THE ROLE OF STEROID HORMONES IN SKELETAL MUSCLE METABOLISM

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To my family

Although my mind did go around this wilderness a lot did not understand a thing but discovered many things in my mind thousands of ideas surfaced at the end it did not get to the tiniest perfection

Avicena
ABSTRACT

Steroid hormones play important roles in the regulation of whole body metabolism. Skeletal muscle is an insulin-responsive organ with a key role in overall substrate metabolism. Disturbances in skeletal muscle metabolism, as a result of hormonal imbalance may be an underlying defect in metabolic disease. Reduced insulin-responsive glucose disposal in skeletal muscle is a characteristic feature of metabolic syndrome. The overall aim of this thesis work is to identify the role of steroid hormones on glucose and lipid metabolism; and to dissect the impact of sex steroid hormones on insulin signaling pathways in human skeletal muscle. A further goal is to understand how sex differences impact on skeletal muscle metabolism.

Whole body metabolism differs between men and women, and sex-dependent differences in gene expression are evident in skeletal muscle biopsies. Some sex-dependent differences in gene expression are retained in vitro in cultured human skeletal muscle. In contrast, glucose and lipid metabolism did not show any sex-dependent differences. Chronic exposure of muscle cell cultures to physiological doses of testosterone or 17β-estradiol resulted in sex-dependent responses. Exposure to testosterone enhanced palmitate oxidation, AMP dependent protein kinase phosphorylation and IRS2 gene expression in myotubes from both sexes, while 17β-estradiol exposure increased palmitate oxidation in myotubes from male donors only and PDK4 gene expression from female donors only. Testosterone or 17β-estradiol treatment enhanced insulin-stimulated glucose incorporation into glycogen and AKT phosphorylation only in myotubes from female donors. Acute supra-physiological doses of testosterone or 17β-estradiol reduced glucose metabolism, independent of sex origin of the cells. Moreover, acute testosterone treatment increased basal palmitate oxidation and disrupted the insulin-suppressive effect on palmitate oxidation.

Increased glucocorticoid action leads to reduced whole body insulin action and may predispose to type 2 diabetes. Local conversion of cortisone to active cortisol by the enzyme 11β-hydroxysteroid dehydrogenase in target tissues may regulate tissue-specific roles of glucocorticoids in patho-physiological states. Chronic high dose exposure to cortisol or cortisone reduced glucose metabolism, and enhanced palmitate oxidation, via induction of PDK4 expression in myotubes. siRNA-mediated reduction or pharmacological inhibition of HSD1 prevented the effects of cortisone, but not cortisol, on metabolic responses.

In conclusion, steroid hormones exert diverse effects in a dose and time dependent manner. Modulation of steroid hormone actions at specific regulatory steps may provide potential therapeutic entry points for metabolic disease and Type 2 diabetes. Moreover, attention should be focused on understanding sex-dependent differences in metabolic disease, and sex-origin of cells is important to consider when assessing hormonal responses in culture.
LIST OF PUBLICATIONS INCLUDED IN THIS THESIS


LIST OF PUBLICATIONS NOT INCLUDED IN THIS THESIS


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<th>Definition</th>
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<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamine-1-β-D-ribonucleoside</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CD36</td>
<td>Fatty acid translocase</td>
</tr>
<tr>
<td>CPT1B</td>
<td>Carnitine palmitoyltransferase 1B</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>Enzyme P450 aromatase (P450)</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydroxytestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential media</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Esterogen receptor α</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FABP3</td>
<td>Fatty acid binding protein 3</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GYS1</td>
<td>Glycogen synthase 1</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HSMC</td>
<td>Human skeletal muscle cell</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus–pituitary-axis</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>11β-HSD</td>
<td>11β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Association</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun n-terminal kinase</td>
</tr>
<tr>
<td>LH</td>
<td>Releases luteinizing hormone</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
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1 INTRODUCTION

1.1 METABOLISM AND HOMEOCA"STASIS

Energy homeostasis and metabolism is determined by the balance between food intake and energy expenditure. Glucose and fatty acids are the major metabolic fuels which are used for energy production. However, the body maintains a strict glucose level, which is essential for brain function. Normal physiological glucose level (4.3–6.5 mmol/l) is controlled through a complex network between the pancreas, liver, adipose tissue, muscle, and brain. In the basal state glucose is stored (as glycogen) mainly in liver and partly in skeletal muscle, and it is further oxidized in adipose tissue and skeletal muscle (Figure 1)(Ferrannini et al, 1988; Kelley et al, 1990). During shortage of glucose or excess of fatty acids, a metabolic shift may occur towards fat metabolism in order to protect whole body glucose homeostasis. In fact the shift to different fuel energy source has been termed metabolic flexibility (Frayn, 2003; Randle et al, 1963). Thus, diverse nutrient availabilities during environmental alterations such as fasting, starvation and exercise impact on metabolic flexibility (Ahlborg et al, 1974; Ahlborg et al, 1986; Cahill et al, 1966; Drury et al, 1959).

1.1.1 Hormonal regulation of metabolism and homeostasis

Hormones regulate various human functions, including whole body metabolism, growth and development. Insulin is one essential hormone, involved in the coordination of glucose homeostasis, and exerts several actions on skeletal muscle, liver, and adipose tissue (Figure 1) (DeFronzo, 1988). Hormones other than insulin may also have major effects on energy metabolism such as the 'fight-and-flight' hormones epinephrine and norepinephrine, growth hormone, steroid hormones (glucocorticoids) and the thyroid hormones (tri- and tetraiodothyronine). Interestingly, evidence also suggests that sex hormones, such as testosterone and estradiol, play a role in whole body lipid and glucose metabolism (Haffner & Valdez, 1995; Landau & Poulos, 1971).
Figure 1: A postabsorptive change of substrate fluxes between organs in whole body is mainly regulated by insulin. Inputs to serum glucose and fatty acid levels include absorption from the intestine and release from the liver, increased insulin release from the pancreatic beta cell. Insulin inhibits glucose production from the liver, stimulates glucose uptake in skeletal muscle and adipose tissue.

1.2 INSULIN ACTION AND METABOLISM

Insulin is a 51-amino acid peptide hormone that is synthesized and secreted by pancreatic beta cells. Under normal conditions after a meal (i.e. in the postparandial state), insulin is released into the bloodstream (Felig et al, 1976). Insulin’s major effect is to enhance glucose uptake and storage into glycogen. Glucose uptake occurs via specific glucose transporters (Gluts) and sodium–glucose transporters (SGLTs). GLUT4 is unique such that it is expressed only in insulin sensitive tissues such as skeletal muscle and adipose tissue (Hainault et al, 1991; Klip & Paquet, 1990).

Insulin activates, by auto-phosphorylation, its own receptor (IR) (Kasuga et al, 1983; Zick et al, 1983), which phosphorylates insulin receptor substrate proteins (IRS proteins). IRS proteins are linked to the activation of two main signaling pathways: the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, which is responsible for GLUT4 translocation to cell membrane and for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of several genes and it also cooperates with the PI3K pathway to control cell growth and differentiation (Figure 2)(Avruch, 1998; Taniguchi et al, 2006). IRS1 appears to have its major role in skeletal muscle and adipose tissue (Sesti et al, 2001). IRS1 contains several potential tyrosine phosphorylation sites and serine/threonine phosphorylation sites. In response to insulin, IRS1 becomes tyrosine phosphorylated whereas the role of serine/threonine phosphorylation has shown to play
negative regulatory role on IRS1 tyrosine phosphorylation (Ullrich & Schlessinger, 1990). Thus a delicate balance between positive IRS1 tyrosine phosphorylation and negative IRS1 serine phosphorylation regulates the IRS1 function (Gual et al, 2005).

The activation of AKT/PKB in response to insulin regulates phosphorylation of IRS1 on serine residues mediating a positive-feedback loop for insulin action (Giraud et al, 2004; Paz et al, 1999). Whereas activation of JNK, ERK, PKC and mTOR mediated by insulin, controls insulin action through a negative-feedback loop (Aguirre et al, 2000; Gual et al, 2003).

Thus, insulin signaling is mediated by a complex, highly integrated network that controls several processes. As in general, insulin stimulates glucose uptake into peripheral insulin-sensitive tissues, increased protein synthesis, gene expression, decreases hepatic glucose production, increases glucose utilization in adipose and muscle tissue and prevents lipolysis and free fatty acid release in adipose tissue (Figure 1).

Figure 2. Schematic view of insulin-signaling pathway in skeletal muscle. Insulin triggers series of signaling cascade via autophosphorylation of insulin receptor result in the phosphorylation of the IRS family. These proteins interact with molecules leading to a diverse series of signaling pathways, including activation of PI3K and downstream protein kinases, ras and the MAP kinase cascade, and the activation of glucose transport. These pathways act in a harmony to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which results in the regulation of glucose, lipid and protein metabolism.
1.3 METABOLISM: SEX MATTERS

There is increasing evidence that metabolic control varies between men and women. Sex differences which stand for biological differences between males and females, excluding interaction with environment, exist at the molecular (e.g. imprinting, X-inactivation) and cellular (e.g. sex-specific receptors) (Davies, 2010; Jablonka, 2004; Roy & Chatterjee, 1983) level. These differences may in turn influence the synthesis and release of signaling compounds (hormones, cytokines, and intracellular signaling elements) and their actions, which leads to differences in the control of substrate metabolism between men and women. In addition, substrate metabolism may differ between the sexes as secondary effect of different body fat distribution in women and men (Krotkiewski et al, 1983).

The terms sex and gender are often used interchangeably. Despite sounding similar, they actually have distinct meanings. The term gender defines behavioral, cultural, and psychological differences between men and women, while a sex difference stands for only biological differences between males and females, excluding an interaction with the environment.

1.3.1 Intrinsic sex differences in substrate metabolism

Men have little subcutaneous abdominal fat, whereas women have generally much less visceral fat (Krotkiewski et al, 1983). Women store fatty acids after a meal to greater extent in subcutaneous adipose tissue (40% versus 25% in men), whereas in men, a greater proportion is directly oxidized or stored in visceral fat (Romanski et al, 2000). Microarray data from liver tissue obtained from female or male rats shows higher expression of genes involved in the synthesis of fuel metabolism regulatory proteins (e.g. glucose and lipid oxidation) in male rats. Nevertheless fatty acid translocase (FAT/CD36) gene expression was remarkably higher in liver tissue from female rats, and this sex-specific expression was similarly confirmed in human samples with smaller magnitude (Kiens et al, 2004; Stahlberg et al, 2004).

Exercise increases skeletal muscle insulin sensitivity and the rate of glucose uptake into the contracting skeletal muscle in an insulin-independent fashion. During sub-maximal endurance exercise, women oxidize more lipids and less carbohydrate than men. Furthermore, women show lower glycogen utilization in skeletal muscle and lower hepatic glucose production as compared with men (Tarnopolsky & Ruby, 2001). mRNA analysis of muscle biopsies obtained from women after endurance exercise show greater expression of genes related to fatty acid oxidation when compared to skeletal muscle from men (Maher et al, 2010b).

Women appear to have higher basal and post-absorptive fatty acid turnover and show a greater increase in appearance and disappearance of fatty acids from plasma in response to exercise as compared to men (Mittendorfer et al, 2002; Nielseni et al, 2000). Thus, different inherited genetic components and sex-linked gene expression might explain the differential substrate metabolism in men and women. In addition, body systemic factors and hormonal milieu such as steroid hormones further accentuates these differences in metabolism.
1.3.2 Sex Differences in insulin action and metabolism

Differences in body composition and fat distribution between men and women have raised the interest to study sex differences in the regulation of substrate metabolism. Sex differences in insulin sensitivity have been observed in substrate metabolism (Ferrara et al, 2008). Women show higher glucose uptake in skeletal muscle in response to insulin as compared to men (Nuutila et al, 1995; Paula et al, 1990). Moreover, women appear to be more sensitive to insulin in the liver, such that insulin-mediated suppression of endogenous glucose production happens to a greater extent in women than men at low plasma insulin concentrations (approximately 90-120 pmol/L) (Amiel et al, 1993). While at high plasma insulin concentrations, insulin-mediated suppression of endogenous glucose is similar in women and men (Basu et al, 2006).

The response of adipose tissue lipolysis to insulin appears to be the same in both sexes (Perseghin et al, 2001). Studies performed on young men and women provide no evidence for sex differences in the inhibitory effect of insulin on whole body proteolysis, but women appeared to be resistant to the stimulatory effect of insulin on protein synthesis (Chevalier et al, 2005; Smith et al, 2009).

Results from intervention studies reveal that men have larger improvements in insulin sensitivity than women in response to a physical fitness program (Boule et al, 2005), whereas moderate-intensity physical activity combined with caloric reduction improves insulin sensitivity in both men and women (Goodpaster et al, 2003). In addition, men have a lower rate of glucose utilization after acute exercise as compared to women (Perreault et al, 2004).

1.4 METABOLISM IN SKELETAL MUSCLE

Skeletal muscle comprises 40-50 % of body mass and is the major site of substrate metabolism. Glucose clearance in postprandial state in response to insulin is primarily mediated by skeletal muscle, which accounts for 75-95% of all insulin-mediated glucose disposal (DeFronzo et al, 1985). Thus metabolic flexibility and insulin sensitivity of skeletal muscle contributes greatly to glucose homeostasis and whole body metabolism (Kelley et al, 1999).

Skeletal muscle utilizes both glucose and fatty acids as fuel sources for energy production. The fuel selection of muscle fibers at rest is dependent on substrate availability. Once in cytoplasm, glucose is phosphorylated to glucose-6-phosphate by hexokinases (HKs) in the glucose utilization pathway (Figure 3). During aerobic and anaerobic conditions, glucose may have different cellular fates. Under anaerobic conditions, pyruvate is converted to lactate by lactate dehydrogenase enzyme (Bouche et al, 2004; McLane & Holloszy, 1979).

Under normal aerobic conditions, glucose is oxidized to pyruvate, which is converted to acetyl-CoA. Acetyl-CoA undergoes further oxidation in the Krebs cycle (citric-acid cycle) to carbon dioxide and water (final products) or stored as glycogen for rapid utilization through the glycoen synthesis pathway (Sugden et al, 2000). The pyruvate dehydrogenase (PDH) complex is the responsible enzyme that determines transformation of pyruvate to acetyl-CoA in mitochondria. The acetyl-CoA concentration level could be a regulatory key factor for skeletal muscle to switch from glucose to fatty acid metabolism. The activity of the PDH complex can also be regulated by another rate limiting enzyme, pyruvate dehydrogenase kinase isoformes1-
4 (PDK1-4), through inhibitory phosphorylation. PDK2 and PDK4 isoforms are expressed in most of the tissues, while PDK1 and PDK3 isoforms are tissue specific (Wu et al, 2000). PDK4 content is up-regulated during fasting and starvation in skeletal muscle (Wu et al, 2000).

Glucose and fat metabolism has a reciprocal relationship in skeletal muscle through the Randle cycle, suggesting that an increase in fatty acid flux would result in enhanced fatty acid metabolism and reduced glucose metabolism (Pehleman et al, 2005; Randle et al, 1963; Randle et al, 1994). This process is mediated by three different mechanisms including: increased concentration of Acetyl-CoA from oxidation which in turn inhibits PDH complex; fatty acid accumulation of citrate to inhibit phosphofructokinase (PFK); and accumulation of glucose-6-phosphate to reduce hexokinase activity (Figure 3) (Jeukendrup, 2002; Rasmussen & Wolfe, 1999). During starvation and fasting conditions, fatty acids are the major fuel source in skeletal muscle. Fatty acid uptake by skeletal muscle occurs via simple diffusion across the cell membrane or via transportation by fatty acid binding proteins (FABPs) and fatty acid translocase protein (FAT/CD36) (Bonen et al, 1998). Fatty acids are converted to acyl-CoA, which is transferred to the mitochondria by carnitine palmitoyl transfrase I (CPT1). Acyl-CoA will be then converted to Acetyl-CoA and enter Krebs cycle (Figure 3) (Kerner & Hoppel, 2000).

**Figure 3**: Schematic view of glucose and fatty acid metabolic regulation in skeletal muscle. HK, hexokinase; GS, Glycogen synthase; PFK, phosphofructase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; CD36, fatty acid translocase; CPT1, carnitine palmitoyl transfrase 1; CPT2, carnitine palmitoyl transfrase 2; TCA, tricarboxylic acid cycle (krebs cycle).
1.5 STEROID HORMONES

Steroid hormones are generally synthesized from cholesterol in the gonads and adrenal glands and share a sterol group in their chemical structure. The biosynthesis of steroid hormones (estrogens, androgens and glucocorticoids) is regulated by hypothalamic-pituitary-gonad axis in response to numerous neuroendocrine signals from hypothalamus, the central nervous system region. Gonadotropin-releasing hormone (GnRH); and corticotrophin-releasing hormone (CRH) are released from the hypothalamus in a cyclic manner. These hormones respectively stimulate the pituitary gland to release luteinizing hormone (LH), follicle-stimulating hormone (FSH) and adrenocorticotropic hormone (ACTH). Furthermore these hormones respectively target gonads to produce sex steroids (testosterone, and estrogen, progesterone); and adrenal gland to release glucocorticoids (cortisol) mineralocorticoids, (aldosterone) and androgens (testosterone, dehydroepiandrosterone (DHEA)) (Figure 4) (Filocori et al, 1986; Tsigos & Chrousos, 1994). Since the chemical composition of steroid hormones is hydrophobic, they can pass across the cell membrane and bind to steroid hormone receptors. Steroid hormones are generally carried in the blood bound to specific carrier proteins such as sex hormone-binding globulin (SHGB), corticosteroid-binding globulin (CBG), carrier proteins like albumin (Breuner & Orchinik, 2002; Burton & Westphal, 1972; Cunningham et al, 1985).

Figure 4: Schematic hypothalamus–pituitary-axis regulation of steroid hormones. GnRH, Gonadotropin-releasing hormone; CRH, corticotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone. Effect of glucocorticoids and sex steroids itself keeps endogenous synthesis and release of the hormones in balance via a negative feedback loop on the hypothalamus–pituitary-axis. Figure has been adapted from (Wierman, 2007).
Steroid receptors include three groups of receptors: class I receptors (glucocorticoid (GR), progesterone (PR), estrogen (ER), androgen (AR) receptors); class II receptors (vitaminD, thyroid hormone, peroxisome-proliferator, and retinoid receptors); orphan receptors (steroidogenic factor-1 and estrogen-related receptor) (Baulieu et al, 1975). Steroid hormone receptors (SRs) act as transcription factors regulating the expression of several genes (Beato & Klug, 2000). Steroid hormones bind to respective specific steroid hormone receptor which leads to a conformational modification of the receptor. In following, steroid receptor separates from cytoplasmic chaperone proteins such as heat shock protein 90 (Hsp90). As a consequence, nuclear localization sequences will be exposed followed by nuclear translocation and homo/heterodimerization of the ligand-binding receptor to steroid response elements on the promoter regions of the target gene (i.e. nucleotide sequences specifically recognized by steroid hormone receptors). Furthermore, this complex regulates gene expression via interaction with the transcription machinery. This process is the so-called classical or genomic action of steroid hormones (Figure 5) (Chan & O'Malley, 1976; Truss & Beato, 1993).

Not all the effects of steroid hormones are mediated by transcriptional activation of the classical nuclear hormone receptors, but a non-classical or non-genomic effect may occur. The non-genomic action of steroid hormones occurs at a much faster rate than that of RNA and protein synthesis (i.e. within seconds to minutes from exposure to the hormone), and is mostly mediated by the localization of a pool of steroid hormones at the cell membrane (Lu et al, 2004; Simoncini & Genazzani, 2003). Plasma membrane steroid receptors are able to exert their non-genomic actions through regulation of intracellular signaling molecules such as PI3K/AKT pathway (Baron et al, 2004; Simoncini et al, 2000), the mitogen-activated protein kinase (MAPK) family (ERK1/2, p38 and JNK phosphorylation) (Kato et al, 1995; Nguyen et al, 2005; Srivastava et al, 1999), cell membrane ion channels and G-protein-coupled receptors (GPCRs) and tyrosin kinases by transcription-independent mechanisms (Figure 5) (Estrada et al, 2003; Foradori et al, 2008; Migliaccio et al, 1996). Humans studies have provided evidence that single doses of estrogen administration in post menopausal women attenuate abnormal coronary vasomotor responses to acetylcholine and play a cardioprotective role (Reis et al, 1994). Testosterone has been shown to rapidly increase intracellular calcium concentration in osteoblasts (Vicencio et al, 2011).
Figure 5: Schematic view of classical and non-classical cycle of steroid hormone action. Steroid hormone receptor in green color, illustrates different domains as NTD, N-terminal transactivation domain; DBD, DNA-binding domain; LBD, ligand-binding domain. The numbers in the figures represent: 1) The steroid hormone binds to steroid hormone receptor; 2) The ligand-bound steroid hormone receptor induces a conformational alteration that results in the disassociation of the heat shock protein from the steroid hormone receptor; 3) A ligand-bound receptor translocates to nucleus and is further dimerized; 4) A ligand-bound receptor binds to the steroid response elements; 5) Direct interaction of ligand-bound receptors with transcription factors; 6) Non-genomic action of sex steroids mediated by steroid receptor in the plasma membrane or G protein coupled receptors (GPCRs); 7 and 8) Phosphorylation of the ligand and non-ligand bound receptor prevents steroid receptor degradation, and enhances steroid receptor translocation and activity; 9) Steroid hormone non-genomic activity induce signaling cascades which regulate other nuclear transcription factors. Figure has been adapted from (Bennett et al, 2010).

1.5.1 Sex steroid hormones action and substrate metabolism

Sex hormones are produced from adrenal glands and endocrine gonads. In sexually mature females, the ovary, in response to FSH, produces and secretes estrogens (mainly 17β-estradiol and estrone) and in males, the testes secrete androgen (testosterone). Estrogens are produced from androstenedione by the aromatase enzyme, a cytochrome P450 enzyme primarily expressed in the ovaries. Estrone and 17β-estradiol are in reversible equilibrium (Matocha & Waterman, 1985). Testosterone is also derived from androstenedione through the actions of 17β-hydroxysteroid
dehydrogenase, primarily expressed in the testis. Sex hormones also produce locally by peripheral conversion in target tissues such as skeletal muscle, adipose tissue and liver. The 5α-reductase enzyme irreversibly converts testosterone to dihydroxytestosterone (DHT), whereas the aromatase enzyme irreversibly converts testosterone into 17β-estradiol (Figure 6) (Spelsberg, 2004). The conversion of testosterone to the more active androgen metabolites occurs in target tissues such as skeletal muscle (Normington & Russell, 1992).

![Figure 6. Biosynthesis of estrogen and testosterone.](#)

Estrogens regulate many physiological processes, including normal cell growth, development, and tissue-specific gene regulation in the reproductive tract and in the central nervous and skeletal systems. Estrogens also influence the pathological processes of hormone-dependent diseases, such as breast, endometrial, and ovarian cancers, as well as osteoporosis (Pettersson & Gustafsson, 2001). Estrogens are necessary in males for the regulation of male sexual behavior, maintenance of the skeleton and the cardiovascular system, and for the normal function of the testis and prostate. Estrogens are important regulators of metabolic homeostasis and lipid metabolism in different organs. Most of the information regarding the action of estrogens in metabolic homeostasis has been unraveled using estrogen or estrogen receptor (ER) depletion or estrogen replacement models. Estrogen exerts a wide range of actions in different organs including increased insulin secretion from pancreatic beta cells (Ripoll et al, 2008); induction of lipolysis and inhibition of lipogenesis in adipose tissue (Pedersen et al, 2004); inhibition of glucose production and lipogenesis in liver(Bryzgalova et al, 2006) decreased food intake and increased energy expenditure in central nervous system (CNS)(Vilberg & Keesey, 1984); increased glucose uptake in skeletal muscle (Deng et al, 2008).

Androgens exert a widespread pattern of effects on metabolism, muscle growth and body composition (Herbst & Bhasin, 2004). Testosterone is a classic anabolic sex-steroid (West & Phillips, 2010) which plays a role in the regulation of insulin sensitivity and glycogen storage in skeletal muscle (Holmang & Bjorntorp, 1992). The
importance of androgen hormones in females is well known, since androgen is the precursor of estrogen as the substrate for the aromatase enzyme.

Many human studies provide evidence that sex steroids can affect insulin sensitivity (Cook et al, 1993; Dhaturiya et al, 2005; Lee et al, 2004; Rajkhowa et al, 1994). At physiological concentrations, sex hormones have positive effect in maintaining insulin sensitivity. While an elevated serum level of sex hormones during puberty (Amiel et al, 1991; Bloch et al, 1987), pregnancy (Hollingsworth, 1983) or during the menstrual cycle (Bruns & Kemnitz, 2004), have been reported to be associated with a reduction in insulin sensitivity in peripheral glucose metabolism. Moreover, the physiological estrogen level in pre-menopausal women exerts a protective effect against metabolic disorders, and after menopause adverse effects have been observed (Gaspard, 2009).

Sex steroid hormones exert their actions by binding to the specific sex hormone receptors. Two isoforms of the estrogen receptor have been identified in humans and rodents, and they are encoded by two separate genes on separate chromosomes. ERα is expressed primarily in the uterus, liver, kidney, and heart, whereas ERβ is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (Katzenellenbogen, 1996; Koehler et al, 2005). Estrogen receptor knockout mice, lacking either ERα or ERβ, reveal a regulatory role of these receptors in the control of energy homeostasis. ERα knockout mice are obese and insulin resistant (Heine et al, 2000). Skeletal muscle GLUT4 expression is reduced in these animals. Both ERα and ERβ are involved in the action of 17β-estradiol in body fat distribution and adipose tissue metabolism (Cooke et al, 2001).

Androgen hormones bind to the androgen receptor (AR) encoded on the X chromosome (Brinkmann et al, 1989). So far, only one isoform is known for the androgen receptor, whereas most of other ligand-dependent hormone receptors have two isoforms. Androgen receptors are expressed in variety of different tissues in human and rodent, including the central nervous system (CNS), bone, prostate and skeletal muscle, as well as reproductory organs (Keller et al, 1996; Li & Al-Azzawi, 2009). Androgen receptor (AR) null male mice display late-onset obesity, but overall these mice are insulin sensitive possibly due to the enhanced adiponectin levels. Thus the androgen receptor plays an important role in metabolism and energy balance in males. Androgen receptors also have a negative effect on insulin sensitivity and adiposity (Sato et al, 2003).

1.5.2 **Glucocorticoid action and substrate metabolism**

Glucocorticoid hormones (cortisol, cortisone) modulate a number of metabolic, cardiovascular, immune, and behavioral functions (Qi & Rodrigues, 2007). Glucocorticoid hormones are produced and released from the cortex of the adrenal gland. In humans, the main endogenous glucocorticoid is cortisol (active form), and its basal daily secretion is 6–8 mg/m² to maintain normoglycemia and to prevent arterial hypotension (low arterial pressure). In response to stress, cortisol release is increased up to 10-fold over the basal value (Delbende et al, 1992). Similar to other steroid hormones, glucocorticoid hormones exert their action mostly by modulation of gene expression. This occurs by hormone diffusion through the cell membrane, followed by binding to the glucocorticoid receptors in the cytoplasm and translocation to nucleus. In addition to genomic effects, glucocorticoids also act via a non-genomic mechanism,
which only takes a few seconds to minutes (Cato et al, 2002). A diverse mechanism of glucocorticoid non-genomic action has been studied in immune cells. Glucocorticoids rapidly reduce calcium and sodium cycling across the plasma membrane in immune cells, and lead to immune suppression and reduction of inflammation (Buttgereit & Scheffold, 2002). Glucocorticoid receptors play an important role in the regulation of glucocorticoid effects. The glucocorticoid receptors include three different isoforms, α, β, and δ but only the GRα isoform modulates the expression of the glucocorticoid response element (GRE) (Rosmond, 2002).

After hypothalamic-pituitary-gonad axis (HPA)-mediated release, glucocorticoids go through a further intracellular conversion in peripheral tissues. In this process, active cortisol is converted to its inactive form, cortisone or in reverse by the 11 β-hydroxysteroid dehydrogenase enzymes (11 β-HSDs) (Figure 7) (Amelung et al, 1953; Lakshmi & Monder, 1988). The 11 β-HSD enzymes belong to a family of microsomal enzymes, which play crucial role in glucocorticoid conversions (Stewart & Krozowski, 1999). Two separate isoforms of 11 β-HSD enzymes have been identified in mammalian tissues. The 11β-HSD1 is widely expressed in insulin target tissues, such as liver, adipose tissue, skeletal muscle and central nervous system, which converts inactive cortisone to cortisol (Ricketts et al, 1998; Whorwood et al, 2002). The 11β-HSD2 is highly expressed in classical aldosterone-selective target tissues, such as the kidney and placenta and convert active cortisol to inactive form (Albiston et al, 1994; Whorwood et al, 1995).

Steroid hormones play critical roles in overall energy homeostasis. In target tissues, glucocorticoids exert numerous metabolic actions including stimulation of gluconeogenesis in the liver and amino and fatty acids metabolism. The effect of glucocorticoids on metabolism oppose the action of insulin on glucose production from liver during normal feeding, but the metabolic effects of glucocorticoids are required under changing environmental conditions such as fasting and starvation (Schacke et al, 2002). Glucocorticoids mediate enhanced whole body lipolysis, leading to an increased plasma level of free fatty acids. In turn, this latter effect enhances the accumulation of intra-myocellular lipids and ceramids and diacylglycerol, which are negative regulators of glucose uptake and disposal in skeletal muscle (Perseghin et al, 2003). Glucocorticoids are also known as a diabetogenic hormones, as their effect on metabolism opposes the action of insulin on glucose production from liver during normal feeding. Healthy volunteers treated with dexamethasone (an artificial glucocorticoid) displayed reduced glycogen synthesis rates, with a parallel decrease in the concentration and activity of glycogen synthesis in skeletal muscle biopsies (Henriksen et al, 1999).
1.6 DISEQUILIBRIUM IN BODY METABOLISM

Genetic and environmental factors such as sedentary lifestyle, weight gain, high cholesterol diet and aging are factors which can disturb energy homeostasis and metabolic balance in regulatory tissues and organs (Pacholczyk et al, 2008). These factors may therefore, contribute to whole body metabolic disorder. Moreover, thyroid hormones, cortisol, estradiol, and testosterone are needed to work in harmony to keep insulin and glucose levels in balance. Any slight hormone imbalance in the body may lead to a disorder in cellular signaling and metabolism and lead to the development of metabolic disorders and metabolic syndrome.

1.6.1 Metabolic syndrome

The metabolic syndrome, sometimes referred to as Syndrome X, is a widespread and growing public health concern. The metabolic syndrome is associated with Type 2 diabetes and atherosclerotic cardiovascular disease (CVD), and visceral obesity (Hanson et al, 2002; Kendall & Harmel, 2002; Mottillo et al, 2010). The metabolic syndrome, based on the International Diabetes Association (IDF) definition, is a coexistence of several Type 2 diabetes and cardiovascular risk factors including impaired glucose regulation, increased triglycerides, decreased high-density lipoprotein cholesterol (HDL-C), elevated blood pressure (BP), and hyperinsulinemia (Magliano et al, 2006; Zimmet et al, 2005). The metabolic syndrome is also a common feature of many other endocrine diseases such as polycystic ovary syndrome (PCOS) and Cushing’s syndrome (Chanson & Salenave, 2010; Teede et al, 2010).

1.6.2 Type 2 diabetes

Diabetes mellitus is a metabolic disorder associated with a chronic increase in blood glucose levels in the context of insulin resistance and relative impaired insulin signaling (Goldberg & Coon, 1987; Sato, 2000). Type 2 diabetes is also characterized
by impaired glucose tolerance (Zimmet et al, 1984), dyslipidaemia (Kelley et al, 2001) and beta-cell dysfunction (Raskin, 1985). The development of Type 2 diabetes is due to combined environmental and genetic factors. The onset of this disease can be prevented through, improving personal lifestyle, such as a healthy diet and exercise (Sato, 2000).

1.6.3 Insulin resistance

Insulin resistance is a key component of the metabolic syndrome. The reduced peripheral tissue response to insulin is a characteristic feature of insulin resistance and this leads to hyperglycemia. Insulin action is reduced in adipose tissue and skeletal muscle, which may lead to decreased glucose uptake and storage. Moreover, in the insulin resistant state, an increased release of fatty acids from adipose tissue may decrease insulin secretion from pancreatic beta cells and further reduce insulin signaling and action on adipose tissue, liver and skeletal muscle (Bogardus et al, 1984; DeFronzo et al, 1985; Iozzo et al, 2003).

1.7 INTRINSIC SEX DIFFERENCES AND INSULIN RESISTANCE

The current understanding of sex differences in insulin resistance and the molecular mechanisms associated with development of metabolic syndrome is limited, since most studies have been conducted in men or male animals. However, there is evidence suggesting sex differences play a role in the development of metabolic disorders. The prevalence of Type 2 diabetes and impaired glucose metabolism is higher in men when compared to women (Blaak, 2008). Abdominal fat (visceral fat) shows a greater association with insulin resistance so sex differences are likely to play a role. Indeed men have little subcutaneous abdominal fat and women generally have much less visceral fat (Krotkiewski et al, 1983). Greater amounts of visceral and hepatic adipose tissue are related to a higher degree of insulin resistance in men as compared to women with similar body mass index (Geer & Shen, 2009). The regional fat distribution in women, might explain the complexity of increased total fat mass in women compared to men, but with less risk for the development of metabolic syndrome. However higher intra-myocellular lipid content in women as compared to men increases the potential risk of insulin resistance (Steffensen et al, 2002). Women with a history of diabetes from the maternal side have a similar risk for developing Type 2 diabetes as men with family history on both the maternal and parental side (Kuhl et al, 2005).

1.8 SKELETAL MUSCLE INSULIN RESISTANCE

Insulin resistance in skeletal muscle is a major contributor to the metabolic deregulation, which is associated with obesity and physical inactivity, and also contributes to the development of the Type 2 diabetes (Nistala & Stump, 2006; Zierath et al, 2000). Lean healthy individuals, challenged by hyperinsulinemic-euglycemic clamps, are able to increase the glucose storage and oxidation compared to fasting conditions. In contrast, this ability is attenuated in obese insulin-resistant individuals. Moreover, an insulin-mediated reduction of fatty acids was observed in the lean control group but not in obese individuals (Kelley et al, 1999). Thus reduced metabolic flexibility and skeletal muscle insulin insensitivity contributes to the development of
metabolic syndrome and Type 2 diabetes (Corpeleijn et al, 2009; Phielix & Mensink, 2008).

1.9 SEX STEROID HORMONES IN INSULIN RESISTANCE

Alteration in serum levels of sex steroid hormones in aging or disease is associated with the development of the metabolic syndrome. Estrogens or testosterone replacement therapy in postmenopausal women with Type 2 diabetes or obese men, respectively improves insulin sensitivity (Andersson et al, 1997; Marin et al, 1992). Therefore, the diverse effect of sex hormones on insulin action suggests that there is a ‘physiological window’ for the action of sex steroids on insulin sensitivity (Holte, 1996). The endocrine disease, polycystic ovary syndrome (PCOS) is a disorder that is associated with female reproductive abnormalities. Patients with PCOS display several metabolic abnormalities including increased testosterone levels, impaired glucose metabolism and reduced insulin action in peripheral tissues (Dunaif, 1997; Venkatesan et al, 2001). As women with PCOS have increased risk of developing Type 2 diabetes and hypertension (Zacur, 2001), testosterone deficiency in men is defined by changes in body composition, increased body fat content and reduced muscle mass (Zitzmann, 2008).

Many studies on supra-physiological administration of sex steroids to female or male transsexuals (Polderman et al, 1994) or bodybuilders (Cohen & Hickman, 1987) have provided evidence that sex steroids induce peripheral insulin resistance. Moreover, the use of oral contraceptives containing estrogens and progestin is associated with the development of insulin resistance, especially in women with a history of gestational diabetes (Buffington et al, 1993; Godsland et al, 1992). Thus, evidence suggests that sex steroids have a direct action on insulin sensitivity, and it is tempting to speculate that skeletal muscle may be a target.

1.10 GLUCOCORTICOIDS IN INSULIN RESISTANCE

Disturbance of the hypothalamus-pituitary-adrenal cortex axis (HPA), results in increased levels of glucocorticoids, which in extreme situations results in Cushing’s syndrome (Hermus et al, 1988). Cushing’s syndrome is associated with metabolic disorders including insulin resistance with increased risk of Type 2 diabetes (Hermus et al, 1988). In general, glucocorticoid excess reduces insulin sensitivity and consequently insulin-mediated glucose uptake in skeletal muscle by directly disturbing insulin signaling and glycogen synthesis (Henriksen et al, 1999).

1.11 11β-HSD1 AND INSULIN RESISTANCE

The enzyme 11β-HSD1, which reduces the inactive hormone cortisone to the active hormone cortisol, has an important role in metabolism (Morton, 2010; Paterson et al, 2004). Study of 11β-HSD1-knockout mice has shown that these animals are protected from high-fat diet-induced pre-adipocyte differentiation and obesity, and specific aspects of glucocorticoid-mediated diabetes are improved. In contrast, over-expression of hepatic 11β-HSD1 triggers insulin resistance and hepatic/lipid synthesis flux (Paterson et al, 2004). Enhanced expression of 11β-HSD1 and an increased local generation cortisol in skeletal muscle have been identified in obese insulin resistant patients, although cortisol plasma concentration was in the normal range (Whorwood et al, 2002). Consistently, elevated levels of adipose tissue 11β-HSD1 mRNA, and
decreased levels of 11β-HSD2 mRNA have been found in clinical studies in obese patients (Desbriere et al, 2006; Engeli et al, 2004; Paulsen et al, 2007), and human obesity has been associated with locally increased cortisol levels in adipose tissue as compared to lean counterparts (Baudrand et al, 2010; Sandeep et al, 2005).

Increased 11β-HSD1 activity has been reported in adipose tissue and liver obtained from postmenopausal normal weight women, which may contribute to metabolic dysfunction with menopause and aging (Andersson et al, 2009). Together, these studies highlight the critical importance of intracellular cortisol bioactivity, which is mediated by 11β-HSD1 expression in specific target tissues during the development of the metabolic syndrome.

1.12 PRIMARY SKELETAL MUSCLE CELL CULTURE

The molecular pathways and mechanism which can clarify the role of skeletal muscle in glucose disposal and metabolism in physiological and pathophysiological states are incompletely understood. Different methods have been used to study human skeletal muscle metabolism, such as intact muscle obtained from surgical procedures or animal models (Dohm et al, 1988; Zierath, 1995). However, limitation in incubation times, tissue access, surgical difficulties to get intact sample and metabolic viability of isolated skeletal muscle highlight a need for additional platforms to study this tissue. Therefore, it is of great interest to use a cellular model which closely mimics intact skeletal muscle. Human skeletal muscle cells (HSMC) are a useful tool to study insulin-mediated glucose metabolism and molecular mechanisms of insulin resistance in Type 2 diabetes (Henry et al, 1995). However, these cells may not fully present a mature skeletal muscle phenotype, as they have a low mRNA expression level of the specific skeletal muscle glucose transporter, GLUT4 and an increased expression of GLUT1, which is low expressed in adult skeletal muscle. However, GLUT4 content increases in differentiated myotubes, highlighting the possibility that myotubes might be preferable to myoblasts in the study of insulin action (Al-Khalili et al, 2003).

Genetically modified animal models including knock-out or transgenic animals have provided a useful tool to advance the understanding of skeletal muscle metabolism. In skeletal muscle cell cultured cells, over expression or down-regulation of target genes using siRNA technology has provided an opportunity to investigate the specific underlying molecular mechanism for insulin resistance in skeletal muscle (Bouzakri et al, 2006; Krook & Zierath, 2009). The application of these technologies may help to advance the understanding of the molecular mechanism for the development of metabolic diseases such as obesity and Type 2 diabetes. Inherited genetic components have been investigated in primary muscle cell cultures obtained from skeletal muscle from Type 2 diabetes patients, and obese people (Bouzakri et al, 2005; Henry et al, 1996; Hulver et al, 2003).

Human skeletal muscle cells also provide a suitable model to study more specific local action of hormones, and metabolic drugs (agonists/antagonists) to modulate insulin signaling and substrate metabolism in skeletal muscle. Primary muscle cell cultures can be derived from relatively small amounts of human skeletal muscle. Thus, these cultures are useful tools for studying aspects of skeletal muscle metabolism in human tissue influenced by disease or sex origin and aging since the metabolic characteristics of the donor are retained and experimental manipulations can be easily performed.
2 AIMS

The overall goal of this thesis work is to identify the role of steroid hormones on glucose and lipid metabolism; and to dissect the impact of these hormones on signaling pathways in human skeletal muscle. A further aim is to understand whether glucose and lipid metabolism in skeletal muscle is sex dependent. These investigations will provide further insight into the molecular actions of steroid hormones and the possible cross-talk with the insulin signaling pathway.

The specific aims of this thesis are to:

• Investigate sex differences with respect to glucose and lipid metabolism in human skeletal muscle.
• Identify the role of sex hormones on metabolic outcomes in skeletal muscle and determine if their actions are sex dependent.
• Determine acute effect of sex hormones on glucose and lipid metabolism.
• Investigate the effect of glucocorticoids on skeletal muscle metabolism and gene expression by targeting 11 β-HSD1.
3 EXPERIMENTAL PROCEDURES

3.1 SUBJECTS CHARACTERISTICS

Biopsies were taken with a Weil-Blakesley conchotome from vastus lateralis skeletal muscle under local anesthesia (Lidocaine hydrochloride 5 mg/ml), approximately 100mg (Study I-III) or under general anesthesia from rectus abdominis during scheduled abdominal surgery, approximately 1-3g (Study IV). In Study I-III human skeletal muscle cell cultures were established from vastus lateralis muscle biopsies. In Study I and II muscle cells originated from post-menopausal women and age-matched men, which were selected to ensure a relatively comparable hormonal milieu. From this latter cohort, one of the female participants received estrogen replacement therapy (Vagifem 0.25mg 2x/w). Data was calculated with or without including these subjects, and since the values obtained from this subject fell within the normal range, this individual was included in all data analysis.

In Study IV, human skeletal muscle cell cultures were established from rectus abdominis muscle biopsies from subjects scheduled for abdominal surgery. In contrast to satellite cells obtained from skeletal muscle from birds and rodents, human satellite cells are not distinct to fast and slow lineages and myotube cultures prepared from human skeletal muscle co-express slow and fast myosin heavy chains independently of the source of the biopsy (Bonavaud et al, 2001). Therefore, we hypothesize that data from cells established from vastus lateralis (Study I-III) and rectus abdominis (Study IV) are generally comparable.

3.2 ETHICAL APPROVAL

Informed written and verbal consent was received from all participating subjects. The Ethical Committee at the Karolinska Institutet approved all study protocols.

3.3 DETERMINATION OF SERUM CYTOKINE AND APOPROTEIN CONCENTRATIONS

HADK2-61K-B04 (cytokine) and APO-62K-06 (apolipoprotein) assay kits from Linco Research (Electra-box Diagnostica AB, Tyresö, Sweden) were used to assess cytokine and apolipoprotein concentrations in plasma samples, according to the manufacturer’s protocol. Plasma samples from each subject were analyzed in duplicate and quantified using a Luminex Bio-Plex 200 System (Bio-Rad, Stockholm, Sweden). The Luminex technology utilizes flow cytometry in combination with color-coded beads coated with antibodies against each respective target analyte. Constructing a standard curve (supplied by the manufacturer) for each analyte makes it possible to translate the intensity of the fluorescence to a concentration. Calculations were done with the Bio-Plex Manager program (Bio-Rad) (Study I).

3.4 HUMAN SKELETAL MUSCLE CELL CULTURE

3.4.1 Isolation of satellite cells

Skeletal muscle biopsies were collected in cold phosphate-buffered saline (PBS) containing 1% PeSt (100 units/ml penicillin, 100 µg/ml streptomycin). Collected biopsies were stored at 4°C for one day, in order to increase the ratio of satellite cells to
possible contaminating cells (in particular fibroblasts). Biopsies were dissected free from visible connective and adipose tissue, finely sliced and transferred to a digestion solution including trypsin-EDTA, followed by incubation with gentle agitation at 37°C for 10 min. Undigested tissue was allowed to settle and then liberated satellite cells were transferred to growth media (DMEM/Ham’s F12 medium supplemented with 20% fetal bovine serum (FBS), 1% PeSt and 1% Fungizone; Gibco, Invitrogen, Stockholm, Sweden). The remaining tissue was repeatedly digested with new trypsin-EDTA for 10 min, and thereafter for 15 min. Media containing liberated satellite cells were pooled and subjected to centrifugation for 10 min at 2000 g. The cell pellet was resuspended in growth media and left in a non-coated petri dish for 1h at 37°C to eliminate possible contamination of fast adherent (non-myogenic) cells. Finally, the satellite cell-containing media was transferred to cell culture flasks (Costar, Nordic Biolabs, Täby, Sweden) and kept at 37°C, in 7% CO₂. Media was changed every second to third day and the cells were sub-cultured when reaching approximately 70% confluence. Subculture passages 3-5 were used for experiments.

3.4.2 Human skeletal muscle cell culture

Cells were seeded in 6-well plates, petri dishes, or 25 cm² flasks (~10000 cells per cm²). Upon reaching 90% confluence, the differentiation of myoblasts into multinucleated myotubes was initiated by changing to differentiation media (DMEM supplemented with 4% FBS, 1% PeSt, 1% Fungizone). Two days later, serum levels were further decreased to 2% FBS for an additional three days, giving a total differentiation time of seven days. At this time point the expression of desmin, myocyte enhancer factor 2C (MEF2C) and the insulin sensitive GLUT4 transporter are significantly increased in myotubes after cell fusion and glucose metabolism can be readily determined (Al-Khalili et al, 2003b). Hormone treatment was started 15 min before insulin stimulation in myotubes in each assay (Study I-IV). In addition, pre-incubation with inhibitors was performed 30 min before hormone exposure in myotubes, as appropriate (Study II, III).

3.4.3 Giemsa/Wright staining

To ensure proper formation of multinucleated myotubes after differentiation, Giemsa/Wright staining was used on a regular basis. Cells were washed once with PBS, fixed in methanol for 10 min, incubated in 1:10 Giemsa for 15 min and 1:10 Wright for 20 min (Sigma Aldrich, Stockholm, Sweden). Thereafter, cells were washed with water and observed in light microscope.

3.5 GLUCOSE METABOLISM

Different assays have been used to measure glucose metabolism. Glucose incorporation into glycogen, glucose uptake and glucose oxidation were performed in different studies.

3.5.1 Glucose uptake

In Study IV, radioactively-labeled 2-deoxy-D-glucose was used to determine glucose uptake in myotubes. After incubation, radioactivity was measured in the cell lysates. 2-deoxy-D-glucose is a glucose analogue where the 2-hydroxyl group has been replaced by hydrogen. Like glucose, 2-deoxy-D-glucose is taken up by cells and
phosphorylated by hexokinase, but not further metabolized. Thus, the molecule is trapped inside the cell. By measuring the accumulation of the radio-labeled isotope, an estimate of glucose uptake can be determined.

Myoblasts were grown and differentiated in 6-well plates as described above. To measure glucose uptake, myotubes were serum starved overnight and thereafter were stimulated with or without insulin and as incubated in KREBS buffer (20 mmol/l HEPES, pH 7.4; 140 mmol/l NaCl; 5 mmol/l KCl; 2.5 mM MgSO4; 1 mM CaCl2). Thereafter, cells were incubated with 10 µM 2-deoxy-[3H] glucose (1Ci/ml) for 10 min. In the following step, cells were washed several times with cold PBS and lysed with 1 ml 0.4 N NaOH and radioactivity was determined in a WinSpectral 1414 liquid scintillation counter (Wallac). To determine non-specific uptake, parallel incubations were performed in the presence of 50 µmol/l cytochalasin B (Sigma Aldrich) and non-specific uptake was subtracted from the total. Cytochalasin B exposure reduced glucose uptake approximately 15%. Each experiment was performed in triplicate and normalized by protein concentration, as assessed by the Pierce method (BSA Protein Assay Kit, Thermo Scientific, Fisher Scientific GTF AB, Västra Frölunda, Sweden). The results were calculated as counts per minute.

### 3.5.2 Glucose incorporation into glycogen

Glucose incorporation into glycogen performed in Study II, III and IV. Glycogen synthesis was determined as 14C-glucose incorporation into glycogen. Myoblasts were grown and differentiated in 6-well plates. After overnight serum starvation cells were incubated with or without insulin for 30 min, followed by incubation with D-[U-14C] glucose (1 µCi/ml, final specific activity 0.18 µCi/µmol; Amersham) and other stimuli for 90 min. Cells were washed five times with ice-cold PBS and lysed in 1 ml 0.03% SDS. An aliquot (850 µl) of cell lysate was transferred to 10 ml tubes. The remaining cell suspension was used to determine protein concentration using the Pierce method. An aliquot (100 µl) of carrier glycogen (2 mg/sample) was added to the cell lysates and samples were heated at 95°C for 2 h. Glycogen was precipitated by adding 3 ml of 95% ethanol to the samples and incubated overnight at 4°C. Glycogen pellets were collected by centrifugation for 30 min at 2600 g, washed once with 3 ml 70% ethanol and resuspended in 400 µl water. Radioactivity was determined in a WinSpectral 1414 liquid scintillation counter. Each experiment was performed in triplicate and normalized by the protein concentration. The rate of glucose incorporation into glycogen was calculated by dividing the radioactivity counts by incubation time followed by translation into moles. Conversion of radioactivity counts to moles was done by measuring radioactivity in triplicate samples with a known concentration of glucose (5.56 mM glucose in media plus tracer level of radioactively marked glucose).

### 3.5.3 Glucose oxidation

Glucose oxidation was determined in Study III. This method is based on measuring the released 14CO2 as final glucose oxidation product in muscle cells. Myoblasts were cultured in 6 well plates and incubated with D-[U-14C]-Glucose (1 µCi/ml) with or without insulin and other stimuli. To avoid contamination from the radioactive medium, a small cup was placed in bigger cup in each respective well. The plate was covered with plastic film and fixed in a specially designed plate holder and
incubated for 4 hours at 37 °C. Thereafter, the 14C labeled CO2 was released from the medium by the addition of 2 M HCl (150 µl) with syringe. To avoid gas leakage, few drops of mineral oil was added to the plastic film. The released 14C labeled CO2 was thereafter trapped in the small cup after the addition of 2M NaOH (300 µl) with a syringe. Plates were incubated for further 1 hour at 37 °C. The small cups containing 14C labeled Na2CO3 were transferred to scintillation vials and the total 14CO2 was counted in a liquid scintillation counter. Each experiment was carried out in triplicate.

3.6 LIPID METABOLISM

Two different assays have been used to measure fatty acid β-oxidation. Both of these assays have been developed based on the end products of fatty acid oxidation, water and CO2. In the first method (Study II, IV) [14C]-labeled palmitate was used as substrate and the released [14C]-CO2 was trapped in a filter-compress and measured. In the second method (Study I, II) [3H]-labeled palmitate was used and the [3H]-labeled water was separated from the non-metabolized palmitate by activated charcoal and measured by scintillation counting.

3.6.1 14C palmitate oxidation assay, method 1

Cells were cultured in 25 cm² flasks and differentiated to myotubes. Before starting the experiment, a hole was made in each lid and two 24 mm Whatman ® filters were wrapped in compresses and attached to the inside of the lid. Following overnight serum starvation cells were incubated in 2 ml serum-free DMEM supplemented with 4% fatty acid free BSA, 1.0 mmol/l palmitate, and 0.4 µCi [U-14C] palmitic acid (200 µCi/ml, final specific activity 793 mCi/mmol; Amersham), with or without insulin for 120 min at 37°C. Thereafter, a 300 µl aliquot of 70% perchloric acid was added to the medium to move the equilibrium of CO2 to the gas phase. The lids were secured with Parafilm®, and the flasks were incubated under slight agitation for 1 h at room temperature. The filter-compress was transferred to a scintillation tube with 200 µl of ice-cold methanol. The trapped [14CO2] in the filter-compresses was measured in a liquid scintillation counter. All results were normalized by protein concentrations and expressed as counts per mg protein per min.

3.6.2 3H palmitate oxidation assay, method 2

Myotubes cultured in 6-well plates were washed once with PBS and incubated in 1 ml of DMEM (1g glucose/L) supplemented with 0.2% fatty acid free BSA and 0.5 µCi 3H palmitic acid [9-10(n)-3H] (5 mCi/ml, final specific activity 52 Ci/mmol; Amersham), with or without insulin or 1 mmol/l 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) (Toronto Research Chemicals Inc., North York, Canada) (in Study I) for 4-6 h. To absorb non-metabolized palmitate 0.2 ml cell supernatant was mixed with a 0.8 ml charcoal slurry (0.05 g charcoal powder in 1 ml 0.02 mol/l Tris-HCl buffer, pH 7.5) and shaken for 30 min. Samples were centrifugated for 15 min at 15,700 g, after which 0.3 ml supernatant with tritium-bound water was withdrawn and radioactivity was determined in a liquid scintillation counter. Each experiment was performed in duplicate or triplicate wells and normalized by protein concentration. Results are expressed as counts per mg protein per min.

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An advantage of this method is that the cells themselves remain intact and therefore it is possible to determine mRNA or protein expression of the target gene. Simultaneous measurement of HSD1 protein expression in myotubes used for determination of $^3$H palmitate oxidation showed that siRNA-targeting 11 $\beta$-HSD1 prevented cortisone, but not cortisol-mediated effects on palmitate oxidation (Figure 8).

![Image](image_url)

**Figure 8**: Simultaneous determination of protein expression of 11 $\beta$-HSD1 and palmitate oxidation in cultured human muscle cells. Scrambled (Dark bar), HSD1 siRNA (Light bar).

### 3.6.3 Lipid uptake and accumulation

As an estimation of free fatty acid uptake, myotubes used for the $\beta$-oxidation experiments (Study I) were washed five times with Tris-buffered saline supplemented with 0.02% Tween-20 (TBST) and lysed in 0.4 mol/l NaOH. Accumulated $[^{14}C]$- or $[^3H]$-palmitic acid and its intra-myocellular lipid products were measured in the cell lysate in a liquid scintillation counter. Protein concentration was determined in each sample using the bicinchoninic acid (BCA) assay. Results are expressed as counts per mg protein per min.

### 3.7 QUANTITATIVE PCR

mRNA expression was measured by quantitative polymerase chain reaction (QPCR), also called real-time polymerase chain reaction (real-time PCR) using TaqMan technology in Study I, II and IV. This method is considered the most sensitive method for detection of low-abundance mRNA, obtained from limited sample materials.

TaqMan probes consist of a fluorophore which is used to add specificity to the reaction. The probe has a reporter fluorescent dye at the 5’end and a quencher dye in the 3’end, and probe will anneal downstream of the primer on the target sequence. As long as the quencher and reporter are in close proximity, no fluorescence signals can be emitted. During the PCR reaction the Taq DNA polymerase will cleave the probe and

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the reporter dye will no longer be inhibited by the quencher dye, giving a signal responding to the amplification of the target sequence.

Two methods of calculation are commonly used to quantify the results obtained from QPCR: the standard curve method and $C_T$ comparative method. The standard curve method is based on calculating the absolute ng amount of cDNA in each sample, by comparison to a known standard (Bustin, 2000). For this, running a standard curve is required for both the gene of interest and the endogenous control. To quantitate of mRNA level by the standard curve method a serial dilution of cDNAs from control sample (sample with no treatment) should be prepared. The dilution curve is then plotted for $C_T$ values vs. log ng of cDNA dilution. The best fit of standard curve data should have a correlation coefficient of 1.000 (Figure 9). To obtain a correct estimation of the amount of cDNA in the samples, the expression level of each sample should fall within the limits of the standard curve. The relative mRNA expression of target genes was calculated by normalization to a housekeeping gene.

![Figure 9: Standard curve for primers in real-time PCR method.](image)

The comparative $C_T$ method is based on the assumption that the primer efficiencies are relatively similar. The $\Delta C_T$ value is the $C_T$ value of any sample normalized to housekeeping gene [$\Delta C_T$ (sample) = $C_T$ (target gene) - $C_T$ (housekeeping gene)]. The mRNA expression of target genes was calculated based on the following formula (Livak & Schmittgen, 2001).

$$2^{-\Delta C_T}$$
### 3.7.1 PRIMERS AND PROBES

All TaqMan probes (Applied Biosystems) were chosen to span an exon-exon junction to avoid detection of genomic DNA (Table 1).

Table 1. TaqMan primer and probes used in the studies included in this thesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Primer and Probes name</th>
<th>Gene symbol</th>
<th>Order no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>Acetyl-CoA carboxylase</td>
<td>ACCa</td>
<td>Hs01046024_m1</td>
</tr>
<tr>
<td>IV</td>
<td>Acetyl-CoA carboxylase</td>
<td>ACCb</td>
<td>Hs0153715_m1</td>
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<tr>
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<td>AMP-activated protein kinase alpha 2</td>
<td>AMPKa2</td>
<td>Hs00178903_m1</td>
</tr>
<tr>
<td>II</td>
<td>Androgen receptor</td>
<td>AR</td>
<td>Hs00907243_m1</td>
</tr>
<tr>
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<td>Carnitine palmitoyltransferase 1B (muscle)</td>
<td>CPT1B</td>
<td>Hs00993896_m1</td>
</tr>
<tr>
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<td>Carnitine palmitoyltransferase 2</td>
<td>CPT2</td>
<td>Hs00988962_m1</td>
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<td>ERα</td>
<td>Hs01046816_m1</td>
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<td>Esterogen receptor b</td>
<td>ER2</td>
<td>Hs01112040_m1</td>
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<td>Fatty acid translocase</td>
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<td>Hs00178903_m1</td>
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<td>I, IV</td>
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<td>GYS1</td>
<td>Hs0157863_m1</td>
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<td>HK2</td>
<td>Hs01034050_g1</td>
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<tr>
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<td>Hydroxysteroid (11-beta) dehydrogenase 1</td>
<td>HSD11B1</td>
<td>Hs01547870_m1</td>
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<tr>
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<td>HSD11B2</td>
<td>Hs00388669_m1</td>
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<td>IRS1</td>
<td>Hs00388292_m1</td>
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<td>II</td>
<td>Insulin receptor substrate 2</td>
<td>IRS2</td>
<td>Hs01559472_m1</td>
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<tr>
<td>IV</td>
<td>Myocyte enhancer factor 2A</td>
<td>MEF2A</td>
<td>Hs01050409_m1</td>
</tr>
<tr>
<td>IV</td>
<td>Myocyte enhancer factor 2B</td>
<td>MEF2B</td>
<td>Hs04188747_m1</td>
</tr>
<tr>
<td>IV</td>
<td>Myocyte enhancer factor 2C</td>
<td>MEF2C</td>
<td>Hs00231149_m1</td>
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<tr>
<td>IV</td>
<td>Pyruvate dehydrogenase kinase, isozyme 4</td>
<td>PDK4</td>
<td>Hs0176875_m1</td>
</tr>
<tr>
<td>IV</td>
<td>Peroxisome proliferator-activated receptor δ</td>
<td>PPARD</td>
<td>Hs00602622_m1</td>
</tr>
<tr>
<td>IV</td>
<td>Peroxisome proliferator-activated receptor γ</td>
<td>PPARG</td>
<td>Hs00234592_m1</td>
</tr>
<tr>
<td>II, IV</td>
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<td>PGC1α</td>
<td>Hs0173304_m1</td>
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<tr>
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<td>Steroyl-CoA desaturase (delta-9-desaturase)</td>
<td>SCD</td>
<td>Hs01682761_m1</td>
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<tr>
<td>II</td>
<td>Sex hormone globulin binding</td>
<td>SHBG</td>
<td>Hs0168927_m1</td>
</tr>
<tr>
<td>II</td>
<td>Steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1</td>
<td>SRD5A1</td>
<td>Hs00602694_mH</td>
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<tr>
<td>II</td>
<td>Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2</td>
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<td>Hs03003719_m1</td>
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<td>II</td>
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<td>SREBP1</td>
<td>Hs01088691_m1</td>
</tr>
<tr>
<td>LII</td>
<td>TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1</td>
<td>TBC1D1</td>
<td>Hs00989083_m1</td>
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<tr>
<td>II, IV</td>
<td>Uncoupling protein 3 (mitochondrial, proton carrier)</td>
<td>UCP2</td>
<td>Hs01075225_m1</td>
</tr>
<tr>
<td>I, IV</td>
<td>Uncoupling protein 3 (mitochondrial, proton carrier)</td>
<td>UCP3</td>
<td>Hs01106505_m1</td>
</tr>
</tbody>
</table>
3.7.2 RNA extraction, cells

Total RNA was purified from cultured myotubes using Qiagen RNAeasy Kit (Qiagen, Solna, Sweden) according to the manufacturer’s protocol. Myotubes were grown in 60 mm² dishes. Cells were washed twice with ice-cold PBS, harvested in RNAeasy lysis buffer (RLT) buffer and mixed with an equal volume of ethanol. Samples were directly added to RNAeasy columns, which bind RNA and allows for several washing steps before the purified RNA is eluted in RNase-free water. RNA concentration was measured at 260 nm with a BioPhotometer (Eppendorf, VWR International, Spånga, Sweden).

3.7.3 cDNA synthesis and real-time PCR analysis

C DNA synthesis was performed with 1-3 µg RNA, using High Capacity cDNA RT kit (Applied Biosystems) (human biopsies, Study I) or SuperScript First-Strand Synthesis System for real-time PCR (Invitrogen) (myotubes, Study I, II and Study IV), according to manufacturer’s instructions. All RNA was DNase treated before reverse transcription (RQ1 RNase-free DNase, Promega, and Southampton, UK). mRNA from human biopsy samples (Study I) were loaded onto a TaqMan Low Density Array custom designed card (Applied Biosystems) and real-time PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system. mRNA expression in cultured myotube (Study I, II and IV) was quantified with the ABI PRISM 7000 Sequence Detector System (Applied Biosystems). Real-time PCR was performed in a final volume of 25 µl and thermal cycling conditions were set to 50°C for 2 min and 95°C for 10 min, followed by 40 cycles with 95°C for 15 sec and 60°C for 1 min.

3.8 SHORT INTERFERING RNA

Short interfering RNAs (siRNAs) are small double stranded RNAs which interfere with expression of specific target genes mediated by siRNA pathway (Fire et al, 1998).

3.8.1 siRNA constructs

A pool of siRNA was used to knock down expression of targeted genes in myotubes (Table 2).

Table 2. siRNA constructs used in Study IV.

<table>
<thead>
<tr>
<th>Study</th>
<th>siRNA construct</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>SMART pool siRNA HSD11B1</td>
<td>Darmacon</td>
</tr>
<tr>
<td>IV</td>
<td>SMART pool siRNA PDK4</td>
<td>Darmacon</td>
</tr>
</tbody>
</table>
3.8.2 Transfection

siRNA transfection was performed as previously described (Al-Khalili et al., 2003a) using the transfection agent Lipofectamine 2000 (Invitrogen). In Study IV, HSD1 and PDK 4 siRNAs were transfected into cells following four days of exposure to glucocorticoid hormones to determine. In general, myotubes were washed once with PBS before transfection and kept in antibiotic-free DMEM. The siRNA mixture (4 µl of 20 nmol/l stock solution plus 46 µl of DMEM, mixed for 5 min) was incubated with the Lipofectamine 2000 mix (1 µl plus 49 µl of DMEM, mixed for 5 min), agitated for 30 min and 100 µl/ml was added to the myotubes. After 16-18 hours, transfection media was changed to fresh differentiation media. Myotubes were used for specific assays, at the 3rd day after transfection, following overnight serum starvation.

3.8.3 Calculations

The relative abundance of target transcripts was calculated from duplicate samples after normalization of the data against an endogenous control (housekeeping gene). The endogenous control corrects for errors arising from an unequal amount of starting material, different efficiency of cDNA synthesis or loading. In total, two to three different housekeeping genes were tested (GAPDH, 18S ribosomal RNA, 18S rRNA), β-actin or β2-microglobulin) in each study. The mRNA expression of the housekeeping genes were unchanged between the samples analyzed. In Study I, II and IV, 18S rRNA was chosen as the most stable housekeeping gene in human myotubes, while β2-microglobulin was chosen in human biopsy samples (Study I). The quantity of mRNA expression was calculated using the standard curve method (Study I and IV) or comparative Ct method (Study I, II).

3.9 Western Blot

Protein expression was determined by Western blot analysis. Cells were cultured in 100 mm² dishes, washed once with ice-cold PBS and harvested by scraping into ice-cold lysis buffer (137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l MgCl₂, 0.5 mmol/l Na₃VO₄, 1% Triton X-100, 10% [vol/vol] glycerol, 20 mmol/l Tris [pH 7.8], 10 µg/ml leupeptin, 0.2 mmol/l phenylmethysulfonyl, 10 mmol/l NaF, 10 µg/ml aprotinin, 1 mmol/l EDTA, 1 mmol/l DTT, 5 mmol/l natriumpyrophosphate, 1 mmol/l benzamide). Lysates (10-20µg protein) were applied to a 12-6% SDS-PAGE gradient gel and separated proteins were transferred to Immobilon-P membranes (Millipore, Solna, Sweden). The membranes were blocked with 7.5% nonfat milk and probed with primary protein-specific antibodies and appropriate secondary horseradish peroxidase-conjugated antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL Western blotting detection reagent, GE Healthcare, Amersham) and quantified by densitometry using Quantity One computer software (Bio-Rad). To assess equal loading GAPDH protein expression was determined (Table 3).
Table 3. List of antibodies used in the studies included in this thesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>Actin</td>
<td>Cell Signaling Technology, MA, USA</td>
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<tr>
<td>III</td>
<td>ACC Ser79</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>II, III</td>
<td>AMPK Thr172</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>II</td>
<td>AMPK</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>II, III</td>
<td>Akt Ser473</td>
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</tr>
<tr>
<td>III</td>
<td>Akt Thr368</td>
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<tr>
<td>II</td>
<td>AKT</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>II, III</td>
<td>ERK1/2 Thr202/204</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>II, III</td>
<td>GAPDH</td>
<td>Cell Signaling Technology, MA, USA</td>
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<tr>
<td>IV</td>
<td>HSD1</td>
<td>Gift from Biovitrum, Stockholm, Sweden</td>
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<tr>
<td>III</td>
<td>IRS1 pY612</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>III</td>
<td>IRS1 Ser636</td>
<td>Cell Signaling Technology, MA, USA</td>
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<tr>
<td>IV</td>
<td>PDK4</td>
<td>Abgent, CA, USA</td>
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<td>II, III</td>
<td>p38 Thr180/Tyr182</td>
<td>Cell Signaling Technology, MA, USA</td>
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<tr>
<td>II</td>
<td>P38</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
<tr>
<td>III</td>
<td>S6K1 Ser389</td>
<td>Cell Signaling Technology, MA, USA</td>
</tr>
</tbody>
</table>

3.10 STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical differences were determined by 2-way ANOVA, one factor or two factor repeated measurements and Student-Newman-Keuls method in Study II, III, IV or Student t-test in Study IV as appropriate. Before performing statistical analysis outliers were removed. Because of the experimental or measurements error some values could fall out of the normal range which can inappropriately skew the data. Quartile method is one of the simplest methods to calculate outliers. To apply this method, the median of the data set should be calculated, and then the upper and lower quartiles should be determined. Upper quartiles (Q3) is the data point which 25 percent of data are larger than median and lower quartile (Q1) is the data point that 25 percent of the data are smaller than median. By subtracting Q1 from Q3, the internal quartile (IQ) is calculated (Devore, 2000). The upper and lower outliers were calculated in excel by following the formula below.

\[ Q1-1.5\times(Q3-Q1) > \text{lower outlier} \]

\[ Q3+1.5\times(Q3-Q1) < \text{upper outlier} \]
4 RESULTS
4.1 INTRINSIC SEX DIFFERENCES IN HUMAN MYOTUBES

As primary skeletal muscle cell culture is a useful cell system in the study of metabolism and insulin action, it is of interest to take into consideration the sex origin of these cells. There is ample data to suggest that metabolic control is different between men and women. Therefore the aim of Study I was to investigate the possible existence of intrinsic sex differences in metabolism at base line in human skeletal muscle cells obtained from men and women, and whether isolated human skeletal muscle cell cultures retain genetic memory of their sex origin when compared to the muscle biopsies from which they were derived.

4.1.1 Subject characteristics

The clinical characteristics of 11 age-matched men and 10 postmenopausal women and serum cytokine and adipokine levels are presented in Study I, Table 1 and Table 2, respectively. All the subject characteristic was similar between females and males, except height and hemoglobulin level. Leptin levels in female subjects tended to be higher than male subjects, and that is consistent with the body fat content, which was higher in the female subjects, compared with the male subjects.

4.1.2 Metabolic assays and gene expression

To study intrinsic differences in metabolism between myotubes from female and male donors, assays to determine glucose incorporation into glycogen and palmitate oxidation were performed. In addition palmitate uptake and accumulation was also analyzed. Basal palmitate oxidation (Study I, Figure 2c) or uptake (Study I, Figure 2d) was similar between the sexes. Moreover the effect of AICAR (an AMP mimetic (ZMP), activator of AMPK) on palmitate oxidation (Study I, Figure 2c) or palmitate uptake (Study I, Figure 2d) was similar between myotubes from female and male donors. Furthermore, basal and insulin-stimulated glucose uptake (Study I, Figure2a) and incorporation into glycogen was similar between the sexes (Study I, Figure2b). Thus, this study provides evidence against sex-specific differences in glucose and lipid metabolism in skeletal muscle cell cultures obtained from older women and men.

The mRNA expression of several genes involved in both glucose and lipid metabolism were differently expressed in muscle biopsies obtained from men as compared to women. This was not evident in cultured myotubes. Although a trend towards a sex-dependent difference in the mRNA expression of some genes such as PPARG was observed (Study I, Figure 1). In this study, data was calculated using the standard curve method. However, when calculation of the gene expression data was performed using C_T comparative method (Study II), some of these trends became statistically significant (Study II, Table 1). Furthermore sex-related mRNA expression of several proteins involved in glucose metabolism such as GYS1, and PDK4 or lipid metabolism such as PPARD, PPARG and CPT1 was detected at the baseline in myotubes established from female and male donors. Hence Study I was likely underpowered to detect these differences. In addition, we found that using a similar QPCR quantifying method to calculate gene expression in myotubes and biopsies provides a better platform for mRNA expression comparison between the two systems.
4.2 SEX HORMONE EXPOSURE UNMASKS SEX-RELATED DIFFERENCES IN METABOLISM

Difference in systemic factors and sex hormones levels between men and women could be one of the possible contributing factors to the sex differences observed in vivo. Consequently, in Study II, role of testosterone or 17β-estradiol on metabolism and gene expression was investigated.

4.2.1 Clinical parameters

Myotubes obtained from a similar cohort of female and male subjects as reported in Study I were included in Study II. None of the male donors used hormone replacement therapy (HRT). One out of the 10 studied women received hormonal replacement therapy (Vagifem 0.25mg 2x/w). Analysis of data after excluding this subject did not alter results obtained for palmitate oxidation, glycogen synthesis, mRNA expression or protein phosphorylation when compared to the collected data of all female subjects. Therefore the data presented in Study II are shown from all women including the HRT-treated subject.

4.2.2 Sex hormone-mediated effects on glucose and palmitate metabolism

To elucidate the possible role of sex hormones in skeletal muscle metabolism, glucose incorporation into glycogen and palmitate oxidation was determined. Two different physiological concentrations (1 and 10 nM) of either testosterone or estradiol were tested. Incubation of cultured cells in the presence of either hormone (10 nM) revealed a clear effect on palmitate oxidation in skeletal muscle myotubes (Study II, Figure I). Differentiated myotubes (3 days) were exposed to testosterone and 17β-estradiol for 4 days.
Testosterone enhanced palmitate oxidation in myotubes from female and male donors, while 17β-estradiol increased palmitate oxidation in myotubes only from male donors. To determine whether the effects on palmitate oxidation by sex hormones are mediated by the nuclear receptors, specific antagonist of androgen receptor (CDX) and estrogen receptor (ICI182, 780) were used in human skeletal muscle myotubes. Our data in study II provide evidence that inhibition of the androgen receptor reversed the testosterone-mediated effect on palmitate oxidation (Study II, Figure 3).

Using the ER antagonist (ICI) to determine the role of estradiol on palmitate oxidation, we observed a trend towards inhibitor-dependent blocking of the estradiol-mediated effects on palmitate oxidation in myotubes from male and female donors (Figure 10A) or only in male donors (Figure 10B). Interestingly this pattern was observed only in myotubes from male donors when compared with myotubes from female donors. The lack of significance is most likely due to the small n-size that we investigated, and thus we did not have sufficient power to detect an effect.

Figure 10: Effect of estrogen receptor inhibitor on estrogen-mediated increase in palmitate oxidation. Palmitate oxidation in myotubes from A, female (n=3) and male (n=3), or B, male (n=3). Results are mean ± SEM.

30
Testosterone treatment increased insulin-stimulated glucose incorporation into glycogen in myotubes in a sex-dependent manner. This response was only evident in myotubes obtained from female donors. Moreover exposure of myotubes to 17β-estradiol tended to enhance insulin-stimulated glucose incorporation into glycogen in myotubes established from female donors. Also the effect of either sex hormone to enhance insulin-stimulated pAKT was more potent in myotubes obtained from female donors (Study II, Figure 4, 5). We can therefore conclude that there is a strong-sex dependent effect on glucose incorporation into glycogen in response to both sex hormones, which is more potent in myotubes from female donors. However the molecular mechanism underlying this sex-dependent effect warrants further investigations.

Testosterone treatment increased AMPK and p38 phosphorylation in myotubes from both female and male donors. Thus in myotubes, the energy-sensing kinase (AMPK) and stress-sensing MAP kinase (p38) responded more readily to testosterone, with no clear sex-specific differences noted (Study II, Figure 6, 7).

### 4.2.3 Sex hormone-mediated effects on gene expression

The effect of 4 days treatment of cultured myotubes from female or male donors with either testosterone or 17β-estradiol was determined on mRNA expression of several metabolic enzymes (Study II, Table 1, 2). In addition mRNA expression of sex-related genes, such as sex hormone receptors (estrogen receptor α [ERα], estrogen receptor β [ERβ], and androgen receptor [AR]) and sex hormone regulatory genes (enzyme P450 aromatase (P450arom) [CYP19A1], the sex hormone-binding globulin [SHBG], steroid- 5-alpha-reductase, alpha polypeptide 1 [SRD5A1] and steroid- 5-alpha-reductase, alpha polypeptide 2 [SRD5A2] was analyzed (Study II, Table 1, 2). The sex hormones enhanced mRNA expression of SHGB, compared to the baseline in myotubes from female donors. In addition mRNA expression of the androgen receptor was more potently increased in myotubes established from male donors (Study II, Table 1). Thus both sex hormones, altered the mRNA expression of some key regulatory genes, involved in glucose or lipid metabolism in a sex-dependent manner (Table 5).

<table>
<thead>
<tr>
<th>Genes</th>
<th>17β-Estradiol</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>CPT1</td>
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</tr>
<tr>
<td>IRS2</td>
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<td>PDK4</td>
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<td></td>
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<tr>
<td>PPARγ</td>
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</table>

Table 5: Sex-related mRNA expression of regulatory genes in glucose and lipid metabolism.
Taken together, in Study II, we provide evidence that sex hormone exposure unmask sex-related differences in human skeletal muscle metabolism, and gene expression.

4.3 ACUTE EFFECT OF SEX HORMONES ON SKELETAL MUSCLE METABOLISM

High serum sex steroids levels contribute to the development of insulin resistance (Gonzalez et al, 2000). Consequently, in Study III, we investigated the acute effects of high-dose testosterone or 17 $\beta$-estradiol treatment on metabolism in primary muscle cells.

4.3.1 The effect of acute sex hormones exposure on glucose and lipid metabolism

To elucidate the effects of sex hormones on skeletal muscle cell metabolism, glucose incorporation into glycogen, glucose oxidation and palmitate oxidation were performed. The effect of acute exposure to testosterone or estradiol was determined on glucose incorporation into glycogen at different concentrations, (10 nM-10 $\mu$M) as shown in Study III, Figure 1. Based on these results, a hormone concentration of 10 $\mu$M was selected as the most effective dose. Acute exposure to either testosterone or 17 $\beta$-estradiol reduced basal and insulin-stimulated glucose incorporation into glycogen (Study III, Figure2a) and insulin-stimulated glucose oxidation (Study III, Figure 2b). In addition, the acute effect of supra-physiological doses of sex hormones on skeletal muscle metabolism was sex-independent.

To determine whether the negative effect on glucose incorporation into glycogen by sex hormones is mediated by the respective nuclear receptors, the role of androgen receptor antagonist (CDX) or estrogen receptor antagonist (ICI 182, 780) was assessed. We provide evidence that the effect of testosterone or 17 $\beta$-estradiol to reduce glucose incorporation into glycogen was prevented following inhibition of the androgen or estrogen receptor respectively (Figure 11). However, the negative effect of sex hormones on glycogen synthesis was not restored to the control level. The lack of a conclusive result is most likely due to the small n-size (n=4) that we have investigated thus far, and therefore we did not have sufficient statistic power to detect an effect. Further investigation is warranted to determine the role of sex steroid receptor-mediated signaling in glycogen synthesis and glucose metabolism.
Figure 11: Acute effect of sex hormones and inhibitors on glucose incorporation into glycogen. Myotubes were treated for 2 hours with 10 µM testosterone or 10 µM 17β-estradiol combined with sex hormone receptor inhibitors in the absence (white bar) or presence of 120 nM insulin (dark bar). Results are mean±SEM, n=4-19. #p<0.05; sex hormone treatment vs. control and *p<0.05; insulin vs. basal in each group.

To further determine the causes behind the negative role of high dose of sex hormones on glycogen synthesis, we have investigated several possible hypothesis, such as a metabolic shift from glycogen synthesis towards lipid oxidation. Our data in Study III demonstrates that acute testosterone exposure increased basal palmitate oxidation, whereas insulin-mediated suppression of palmitate oxidation was detained by testosterone treatment in myotubes (Study III, Figure 3).

4.3.2 Acute effect of sex hormones on signaling pathways

The impact of sex hormones on intracellular signaling in myotubes was measured by assessing phosphorylation of key proteins in the insulin signaling and stress kinase pathways (Study III, Figure 4). The sex hormones enhanced insulin-stimulated AKT ser\(^{473}\) phosphorylation. In contrast the phosphorylation of p38, JNK and S6K were reduced after acute testosterone exposure (Study III, Figure 4C, D, E). 17 β–estradiol treatment decreased basal JNK phosphorylation (Study III, Figure 4D) and increased insulin-stimulated IRS1 ser\(^{636}\) phosphorylation (Study III, Figure 4B). Data from Study III indicate a negative effect on glycogen synthesis after an acute exposure to the sex hormones. However, it was not possible to directly link this effect to alterations in canonical insulin signaling in skeletal muscle cells. Further experiments should be performed to dissect the underlying molecular mechanism for the effect of an acute sex hormone exposure on gene expression regulation, protein synthesis, as well as the possible role of sex steroid receptors. Moreover, investigations of the role of the signaling molecules AKT and JNK on the sex hormone effect on metabolism would be of interest. Whether acute sex hormone action occurs through the signals from the cytoplasm or from a secondary response to rapid actions of hormones via genomic pathways remains to be determined.
4.4 EFFECT OF 11B-HSD1 ON GLUCOCORTICOID ACTION IN SKELETAL MUSCLE CELLS

Glucocorticoids have essential effects on whole body metabolism, yet the impact of glucocorticoids on metabolism in skeletal muscle is poorly understood. Therefore, in Study IV, we aimed to study the effect of cortisone or cortisol on glucose and lipid metabolism in human skeletal muscle cell cultures and to determine the role of the intracellular enzyme 11β-HSD1 on glucocorticoid-mediated effects on metabolism.

4.4.1 Effect of glucocorticoids on skeletal muscle metabolism:
no evidence for sex difference

Effect of chronic exposure of myotubes from female and male donors to either active or inactive glucocorticoids, cortisol or cortisone, respectively was determined on glucose metabolism. Chronic treatment of myotubes with either cortisone or cortisol reduced basal glucose uptake (Study IV, Figure 1b) and incorporation into glycogen (Study IV, Figure 1c). Since an aim of this thesis is to understand whether the sex of the donor influences the hormonal response, we first determined the mechanism by which cortisone or cortisol impacts metabolism in myotubes derived from male or female donors. The effect of cortisone and cortisol on substrate metabolism was similar between the sexes (Figure 12a, b). These results also indicate that glucocorticoids induce a shift from glucose towards lipid oxidation.

![Figure 12: Effect of glucocorticoids exposure on substrate metabolism in skeletal muscle cells obtained from male and female donors. Primary skeletal muscle cells were exposed to 0.5 µM cortisone or cortisol for 8 days. Glucose incorporation into glycogen (a) or palmitate oxidation (b) was determined. Results are mean ±SEM, n=4-5 in each group. Myotubes from female donors (light bar), and myotubes from male donors (dark bar). #p<0.05 vs. basal for each condition.](image-url)
4.4.2 11β-HSD1 siRNA reversed glucocorticoids effects on metabolism and gene expression.

11β-HSD1 is a key enzyme regulating glucocorticoid effects in target tissues. The role of 11β-HSD1 in glucocorticoid-mediated effects on skeletal muscle metabolism is poorly understood. We demonstrate that the effect of chronic exposure to cortisone, but not cortisol, on glucose and lipid metabolism was reversed using specific siRNA against 11β-HSD1 (Study IV, Figure 3). Cortisone and cortisol treatment independently increased mRNA and protein expression of 11β-HSD1. However, the effect of glucocorticoids on 11β-HSD1 mRNA and protein expression was reduced to below basal level after silencing the 11β-HSD1 with siRNA transfection (Study IV, Figure 2a, b). Similarly, the cortisone-mediated effect on glucose and lipid metabolism was prevented by using a specific inhibitor of 11β-HSD1 in myotubes. Therefore 11β-HSD1 is a candidate target to reduce the negative actions of chronic cortisone exposure on skeletal muscle glucose metabolism. Moreover cortisol can mediate metabolic derangements in skeletal muscle tissue via induction of 11β-HSD1 activity at the mRNA and protein expression level. To validate the siRNA specific mediated knockdown of 11β-HSD1 and detect possible siRNA off-target effects in skeletal muscle cells, mRNA expression of 11β-HSD2 was measured after exposure to glucocorticoids (Figure 13). However 11β-HSD2 expression was unaltered by 11β-HSD1 siRNA transfection.

![Graph showing the effect of HSD1 siRNA on mRNA expression of 11β-HSD2. Myotubes were treated with glucocorticoids and were transfected with scrambled sequence (Dark bar) and HSD1 siRNA (Light bar). Results are mean±SEM, n=7-8.](image)

Glucocorticoids act as nuclear transcription factors and mediate cellular effects via gene expression changes in nucleus. The mRNA expression of some key regulatory genes in glucose and lipid metabolism was unaltered in response to glucocorticoids in skeletal muscle cells (Study IV, Table 2). The mRNA expression of glucocorticoid receptor alpha (GRα) was decreased by cortisol exposure, also glucocorticoids profoundly enhanced PDK4 expression at mRNA and protein level in myotubes.

The role of 11β-HSD1 on the glucocorticoid-induced effect on PDK4 expression was determined. PDK4 expression was reduced in myotubes by HSD1 siRNA after
cortisone exposure. To understand the molecular mechanism for the negative effect of glucocorticoids on glycogen synthesis, PDK4 expression was inhibited by specific siRNA. The inhibitory effect of cortisol on basal glucose incorporation into glycogen was retained to the basal level via a PDK4 dependent down-regulation in myotubes (Study IV, Figure 4c). Therefore PDK4 is a key molecule responsible for the cortisol-mediated metabolic shift in substrate metabolism in skeletal muscle cells.
5 DISCUSSION

5.1 INTRINSIC SEX DIFFERENCES IN HUMAN MYOTUBES

Skeletal muscle is an important organ in metabolism and insulin action (DeFronzo et al, 1985). Using human skeletal muscle biopsies to study skeletal muscle metabolism has some restrictions due to limitation in incubation time or the influence of extracellular factors (hormones, substrates, cytokines) released from the environment of the isolated biopsy. Thus in vitro cell culture systems are valuable for studies of metabolic regulation to eliminate any whole body hormonal effect. We have used cultured human skeletal muscle cells as an in vitro model to study the effect of insulin or other stimuli such as sex steroid hormones (Study I-III) and steroid glucocorticoids (Study IV). Sex differences between men and women exist in body composition and metabolism at rest or during exercise, therefore first we aimed to identify whether intrinsic sex differences exist in primary muscle cell cultures obtained from male and female donors (Study I).

In Study I, we provide evidence against apparent intrinsic sex differences with respect to glucose and lipid metabolism in skeletal muscle cell culture. Since we found that glucose and lipid metabolism in cultured muscle obtained from either female or male donors were similar. This may indicate that studies performed in human myotubes from one sex are applicable to both males and females. To avoid large differences in the in vivo hormonal metabolic milieu, and to isolate the role of sex hormones from other hormones in the cell culture system, we selected post-menopausal women and age-matched men to be included in Study I-II. Female and male sex hormones levels are known to decline with aging (van den Beld et al, 2000). However, testosterone and estrogen levels were not assessed in our study participants. Furthermore, we have not determined metabolic parameters in vivo in Study I. For this we could have performed a euglycemic-hyperinsulinemic clamp to measure glucose metabolism before the muscle biopsies were taken. Additionally, an assessment of glucose uptake in isolated muscle would have strengthened our data to determine intrinsic sex differences in metabolic responses between men and women both in the in vivo and in vitro systems.

Furthermore we are able to show that there is a sex difference in myotubes at the base line by using C_T comparative method (in Study II) instead of the standard curve method (in Study I). This finding may suggest that our mRNA expression data has been underpowered to detect sex differences in Study I. The mRNA expression of some specific genes determined in skeletal muscle cell cultures was not similar to the mRNA expression determined in skeletal muscle biopsies (Table 4). Whether these differences have been identified due to the use of different housekeeping genes as internal controls is unclear. Moreover, these sex differences were only found in the mRNA levels, but intrinsic sex differences in glucose and lipid metabolism were not observed between the sexes.

Evidence suggests that men and women differ in substrate metabolism. The rate of basal lipid oxidation is lower in women than in men, independent of fat mass (Nagy et al, 1996). The fasting glucose level is greater in men than women, while women have a higher prevalence of glucose intolerance (Blaak, 2008). Thus, sex-related differences observed for in vivo metabolism may be influenced by the systemic milieu at the whole body level, and therefore sex-related differences in skeletal muscle cells might appear following sex hormone exposure as external factor. Sex hormones are possible contributing factors influencing in vivo gene expression patterns and metabolic
differences observed between men and women (Fu et al, 2009; Hamadeh et al, 2005; Muthusamy et al, 2007). Therefore, in Study II we investigated the direct role of testosterone or estradiol on skeletal muscle cell and hypothesized that treatment with either sex steroid hormone may reveal sex differences in metabolism and gene expression.

5.2 SEX HORMONE EXPOSURE UNMASKED SEX-RELATED DIFFERENCES IN METABOLISM IN MYOTUBES

Serum levels of sex hormones alter with aging and illness, and changes in the ratio of sex hormones may lead to development of metabolic disorders. Moreover, premenopausal women have a lower risk of developing cardiovascular disease and Type 2 diabetes as compared to age-matched men. However, this advantage is not evident after menopause in women (Mercuro et al, 2003). In Study II, we aimed to study whether testosterone or 17 β-estradiol hormone treatment plays a role in metabolism in primary skeletal muscle cells, and whether an intrinsic sex difference is evident after exogenous sex hormone exposure in skeletal muscle cultures.

In Study II we provide evidence that exposure to chronic testosterone or 17 β-estradiol at a lower (physiological) concentration, revealed sex differences in metabolism in human skeletal muscle cells. Moreover, each sex steroid hormone had a clear role in glucose and lipid metabolism. In other study, treatment with 17 β-estradiol in young men increased the beta oxidation capacity in skeletal muscle (Maher et al, 2010a). This is consistent with our finding of enhanced palmitate oxidation in myotubes from male donors after 17 β-estradiol exposure. Interestingly testosterone exposure enhanced palmitate oxidation in myotubes obtained from both sexes. Enhanced-palmitate oxidation was observed in testosterone-treated myotubes in concordance with increased AMPK$^{\text{Thr172}}$ phosphorylation. This finding indicates that the testosterone-mediated effects on lipid metabolism may be mediated via AMPK signaling. Testosterone might also regulate palmitate oxidation by inducing changes at the mRNA level. For example, IRS2 is an important regulator of skeletal muscle lipid metabolism in cultured myotubes (Bouzakri et al, 2006) and the $\text{IRS2 mRNA}$ expression was increased in response to testosterone treatment independent of the sex origin of muscle cells. Thus, we found that myotubes established from both men and women respond more potently to testosterone as compared to 17 β-estradiol with respect to lipid metabolism.

Nuclear hormone receptors are generally accepted as one of the mediators of sex steroid hormones action (Henderson & Hale, 1990). Steroid hormones may also mediate a more rapid effect via “non-genomic” signaling pathways (see section 1.6) (Baulieu & Robel, 1995). Thus, to elucidate the molecular mechanism mediating the effect of sex hormones on lipid oxidation, myotubes were treated with anti-androgen or anti-estrogen receptor inhibitors, CDX and ICI182 780, respectively. The sex hormone receptor inhibitors bind to the receptor and competitively inhibit sex hormone binding to the classical receptor, and in this way block their action via nuclear translocation (Wakeling, 1992). Pre-treatment with CDX prevented the positive effect of testosterone on palmitate oxidation in human myotubes. Therefore we conclude that testosterone exerts its effect on palmitate oxidation via nuclear androgen receptor. However, the effect of the estrogen hormone receptor inhibitor on the 17 β-estradiol-mediated effects on palmitate oxidation was inconclusive, due to the sex-dependent
effect of estradiol on palmitate oxidation. Thus, further investigation is warranted to determine the molecular mechanism of the estrogen-dependent signaling and the cause of sex differences in lipid oxidation (Study II).

Low testosterone levels in men, and higher testosterone level in women, are associated with increased risk of developing Type 2 diabetes (Ding et al, 2006). Thus the level of testosterone plays a critical role in insulin sensitivity. In Study II, a sex-dependent effect was evident on the ability of testosterone to influence glucose metabolism in myotubes from female subjects only. This was somewhat counterintuitive, and the reason for the enhanced sensitivity to testosterone in myotubes derived from female subjects remains to be identified. Consistent with our results noted for glucose metabolism, both sex hormones increased insulin-stimulated AKT phosphorylation in a sex-dependent manner. AKT is a key modulator of insulin signaling-related glucose metabolism (Datta et al, 1996; Krook et al, 1998). Therefore the enhanced glycogen synthesis mediated in response to testosterone exposure might be mediated by activation of AKT signaling in myotubes from female donors, but the underlying molecular mechanism should be further examined.

The role of MAPK’s in regulation of cellular homeostasis is complex and varies depending on tissues, the nature of the stimulus, and duration of activation (Johnson & Lapadat, 2002). Activation of p38 MAPK is involved in glucose uptake and is enhanced in response to cellular stress, possibly via interaction along a PI3-kinase-dependent signaling pathway (Kim et al, 2006). However, the activation of p38MAP kinase has also been suggested to have a direct link to insulin resistance in skeletal muscle (Henriksen et al, 2010). In Study II, the effect of testosterone on p38 MAPK phosphorylation was noted in myotubes from either sex. Whether this effect of testosterone on p38 MAPK phosphorylation plays a positive role on skeletal muscle metabolism to enhance insulin sensitivity in myotubes requires further investigation.

Sex–dependent differences in gene expression have been identified between men and women (Welle et al, 2008). In Study II, significant sex differences were notified in baseline mRNA expression of CPT1, PPARD, PPARG and SCD in cultured muscle cells. This is in agreement with previous reports providing evidence that mRNA of CPT1 and PPARD is higher in skeletal muscle biopsies from young women as compared to men (Maher et al, 2010b). We also noted that sex-dependent mRNA expression of CPT1 was enhanced after testosterone treatment, and this was higher in myotubes from female subjects. Furthermore mRNA level of PDK4 after 17β-estradiol treatment was potently increased to a greater extent in myotubes from female donors. The sex-dependent regulation of PDK4 in response to 17β-estradiol requires further investigation. PDK4 is a key enzyme in regulation of lipid oxidative flux. A positive effect of 17β-estradiol treatment on PDK4 gene expression has been reported in ovariectomized female rats (Campbell et al, 2003). PPARG mRNA expression was also altered by 17β-estradiol, but in myotubes from male donors only. Taken together, our current data suggests a ‘sex memory’ at the level of mRNA, which is further pronounced following exposure to sex hormones.

Sex hormones can be further metabolized to a different sex steroid hormone, in peripheral tissues (Jin & Penning, 2001; Longcope, 1987). Thus we have investigated whether testosterone can be aromatized to 17β-estradiol via the P450 aromatase or into its "bioactive" metabolite dehydrotestosterone, DHT, via the 5-alpha reductase SRD5A1 or SRD5A2. Our data indicates P450 aromatase and SRD5A2 mRNA expression was almost undetectable. However the mRNA expression of SRD5A1 was readily observed
in human myotubes. Thus we cannot exclude a possible local effect of converted DHT on skeletal muscle metabolism.

So far our findings provide evidence that the response to external exposure to sex steroid hormones depends on the sex origin of the cells with respect to glucose and lipid metabolism. This may then, suggest that sex hormones are one of the pivotal factors found in the *in vivo* systemic milieu which influences the different metabolic response between men and women.

### 5.3 ACUTE EFFECT OF SEX HORMONES ON SKELETAL MUSCLE METABOLISM

Sex steroid hormones play an important role in insulin sensitivity and whole body metabolism. However, several lines of evidence indicate that an increase in sex steroid hormones levels in the body may lead to pathological conditions such as insulin resistance (Livingstone & Collison, 2002). Therefore, our aim in Study III was to investigate the acute effect of a high dose of sex hormones on metabolism in skeletal muscle cells. Interestingly, an acute exposure of myotubes to either testosterone or 17 β-estradiol reduced basal and insulin-stimulated glucose incorporation into glycogen, and insulin-stimulated glucose oxidation. This finding provides evidence that sex hormones can cause a rapid negative effect on insulin-induced glucose metabolism. Accordingly, elevated level of sex hormones during pregnancy, puberty (Amiel et al, 1991; Bloch et al, 1987), or after administration of high doses of sex hormones to power lifters (Cohen & Hickman, 1987) is associated with insulin resistance and metabolic alterations in skeletal muscle. In contrast to our result from the chronic low dose exposure sex hormone treatment (Study II), an acute exposure of myotubes to a higher dose of sex hormones was independent of sex. This might suggest that acute sex hormone action at high concentrations may occur rapidly in a non-transcriptional manner.

We provided evidence that an acute testosterone treatment increases basal palmitate oxidation and disrupts the insulin-suppressive effect on palmitate oxidation. Furthermore increased basal palmitate oxidation might be indicating a substrate metabolic shift from glucose to lipid as fuel source in skeletal muscle cells. Moreover, treatment of human myotubes with ligand-bound sex hormone receptor inhibitors failed to rescue the inhibitory effect of sex hormones on glucose incorporation into glycogen. However, the data are preliminary and in order to draw any firm conclusions, further experiments are warranted. In addition, sex steroid hormone receptors can be differently localized within the cells: in the nucleus, cytoplasm or near the plasma membrane. Modification of diverse sex hormone receptors can induce alterations of different intracellular signaling cascades. Since the non-nuclear receptors are insensitive to nuclear-sex hormone receptor antagonists, use of anti-sex hormone receptors can provide evidence regarding which forms of receptors are modified during acute exposure to sex hormones.

Insulin-stimulated AKT phosphorylation was increased after 17 β-estradiol and testosterone treatment. This is similar to our signaling results following a chronic, low-dose exposure of human myotubes to sex hormones. In 3T3L1 adipocytes, exposure to a high concentration of estradiol (10µM, 16 hours) increases insulin sensitivity via alterations in IRS1 tyrosine and AKT phosphorylation. Estradiol reduced insulin-stimulated glucose uptake and p-AKT in adipose cells in dose dependent manner.
Furthermore, a low-dose of estradiol enhanced glucose uptake via IRS1 tyrosine phosphorylation. The latter effect was abolished by exposure to a specific estrogen receptor inhibitor (ICI 182, 780), which indicated that effect of estradiol at lower concentration on insulin signaling was mediated by nuclear estrogen receptors. Treatment of 3T3L1 adipocytes with 10 µM E2-BSA (impermeable estradiol) inhibited IRS1 tyrosine phosphorylation and serine phosphorylation of AKT. These results in adipocytes provides additional evidence that the estradiol effect at high concentration is mediated by cell membrane estrogen receptors (Nagira et al, 2006). Therefore to understand the diverse effect of an acute high dose sex hormone exposure on AKT signaling and glucose metabolism in skeletal muscle cells, further studies are necessary. However, future experiments will be performed using sex hormone receptors antagonists or sex hormone mimicking compounds in a time and dose dependent manner.

An acute exposure of cultured human muscle cells to sex hormones reduced JNK phosphorylation. Increased JNK activity has an inhibitory effect on glucose metabolism via phosphorylation of IRS1 on Ser307 (Aguirre et al, 2000). Therefore phosphorylation of IRS1 at this site and other serine sites should be investigated following acute sex hormone treatment. Acute testosterone treatment reduced basal and insulin stimulated p38 MAPK phosphorylation. This finding contrasts the increased p38 MAPK observed after a chronic low-dose exposure of myotubes to testosterone. Our result provides evidence that both acute and high-dose sex hormone treatment is associated with a negative effect on phosphorylation of stress MAP kinases. The role of p38 MAPK and interaction with PI3-kinase-dependent signaling pathways on glucose uptake was not determined in Study II. Thus a further treatment with specific p38-MAPK or JNK inhibitors in combination with acute sex hormone exposure may reveal the molecular mechanisms underlying the role of sex hormone on p38 MAPK and JNK in skeletal muscle metabolism.

Acute testosterone treatment reduced both basal and insulin-stimulated S6K phosphorylation. The anabolic effects of androgens are well established in skeletal muscle and testosterone has been used to enhance muscle growth and protein synthesis (Sinha-Hikim et al, 2002). Increased activity of mTOR signaling through phosphorylation of 4EBP1 and p70S6 (pS6K) kinase is a known mechanism which plays a critical role in determining cell size. Administration of DHT (the bioactive metabolite of testosterone) to castrated rats induced S6K phosphorylation after 6 hours in androgen-sensitive skeletal muscle (Xu et al, 2004). However our data provide evidence that S6K phosphorylation declined after a 20 min acute testosterone exposure. Further experiments should be performed to investigate the time-dependent effects of sex hormones on mTOR signaling. Assessing amino acid incorporation and protein synthesis might also highlight the anabolic effects of sex hormones on skeletal muscle.

5.4 EFFECT OF 11B-HSD1 ON GLUCOCORTICOIDS ACTION IN SKELETAL MUSCLE CELLS

Steroid hormones and systemic factors coordinately play important role to maintain metabolic balance. Steroid glucocorticoids and sex hormones imbalance is considered to be an important factor for the pathophysiology of obesity and metabolic syndrome (Pasquali et al, 2008). Glucocorticoids are essential hormones and thus, complete absence is incompatible with life (Lee & Gumowski, 1992). Physiological
levels of glucocorticoid are required for proper metabolic control, while an increased glucocorticoid level is linked to the development of the metabolic syndrome, Type 2 diabetes and obesity (Phillips et al, 1998). Local conversion of cortisone to cortisol in target tissues may determine the tissue specific role of glucocorticoids in pathophysiological states. Ablation of 11β-HSD1 in knockout mice suggests that 11β-HSD1 effectively amplifies glucocorticoid action in the liver, adipose tissue, and brain (Chapman et al, 1997; Kotelevtsev et al, 1997; Moisan et al, 1990). Therefore in Study IV, we investigated the effect of chronic glucocorticoid exposure on skeletal muscle metabolism, as well as the regulatory role of 11β-HSD1 on glucocorticoid-mediated effects on skeletal muscle metabolism and gene expression.

We show provide evidence that a chronic exposure of skeletal muscle cells to a high dose of glucocorticoids exerts negative effects on glucose metabolism. Clinical studies reveal that a long-term, exposure to a high-dose of glucocorticoids after renal transplantation reduces the rate of glycogen synthesis in parallel with decreases in glycogen synthase (GS) protein and activity in skeletal muscle biopsies of healthy volunteers and in kidney transplant patients (Ekstrand et al, 1996; Henriksen et al, 1999). 11β-HSD-1 expression and cortisol levels are increased in visceral adipose tissue as compare to subcutaneous adipose tissue in obese women (Veilleux et al, 2010). The peripheral actions of glucocorticoids are dependent on enzymatic interconversion by 11β-HSD-1 (Stewart & Kroowski, 1999). Physiological serum levels of the glucocorticoid hormones in obese subjects with concomitant enhanced peripheral action of these hormones in adipose tissue and skeletal muscle has highlighted 11β-HSD1 as a possible target for therapeutic intervention. Application of a selective 11β-HSD1 inhibitor in diabetic mice improved glucose homeostasis (Sundbom et al, 2008). Therefore the effect of an HSD1 inhibitor was studied in myotubes treated with both cortisol and cortisone. In line with our previous results for siRNA-mediated gene silencing of 11β-HSD1, we observed that an exposure of human myotubes to the HSD1 inhibitor reversed the deleterious effects of glucocorticoids on glucose and lipid metabolism. In Study IV we provide evidence that a chronic exposure of myotubes to either cortisol or cortisone highly upregulated 11β-HSD1 mRNA and protein expression. siRNA targeted against 11β-HSD1 reversed the cortisone-mediated reduction in glycogen synthesis. Reduced 11β-HSD1 expression mediated by siRNA also plays a role in glucocorticoid action on vascular smooth muscle cell proliferation (Michas et al, 2011). Taken together, application of siRNA technique provides a useful approach to study local glucocorticoid actions that are regulated by 11β-HSD1.

In Study IV, we provide evidence that the cortisone-mediated enhancement in palmitate oxidation was reduced following gene silencing of 11β-HSD1. Therefore, peripheral actions of cortisol are dependent on elevated 11β-HSD1 o xo-reductase activity, converting cortisone to cortisol. In contrast to our results for testosterone, no clear sex-specific difference in metabolic endpoints mediated by glucocorticoids in myotubes was observed. However, to address this matter more thoroughly, a larger study is warranted. Glucocorticoid hormones, like other steroid hormones, modulate gene expression, which has subsequent effects on metabolism. In Study IV, mRNA expression of GRα and PDK4 was altered following glucocorticoid exposure in myotubes. Peripheral tissue sensitivity to glucocorticoids is also regulated by the expression of glucocorticoid receptor isoforms (GRα and GRβ). There is some evidence
that treatment of myoblasts with increasing concentrations of cortisol (0.05-1µM) leads
to a dose-dependent decline in \( GR\alpha \) expression and a dose-dependent increase in \( GR\beta \) expression. (Whorwood et al, 2001). The expression of \( GR\alpha \) is known to be regulated
by its own ligand, such that cortisol down-regulates \( GR\alpha \) mRNA expression and
stability and increases the post-translational turnover of \( GR\alpha \) protein (Oakley & Cidlowski, 1993). In line with this, Study IV provides evidence for a reduced mRNA expression of \( GR\alpha \) following chronic exposure to cortisol. However, the mRNA expression level of \( GR\beta \) was undetectable in human myotubes.

\( PDK4 \) is a major target for controlling glucocorticoid-induced gene expression. In Study IV we provide evidence that \( PDK4 \) mRNA expression was highly elevated following either cortisone or cortisol treatment. \( PDK4 \) plays an important role in lipid metabolism (Rosa et al, 2003), and is under the regulation of pyruvate production. In general \( PDK4 \) activity is enhanced when rates of fatty acid oxidation are high, which consequently inhibits glucose oxidation (Sugden & Holness, 2006). Several lines of evidence indicate that reduced insulin levels and increased levels of fatty acids and glucocorticoids promote \( PDK4 \) gene expression in starvation and Type 1 diabetes (Huang et al, 2002). We provided evidence in Study IV that an RNA-mediated reduction of \( PDK4 \) can prevent the glucocorticoid-mediated changes in skeletal muscle metabolism. Thus, the effects of glucocorticoids to increase lipid oxidation and reduce glucose metabolism appear to be dependent on the induction of \( PDK4 \) expression. An aberrant local increase of cortisol in skeletal muscle mediated by increased activity of \( 11\beta\)-HSD1 may be an important factor in the development of metabolic diseases.

### 5.5 SEX DEPENDENT HORMONAL RESPONSES IN SKELETAL MUSCLE CELLS

Established muscle cell cultures from Type 2 diabetic patients maintains an
insulin-resistant metabolic memory, even after several passages of culture (Bouzakri et al, 2003; Henry et al, 1995). Reduced insulin-mediated glucose uptake has also been reported in myotubes from insulin resistance non-diabetic relative of Type 2 diabetes patients. In Study IV, the sex origin of the donor did not influence the response of primary myotubes to insulin or glucocorticoids. However, the muscle cells established from female donors respond to a low dose chronic testosterone exposure more readily in terms of glucose metabolism. Whereas the effect of testosterone on palmitate oxidation was sex-independent. The sex-specific effect of 17 \( \beta\)-estradiol on mRNA expression was more potent in myotubes. In contrast to our result from a chronic low dose exposure of myotubes to sex hormones (Study II), an acute exposure to a high concentration of sex hormones (Study III) was independent of the sex origin of the cell culture. This might indicate that the action of sex hormones at higher concentration over a shorter time is mediated rapidly at the non-transcriptional level.

In conclusion, the persistence of metabolic memory in skeletal muscle cells after several passages supports the role of inherited factors in insulin resistance of Type 2 diabetes; whereas the sex-related metabolic differences may be predominantly occur due to external hormonal factors.
6 SUMMARY

The aim of this thesis was to identify the role of steroid hormones in glucose and lipid metabolism in human skeletal muscle, to unmask intrinsic sex differences in metabolism, and to investigate the underlying molecular mechanism that is activated by steroid hormones in skeletal muscle cells. The results presented in this thesis are summarized as follows:

• Human myotubes are not characterized by intrinsic sex differences in glucose incorporation into glycogen and palmitate oxidation at the base line, although the mRNA expression of select genes involved in metabolism were different between men and women.
• Sex-dependent differences in metabolic outcomes are revealed after sex hormone exposure in skeletal muscle cells. A metabolic “memory” of the sex of the donor exists at the mRNA level, which is unmasked by chronic exposure to sex hormones. Testosterone and 17β-estradiol have differential sex-dependent effects on signaling pathways and metabolism in skeletal muscle cells.
• Acute sex hormone treatment causes insulin resistance in skeletal muscle cells. This effect of sex hormones is independent of the sex origin of the donor of the cell culture.
• Cortisol increases palmitate oxidation and reduces glucose incorporation into glycogen in human muscle cells. Targeted inhibition of 11β-HSD1 by siRNA or specific inhibitors prevents the effects of cortisone, but not those of cortisol on metabolic readouts in skeletal muscle cells. These effects indicate a PDK4-dependent mechanism.
7 CONCLUSIONS AND FUTURE PERSPECTIVES

The main focus of this thesis was to identify the molecular mechanisms underlying steroid hormone-mediated effects on skeletal muscle metabolism in a primary cell culture system. Altered circulatory levels of steroid hormones associated with obesity, Type 2 diabetes and aging have highlighted the need for further investigation regarding the role of these hormones. Furthermore, hormonal effects may unmask sex-dependent differences in metabolism in skeletal muscle cell. To find better approaches for treatment of metabolic syndrome, it is important to understand the metabolic differences between men and women, and the molecular mechanisms that underlie these differences. Steroid hormones are one important factor that influences the metabolic responses, as well as gene profile, in men and women. Steroid hormones have been administered as therapeutic agents; therefore it is pivotal to delineate the molecular mechanism of their broad actions in target tissues.

Cultured myotubes are a useful tool to study skeletal muscle metabolism. This approach allows for controlled conditions and eliminates potential interference of whole body systemic stimuli, such as hormones, cytokines or altered fuel sources. We present evidence against intrinsic sex difference in skeletal muscle metabolism since metabolic responses and gene expression were similar between men and women in cultured myotubes in the absence of hormonal stimulation.

In this thesis evidence is provided that sex steroid hormones at physiological doses unmask sex-based differences in metabolic outcomes and gene expression. In this respect, cultures of muscle cells obtained from aged-matched men and women provided a useful system, since it avoids differences in whole body milieu between men and women. Although our data provided evidence against a sex-related difference in response to glucocorticoids at the base line, with different metabolic assays, the chronic response to sex-steroids was dependent on the sex origin of the human skeletal muscle cell cultures. Somewhat surprisingly, cells derived from female subjects where more sensitive to testosterone than cells derived from male subjects. This highlights the need to consider the sex-origin of cells when interpreting experimental data in response to diverse stimuli. In the case of many commonly used cell lines, the sex origin is very rarely mentioned, but based on our data provided in this thesis, it may influence the results that are obtained. Thus, the molecular mechanisms mediating the sex-memory in cells are currently unknown.

Result presented in this thesis revealed that supra-physiological doses of sex steroid hormones lead to insulin resistance. The metabolic effects of low dose chronic, and high dose acute sex hormones is different, highlighting that sex hormones can play positive and negative roles in a dose and or time dependent manner. However the molecular mechanism of acute sex hormones action in metabolism and signaling pathways needs further investigations, although differences may be due to differential activation of genomic and non-genomic pathways in a time and/or dose dependent manner.

Finally, the work in this thesis has highlighted siRNA as a powerful approach to understand peripheral actions of steroid hormone such as glucocorticoids. Therefore, inhibition of regulatory enzymes of steroid hormones could be considered as an potential approach to identify new drug target for the prevention or treatment of metabolic disease.
In conclusion, the results presented in this thesis provide evidence for an important role of steroid hormones in the regulation of skeletal muscle metabolism, and the mechanisms governing the sex-dependent control of metabolic responses in skeletal muscle.
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