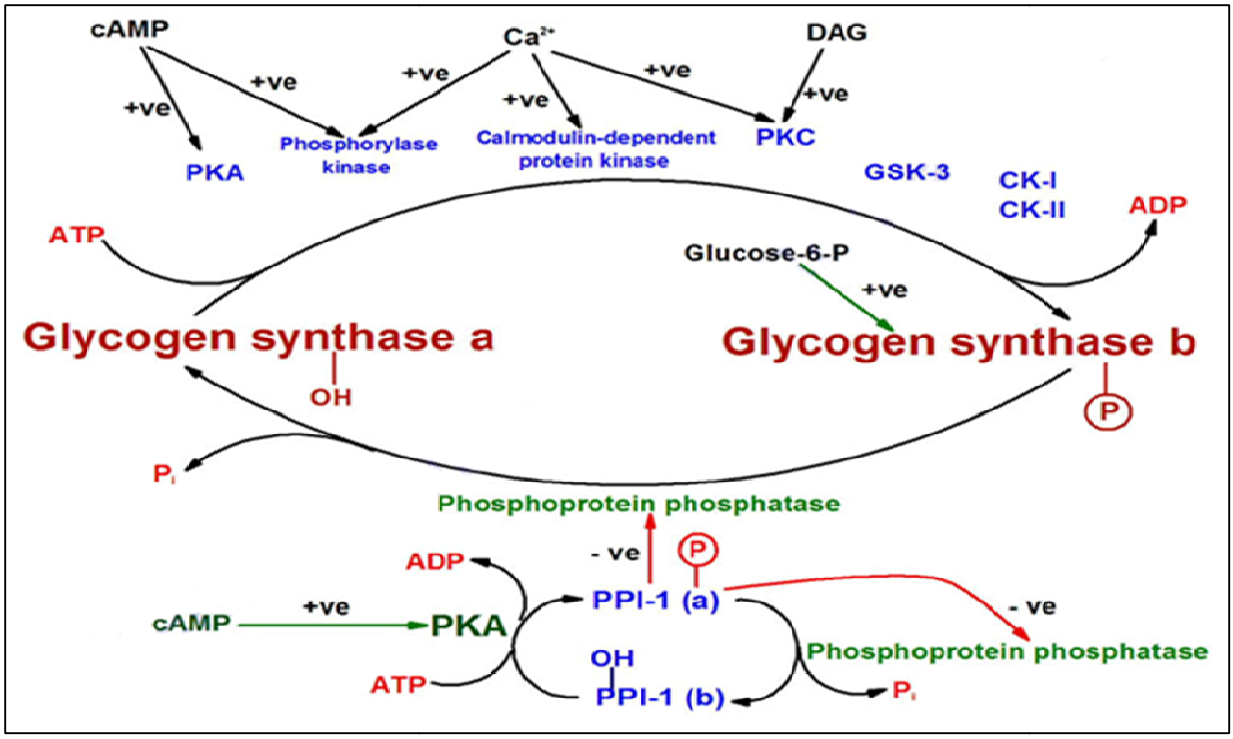

Figure B: Pathways involved in the regulation of glycogen synthase.

Glycogen synthase a is phosphorylated, and rendered much less active and requires glucose-6-phosphate to have activity. Phosphorylation of glycogen synthase is accomplished by several different enzymes such as protein kinase C (PKC), calmodulin-dependent protein kinase, glycogen synthase kinase-3 (GSK-3) and two forms of casein kinase (CK-I and CK-II). The enzyme PKC is activated by Ca2+ ions and phospholipids, primarily diacylglycerol (DAG) (reproduced from themedicalbiochemistrypage.org with a special permission from Dr. Michael W. King).

Glycogen concentration participates in the regulation of glycogen synthase activity (Danforth, 1965), the low concentration of glycogen influences the effect of adrenaline after the contractile effect (Franch et al,. 1999)

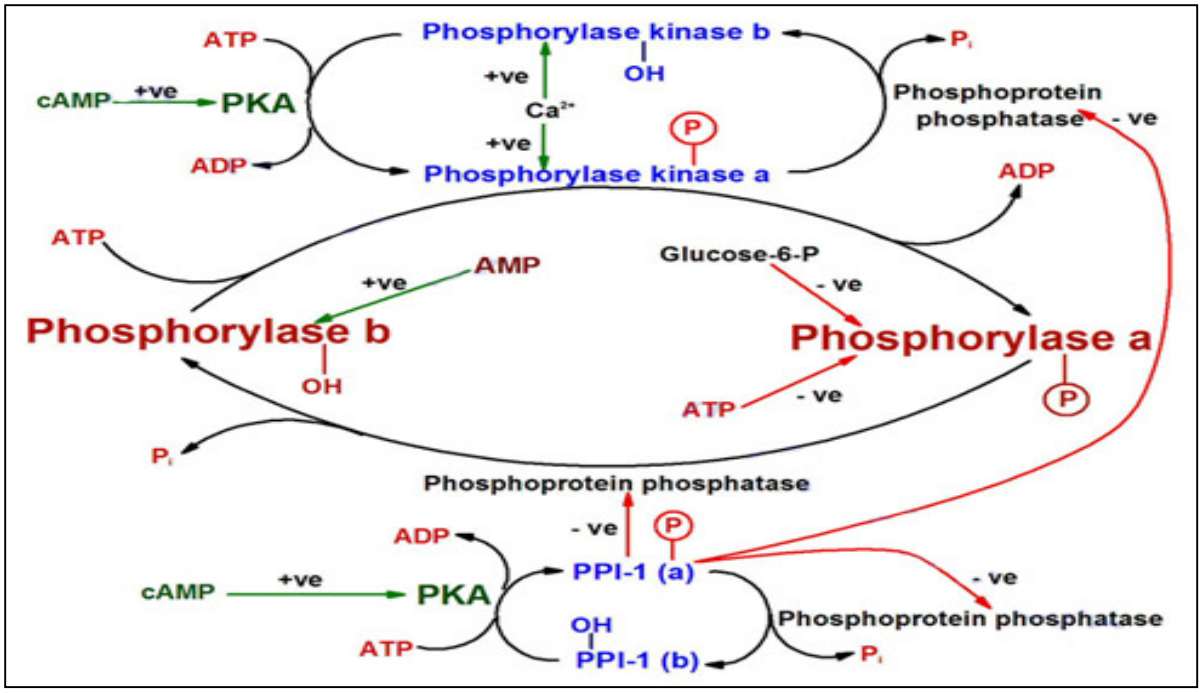

Figure C: Pathways involved in the regulation of glycogen phosphorylase.

PKA is cAMP-dependent protein kinase. PPI-1 is phosphoprotein phosphatase-1 inhibitor. Phosphorylase a is phosphorylated, and rendered highly active, by phosphorylase kinase. Phosphorylase kinase is itself phosphorylated, leading to increased activity, by PKA (itself activated through receptor-mediated mechanisms). Calcium ions can activate phosphorylase kinase even in the absence of the enzyme being phosphorylated. This is carried out predominately by protein phosphatase-1 (PP-1) the same phosphatase involved in dephosphorylation of phosphorylase (reproduced from themedicalbiochemistrypage.org with a special permission from Dr. Michael W. King).

In addition to glycogen synthase, phosphorylase kinase is an important enzyme in the regulation of glycogen synthesis .Phosphorylase kinase activity is affected by two distinct mechanisms involving Ca^{2+} ions and PKA. The ability of Ca^{2+} ions to regulate phosphorylase kinase is through the function of one of the subunits of this enzyme. One of the subunits of this enzyme is the ubiquitous protein, calmodulin. Calmodulin is a calcium binding protein. Binding induces a conformational change in calmodulin which in turn enhances the catalytic activity of the phosphorylase kinase towards its substrate, phosphorylase-b. This activity is crucial to the enhancement of glycogenolysis in muscle cells where muscle contraction is induced via acetylcholine stimulation at the neuromuscular junction. The effect of acetylcholine release from nerve terminals at a neuromuscular junction is to depolarize the muscle cell leading to increased release of sarcoplasmic reticulum stored Ca^{2+}, thereby activating phosphorylase kinase. In addition to the increase in the intracellular calcium, the rate of muscle contraction increases glycogenolysis which

provides the muscle cell with the ATP it also needs for contraction. The second mechanism to control the activity of phosphorylase kinase is the accumulation of cAMP through the activation of β-adrenergic receptors by adrenaline. cAMP controls the phosphorylation of phosphorylase kinase b to phosphorylase kinase a (Fig. C).

1.7 Glycogenolysis (Adrenaline effect in skeletal muscles)

The glycogenolytic cascade begins by adrenaline stimulation to β-adrenergic receptors which stimulate adenylate cyclase to produce cAMP. The second step is the activation of glycogen phosphorylase and inactivation of glycogen synthase. The production of c-AMP by adrenaline leads the sequential activation of c-AMP dependent protein kinase and phosphorylase kinase. The latter kinases convert glycogen phosphorylase from the inactive dephosphorylated b form to the active phosphorylated a form, stimulating glycogenolysis in advance of an increased energy demand. It's important to mention that calcium ions are important for the activity of phosphorylase kinase and the calcium channel must be open to sustain muscle contraction by providing energy via the breakdown of glycogen (Cohen, 2002).

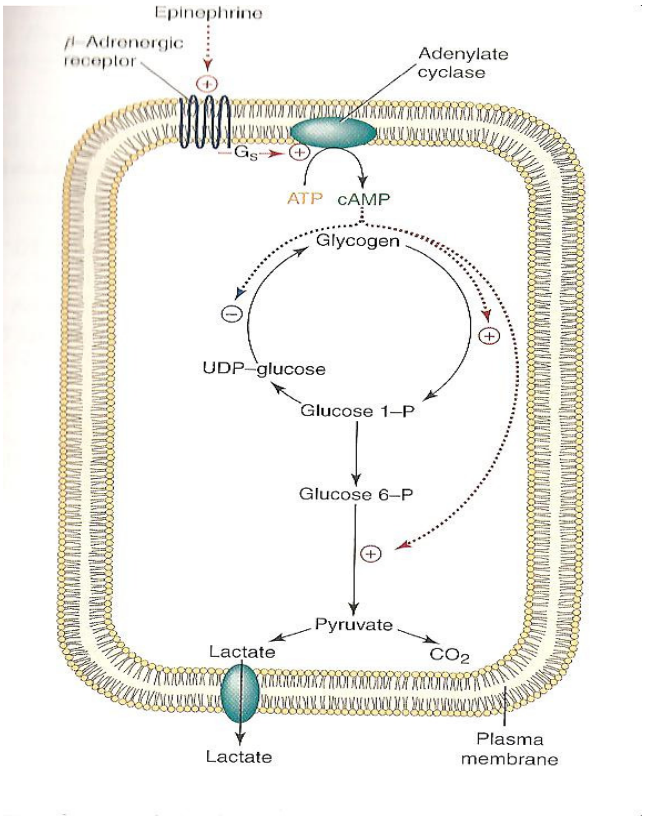

Figure D: cAMP mediates stimulation of glycogenolysis in muscle by β agonists (Adrenaline)

The β-adrenergic receptor is an intrinsic component of the plasma membrane that stimulates adenylate cyclase by stimulatory G-protein (Gs) (Devlin, T. Textbook of Biochemistry with Clinical Correlations 6th edition).

1.8 Insulin and adrenaline effects in relation to glucose kinetics

Adrenaline normally contrasts the effect of insulin in metabolic regulation. Insulin stimulates glucose uptake and synthesis of glycogen and lipids leading to energy accumulation whereas adrenaline decreases insulin-stimulated glucose uptake, increases energy expenditure and stimulates breakdown of glycogen and lipids. Glucose uptake is the transport and phosphorylation of glucose by hexokinase that results in its clearance from the surrounding medium while glucose transport is the movement of glucose across the plasma membrane.

Insulin enhances glucose uptake through a special signaling pathway. Insulin signaling pathway begins when insulin binds to its receptor (IR) on the cell surface. IR is consisting of α and β subunits, when insulin binds to the α-subunits of the IR, the β-subunits become phosphorylated automatically and

the tyrosine kinase activity stimulated. After that, insulin receptor substrate-1 phosphorylated and activated, IRS-1 activates phosphatidylinositol 3-kinase (PI3-kinase) and leading to the formation of phosphatdiylinositol (3,4,5)-triphosphate which activates phosphoinosetide-dependent protein kinase (PDK) and activates protein kinase-B (PKB). PKB translocates insulin-stimulated glucose trasporter protein (GLUT4) which mediates glucose uptake in skeletal muscles. Insulin also stimulates glycogen synthesis by activating glycogen synthase. Activation of glycogen synthase is important in the regulation of blood glucose, this process involves the phosphorylation of GSK-3 which is mediated by PKB.

To clarify the mechanism by which adrenaline decreases glucose uptake, some studies have reported that acute exposure of isolated muscle to adrenaline or the β adrenoceptor agonist isoprenaline causes an activation of glycogenolysis (glycogen breakdown), and an elevation of hexose monophosphate concentrations and a consequent inhibition of the rate of glucose phosphorylation (Chiasson et al., 1981; Challiss et al., 1986). Furthermore the higher plasma lactate levels, is another mechanism that may account for the reduction in muscle glucose uptake by adrenaline include effects on muscle glucose transport and/or fat metabolism (Howlett 1998). Under resting conditions adrenaline has been shown to decrease muscle glucose transport despite an increase in glucose transporter GLUT4 translocation to the plasma membrane, which suggests a reduction in GLUT4 intrinsic activity (Bonen et al. 1992).

Adrenaline stimulates glycogen breakdown and causes accumulation of lactate in skeletal muscles (Aselen & Jensen, 1998; Jensen & Dahl, 1995; Jensen et al., 1997). This process begins with the activation of glycogen phosphorylase and inactivation of glycogen synthase as a result of accumulation of cAMP which results from β-adrenoceptor stimulation by adrenaline. Stimulated β-adrenergic receptors activate adenylate cyclase (AC) via stimulatory G-protein (Gαs), and activated AC will produce cAMP. AC activity is also regulated by inhibitory G-protein (Gαi). Jensen et al have found that β-adrenergic stimulation by adrenaline stimulated glycogen breakdown in fast twitch muscles (type IIA and type IIB fibers in EDL) but not in slow twitch muscles (type I and type IIA fibers in soleus) (Jensen et al., 1989). In addition to the fiber type, glycogen content is of importance in the effect of adrenaline on glycogen breakdown in skeletal muscles. Glycogen content in type IIA fibers in EDL muscles is higher than glycogen content in type IIA soleus muscles and glycogen breakdown in EDL muscles stimulated by adrenaline infusion (Jensen et al., 1989). In 1989 Jensen et al have also reported that muscle glycogen content in type I soleus was higher than that in the corresponding fiber type in EDL muscles, but still there was no glycogen breakdown in type I soleus after adrenaline infusion (Jensen et al., 1989). In a later study Jensen et al. have reported a decrease in glycogen content in soleus muscles with high

glycogen content after 30 min incubation with adrenaline (Jensen et al., 1999). This finding indicated a relationship between glycogen content and adrenaline stimulated glycogen breakdown in skeletal muscles depending on the fiber types of these muscles.

It is well known that adrenaline secretion increases in response to exercise. Kjær et al (Kjær et al. 1993) have found that adrenaline inhibits glucose clearance during exercise, they found that glucose clearance was higher and plasma glucose concentration decreased more rapidly during exercise when adrenaline secretion was inhibited by pharmacological coeliac ganglion blockade than when compared with exercise where adrenaline secretion was physiologically normal (Kjær et al., 1993). The mechanism responsible for this reduction in glucose clearance may be related to the reduced glucose utilization in muscle secondary to an increase in glycogenlysis, as a result of adrenergic receptor stimulation.

1.9 β-adrenergic signaling and insulin resistance in skeletal muscles

Adrenergic receptors are devided into β and α adrenergic receptors. β2 and α1 are the most expressed adrenergic receptors in skeletal muscles. As we mentioned before, the most important type of these receptors in glucose uptake and phosphorylation is β-adrenoceptors (Jensen et al., 1995). The first step in adrenergic signaling is the binding of adrenaline to the β-adrenergic receptors, which stimulate the adenylate cyclase and leads to the accumulation of cAMP and activation of PKA (Cohen, 2002). PKA is an enzyme consisting of two catalytic subunits and two regulatory subunits, which binds to the cAMP and the catalytic subunit dissociates and phosphorylates substrate proteins. The continuous production of cAMP causes insulin resistance and decreases glycogen synthesis, this process is controlled by adenylate cyclase which balances its activity by the G-protein subunits Gαs and Gαi.

Many previous studies have reported that adrenergic system can attenuate the effect of insulin. Katz et al. have found β2 agonists markedly reduced glucose tolerance and reduced the insulin response to a glucose challenge without altering insulin-stimulated muscle glucose uptake or transport. (Katz et al., 1983). Another study has found that selective β2 agonist treatment also significantly reduced the fasting plasma insulin levels in obese zucker rats. (Torgan et al., 1993).

1.10 β-adrenergic signaling (the role of receptor content and exercise)

Expression of β-adrenergic receptor is fiber type dependent, the density of β-adrenergic receptors is twofold higher in soleus muscles than in epitrochlearis muscles (Jensen et al., 2002).The effect of

adrenaline on metabolic regulation differs between muscles composed of type I and type II fibers. Slow twitch muscles have higher insulin-stimulated glucose uptake than fast twitch muscles, also slow twitch muscles have about twofold higher PKB expression and activation during insulin stimulation than in fast twitch muscles.

Exercise increase β-adrenoceptor density in skeletal muscles (Williams et al., 1984). Insulin sensitivity is also increased after exercise, and β-adrenergic signaling is modulated in concert with insulin. In several physiological and pathophysiological settings, altered functional responsiveness to β-adrenergic receptors stimulation is associated in corresponding differences with β-adrenergic receptors density (Stob et al., 2007). In the sedentary compared with the exercise-trained state , Stob et al suggested that skeletal muscle β-adrenergic receptors density does not increase following endurance exercise training in healthy adult men (Stob et al., 2007).

Skeletal muscles need energy for its mechanical action, this energy expenditure is also affected by β-adrenergic system. The thermogenic response to the stimulation of peripheral β-adrenergic receptors is mediated by activation of the adenyl cyclase system, leading to increased production of cAMP (Nonogaki, 2000; Stob et al., 2007). Other studies have found that the β2-adrenergic receptors subtype has been shown to contribute significantly to β-adrenergic-mediated thermogenesis (Blaak et al., 1993, Stob et al. 2007). The last finding suggested that the greater thermogenic responsiveness to the β-adrenergic receptor stimulation in trained humans is not mediated by increased expression of β-adrenergic receptors in thermogenic tissues (Stob et al., 2007).

2. Aims

The first aim of this study was to compare insulin-stimulated glucose uptake and glycogen content in soleus and EDL muscles from lean and obese Zucker rats.

The second aim was to investigate the effect of adrenaline on insulin-stimulated glucose uptake and glycogen breakdown in normal and insulin resistant soleus and EDL muscles.

The third aim was to compare liver glycogen content in lean and obese Zucker rats.

3. Materials and methods

3.1 Rats and muscles

Female lean Zucker (fa/-) and obese Zucker (fa/fa) rats (Charles River) (delivered by Folkehelsa, Oslo, Norway) were received at 5–6 wk of age. The animals were housed in a temperature-controlled room (21°C) at the Animal Facility of the National Institute of occupational health (STAMI) for 6-7 weeks. Rats had free access to rat chow (RM1; BK Universal, Nittedal, Norway) and water. A reversed 12:12-h light-dark cycle was maintained (lights on 06:00 am – 0600 pm). The experiments were performed in the light cycle (between 10:00 am and 02:00 pm). At the day of experiments the weights of the lean rats were 200-250g and the weights of the obese rats were 300-400g. The experiments were conducted in conformity with the laws and regulations controlling experiments on live animals in Norway and European Convention for the protection of vertebrate animals used in experimental and scientific purposes.

The rats were anesthetized with an intraperitoneal injection of ~7.5 mg pentobarbital (50mg/ml) per 100 g rat weight. Both soleus and extensor digitorium longus (EDL) muscles were surgically and carefully removed, soleus muscles were split by scalpel into two relatively homogenous muscles (Fig E). The soleus muscle was chosen for study because of our ability to section this tubular muscle longitudinally into ~25-mg strips, which can be incubated with minimal limitations because of diffusion and its predominantly slow-twitch fiber composition: 84% type I, 16% type IIa, 0% type IIb (Ivy et al. 1996). EDL muscles were split by tearing along the tendons stretching to each toe resulting in deep rEDL and superficial wEDL (Fig F). The muscles (soleus and both EDL parts) were mounted on a contraction apparatus between two platinum electrodes at their resting length. The electrode was then placed in a test tube with 3.5 ml Krebs Henseleit buffer solution containing 5.5 mM glucose, 2 mM pyruvate, 5 mM HEPES and 0.1% bovine serum albumin, and gassed continuously with a gas containing 95% O_2 and 5% CO_2 through the incubation buffer for 30 min. To determine the effect of insulin concentration on glucose uptake and glycogen content, soleus muscles were incubated additional 30 min with insulin 0.2 mU/ml, 10 mU/ml (Monotard, Novo Nordisk, Denmark) or kept in the buffer solution as control. To determine the effect of adrenaline either on insulin-stimulated glucose uptake or glycogen content, soleus muscles were incubated for 30 min with insulin (10 mU/ml), adrenaline 10^{-6} M, epinephrine and insulin, or kept in the buffer solution as control (Fig E). For determination of glucose uptake and glycogen content in EDL muscles, after pre-incubation, wEDL muscles were then incubated with or without adrenaline 10^{-6} M {(-)-epinephrine; Sigma} for 30 min (Fig F).

rEDL muscles were either incubated with insulin 10 mU/ml (Monotard, Novo Nordisk, Denmark) or contracted electrically for 30 min with 200 ms trains (100 Hz), square wave pulses of 0.2 ms duration, 10 V) delivered every 2 seconds. All incubations were placed in a waterbed (30° C) and gas was bubbled continuously through the buffer. After incubations, muscles were removed from the apparatus, blotted on filter paper, fixed in marked polystyrene, frozen in liquid nitrogen and stored at -70 °C until analysis day.

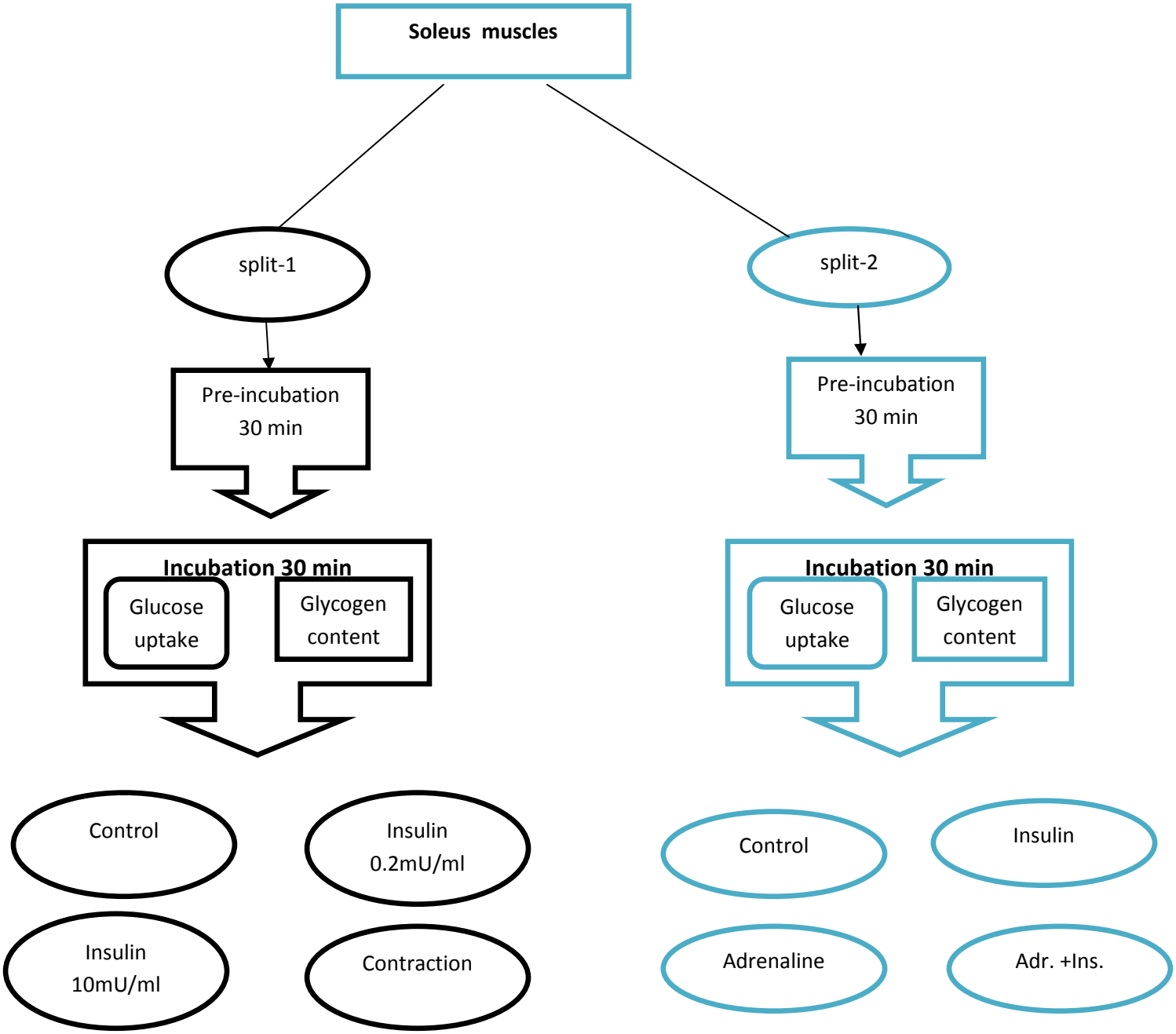

Fig. E. Soleus muscles incubation procedure. Muscles split into two splits, pre-incubated in the buffer solution for 30 min and incubated in the required medium for 30 min with continues (Co_2 and O_2) gassing, and temperature adjusted on 30°C.

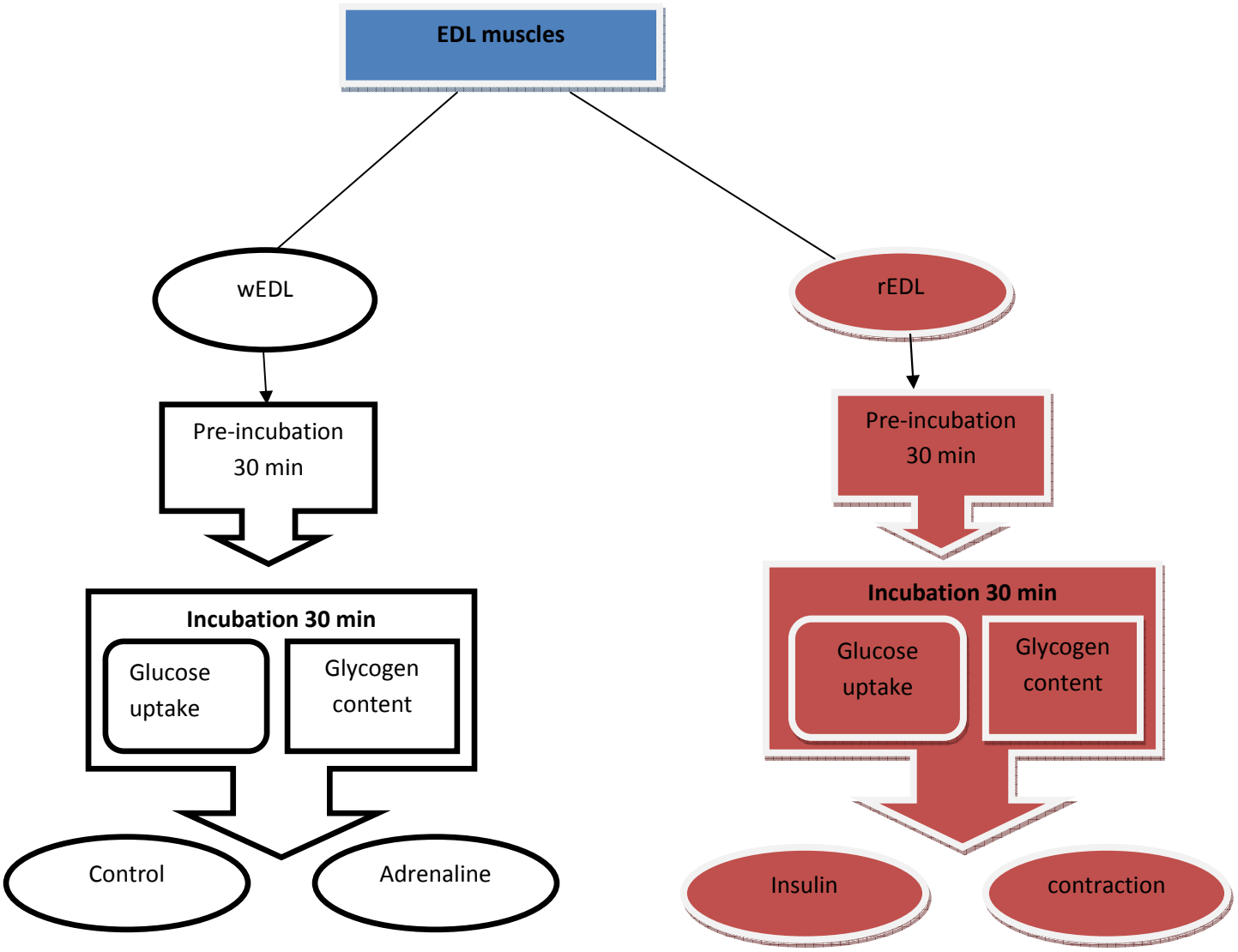

Fig. F. EDL muscles incubation procedure. The superficial part of the EDL (wEDL) were pre-incubated in the buffer solution for 30 min and incubated with or without adrenaline for 30 min, while the deep part of the EDL muscles (rEDL) were incubated in insulin or electrically stimulated for 30 min after 30 min pre-incubation in buffer solution.

3.2 Glucose uptake and glycogen content

For determination of glucose uptake, 0.25 µCi/ml 2-[1,2H(N)]deoxy-D-glucose (30.6 Ci/mmol; NEN) (NET 549 PerkinElmer) and 0.1 µCi/ml D-[1-14C]mannitol (54.5 mCi/mmol; NEN) (NEC 314 PerkinElmer) were added to the buffer (containing 5.5 mM glucose). Glucose uptake was calculated from the intracellular accumulation of 2-[^{3}H]deoxy-D-glucose during 30 min of incubation assuming similar uptake kinetics for glucose and 2-deoxyglucose. Muscles were removed from the ultra freezer (-70 °C or lower) and cut into two pieces at -20°C. For glucose uptake, one part of the muscle is freeze-dried for 3 hours and weighed at -20°C, we used 2-3 mg of the dry weight which was enough for measurement of glucose uptake and glycogen content (concentrations between 20 and 250 mmol/kg dw) in the same sample. The freeze dried and weighed muscle samples are dissolved in 600 µl 1 M KOH for 20 min at 70 °C. After cooling down, for counting for radioactivity, 400 µl of the muscle digest were mixed well with 3 ml of Hionic Flour scintillation cocktail (Hionic-Flour 6013319, PerkinElmer) in counting tube (Pony Vial 6000292, PerkinElmer). After one hour, samples were counted for ^{3}H and ^{14}C (Tri-Carb 1900 TR, protocol #28, 2 cycles and 5 min counting). (Region A: 0.0-12.0 KEV for ^{3}H, window B: 12.0-156 KEV for ^{14}C).

For determination of glycogen content, glycogen in muscle samples is hydrolysed to glucose with amyloglucosidase (AGS) at pH 4,8, the glucose formed is specifically measured with the enzyme hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH), (a fluorometric method published by Lowry and Passonneau, 1972). Amyloglucosidase hydrolyses the α-D-(1-4) and α-D-(1-6)-linkages of glycogen, the product is D-glucose, the glucose content after hydrolysis corresponds to the glycogen content of the sample. G6P-DH is specific for glucose-6-phosphate formed from glucose in the enzymatic reaction, the glycogen content is determined as glycosyl units.

1) Glycogen $\xrightarrow{\text{amyloglucosidase}}$ glucose

2) Glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

(3) Glucose-6-P + $NADP^+$ $\xrightarrow{\text{G6P-DH}}$ 6-P-gluconolactone + NADPH + H^+

Glucose is phosphorylated in reaction 2 to glucose-6-phosphate and G-6-P oxidises with $NADP^+$ in reaction 3. The equilibrium in reaction 3 lies on the side of G-6-P reduction and the glucose liberated after hydrolysis of glycogen is proportional to the increase of NADPH measured as change in fluorescence at wavelength 340 nm (excitation) and 460 nm (emission). The muscles sample is dissolved in 600 µl 1 M KOH and analysed together with controls (10 µl glycogen control solution {glycogen Sigma

G-8876 rabbit liver} ~50 mM into 600 µl 1 M KOH{Merck 5021, MW 56,11, min 85%}), for hydrolysis of muscles, 100 µl of muscle digest samples and 25 µl 7 M acetic acid were mixed with 500 µl 0.3 M acetate buffer (35,4 ml 0.3 M Na-Acetate {Merck 6268, MW 82.03, 99%} + 24,6 ml 0.3 M acetic acid {Merck, MW 60.05, 100%, d=1,05}), incubated for 3 hrs at 37 °C and the hydrolysates stored at -20 °C or analysed directly for glucose. In glucose analysis we used fluorescence range 0.5-10 µM in the cuvette and the wavelength was 340 and 460 nm. For calculation of glycogen concentration in muscles samples, we made a standard curve from 4-6 concentrations which depends on the glycogen content in the muscle samples, we calculated the regression line from the standard curve y= Ax + B, where A is the slope of regression line, x=µM in the cuvette and B is the intercept with the y axis, the rest of the calculations were done upon the dilutions used in this protocol.

3.3 Liver glycogen

The liver tissue samples weighed ~ 5-10 mg at -20°C and freeze dried up to ~ 3 hrs, the relation between the dry weight and the wet weight for liver is 0.3. 1-3 mg dry weight was enough for calculation of the glycogen concentration between 500 and 1500 mmol/kg dw. Each liver sample of the two freeze dried and weighed muscle samples added to 3000 µl 1 M HCl (Merck p.a. 317 (~37 %)), samples were incubated 2.5 hrs in 100 °C heating block, mixed gently and cooled down, the evaporated water compensated. HCL broke glycogen down to glucose. The glucose analysis is done just as mentioned in the determination of glycogen content in muscles except that the hydrolysates were centrifuged at +4 °C, 3000 g for 5-10 min and diluted (100 µl hydrolysate + 300 µl 1 M HCl) as we expected high levels of glycogen.

3.4 Statistics

Data are presented as means ±SEM. One way ANOVA was performed to determine the P-value, the level of significance been chosen is 0.05, when ANOVA revealed significant differences, Least Significant Differences were performed to compare groups. All tests have been performed using SPSS, $p<0.05$ considered statistically significant.

4 Results

4.1 Glucose uptake and glycogen breakdown

Glucose uptake in control soleus muscles from lean Zucker rats was 3.48 ± 0.39, and 2.00 ± 0.11 mmol/kg dw/30 min for obese rats. Glucose uptake in the presence of 0.2 mU/ml insulin was more than twice as high in muscles from lean rats compared to muscles from obese rats ($p<0.01$ Fig. 1A). In muscles treated with 10 mU/ml insulin, glucose uptake was ~ 5 times as high as basal glucose uptake in lean rats and was twice as high as in muscles from obese Zucker rats, ($p<0.01$ Fig. 1A).

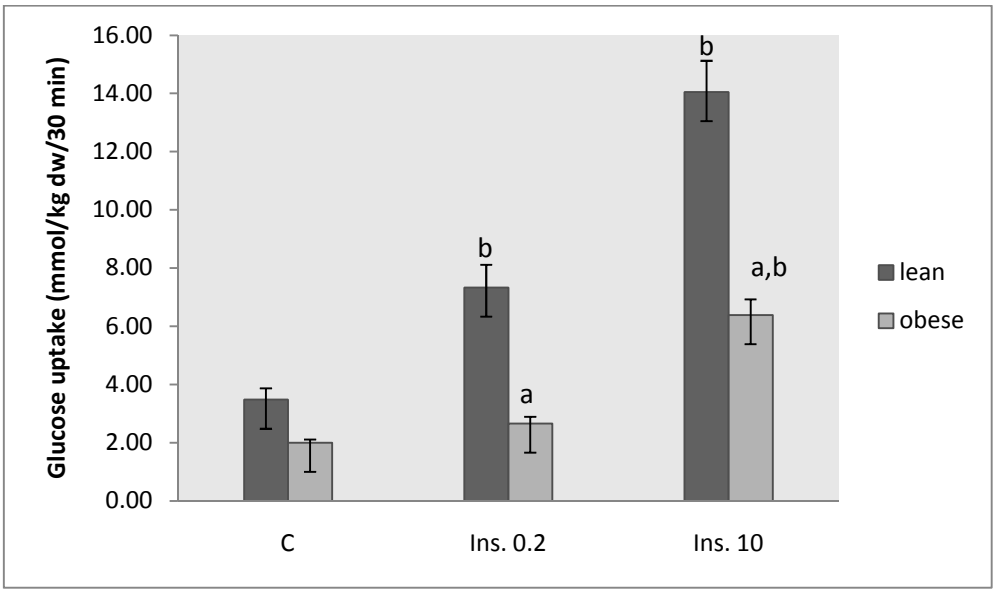

Fig. 1A Glucose uptake in soleus muscles from lean and obese Zucker rats incubated with different insulin concentrations.

Data presented are means ± SEM, n=12.

(a) Significantly different from muscles taken from lean rats treated similarly.
(b) Significantly different from control muscles.

Glycogen content in control soleus muscles from lean Zucker rats was 126.1 ±6.7 mmol/kg dw, in obese rats, glycogen content was ~15% higher than that in muscles from lean Zucker rats ($p<0.05$). With physiological insulin concentration, glycogen content was 121.3 ± 7.5 mmol/kg dw in muscles taken from lean rats, in muscles from obese Zucker rats, glycogen content was ~24% higher than that in lean Zucker rats ($p<0.05$ Fig. 1B). High insulin concentration showed no significant effect on glycogen content in both lean and obese rat muscles ($p>0.05$).

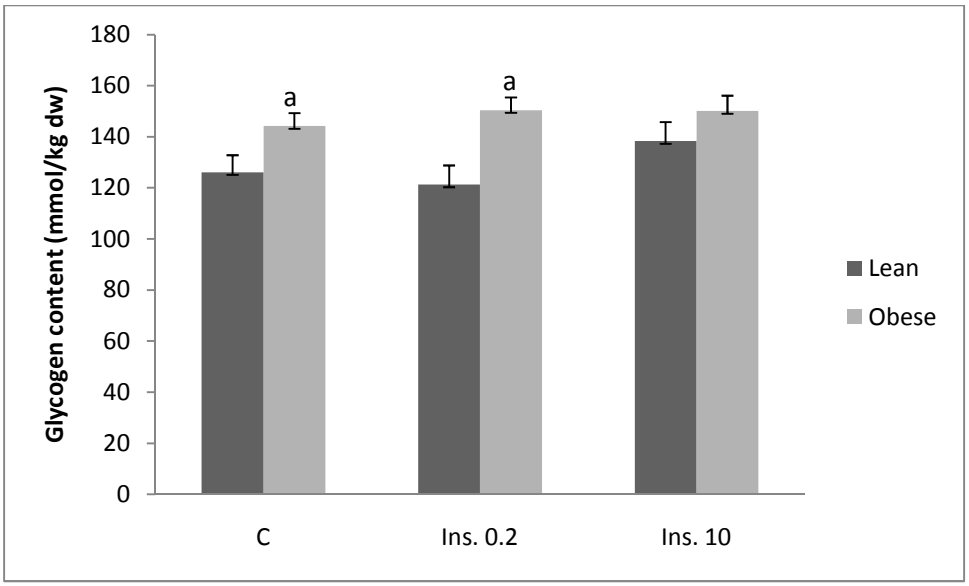

Fig. 1B Glycogen content in soleus muscles from lean and obese Zucker rats. Muscles were treated with physiological insulin concentration 0.2mU/ml, with maximal insulin concentration 10mU/ml or kept rested. All muscles were incubated for 30 min. at 30° C with continues CO_2 and O_2 gassing.

Data presented are means ± SEM, n=12.

(a) Is significantly different from lean rat muscles treated similarly.

Table 1

The effect of electrical stimulation on glycogen content in soleus muscles from lean and obese Zucker rats.

	Glycogen content (mmol/kg dw)	
	Basal	Electrical stimulation
Lean	126.1 ± 6.72	75.2 ± 4.57*
Obese	144.2 ± 5.09†	97.6 ± 4.58*†

Muscles from lean and obese rats were electrically stimulated for 30 min or kept rested.

Data presented are means ± SEM, n=12.

*significantly different from control

†significantly different from lean rat muscles treated similarly.

In another set of experiments glucose uptake in control soleus muscles from lean Zucker rats was 3.19 ± 0.41 and 1.71 ± 0.27mmol/kg dw/30 min in soleus muscles from obese Zucker rats. Insulin increased glucose uptake in lean rat soleus muscles ~ 5 times, and glucose uptake was more than twice as high in solues muscles from obese rats than in control muscles ($p < 0.01$ Fig. 2A). Muscles incubated with adrenaline alone showed no difference in glucose uptake from control muscles. Adrenaline

decreased insulin-stimulated glucose uptake in lean rat muscles by ~35%, and by ~15% in obese rats muscles ($p<0.01$). However the glucose uptake doubled by the combination action of insulin and adrenaline in both lean and obese rats compared to control.

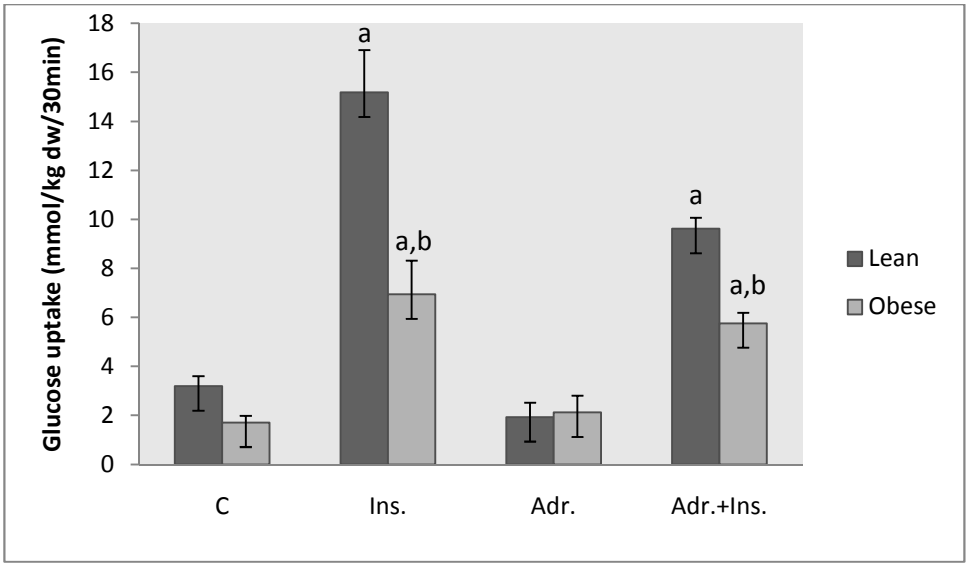

Fig. 2A Effect of adrenaline on Insulin-stimulated glucose uptake in soleus muscles from lean and obese Zucker rats. The same insulin concentration used 10 mU/ml, muscles were incubated for 30 min at the same temperature 30°C.

Data presented are means ± SEM n=6-7.

(a) Significantly different from control muscles.

(b) Significantly different from lean rat muscles.

In control rested soleus muscles glycogen content was 117.6 ± 10.1 mmol/kg dw/30 min for muscles taken from lean Zucker rats (Fig. 2B). In muscles taken from obese Zucker rats glycogen content was ~30% higher than that in control muscles from lean rats, ($p<0.05$ Fig. 2B). In insulin incubated muscles, glycogen content was not significantly different from control in both muscles from lean and obese rats, ($p = 0.085$). Glycogen content in adrenaline incubated muscles from lean rats was 112.5 ± 9.6 mmol/kg dw. In muscles taken from obese Zucker rats, glycogen content was ~25% higher than that inmuscles from lean rats, ($p< 0.05$ Fig 2B). Adrenaline and insulin showed no significant effect on glycogen content in both lean and obese Zucker rats, ($p>0.05$; Fig 2B).

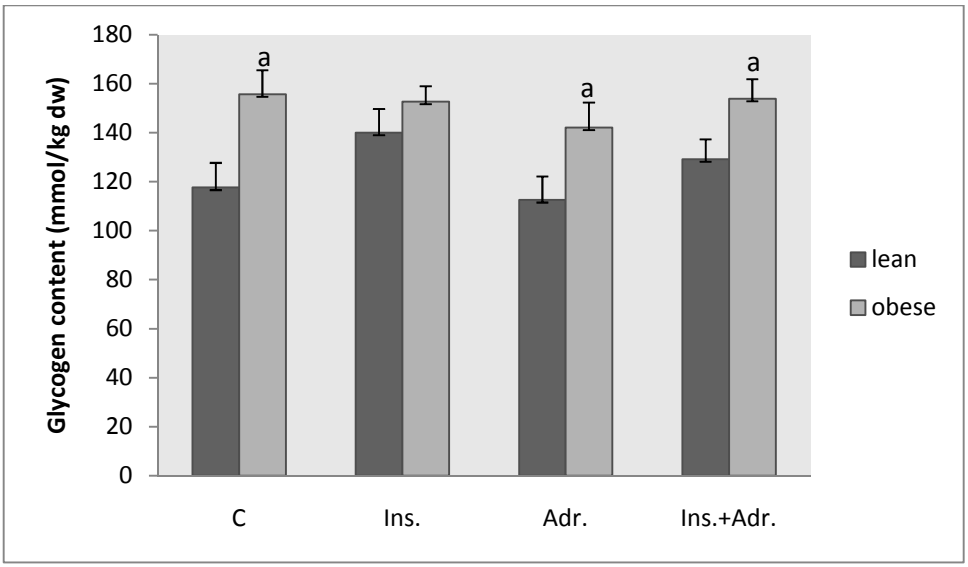

Fig. 2B Effect of adrenaline on glycogen content in soleus muscles from lean and obese Zucker rats.

Muscles were incubated for 30 min. Maximum insulin concentration 10mU/ml and 10^{-6} M adrenaline were used.

Data presented are means ± SEM n=6-7.

(a) Significantly different from lean muscles treated similarly.

Table 2

The effect of electrical stimulation on glucose uptake in soleus muscles from lean and obese Zucker rats.

	Glucose uptake (mmol/kg dw/30min)	
	Basal	Electrical stimulation
Lean	3.48 ± 0.39	8.34 ± 0.49 *
Obese	2.00 ± 0.11 †	5.18 ± 0.39 *†

Muscles contracted isometrically for 30 min or kept rested in the continuous gassed buffer solution. The temperature were kept on 30°C.

Data presented are means ± SEM where n=12.

*Significantly different from control muscles.

† Significantly different from lean rat muscles treated similarly.

In white EDL muscles from lean Zucker rats, basal glucose uptake was 4.12 ± 0.43 mmol/kg dw/30 min. Basal glucose uptake in muscles from obese Zucker rats was ∼30% lower than that in lean rat muscles ($p>0.05$ Fig. 3A). Compared to control muscles, adrenalin decreased glucose uptake by ∼40% in muscles from lean Zucker rat ($p<0.05$) while the effect of adrenaline showed no significant decrease in glucose uptake in white muscles from obese Zucker rat compared to control muscles (Fig. 3A). Insulin-stimulated glucose uptake in lean rat muscles was 12.27 mmol/kg dw/30min.Glucose uptake in obese rat muscles was ∼50% lower than that in lean rat muscles (Fig. 3B). Contraction-stimulated glucose uptake in lean rat muscles was rather similar to insulin-stimulated glucose uptake. In obese Zucker rat muscles, contraction-stimulated glucose uptake was 35% lower than in muscles from lean Zucker rats ($p<0.01$; Fig 3B).

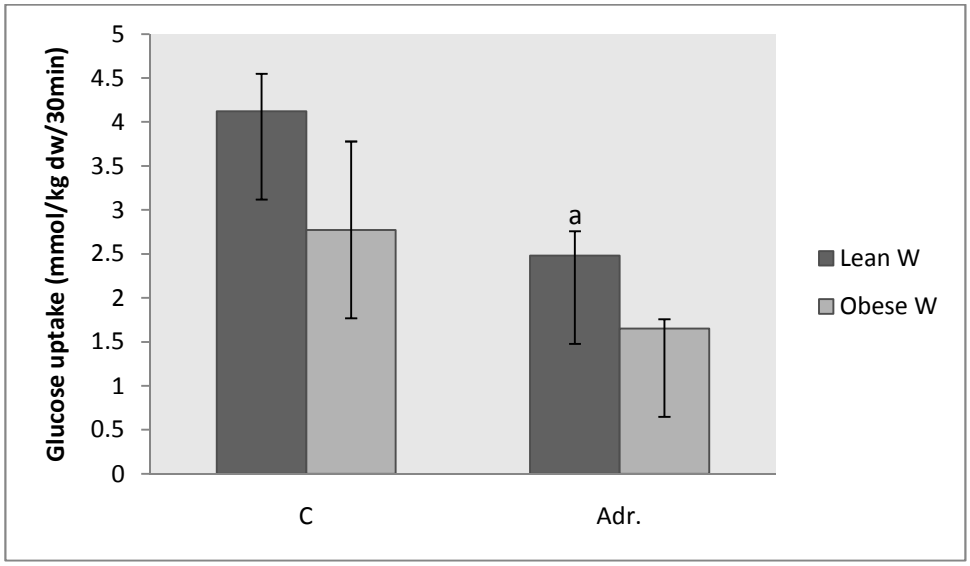

Fig. 3A The effect of adrenaline on glucose uptake in white EDL muscles from lean and obese Zucker rats. Muscles were incubated for 30 min with or without 10^{-6} M adrenaline.

(a) Significantly different from control muscles.

Data used are means ± SEM n=7-8.

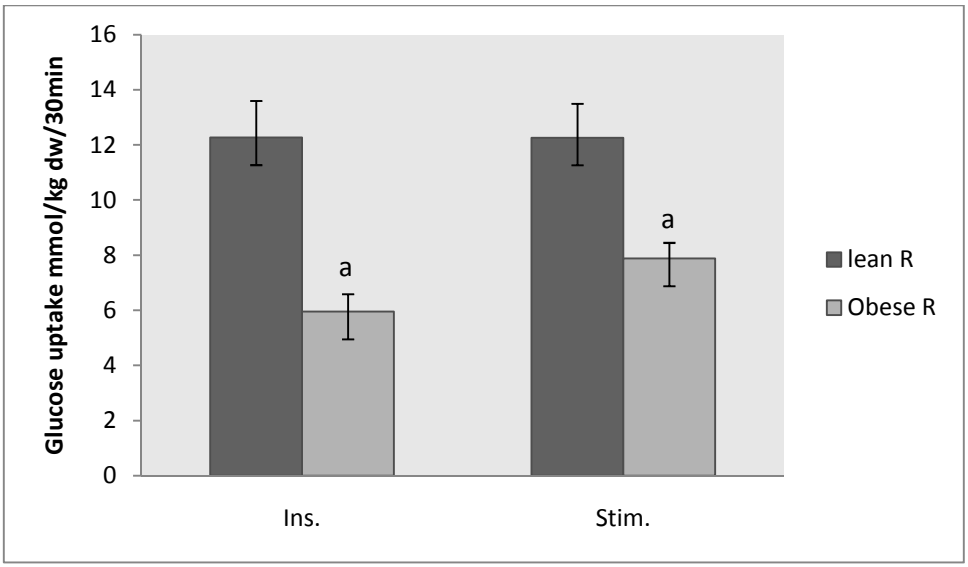

Fig. 3B The effect of insulin and electrical stimulation on glucose uptake in red EDL muscles from lean and obese Zucker rats. Muscles were incubated in 10 mU/ml insulin solution or electrically stimulated, all muscles were incubated for 30 min.

(a) Significantly different from lean rat muscles treated similarly.

Data presented are means ± SEM n=7-8.

Glycogen content in control white EDL muscles from lean Zucker rats was 154.0 ± 7.6 mmol/kg dw. In obese Zucker rats glycogen content was ~20% higher ($p<0.05$ Fig. 4A). Adrenaline slightly decreased glycogen content in muscles from lean rats (~ 13%; $p=0.078$), while in obese rats, glycogen content decreased by ~15% ($p<0.05$; Fig 4A). In red EDL muscles from lean rats incubated for 30 min with insulin, glycogen content was 134.1±11.7 mmol/kg dw, while in red EDL muscles from obese Zucker rats treated similarly, glycogen Content was ~10% higher than in muscles from lean rats (Fig. 4B). Electrical stimulation decreased glycogen content by ~40% in lean rat red EDL muscles and ~45% in obese rats ($p<0.05$; Fig 4B).

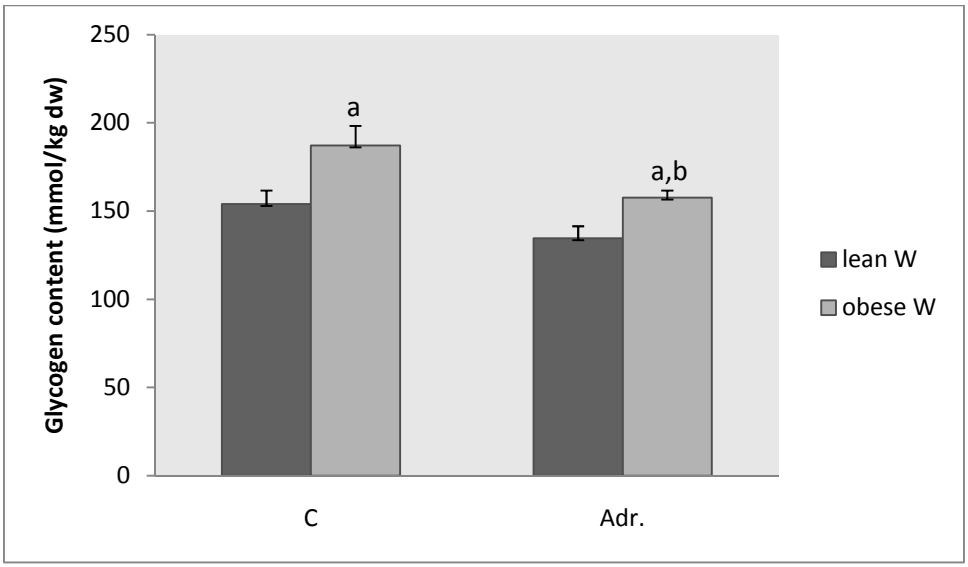

Fig. 4A The effect of adrenaline on glycogen content in white EDL muscles from lean and obese Zucker rats. Muscles were incubated with or without 10^{-6}M adrenaline for 30 min at 30° C.

(a) Significantly different from lean rat muscles treated similarly.
(b) Significantly different from control muscles.
Data presented are means ± SEM n=7-8.

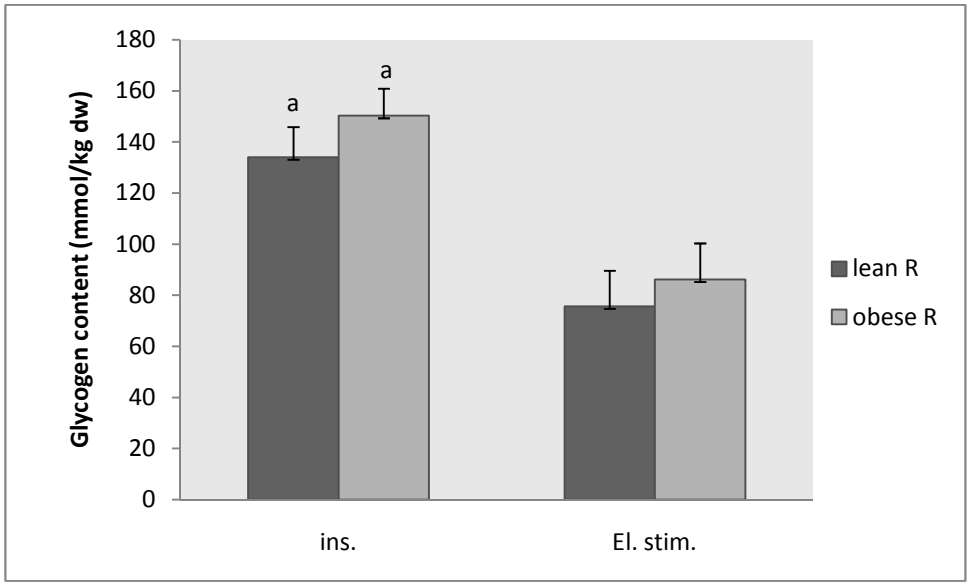

Fig. 4B The effect of insulin and contraction on glycogen content in red EDL muscles from lean and obese Zucker rats. Muscles were incubated in 10mU/ml insulin or contracted for 30 min.

Data presented are means ± SEM n=7-8.

(a) Significantly different from electrically stimulated muscles.

4.2 Liver glycogen and muscle weight

In lean rats, liver weight was 9.17 ± 0.32 g but the liver weight in obese Zucker rats was ~ 60% higher. Liver glycogen contents were not significantly different between genotypes, although they tended to be lower in lean Zucker rats (Table 3). Lean rats body weight was 226.1 ± 4.0 g while obese Zucker rat weight was ~60% higher, despite this result the EDL muscle weight in lean Zucker rat was 116.4 ± 2.9 mg which is ~20% higher than EDL muscle weight in obese Zucker rats (Table 3).

Table 3

Lean and obese rats liver weight, glycogen content and EDL muscles weights.

	Body wt. (g)	Liver wt. (g)	Liver glycogen (mmol/kg dw)	Body wt. (g)	Liver/BW	EDL wt. (mg)
Lean	226.1±4.0	9.17±0.32	1212.0±53.0	226.1±4.0	0.041±0.001	116.4±2.9
Obese	359±4.8	14.91±0.63*	1335.8±55.6	359±4.8†	0.042±0.001	97.5±2.1‡

*Significantly different from lean rat liver weight.

†Significantly different from lean rat weight.

‡Significantly different from lean rat EDL weight.

Data presented are means ± SEM, EDL weights are means of EDL left and EDL right. n=12.

5 Discussion

Insulin resistance is the most common feature in most types of human and animal obesities. we have investigated insulin resistance in skeletal muscles because skeletal muscles make up about 40% of the body weight and nearly 70-90% of insulin stimulated glucose disposal is incorporated into muscles glycogen (Shulman et al., 1990). Skeletal muscles are also responsible for at least 80% of glucose uptake in humans during insulin stimulation (DeFronzo et al., 1981). Skeletal muscle is a specialized tissue for movement where chemical energy is transformed into mechanical work. Skeletal muscles have also an important role for regulation of blood glucose. Skeletal muscle insulin resistance is of special interest as far as the chronic insulin resistance is believed to be in large part, due to insulin resistant muscle tissue (Ivy et al., 1986). Skeletal muscles insulin resistance can be described as predisposing force and an underlying feature of type-2 diabetes. This fact has led to many researches on the insulin resistance of the skeletal muscles of the genetically obese Zucker rats (Ivy et al., 1986).

The obese Zucker rat is a preferred rat model for insulin resistance due to a combination of reduced insulin sensitivity and responsiveness. Obese Zucker rats have a reduced number of insulin receptors at the plasma membrane and a defect in the insulin-stimulated glucose transporter 4 (GLUT4) translocation process (Sherman et al., 1988; Etgen et al., 1996). Some studies have reported that, the insulin resistance in the obese Zucker rats is associated with a decreased insulin binding (Crettaz et al., 1980), rate of glycogen synthesis (Ivy et al., 1986), and rate of glycolysis (Creattaz et al., 1983) when compared with lean littermates. Other factors may play a major role in bringing about insulin resistance in the obese Zucker rats, such as the decreased ability of liver, adipose tissue, and muscle to bind insulin (Crettaz, et al., 1980). Previous studies have characterized the insulin receptor signaling system in skeletal muscle of the obese Zucker rat and found extreme insulin resistance consistent with the decrease in insulin-stimulated glucose uptake into skeletal muscle (Ivy, J. 2001; Sherman et al., 1988). Furthermore, Sherman et al (Sherman et al., 1988) have demonstrated that the glucose transport process is the major site of skeletal muscle insulin resistance in the obese Zucker rats.

Skeletal muscles of the obese Zucker rats differs from those of lean animals in two respects; 1) lower insulin sensitivity associated with reduced insulin binding, and 2) lower response to insulin which may also decreased upon the additional changes in the utilization of glucose beyond the interaction of insulin with its receptor (Crettaz et al 1980; Smith & Czech, 1983). Insensitivity to insulin in large portion of the peripheral tissues could contribute to insulin resistance in Zucker fatty rat in vivo (Smith & Czech,

1983). Because insulin controls its own receptors, hyperinsulinemia has been blamed for the reductions in binding and sensitivity in adipocytes as well as for the overeating and excess fat storage that cause obesity itself. Previous studies have reported that the response of the peripheral tissues to the maximal insulin concentration, which tests glucose disposal beyond the insulin-receptor interaction, was also impeded. Therefore, defective transport or metabolism of glucose in skeletal muscles throughout the body can play a major role in the production of whole body resistance to insulin (Crettaz et al., 1980; Ivy et al., 1989).

Other studies have characterized the insulin receptor signaling system in skeletal muscles of the obese Zucker rat and found extreme insulin resistance consistent with the decrease in insulin-stimulated glucose uptake into skeletal muscles (Sherman et al., 1988). In skeletal muscles, insulin stimulates glucose uptake via translocation of GLUT4 from intracellular vesicles to the cell membrane. Upon insulin binding, the insulin receptor becomes tyrosine phosphorylated, and recruits IRS for tyrosine phosphorylation. After tyrosine phosphorylation, IRS-1 binds and activates the enzyme phosphatidylinositol 3-kinase (PI3K). PI3K activation results in serine phosphorylation of protein kinase B (PKB), and ultimately, stimulation of glucose transport in skeletal muscles. IRS-1 acts as metabolic switch for the insulin signaling pathway. In insulin resistant muscles a Serine/threonine phosphorylation of IRS-1 can result in suppression of the insulin signaling pathway (Gupte et al., 2008). This pathway suppression results in insulin inability to translocate GLUT4 to the cell membrane and to activate glycogen synthase (Etgen et al., 1996; Etgen et al., 1997). In addition, activation of key proteins in the insulin signaling pathway is reduced, as well as a reduction in PI 3-kinase and PKB activation have been associated with insulin resistance (Widegren et al., 1998; Christ et al., 2001).

In the present study, glucose uptake in the obese Zucker rat muscles was lower than that in lean littermates. Our data showed very low glucose uptake in soleus muscles from obese Zucker rats incubated with physiological insulin concentration, and the physiological insulin concentration did not significantly increase glucose uptake. In contrast, glucose uptake was doubled at physiological insulin concentration in muscles from lean Zucker rats. These results are comparable with other previously reported results such as Crettaz et al. (Crettaz et al., 1980) who studied insulin sensitivity in vitro and found that muscles from obese Zucker rats were less insulin-sensitive than muscles from lean rats. They also suggested that glucose uptake in muscles of obese Zucker rats was characterized by a decrease in both insulin-sensitivity and insulin-responsiveness (Crettaz et al., 1980). Agreeing with this suggestion, my results showed that glucose uptake in lean rat muscles incubated with maximal insulin concentration was much higher than glucose uptake in obese Zucker rat muscles (fig 1A & 1B).

Other agreeing In vivo studies have shown that, insulin-stimulated hind-limb glucose uptake was significantly reduced in the obese Zucker rats compared with lean Zucker rats in the absence of insulin and at physiological, sub-maximal and maximal insulin concentrations (Ivy et al., 1988; Ivy, J. 2001). Ivy et al. (Ivy et al., 1988) have suggested that the muscles insulin resistance of the obese rat is due to reduced insulin responsiveness and not sensitivity. Leonard et al. have used Zucker diabetic fatty (ZDF) rats and found that the decrease in glucose uptake in skeletal muscle of obese animals is paralleled by a decrease in glycogen synthesis and storage in these tissues (Leonard et al., 2005). I suggest that impaired glucose uptake in the obese rat muscles is due to a defect in insulin sensitivity and responsiveness as glucose uptake was lower in physiological and high insulin concentrations.

I have demonstrated that adrenaline decreased insulin-stimulated glucose uptake in soleus muscles from lean and obese Zucker rats and wEDL muscles from lean Zucker rats. Aslesen and Jensen (Aslesen & Jensen, 1998) agreed with these results as they found that adrenaline decreased insulin-stimulated glucose uptake, but adrenaline did not influence glucose uptake in the absence of insulin. This result is consistent with an in vivo study done by Chiasson et al. (Chiasson et al. 1981) where insulin-stimulated glucose uptake in hind-limb of fed rats decreased by adrenaline. Another study supporting my findings is Rattigan et al. (Rattigan et al., 2001) who have shown that nor-adrenaline reduces glucose uptake via α-adrenergic regulation of blood flow. But blood flow is without effect in the present study since muscles were incubated in vitro. Furthermore, I did not investigate which subtype of adrenergic receptors that mediated the effect. It`s most likely via β-adrenergic effect on the muscle fibers. I suggest that β-adrenergic signaling is not impaired in insulin-resistant muscles. Stimulation of β-adrenergic receptors by adrenaline increases the concentration of glucose 6-phosphate which inhibits hexokinase activity. Inhibition of hexokinase activity decreases glucose phosphorylation which results in the reduction of glucose uptake (Aslesen & Jensen 1998). Some studies have also found that adrenaline has an indirect effect on glucose level through stimulation to hepatic glucose production by increased gluconeogenesis as a result of accelerated muscle glycogenolysis and lactate release (Sonne et al., 1987; Wasserman et al., 1987; Howlett et al., 1999).

In this study I investigated the effect of in-vitro electrical stimulation on glucose uptake in soleus and EDL muscles from lean and obese Zucker rats. Contraction-stimulated glucose uptake in obese Zucker rat EDL muscles was lower than that in lean rat muscles. While contraction-stimulated and insulin-stimulated glucose uptakes were the same in soleus muscles from lean Zucker rats, contraction-stimulated glucose uptake was higher than insulin-stimulated glucose uptake in muscles from obese Zucker rats. The well known treatment to enhance skeletal muscles insulin action is exercise

training (Ivy et al., 1986). This study suggested that the exercise training in combination with the high-carbohydrate diet functioned synergistically to improve the insulin-stimulated glucose uptake in the muscle of the obese Zucker rat (Ivy et al., 1986). Our results agree with many previous studies who have documented that exercise training enhances insulin stimulation of skeletal muscle glucose uptake in both normal and obese rats (Mondon et al., 1980; Ivy et al., 1983). Also, some studies have reported a slight reduction in contraction-stimulated glucose transport in muscles from obese Zucker rat compared to lean phenotypes (Henriksen & Jacob, 1995; Dolan et al., 1993). Others have reported that contraction normally stimulates glucose uptake in muscles from obese Zucker rats (Brozinick et al., 1992; Etgen et al., 1996). In a submitted study from the laboratory where I did my experiments, Lin et al (Lin et al., submitted) have reported that contraction stimulated glucose uptake and GLUT4 expression in the obese Zucker rat skeletal muscles was lower than that in the lean phenotypes. Ivy et al (Ivy et al., 1983) concluded that in vivo aerobic exercise training improved glucose tolerance and insulin sensitivity in the obese Zucker rat. This improvement is propably due to the reduction in the size of the adipocytes, this improvement did not persisted for a long time and the adipocytes regain their large size after cessation of the exercise (Ivy et al., 1983).

In order to further investigate the effect of the muscle type in the glucose uptake and glycogen content I used slow twitch (Soleus) and fast twitch muscles (EDL). Rodent's fast-twitch white fibers (type IIb) are considered to be glycolytic, metabolizing glycogen to lactate as the primary fuel for contraction and showing signs of fatigue when glycogen stores are depleted. Fast-twitch red fibers (type IIa) are both glycolytic and oxidative in that glucose is metabolized both aerobically and anaerobically. Jensen and Dahl (Jensen & Dahl, 1995) have indicated that the ability of adrenaline to stimulate glycogen breakdown in resting muscles in vivo seems to depend on the muscle fiber type composition of the intended muscle. They found that glycogen breakdown was more pronounced in type IIB fibers than in type I and type IIA (Jensen & Dahl, 1995).

In this study, glycogen content was higher in soleus muscles from obese Zucker rats than that in lean littermates. This finding agrees with Ruzzin & Jensen (Ruzzin & Jensen 2005) who used dexamethasone-treated rat muscles as insulin resistant muscles and found glycogen content in insulin resistant muscles was higher than that in normal muscles. This study showed no effect of adrenaline on glycogen content in soleus muscles from lean and obese Zucker rats which agree with Jensen et al (Jensen et al., 1989; Jensen et al., 1999). In consistent results, my study showed no effect of adrenaline on glycogen content in soleus muscles from lean and obese Zucker rats. Some previous studies have reported that, slow twitch (soleus) muscles exhibit little glycogen phosphorylase activation in response

to adrenergic stimulation and no glycogen breakdown is seen (Rennie et al., 1982; Richter, EA. 1984; Peters et al., 1998). Several studies have found that adrenaline stimulated glycogen breakdown in fast twitch rat skeletal muscles (Chasiotis, 1985; Jensen et al., 1989; Jensen & Dahl, 1995). The present study is the first to report that adrenaline stimulated glycogen breakdown in EDL muscles from obese Zucker rats. Adrenaline slightly stimulated glycogen breakdown in the correspondent muscles from lean littermates, but during adrenaline stimulation, the major part of glycogen phosphorylase is phosphorylated and transformed from the b (inactive) form to the a (active) form in fast-twitch (EDL) muscles. Adrenaline is also a strong inhibitor of glycogen synthase and decreases its activity to low level, adrenaline also blocks insulin-mediated activation of glycogen synthase. This notion been proved by Jensen et al (Jensen et al. 1999) who found that adrenaline decreased the fractional activity of glycogen synthase to low levels in insulin-stimulated non-contracted muscles. It is important to remember that glycogen synthase activity in vivo is regulated by phosphorylation as well as the concentration of glucose 6-phosphate, but in the absence or low concentrations of glucose 6-phosphate, glycogen synthase activity is regulated by insulin, adrenaline, glycogen content and muscle contraction.

My data showed that electrical stimulation decreased glycogen content in rEDL muscles in lean and obese Zucker rats. Previous studies supported this finding either directly by measuring glycogen content (Chesley et al., 1995) or indirectly by measuring glycogen synthase fractional activity (Nielsen et al., 2001). Chesley et al. have found that intense aerobic exercise is likely to fully activate glycogenolysis via increased calcium release (Chesley et al., 1995). But glycogen synthase fractional activity increased after contraction and Nielsen et al., suggested that this result is a feed-back mechanism to the glycogen breakdown resulted from contraction and furthermore they suggested that this mechanism is the only mechanism which control glycogen synthase fractional activity (Nielsen et al., 2001). But another study indicates that contraction increased glycogen synthase fractional activity more than expected from the decrease in glycogen content (Jensen & Lai, 2009). Lin et al (Lin etal., submitted) have found that glycogen synthase total activity was slightly reduced by muscle contraction and glycogen content was reduced to similar level in muscles from lean and obese Zucker rats.

Although the body and liver weights of the obese Zucker rats were significantly higher than in lean littermates, liver glycogen content in obese Zucker rats was not significantly different from that in lean phenotypes. This result is comparable with Bruce et al (Bruce et al., 2001) who found that liver glycogen content at rest in obese Zucker rats was not significantly higher than that in lean phenotypes. In the same study, carbohydrates refeeding did not restore the depleting liver glycogen in obese Zucker rat after exercise (Bruce et al., 2001). These results lead us to conclude that, the obese Zucker rat is

hyperphagic and usually converts carbohydrates to fat not glycogen or protein. As far as the fat production occurs only in the liver, this mission needs a larger liver size in obese Zucker rats than lean phenotypes. Also the obese Zucker rat liver colour was pale which indicates more fat content. These results can clarify that the obesity is a result of failure in the process of the gluconeogenesis in the liver as well as a decreased glucose uptake and decrease muscle gluconeogenesis. In conclusion, adrenaline decreased insulin-stimulated glucose uptake in normal and insulin resistant rat skeletal muscles, also adrenaline found to enhance glycogen breakdown in the insulin resistant EDL muscles while slightly enhanced glycogen breakdown in the correspondent muscles of the lean rat. I suggest that β-adrenergic signaling is not impaired in insulin resistant rat muscles.

6 Conclusions

1- Insulin-stimulated glucose uptake was lower in soleus muscles from obese than in muscles from lean Zucker rats at physiological and supra physiological insulin concentrations.

2- Glycogen content was slightly higher in soleus and EDL muscles from obese rats compared to muscles from lean rats.

3- Adrenaline decreased insulin-stimulated glucose uptake in both normal and insulin resistant muscles. However, the reduction was higher in muscles from lean rats than in muscles from obese rats.

4- Adrenaline decreased glycogen content in insulin resistant muscles but did not significantly decrease glycogen content in normal muscles.

5- Despite the higher weight of obese rats than lean rats, the weight of the EDL muscles from obese rats were lower than weight of EDL muscles from lean Zucker rats.

6- The liver weight was higher in obese Zucker rats than liver weight in lean Zucker rats but the glycogen content was similar.

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Appendices

Appendix 1: Glycogen content

REAGENTS AND SOLUTIONS

1 M potassium hydroxide,KOH:

16,5g KOH Merck 5021 (MW 56,11 , min 85%) + $H_2O_{millipore}$ up to 250 ml.

Store at + 4°C.

50 mM glycogen:

40,5 mg glycogen Sigma G-8876 (rabbit liver) + 5 ml $H_2O_{millipore}$.

Mix well, make up portions and store at -20°C.

0,3 M sodium acetate, CH_3COONa:

12,43 g Na-Ac Merck 6268 (MW 82,03 , 99%) + $H_2O_{millipore}$ up to 500 ml.

Store at + 4°C.

0,3 M acetic acid, CH_3COOH:

8,58 ml concentrated acetic acid Merck (MW 60,05 , 100% , d=1,05) + $H_2O_{millipore}$ up to 500 ml. Store at + 4°C.

Amyloglucosidase:

Boehringer 208 469 (EC 3.2.1.3) from Aspergillus niger - about 6 U/mg solid (25°C - glycogen as a substrate). Store dry at + 4 °C and take out the right amount when preparing the AGS mixture. Not produced anymore. We recommend to try Roche cat.No.1 202 332.

7 M acetic acid, CH_3COOH:

2 ml concentrated acetic acid Merck (MW 60,05 , 100% , d=1,05) + 3 ml $H_2O_{millipore}$.

Standard solution, D-glukose, 10 mM:

495,42 mg D(+)glucose Merck 8342 (monohydrate MW 198,17) + $H_2O_{millipore}$ up to 250 ml. Make up portions and store at -20°C.

1 M Tris/HCl-buffer pH 8,1:

12,1 g Trizma Base Sigma T-1503 (MW 121,1) + 4 ml concentrated hydrochloride (HCl, Merck p.a. 317, min. 37%). Add $H_2O_{millipore}$ up to 75 ml.

Mix well and adjust to pH 8,1.

Add $H_2O_{millipore}$ up to 100 ml. Store at - 20°C.

1 M magnesium chloride, $MgCl_2$:

2,03 g $MgCl_2 \cdot 6H_2O$ Merck 5833 (MW 203,3) + $H_2O_{millipore}$ up to 10 ml.

Store at - 20°C.

0,5 M dithiothreitole, DTT:

77,2 mg DTT (Cleland reagens) Sigma D-0632 (MW 154,3 ca.99%) + 1 ml $H_2O_{millipore.}$ Store at -20°C.

100 mM adenosine-5-triphosphate, ATP:

123 mg ATP Sigma A-2383 (with teoretical MW 614,2 , 99% pure - 3,5 H_2O and 2 Na/mol) + 2ml $H_2O_{millipore}$. Mix and add 6 N NaOH (50 - 60 µl) to pH 7 - 7,5. See datasheet for information.

Store at - 20°C.

50 mM nicotinamide-adenine dinucleotide phosphate, $NADP^+$:

21 mg $NADP^+$ Sigma N-0505 (MW for free acid 743,4 - see datasheet and label for exact information on each batch). Add 500 µl $H_2O_{millipore}$. Mix and store at - 20°C, 100 µl in each tube.

1% Bovine Serum Albumin:

100 mg albumin Sigma A-4503 + $H_2O_{millipore}$ up to 10 ml.

Mix gently, to avoid foam. Dissolve slowly. Store at - 20°C.

1 mg/ml glucose-6-phosphat dehydrogenase, G6P-DH:

Boehringer 127 035,type I (EC 1.1.1.49) from yeast - about. 350 U/mg.

10 mg/ml hexokinase, HK:

Boeringer 127 809 (EC 2.7.1.1) from yeast - about 140 U/mg.

Mix well before use.

Enzyme dilution buffer (20 mM Tris/HCl w/0,02% BSA pH 8,1):

1 M Tris/HCl pH 8,1	100 µl	
1% Bovine Serum Albumin	100 µl	
$H_2O_{millipore}$ up to	5 ml	Store at -20°C.

Appendix2: E-mail from Dr. Michael W. King

Nader Alhaj <nader.alhaj@gmail.com> Wed, Dec 16, 2009 at 8:13 PM

To: "King, Michael W" <miking@iupui.edu>

Dear Mr. King,

I've talked to you about glycogen synthase and the information from your web page medicalbiochemistrypage,

I've got the text book you recommended to me, it was very helpfull and I really thank you for this great recommendation, I am sending this mail to kindly ask you for a permission to use some figures from your

page in my thesis.

Kindest regards.

Nader Alhaj

Master student

School of pharmacy

University of Oslo

Norway

+47 97970747

[Quoted text hidden]

King, Michael W <miking@iupui.edu> Mon, Dec 21, 2009 at 8:59 PM

To: Nader Alhaj <nader.alhaj@gmail.com>

Dear Nader:

If you ensure that you include the proper citation as to the source of the images from my web site then you may reproduce them in your thesis. Please indicate that the images are reproduced from http:// themedicalbiochemistrypage.org with permission from Dr. Michael W. King

Sincerely,

Dr. King

_____

Michael W King, PhD

Professor

IU School of Medicine and

Center for Regenerative Biology and Medicine

620 Chestnut St, Room 135HH

Terre Haute, IN 47809

812-237-3417

miking@iupui.edu

From: Nader Alhaj [nader.alhaj@gmail.com]

Sent: Wednesday, December 16, 2009 1:13 PM

To: King, Michael W

Subject: Re: Glycogen synthase

[Quoted text hidden]

Nader Alhaj <nader.alhaj@gmail.com> Mon, Dec 21, 2009 at 10:53 PM

To: "King, Michael W" <miking@iupui.edu>

Dear Dr. King,

thank you too much, I will do it properly.

Kindest regards.

Nader Alhaj.

[Quoted text hidden]