Regulation of Glucose Metabolism in Muscles from Dexamethasone Treated Rats

Effect of insulin, adrenaline and contraction

Anders Rasmushaugen

Master thesis in pharmacology for the degree Master of Pharmacy

Department of Pharmaceutical Biosciences,

School of Pharmacy, Faculty of Mathematics and Natural Sciences

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The thesis was conducted between July 2008 and May 2009 at the

Department for Work-related Muscoskeletal Complaints,

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Advisors

Senior scientist Dr. scient Jørgen Jensen

Professor Arild C Rustan

Preface

This thesis has been my biggest challenge in my years at the Institute of Pharmacy, and I want to thank those who have supported me during it.

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I direct a warm gratitude to my family for positive thoughts and support. Friends, both off- and on-campus has also been most supportive.

Last, but not least, I thank my boyfriend Michael. Not only am I thanking him for his proof-reading but also for being who he is and the support he has given me in this period. He has also been able to deal with the increased stress levels in the last period and long hours.

Good luck in the future!

Anders Rasmushaugen

Oslo, 14.05.2009

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Abbreviations

11βHSD	11β-hydroxysteroid dehydrogenase	HUNT	Helseundersøkelsen i Nord-Trøndelag
ACTH	Adrenocorticotropic Hormone	i.p.	Intraperitoneal (injection)
ADP	Adenosine diphosphate	IDDM	Insulin-dependent diabetes mellitus
AmG	Amyloglucosidase		
ATP	Adenosine triphosphate	IFG	Impaired fasting glucose
BMI	Body mass index	IGT	Impaired glucose tolerance
BSA	Bovine serum albumin	IRS-1	Insulin receptor substrate – 1
cAMP	Cyclic adenosine monophosphate	LDH	Lactate dehydrogenase
CI	Confidence Interval	NADH	Nicotinamide adenine dinucleotide
CNS	Central Nervous System		
CRH	Corticotropin releasing hormone	NIDDM	Non-insulin-dependent diabetes mellitus
CVD	Cardiovascular disease		
d.p.m.	Disintegrations per minute	OD	Optical density
DDD	Defined Daily Dosages, turnover by dosage	PBS-T	Phosphate buffered saline/0.1 % Tween-20
dex	dexamethasone	PDK1	phosphoinositide dependent kinase 1
DG	Deoxy-glucose		
DM	Diabetes mellitus	p-GS	Phospho-Glycogen Synthase
dw	Dry weight (freeze-dried)	p-GSK-3	Phospho - Glycogen Synthase Kinase 3
EDL	Extensor Digitorum Longus		
epi	Epitrochlearis	PI 3-kinase	Phosphatidylinositol 3-kinase
FFA	Free fatty acids	PKA	cAMP dependent Protein kinase
GC	Glucocorticoid	PP1	Phosphatase 1
GP	Glycogen phosphorylase	p-PKB	Phospho-Protein Kinase B (AKT)
GPT	Glutamate pyruvate transaminase	PVDF	Polyvinylidene fluoride
		SAM	Symphatetic-adrenomedullary system
GR	Glucocorticoid receptor		
GS	Glycogen synthase	Ser	Serine
HbA$_{1c}$	Glycosylated hemoglobin	sol	Soleus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid	Thr	Threonine
		WHO	World Health Organization
HPA	Hypothalamic-pituitary-adrenal axis	WHR	Waist hip ratio
		AS160	Akt Substrate 160 kDa
HRP	Horseradish peroxidase	AMP	Adenosine monophosphate
		ww	Wet weight

Abstract

Diabetes is one of the world's greatest health related concerns, and it affects more and more people every year.

The objective of this thesis was to investigate the various effects that the synthetic glucocorticoid dexamethasone exerted in rats. Dexamethasone in high concentration has been shown to induce insulin resistance in rat models, the models showing similar signs of disease as in patients affected by Cushing's syndrome.

The purpose of this thesis was to investigate how dexamethasone treatment affected glucose metabolism in muscles. Skeletal muscles were incubated under the influence of different mediators of glucose and glycogen metabolism; insulin, adrenaline and contraction. Two experiments were conducted; I and II.

The purpose of Experiment I was to investigate the effects of dexamethasone treatment during five days of treatment. Insulin sensitivity was measured with glucose uptake and western blots. Glycogen content and lactate release were also measured.

The purpose of Experiment II was to investigate the effects of dexamethasone treatment after eleven days, and compared how a fed and fasted state affected the dexamethasone-induced state of insulin insensitivity. Glycogen content, western blots, lactate release and glucose uptake were measured.

Experiment I and II show dexamethasone-mediated insulin insensitivity based on alterations in insulin-responsive tissues as skeletal muscles and liver.

1 Background

1.1 Diabetes

1.1.1 Prevalence

The incidence of diabetes is rising in most parts of the world, WHO estimate the prevalence in all age groups worldwide to be 2.8 % in 2000, projected to reach 4.4 % by 2030 [1]. The numbers of patients with diabetes was 171 million worldwide in 2000, WHO estimates it to increase to 366 million in 2030 [1], while the International Diabetes Federation (IDF) is projecting 246 million in 2007 and 333 million in 2025 [2]. The cost of diabetes care is growing fast and IDF claims diabetes care is going to be a dominating contributor to most countries' health budgets by the end of 2025 [2]. The American yearly expense caused by diabetes patients was $US132 billion in 2002 increasing to $US192 billion in 2020 [3]. Current approximations show that 90-120 000 patients have diabetes mellitus (DM) in Norway, of which 90 % type 2, many studies claim there may be just as many un-diagnosed patients [4]. The Norwegian Diabetes Association suggests a comparable figure of DM; 250 000 in total, where half of these may be un-diagnosed [5]. There are few signs that this epidemic of diabetes and metabolic diseases will cease to develop during our life-time, mainly due to an aging population and a modern, sedentary lifestyle with high-energy diets. It is also a grave concern that the developed world progress to adopt `western´ lifestyles as well.

"In developing countries, as their economies grow, non-communicable diseases will become more prevalent largely because of the adoption of `western´ lifestyles and their accompanying risk factors – smoking, high-fat diets, lack of exercise"

The World Health Report 1998, WHO

1.1.2 Pathophysiology

Diabetes is a variety of conditions that manifests itself in hyperglycemia. The reasons for hyperglycemia are diverse and results in classification of diabetes into different sub-groups. The most common type of diabetes is type 2 (type 2 DM), which is also called non-insulin-dependent diabetes mellitus (NIDDM). This is caused by lack of insulin production, reduction in the response of insulin (insulin resistance) or a combination of these. The other type is diabetes type 1 (type 1 DM, insulin-dependent diabetes mellitus, IDDM) where the auto-immune system attacks the insulin-producing β-cells in the pancreas, and hence ceases insulin production in vivo.

The focus in this thesis is type 2 DM, as dexamethasone (dex) are known to induce insulin resistance and mimic this type of diabetes, and not the autoimmune type 1 DM. The view of type 2 DM has developed in the last 30 years from being seen as a relatively mild ailment seen in the elderly to one of the leading causes of morbidity and mortality in most countries[2]. Complications from DM are micro- and macrovascular and hence one of the leading causes of death through its effects on cardiovascular disease (CVD) [2]. Elevated blood sugar levels are directly toxic and will over time lead to tissue damage (Figure 2)**.**

There are some intermediate steps between normoglycemia and type 2 DM as well which are in focus when treating diabetes. Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) are two of those [3]. Consult Appendix 1 – WHO Summary of Technical Report and Recommendations [3] definition of these different stages.

The research in this field shows that conditions previously considered to have different etiology really are related to each other, in fact this is of such importance that the term `metabolic syndrome´ was developed. Metabolic syndrome consists of various clinical findings, the criteria from WHO (2006) specifies these (Table 1) [6]. It must be noted that other guidelines exist as well, but these are not mentioned here. The term `diabesity´ is also used to describe the clustering of symptoms found in patients at risk of type 2 DM. Energy intake per capita has increased from 2 300 kcal in 1 963 to 2 720 kcal in 1992 [7], this combined with a sedentary lifestyle and possibly psychological stress instead of physiological stress might all lead to higher risk of any metabolic disease.

Table 1: WHO diagnostic criteria for the metabolic syndrome*

The diagnosis of metabolic syndrome require at least 3 of the 5 criteria to be met:	
Criteria	Defining level
Abdominal obesity	
- Men Waist circumference	>102 cm (>40 inches)
- Women Waist circumference	>88 cm (>35 inches)
High levels of triglycerides	At least 150 mg/dL
Low HDL cholesterol	
- Men	<40 mg/dL
- Women	<50 mg/dL
High blood pressure	At least 130/>85 mmHg
High fasting glucose	At least 110 mg/dL

*Adapted from a WHO publication (2006) [6]

1.1.3 Diabetic risk factors

Obesity and the hormonal dysregulation in adipose tissue are considered to be two of many risk factors of type 2 DM, and especially abdominal obesity [2, 8, 9]. A causal relationship between visceral adipose tissue aggregation and insulin resistance was established by Gabriely *et al.* [10]. Removal of adipose tissues from various anatomical sites showed markedly different effects on metabolic outcomes. Removal of visceral fat (peri-renal and peri-epididymal) from various insulin-resistant rat models markedly improved peripheral and hepatic insulin sensitivity and glucose tolerance[10]. High BMI correlates with increased cortisol excretion, however, plasma cortisol levels are usually lower in obese patients compared to non-obese [9]. This could be due to enhanced peripheral metabolism of glucocorticoids (GCs) or increased activity in the HPA axis as a result of decreased sensitivity to GC feedback [9]

There is evidence that diabetics have a two to four times larger risk of CVD than the general population [2, 11]; one study conducted in Norway compared CVD mortality rates for two periods of time; from 1984-86 and from 1995-97. The study discovered a major decrease in mortality from CVD in the general population, a decrease which also benefited patients with diabetes, but the more than 2-fold higher mortality rate in diabetics persisted[11]. Risk factors such as hypertension and dyslipidemia add up and worsen the morbidity/mortality of diabetics [2].
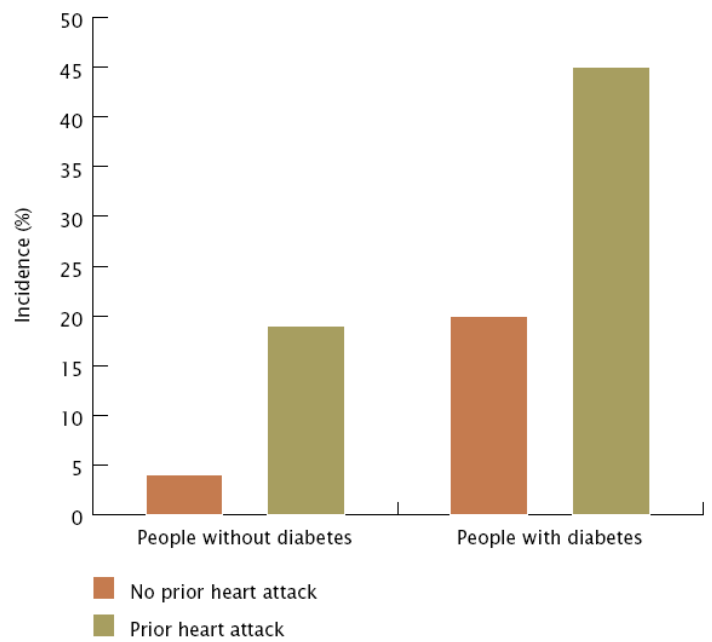
Figure 1: Heart attacks in people with and without diabetes over a period of seven years; adapted from [2]

Many studies show that life style and socio-economic status correlates with obesity, and hence increases the risk of type 2 DM [7, 12]. This effect might be due to a lot of factors; stressful and long hours at work, constantly having a feeling of being an `under-dog´ in addition to the cheap, but unhealthy, fast-food are all mentioned as plausible causes. It has been calculated that if a whole population avoids BMI > 25, the risk of type 2 DM will decrease by 65 – 76 % (in a Caucasian population that is) [7].

Genetic predispositions is inevitably linked to many type 2 DM conditions, as well as HPA axis dysregulation [13]. A direct correlation between some genetic predispositions and type 2 DM was found in the Norwegian HUNT project [14]. Specific type 2 DM-related loci were investigated in patients diagnosed with type 2 DM and compared to patients without the diagnosis. The HUNT study is the first to use a large population-based body of material, without any selection in patient material. Triglyceride levels, BMI, cholesterol and WHR were available and correlated with genotyping comparing undiagnosed and diagnosed patients with type 2 DM. The study concludes many SNPs related to type 2 DM, and is important to broaden our scope of genetic predispositions involved in the development of type 2 DM.

Other studies show how an adjustment of diet and exercise in risk groups can benefit the patients in terms of decreasing the risk of type 2 DM and associated complications. A study conducted in Chinese patients with IGT compared the relative risk for complications in an intervention group (intervention is diet and/or exercise) with a group of patients receiving regular treatment; the relative risk for DM in the intervention group was 38 % lower (CI 95 % 17 to 55) than the control group after the 6 years treatment [15]. The same study also shows the longevity of the beneficial effects; 20 years after study closure the patients in the intervention group experienced a mean of 3.6 fewer years with diabetes. The most alarming finding was that 93 % of the patients in the control group were diagnosed with diabetes after 20 years, compared to 80 % in the intervention group; this emphasize the importance early intervention. The study did not find any significant difference in CV complications between the two groups. Another study with patients suffering from IGT was conducted in Finland [16]. The patients in this study received similar guidance in terms of exercise and diet as the Chinese study, but specifically focused at reducing total fat intake and saturated fat intake. The cumulative incidence of diabetes during the 4 years was 11 % in the intervention group, compared to 23 % in the control group (CI 95 %; 6-15 % vs. 17-29 % respectively). During the 4 years the risk of DM was decreased by

58 % in the intervention group. The authors state that data may be conservative since it was done on an `intention to treat´-basis and since the control group, for ethical reasons, received advice on lifestyle changes as well. The authors state that the reduction in diabetes incidence was directly associated with lifestyle changes and that type 2 DM can be prevented by lifestyle changes in high risk patients [16]. All these studies show how type 2 DM is caused by a multifaceted and intricate mix of many factors, and most of all; the disease is preventable in many of the cases if treated early.

Figure 2: The major diabetic complications [2]

The importance of a closely monitored blood glucose level as well as monitoring of HbA_{1c} is critical; results from the UK Prospective Diabetes Study (UKPDS) show that patients treated intensively differ in HbA_{1c} levels compared to the conventionally treated group; the intensively treated group had a mean HbA_{1c} 0.9 % lower than the conventionally treated group[17]. This resulted in a reduction in risk of any diabetes-related endpoint (12 %), diabetes-related death (10 %) and a reduction in all-cause mortality (6 %) during the study period of 10 years. The risk reduction of any diabetes-related endpoint was mainly due to a 25 % reduction in microvascular endpoints. Contradictory to this, a recent study published in NEJM (12th June 2008) showed an increase in mortality and weight gain in an intensively treated patient group (reached an HbA_{1c} of 6.4 %) compared to the control group (reached an HbA_{1c} of 7.5 %) [18]. An implication of increased mortality in this study led to an early abortion after 3.5 years due to ethical reasons.

It must be noted that patients in the UKPDS study only received either sulfonylureas or insulin, while patients in the American study received various classes of antidiabetics and a combination of these. This might in part be an explanation of the contradictory results. The
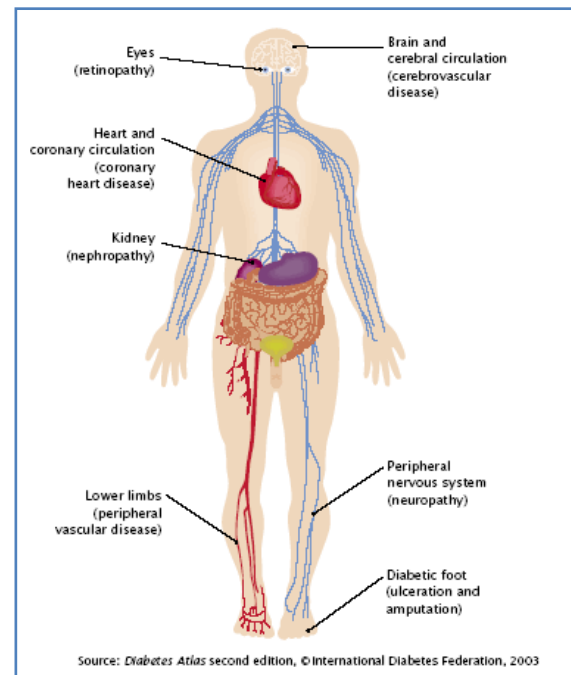
American study highlights the hypoglycemic events and its effect on mortality ratios. The study also describes the importance of a controlled and reasonable drug regimen.

1.1.4 Treatment

Diabetes has existed as an untreatable disease in all times, first described by the ancient Egyptians. A treatment for type 1 DM arose in 1921 as the hormone named insulin was discovered. Administration of insulin itself has changed from human to porcine or bovine insulin harvested from pancreas and then to synthetic insulin with altered qualities in terms of initiation and duration of effect, stability and reduction in risk of immune reactions. The availability of oral treatments for type 2 DM has also developed; the sulfonylureas, developed in the 50's, were the first oral treatment to arise. The introduction of the biguanids, thiazolidindiones and the alpha-glucosidase inhibitor came next. A new class of antidiabetica emerged recently as the two (as per May 2009) substances related to a gut hormone was developed and termed incretine mimetics. It is easy to forget the benefits one can achieve by changing lifestyle (i.e. diet, exercise) in patients with type 2 DM, especially from a pharmaceutical point of view given all the new drugs on the market. One might soon see a change where the aim of type 2 DM therapy is not the treatment of clinical findings but the prevention of such in risk groups through lifestyle changes.

1.1.5 Drug-induced hyperglycemia

It is well known that many drugs interfere with the metabolic pathways, and hence change glucose concentrations in blood. Many of the most commonly used drugs in Norway today can increase blood concentrations to such an extent that diabetics can experience difficulties controlling their disease [19, 20]. Many articles are discussing the most used drugs, and their risk of hyperglycemia, the two articles from B Luna and M N Feinglos [19] and M Pandit et al [20] presents many concerns. The two groups most interesting for this thesis is the effect of β-blockers and glucocorticoids (GCs), which are both two of the most commonly prescribed groups of drugs in Norway; a search in the Norwegian Prescription Database from the Norwegian Institute of Public Health [21] using search criteria; ATC-code C07 (β-blockers) found a prevalence of use in 2007 to be 72.98/1,000 inhabitants, while a search on ATC-code H02 (GCs for systemic use) found a prevalence of use in 2007 to be 35.99/1,000 inhabitants. It is important to note that the patients getting these drugs on a daily basis often suffer from other diseases as well, increasing the risk of diabetes development over time. A recent

prospective cohort evaluated the increased risk of DM after initiation of a β-blocker or a thiazide diuretic [22]. The results show a vastly increased risk of DM after the 6-year follow-up; 28 % increased risk of developing DM in subjects receiving β-blocker therapy, the data are independent of the degree of hypertension or family history of type 2 DM. The pro-diabetic effect of GCs has been known for decades; first showed by Long *et al.* in 1940, and recent evidence show that insulin resistance occur both at pre- and post-receptor sites [20]. It is also shown that GCs as hydrocortisone, prednisone and prednisolone are more diabetogenic than other GCs used, due to molecular differences. Prednisolone is the most frequent orally administered GC in Norway, its share of the total DDD in ATC-code H02 was approximately 81.9 % in 2007 (ca 16,71 out of a total of 20.39 million DDD) [21].

1.2   Glucose and glycogen; metabolism

Glucose is stored as glycogen in all vertebrates and the regulation of the metabolism of these substances is intricate [23]. Excess glucose is stored in a polymeric form as glycogen; and can in itself represent up to 10 % and 1-2 % of liver and muscle weight respectively [24]. To relieve the cells from the shear osmotic pressure of the vast amounts of glucose stored, a polymer is formed in large cytosolic granules to form an α-rosette. Glycogen itself is synthesized from glucose-6-phosphate via glucose-1-phosphate and UDP-glucose. Under conditions of insulin stimulation, the majority of muscle glucose cleared by the skeletal muscle are incorporated into glycogen [25]. It is showed that nearly 35 % of carbohydrates in a meal are stored as muscle glycogen, 20 % as liver glycogen [24]. Skeletal muscles cannot release glucose molecules as they lack glucose 6-phosphatase, hence muscles or adipocytes are prone to contribute glucose in a situation of e.g. fasting through other means, such as lactate release for instance [24]. Glycogen can be broken down as a response to adrenaline activation of cAMP dependent protein kinase (PKA) and the following amplification, or due to Ca^{2+} or adenosine monophosphate (AMP) (due to vigorously contractions) activation of protein kinase B (PKB/Akt), both leading to activation of glycogen phosphorylase (GP) and glycolysis. These reactions are allosteric and take place in the passing of milliseconds. Hormones as insulin, glucagon (too high or too low levels of blood glucose, respectively) and adrenaline (`fight or flight´ hormone) are somewhat slower in mediation of effect and takes place in the passing of seconds or minutes.

It is said that this glycolysis in myocytes probably is mediated through glycogen-bound phosphatase, particularly phosphatase 1 (PP1) [26]. If sufficient oxygen is present, the end product pyruvate will be fully oxidized in the mitochondria to H_2O and CO_2. At high intensity or if insufficient levels of oxygen are present pyruvate is converted to lactate, and the majority of lactate is released in the blood [24]. Lactate in the blood stream is available as an energy source for other tissues (e.g. heart tissue) once released or function as precursors for gluconeogenesis in liver or the kidneys [24].

The catabolic pathway from glycogen is catalyzed by GP, while the anabolic pathway to glycogen is catalyzed by Glycogen Synthase (GS) [23, 27]. The glycogen molecule is bound in a complex with GS, GP and several other enzymes that regulate the synthesis and metabolization of glycogen [25, 27, 28]. Glucose uptake and GS are the rate-limiting steps in glycogen synthesis in skeletal muscles [29], and regulation of these are of interest. The glucose-6-phosphate activates GS allosterically, however to achieve full activation GS demands covalent phosphorylation at some of nine different serine residues [24, 30, 31]. Although the inverse relationship between glycogen concentration and GS activity is well established (first by Danforth *et al.* 1965 [32]), the mechanisms of GS regulation needs further elucidation[28, 33]. Glycogen content strongly regulated GS activity [25, 27, 28, 32, 33], and has even been shown to regulate GS fractional activity to a higher extent than insulin[30]. Contraction also strongly influences GS activity, it has been reported that contraction reduces GS Ser[641] and Ser[645,649,653,657] phosphorylation, but the exact pathway is elusive. It has been suggested that this activation is mediated through lower glycogen content as contraction leads to this, however, a recent experiment suggest this not to be the case and that contraction regulates GS activity independently of glycogen content [30]. It has been shown that Ser[641] and Ser[645] are the two most important serine residues responsible for GS activation [29]. These two residues are mainly phosphorylated by GSK-3 as discussed in the next section.

1.3 Insulin signaling

Insulin binding to the insulin receptor (IR) leads to spontaneous auto-tyrosine-phosphorylation – the first step in the phosphorylation cascade following binding of insulin [34]. The IR can itself be phosphorylated and hence deactivated by regulatory proteins such

as tyrosine phosphatases [35]. Another protein important to mention in this context is the suppressor of cytokine signaling-1 (SOCS1), which has been seen to be upregulated in states of insulin resistance such as obesity [35]. Insulin receptor substrate – 1 (IRS-1) is then phosphorylated, amongst various other substrates, and binds the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). The regulatory subunit is p85 α, while the catalytic subunit is p110. Both of these subunits have a protein module known as Src homology (SH2) domains that interact with the phosphotyrosine-part of IRS-1. It is through PI 3-kinase activation that recruitment of PI 3-kinase to signaling complexes adjacent to membranes takes place [12, 31, 34, 36]. The p110 subunit of PI 3-kinase is responsible for the activation of PIP_2 to PIP_3 in the plasma membrane. PIP_3 is a second messenger that mediates critical regulation of insulin signaling in the cell [35]. Hence PI 3-kinase is responsible for modulation of several proteins through PIP_3 activation; perhaps the most essential being through PDK-1 mediation. PDK-1 phosphorylates the PKB Thr^{308} residue and enhancing its activity [31, 35], however, PKB must be phosphorylated at Ser^{473} for full activation. This is mediated through what was previously called PDK2; mammalian target of rapamycin (mTOR) complexed with the rapamycin-insensitive companion of mTOR (RICTOR) [37]. When PKB is phosphorylated at both residues, the enzyme exhibits a 4- to 5-fold higher activity compared to Thr^{308} alone [38]. PKB is found in three different isoforms, the PKBα is the isoform responding to insulin in muscles and in liver [31], PKBβ being the second one to be found in skeletal muscles. PKB will when activated phosphorylate GSK-3 (GSK-3 α at Ser^{21}, and GSK-3 β at Ser^9), deactivating the GSK-3 kinase and hence decreasing its activity towards GS [31, 35, 36]. Impaired GSK-3 effect promotes the active de-phosphorylated version of GS and an increase in glycogen synthesis [35]. GSK-3 β is more important than GSK-3 α in regulation of GS in skeletal muscles [39]. The activity of GS is, as previously discussed, regulated by glucose-6-phosphate and phosphorylation at nine different serine residues, in which GSK-3 is the single most important regulator of [34, 36].

There are many different isoforms of the GLUT transporter, but in skeletal muscles two exist; the GLUT1, which is omnipresent, and GLUT4, which is restricted to insulin-sensitive tissues [40]. Insulin recruits GLUT4 from intracellular vesicles, in which ≈90 % of the transporters are stored in the basal state [41]. The GLUT4 is recruited to the cell membrane; this mechanism is thought to be the major mechanism responsible for insulin-mediated glucose uptake [34,

36, 40-42]. 70-90 % of glucose taken up in the myocytes following insulin stimulation is incorporated into muscle glycogen [24]. Insulin and contraction are the two most important inducers of glucose uptake in skeletal muscles [25, 43]. PKB stimulates GLUT4 translocation to the cell membrane [31, 36], hence increasing glucose uptake [28]. This is mediated through PKB's phosphorylation of Akt Substrate 160 kDa (AS160); a Rb GTPase activating
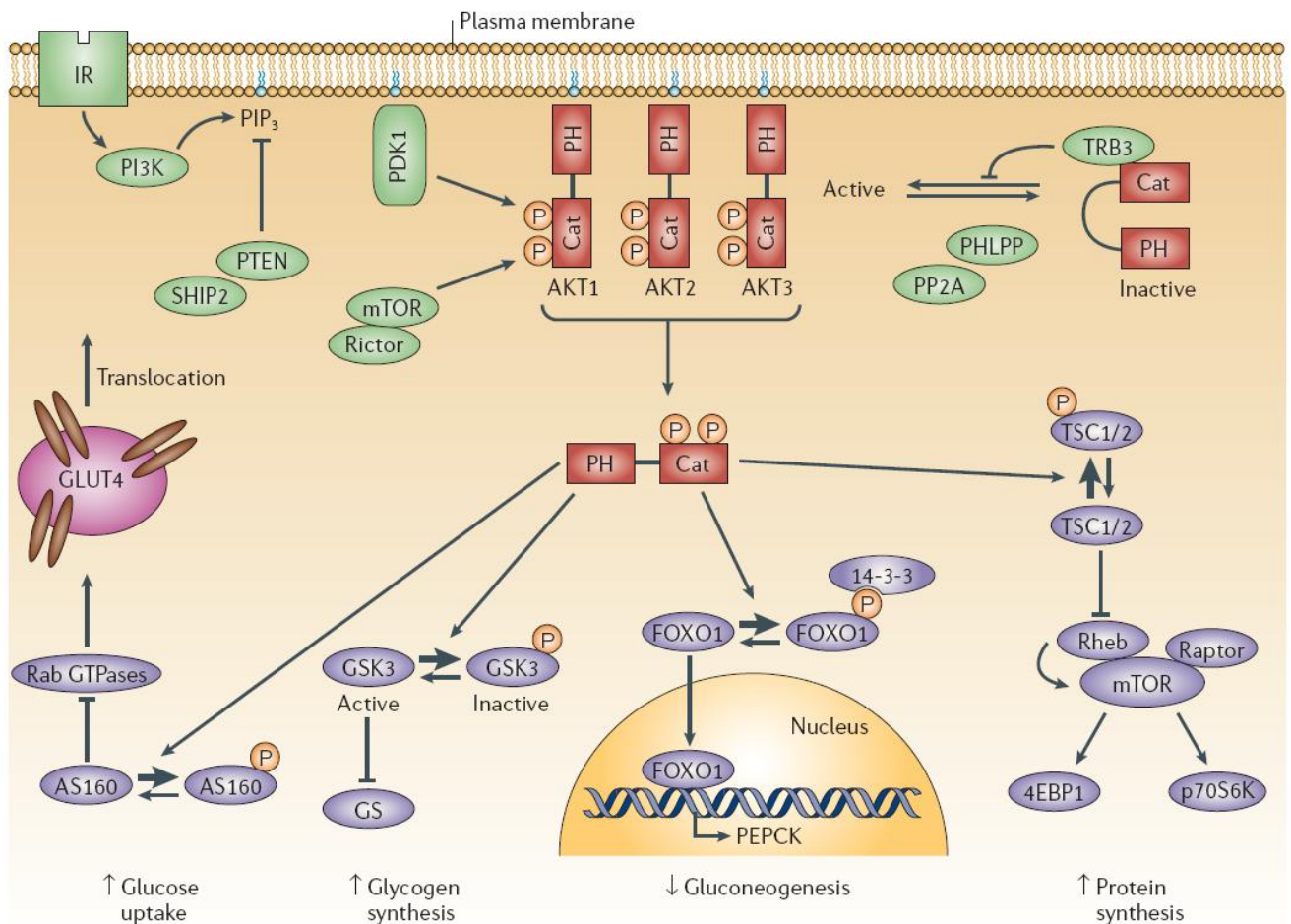
protein [24, 29, 31, 44].

Figure 3: Insulin signaling pathway through the PKB pathway and its regulation of glucose **uptake [35].** The three AKT/protein kinase B (PKB) isoforms (in red) share the same structural organization: a pleckstrin-homology (PH) domain, which is required for binding to phospholipids, and a catalytic domain (Cat), which becomes active on phosphorylation (P) of two residues (Tyr308 and Ser473 for AKT1, Tyr309 and Ser474 for AKT2, and either Tyr305 alone or Tyr305 and Ser472 for AKT3). The upstream regulators of AKT/PKB activity are represented in green. Positive regulators are phosphatidylinositol 3-kinase (PI3K), which produces phosphatidylinositol-3,4,5-triphosphate (PIP3) in response to insulin. PIP3 recruits phosphoinositide-dependent kinase-1 (PDK1) and AKT/PKB at the plasma membrane, where AKT/PKB is phosphorylated by PDK1 and PDK2 (this is thought to be a complex between the mammalian target of rapamycin (mTOR) and the rapamycin-insensitive companion of mTOR (rictor)). Negative regulators include the phosphatases phosphatase and tensin homologue (PTEN), Src-homology-2 domain-containing inositol phosphatase-2 (SHIP2), phosphatase-2A (PP2A) and the PH-domain leucine-rich-repeat protein phosphatase (PHLPP). Tribbles-3 (TRB3) is another protein that can bind to and inhibit AKT/PKB. The downstream targets of AKT/PKB are shown in purple. The GTPase activating protein AKT substrate of 160 kDa (AS160) is one of the AKT/PKB targets that is responsible for the translocation of the glucose transporter-4 (GLUT4). Glycogen synthase kinase-3 (GSK3), forkhead box O1 (FOXO1) and the tuberous sclerosis complex-1 and -2 (TSC1/2) complex are direct targets of AKT/PKB. GSK3 inhibits glycogen synthase (GS). Phosphorylated FOXO1 is sequestered in the cytoplasm on binding to 14-3-3 proteins. TSC1/2 inhibits the small GTPase Ras homologue enriched in brain (Rheb), an activator of mTOR. mTOR, associated with regulatory associated protein of mTOR (raptor), phosphorylates

its substrates eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6K). Plain arrows represent an activation process, and blocked arrows represent an inhibition process. IR, insulin receptor; PEPCK, phosphoenolpyruvate carboxykinase.

1.4   Stress hormones

1.4.1   Anatomy

Stress as a definition is easy to understand and surrounds us in our day to day life. Psychological stress versus physiological stress will affect the body's hormone balance, through the HPA axis (Hypothalamic-pituitary-adrenal axis) or SAM system (symphatetic-adrenomedullary) as explained later [45]. Stress perceived as either threatening or challenging has shown to induce different responses in humans. Threat stress will induce a reaction from the HPA axis (GC release), compared to challenging stress which induce the SAM system (catecholamine release) [45]. Stress in rats seem to act differently than in humans; rats are almost always prone to eat less (but not in cases where the rats are given a diet rich in fat) while 30 % of humans are prone to eat less, while the rest are prone to eat more [45].

Adrenal GC secretion is normally controlled by the HPA axis, striving homeostasis, and occasionally also from ACTH-independent mechanisms (Adrenocorticotropic hormone; also known as corticotropin)[8]. Corticotropin-releasing hormone (CRH) initiates the stress response in the HPA axis and is the most important regulator as it stimulates release of ACTH, which in turn stimulates steroid release; cortisol or corticosterone in mammals and rodents respectively [8]. Consult Figure 4 for a better view of the pathway in regulation of GC production. The adrenal cortex synthesize and secretes the GCs, while the adrenal medulla produces catecholamines (adrenaline and noradrenaline), and several neuropeptides [8]. The secretion of catecholamines is mediated through activation of the sympathetic nervous system as well as through ACTH mechanisms.
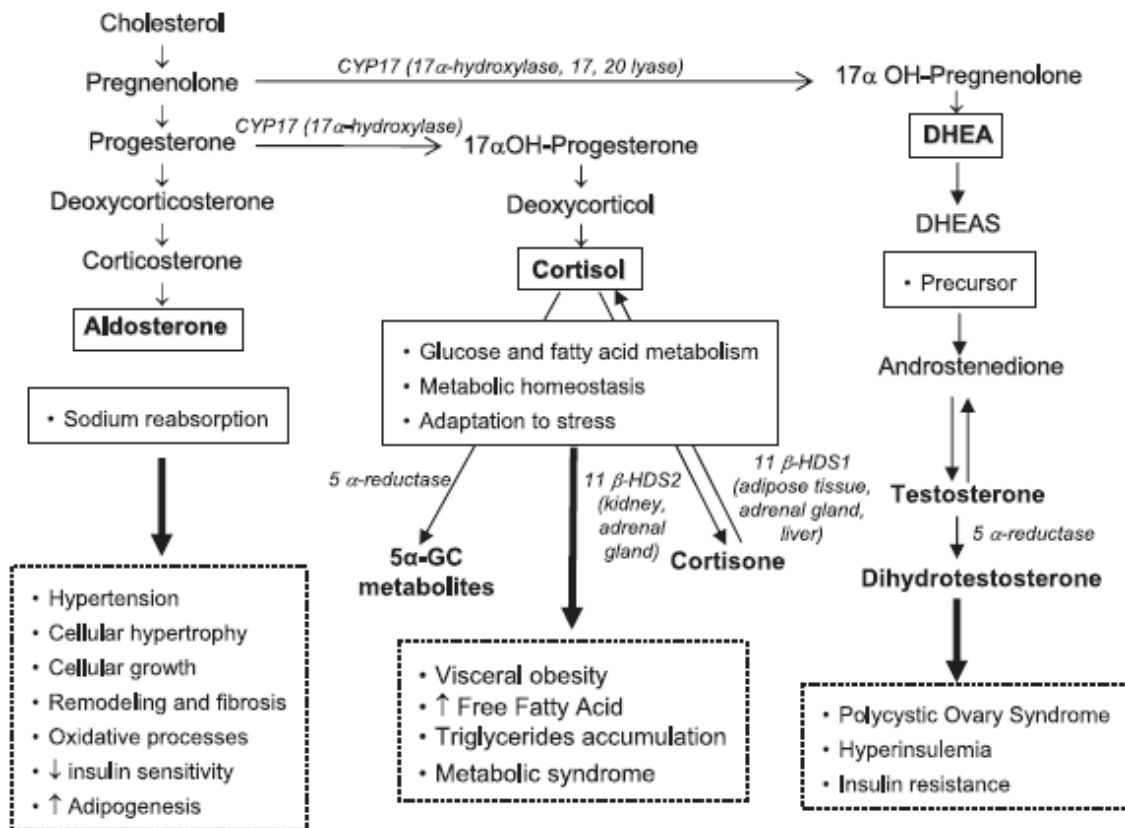
Figure 4: Adrenal gland steroids and their respective actions, adapted after Roberge *et al.* [8]

1.4.2   Glucocorticoids

The understanding of GCs in this already intricate puzzle of the metabolic syndrome and stress is built upon the studies of Cushing's syndrome – sustained elevation of cortisol secretion [8, 34, 46]. The acute effect of GCs is described as one of the `fight and flight´ responses is responsible for many homeostatic actions; liberation of fuel through effects on glucose and fatty acid metabolism, shock protection through effects on haemodynamics and fluid balance and effect on acute immune responses through anti-inflammatory action [45, 46]. It is clear that the effects of GCs are vital for humans to adapt to stress, although these effects are only of benefit if transient. Sustained elevated levels of GCs will lead to symptoms seen in Cushing's syndrome; central obesity, hypertension, glucose intolerance and dyslipidemia [8, 9, 46]. GCs in general oppose insulin action [9, 47] and causes insulin resistance [34, 36]; they mediate adipose tissue differentiation, function and distribution, and in excess causes visceral obesity [12]. GCs also have a direct inhibitory effect on glucose-induced insulin release from the pancreatic β-cells[41]. The combination of high GC levels, dense calories and in turn elevated insulin contributes to this visceral fat distribution [45].

 A Swedish research group studied the regulation of GC levels in humans in relation to somatic symptoms. GC levels were tightly regulated, with an efficient feedback mechanism in healthy subjects – making them able to respond to everyday stress [13]. Contrary to this group was a group not able to control their GC levels tightly and experienced consistent mal-secretion; the group introduced the term `hypothalamic arousal syndrome´ and described a parallel activation of both the HPA axis and the SAM system. There was found correlating somatic symptoms to `hypothalamic arousal syndrome´: inhibited secretion of sex steroids and growth hormone, insulin resistance, abdominal obesity, elevated leptin levels, hyperglycemia, dyslipidemia and hypertension with elevated heart rate [13]. The findings suggest the syndrome is responsible for many of the metabolic symptoms, caused by environmental factors and genetic predispositions. Many studies point at GC dysregulation as one of the key issues understanding pathophysiology in diabetes and metabolic syndrome [8, 12, 45, 46]. Skeletal muscles and the liver are organs are both insulin- and GC-responsive, and hence crucial organs in regulation of adipose tissue, as well as obesity and diabetes [9].GCs enhance glucose production in the liver both directly by transactivating crucial genes involved in the process and indirectly by stimulation of other hormones, e.g. glucagon [9]. On the other hand, insulin opposes GC action through decrease of GC receptor expression in skeletal muscle [9].

GC metabolites in adipose tissue, colon and kidneys are recycled by the enzyme 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1) through conversion from cortisone to cortisol. This specific enzyme has thus been connected to the development of obesity and insulin resistance [9, 48, 49], as shown in Figure 5. Excess of GCs have shown to lead to growth of visceral adipose tissue, and hence an evil circle where more adipose tissue seem to develop as the local recycling of GC is increased. The action of the two 11β-HSDs, type 1 and 2, is mainly in insulin-responsive central adipose tissue and in the kidneys respectively [46]. The reason why visceral adipose tissues are linked to diabetes, compared to how subcutaneous adipose tissues are not, might be due to the higher responsiveness and sheer number of GC receptors.

GCs enhancement of 11β-HSD1 expression happens to a higher degree in visceral adipose tissue than normal tissue [8, 9, 46]. In addition to this Roberge *et al.* points out that since the GC recycling may be of similar amplitude as the original GC secretion, as well as the recycled hormones have direct access to the liver via the portal vein [8].
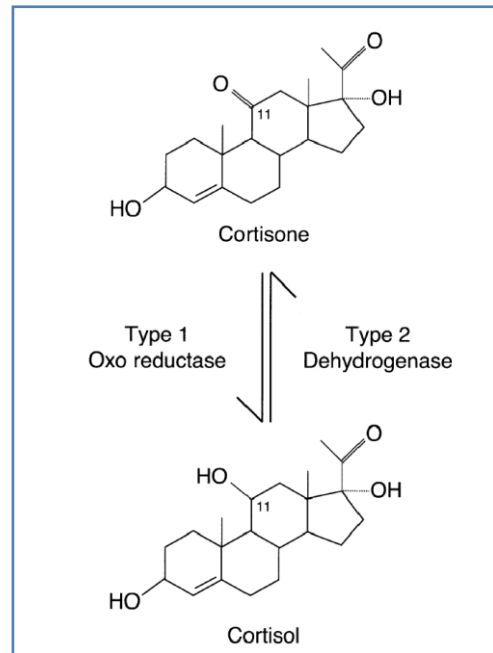

Figure 5: Interconversion of cortisone to cortisol, catalyzed by 11β-HSD1 [9, 46]

1.4.3 Synthetic cortisone; dexamethasone

Dex is a synthetic GC and is more potent than cortisol, though not exerting any mineralcorticoid properties. The substance is frequently used in animal models, and has been shown to inhibit insulin-stimulated glucose transport in rats at a concentration of 1.0 mg/kg [50]. This concentration is based on the secretion found in adult male humans; which under normal conditions is 0.2 mg/kg, but during stress can increase 10-fold to 2.0 mg/kg [51]. Qi *et al.* have proven insulin resistance after 4 hours of dex treatment using the euglycemic-hyperinsulinemic clamp method in rat [50, 51]. Literature show that the time span for which dex is given normally is 4-12 days when investigating dex's effect on glucose metabolism, consult Appendix 8 for a list of experiments using dex treatment (page). Experiments at STAMI has previously conducted dex treatment for 12 days [36] and 11 days [34].

One of the clinical effects of dex treatment in high doses is growth retardation, dex treated rats are expected to either lose or slowly gain weight as this is reported in several publications. A large weight difference can introduce bias to the data for many reasons, rats with more adipose tissue can exhibit other metabolic responses compared to lean rats. There is especially seen that larger rats exhibit lower insulin sensitivity as they grow larger,

while the dex treated rats are leaner. Consult the tables below for the weight differences experienced in some published studies. Dimitriadis *et al.* found that proteolysis in muscle arose after 5 days dex treatment, thus increasing alanine-levels. The increased amount of alanine leads to increased turnover via the alanine-cycle, producing lactate, then yielding pyruvate [42] that potentially affects results as it will convert to glucose. Dimitriadis *et al.* discuss the clinical relevance of this to be low, especially since the buffer used for incubation contains 2 mM of pyruvate.

Table 2: Average rat weight, Ruzzin et. al(Control n=29, dex n=28)[36].

	Day 12*
Control	321.9 ±5.0 g
Dex (1.0 mg/kg)	233.4 ±3.2 g

* Significant weight-difference between the two groups, p <0.006.

Table 3: Average rat weight, Coderre, L., *et al.* (n=10)[26].

	Day 1	Day 7
Control	201 ± 2 g	236 ± 2 g
Dex (1.0 mg/kg)	209 ± 2 g	154* ± 2 g

* Significant weight-difference between the two groups, p<0.001

Table 4: Average rat weight, Coderre, L., *et al.* (n=10)[40].

	Day 1	Day 7
Control	201 ± 2 g	240 ± 3 g
Dex (0.4 mg/kg)	198 ± 2 g	185* ± 4 g
Dex (1.0 mg/kg)	211 ± 3 g	154* ± 1 g

* Significant weight-difference between the two groups, p<0.001

1.4.4 Adrenaline

Activation through the SAM system increases secretion of catecholamines, one of these are adrenaline. Glucose uptake is regulated by adrenaline but, unlike muscle contraction and insulin, it is seen as the single most important inhibitor of glucose uptake [43]. Studies showing adrenaline-mediated inhibition of insulin-stimulated glucose clearance in vivo [43] and inhibition of insulin-stimulated glucose uptake in skeletal muscles [25] support this. Adrenaline activates GP and stimulates glycolysis [24] via β-adrenoceptors, this is especially evident in type II (fast-twitch) muscles such as Epi and EDL [25]. In type I muscles (slow-twitch), that normally have a lower glycogen content, adrenaline have minimal ability to stimulate glycogen breakdown [25]. The phosphorylation cascade downstream of β-adrenoceptors is described shortly; adrenaline binding to these receptors release cAMP, hence activating PKA and glycogen phosphorylase kinase [25, 52]. Glycogen phosphorylase kinase phosphorylates GP *b* and transforms it to the active *a* form [25]. GS is also phosphorylated by adrenergic stimulation, this will hence inactivate the enzyme and start

glycogen breakdown [27, 52]. It has also been shown that while most of GP is phosphorylated to the active form during adrenergic stimulation in fast-twitch muscles, this is not the case in slow-twitch muscles – although the latter muscles inhibit a β-adrenoceptor density almost twice as high as in fast-twitch muscles [27].

There is a cross-link between PKB phosphorylation and adrenaline-stimulated cAMP release as well, this cross-link is called Epac. Epac is a GTPase exchange factor activating Rap1, and there is growing evidence that cAMP-mediated PKB activation requires the presence of Epac [52]. Brennesvik *et al.* showed additive effect on PKB phosphorylation of insulin and adrenaline supporting this [52].
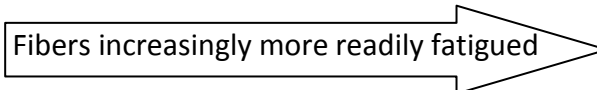
1.5   The role of skeletal muscles in glucose homeostasis

Insulin is not the only factor in the crucial task of glucose uptake. Contractile activity is along with insulin the two most important stimuli of glucose uptake [25, 43] through GLUT4 translocation [53]. Contractile activity mediate GLUT4 translocation through a pathway different from insulin; contraction activates AMP-activated kinase (AMPK) independent of PI 3-kinase [24]. Skeletal muscles are important in the regulation of blood glucose levels, and many claim emerging insulin resistance in skeletal muscles is one of the major causes of type 2 DM development [26, 40]. Skeletal tissue comprises up to 40 % of body weight in an adult human being, and is responsible for 70-90 % of insulin-stimulated glucose uptake. This glucose is mainly stored as muscle glycogen [28]. The continuous metabolism and catabolism of glycogen is closely regulated, the metabolism through GS and GP are the major contributors to this homeostasis [27].

Muscle fibers have been divided into type I and II through histochemical staining of the fiber's ATPase activity; acid-stable and alkali-labile type I (slow-twitch) and acid-labile and alkali-stable type II (fast-twitch). Type II fibers is then again divided into type IIA and IIB [54]. Both type IIA and IIB contract more rapidly, but type IIB is fatigued at an earlier stage than type IIA. Type I fibers contract more slowly, and are fatigued less readily than type IIA and IIB. The contractile property of a muscle depends on what fibers the muscle is constituted of [54] (Table 5). T Nakatani *et al.* indicate that fibers of a high-oxidative nature (fast-twitch muscles) has a higher degree of mitochondrial activity compared to fibers of less oxidative activity [54].

Different muscle fiber types have different roles; hence the muscles dissected out in these experiments performed at STAMI are expected to give somewhat different results. The two pieces obtained from one sol results in two relatively similar muscles both in size and composition (mainly slow-twitch)[54]), while the two EDLs are somewhat different from each other; one white (superficial part of muscle) that has a higher majority of type II fibers while the other one (red, deep part of muscle) has a higher majority of type I fibers[54] (Table 5). The two muscle strips are consistently differed between and referred to as respectively rEDL and wEDL. The epi muscle, is fast-twitch and contains mostly of type II fibers [25].

Table 5: Specifications of various fiber types and muscles, presented in % (mean ± SEM) [27, 54, 55]

Fibers increasingly more readily fatigued ▷

	Type I (slow-twitch)	Type IIA (fast-twitch)	Type IIB (fast-twitch)
wEDL (superficial)	0	11.2 ± 4.1	88.8 ± 4.1
rEDL (deep)	10.0 ± 3.1	26.7 ± 6.9	63.3 ± 8.5
Soleus	87	13	0
Epitrochlearis [56]	10-15.0	15-23	67-70
Glycogen content	Low	High	High
Oxidative capacity	High	High	Low
Mitochondrial density	High	High	Low

2 Purpose

2.1 Experiment I

The purpose of Experiment I was to investigate the onset of dex-mediated effects.

The following questions were tested:

1) How will dex treatment affect glycogen content?
    a. In skeletal muscle
    b. In liver
    c. In cardiac muscle
2) How will dex treatment affect activation of the insulin signaling pathways?
3) How will dex treatment affect insulin-stimulated glucose uptake in skeletal muscles?

2.2 Experiment II

The purpose of Experiment II was to investigate the dex-mediated effects after 1 and 11 days. Another purpose was to compare the effect of fasting (24 hours)

The following questions were tested:

1) How will dex treatment affect glycogen content?
    a. In skeletal muscle
    b. In liver
2) How will dex treatment affect the rate of lactate release?
3) How will dex treatment affect activation of the insulin signaling pathways?
4) How will dex treatment affect the efficiency of different mediators on glucose uptake in skeletal muscle?
5) How will fasting for 24 hours differ from fed status, and how will fasting status affect purposes 2-5?

3 Methods and materials

3.1 Rats

Male Wistar rats (Bk1:Wist) from B & K Universal AS (Nittedal, Norway) were used in all experiments. The rats were kept in our laboratory animal facilities for at least a week prior to experiment. Room temperature was kept at 21 °C, humidity was kept at 55 %, and a 12:12 hour light/dark cycle (from 6 AM to 6 PM) was obtained throughout the housing. The rats had free access to standard rat chow (B & K Universal, Grimston UK) and tap water. The experiments were performed during the light cycle (between 10 AM and 3 PM). The rats were all treated with either dex (1.0 mg/kg, dissolved in saline) or saline (9 mg/kg) by intraperitoneal injection (i.p.) at 10 AM. Experiments and procedures were approved by official authorities and performed in accordance with the laws and regulations controlling experiments on live animals in Norway and the European Convention for the Protection of Vertebrate Animals used in Experimental and Other Scientific Purposes.

3.2 Muscle preparation and incubation

Muscles where prepared and handled as described earlier in articles by Jensen *et al*. [28, 34, 43]. All rats were anesthetized with an IP injection of ≈ 10 mg of pentobarbital sodium (50 mg/ml) per 100 g rat. Epitrochlearis (epi), soleus (sol) and extensor digitorium longus (EDL) were dissected first. The epi were studied intact, while sol and EDL were both split in two; sol resulting in two relatively homogenous muscles, while EDL was split in wEDL and rEDL. The sol is cut with a scalpel, while the EDL is split by tearing along the tendons stretching to each toe. The muscles were mounted at their approximate resting length on electrodes, consult Figure 8. The electrodes were placed in test tubes with 3.5 ml buffer and gassed continuously (95 % O_2, 5 % CO_2) throughout incubation, as well as at least 15 minutes before mounting. All incubation was conducted in a water bed (30 °C). After preincubation (30 minutes, 3.5 ml Krebs-Henseleit buffer), the electrodes were taken out and placed in vials with the radioactive buffers, consult flow charts presented below. The muscles were removed from the electrodes after incubation (½ hour and 1 hour in Experiment I and II respectively), blotted on filter paper to remove excess buffer and frozen in liquid N_2. Incubation buffer was saved in experiment II for lactate analysis. Muscles were cut in cryo-stat before analysis (-20 °C), samples for western blot were weighed (ww) and frozen (-70

°C), while samples for glucose uptake and glycogen content were freeze dried (3 hours), weighed (dw) and frozen (-70 °C).
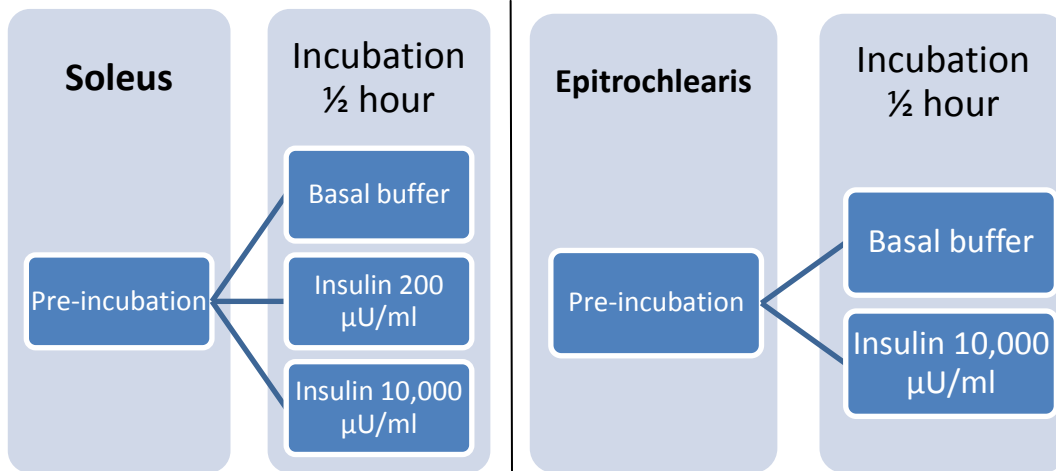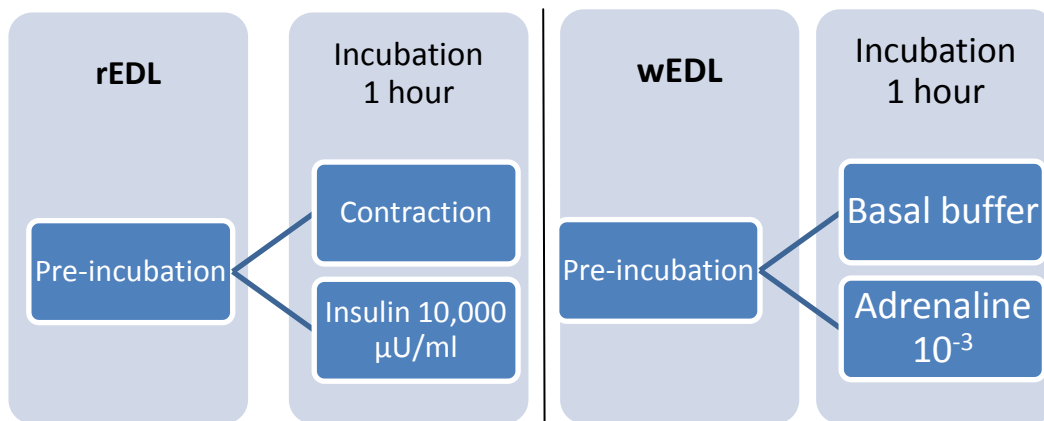

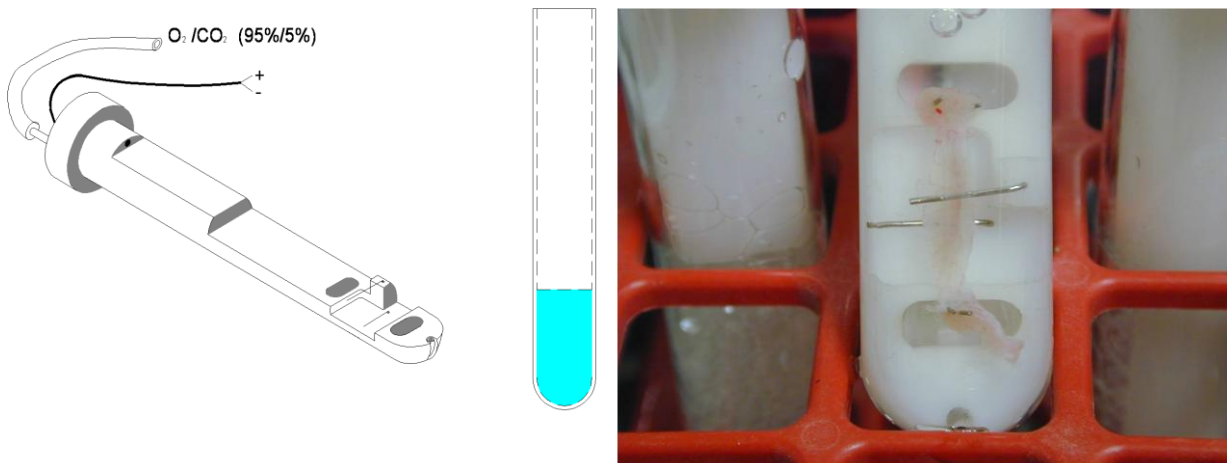Figure 6: Flowchart of Experiment I

Figure 7: Flowchart of Experiment II

Figure 8: Schematic presentation of the electrodes used and test tube (left), mounted epi muscle (right)

3.3   Muscle contraction

Some of the muscles were contracted isometrically in the basal buffer. The muscles were stimulated with impulse trains of 200 ms at a frequency of 100 Hz (square wave pulses of 0.2 ms duration and 10 V amplitude) delivered at a rate of one train per 2 s for 60 minutes in experiment II.

3.4   Statistics

All data sets were entered into Word Excel standard sheets prepared by Jorid Thrane Stuenæs for the respective methods. All p-values in Experiment I was found using Word Excel formulas; due to a small test size unpaired student's t-test assuming unequal variance were performed. All figures are prepared using SigmaPlot version 10.0.

Experiment II has a bigger test size and analyses of variances (ANOVA) were used, as well as a least significant difference (LSD) post hoc test. The software used to perform these analyses was SPSS 16.0. Unpaired student's t-test assuming equal variance was performed to compare responses on day 1 versus day 11 using Word Excel formulas.

P-values were acknowledged with an alpha significance level =0.05. p-values > 0.05 were termed as `borderline´ significant and discussed where appropriate.

3.5   Glucose uptake analysis

Glucose uptake was conducted as described in articles from J. Jensen *et al.* [28, 43]. In brief 0.25 µCi/ml 2-[1,2^{3}H(N)]deoxy-D-glucose (30.6 Ci/mmol; NET 549 PerkinElmer) and 0.1 µCi/ml D -[1-^{14}C]mannitol (54.5 mCi/mmol; NEC 314 PerkinElmer) were added to the Krebs-Henseleit buffer and counted for radioactivity (d.p.m, Tri-Carb 1900 TR, Packard). A freeze-dried muscle sample (2-3 mg dw) was dissolved in 600 µl 1 M KOH for 20 minutes at 70 °C, samples were mixed several times. Samples were cooled and centrifuged (3000 G, 10 minutes). Blanks were prepared and handled together with the samples; 2 vials 600 µl 1 M KOH and 2 vials 600 µl 1 M KOH with 50 mM glycogen control solution (Seronorm 20 µl, precipitated in 500 µl 0.4 M perchloric acid). 400 µl of the homogenate/blanks and 3 ml Hionic Fluor scintillation cocktail (Hionic-Fluor 6013319, Perkin-Elmer) were pipetted into counting tubes (Pony Vial 6000292, PerkinElmer) and then counted for radioactivity; two different pre-programmed settings count the two isotopes in 2 cycles x 5 minutes.

Background for analysis of glucose uptake

Some assumptions were made; similar uptake kinetics for glucose and 2-^{3}H-deoxyglucose (DG) during incubation, hence the relation between glucose- and 2-^{3}H-DG -uptake was equal to the relation between the concentrations of the two in the incubation buffer. The amounts of radio-marked glucose analogues were assumed equal in the extracellular compartments as in the incubation buffer, and ^{14}C mannitol was assumed not to be subject to transport into the cells. Since the amount of 2-^{3}H-DG added, dw and specific activity of the buffer are known, the uptake of 2-^{3}H-DG can be calculated. Glucose uptake is equal to 2-^{3}H-DG -uptake multiplied by the concentration relationship between glucose and 2-^{3}H-DG in the incubation buffer.

3.6 Glycogen content analysis; muscles

The method for measurement of glycogen content in muscle follows what is described by Aslesen *et al.* [43]. 100 μl of the muscle homogenate/controls from the dissolution in KOH was hydrolyzed by amyloglucosidase (Amylase, AmG), in this case a γ-amylase that hydrolyzes glycogen. 100 μl of muscle homogenate/controls were pH-adjusted to 4.8 using 20-25 μl 7 M acetic acid and 500 μl 0.3 M acetate buffer (with AmG, 30 μg/ml). Homogenate/blanks were mixed and incubated for 3 hours at 37 °C. Reaction solution were made daily and kept on ice, composition of which is presented in `Appendix 5´, page 86. 25 μl sample/blanks are pipetted into acid-washed vials (10 x 75 mm) together with 750 μl reaction solution and mixed. Glycogen contents were measured on Shimadzu spectrofluorophotometer RF-5000 (which delivers valid results in the fluorescence area 0.5 – 10 μM), and all solutions were kept at a stable temperature of 23-25 °C during counting. Background emission were measured first; then added 5 μl hexokinase and mixed. Glycogen contents in the vials were measured as Δ fluorescence after 20-30 minutes.

Background for analysis of glycogen content;

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

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

3) glucose-6-P + NADP$^+$ $\xrightarrow{\text{G6PDH}}$ 6-P-gluconolacton + NADPH + H$^+$

Glucose is being phosphorylated in reaction 2) to glucose-6-phosphate that is oxidized by NADP$^+$ in reaction 3). Reaction 3) is heavily skewed to the right, hence levels of reacted

glucose is proportional with levels of NADPH made. NADPH is measured as the increase in fluorescence after addition of Hexokinase, wavelengths 340 nm (excitation) and 460 nm (emission).

3.7 Glycogen content analysis; liver and heart

Direct acid hydrolysis was used to dissolve the freeze-dried liver and heart (1-2 mg dw) in respectively 3000 and 1000 µl 1M HCl for 2.5 hours at 100 °C, and mixed several times. Samples were cooled, and centrifuged (3000 G, 10 minutes). Blanks were prepared and handled together with the samples; 2 vials 1 M HCl, and 2 vials 1 M HCl/ 50 mM glycogen control solution (Seronorm 20 µl, precipitated in 500 µl 0.4 M perchloric acid). Samples were cooled and centrifuged 2 vials of 1M HCl and 2 vials of 1M HCl were added 30 µl glycogen standard solution (Seronorm 20 µl, precipitated in 500 µl 0.4 M perchloric acid). 100 µl of liver samples were diluted in 300 µl 1M HCl, heart samples were not. Reaction solution were made daily and kept on ice, composition of which is presented in `Appendix 5´ on page 86. 10 µl sample/blanks were pipetted into acid-washed vials (10 x 75 mm) together with 750 µl reaction solution. Glycogen contents were measured on Shimadzu spectrofluorophotometer RF-5000 (which delivers valid results in the fluorescens area 0.5 – 10 µM), and all solutions were kept at a stable temperature of 23-25 °C during counting. Background emission was measured first, then added 5 µl hexokinase and mixed. Glycogen contents in the vials were measured as Δ fluorescence after 20-30 minutes.

3.8 Glycolytic flux

Glycolytic flux measurements were conducted in several pilot experiments, the method is added in `Appendix 6´, page 88. This method is not described closer as no results from these pilot studies are presented in this thesis due to failure to provide certain results. This method would be interesting as it would provide information of to what extent glycogen would undergo glycolysis and in addition make it possible to conduct measurements of glycogen synthesis in the same samples.

3.9 Glycogen synthesis ^{14}C and ^{3}H

The method used at STAMI for measurement of glycogen synthesis is usually including ^{14}C-glucose as the radioactive compound. However, to be able to measure glycogen synthesis and glycolytic flux in the same samples, a series of pilot studies was done to compare results

in glycogen synthesis between the two radioactive substances. Our pilot studies did not find equal findings when comparing glycogen synthesis with ^{14}C and ^3H and was hence abandoned. This method is not described closer as no results from these pilot experiments are presented in this thesis. A detailed description of this method is provided in `Appendix 7´, page 89.

3.10 Lactate release in buffer

The method for measurement of lactate in muscles was adapted to measure lactate release in incubation buffer. It was crucial that all electrodes, vials and equipment used were washed in $H_2O_{millipore}$. Lactate levels in sweat are many times higher than in blood, thus clean equipment and latex gloves were crucial. In addition vials with electrodes (but without muscles) were incubated (30 °C) for 1-2 hours together with the vials containing muscles to be used as blanks. After incubation, and freezing of the muscles in liquid N_2, the buffer was kept on ice (for as short time as possible) until it was pipetted into vials and frozen (-20 °C).

Reaction solution were made daily and kept on ice, composition of which is presented in `Appendix 5 ´ page 86. 10 µl sample/blanks were pipetted into acid-washed vials (10 x 75 mm) together with 750 µl reaction solution. 2 blanks containing 10 µl 10 mM L(+)-lactate (Sigma L-1750) were prepared as parallels. Lactate concentration was measured on Shimadzu spectrofluorophotometer RF-5000, and samples were kept at a stable temperature of 23-25 °C during measurement. Background emission was measured first; then added 5 µl 10 U/ml LDH and mixed. Lactate release were measured as Δ fluorescence after 45-60 minutes.

Background for analysis of lactate release;

1) L-(+)-lactate + NAD$^+$ $\xrightarrow{\text{lactate dehydrogenase (LDH)}}$ pyruvate + NADH + H$^+$

2) pyruvate + glutamate $\xrightarrow{\text{glut-pyr-transaminase (GPT)}}$ alanine + a-ketoglutarate

Lactate is oxidized by NAD$^+$ in 1). To ensure the reaction is skewed to fulfillment towards NADH, pyruvate is removed from the solution in the reaction catalyzed by addition of glutamate and GPT (both added in abundance), as well as ensuring basic environment. It is crucial to sustain as high a pH as possible, without induction of denaturation. The production of NADH is measured as an increase in fluorescence, which is directly proportional to lactate release in the samples. The wavelengths measured are at 340 nm (excitation) and 460 nm

(emission). The method is specific for L-(+)-lactate, as the enzyme LDH is not reacting with D-(-)-lactate.

3.11 Western blot

A sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) method was used to do western blotting, all solutions used are declared in `Appendix 5 ´ on page 86.

3.11.1 Homogenization of muscle samples

Muscles for homogenization were weighed (ww, ≈10 mg) in -20 °C. Homogenization buffer was made the same day and added to the vial containing the muscle (ratio 1:30). The muscle was homogenized immediately, 2 x 15 seconds (Polytron PT1200, +4 °C). 1 % Triton X-100 was added after homogenization and the samples rotated in +4 °C for 1 hour. Samples were centrifuged (10 minutes, +4 °C, 11,500 g) and the supernatant was pipetted and the volume measured with an electronic pipette. 30 µl of the supernatant was diluted in 200 µl $H_2O_{millipore}$. The method measuring total protein levels follows the instruction manual of *DC* Protein Assay (Bio-Rad laboratories). DC Protein Assay reagents was added and transferred to plastic cyvettes for determination of Optical Density (OD) at 750 nm in a spectrophotometer (Shimadzu MPS-2000). Additional buffer based on the results from the total protein assay was added to the samples to a uniform protein concentration (in the range 1.0 - 2.5 µg/µl). 100 µl of the homogenates with equal protein concentration were added to a solution of 6.2 µl 2-mercapto ethanol and 25 µl 5xSDS. The samples were left in room temperature for at least 2 hours, then frozen until used (-20 °C).

3.11.2 Gel making

Western blot is a method used for a semi-quantitative measurement of protein expression and phosphorylation with the help of antibodies. The proteins are in this method (SDS-PAGE) denatured and divided via electrophoresis, depending on molecular weight. The gel used in this method is an SDS-polyacrylamide gel consisting of a stacking gel (top, 4 %) and a separation gel (bottom, 10 %). The stacking gel will concentrate the added sample volume (10 – 14 µl); concentrating the solution, and narrowing the bands. The function of SDS is to destroy secondary and tertiary protein structure through breakage of disulfide bonds, and cover the proteins giving the proteins a uniform negative charge. The only factor separating

the proteins will hence be the size as SDS makes the charge:mass ratio even in all proteins. All equipment used is from BioRad (Model no. Mini-PROTEAN® 3 Cell).

3.11.3 Electrophoresis

Samples were mixed gently after defrost, and applied to the gels' 15 wells. A marker was added to visually indicate protein bands with specified molecular weight. The amount of sample added was 10-14 µl. The electrophoresis was run on 4 gels at a time, and the electrophoresis buffer filled the chamber in which the gels were placed. Voltage of 100 V was added to the system to compress the samples in the stacking gel (10 minutes) then increased to 180 V (70 minutes total).

3.11.4 Blotting

This method uses polyvinylidene fluoride (PVDF) membranes. The transmission of proteins from the gel to the membrane (blotting) is based on hydrophobic interactions as well as charged binding between membrane and protein. The membranes are equilibrated in 100 % methanol for 15 seconds, water for 5 minutes and transfer buffer for at least 15 minutes, the gels are added to the transfer buffer together with the membranes (minimum 10 minutes). A blotting sandwich was made, consisting of; filter pad, filter paper x 2, gel, membrane, filter paper x 2 and filter pad. It was important to remove excess air from between the layers before blotting. Blotting was commenced in cold blotting buffer with constant stirring with a constant power of 250 mA added to the system for 1 hour.

3.11.5 Blocking and immune reactions with antibodies

The membranes were washed 3x10 minutes in PBS/0.1 % Tween-20 (PBS-T). Blocking of the membranes in 5 % skimmed milk (Tine unspecified dry milk) dissolved in PBS-T, for 2 hours in room temperature was done to reduce unspecific binding of the antibodies. The membranes were washed in PBS-T for 2x30 seconds after blocking, and then incubated in the primary antibody. The primary antibody was added in a solution of 3 % BSA in PBS-T, the primary antibody was used several times (stored in -20 °C). A complete list of all antibodies used presented in `Appendix 5´ (page 85). All these antibodies require overnight incubation in +4 °C with constant motion, then washing (6 x 10 minutes in PBS-T). The secondary antibodies (1 % BSA, PBS-T) were made the same day, at least 15 minutes prior to use. The membranes were incubated for 1 hour in room temperature, and washed (6 x 10 minutes in PBS-T).

3.11.6 Exposure, interpretation

PBS-T buffer from the washing were carefully dried off and ECL (Enhanced Chemiluminescent)-liquid was applied to the membranes for 5 minutes. Membranes were carefully dried and put in a plastic folder on a Teflon plate. ECL detection uses the enzyme Horseradish peroxidase (HRP) which catalyzes the oxidation of luminol by peroxide. Oxidized luminol emits light when returning to its stable state. The HRP enzyme is connected to a secondary antibody; hence the enzyme will only adhere to bands on the membrane where the secondary antibody is attached to a primary antibody. The reaction is called Horseradish peroxidase chemiluminescent reaction.

The protein bands were detected in Fuji las 4000 mini and interpreted (as OD) in the software provided by the manufacturer (Multi Gauge). Data is processed in Windows Excel. The background of each band was subtracted from the OD. Samples from saline treated insulin-incubated muscles were used as comparison for all the other groups; the values of the other samples are calculated as % of these values. This was to be able to make comparisons between the different gels.

4 Results

4.1 Experiment I

Consult `Time-course Experiment I´, page 83 for further information regarding the experiment. The muscles studied in this experiment were the sol and epi muscles, 3 and 2 harvested per rat respectively. The heart and liver were also analyzed.

4.2 Rat and muscle weight

The weight of dex treated rats are lower than the saline treated rats after 5 days treatment with a difference between the groups of ≈20 grams (p<0.04) (Table 6). There were no differences in weight between saline and dex treated rat muscles during this experiment (Table 7).

Table 6: Mean rat weight on the day of experiment, (n=4).

Treatment	Day 1	Day 2	Day 3*	Day 5*
Saline	165.3 ±7.9 g	168.5 ±3.5 g	173.8 ±8.7 g	186.8 ±9.0 g
Dex (1.0 mg/kg)	161.8 ±4.6 g	159.0 ±9.4 g	159.3 ±7.4 g	166.5 ±12.6 g

*Significant weight-difference between the two groups, P <0.05 and <0.04 respectively

Table 7: Mean muscle weight on the day of experiment

Treatment	Muscle	Day 1	Day 2	Day 3	Day 5
Saline	Epi (n=8)	28.4 ± 1.9 mg	28.4 ± 1.6 mg	28.3 ± 1.2 mg	33.9 ± 1.8 mg
	Sol (n=11-12)	29.6 ± 1.9 mg	31.7 ± 1.6 mg	36.8 ±1.4 mg	34.0 ± 1.9 mg
Dex	Epi (n=8)	27.6 ± 1.1 mg	29.7 ± 2.2 mg	27.0 ± 1.8 mg	32.6 ± 1.4 mg
	Sol (n=11-12)	30.7 ± 1.1 mg	31.0 ± 1.5 mg	32.4 ± 1.6 mg	32.9 ± 1.5 mg

No significant difference between saline and dex treated muscles

4.3 Glycogen content – epi and sol

Glycogen contents in epi (**Figure 9**) are higher in dex than saline treated groups on days 2, 3 and 5 (p<0.02). There is no significant difference in glycogen content on day 1.

Dex's effect on glycogen content in skeletal muscles was strengthened as time progressed: glycogen content in epi was approximately 30 % higher in dex versus saline treated rats on day 1, but ≈55 % higher on day 5 (p<0.02).

Glycogen contents in sol (**Figure 10**) are higher in dex than saline treated groups on days 2, 3 and 5 (p<0.03). Glycogen content is not significantly higher in dex compared to saline treated groups on day 1. Glycogen content was ≈10 % higher in dex versus saline treated rats on day 1, but ≈60 % higher on day 5 (p<0.03).
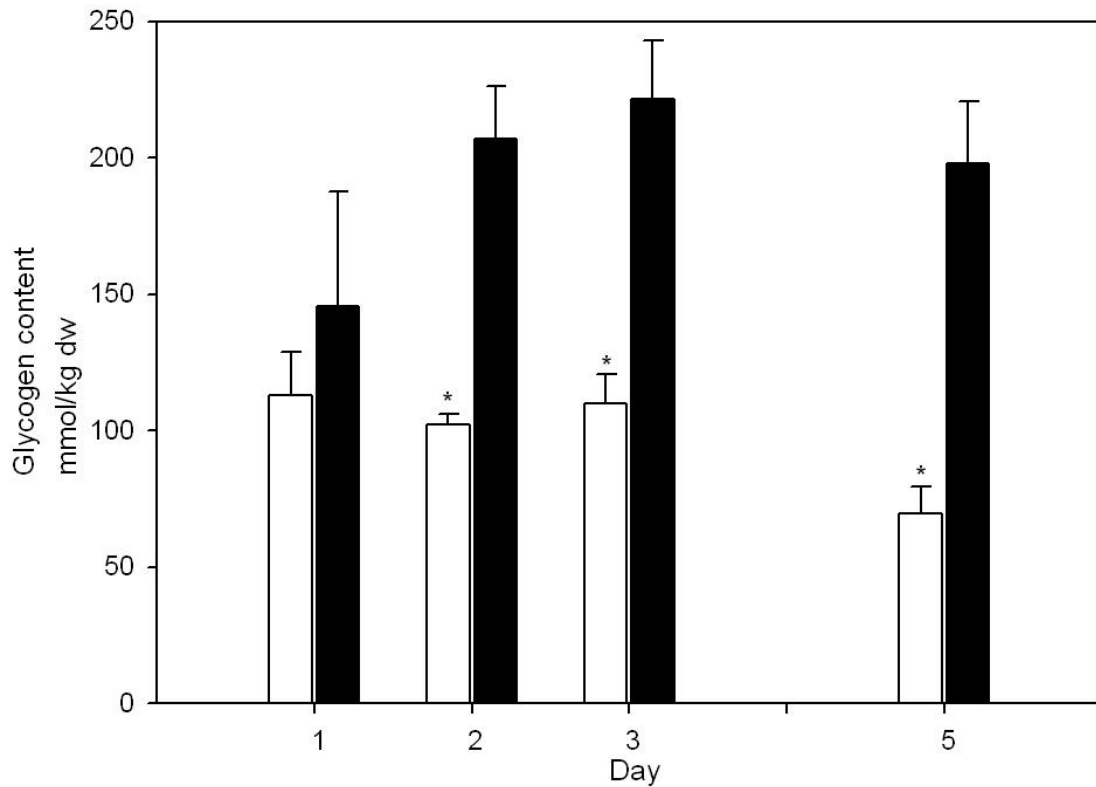
Figure 9: Effect of dex treatment on glycogen content in epi.
Epi from saline and dex treated rats (open and filled bars respectively). Treatment was given for 1-5 days, and rats were fasted 12 hours prior to experiment. Muscles were incubated for 30 minutes without insulin.
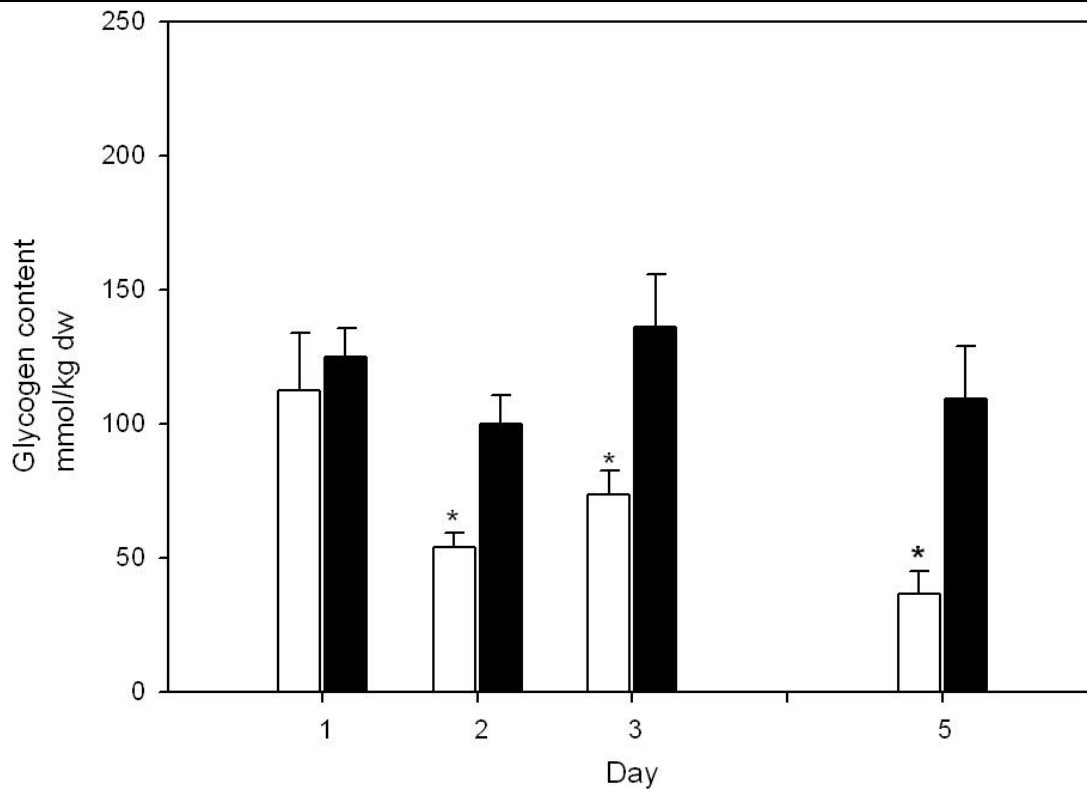Data are presented as means ± SEM, n=4. *$p<0.02$.

Figure 10: Effect of dex treatment on glycogen content in sol.
Sol from saline and dex treated rats (open and filled bars respectively). Treatment was given for 1-5 days, and rats were fasted 12 hours prior to experiment. Muscles were incubated for 30 minutes without insulin.
Data are presented as means ± SEM, n=3-4, *$p<0.03$.

4.4   Glycogen content - liver and heart

Liver from saline treated rats shows nearly complete depletion of glycogen storages after 12 hours fast (average glycogen content ≈25 mmol/kg) (**Figure 11**). Liver glycogen content in dex treated shows no such depletion compared to the saline treated rats (average glycogen content ≈530 mmol/kg). Liver glycogen content in dex treated rats are higher than saline treated rats ($p < 0.03$ on day 1, 2 and 5; $p < 0.06$ on day 2).

Average liver weight is stable in saline treated rats, while it increases in dex treated groups from day 1 to 5. Keep in mind that dex rats weighed approximately 20 g less than control rats on day 5, yet some of the dex treated rats had a liver twice as heavy as the control rats.

The heart glycogen content (**Figure 12**) on day 1 is higher in dex treated compared to saline treated rats ($p < 0.02$), as well as on day 5 ($p < 0.06$). Heart glycogen tended to be higher in dex than saline treated rats on days 2 and 3 as well, though not significantly higher.
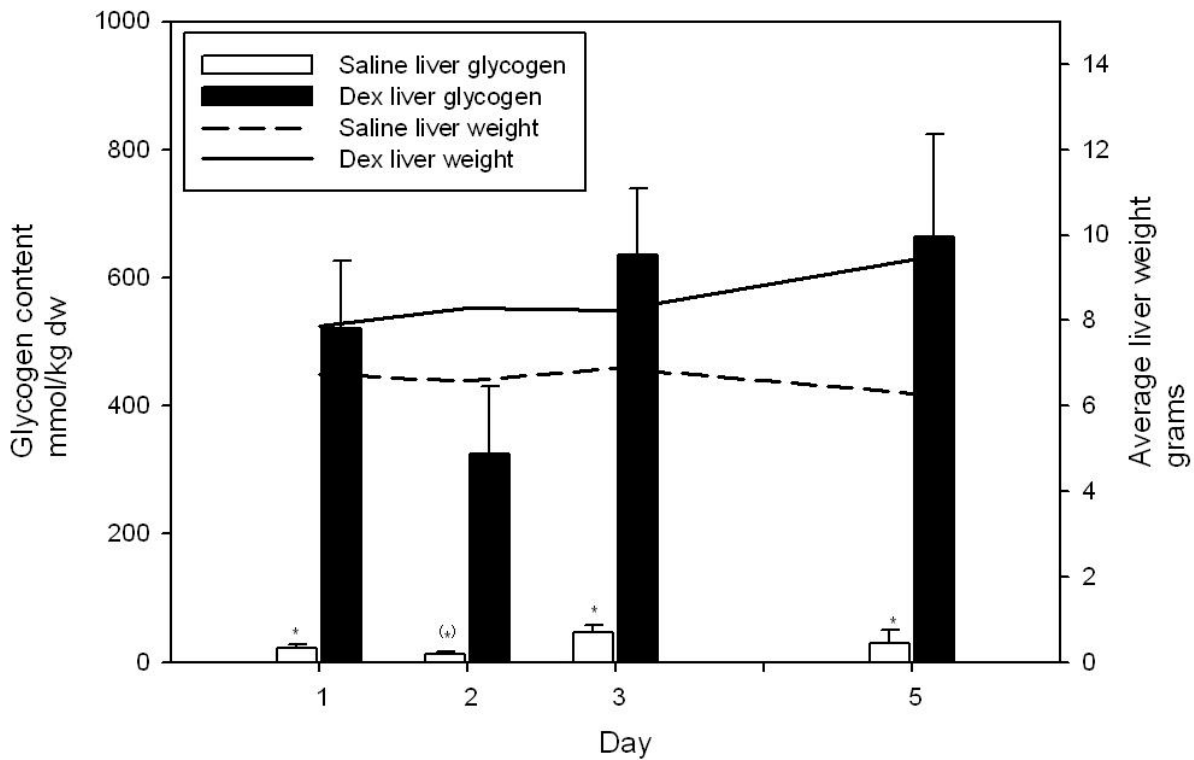
Figure 11: Effect of dex treatment on liver weight and glycogen content.
Liver from saline and dex treated rats. Treatment given for 1-5 days, rats fasted 12 hours prior to experiment. Mean weights of the livers are plotted as lines. Bars are presented as means ± SEM, n=4. *p<0.03, (*)p<0.06.
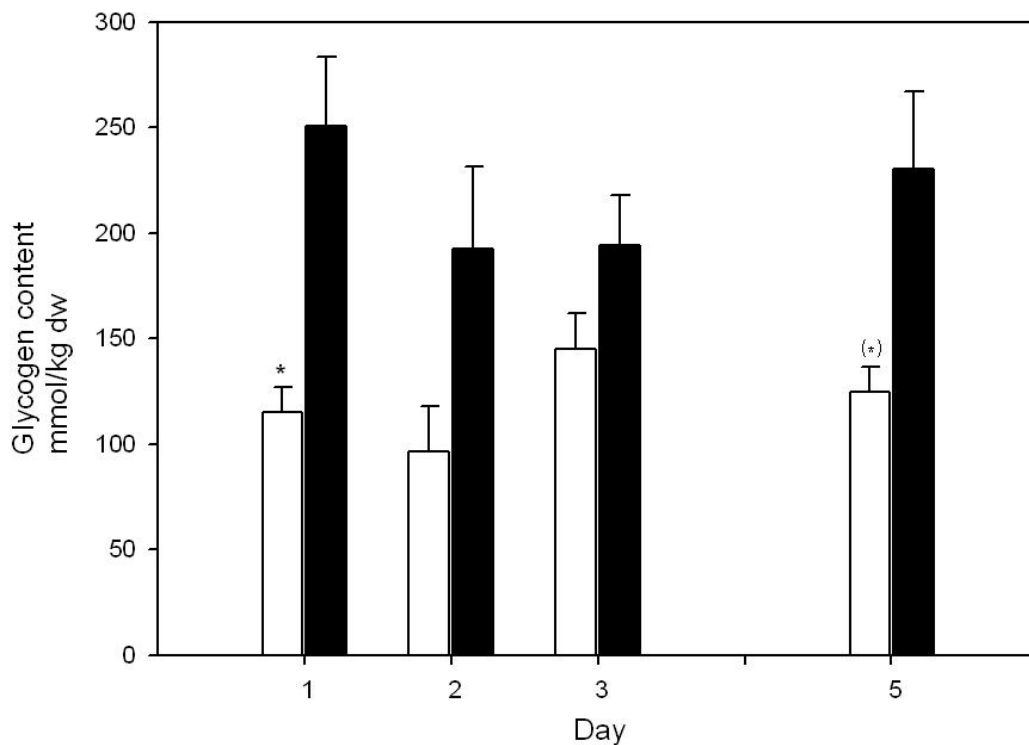

Figure 12: Effect of dex treatment on glycogen content in heart.
Heart from saline and dex treated rats (open and filled bars respectively). Treatment given for 1-5 days, and rats fasted 12 hours prior to experiment. Data are presented as means ± SEM, n=3-4. *p<0.02, (*)p<0.06.

4.5 Western blots

4.5.1 PKB Ser^{473} phosphorylation

The blots show decreased insulin-stimulated phosphorylation of PKB Ser^{473} in dex treated compared to saline treated groups (**Figure 13**, and blots in **Figure 16**). The decrease in dex treated insulin-stimulated phosphorylation at Ser^{473} is ≈25 % on day 5 (p<0.03), and ≈50 % on day 1 (p<0.14). Muscles incubated in basal buffer show a trend of higher basal phosphorylation of PKB Ser^{473} when comparing saline to dex treated groups. The PKB Ser^{473} phosphorylation in all basal groups is less than 15 % of the insulin-stimulated saline treated group.

Figure 13: Effect of dex treatment on PKB Ser^{473} phosphorylation:
Epi from saline and dex treated rats (open and filled bars respectively). Treatment was given for 1 or 5 days, rats were fasted 12 hours prior to experiment. Level of phosphorylation is calculated as % of the insulin-incubated muscles from saline treated rats. Data are presented as means ± SEM, n=3-4 in insulin groups, n=2-3 in basal groups. *p<0.03, (*)p<0.14.

4.5.2 PKB Thr^{308} phosphorylation

The western blots of PKB Thr^{308} phosphorylation (**Figure 14** and blots in **Figure 16**) coincides with the western blots of PKB Ser^{473} phosphorylation (**Figure 13**).

The blots show significantly decreased insulin-stimulated phosphorylation of PKB Thr^{308} in dex treated compared to saline treated rats on both day 1 and day 5 ($p < 0.04$), the reduction from saline-treated muscles being ≈50 and ≈30 % respectively. Muscles incubated in basal buffer show a trend of higher basal PKB Thr^{308} phosphorylation in saline treated compared to dex treated groups, but the phosphorylation in all these groups are less than 15 % of the insulin-stimulated saline treated group.
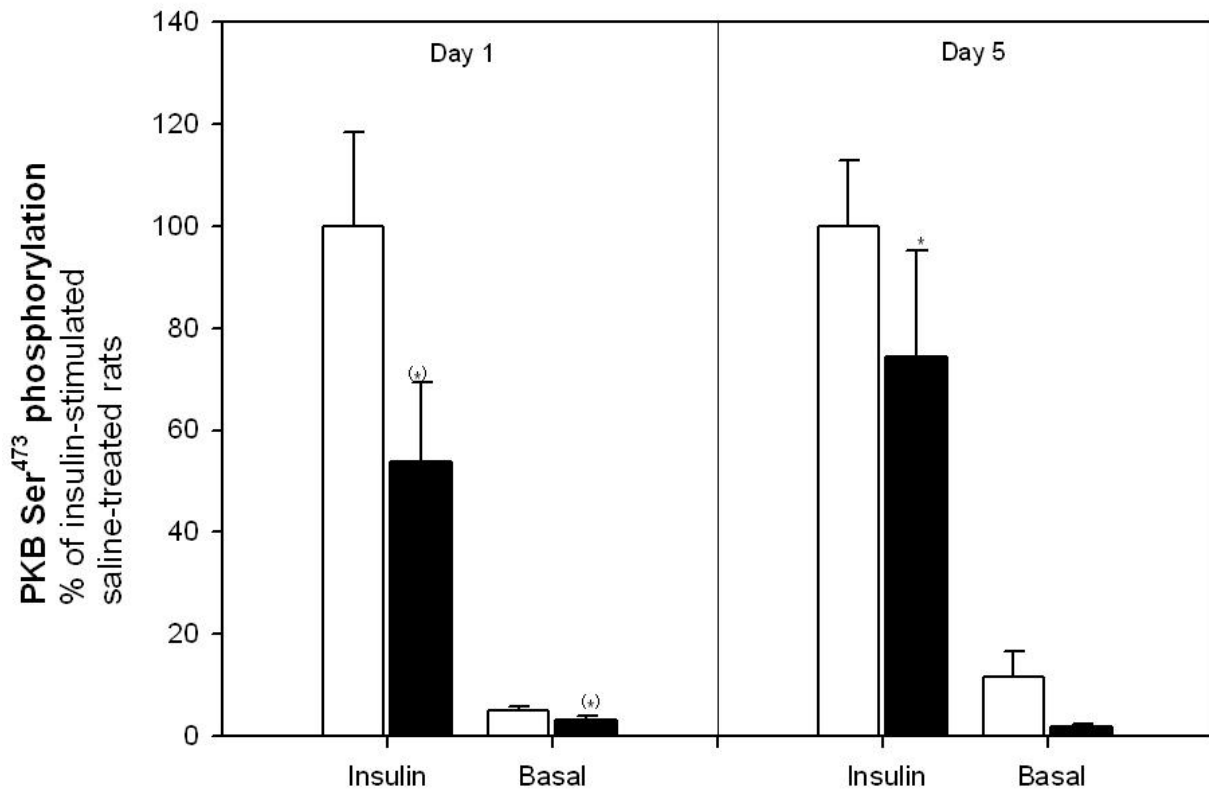

Figure 14: Effect of dex treatment on PKB Thr^{308} phosphorylation:
Epi from saline and dex treated rats (open and filled bars respectively). Treatment was given for 1 or 5 days, rats were fasted 12 hours prior to experiment. Level of phosphorylation is calculated as % of the insulin-incubated muscles from saline treated rats. Data are presented as means ± SEM, n=3-4 in insulin groups, n=2-3 in basal groups. *$p < 0.04$, $^{(*)}p < 0.12$.

4.5.3 GSK-3β Ser^9 phosphorylation

The blots of insulin-stimulated GSK-3β Ser^9 phosphorylation show no significant differences when comparing dex and saline treated groups (**Figure 15** and blots in **Figure 16**). Insulin-stimulated GSK-3β Ser^9 phosphorylation shows a tendency to have decreased from day 1 to day 5 in the dex treated group compared to the saline treated groups, the decrease is ≈ 15 %. Muscles incubated in basal buffer show a trend of higher basal phosphorylation in the saline than the dex treated groups, especially on day 5.
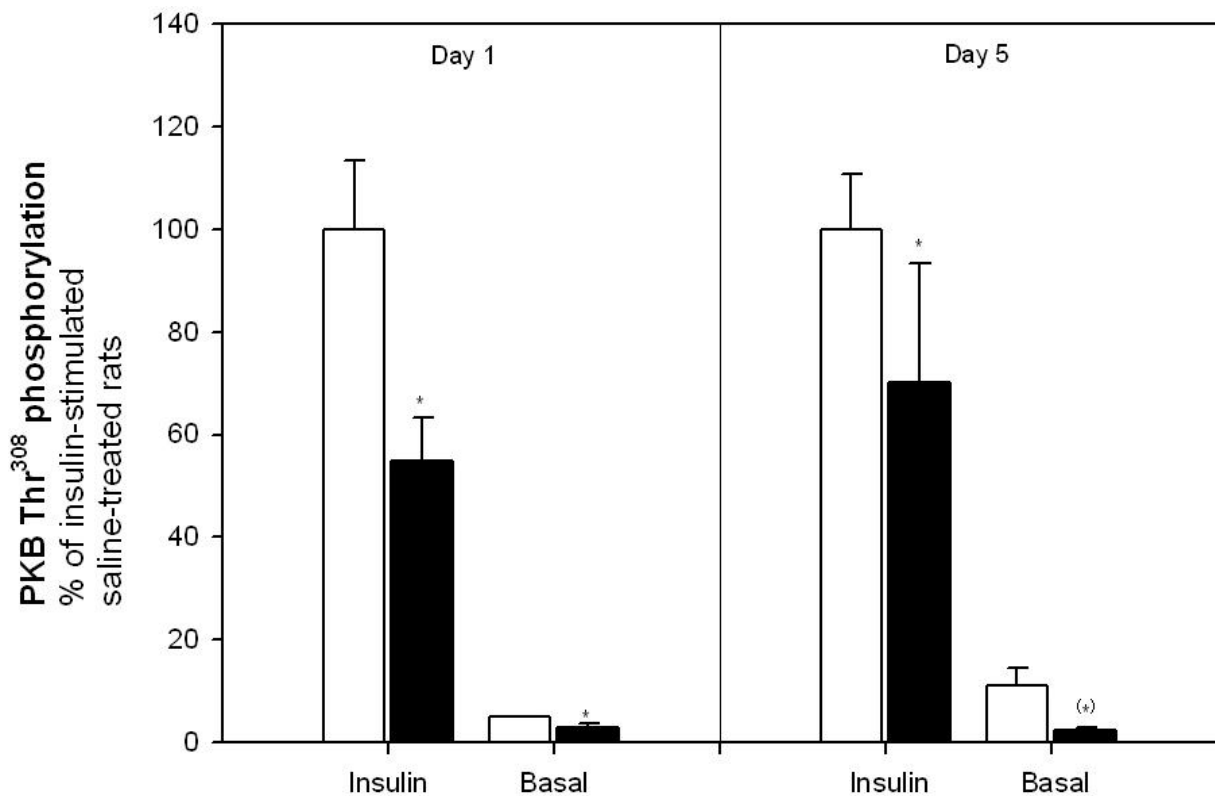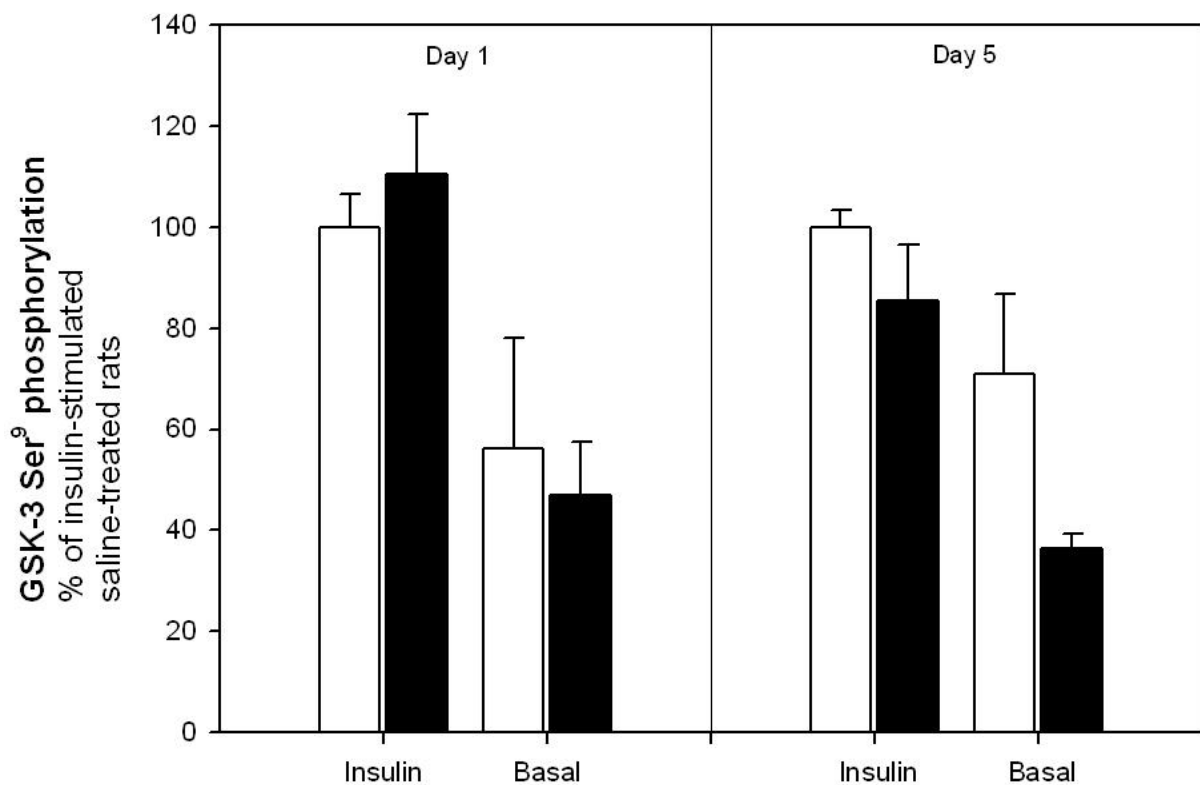

Figure 15: Effect of dex treatment on GSK-3β Ser^9 phosphorylation:
Epi from saline and dex treated rats (open and filled bars respectively). Treatment given for 1 and 5 days, rats fasted 12 hours prior to experiment. Level of phosphorylation is calculated as % of the insulin-incubated muscles from saline treated rats. Data are presented as means ± SEM, n=3-4 in insulin groups, n=2-3 in basal groups

	Day 1				Day5			
Insulin	+	+	-	-	+	+	-	-
Dex	-	+	-	+	-	+	-	+
PKB Ser^{473}								
PKB Thr^{308}								
GSK-3β Ser^9								
GSK Total protein								

Figure 16: Blots showing phosphorylation levels for the PKB Ser^{473}, PKB Thr^{308}, GSK-3 Ser^9 and GSK total protein

4.6   Glucose uptake – epi and sol

Glucose uptake in dex treated epi groups are lower compared to the saline treated groups (**Figure 17**). Dex treated rats had a significantly lower glucose uptake on day 5 compared to saline treated groups when incubated in basal buffer (≈70 % lower, $p<0.05$) but not significant when incubated in supraphysiological insulin buffer (10,000 µU/ml)(≈35 % lower, $p<0.20$).

The dex treated sol show significantly lower basal glucose uptake on day 3 compared to the saline treated group ($p<0.04$) (**Figure 18**). The dex treated group shows no significant difference in basal glucose uptake on day 5, but glucose uptake is ≈35 % lower compared to the saline treated group. Dex treatment showed lower level of insulin-stimulated glucose uptake (200 µU/ml) than saline treatment on days 1, 2 and 5 ($p<0.10$ in all groups, glucose uptake ≈55 % lower on day 5). Dex treatment showed lower level of insulin-stimulated glucose uptake (10,000 µU/ml) than saline treatment on day 5 compared to the saline treated group ($p<0.09$, glucose uptake ≈40 % lower).

Glucose uptake decreases from day to day, even in the saline treated muscles, but the decrease in glucose uptake from day 1 to 5 is only significant in the insulin-stimulated muscles (10,000 µU/ml)(**Table 8**).

Table 8: % change in glucose uptake from day 1 to day5 in the respective groups, p-values

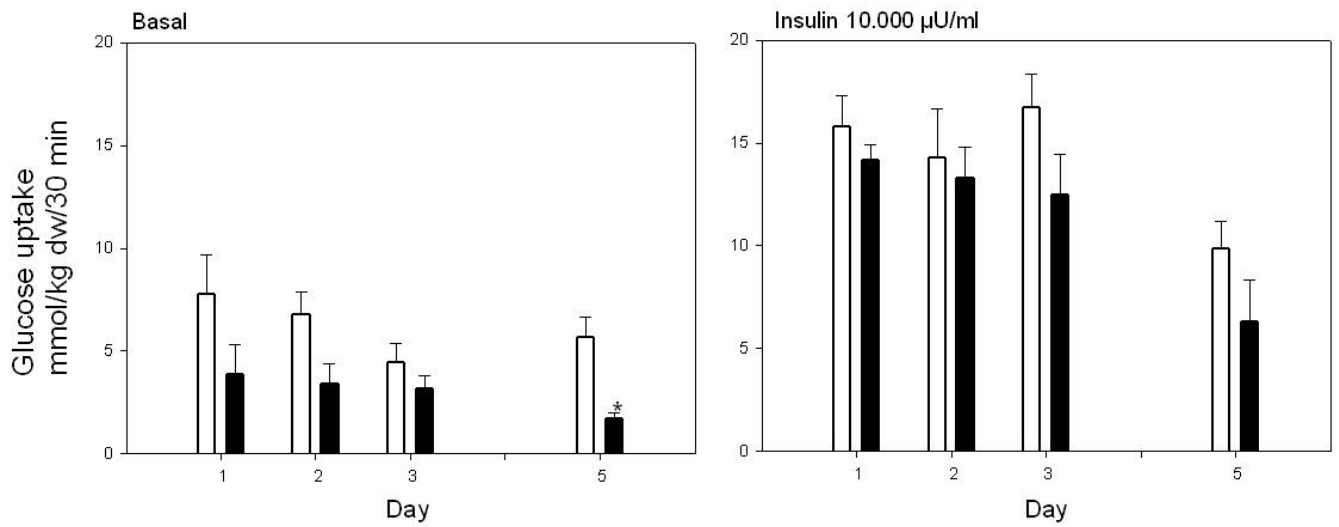| Sol	Basal	Ins 200 µU/ml	Ins 10,000 µU/ml
Saline treated	-50 %,p<0.12	-25 %, p<0.25	-25 %, p<0.28
Dex treated	-50 %, p<0.20	-35 %, p<0.30	**-40 %, p<0.04***
Epi	Basal		Ins 10,000 µU/ml
Saline treated	-25 %, p<0.36		**-40 %, p<0.03***
Dex treated	-55 %, p<0.20		**-55 %, p<0.01***

*p<0.05 = significant

Figure 17: Effect of dex treatment on basal and insulin-stimulated glucose uptake in epi:
Epi from saline and dex treated rats (open and filled bars respectively). Treatment given for 1-5 days, and rats fasted 12 hours prior to experiment. Incubated in basal buffer and insulin buffer (10,000 µU/ml) for 30 minutes. Data are presented as means ± SEM, n=4. *p<0.05.

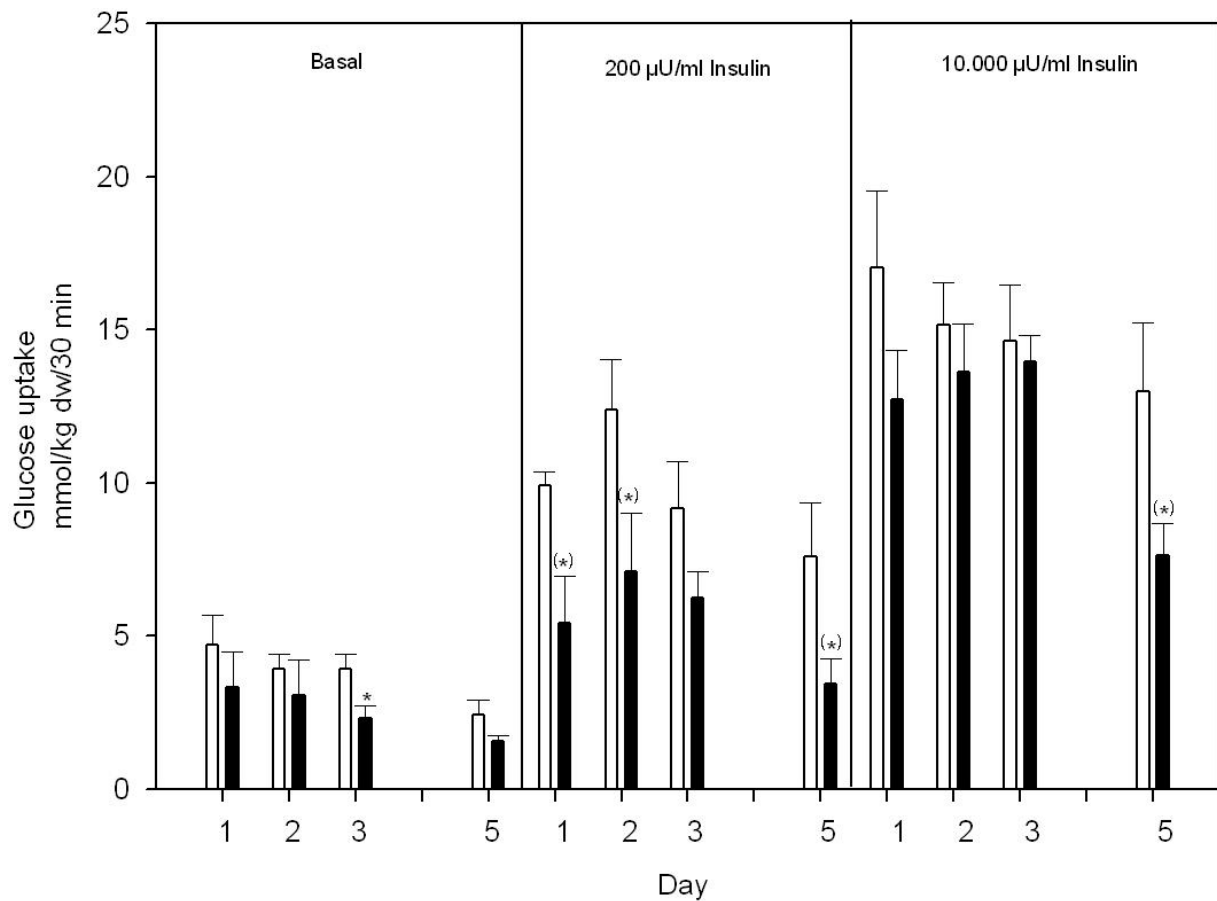**Figure 18: Effect of dex treatment on basal and insulin-stimulated glucose uptake in sol:**
Sol from saline and dex treated rats (open and filled bars respectively). Treatment given for 1-5 days, and rats fasted 12 hours prior to experiment. Incubated in basal buffer, physiological and supraphysiological insulin buffer (200 and 10,000 µU/ml respectively) for 30 minutes. Data are presented as means ± SEM, n=4. *p<0.04, (*)p<0.10.

4.7 Experiment II

Consult the `Time-course Experiment II´, page 84 for further information regarding the specifics of the experiment. Two rEDL and wEDL were harvested per rat respectively, in addition to the liver.

4.8 Rat and muscle weight and ratios

The weight and food intake in the saline and dex treated rats were measured for a period of 14 days, and the rat weight was higher in saline treated than dex treated rats after 1 day of treatment ($p < 0.005$) (**Figure 19**). Saline treated rats gained a mean of 35 ± 1.8 % during the 11-days treatment, dex treated rats lost 15 ± 2.1 % of total body weight in the same period.

Food intake in saline treated rats had a slight increase during the 14 days of measurement from 20 to 25 grams of food consumed daily per rat. Food intake in dex treated rats followed the same pattern as seen in saline treated rats before injection started with a daily food intake of 20 grams but dropped instantly when dex was administered (Figure 19C and D), equaling a ≈40 % drop in food intake on day 1. The `food intake:100 g body weight´ ratio show the sudden drop in food intake due to dex treatment, and then normalization of the dex treated group on day 7 and onwards (**Figure 20**).

Saline treated rats show a ≈40.0 % increase in EDL weight from day 1 to day 11. Dex treated rats on the other hand show a ≈5.5 % decrease in EDL weight from day 1 to 11 (Table 9).

EDL muscle (mg):rat weight (g) ratio is presented for the different groups (Table 10). Ratios between dex and saline treated rats are equal on day 1, and higher in the fasted groups. Ratios in dex treated rats are higher than saline treated rats on day 11, and higher in the fasted groups.

Table 9: Mean EDL muscle weight on the day of experiment (mg) (n=7 rats in each group)

	Fed		Fasted	
	Saline	Dex	Saline	Dex
Day 1	40.58 ± 3.7	33.55 ± 3.9	33.44 ± 1.1	32.63 ± 1.0
Day 11	51.84 ± 0.8	32.56 ±1.6	49.66 ± 1.9	30.06 ± 1.8

Table 10: Mean EDL `muscle (mg):rat weight (g)´ ratios on the day of experiment (n=7 rats in each group)

	Fed		Fasted	
	Saline	Dex	Saline	Dex
Day 1	0.21	0.21	0.22	0.22
Day 11	0.22	0.23	0.23	0.24
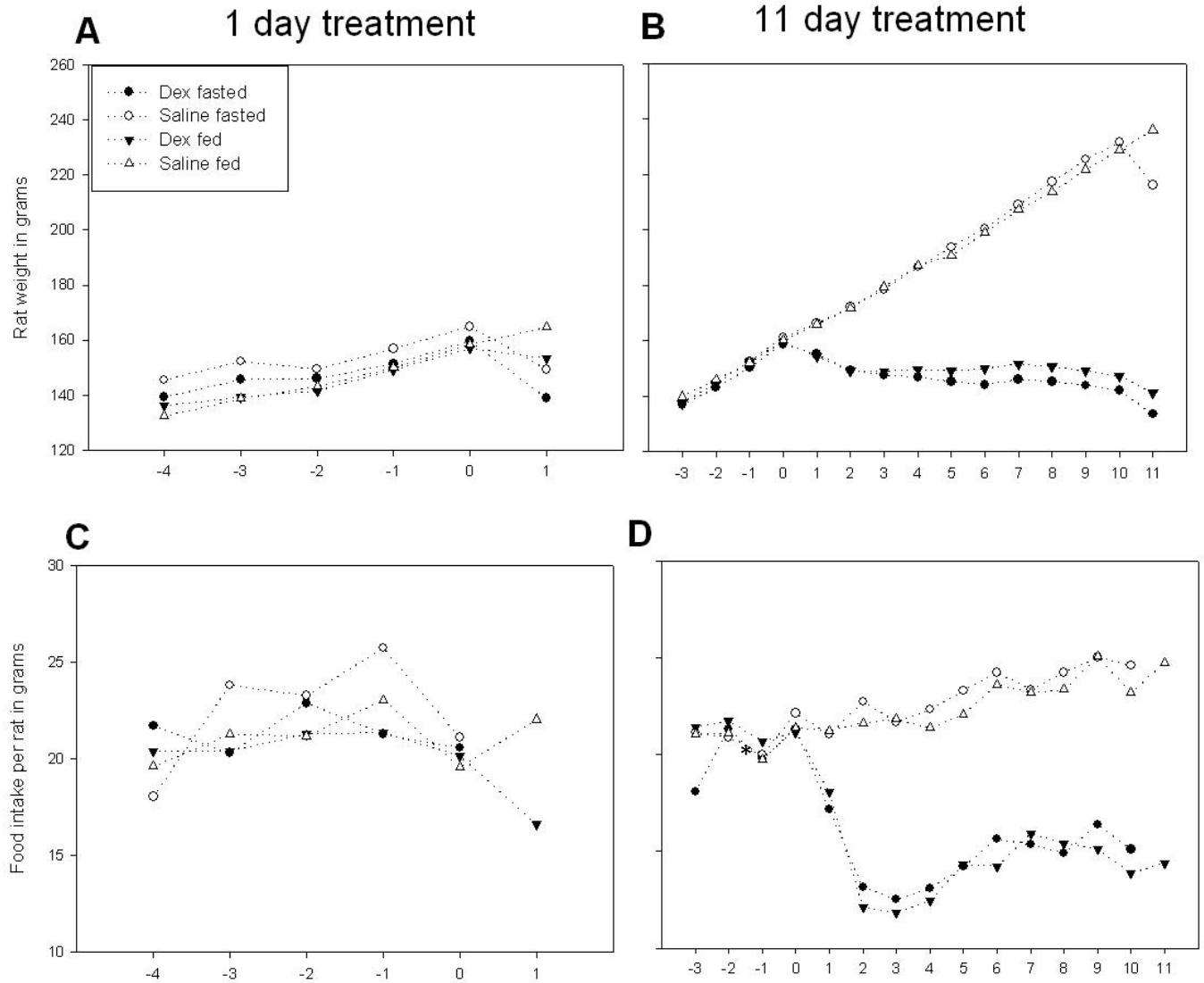
Figure 19: Effect of dex treatment on rat weight and food intake.
Rat weight and food intake in saline and dex treated male Wistar rats. Treatment was given either 1 or 11 days, rats were either fasted or given free access to chow ad libitum for 24 hours prior to experiment. A; Rat weights during 1 day of treatment, injection administered at day 0. B; Rat weights during 11 days of treatment, injection administered at day 0 and onwards. C; Food intake per rat, 1 day of treatment. D; Food intake per rat, 11 days of treatment. *p<0.005 between dex and saline treatment from this day.

Figure 20: Effect of dex treatment on `food intake:100 g body weight´ ratio.
`Food intake:100 g body weight´ ratio in saline and dex treated male Wistar rats. Rats were either fasted or given free access to chow ad libitum for 24 hours prior to experiment. P<0.03 from day 3-6.

4.9 Glycogen content rEDL

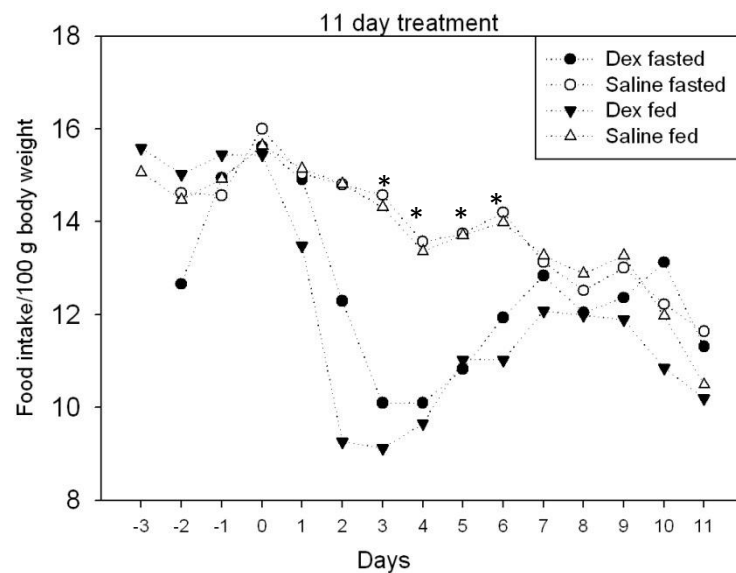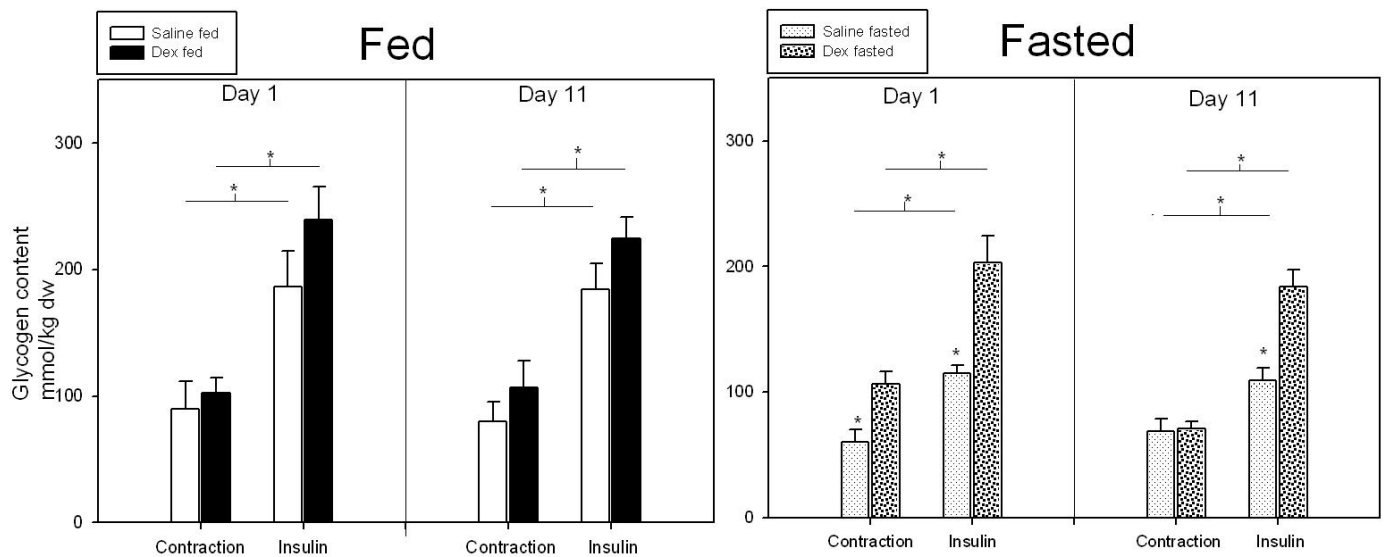**Figure 21: Effect of dex treatment on glycogen content in contracted and insulin-incubated rEDL:**
rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 µU/ml) for 1 hour. Data are means ± SEM, n=7. *p<0.02. Lines indicate significantly higher glycogen content in insulin-incubated than contracted groups *p<0.007.

Fasting decreased glycogen levels compared to the fed status by ≈ 40 % in the insulin-incubated saline treated muscles on both day 1 (p<0.004) and day 11 (p<0.003).

Fasting did not decrease glycogen levels to the same degree in insulin-incubated dex as in saline treated muscles. Fasting decreased glycogen levels in dex treated muscles by 15 % on both day 1 and 11 (non-significant).

Contraction decreased the glycogen contents similar levels (60-100 mmol/kg) in all treatment groups on all days in the rEDL.

Contraction decreased the glycogen content by ≈ 50 % when compared to basal glycogen content in wEDL (**Figure 22**). Glycogen content in insulin-incubated saline and dex treated muscles was not different from basal glycogen content in wEDL.
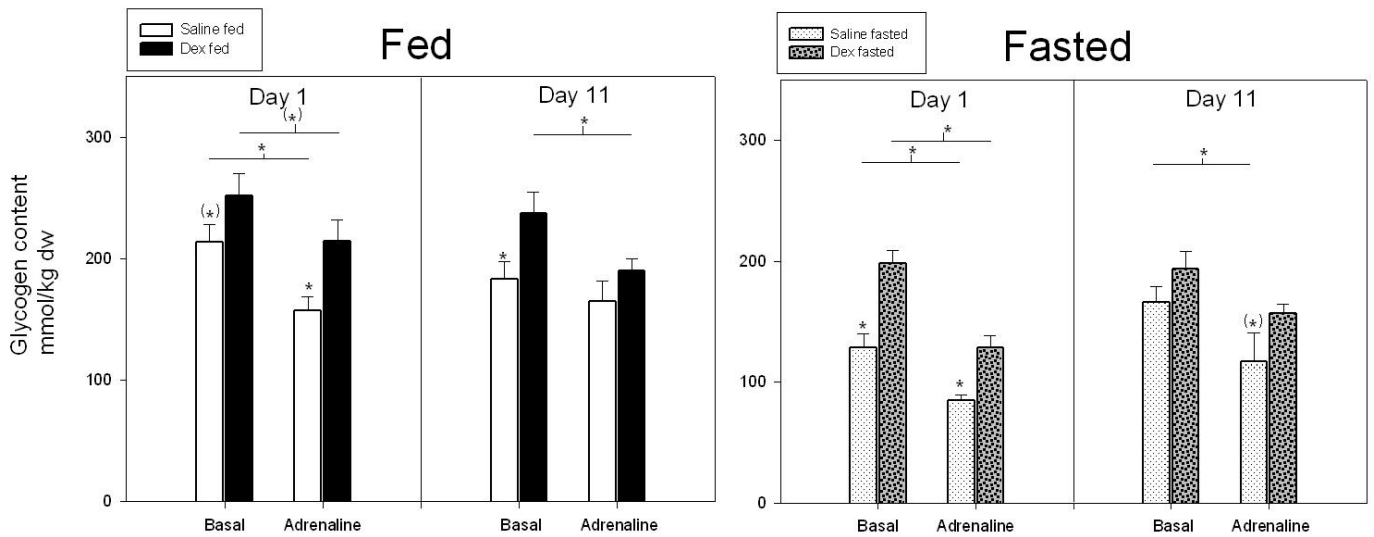
4.10 Glycogen content wEDL

Figure 22: Effect of dex treatment on glycogen content in basal- and adrenaline-incubated wEDL: wEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either incubated in a basal or an adrenaline buffer (10^{-3} mg/ml) for 1 hour. Data are means ± SEM, n=7.*p<0.04, (*)p<0.10. Lines indicate significantly higher glycogen content in basal than adrenaline-incubated groups *p<0.02, (*)p<0.10.

Fed basal glycogen content in wEDL has a mean of ≈200 mmol/kg in the saline treated muscles. Fed basal glycogen content in dex treated muscles had a mean of ≈240 mmol/kg, ≈20 % higher than what was found in saline treated muscles (p<0.10 on day 1, p<0.02 on day 11).

Fasting reduced the basal glycogen content from the fed state; in saline treated muscles the reduction from the fed state was ≈40 % on day 1 (p<0.0005), while only by ≈10 % on day 11 (p<0.4). This indicates less effect of fasting on day 11 than on day 1. In dex treated muscles the reduction from the fed state was ≈20 % on both day 1 (p<0.008) and day 11 (p<0.031).

Adrenaline-incubation markedly decreased glycogen levels from basal levels, this effect was more pronounced on day 1 than day 11 as the lines show (**Figure 22**). Adrenaline led to a mean decrease in glycogen content of 25 % compared to the basal glycogen content across all groups.

4.11 Glycogen content liver
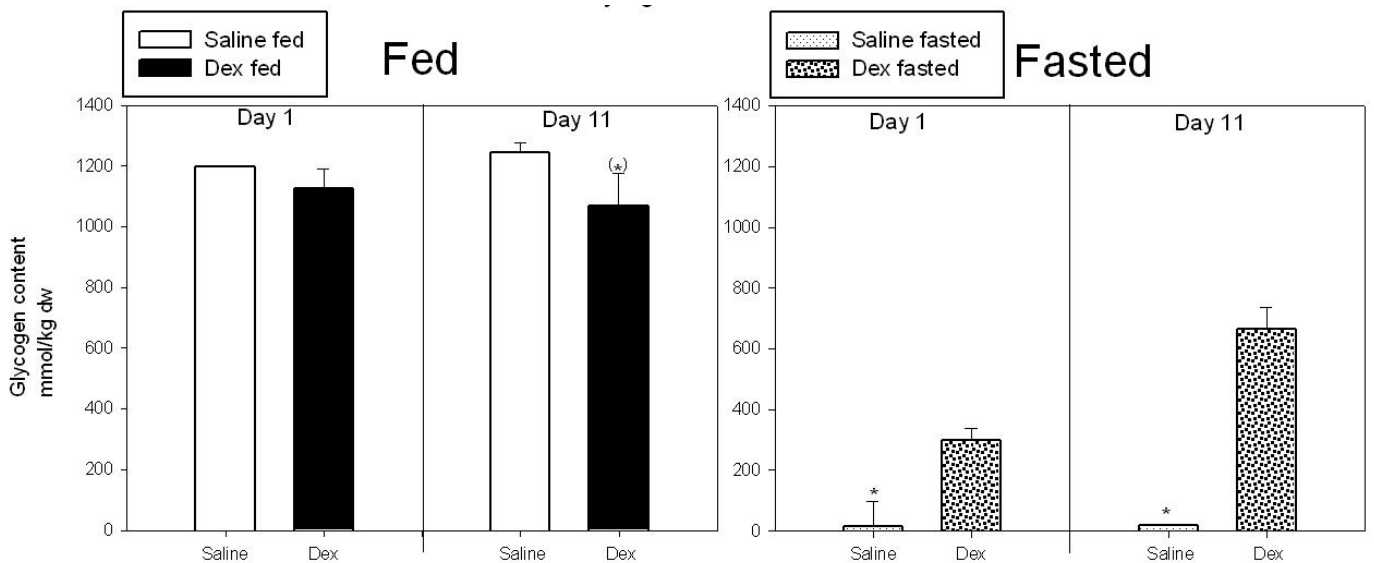
Figure 23: Effect of dex on liver glycogen content.
Liver from saline or dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment of given free access to chow ad libitum. Data are meand ± SEM. *$p<0.0005$, (*)$p<0.12$.

Fed liver glycogen content was similar in dex and saline treated groups. Fed liver glycogen levels were lower in dex than saline treated groups on both day 1 (≈5 % lower) and on day 11 (≈15 % lower, $p<0.12$).

Fasted liver glycogen content in saline treated groups was depleted on both day 1 and day 11 (<20 mmol/kg on both days). Fasted liver glycogen content was higher in dex than saline treated rats on both day 1 (≈300 mmol/kg) and day 11 (≈650 mmol/kg) ($p<0.0005$ on both days).

The difference in liver weight is only different between saline and dex treated rats on day 11 in the fed status ($p<0.001$). Only the livers from saline treated rats increase in weight from day 1 to day 11 ($p<0.03$ in both fed and fasted status). Livers from dex treated rats did not increase in weight from day 1 to day 11.

Table 11: Effect of dex treatment on liver weight (n=7).

	Fed		Fasted	
	Saline	Dex	Saline	Dex
Day 1	8.01 ± 0.2 g	8.46 ± 0.2 g	6.14 ± 0.6 g	6.11 ± 0.4 g
Day 11	11.13 ± 0.2 g	8.49 ± 0.6 g	7.89 ± 0.5 g	7.06 ± 0.4 g

4.12 Lactate rEDL

Figure 24: Effect of dex treatment on lactate release from contracted and insulin-incubated rEDL:
rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 µU/ml) for 1 hour. Data are means ± SEM, n=7. *p<0.006. Lines indicate significantly higher lactate release in contracted than insulin-incubated groups *p<0.001.

Contraction increased lactate release from rEDL 3-fold compared to basal lactate release from wEDL (≈11 in saline, ≈14 mmol/kg in dex treated groups). Contraction-stimulated lactate release from dex treated rEDL was ≈50 mmol/kg, which was higher than saline treated rEDL of ≈30 mmol/kg (p<0.006).

Insulin-stimulated lactate release from rEDL was somewhat similar with basal lactate release in wEDL. Insulin-stimulated lactate release from dex treated rEDL was ≈15 mmol/kg, which was higher than lactate release from saline treated rEDL ≈12 mmol/kg (non-significant).

Fasting decreased contraction-stimulated lactate release by ≈25 % in the saline treated groups on day 1 and 11 (p<0.05 in both groups), and by 15 % in the dex treated groups on day 1 and 11 (p<0.008 on day 1, non-significant on day 11).

Fasting did not alter insulin-stimulated lactate release in any of the groups
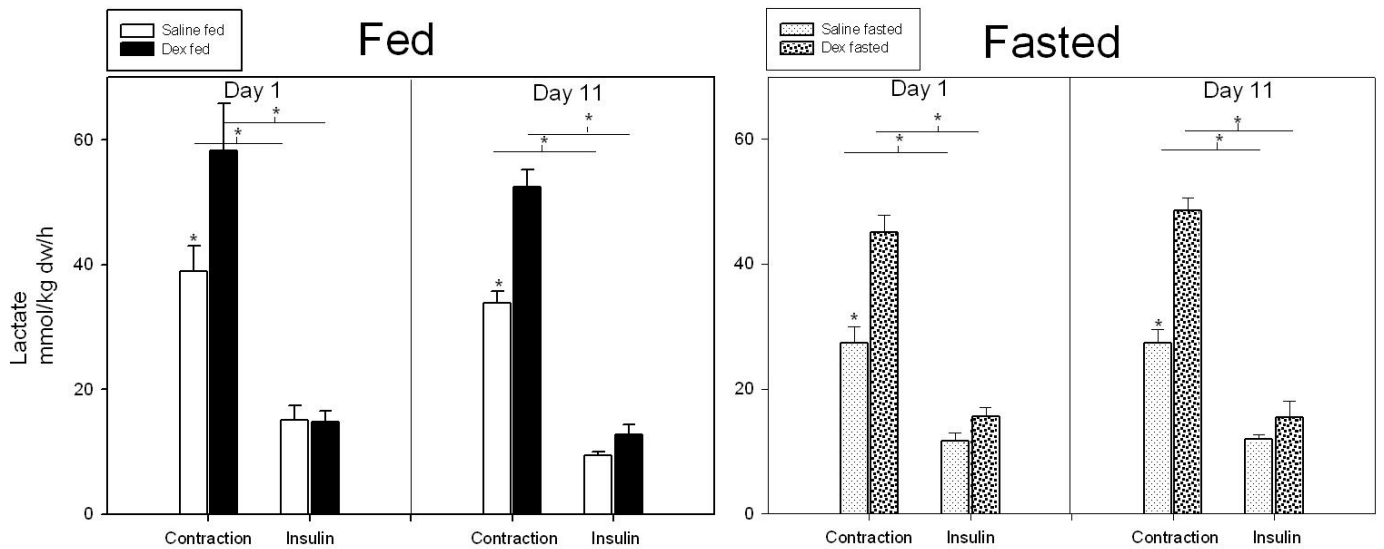
4.13 Lactate wEDL
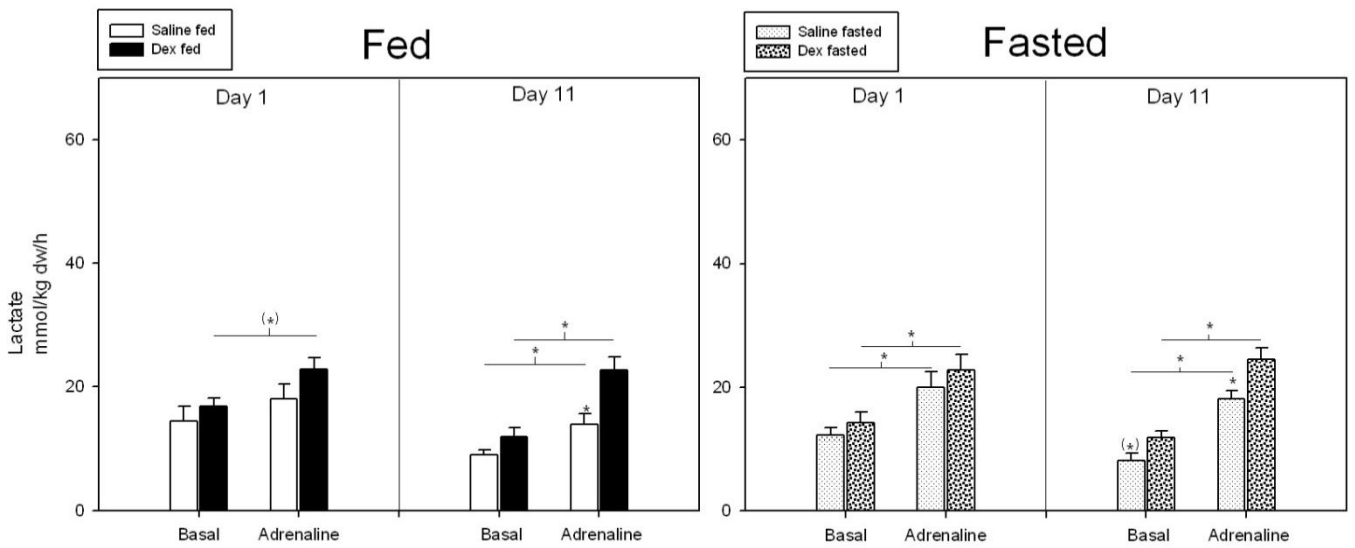
Figure 25: Effect of dex treatment on lactate release in basal- and adrenaline-incubated wEDL:
wEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either incubated in a basal or an adrenaline buffer (10^{-3} mg/ml) for 1 hour. Data are means ± SEM, n=7. *p<0.05, (*)p<0.08. Lines indicate significantly higher lactate release in adrenaline-incubated than basal groups.

Basal lactate release in saline treated groups was ≈11 mmol/kg in saline treated muscles and ≈14 mmol/kg in dex treated groups. Overall dex basal lactate release was ≈20 % higher than in saline treated groups.

Adrenaline increased lactate release from basal lactate release in a stronger manner on day 11 compared to day 1; mean increase across all adrenaline stimulated groups were ≈45 % on day 1 versus ≈95 % increase on day 11. Adrenaline increased lactate release with a mean of ≈70 % across all groups when compared to basal lactate release (p<0.05).

The adrenaline-incubated lactate release was increased by fasting, the mean increase from basal lactate release was almost twice as high when fasted compared to fed; mean increase in fed was ≈50 %, while mean increase in fasted was ≈90 %.

4.14 Western blots

The western blots were conducted in the rEDL muscles; these were isometrically contracted and insulin-incubated. The results are based on 4-7 samples, the insulin-incubated samples were prioritized and results from the insulin-incubated groups are based on 6-7 samples while contracted groups are based on 4-6 samples.

4.14.1 PKB Ser^{473} phosphorylation – rEDL

Figure 26: Effect of dex and fasting on insulin-stimulated PKB Ser^{473} phosphorylation.
rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 µU/ml) for 1 hour. Data are means ± SEM, means are calculated as % of the insulin-stimulated PKB Ser^{473} phosphorylation in the fed saline treated group on day 1. n=4-7 in stimulated, n=6-7 in insulin-incubated groups, *p<0.05, $^{(*)}$p<0.15.

The fed dex treated groups show impaired insulin-stimulated PKB Ser^{473} phosphorylation on day 1 (p<0.05) equaling a ≈40 % decrease compared to the saline treated groups. Impaired insulin-stimulated PKB Ser^{473} phosphorylation in the dex treated muscles is also evident on day 11 equaling ≈30 % decrease compared to saline treated groups (p<0.15).

Fasting increased insulin-stimulated PKB Ser^{473} phosphorylation in all groups compared to the fed groups. Saline treated groups show increased insulin-stimulated PKB Ser^{473} phosphorylation of ≈12 % on day 1 and 11. The fasted dex treated groups show an increase in insulin-stimulated PKB Ser^{473} phosphorylation of ≈40 % on day 1, and ≈5 % on day 11.

Contraction-stimulated PKB Ser^{473} phosphorylation is less than 12 % of the insulin-stimulated saline treated group on day 1.

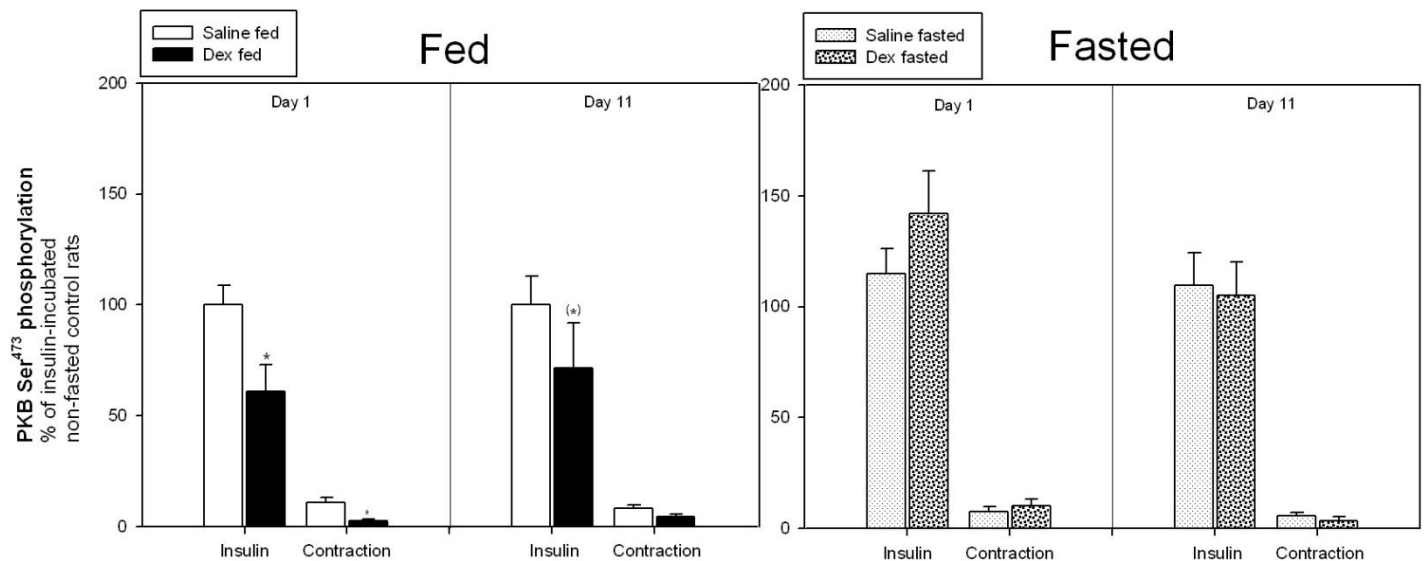## 4.14.2 GSK-3 Ser^9 phosphorylation – rEDL

Figure 27: Effect of dex and fasting on insulin-stimulated GSK-3 Ser^9 phosphorylation.
rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 μU/ml) for 1 hour. Data are means ± SEM, means are calculated as % of the insulin-stimulated GSK-3 Ser^9 phosphorylation in the fed saline treated group on day 1. n=4-7 in stimulated, n=6-7 in insulin-incubated groups, *p<0.02, $^{(*)}$p<0.12. Lines indicate higher levels of phosphorylation in dex treated rats.

The level of insulin-stimulated GSK-3 Ser^9 phosphorylation is similar in saline treated rats on all days, in both fed and fasted groups. Insulin-stimulated GSK Ser^9 phosphorylation is of similar magnitude in dex treated rats on day 1 compared to saline treated rats.

GSK-3 Ser^9 phosphorylation levels in dex treated rats on day 11 (≈15 % in fed and 40 % in the fasted group compared to saline).

Fasting status and treatment does not affect the level of contraction-stimulated GSK-3 Ser^9 phosphorylation on any of the days.

Contraction decreases all GSK-3 Ser^9 phosphorylation to ≈50 – 60 % of the insulin-stimulated phosphorylation in the fed saline treated group on day 1.

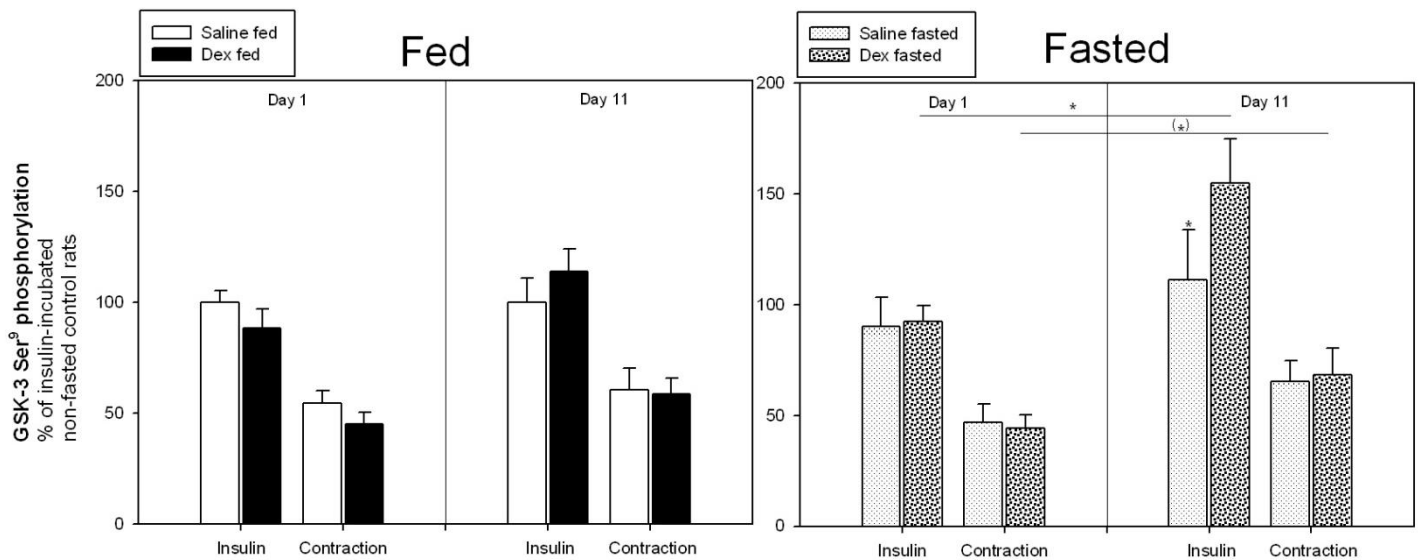### 4.14.3 GS Ser^{641} phosphorylation – rEDL
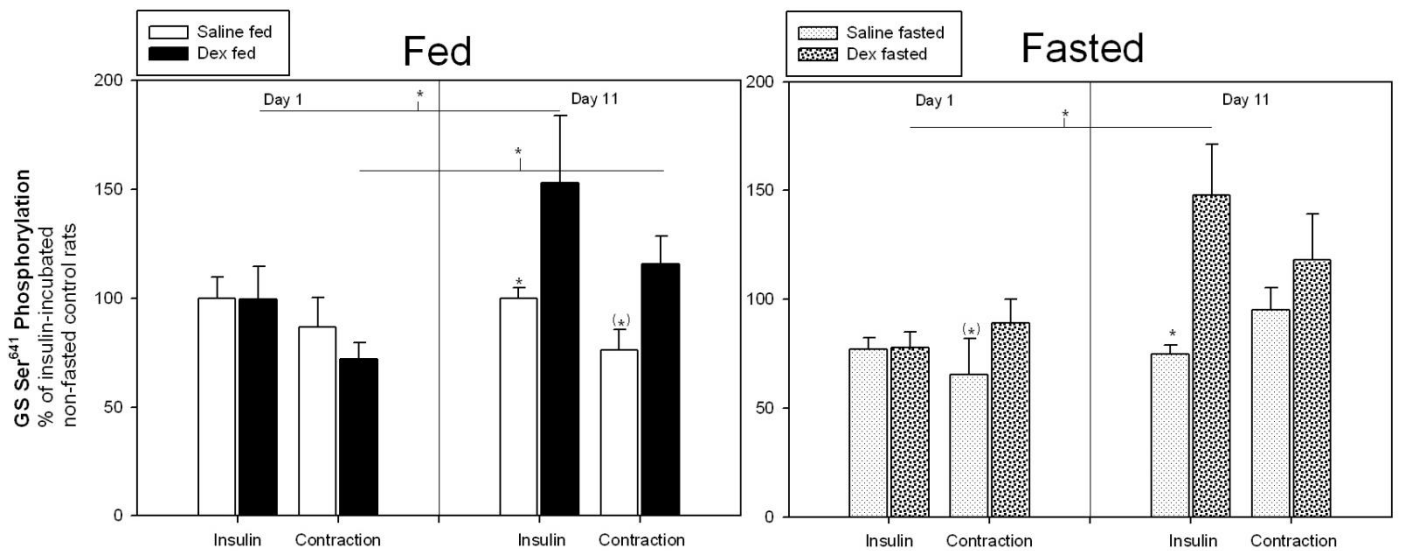
Figure 28: Effect of dex and fasting on insulin-stimulated GS Ser^{641} phosphorylation.
rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 µU/ml) for 1 hour. Data are means ± SEM, means are calculated as % of the insulin-stimulated GS Ser^{641} phosphorylation in the fed saline treated group on day 1. n=4-7 in stimulated, n=6-7 in insulin-incubated groups, *p<0.05, $^{(*)}$p<0.07. Lines indicate higher levels of phosphorylation on day 11 than day 1.

Insulin-stimulated GS Ser^{641} dephosphorylation happen to the same degree in saline and dex treated groups on day 1 regardless of fasting status. However, insulin-stimulated GS Ser^{641} dephosphorylation is impaired in both dex treated groups on day 11. This impairment is evident on day 11 where the magnitude of phosphorylation in the fed dex treated group is ≈50 % higher, while the fasted dex treated group is ≈100 % higher than the saline treated group (p<0.05).

Contraction-stimulated GS Ser^{641} dephosphorylation happen to the same degree in saline and dex treated groups on day 1 in the fed group. However, contraction-stimulated GS Ser^{641} dephosphorylation is impaired in the fasted dex compared to the saline treated group on day 1 (p<0.07). The impairment of contraction-stimulated dephosphorylation is more evident on day 11 than day 1, where the fed dex treated group is ≈50 % higher (p<0.07) and the fasted group is ≈25 % higher than the saline treated group.

4.15 Glucose uptake rEDL

Figure 29: Effect of dex treatment on stimulated and insulin-incubated glucose uptake in rEDL: rEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either isometrically contracted or insulin-incubated (10,000 µU/ml) for 1 hour. Data are means ± SEM, n=7. *p<0.05, †p<0.001. Lines indicate higher contraction-mediated glucose uptake than insulin-mediated in dex treated groups.

Insulin increase glucose uptake 2-3-fold compared to the basal glucose uptake seen in wEDL (≈10 mmol/kg). Contraction increased glucose uptake 3-fold from the basal glucose uptake in wEDL.

Insulin-stimulated glucose uptake in dex treated muscles decreased from day 1 to day 11 by ≈25 % in the fed group (p<0.04) and ≈40 % in the fasted group (p<0.001). Insulin-stimulated glucose uptake also decreased in saline treated muscles from day 1 to day 11 by ≈25 % in the fed group and also by ≈25 % in the fasted group (p<0.02).

Contraction-stimulated glucose uptake in dex treated muscles was slightly increased from day 1 to day 11. Contraction-stimulated glucose uptake in the saline treated muscles decreased from day 1 to day 11 by ≈20-25 % in fed and fasted group (p<0.05).

Fasting did not alter insulin- or contraction-stimulated glucose uptake in any group.
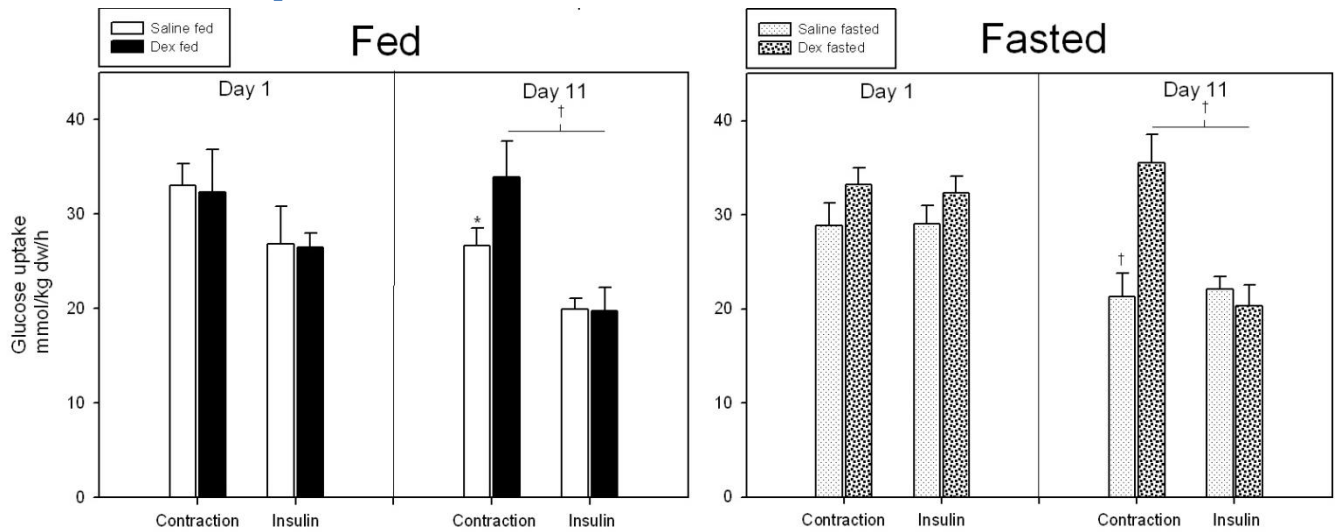
57

4.16 Glucose uptake wEDL
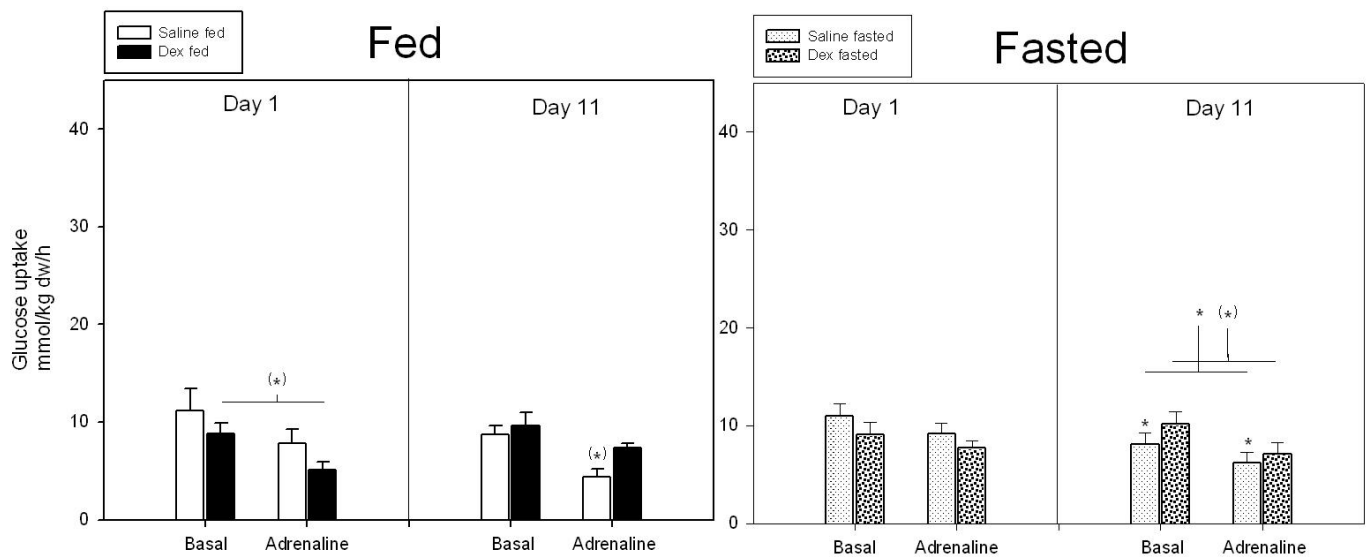
Figure 30: Effect of dex treatment on basal and adrenaline-incubated glucose uptake in wEDL.
wEDL from saline and dex treated male Wistar rats. Treatment was given for either 1 or 11 days, rats were either fasted for 24 hours prior to experiment or given access to chow ad libitum. Muscles are either incubated in a basal or an adrenaline buffer (10^{-3} mg/ml) for 1 hour. Data are means ± SEM, n=7. *p<0.05, $^{(*)}$p<0.09. Lines indicate decreased adrenaline-stimulated glucose uptake than basal glucose uptake.

Basal glucose uptake in wEDL was seen to be ≈10 mmol/kg. Adrenaline decreased this glucose uptake to 4-9 mmol/kg, which were a 40 % reduction in the fed and 20 % reduction in the fasted group compared to basal levels.

Adrenaline-stimulated glucose uptake in dex treated muscles increased in the fed group from day 1 to day 11 by 45 % (p<0.03), while decreased in the fasted group by 8 %. Adrenaline-stimulated glucose uptake in saline treated muscles decreased from day 1 to day 11 in both the fed and fasted groups by ≈35 % (p<0.07).

5 Discussion

5.1 Experiment I

Experiment I was set up to investigate whether 5 days treatment resulted in evidence of insulin resistance, related previous findings at STAMI and by others in 5 to 12-day experiments.

5.2 Rat weight

Dex treatment led to weight retardation

Experiment I show higher body weight in saline than dex treated rats after 3 and 5 days of treatment. It is a well-known effect of GC treatment at high concentration that weight retardation occurs. This weight reduction is discussed in several publications – all showing impaired weight gain in dex treated compared to saline treated rats [26, 34, 36, 40]. The weight increase in saline treated rats might lead to a substantial impairment of insulin sensitivity when compared to smaller dex treated rats; this is discussed by Jensen *et al.* and Burén *et al.* [28, 34]. There is evidence from Experiment I and other experiment at STAMI that epididymal fat pads of dex treated rats weigh more than those from saline treated rats, but are smaller in dex treated rats than saline treated rats when related to rat body weight [34]. Pitombo *et al.* shows that surgical removal of visceral fat (epididymal and perinephric fat pads) abolished diet-induced DM in rats [57], this is also showed by Gabrielly *et al.* [10]. This articles carries strong evidence regarding the effect of extra adipose tissue as a result of the heavier saline treated rats. Results in these publications suggest that weight difference might lead to underestimation of the degree to which dex treatment affects insulin sensitivity [34]. There is, however, a smaller weight difference between the two treatment groups after 5 days treatment compared to what is normally seen after 11 days treatment in other experiments. The smaller weight difference is an advantage when insulin sensitivity is to be measured.

5.3 Glycogen content

5.3.1 Glycogen content in skeletal muscle – epi and sol

Dex treatment led to higher glycogen content in skeletal muscles

Glycogen content was higher in dex treated epi and sol than saline treated muscles on days 2, 3 and 5 (p<0.03). Glycogen content in epi and sol was ≈55-60 % higher in dex than saline

treated rats on day 5. Ruzzin *et al.* found glycogen levels of ≈155 and ≈200 mmol/kg in saline and dex treated epi respectively. The authors also found glycogen levels of ≈116 and ≈160 mmol/kg in saline and dex treated sol respectively, though not during complete fasting; rats had access to 5 g chow in the 18 hours prior to the experiment [36].

The glycogen content in saline treated epi found in Experiment I (≈100 mmol/kg) coincides with publications by Jensen *et al.* where epi glycogen content from fasted rats (12 hours) was ≈100 mmol/kg [25]. Jensen *et al.* and Lai *et al.* have shown glycogen content in epi to be similar after both 24 and 12 hours fasting in multiple publications. All articles show glycogen levels of ≈100 mmol/kg [25, 28-30].

The glycogen content in our saline treated sol in Experiment I (≈ 70 mmol/kg) coincides with findings previously done at STAMI, but to my knowledge no publications with 12 hour fasting are available. Jensen *et al.* refer to glycogen content in sol in a fed state as ≈100 mmol/kg [27], while Aslesen *et al.* showed ≈85 mmol/kg in a fed state [43].

5.3.2 Glycogen content in liver and heart

Dex treatment abolish glycogenolysis induced by fasting in liver

Saline treated rats depleted the liver glycogen storages (mean of ≈30 mmol/kg on days 1 to 5) whereas the dex treated glycogen storages seemed unaffected as a response to 12 hours fasting (mean of ≈540 mmol/kg on days 1 to 5). Ruzzin *et al.* showed comparable figures in liver glycogen content of 182.2 mmol/kg in saline treated versus 569.2 mmol/kg in dex treated rats (1.0 mg dex/kg, 12 days) [36]. The higher liver glycogen content in saline treated rats can be explained by the limited access to food as discussed previously as well as larger rats were used in this experiment (280 g) [36].

Dex treatment led to higher cardiac glycogen content

Dex treatment led to higher cardiac glycogen content than saline treatment on all days (days 1-5). Dex administered one day, then followed by 12 hours fasting, led to higher cardiac glycogen content than in saline treated rats (≈250 versus ≈115 mmol/kg respectively, $p<0.02$). The reason for the non-significant higher cardiac glycogen levels on day 2 and 3 are probably linked to that there were only 3 rats in the dex treated groups on these days. There is a higher concentration of cardiac glycogen content in the dex treated rats on day 5 (≈230 versus ≈125 mmol/kg respectively, $p<0.06$). Qi *et al.* administered dex (1.0 mg/kg) acutely

which after 4 hours led to higher cardiac glycogen levels in dex than in saline treated rats (≈125 versus ≈180 mmol/kg respectively) [50]. This was proven again by the same research team by Puthanveetil *et al* [51]. Ruzzin *et al.* found equal cardiac glycogen content after 12 days of dex treatment, suggesting that the dex-induced elevation in cardiac glycogen content is only seen when dex is administered acutely [36], or that 5 g of chow in the 18 hours prior to the experiment satisfies the energy needs, hence halting glycogenolysis. Qi *et al.* concluded that dex is capable of inducing insulin resistance and switching cardiac glucose disposal from oxidation to storage, likely compromising the energy production in heart – in the span of 4 hours [50].

5.4   Western blots

Dex treatment decreased insulin's ability to phosphorylate PKB Ser^{473} and Thr^{308} from day 1
Western blots for two steps downstream of IR were tested for level of basal and insulin-stimulated phosphorylation in epi muscles. As has been shown earlier [49], blots of PKB Ser^{473} and Thr^{308} phosphorylation was barely detectable in the absence of insulin in both dex and saline treated rats. Blots of insulin stimulated PKB Ser^{473} and Thr^{308} showed severely reduced phosphorylation in dex treated rats at both residues. The reduction in both Ser^{473} and Thr^{308} were ≈50 % on day 1 and ≈25-30 % on day 5. Burén *et al.* report of a dex-mediated decrease in insulin stimulated PKB Ser^{473} and Thr^{308} phosphorylation by ≈40 and ≈ 60 % respectively compared to saline treatment. Burén *et al.* treats the rats for 11 days, but the rats are of similar weight as in Experiment I and are fasted for 12 hours. The coinciding decrease at both residues has also been shown by Ruzzin *et al.* in epi [36], the authors reported a dex-mediated reduction in insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation by ≈40 % from saline treatment.

Experiment I shows a fast onset of dex-mediated impairment of insulin-stimulated phosphorylation at both PKB Ser^{473} and Thr^{308} residues.

Dex treatment decreased insulin-mediated GSK-3β Ser^{9} phosphorylation after 5 days
Phosphorylation of GSK-3 deactivates the enzyme, its single most important task being phosphorylation, hence deactivating the GS. High levels of GSK-3 phosphorylation can be related to activated GS, hence enabling it to synthesize glycogen [34].

Experiment I shows that insulin-stimulated and basal GSK-3β Ser^9 phosphorylation in the dex treated groups decreased from day 1 to day 5. Both insulin-stimulated and basal GSK-3β Ser^9 phosphorylation in the saline treated groups increased from day 1 to day 5. All basal GSK-3β Ser^9 phosphorylation were found to be between 40-60 % of insulin-stimulated phosphorylation level in the saline treated group on day 1.

Burén *et al.* support our findings of decreased GSK-3β Ser^9 phosphorylation in dex treated muscles. The authors suggest this after a similar experiment, but it was conducted for 11 days. Burén shows dex treatment to decrease GSK-3β Ser^9 phosphorylation by ≈20 % compared to saline treatment [34].

Ruzzin *et al.* found decreased insulin-stimulated GSK-3β Ser^9 phosphorylation in dex treated epi compared to saline treated epi (≈20 % decrease) [49] – the authors have previously found matching effects in dex treated sol muscles (≈20 % decrease) [36, 49]. Ruzzin *et al.* found basal GSK-3β phosphorylation levels to be elevated in epi compared to sol (40-50 % versus 10 % respectively), which matches the basal phosphorylation levels seen in Experiment I [49].

There is strong evidence that PKB is a major mediator of insulin-stimulated glucose uptake, and that PKB phosphorylation levels are important supplements to glucose uptake in the investigation of insulin insensitivity, especially when GSK-3 phosphorylation can support this finding. Experiment I indicates that impairment of insulin-stimulated PKB and GSK-3 phosphorylation had taken place after only 5 days. Experiment I also suggests that the dex-mediated impairment of insulin-stimulated PKB phosphorylation has an earlier onset than what is found for GSK-3.

5.5 Glucose uptake

Dex treatment leads to impaired glucose uptake, which is worsening day by day

Experiment I showed a lower insulin-stimulated glucose uptake in the dex treated compared to the saline treated groups. Glucose uptake shows a widening gap between dex and saline treated muscles as the difference becomes larger and larger further into Experiment I. This shows that 5 days dex treatment impairs glucose uptake. The effects are not as evident in all muscle types, however, or at supraphysiological insulin concentrations.

Dex treatment led to lower basal glucose uptake in both epi and sol

The decrease in basal glucose uptake from day 1 to day 5 was ≈35 % in both saline and dex treated epi muscles, and ≈50 % in both saline and dex treated sol muscles. The lower basal glucose uptake in dex treated groups was not expected, as similar experiments have not found lower basal glucose uptake in dex treated versus saline treated groups to this extent [34, 36, 49, 58]. Dimitriadis *et al.* did find this after 5 days dex treatment, however [42]. It must also be noted that the basal glucose uptake found in Experiment I was higher than what has been found in previous experiments at STAMI.

Dex treatment led to lower insulin stimulated glucose uptake in both epi and sol

The decrease in insulin stimulated glucose uptake at supraphysiological concentration from day 1 to day 5 was ≈40 and ≈55 % in saline and dex treated epi respectively. The decrease in insulin stimulated glucose uptake at physiological concentration from day 1 to 5 was ≈25 and ≈35 % in saline and dex treated sol respectively. The decrease in the supraphysiological group was ≈25 and ≈40 % in saline and dex treated sol respectively. The higher reduction in insulin-stimulated glucose uptake in dex than in saline treated muscles from day 1 to 5 suggests an onset of insulin insensitivity.

Data from experiment I is in agreement with other studies showing impaired glucose uptake due to dex treatment during the same time, in a similar manner [34, 36, 49, 58]. Holmäng *et al.* discuss the effect of dex treatment as stepwise, where impairment of the glycogen synthesis system is first impaired in insulin-sensitive muscles by acute dex treatment. The rest of the muscles, as well as an impairment of glucose uptake is, however, evident after two days treatment [59].

The department at STAMI has not previously experimented with 5 days dex treatment, though other research groups have. Dimitriadis *et al.* showed impairment of insulin-mediated glucose uptake in physiological, but not at supraphysiological, insulin levels in sol after 12 hours fasting and 5 days treatment [42]. Dimitriadis *et al.* showed this, though using a higher dose of dex (0.5 mg/rat per day – rats 160-180 g) [42]. This strongly supports the findings in Experiment I that suggests that the impaired insulin sensitivity is correct.

Dex reduces insulin sensitivity in various tissues in the span of 5 days treatment

The impaired glucose uptake and the impaired phosphorylation of important steps within the insulin signaling cascade suggest onset of insulin insensitivity after 5 days of dex treatment. Due to PKB's effect on translocation of GLUT4, decreased phosphorylation of PKB will support the findings that glucose uptake is decreased [36]. This seems to coincide with our experiment, as the dex treated rats had a decreased glucose uptake as well as reduced insulin-stimulated PKB phosphorylation at both Ser^{473} and Thr^{308}.

The lack of significant differences between quite large differences in dex and saline treated rats might indicate too few rats in each group (n=3-4). There is no reason to believe that weight difference between saline and dex treated rats is too large.

5.6 Experiment II

Experiment II was set up to investigate the effects of 11 day dex treatment, and compare this to 1 day of treatment, as well as how fasting status affected the results.

5.7 Rat and muscle weight and ratios

Dex treatment led to acute drop in food intake, body and muscle weight retardation

The weight retardation in the dex treated rats follow the same pattern as seen in Experiment I; though the dex treated rats lost weight in the period from day 1 to 11. At the end of the 11 day period saline treated rats had gained ≈ 35 % while dex treated had lost ≈ 15 % of their own weight on day 1.

We measured food-intake as well in this experiment, and saw a 40 % drop in food intake after the first dex injection, saline treated rats showed no change in food intake. In the dex treated group average food intake plummeted from ≈20-25 grams per rat daily initially to ≈15 and ≈12 on the two days following dex injection. The daily food intake per rat stabilized at ≈15 g per rat from day 3 and onwards. `Food intake:100 g body weight´-ratio showed that dex treated rats ate less from day 3 to 6 after injection while saline treated rats remained on the same ratio level. When related to the `food intake:100 g body weight´-ratio the dex treated rats catched up with the saline treated rats on day 6 and remained similar from this day on. This indicates an acute drop in food intake as a result of dex treatment, then normalization after 6 days. He *et al.* [60] did not report of a sudden drop in food intake, but

rather that dex ate less than saline treated rats (male 15% less, females 45 % less on day 35), but did not show a lower `food intake:100 g body weight´ ratio compared to saline treated rats. He *et al.* used dex administration from a very early age (0.2 mg/kg from 2 days of age) in Sprague Dawley rats in a period from 2 to 120 days of age.

The `muscle weight:rat weight´ differ slightly between the different groups, hence indicating one or both factors to have changed. Ratios between saline and dex treated rats are equal on day 1. Dex treated ratios are 0.01 higher than saline treated in both the fed and fasted group on day 11. This is not a result of the decreased EDL weight, but rather a sign of a higher decrease in body weight than the decrease in muscle weight. EDL weight differ between dex and saline treated rats; the EDL from dex treated rats weighed an average of 31 mg on day 11, compared to the EDL from saline treated rats weighing an average of 50 mg the same day. This might affect the rates of diffusion in to the muscle during incubation; probably affecting some of the measurements (e.g. glucose uptake) as the surface areas are approximately equal in saline and dex treated muscles, though weight and thickness differ. Dimitriadis *et al.* discuss the higher rates of proteolysis in dex treated muscles than saline treated muscles [42], and this might be one of many explanations for the weight decrease.

5.8   Glycogen content

5.8.1   Glycogen content in skeletal muscles

Dex treatment impairs glycogenolysis initiated by fasting compared to saline treatment
Fasting initiate processes that liberate energy, glycogenolysis is one of these, and is said to be one of the most important processes to redistribute energy. As skeletal muscles contain 80 % of the body's carbohydrates, this redistribution is often necessary, lactate from glycogenolysis is thought to play a key role [24].

Fasting decreased glycogen content in all saline treated groups, but to different extent depending on incubation buffer as discussed in the following paragraphs. There is though some general conclusions to be drawn from the fed versus fasted data. Fasting decrease glycogen levels in basal, insulin and adrenaline buffers, glycogen content in contracted muscles on the other hand is not affected by fasting status and is reduced to the same levels as in fed status. Glycogen content in dex treated rats is not decreased to the same degree as glycogen content in saline treated rats.

Coderre *et al.* showed that dex treatment abolished the decrease in glycogen content as a response to fasting, leading to higher glycogen content in dex treated muscles when fasted which support our data [58, 61]. However, our data show higher glycogen content in dex treated rats in the fed status as well, ≈20 % higher on day 1 and ≈30 % higher on day 11 across all groups. The elevated glycogen content in dex treated EDLs are similar to the findings in Experiment I in both epi and sol. Coderre *et al.* showed that glycogen content did not differ between saline and dex treated sol when rats were in a fed state [58, 61]. There is, however, a tendency in Coderre *et al.'s* data of higher glycogen content in all dex treated groups compared to saline treated groups, and might relate to the dosage dex administered (0.4 mg/kg for 14 days). Coderre *et al.* reports that the higher glycogen contents found in dex treated rats possibly is a result of impaired fasting-induced glycogenolysis than glycogen supercompensation [26]. Data from Experiment II, and data from others, suggest a mix of these two as fed dex treated rats also have higher glycogen content than fed saline treated rats. Coderre *et al.* compared one group receiving dex to a group receiving both dex and 10 % sucrose solution and found that glycogen content dropped to the same level during fasting as in saline treated rats. Thus dex may generate various effects on glycogen metabolism depending on physiological conditions [26].

Dex treatment led to higher glycogen content in all groups compared to saline treatment
Dex treated rats had basal glycogen content 23 % higher than the saline treated rats, regardless of length of treatment and fasting status. Experiment II found glycogen content in resting saline treated wEDL to be ≈200 mmol/kg, while dex treated glycogen content was ≈240 mmol/kg. This increased glycogen level in dex treated rats is found in experiments done at STAMI previously [27, 36]. Coderre *et al.* discuss in several publications how dex does not lead to increased glycogen levels in skeletal muscles compared to saline in a fed state [26, 58], however, tendencies in Coderre *et al's* data show higher glycogen levels in dex than saline treated rats in the fed state as well.

Insulin did not prevent glycogenolysis during fasting, nor increase glycogen levels when fed
Insulin incubation did not prevent glycogen breakdown due to fasting in any of the groups, nor did insulin incubation increase glycogen content in any of the rEDL compared to basal glycogen content in the wEDL. The effect of higher glycogen breakdown in saline treated muscles than in dex treated muscles is probably not linked to the anabolic effect of insulin.

This is shown as fed muscles did not increase glycogen content while incubated in insulin compared to basal glycogen levels; thus effect of sustained glycogen levels in fasted dex treated muscles are probably due to impaired glycogenolysis by dex treatment. There is no change in insulin-incubated glycogen content between day 1 and 11. Insulin has previously showed to increase glycogen content slightly in saline treated muscles, this equals ≈9 % increase in epi and ≈6 % in sol [43]. This higher glycogen content is too small to be of significance, though this is supported by **REF** . There are some discrepancies between the methods used, Aslesen *et al.* uses 30 minutes incubation, as well as different muscles.

Dex treatment decreased adrenaline-mediated glycogenolysis

Adrenaline incubation decreased glycogen content to lower levels than basal glycogen content in all groups. Fasting status reduced the adrenaline-mediated glycogenolysis further in saline than dex treated groups. Peters *et al.* found decreased glycogen levels in adrenaline-treated epi, but not sol [62], this is reflected by Jensen *et al.* that found adrenaline stimulated glycogenolysis to be reduced in EDL, but not in soleus [63]. Jensen *et al.* discuss the β_2-adrenoceptor density as twice as high in type I muscles, and the paradox that these muscles are shown to have a lower rate of glycogenolysis when influenced by adrenaline. Aslesen *et al.* found adrenaline-mediated glycogenolysis equaling ≈15 % in epi and ≈20 % reduction from basal glycogen content in the fed state [43].

Contraction reduced glycogen content in dex and saline treated muscles equally

Glycogenolysis as a result of contraction is a well-known effect. Contraction mediates decrease in glycogen levels in Experiment II to the same degree in all muscles, regardless of fasting status and whether dex or saline treatment was given. Even though glycogen content in basal dex treated groups are higher, contraction reduces glycogen contents to the same extent. Contraction also reduces glycogen content to almost equal levels; ≈95 and ≈75 mmol/kg in dex and saline treated muscles respectively. Basal glycogen content in wEDL is roughly two-fold to glycogen content found in contracted groups from rEDL. This reduction in glycogen content due to contraction is verified by Ruzzin *et al.* in sol and epi muscles. The authors show a significantly higher glycogen content in dex than in saline treated rats after contraction. Ruzzin *et al.'s* experiment did not completely fast the rats as discussed earlier, and contracted the muscles for 30 minutes [49]. The fact that contraction was performed for 60 minutes in Experiment II could be the reason for the equal glycogen contents found in

saline and dex treated groups. Ruzzin *et al.* authors further suggest that epi muscles had a similar contraction-mediated glucose uptake in dex and saline treated muscles, yet higher glycogen content in dex treated after contraction (≈50 % higher in dex versus saline treated rats). The effects seen in epi were somewhat different from those found in sol, which showed equal glycogen content in dex and saline treated muscles after contraction [49]. Aslesen *et al.* showed contraction-stimulated glycogen reduction in saline treated rats equaling ≈47 and ≈37 % in epi and sol muscles respectively, the level of decrease is therefore similar to what is found in Experiment II.

5.8.2 Glycogen content in liver

Dex treatment completely abolish the liver glycogenolysis expected when fasted

Experiment I did not compare liver glycogen content in fed rats to fasted rats. Experiment II on the other hand explores the effect of fasting status as well as dex and saline treatment on liver glycogen content. The findings from the fasted rats coincide with the findings from Experiment I; liver glycogen storages are completely depleted in saline treated rats, but sustains elevated in dex treated rats even after a 24 hour fast (p<0.0005). Surprisingly Experiment II found no higher liver glycogen content in the fed state in dex compared to the fed state in saline treated rats. Liver glycogen content in fed dex treated rats was even found to be lower than in fed saline treated rats (borderline significantly lower on day 11, p<0.12). This suggests impairment in fasting mediated glycogenolysis in liver due to dex treatment. Bruce *et al.* found 35 % higher liver glycogen content in obese Zucker rats than in lean Zucker rats [64], and nearly depletion after exercise in the lean phenotype, while only 50 % reduction in liver glycogen in the obese. Glycogenolysis in liver from obese Zucker rats seems impaired compared to the lean phenotype, this further supports the data from Experiment II.

5.9 Lactate release

Dex treatment increased lactate release

Lactate release is an important response to many factors and is crucial in the interchange of energy between tissues [24].

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Dex treatment increased the basal lactate release compared to the saline treated group. This is evident in the adrenaline and contracted muscles as well, and a tendency of slightly increased insulin stimulated lactate release can be seen as well.

Dimitriadis *et al.* used similar methods as in Experiment II to compare lactate release from sol muscle in dex treated rats (0.5 mg/rat daily, rat weight 160 g, for 5 days) [42]. Basal lactate release (1 µU/ml insulin) in the saline treated group was ≈10 mmol/kg, while the dex treated group released ≈ 12 mmol/kg. The insulin-stimulated lactate release (10,000 µU/ml) equaled ≈16 and ≈18 mmol/kg in the saline and dex treated groups respectively. These findings are somewhat similar to what was found in Experiment II, even though there are some discrepancies in methods; the higher dex dose, 12 hour fast, 1mM pyruvate in the buffer and the use of sol. The same research group has previous shown a two-fold increase in insulin-stimulated lactate release (10,000 µU/ml) in saline treated rats compared to basal lactate release (1 µU/ml); ≈15 versus ≈7 mmol/kg respectively) [65].

Bonen *et al.* has measured lactate content in both sol and EDL, and found higher concentrations of lactate in EDL than in sol [66], which support that our findings in EDL really are higher than Dimitriafis *et al.*'s findings in sol.

 Dimitriadis *et al.* found reduced glucose transport and glucose phosphorylation in dex treated rats, and hypothesized that the rate through glycolysis would be decreased as well. This was, however, contradicted by the levels of lactate release that were normal. Dimitriadis *et al.* showed a decrease in the rate of glycogen synthesis and hypothesized that this might have lead to a shift of glucose metabolism towards glycolysis and increased lactate release [42].

Fasting increased the adrenaline mediated lactate release
Adrenaline increased lactate release with a mean of ≈ 70 % across all groups when compared to basal lactate release. Fasting increased the adrenaline-mediated lactate release. This was not seen in any other incubation medium, as basal, insulin and contraction-stimulated lactate release were decreased in fasted groups. Adrenaline is known to stimulate glycogen breakdown, hence increasing lactate release from skeletal muscles [43], adrenaline is, however, known to only increase glycolysis from glycogen, while lactate formation from

external glucose remains unchanged. This is probably through adrenaline-mediated increase in glucose 6-phosphate which blocks hexokinase, thus blocking glucose phosphorylation [43].

Aslesen *et al.* showed that lactate concentration in a muscle incubated in adrenaline in a fed state led to a 2-3-fold increase in sol and epi muscles, insulin increased lactate release by ≈30-40 %, while contraction increased lactate concentration 10-fold [43]. Notice that these results were achieved from muscle tissue and not incubation buffer, hence following different methods than Experiment II. The relative increase is however interesting, at least when considering the fact that most lactate is transported out of the cells and is transferable to our results.

Incremental lactate release has been studied by Leighton *et al.* [67] and Chaliss *et al.* [68]. The method used is based on the incorporation of U-^{14}C-glucose into lactate, the authors uses this as a direct method of measuring glycolysis from glucose uptake in the muscle. Chaliss *et al.* also used methods measuring lactate concentration in muscle tissue.

Contraction increased lactate release

The three-fold contraction-mediated increase in lactate release from basal lactate release is supported by other publications. G. Van Hall discusses the contraction mediated increase in lactate release, however, ^{14}C-lactate uptake in skeletal muscles was increased several-fold, hence no net flow of lactate in and out of the muscles [69]. This support our data of increased glycogenolysis in dex treated rats as levels of lactate release are increased, in concert with initially increased glycogen content in the dex treated group and a possible dex-mediated impairment of GS activation as presented later. G. Van Hall discuss a contraction studies that has seen that light exercise in one hind limb led to increased lactate uptake, while a hind leg in rest led to increased lactate production [69]. Our results does not measure lactate uptake in the incubated muscles, but increased lactate release is definitely evident in our studies.

5.10 Western blots

Dex treatment impaired insulin-stimulated PKB Ser[473] phosphorylation

Contraction-stimulated PKB Ser[473] phosphorylation was low compared to insulin stimulated phosphorylation levels (≈12 %), this coincides with other published articles [36, 49, 52].

Dex treated groups had a lower insulin-stimulated level of PKB Ser^{473} phosphorylation than saline treated groups. This is supported by Experiment I, as well as Burén *et al.* [34], and Ruzzin *et al.* in epi and sol muscles [49]. Burén *et al.* showed that dex treatment impaired insulin-stimulated PKB Ser^{473} phosphorylation both when incubated in insulin at physiological and supraphysiological concentrations.

Fasting seems to increase insulin stimulated levels of PKB Ser^{473} phosphorylation in the dex and saline treated groups, though to a higher degree in dex treated groups. Other publications have shown a more certain decrease in PKB Ser^{473} phosphorylation as a response to fasting, and contradicts the findings in Experiment II. Two separate publications by Lai *et al.* and Jensen *et al.* showed 24 hours fasted saline treated rats to have a two-fold higher level of PKB Ser^{473} phosphorylation versus fed rats [28, 29]. Ruzzin *et al.* showed lower insulin-stimulated PKB phosphorylation in dex than saline treated groups at both Ser^{473} and Thr^{308} residues and supports the impaired insulin-activated PKB phosphorylation in dex treated rats [36, 49].

Dex mediate insulin resistance, this is evident in part through insulin's decreased ability to phosphorylate PKB Ser^{473}.

Experiment II was unable to show that dex treatment impairs insulin-stimulated GSK-3β Ser^9 phosphorylation

Experiment II show equal levels of insulin-stimulated GSK-3β Ser^9 phosphorylation on day 1. However, an increase in the dex treated insulin-stimulated GSK-3β Ser^9 phosphorylation on day 11 compared to saline treated leads to ≈15 % higher levels in the fed, and 40 % higher in the fasted group ($p<0.02$). Burén *et al.* showed that dex decreased insulin-stimulated GSK-3β Ser^9 phosphorylation in dex compared to saline treated rats [34]. Ruzzin *et al.*, however, was not capable to show impaired insulin-stimulated GSK-3β Ser^9 or GSK-3α Ser^{21} phosphorylation in epi, but did so in sol [49]. The results from GSK-3 phosphorylation is found not to coincide with the impaired insulin-stimulated PKB Ser^{473} phosphorylation in epi muscles as presented above [49], and might suggest fiber-type specific differences.

Contraction led to GSK-3β Ser^9 dephosphorylation to same extent in dex as saline treated

Contraction mediated dephosphorylation of GSK-3β Ser^9 to the same extent in both dex and saline treated muscles (≈50-60 % in both dex and saline). This was also found by Ruzzin *et al.*

in epi but not sol, in which the authors concluded that the degree of contraction mediated phosphorylation of GSK-3β Ser[9] is fiber type specific and differed between sol and epi [49]. Epi had a higher level of contraction-mediated GSK-3β Ser[9] phosphorylation at ≈70-80 % of insulin-mediated phosphorylation [49]. Sakamoto *et al.* showed contraction mediated GSK-3β Ser[9] phosphorylation to be ≈80 % of insulin-mediated phosphorylation [70].

Dex treatment led to impairment in insulin-stimulated activation of GS Ser[641]

Dex treatment impaired insulin stimulated GS Ser[641] dephosphorylation on day 11, this was not seen at all in the saline treated rats, or on day 1 of dex treatment. This is similar results as Ruzzin *et al.* published [49], and suggests that insulin is unable to activate GS [28]. Coderre *et al.* discuss the finding that dex treatment was associated with lower GS activity ratio, and concludes that an inhibition of fasting-induced glycogenolysis rather than glycogen supercompensation takes place [26]. This supports the findings in Experiment II of an impairment of insulin stimulated GS Ser[641] activation. Lai *et al.* found that high glycogen increased GS Ser[641] phosphorylation [30]. This might in part be explanation for the findings in Experiment II where dex treated groups had a higher degree of GS Ser[641] phosphorylation, and coinciding higher glycogen content, another interesting finding by Lai *et al* was that contraction on the other hand activates GS independently of glycogen content.

Contraction led to different degree of GS activation between dex and saline treatment

Contracted muscles from saline treated rats showed reduced GS Ser[641] phosphorylation to the equal levels to ≈80 % of the insulin stimulated phosphorylation on day 1. Contracted muscles from dex treated rats on day 1 showed the same level of dephosphorylation compared to saline treated muscles. Contracted muscles from dex treated rats on day 11 on the other hand showed impaired GS Ser[641] dephosphorylation and were ≈50 and ≈25 % higher than the saline treated group in respectively fed and fasted status. Ruzzin *et al.* on the other hand found that contraction led to same levels of GS Ser[641] phosphorylation in both dex and saline treated sol after 12 days treatment [49]. Lai *et al.* showed contraction-mediated dephosphorylation of GS Ser[641] to the same degree as Ruzzin et al. in previous experiments in both fed and fasted saline treated rats [30].

5.11 Glucose uptake

Contraction stimulated glucose uptake to the same extent in dex as saline treated rats

Glucose uptake is regulated by several factors, contraction is one strong mediator to increase glucose uptake. Dex treatment led to similar contraction-stimulated glucose uptake compared to saline treatment on day 1. Dex treated muscles had a higher contraction-stimulated glucose uptake on day 11 than saline treated groups though. Several articles show that contraction leads to normal glucose uptake in otherwise insulin-resistant muscles from dex treated rats [49]. The reason why dex treatment showed significantly higher insulin stimulated glucose uptake on day 11 can theoretically be connected to the mounting of muscles, giving uneven degree of contraction for instance. This is, however, not plausible due to the high number of muscles incubated (n=7) as well as the experience of the one mounting the muscles. However, the weight of the EDLs was higher in the saline treated group and glucose uptake per muscle wet weight will be lower due to a lower `surface:weight´ ratio compared to the smaller dex treated EDLs. There is also the possibility of insulin insensitivity in the saline treated rats as they weigh significantly more than dex treated rats. Other publications, to the best of my knowledge, uses 30 minutes contraction when measuring glucose uptake instead of the 60 minutes used in Experiment II. This will affect glucose uptake. PATHWAY

Insulin stimulated glucose uptake to the same extent in dex as saline treated rats

Dex treatment is known to impair insulin-stimulated glucose uptake in skeletal muscles, and there are presented several reasons for this in literature. Insulin acts mainly on glucose uptake through translocation of the GLUT4 transporters [34, 36, 40, 42] and it is through this insulin-activated pathway one believe dex exerts its effects on glucose uptake. GLUT4 translocation is mediated through both contraction and insulin stimulation. Both hyperglycemia and hyperinsulinemia are seen spontaneous following dex administration, over time this result in insulin resistance – increased glycogen storage is also seen, possibly a response to the increased levels of insulin.

Burén *et al.* describes that dex treatment resulted in lower insulin-stimulated glucose uptake than saline treatment in both sol and epi after 11 days treatment (equaling ≈40 and ≈30 % at physiological and ≈30 and ≈20 % at supraphysiological insulin levels in the respective muscles during 30 minutes of incubation) [34]. Experiment II used 60 minutes of incubation,

this can affect glucose uptake severely. This can be reflected in the insulin-stimulated glucose uptake that is equal in dex and saline treated rats on all days.

Adrenaline led to lower glucose uptake compared to basal glucose uptake

Experiment II show that adrenaline inhibit glucose uptake compared to the basal glucose uptake. A ≈ 40 and ≈ 20 % reduction in glucose uptake is seen from basal levels in the fed and fasted groups respectively. Inhibition of glucose uptake by adrenaline may be caused by reduction of intrinsic activity of the glucose transporter by phosphorylation or indirectly by accumulation of free glucose [25]. Jensen *et al.* argues that adrenaline might inhibit glucose uptake directly; GLUT4 is translocated during insulin stimulation, GLUT4 can however be phosphorylated by β–adrenergic receptor stimulation [25]. The decrease in glucose uptake described by Jensen *et al.* may then be explained by loss of intrinsic activity of the GLUT4 transporter.

Dex failed to show impaired insulin-stimulated glucose uptake, which does not orchestrate the impaired insulin-mediated modulation of the insulin signaling pathway

Glucose uptake is not altered by dex treatment for 11 days in Experiment II, though impairment of insulin actions in the insulin pathway downstream of IR is obvious. Dex, as a steroid hormone affecting nuclear receptors, induces changes in protein expression. These processes develops over time and might be the reason for the various findings concerning expression of GS, PKB α/β, GSK-3 α/β and GLUT4 in dex compared to saline treated rats. Ruzzin *et al.* did not find any differences in expression of GS, PKB α/β, GSK-3 α/β between saline and dex treated rats, nor in GLUT4 expression in sol, but higher in dex treated epi [36]. The results of unaltered GLUT4 expression in sol has been shown earlier [42], but is contradicted by Coderre *et al.* [40]. Burén *et al.*(2008) contradicts the latter experiment when reporting a reduction of PKB expression in dex treated sol and epi (≈ 50 and 40 % reduction compared to saline treated muscles), but supports the increase in GLUT4 expression in both sol and epi that match the publication by Ruzzin *et al.*(≈ 30 and 70 % increase compared to saline treated muscles) [34]. Burén *et al.* (2002) showed, contradictory to the findings in 2008, that dex decreased PI 3-K and PKB content in rat adipocytes after long-term treatment [41]. These contradictory findings make certain conclusions regarding changes in expression downstream of IR as a response to dex treatment difficult. The higher GLUT4 expression as a response to dex treatment is ascertained, but seems odd compared

to the lower glucose uptake in the dex treated muscles. The paradox can be explained by the atrophy experienced by dex treatment, hence concentrating the proteins in the muscle as Ruzzin *et al.* discusses [36]. The muscle atrophy seen in Experiment II is, however, of bigger magnitude than is seen in other studies. *Et al.* report of reduction in dex treated skeletal muscle weight equaling 10 % of saline treated muscle weight.

6 Conclusions

6.1 Experiment I

1) Glycogen content is notably higher in dex treated tissues compared to saline treated tissues after a 12 hour fast
   a. Glycogen content in skeletal muscle was escalated in all dex treated groups
   b. Liver glycogen was nearly depleted in saline treated rats, and was significantly higher in dex treated rats after only 1 day treatment. This effect carries through the 5 days of treatment
   c. Cardiac muscle glycogen content was higher in all dex treated groups on all days, but more evident when acutely administered
2) Dex treatment reduces PKB phosphorylation at both site Ser^{473} and Thr^{308} notably compared to saline treatment
3) Glucose uptake in dex treated rats show impaired insulin-stimulated glucose uptake compared to saline treated rats. This tendency is more evident in sol when incubated in insulin at physiological levels.

Experiment I did show tendencies that dex induced insulin-resistance during 5 days treatment to a similar degree to what is found during longer treatment.

6.2   Experiment II

1) Dex treatment induced both body and muscle weight retardation, and led to an instant cut in food intake that normalized after some days of dex treatment

2) Glycogen content in dex treated rats was elevated in all groups compared to saline treated rats
   a. Glycogen content in dex treated rats was elevated in all groups compared to saline treated rats, neither adrenaline nor contraction managed to decrease glycogen content in dex treated muscles to the same extent as in saline treated muscles. Insulin-mediated glycogen increase was not seen in any of the muscles.
   b. Dex treatment impaired utilization of stored glycogen in liver when subjected to fast on both day 1 and 11 compared to saline treated rats, even though the liver glycogen content between saline and dex treated rats were equal in the fed state.

3) Dex treatment increased lactate release in all muscles, in all incubation mediums. Neither adrenaline, insulin nor contraction managed to increase lactate release in saline treated muscles to similar levels as lactate release in dex treated muscles.

4) Dex treatment significantly impaired insulin-stimulated PKB Ser^{473} phosphorylation in the fed status, however not in the fasted status. Dex treatment was not shown to affect insulin-stimulated GSK-3 Ser^{9} phosphorylation, except from on day 11 in the fasted rats, in which dex increased insulin-stimulated GSK-3 Ser^{9} phosphorylation. Dex increased phosphorylation of both insulin- and contraction-stimulated GS Ser^{641} in all groups, but only significantly on day 11.

5) Dex treatment failed to show impaired glucose uptake compared to saline treated rats. Also when mediators promoting glucose uptake were added; insulin, contraction and adrenaline.

6.2   Experiment II

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Appendices

Appendix 1 – WHO Summary of Technical Report and Recommendations [3]

Since 1965 the World Health Organization (WHO) has published guidelines for the diagnosis and classification of diabetes. These were last reviewed in 1998 and were published as the guidelines for the Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Since then more information relevant to the diagnosis of diabetes has become available. In November 2005 a joint WHO and International Diabetes Federation (IDF) Technical Advisory Group met in Geneva to review and update the current WHO guidelines.

After consideration of available data and recent recommendations made by other organisations, the Group made the following recommendations:

Recommendation 1

The current WHO diagnostic criteria for diabetes should be maintained – fasting plasma glucose ≥ 7.0mmol/l (126mg/dl) or 2–h plasma glucose ≥ 11.1mmol/l (200mg/dl).

Despite the limitations with the data from which the diagnostic criteria for diabetes are derived, the current criteria distinguish a group with significantly increased premature mortality and increased risk of microvascular and cardiovascular complications.

Recommendation 2

Since there are insufficient data to accurately define normal glucose levels, the term 'normoglycaemia' should be used for glucose levels associated with low risk of developing diabetes or cardiovascular disease, that is levels below those used to define intermediate hyperglycaemia.

Recommendation 3

The current WHO definition for Impaired Glucose Tolerance (IGT) should be maintained for the present.

Consideration should be given to replacing this category of intermediate hyperglycaemia by an overall risk assessment for diabetes, cardiovascular disease, or both, which includes a measure of glucose as a continuous variable.

Recommendation 4

The fasting plasma glucose cut-point for Impaired Fasting Glucose (IFG) should remain at 6.1mmol/l.

This decision was based on concerns about the significant increase in IFG prevalence which would occur with lowering the cut-point and the impact on individuals and health systems. There is a lack of evidence of any benefit in terms of reducing adverse outcomes or progression to diabetes and people identified by a lower cut-point eg 5.6mmol/l (100mg/dl) have a more favourable cardiovascular risk profile and only half the risk of developing diabetes compared with those above the current WHO cutpoint. Lowering the cut-point would increase the proportion of people with IGT who also have IFG but decreases the proportion of people with IFG who also have IGT.

Consideration should be given to replacing this category of intermediate hyperglycemia by an overall risk assessment for diabetes, cardiovascular disease, or both, which includes a measure of glucose as a continuous variable.

Recommendation 5

1. Venous plasma glucose should be the standard method for measuring and reporting glucose concentrations in blood. However in recognition of the widespread use of capillary sampling, especially in under-resourced countries, conversion values for capillary plasma glucose are provided for post-load glucose values. Fasting values for venous and capillary plasma glucose are identical.

2. Glucose should be measured immediately after collection by near-patient testing, or if a blood sample is collected, plasma should be immediately separated, or the sample should be collected into a container with glycolytic inhibitors and placed in ice-water until separated prior to analysis.

Recommendation 6

The oral glucose tolerance test (OGTT) should be retained as a diagnostic test for the following reasons:

- fasting plasma glucose alone fails to diagnose approximately 30% of cases of previously undiagnosed diabetes,
- fan OGTT is the only means of identifying people with IGT,
- fan OGTT is frequently needed to confirm or exclude an abnormality of glucose tolerance in asymptomatic people.

An OGTT should be used in individuals with fasting plasma glucose 6.1–6.9mmol/l (110–125mg/dl) to determine glucose tolerance status.

Recommendation 7

Currently HbA1c is not considered a suitable diagnostic test for diabetes or intermediate hyperglycemia.

The following Table summarises the 2006 WHO recommendations for the diagnostic criteria for diabetes and intermediate hyperglycemia.

Diabetes	
Fasting plasma glucose	≥7.0mmol/l (126mg/dl)
2–h plasma glucose*	**or**
	≥11.1mmol/l (200mg/dl)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose	<7.0mmol/l (126mg/dl)
2–h plasma glucose*	**and**
	≥7.8 and <11.1mmol/l
	(140mg/dl and 200mg/dl)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose	6.1 to 6.9mmol/l
2–h plasma glucose*	(110mg/dl to 125mg/dl)
	and (if measured)
	<7.8mmol/l (140mg/dl)

* Venous plasma glucose 2–h after ingestion of 75g oral glucose load

* If 2–h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

Appendix 2 – Time-course experiment I

Time-course for dexamethasone-induced insulin resistance: glucose uptake.

Jørgen Jensen, Ada Ingvaldsen, Fang-Chin Lin, Anders Rasmushaugen; 2008-07-02

Experiment: 3.-8. August 2008

32 rats of 90-100 g will arrive Wednesday 23. of July
32 rats (16 dex and 16 control). Rats live 2 per cage.
Weigh rats and food intake every day at 10 AM

Rat weight when dex injection is started ~180 g.
Dex: I.p. injection (1.0 mg/kg)
Control: I.p. injection of saline (1.0 ml/kg)

Injection of dex between 10-11 h from Sunday 3.8 to Thursday 7.8.
Dex: 0.1 mg/ml saline. Inject 1 ml/100g rat.
(Dexamethasone solubility: 10 mg/100 ml (0.1 mg/ml) 25 ºC.)
Make new solution every 2nd day (min. 10 ml daily). Store in refrigerator

Experiments:

Monday 4.8., Tuesday 5.8., Wednesday 6.8. and Friday 8.8. Rats are fasted 12 h before experiment (from 22.00 h)
Each day: 4 dex and 4 saline treated rats.

Insulin-stimulated glucose uptake measured in epitrochlearis (0 and 10 mU/ml incubation)
Insulin-stimulated glucose uptake measured in soleus muscles (0, 0.02 and 10 mU/ml incubation)
Blood samples for analysis of insulin and cytokines.
(⅓ muscle for glucose uptake and glycogen content, ⅓ for Western Blot and ⅓ for glycogen synthase activity).

Weigh epididymal fat and heart.

Freeze soleus and epi, liver, heart, epididymal fat.

Appendix 3 – Time-course experiment II

Time-course for dexamethasone-induced insulin resistance: glucose uptake.
Jørgen Jensen, Anders Rasmushaugen, Ada Ingvaldsen, Jorid Thrane Stuenæs and Fang-Chin Lin

Purpose: The purpose of this trial is to examine the effect of dex acutely administered versus an 11-day administration; these rats will be compared with control groups as well. We will in addition compare the difference between fasted and fed rats for both groups (24 hour fasting).

Experiment: As soon as the two groups reach ~150 g, (Experiment A9 16th and experiment A10 17th of February). Experiment can be postponed if significantly lower weight than 150g.

16 rats of 85-95 g will arrive at 4th of February,
16 rats (8 dex and 8 controls). **Rats live 2 per cage**

Between 10-11 AM: Inject dex and weigh rats and food intake

Dex/saline injection starts the day prior to experiment Monday 16th of February for 8 rats (Experiment A9).
Dex/saline injection starts the day prior to experiment Tuesday 17th of February (Experiment A10).

Dex: I.p. injection of 1,0 mg/kg.
Control: I.p. injection of saline 1.0 ml/kg.

Dex: 0.1 mg/ml saline. Inject 1 ml/100 g rat.
(Dex solubility: 10 mg/100 ml (0.1 mg/ml) – 25 ºC.)
Make new solution every 2nd day (min. 10 ml daily). Store in refrigerator.

Experiments:
Monday 16th: 1 day dexinjection (8 rats: 4 dex and 4 controls)
2 dex treated rats are fasted 24 h before experiment (from 10.00 h), the other 2 rats are given normal access to chow until experiment the following day.
2 Control rats fasted and 2 are given normal access to chow.

Tuesday 17th: 1 day dexinjection (8 rats: 4 dex and 4 controls)
2 dex treated rats are fasted 24 h before experiment (from 10.00 h), the other 2 rats are given normal access to chow until experiment the following day.
2 Control rats fasted and 2 are given normal access to chow.

Liver, heart, red and white gastro, epididymal fat, brain, Epi, EDL and Soleus are collected for measurement of glucose uptake, glycogen content, glycogen synthesis and buffer is measured for lactate.

Appendix 4 - Chemicals and equipment

Other chemicals not mentioned here are all from Merck or Sigma-Aldrich.

Name of product/equipment	Serial number	Producer
Actrapid Injection solution 100 IE/ml	014398	Novo Nordisk A/S, Dk
Immobilon Western Chemiluminescent HRP Substrate	WBKLS0500	Millipore
Immobilon-P Transfer membrane, PVDF	IPVH00010	Millipore
Pony Vial	6000292	PerkinElmer
Prestained precision protein standards	161-0373	BioRad
Antibody GS Ser^{641}	Cell signaling Tech. #3891	
Antibody GSK Total protein	Upstate #05-412	MedProbe
Antibody p-GSK-3α/β Ser$^{21/9}$	#9331	Cell Signaling Tech. Inc
Antibody PKB Ser^{473}	#9271	Cell Signaling Tech. Inc.
Antibody PKB Thr^{308}	#4056	Cell Signaling Tech. Inc.
Protease inhibitor cocktail	P-8340	Sigma-Aldrich
Secondary antibody Anti-mouse	Upstate #12-349	MedProbe
Secondary antibody Anti-rabbit	Cell signaling Tech. #7074	MedProbe
Lactate dehydrogenase (LDH)	L-2625	Sigma-Aldrich
Equipment		
Muscle incubation equipment	Made at STAMI	
Homogenizator	Polytron PT1200	
Electrophoresis and blotting equipment	BioRad	
Scintillation counter, TRI-Carb 1900 TR	Packard	

Appendix 5- solutions used in experiments

BUFFER FOR MUSCLE INCUBATION, Krebs-Henseleit buffer:

Stock I	Concentration
NaCL	1.16 M
KCl	0.046 M
KH_2PO_4	0.0116 M
$NaHCO_3$	0.253 M

In $H_2O_{millipore}$

Stock II	Concentration
$CaCl_2x2H_2O$	0.025 M
$MgSO_4x7H_2O$	0.0116 M

In $H_2O_{millipore}$

Ready to-use solution is made daily:
100 ml Stock I + 100 ml Stock II + 800 ml $H_2O_{millipore}$
The solution is gassed in 95 % O_2/5 % CO_2 for at least 45 minutes prior to use

100 ml was taken out after gassing to use for lubricant while dissecting muscles, the 900 ml left was added:

Substance	Concentration
BSA	0.1 %
HEPES	5 mM
Glucose monohydrate	5.5 mM
Pyruvate	2 mM

pH adjusted to 7.4 with NaOH if needed.

GLYCOGEN CONTENT, Reaction solution:

Concentrations	
Concentration of reagents	Concentration
Tris/HCl pH 8.1	100 mM
$MgCl_2$	1 mM
Dithiothreitol, DTT	0.5 mM
ATP	300 µM
$NADP^+$	30 µM
G6PDH	0.08 U/ml
H_2O $_{millipore}$	

LACTATE RELEASE, Reaction solution

Concentrations	
Concentration of reagents	Concentration
2a-2m-1p pH 9.9-10.0	50 mM
L-Glutamate	2 mM
NAD^+	500 µM
LDH	10 U/ml
GPT	3 U/ml
H_2O $_{millipore}$	

WESTERN BLOTTING, SDS-PAGE:

Homogenization buffer	Concentration in solution
H_2O millipore	
1 M HEPES pH 7.4	50 mM
3 M NaCl	150 mM
100 mM Na_4P_2O7	10 mM
500 mM NaF	30 mM
100 mM Na_3VO_4	1 mM
200 mM EDTA	10 mM
250 mM Benzamidin	2.5 mM
Protease inhibitor cocktail (Sigma, P-8340)	0.5 µl/10 mg muscle
Triton X-100*	1 %

*(added directly after homogenization)

5xSDS sample buffer	Concentration in solution
1.5 M Tris pH 6.8	313 mM
20 % SDS	10 %
2.5 % Bromphenol blue/glycerol	0.25 %/9.9 %
Glycerol	19 %

Stacking gel, 4 % acrylamide, 4 gels	Total amount
H_2O millipore	6.4 ml
1.5 Tris pH 6.8	0.67 ml
20 % SDS	40 µl
40 % acrylamide/bis (37.5:1)	0.8 ml
TEMED (BioRad, concentrated)	8 µl
10 % Ammonium persulfate	80 µl

Separation gel, 10 % acrylamide, 4 gels	Total amount
H_2O millipore	7.8 ml
1.5 Tris pH 8.8	4 ml
20 % SDS	80 µl
40 % acrylamide/bis (37.5:1)	4 ml
TEMED (BioRad, concentrated)	9.6 µl
10 % ammonium persulfate	160 µl

Methanol	10 %

In H_2O Millipore

Electrophoresis buffer	Concentration
Tris	25 mM
Glycine	192 mM
SDS	0.1 %

In H_2O Millipore

PBS/0.1 % Tween 20	Concentration
Na_2HPO_4 x $2H_2O$	80 mM
NaH_2PO_4 x H_2O	20 mM
NaCl	100 mM
Tween 20	0.1 %

In H_2O Millipore, pH-adjusted to 7.4 with NaOH.

Transfer buffer	Concentration
Tris	25 mM
Glycine	192 mM

Appendix 6 – method for Glycolytic flux measurement

The radioactively labeled 0.25 µCi/ml 2-[^{3}H] -glucose (30.6 Ci/mmol; NET 549 PerkinElmer) were added to the buffer (containing 5.5 mM glucose, 2mM pyruvate, 5 mM HEPES and 0.1 % BSA). Used incubation buffer are frozen (-20 °C) as soon as possible. 750 µl H$_2$O$_{millipore}$ are added to scintillation tubes (Pony Vials 6000292, PerkinElmer) and an eppendorf vial without a lid is placed inside the scintillation tube, the eppendorf vial is filled with 5 µl HCl (to prevent microbial growth) and 50 µl of the sample buffer/blanks, 1 parallel are made for each sample. Before incubation in 37 °C for 3 days blanks are prepared. The blanks are divided in two groups; directly pipetted and incubated with the muscles (30 °C). The blanks incubated together with the muscles consist of 2 H$_2$O$_{millipore}$ parallels, 2 parallels of a solution with the same concentration radioactively labeled 2-[^{3}H] -glucose as in the original buffer, 5 µ HCl and 695 µl H$_2$O. The blanks directly counted consist of 2 parallels of 745 µl H$_2$O$_{millipore}$ and 5 µl HCl, 2 parallels of a solution with the same concentration radioactively labeled 2-[^{3}H] -glucose as in the original buffer, 5 µ HCl and 695 µl H$_2$O. 2 parallels of unused incubation buffer were also incubated. The eppendorf vials were placed within the scintillation tube with tweezers, it was important to avoid spilling of fluids between the compartments. The scintillation tube is closed and placed in 37 °C for a minimum of 72 hours – this will create equilibrium between the water in the eppendorf vial and the scintillation vial. After the incubation, the eppendorf vial was discarded and 4.5 ml Ultima Gold scintillation cocktail was added. The samples were counted for radioactivity (d.p.m.) on Tri-Carb 1900 TR; 5 minutes counting, 2 cycles.

Background for analysis of glycolytic flux;

The method measures at what rate glucose is metabolized in the muscles incubated (rate of glycolysis). The buffer contains radioactive labeled glucose in known concentrations, as well is the glucose concentration in the buffer known (5 mM). Same kinetics are assumed between glucose and the radioactively labeled glucose. During metabolic processes in the muscle, ^{3}H$_2$O will be cleaved from the 2-[^{3}H] –glucose molecule. During equilibrium the amount ^{3}H$_2$O will, by calculating back to the original concentration in the small eppendorf vial, show the rate of glycolysis.

Appendix 7 – method for Glycogen synthesis measurement

A measurement using either [^{14}C(U)]-glucose or 2-[^{3}H] -glucose (30.6 Ci/mmol; NET 549 PerkinElmer) follows the same method.

[^{14}C(U)]-glucose was added to the incubation buffer, incubation time was 1 hour for the muscles. Muscles were handled as described earlier, 2-3 mg dw was used. Due to this, dissolving and preparation of the muscles follow the same steps as in method for measurement of glucose uptake, and the same blanks are prepared as mentioned. The point where this method deviates from the method for glucose uptake is after dissolving of the muscles; 500 µl of the digest was added to a saturated solution of 100 µl Na_2SO_4 and 100 µl glycogen solution (25 mg/ml – dissolved in $H_2O_{millipore}$), then mixed thoroughly. 1.5 ml absolute ethanol stored at -70 °C was added to this solution (quick dispensing of the liquid from a pre-cooled pipette tip was important). Samples was precipitated overnight (-20 °C).

The samples were centrifuged after precipitation at 3000 x g for 20 minutes at +4 °C, the supernatant was discarded. The precipitate was added 500 µl $H_2O_{millipore}$ and dissolved again (10 minutes, 70 °C). Samples were now re-precipitated with 1.0 ml absolute ethanol (-70 °C) and stored for at least 1 hour at -20 °C. Samples were mixed thoroughly at each step. The samples were centrifuged again after precipitation (3000 x g, 20 minutes, +4 °C), supernatant was discarded. Tubes were placed upside-down on filter paper to remove excess solution for ca 5 minutes. The pellet was re-dissolved in 300 µl $H_2O_{millipore}$ (70 °C, 10 minutes). Samples were cooled down, 250 µl of the solution was pipetted into counting vials (Pony Vial, Perkin Elmer) and added 3 ml Ultima Gold scintillation cocktail, thoroughly mixed. Samples were counted for radioactivity (d.p.m.) after 1 hour on Tri-Carb 1900 TR; 5 minutes counting, 2 cycles. The 4 vials containing incubation buffer from the day of experiment was counted again together with the samples.

Appendix 8 – List of experiments conducted with dex treatment

Author, year	Ref	Dex	Fasting status	Days	Weight	Rats	Muscles
Burén, 2002	41	0.3mmol/ml	Fed	0-24 h	150-200	SD	Adipocyts
Burén, 2008	34	1.0 mg/kg	12 h	11	149 vs 216	W	Epi, sol, adip
Coderre, 1992	58	0.4 mg/kg	24 h	14	240-260	SD	RG, WG, sol
Coderre, 1992	61	0.4 mg/kg	24 h	14	200-250	SD	RG, WG, sol
Coderre, 1996	40	0.4/1.0 mg/kg	Fed	7	200-250	SD	Sol, heart, RG, WG
Coderre, 2007	26	1.0 mg/kg	16 h	7	200-250	SD	RG, WG
Dimitriadis, 1997	42	0.5 mg/day	12 h	5	160-180	W	Sol
He, 2003	60	0.2mg/kg	Fed	7		SD	sol edl
Leighton, 1987	67	2.5 mg/kg	16 h	4	140-150/80-90	W	sol/EDL
Puthanveetil,	51	1.0 mg/kg	Fed	Acute	260-300	W	Heart
Qi, 2004	50	1.0 mg/kg	Fed	Acute	270-290	W	Heart
Ruzzin, 2005	49	1.0 mg/kg	5 g last 18 h	12	250	W	Epi, sol
Ruzzin, 2005	36	1.0 mg/kg	5 g last 18 h	12	280-290	W	Heart, Liver

Abbreviations used: W = Wistar rats, SD = Sprague Dawley, RG/WG = Red/White gastrocnemius