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MOLECULAR MECHANISMS OF ESTROGEN ACTION IN RELATION TO METABOLIC DISEASE

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To my family

ABSTRACT

Estrogens have multiple effects on the body. Most studied are the effects on female reproduction. In this thesis, we have focused on the metabolic effects of estrogens. Estrogen treatment decreases adipose tissue mass and has anti-diabetic effects in humans and mice. Changes in food intake and voluntary activity occur upon estrogen treatment but are considered to be of minor importance for the observed effects. Studies on knockout mice have revealed a number of metabolic phenotypes such as obesity, glucose intolerance and insulin resistance as a consequence of disrupted signaling through one of the estrogen receptors (ER), *ERα*.

The aim of this thesis was to characterize the molecular mechanisms that mediate protective effects of estrogens against obesity and diabetes. We have used gene expression profiling to analyze changes in transcription after estrogen treatment in adipose tissue and liver, respectively. A number of target genes have been identified that could be possible mediators of the effects of estrogens on adiposity and glucose metabolism. In **Paper I**, we identified the nuclear receptor liver X receptor (*LXRα*) as an estrogen-regulated gene. The expression of *LXRα* and several of its target genes was decreased in mouse white adipose tissue following 10 h of estrogen treatment. One of the target genes, sterol regulatory element binding protein 1c (*Srebp1c*), is a master regulator of fatty acid synthesis. We also studied changes in gene expression in fat biopsies from postmenopausal women upon estrogen treatment for three months (**Paper II**). Several genes encoding enzymes in the fatty acid synthesis pathway were decreased by estrogen treatment in a subset of women. This was the first report showing estrogen regulation of stearoyl-CoA desaturase (*Scd1*), fatty acid synthase (*Fas*) and acetyl-CoA carboxylase α (*Acc1*) in human subcutaneous abdominal adipose tissue.

In **Paper III**, we investigated effects on estrogen administration on potential central and peripheral target tissues. Gene expression profiling revealed a small number of regulated genes in hypothalamus compared to white adipose tissue after three weeks of estrogen treatment. We focused on glutathione peroxidase (*Gpx3*) and cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*) that were increased and decreased, respectively, in white adipose tissue upon estrogen treatment. *Gpx3* protects against oxidative stress and its expression is low in obesity and high after weight loss. Obesity and diabetes are correlated to oxidative stress, which lead us to hypothezise that *Gpx3* has a potential role in the effects of estrogen on adiposity. *Cidea* might have a role in the regulation of energy expenditure. In **Paper IV**, we confirmed that the anti-diabetic effects of estrogens are mediated via *ERα*. Diabetic *ob/ob* mice were treated with estrogen or the *ERα*-selective agonist PPT. Both compounds improve glucose tolerance, insulin sensitivity and the insulin response to glucose *in vivo*, with suggested action via the liver in this model. The mechanisms behind these effects are likely to be linked to an increased expression of signal transducer and activator of transcription 3 (*Stat3*) and a decreased expression of glucose-6-phosphatase (*G6pc*) as the regulation of these genes coincided with the observed phenotypes.

In conclusion, our studies, based around gene expression profiling, has contributed with a significant knowledge about the molecular mechanisms responsible for the effects of estrogen in relation to obesity and diabetes.

LIST OF PUBLICATIONS

I. **Lovisa Lundholm**, Sofia Movérare, Knut R. Steffensen, Maria Nilsson, Michio Otsuki, Claes Ohlsson, Jan-Åke Gustafsson and Karin Dahlman-Wright. *Gene expression profiling identifies liver X receptor alpha as an estrogen-regulated gene in mouse adipose tissue.* J Mol Endocrinol, 2004, 32(3), 879-92.

II. **Lovisa Lundholm**, Hong Zang, Angelica Lindén Hirschberg, Jan-Åke Gustafsson, Peter Arner and Karin Dahlman-Wright. *Key lipogenic gene expression can be decreased by estrogen in human adipose tissue.* Fertil Steril, in press.

III. **Lovisa Lundholm**, Michio Otsuki, Sandra Andersson, Claes Ohlsson, Jan-Åke Gustafsson and Karin Dahlman-Wright. *Effects of estrogen on gene expression profiles in mouse hypothalamus and white adipose tissue; Target genes include GPX3 and CIDEA.* Manuscript being revised for J Endocrinol.

IV. Galina Bryzgalova[*], **Lovisa Lundholm**[*], Hui Gao, Neil Portwood, Susann Fält, Kurt D. Berndt, Andrea Dicker, Dana Galuska, Juleen R. Zierath, Jan-Åke Gustafsson, Suad Efendic, Karin Dahlman-Wright and Akhtar Khan. *The estrogen receptor alpha-selective agonist PPT improves glucose tolerance in ob/ob mice; potential molecular mechanisms.* Submitted manuscript.

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LIST OF ABBREVIATIONS

Acc	acetyl-CoA carboxylase
AF	activation function
affylmGUI	Affymetrix linear modeling graphical user interface
AP-1	activator protein 1
ArKO	aromatase knockout mice
BERKO	*ERβ* knockout mice
BMI	body mass index
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation
Cidea	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
CoA	coenzyme A
Cpt1	carnitine palmitoyl transferase 1
DPN	diarylpropionitrile
E1	estrone
E2	17β-estradiol
E3	estriol
ER	estrogen receptor
ERE	estrogen response element
ERKO	*ERα* knockout mice
ERR	estrogen-related receptor
Fas	fatty acid synthase
FFA	free fatty acid
G6pc	glucose-6-phosphatase, catalytic
Gpx3	glutathione peroxidase 3
HDL	high-density lipoprotein cholesterol
HRT	hormone replacement therapy
IGT	impaired glucose tolerance
Lce	long-chain elongase
LXR	liver X receptor
PGC	*PPARγ* coactivator
PPAR	peroxisome proliferator activated receptor
PPT	propyl pyrazole triol
RMA	robust multiarray average
ROS	reactive oxygen species
Scd1	stearoyl-CoA desaturase 1
Srebp1c	sterol regulatory element binding protein 1c
Stat3	signal transducer and activator of transcription 3
TG	triglyceride
WAT	white adipose tissue

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

Hormone signaling is important for the maintenance of body homeostasis, involving processes such as development, growth, metabolism and reproduction (1). Hydrophilic peptide hormones and some amine hormones (including insulin and epinephrine, respectively) bind to receptors on the plasma membrane, while small lipophilic steroid hormones (including vitamins) and thyroid hormone can diffuse through the membrane and directly bind receptors that function as transcription factors in the cell nucleus, so called nuclear receptors (1).

Nuclear receptors have a common overall structure with a variable amino (N-) terminal domain containing a hormone-independent activation function (AF-1), a highly conserved DNA-binding domain that recognizes the specific response element on DNA and a carboxy (C-) terminal ligand binding domain which contains a hormone-dependent activation function (AF-2) and silencing regions (2). Both the DNA-binding and ligand-binding domains contain regions for dimerization and nuclear localization (2). The DNA-binding domain contains so called zinc finger motifs (3). These consist of two zinc atoms surrounded by four cysteins. The integrity of this structure is required for proper DNA binding (3, 4). The receptors bind to DNA as monomers or homodimers, or as heterodimers with the retinoid X receptor (5). The hormone response elements are composed of one or two hexamer half-sites separated by a various number of base pairs (5). Dimers can bind to either direct repeats, or inverted or everted repeats (palindromes) (2).

48 nuclear receptors have been identified in humans (Table 1) (5). About half of these are activated by hormone (ligand) and correspond to the classic nuclear receptors or the so called adopted orphans (5). The others are true orphan receptors without ligand (non-existing or not known), although a few orphan receptors exist that may be affected by ligands (5). Most of the adopted orphans bind larger and more diverse ligands than the classic receptors, and their candidate ligands were identified more recently (5). The members of the nuclear receptor superfamily are also classified into subgroups based on dimerization and DNA-binding properties (6).

The steroid hormone receptors bind to inverted repeats as homodimers and include estrogen, androgen, progesterone, glucocorticoid and mineralocorticoid receptors (6). Their ligands are endogenously synthesized and bind with high affinity to the receptors (7). All other ligand-binding receptors form heterodimers with retinoid X receptor and bind mainly to direct repeats (6). The adopted receptors respond to dietary lipids that bind with relatively low affinities, such as oxysterols for liver X receptors (*LXR*) and fatty acids for peroxisome proliferator activated receptors (*PPAR*). The thyroid hormone receptors, retinoic acid receptors and vitamin D receptors have intermediate properties (7). Their ligands are, similar to adopted orphans, synthesized using exogenous elements that are provided via the diet (iodine, vitamin A) or the skin (sunshine) (7). On the other hand, the transcriptional pathways they regulate and their ligand binding affinities resemble those of the steroid hormone receptors (5, 7).

Names	Nomenclature	Ligands
Endocrine receptors		**High-affinity, hormonal lipids**
ERα	NR3A1	17β-estradiol, 4-hydroxytamoxifen, raloxifene
ERβ	NR3A2	17β-estradiol, various synthetic compounds
GR	NR3C1	Cortisol, dexamethasone, RU486
MR	NR3C2	Aldosterone, spirolactone
PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486
AR	NR3C4	Testosterone, flutamide
TRα	NR1A1	Thyroid hormones
TRβ	NR1A2	Thyroid hormones
RARα	NR1B1	Retinoic acid
RARβ	NR1B2	Retinoic acid
RARγ	NR1B3	Retinoic acid
VDR	NR1I1	Vitamin D, 1,25-dihydroxyvitamin D3
Adopted orphan receptors		**Low-affinity, dietary lipids**
RXRα	NR2B1	Retinoic acid
RXRβ	NR2B2	Retinoic acid
RXRγ	NR2B3	Retinoic acid
PPARα	NR1C1	Fatty acids, leukotriene B4, fibrates
PPARβ	NR1C2	Fatty acids
PPARγ	NR1C3	Fatty acids, prostaglandin J2, thiazolidinediones
LXRα	NR1H3	Oxysterols, T0901317, GW3965
LXRβ	NR1H2	Oxysterols, T0901317, GW3965
FXRα	NR1H4	Bile acids, fexaramine
PXR	NR1I2	Xenobiotics, 16α-cyanopregnenolone
CAR	NR1I3	Xenobiotics, phenobarbital
Orphan receptors		**Unknown**
DAX-1	NR0B1	Orphan
SHP	NR0B2	Orphan
Rev-erbα	NR1D1	Orphan
Rev-erbβ	NR1D2	Orphan
RORα	NR1F1	Cholesterol, cholesteryl sulfate
RORβ	NR1F2	Retinoic acid
RORγ	NR1F3	Orphan
HNF4α	NR2A1	Orphan
HNF4γ	NR2A2	Orphan
TR2	NR2C1	Orphan
TR4	NR2C2	Orphan
TLL	NR2E2	Orphan
PNR	NR2E3	Orphan
COUP-TFI	NR2F1	Orphan
COUP-TFII	NR2F2	Orphan
EAR2	NR2F6	Orphan
ERRα	NR3B1	Orphan
ERRβ	NR3B2	Diethylstilbestrol, 4-hydroxytamoxifen
ERRγ	NR3B3	Diethylstilbestrol, 4-hydroxytamoxifen
NGFI-B	NR4A1	Orphan
NURR1	NR4A2	Orphan
NOR1	NR4A3	Orphan
SF1	NR5A1	Orphan
LRH-1	NR5A2	Orphan
GCNF	NR6A1	Orphan

Table 1. Human nuclear receptors. Adapted from (5, 7)

Most orphan receptors bind either to direct repeats as homodimers, or to extended core sites as monomers (6). Many of the orphan receptors are constitutively active (8). However, for example estrogen-related receptor (ERR) γ can be repressed by *ER*-antagonists like 4-hydroxytamoxifen (8). A few other orphans such as retinoid-related orphan receptors, steroidogenic factor 1 and liver receptor homolog might have potential to be pharmaceutical targets (8).

In addition to regulation by ligand, nuclear receptor signaling can be regulated by the level of receptor expression, by coregulator proteins or by post-translational modifications (8). Coregulators, which is the general term for coactivators and corepressors, are receptor interacting proteins that modulate the transcriptional activity of nuclear receptors on target genes (9). Nuclear receptor target genes produce proteins that are the ultimate mediators of the observed biological effects.

1.2 ESTROGEN RECEPTORS

One of the first nuclear receptors to be cloned was an estrogen receptor (ER), now known as *ERα*, in 1986 (10, 11). However, estrogen receptors were identified using biochemical approaches many years prior to their eventual cloning (5). A second estrogen receptor, *ERβ*, was identified in 1996 (12). When comparing amino acid sequences, *ERα* and *ERβ* are 97% homologous in the DNA-binding domain, 56% in the ligand-binding domain and 24% in the N-terminal domain (13). As the homologies indicate, both receptors can bind to a classic estrogen response element (ERE) (13). Moreover, both receptors bind the endogenous ligand 17β-estradiol (E2) with high affinity (14). However, the differences in the ligand-binding domain are sufficient as basis for development of subtype-selective ligands (15, 16). Differences in the N-terminal part include a weaker AF-1 in *ERβ* than in *ERα* (17).

In the regulatory regions of ER target genes, the consensus ERE (GGTCAnnn TGACC), or variants of it, are present in a number of estrogen responsive sites (13). ERs can also bind indirectly to activator protein (AP)-1-, cyclic adenosine monophosphate (cAMP)-, or Sp1-response elements, via AP-1 or Sp1 transcription factor complexes (9).

ERα and *ERβ* are expressed in diverse tissues and cells in the body. *ERα* is mainly expressed in tissues such as the uterus, ovary (theca cells), testes (Leydig cells), epididymis, breast, kidney, bone, white adipose tissue, liver, skeletal muscle and various regions of the brain, including pituitary and hypothalamus (13, 14, 18). *ERβ* is mainly expressed in tissues such as the ovary (granulosa cells), prostate (epithelium), testis, epididymis, colon, lung, bladder, bone marrow, salivary gland, vascular endothelium and regions of the brain, including hypothalamus and cortex (13, 14, 18).

1.2.1 Estrogen receptor ligands

All steroid hormones are derived from cholesterol (5). The enzyme aromatase catalyzes the last step in the formation of estrogens from androgens (19). Estrogens are mainly produced by granulosa cells in the ovaries, with E2 being the most potent ligand

(Figure 1), followed by estrone (E1) and estriol (E3) (20, 21). Estrogens are however also produced by aromatase at extragonadal sites such as the adipose tissue (21). In men and postmenopausal women, this production of estrogens (mainly E1) can constitute a significant fraction of the total production (21). The aromatization to estrogens is increased by obesity and with age (21). Bone and brain are other extragonadal sites where conversion occurs but there the hormone mainly acts locally (19).

Synthetic ER ligands include tamoxifen, which has been used for more than 30 years for the treatment of breast cancer (22). Tamoxifen is metabolized in the liver to the active metabolite 4-hydroxytamoxifen (23). It acts as an antagonist in breast, but as an agonist in other tissues such as the uterus (endometrium) and bone (22).

The *ERα*-selective ligand propyl pyrazole triol (PPT) has 410-fold selectivity for *ERα* versus *ERβ* (16). An *in vivo* study using this compound shows effects similar to those of E2, including increased uterine weight and prevention of the increased body weight and reduced bone mineral density normally associated with ovariectomy (24). The *ERβ*-selective ligand diarylpropionitrile (DPN) has 70-fold selectivity for *ERβ* versus *ERα* (15). DPN has, similar to E2, *in vivo* anti-anxiety effects (25, 26), enhances spatial memory (27), attenuates lung injury (28) and is cardioprotective (29, 30).

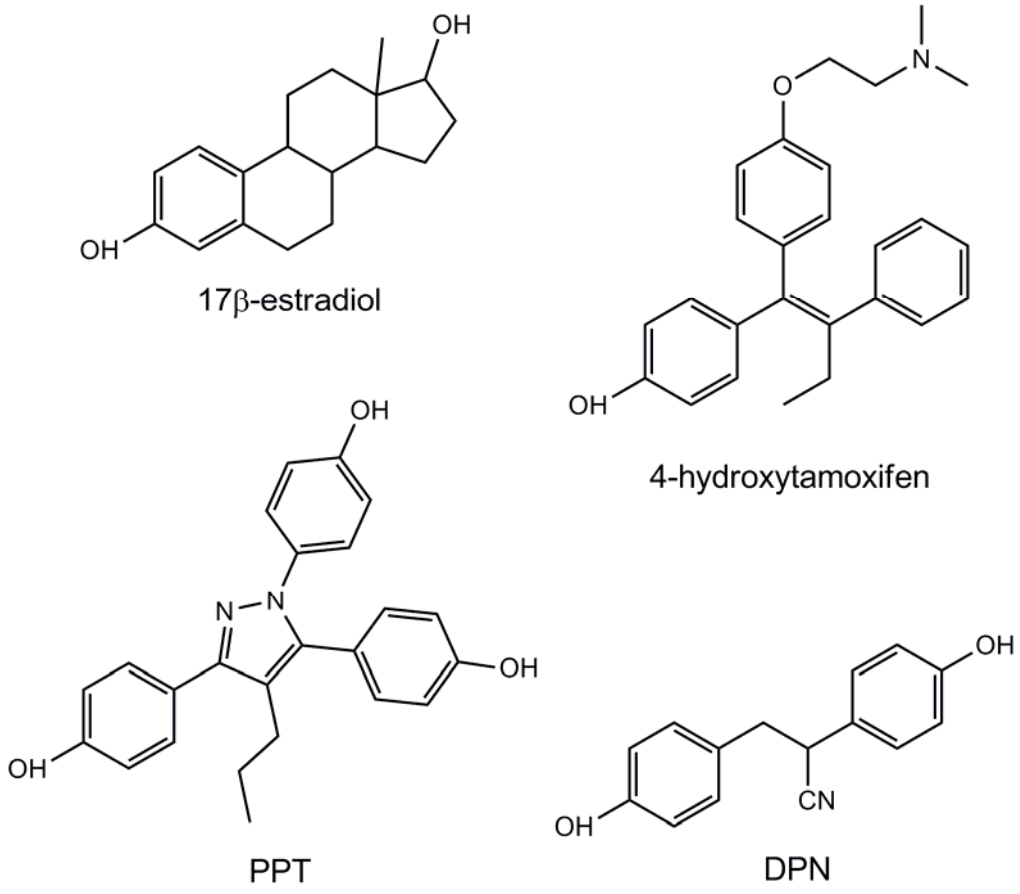

Figure 1. Estrogen receptor ligands.

1.2.2 Estrogen receptor signaling

Unliganded estrogen receptors are, like other steroid receptors, bound to a complex of heat shock proteins, which are believed to repress receptor function (31). When E2 binds to its receptor, conformational changes occur in the receptor protein and the heat shock proteins dissociate (31). The position of an α-helix, helix 12, in the ligand binding domain is altered and this exposes a surface for interaction with coactivators in AF-2 (32). Binding of partial agonists/antagonists like 4-hydroxytamoxifen, on the other hand, displaces helix 12 to a position blocking the coactivator interaction surface, therefore inhibiting transcriptional activation in some tissues (32). In tissues where 4-hydroxytamoxifen acts as an agonist, *ERα* AF-1 but not *ERβ* AF-1 is used instead of AF-2 (32).

The ligand-activated ERs dimerize and bind directly ① or indirectly ② to response elements, as described above (Figure 2). Depending on the relative abundance and binding affinities of coregulators, ERs can associate with different complexes that turn on or inhibit target gene transcription (32). This is the classic action of estrogen

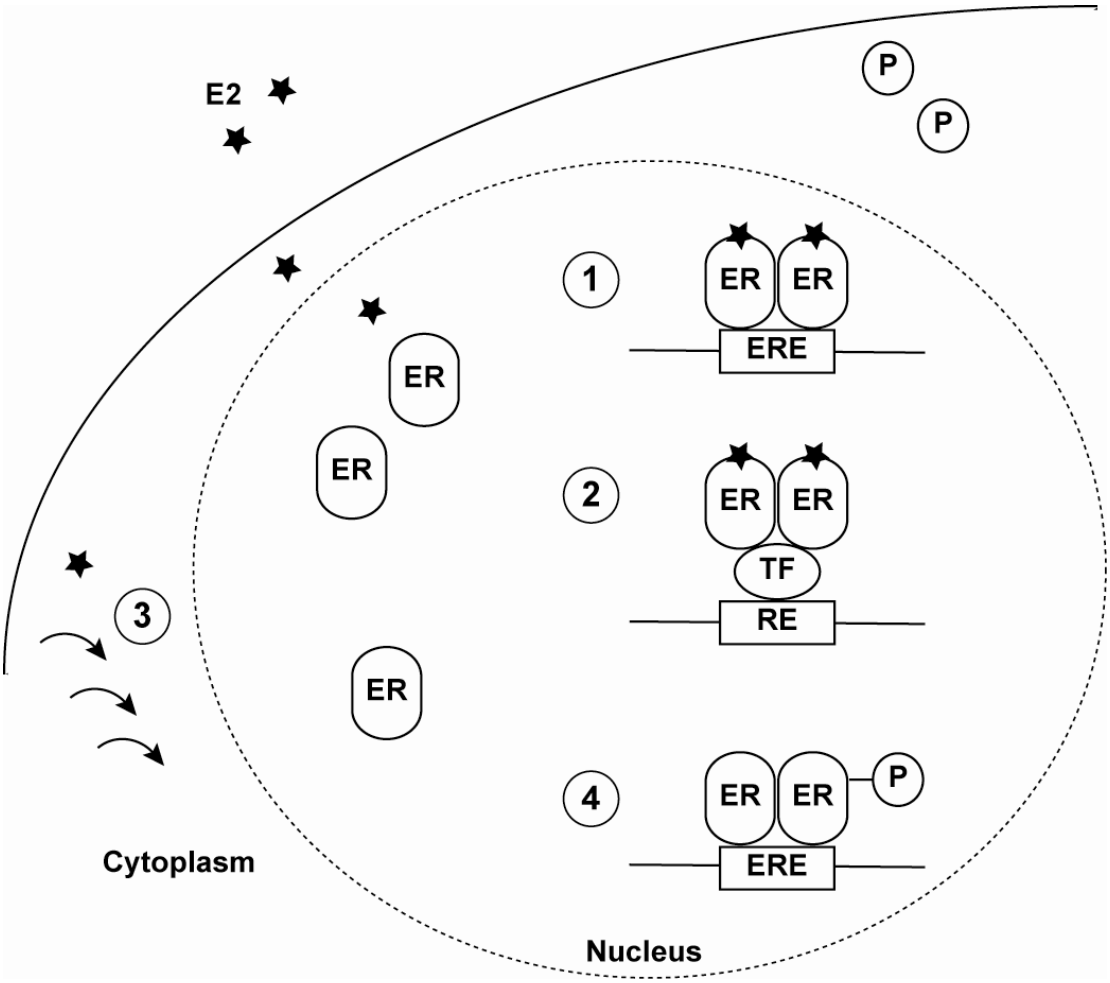

Figure 2. Mechanisms for estrogen signaling. 1: 17β-estradiol (E2) binds the estrogen receptors (ERs) which associate with an estrogen response element (ERE). 2: E2 binds the ERs which associate with a response element (RE) via another transcription factor (TF). 3: E2 activates signaling cascades in a non-genomic mode. 4: ERs are activated by modifications such as phosphorylation (P) and bind to an ERE.

hormone signaling and is referred to as the genomic action. Non-genomic actions of estrogen ③ are very rapid hormone responses, occurring within seconds or minutes, and therefore not involving new synthesis of mRNA or protein (32). However, the non-genomic actions could require the receptor (32). Examples of non-genomic actions are effects on endothelial cells where E2 could induce nitric oxide release by activating protein kinase cascades, or activation of neuroprotection in neurons using similar cascades (32). The transcriptional activity of nuclear receptors and coregulators can be modulated by posttranslational modifications such as phosphorylation ④, acetylation, SUMOylation, ubiquitinylation and methylation (5).

1.2.3 Estrogen receptors as therapeutic targets

Estrogens have multiple effects on the body and are typically known for their importance in the development and maintenance of reproductive functions (33). However, estrogens have biological effects in both men and women, including effects on metabolic tissues, bone and the cardiovascular, immune and central nervous systems (34).

Estrogens are widely used as hormone replacement therapy (HRT) in postmenopausal women and as contraceptives in fertile women (34). The estrogen dose is typically about 1-2 mg/day for HRT and 30 μg/day for contraceptives. Estrogen treatment has many beneficial effects, however adverse effects may include an increased risk of breast cancer, endometrial cancer and venous thrombosis (34). Estrogen receptor antagonists and inhibitors of estrogen synthesis are used for treatment of breast cancer (34).

1.3 ADIPOSE TISSUE

Triglycerides (TGs) constitute ≥85% of the white adipose tissue (WAT) weight (35). The white adipocytes are characterized by a thin cytoplasm containing the nucleus and other organelles, surrounding the lipid droplet (35). Brown adipocytes have a large number of mitochondria that can produce heat by uncoupling oxidative phosphorylation (36). Brown adipose tissue has typically been shown to be present in rodents (particularly in interscapular regions) and in newborns in humans (37). It may however also be present in human adults in for instance supraclavicular and neck regions according to a recent study (37).

WAT is generally present in subcutaneous layers between muscle and dermis or around internal organs (38). In rodents, the gonadal adipose tissue represents a typical WAT depot (38). Depot-specific properties exist; subcutaneous and visceral (intra-abdominal) depots have for instance different susceptibility to obesity (38).

WAT was long considered as merely a storage tissue, where fatty acids are accumulated as TGs after a meal and released when needed as fuel (36). The discovery of the cytokine-like hormone leptin in 1994 initiated the current view of WAT as an endocrine organ, secreting a large number of adipokines such as adiponectin, resistin, tumor necrosis factor α, interleukin 6, transforming growth factor β, angiotensinogen

and haptoglobin (35). Adiponectin is low in obesity and promotes lipogenesis while resistin in high in obesity and stimulates lipolysis (38).

In addition to adipocytes, WAT contains other cell types separated out as a stroma-vascular fraction when performing a basic cell separation (38). These are endothelial cells, monocytes, macrophages and pluripotent stem cells, including preadipocytes. The differentiation of preadipocytes into adipocytes engages a cascade of sequentially activated transcription factors, the most important ones being *PPARγ* and CCAAT/enhancer binding protein (*C/EBP*) α (38).

1.4 REGULATION OF ADIPOSE TISSUE MASS

1.4.1 Lipogenesis

The balance between lipid synthesis (lipogenesis) and lipid breakdown (lipolysis) determines adipocyte size (39). Lipogenesis involves fatty acid synthesis and subsequent TG synthesis, and is mediated by key lipogenic enzymes as shown in Figure 3 (38). Synthesis of fatty acids from carbohydrates, *de novo* lipogenesis, occurs both in adipose tissue and liver (40). The synthesis in liver contributes to about 10% of the circulating and stored TGs; in obese humans this fraction is raised to about 25% (41). When the intake of carbohydrates exceeds the glycogen storage capacity of liver and muscle, they are converted to TGs and stored in adipose tissue (1).

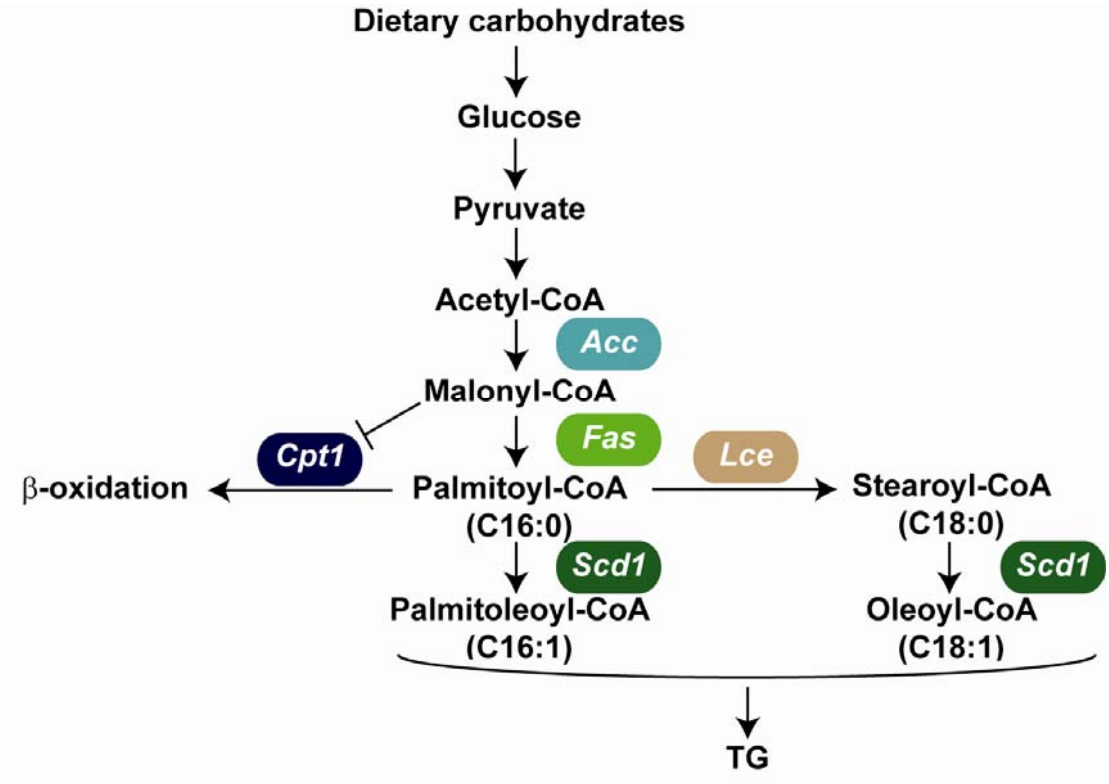

Figure 3. Pathways that mediate fatty acid synthesis and subsequent triglyceride (TG) synthesis as well as mitochondrial fatty acid oxidation are outlined. Abbreviations: acetyl-CoA carboxylase (*Acc*), fatty acid synthase (*Fas*), stearoyl CoA desaturase 1 (*Scd1*), carnitine palmitoyl transferase 1 (*Cpt1*) and long-chain elongase (*Lce*). Adapted from (41).

1.4.1.1 Enzymes and transcription factors relevant for lipogenesis

Enzymes and certain transcription factors relevant for lipogenesis are potentially promising drug targets for metabolic disease (41).

Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, a key molecule controlling intracellular fatty acid metabolism (41). Except being critical for fatty acid synthesis, malonyl-CoA plays an important role in inhibiting carnitine palmitoyl transferase 1, an enzyme regulating the first step in the transfer of long-chain fatty acids into mitochondria for their oxidation (42). Acetyl-CoA carboxylase α (*Acc1*) is expressed in many tissues but at higher levels in adipose tissue and liver (41). Blocking *Acc1* is predicted to reduce the *de novo* fatty acid synthesis in lipogenic tissues and to decrease adiposity (41). The complete knockout of *Acc1* is embryonically lethal (43). However, mice with a liver-specific deletion survive and have reduced triglyceride accumulation in the liver (42). Acetyl-CoA carboxylase β (*Acc2*) is expressed predominantly in muscle and heart (41). A few dual *Acc1* and *2* inhibitors have been tested and were reported to have favorable effects on multiple aspects of the metabolic syndrome in rodents (41).

Fatty acid synthase (*Fas*) catalyzes the *de novo* synthesis of long-chain fatty acids, palmitoyl-CoA, from acetyl-CoA and malonyl-CoA (44). It is highly expressed in liver, adipose tissue and lactating mammary gland, and plays an important role in the conversion of excess energy intake into lipids for storage (45). *Fas* inhibitors decrease body weight in mice, primarily due to reduced food intake (44). This has been connected to altered expression of hypothalamic neuropeptides (46). Present *Fas* inhibitors also activate certain other targets and are thus not suitable for further studies (46). *Fas* knockout mice are not available since they die *in utero* (45). Therefore it has been problematic to pinpoint the precise effects of *Fas* modulation until more specific inhibitors or tissue-specific knockouts are developed (45).

Stearoyl-CoA desaturase 1 (*Scd1*) catalyzes the critical step in the biosynthesis of monounsaturated fatty acids from saturated fatty acids (40). Palmitoyl- and stearoyl-CoA are preferred substrates that are converted to palmitoleoyl- and oleoyl-CoA, respectively (47). These monounsaturated fatty acids are the major components of various lipids, including TGs, but they also function as mediators of signal transduction and cellular differentiation (47). *Scd1* is most abundant in adipose tissue and liver. *Scd1* knockout mice have increased energy expenditure, reduced adiposity, increased insulin sensitivity and are resistant to diet-induced obesity (48). In addition, *Scd1*-deficiency in leptin-deficient ob/ob mice completely corrects their hypometabolic phenotype and hepatic steatosis (49).

The transcription factor sterol regulatory element binding protein 1c (*Srebp1c*) is a master regulator of fatty acid synthesis pathways and activates the lipogenic enzymes mentioned above (50). Overexpression of *Srebp1c* in mouse liver increases fatty acid synthesis and the expression of lipogenic enzymes, producing a fatty liver, while *Srebp1* knockout animals have reduced fatty acids synthesis and expression of these enzymes (50).

The adopted orphan receptor *LXRα* induces transcription of *Srebp1c* (51). *LXRα* regulates genes involved in lipogenesis and cholesterol efflux and clearance (52). *LXRα* is expressed in liver, adipose tissue, intestine and macrophages (53). Mice lacking *LXRα* have lower expression of *Srebp1c*, *Fas* and *Scd1* (54) and *LXRα/β* double knockout mice have decreased adipose tissue mass (55). Conversely, animals fed synthetic *LXR* agonists or a high cholesterol diet have increased *Srebp1c* expression, induced expression of lipogenic target genes and elevated rates of fatty acid synthesis (56). *LXRs* can also regulate *Fas* directly, independently of *Srebp1c* (57).

1.4.2 Lipolysis

Lipolysis occurs when energy requirements increase. Hormone sensitive lipase in adipocytes hydrolyzes TGs to free fatty acids (FFA) and glycerol that are transported to liver, muscle and heart and used for fatty acid oxidation (β-oxidation) (38). Carnitine palmitoyl transferase 1, followed by carnitine palmitoyl transferase 2, catalyzes the first steps in the β-oxidation pathway which is a series of enzymatic reactions (58). In liver, the end product of β-oxidation are ketone bodies that are used in the brain, which has very low β-oxidation capacity (58). The end product in muscle, acetyl-CoA, can be used directly in the citric acid cycle as an energy source (58).

1.4.3 Hypothalamic regulation of appetite and satiety

Neural circuits in the hypothalamus regulate energy homeostasis by signaling using specific neuropeptides. The arcuate nucleus is thought to be important in the integration of signals regulating appetite (59). One population of neurons, anorectic, inhibits food intake via expression of pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript, while the other important neural circuit, orexigenic, stimulates food intake via expression of neuropeptide Y and agouti-related peptide (59). Downstream effects involve other hypothalamic nuclei containing secondary neurons that process the signals (59).

Peripheral hormones can also signal to the hypothalamus. Insulin exerts anorectic effects in the hypothalamus (60). Leptin, expressed from the *ob* gene, is secreted from adipose tissue at levels correlating to adipose tissue mass (60). When the adipose tissue amount increases, leptin acts on leptin receptors in the hypothalamus (59). Leptin activates the anorectic neurons and inhibits the orexigenic neurons (59). Hormones released from the gut include ghrelin, which has hunger promoting effects and is secreted in response to fasting, and cholecystokinin and peptide YY, secreted from the intestine after food intake and with anorectic actions on the hypothalamus (60).

1.5 METABOLIC DISEASE

1.5.1 Obesity

Changes in environment and lifestyle have raised the incidence of obesity and type 2 diabetes considerably (61). Overweight and obesity are defined as excessive fat accumulation originating from an energy imbalance between consumed and expended

calories (62). The present global increase in obesity with an accompanying increase in type 2 diabetes is caused by a combination of an increased intake of food rich in fat and sugar and decreased physical activity because of transportation and less energy-consuming work (62). 50% or more of the variation in body weight between individuals has a genetic basis, mainly of polygenic nature (63).

Obesity is associated with an increased risk for several diseases including cardiovascular disease, type 2 diabetes and cancer in colon, endometrium, breast and prostate (63, 64). Obesity is the result of increases in adipocyte number and size (65). However, when a critical adipocyte size is reached, further fat accumulation only occurs by increases in adipocyte number (65).

The body mass index (BMI), body weight in kg divided by the square of the height in m, is commonly used to assess overweight (BMI>25 kg/m^2) and obesity (BMI>30 kg/m^2) (62). In Sweden, about 10-15% of the population is obese (66) and more than half of the men and over one third of the women are overweight or obese (67). Globally, 400 million adults are obese and 1.6 billion adults are overweight or obese (62). BMI is a useful measure for populations and has the same scale for both sexes (62). However, it is a somewhat rough measure and individuals with a large muscle mass can, incorrectly, be graded as overweight (62). Additional measures such as waist circumference and waist-hip-ratio are also useful measures of obesity which are used in the clinic (63).

Available treatments for obesity are lifestyle interventions, including changes in food intake and physical activity, drugs and surgery. A comparison of randomized trials with a follow-up period of at least two years showed moderate but sustained reductions in body weight using lifestyle interventions (68). Although the average net difference in body weight was only 3 kg compared to a control group, it induced a remarkable reduction in incidence of diabetes and high blood pressure (68). A similar net weight difference was seen after treatment with anti-obesity drugs (68). Orlistat (Xenical®) reduces fat uptake by inhibiting pancreatic and gastric lipases. The other anti-obesity therapies available in Sweden act centrally, rimonabant (Acomplia®) is a cannabinoid-l receptor blocker and sibutramin (Reductil®) inhibits the reuptake of serotonin and norepinephrine (68, 69).

Surgery is an option for individuals with BMI>35-40 where other methods have failed (68). This intervention produces large weight losses, typically 20-50 kg (70). However, the method may include risks for blood clotting in vessels, pulmonary complications, wound infections and death (70).

1.5.2 The metabolic syndrome

The term metabolic syndrome first appeared in 1923, describing a cluster of symptoms (61). The symptoms have been reevaluated several times and today the definition comprises a cluster of clinical observations including central obesity, hyperglycaemia, decreased high-density lipoprotein (HDL) cholesterol, elevated TGs and blood pressure (61). The metabolic syndrome is associated with a 3-fold increase in type 2 diabetes

Central obesity (assumed if BMI>30 kg/m^2)	Waist circumference ≥80 cm in women, ≥94 cm in men (of European origin, it is ethnicity-specific)
Plus any two other factors:	
Raised triglycerides	>1.7 mmol/l (150 mg/dl)*
Reduced HDL-cholesterol	<1.29 mmol/l (50 mg/dl) in women* <1.03 mmol/l (40 mg/dl) in men*
Raised blood pressure	Systolic ≥130 mmHg, diastolic ≥85 mmHg*
Raised fasting plasma glucose	Fasting plasma glucose ≥5.6 mmol/l (100 mg/dl)*

* or previous treatment or diagnosis

Table 2. Criteria used to identify the presence of the metabolic syndrome. Adapted from (71).

and a 2-fold increase in cardiovascular disease, and clearly constitutes a major public health concern worldwide (61).

1.5.3 Type 2 diabetes

Diabetes is diagnosed by fasting glucose levels ≥7 mmol/l or glucose levels 2 h after a glucose load ≥11.1 mmol/l (72). About 90% of people with diabetes have type 2 diabetes, where the body cannot use the insulin efficiently (73). Type 1 diabetes is instead characterized by a lack of insulin production (73). Insulin, together with glucagon and epinephrine, act to keep the glucose level near 4.5 mmol/l (1). Elevated blood glucose levels trigger insulin release from pancreatic β-cells, which stimulates glucose uptake in muscle and liver (glycogen synthesis) and TG synthesis in adipose tissue (1). In addition, insulin reduces the hepatic glucose output and reduces the TG release from WAT (72). The stimulatory effect of insulin on fatty acid synthesis is proposed to be mediated via an increase in *Srebp1c* (50).

In times of starvation, *de novo* synthesis of glucose from lactate, amino acids or glycerol can occur in the liver, termed gluconeogenesis (74). The regulation of hepatic gluconeogenesis is an important process in the adjustment of blood glucose levels, and the process is regulated by the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6pc*). In addition, *G6pc* catalyzes the terminal step in the catabolism of glycogen to glucose, glycogenolysis (75). Pathological changes in hepatic glucose production are central characteristics of type 2 diabetes (74).

Type 2 diabetes was previously only seen in adults but is now also occurring in obese children (73). The prevalence of type 2 diabetes in the Swedish population is 4.4% (76). An intermediate condition between diabetes and the normal state is impaired glucose tolerance (IGT), defined by fasting glucose levels <7 mmol/l and levels 2 h post-glucose load ≥7.8 and <11.1 mmol/l (72, 73). Subjects with IGT are at high risk for developing type 2 diabetes (73).

Type 2 diabetes is characterized by insulin resistance, which is an inadequate ability of insulin to mediate glucose disposal and suppression of endogenous glucose production, and insufficient insulin secretion from the pancreatic β-cells to counteract the insulin resistance (72, 77). The cause of type 2 diabetes is still elusive. However, as obesity, it is a polygenic disease (77). Regarding the basis for type 2 diabetes, increased plasma

FFAs, which is common in overweight and obese people, and accumulation of TGs in muscle and liver (hepatic steatosis) are likely to be mechanisms for development of insulin resistance, and are referred to as "lipotoxicity" (41). The elevated FFAs could impair insulin-signaling pathways in the muscle (via metabolites), increase oxidative stress and affect insulin action in the liver (78). On the other hand, FFAs can stimulate insulin secretion from pancreas. When insulin resistance has developed, pancreatic β-cells compensate by increasing insulin production. However, eventually the β-cells become dysfunctional and type 2 diabetes develops. A genetic predisposition to pancreatic β-cell failure might explain why not all obese, insulin-resistant people develop diabetes (78).

To reduce the incidence of diabetes in high-risk patients, life style changes are effective (79). Exercise significantly decreases diabetes incidence also in subjects that do not lose weight (80). Pharmacological treatments for type 2 diabetes include exogenous insulin administration, metformin (decreases hepatic glucose output), thiazolidinediones (increase insulin sensitivity by stimulating *PPARγ*), α-glucosidase inhibitors (prevent the digestion of carbohydrates) and sulfonylurea derivatives (including nateglinides that enhance insulin secretion from the pancreas) (72, 79).

For both obesity and type 2 diabetes, however, the available treatments are insufficient, and there is a great need for improved therapies.

1.6 OXIDATIVE STRESS AND METABOLIC DISEASE

Normally, mitochondrial respiration produces superoxide (O_2^-) and peroxide (O_2^{2-}) that are necessary for cellular functions, and excess superoxide is neutralized by endogenous antioxidants and antioxidant enzymes (81). Superoxide can be converted to hydrogen peroxide, which can produce extremely reactive hydroxyl radicals (82). Protective antioxidant enzymes include glutathione peroxidase and catalase (scavenges hydrogen peroxide) and superoxide dismutase (scavenges superoxide) (82).

In the metabolic syndrome and its associated diseases, one of the defects is excess cellular oxidative stress with an overproduction of reactive oxygen species (ROS), which causes cellular damage (81). Conversely, a marker of oxidative stress was decreased in two smaller studies using weight loss programs with diet and physical activity (83, 84). A potential approach for treatment of for example obesity and diabetes could be the use of antioxidants, and improvement in metabolic parameters have been seen in a number of clinical studies (85).

1.7 METABOLIC EFFECTS OF ESTROGENS

There are gender differences in the distribution of adipose tissue. Women generally have more body fat than men and a gynoid fat distribution, with fat located mainly around hips and thighs (86). Men typically have an android fat distribution with more body fat in the abdominal (central, visceral) region (86). At menopause when estrogen levels decrease, the adipose tissue mass usually increases in women and fat

accumulates intra-abdominally (87). A central fat distribution is associated with increased health risks (87).

HRT is a combination of estrogen and synthetic progesterone treatment used to alleviate menopausal symptoms. HRT decreases abdominal adipose tissue mass (88, 89), improves insulin sensitivity and lowers blood glucose levels (90) in postmenopausal women. The Women's Health Initiative study involving over 10 000 women demonstrated reduced incidence of diabetes in postmenopausal women on HRT or on estrogen treatment alone (91, 92). A meta-analysis of HRT effects using pooled data from 107 randomized controlled trials with a duration of at least 8 weeks showed an average reduction in abdominal fat of 6.8%, a reduced incidence of insulin resistance of 12.9% and a reduction in onset of type 2 diabetes of 30% (93). In women with diabetes, the fasting blood glucose was reported to be reduced by 11.5% and the insulin resistance by 35.8% upon HRT treatment (93).

Studies in rodents confirm the effects of estrogen on adipose tissue. Ovariectomy (surgical removal of the ovaries) increases adipose tissue mass and estrogen treatment decreases adipose tissue mass (94). This is associated with changes in food intake and voluntary activity (94). However, peripheral metabolic effects appear to be of primary importance for estrogen effects on adiposity, while the effects of food intake and voluntary activity are minor (94, 95). In addition, ovariectomy in rodents increases basal blood glucose levels and causes impaired glucose tolerance (96). Estrogen treatment has strong anti-diabetic effects in rodent models of type 2 diabetes (90, 97).

1.7.1 Lessons from knockout mice and human mutations

Phenotypes of knockout mice devoid of endogenous estrogen synthesis or lacking estrogen receptors provide further evidence for an important role of estrogen signaling in relation to body fat mass and glucose metabolism. Aromatase knockout mice (ArKO mice), with a genetic impairment of endogenous estrogen synthesis, exhibit increased adiposity, decreased glucose tolerance and insulin resistance (98, 99). Similar metabolic effects are seen in $ER\alpha$ but not in $ER\beta$ knockout mice (ERKO and BERKO, respectively), suggesting a critical role of $ER\alpha$ in the metabolic effects of estrogen (100, 101). The obesity in ERKO mice is due to both increased adipocyte size and adipocyte number (100). The phenotype was not related to increased food intake (100).

In humans, a mutation in $ER\alpha$ has been reported in one male. The observed phenotype was impaired glucose tolerance and hyperinsulinemia and a body weight of 127 kg (102). It also included osteoporosis and an abnormal linear growth continuing after puberty with a final height of 204 cm (102). A few cases are present with mutations in the *Cyp19* gene encoding aromatase, the enzyme critical for endogenous estrogen synthesis. These individuals present a phenotype of high serum levels of insulin and triglycerides that can be corrected by estrogen treatment (103, 104).

1.7.2 Mechanisms of estrogen action

1.7.2.1 Obesity

The tissues responsible for estrogen action in relation to metabolic disease are still under investigation. Possible target tissues for effects on adiposity are hypothalamus and adipose tissue. In a recent study, *ERα* was specifically silenced in the ventromedial nucleus of the hypothalamus (105). Obesity was observed as a phenotype. This was preceded by increased food intake but also a reduced energy expenditure (105). However, as discussed above, differences in food intake and voluntary activity are not sufficient to explain the effects of estrogens on body fat mass.

Estrogen increases hormone sensitive lipase, the rate-limiting enzyme in adipocyte lipolysis (106). In addition, estrogen decreases lipoprotein lipase, which is a lipogenic enzyme that provides substrate for deposition of TGs in adipocytes by hydrolyzing plasma TGs to FFAs and glycerol (107). The maintenance of the typical female fat distribution in premenopausal women can be attributed to an estrogen-regulated increase in the anti-lipolytic α2A-adrenergic receptors in subcutaneous adipocytes (108). More recently, estrogen treatment of pair-fed ovariectomized mice was shown to decrease the expression of *Srebp1c*, *Fas* and *Acc1* in adipose tissue, liver and muscle (95). These genes control fatty acid synthesis and reducing this pathway would decrease adipose tissue mass.

1.7.2.2 Type 2 diabetes

The anti-diabetic action of estrogens might be exerted at multiple sites, such as liver, muscle, adipose tissue and the pancreatic β-cells (90). It has been shown that glucose intolerance in ERKO mice is mainly due to hepatic insulin resistance, resulting from a defective insulin-mediated suppression of hepatic glucose production (109). This suggests that the liver is an important target organ for the anti-diabetic effects of estrogen. In addition, ArKO mice develop insulin resistance in the liver, assessed by an insulin tolerance test, which explores suppression of hepatic glucose production rather than stimulation of muscle glucose uptake (90, 99). Activation of lipogenic genes with accumulation of lipids in the liver has been proposed as a molecular mechanism for insulin resistance in ERKO and ArKO mice (109, 110). In *ob/ob* and ArKO mice, estrogen treatment reverses lipid accumulation and downregulates lipogenic genes, supporting a connection between liver lipid accumulation and insulin resistance (97, 110).

However, there is evidence for a role of additional tissues in the effects of estrogen on glucose metabolism. A reduction in the insulin-responsive glucose transporter 4 in ERKO mice could contribute to impaired glucose uptake in muscle (111). Insulin-stimulated glucose uptake in muscle has been reported to be stimulated by estrogen and decreased by ovariectomy (90). However, this might reflect indirect effects via other tissues (90). Furthermore, estrogenic effects on adipose tissue mass can indirectly affect insulin sensitivity, as obesity is associated with insulin resistance and subsequently the risk of type 2 diabetes (90). Estrogen protects pancreatic β-cells in conditions of oxidative stress in a number of rodent models of type 2 diabetes (90) and consistent with this, ArKO and ERKO mice are vulnerable to β-cell apoptosis (112).

2 AIMS OF THE STUDY

The general aim of this thesis was to characterize the molecular mechanisms that mediate the protective effects of estrogen against obesity and diabetes. Specifically, our objectives were:

- To identify novel estrogen-regulated genes in adipose tissue of mice treated with estrogen short-term (**Paper I**) or long-term (**Paper III**) in order to elucidate possible mechanisms behind the effects of estrogen on adipose tissue mass.

- To study gene expression changes occurring in human adipose tissue biopsies from estrogen-treated women and to investigate potential correlations to clinical data (**Paper II**).

- To validate the role of ERα signaling for the anti-diabetic action of estrogens and to compare the molecular events occurring upon treatment with the estrogen receptor alpha-selective agonist PPT and E2, respectively, in ob/ob mice (**Paper IV**).

3 METHODOLOGICAL CONSIDERATIONS

3.1 MICROARRAY TECHNOLOGY

In this thesis, the Affymetrix microarray technology platform was used to determine gene expression profiles. Microarrays were introduced in the late 1990s, mainly for gene expression analysis (113). However, later on applications such as genotyping, chromatin immunoprecipitation on chip (ChIP-on-chip), exon-based expression arrays and tiling arrays were developed (113).

There are several platforms for gene expression profiling. The most common ones are spotted arrays and Affymetrix GeneChips® (114). Spotted microarrays consist of cDNA sequences (in the early days) or smaller 25-70-mer oligonuclotide sequences (most common today) spotted onto glass slides (114). Two differently labeled samples are commonly hybridized to one array. These arrays can be produced in academic laboratories but this requires a lot of equipment and extensive optimization (114). The spotted arrays can also be purchased from commercial producers such as Agilent Technologies. The spotted slides will still have lower density of features than Affymetrix microarrays due to limitations in the number of spots that can be printed on a slide (114). Illumina is a relatively new technology. Advantages are lower costs compared to Affymetrix. Disadvantages are fewer data analysis and annotation options and that arrays for only a limited number of organisms are available (114).

3.1.1 Affymetrix microarrays

Affymetrix microarrays use standardized and pre-validated protocols and analysis tools (114). One sample is hybridized to one array and the technology allows a much greater number of features than with the spotted arrays (114). Affymetrix high-density manufacturing technology uses photochemical synthesis to build 25-mers on a quartz surface (113). A gene can be represented by one or several probe sets on the microarray. In the studies presented in this thesis, probe set sequences are located in the 3’ region of transcripts. For each probe set, the probes are dispersed on the array, and the signal of those that hybridize perfectly to the target (perfect match probe) is compared to those with a single mismatch centrally in the sequence (mismatch probe) to determine the specific binding (113).

3.1.1.1 Experimental design and preprocessing of data

The experimental design is one of the most important parts of a microarray experiment (114). It includes deciding the number of biological replicates and the RNA source. Generally, the more replicates the better, but it has been shown that stable results can be obtained when at least five biological replicates are used (115). The animal studies in this thesis are generally performed on three biological replicates. However, the results for key genes have always been confirmed by real-time PCR for all individual samples included in the studies (n=5-9).

Using the standard protocol for target preparation, total RNA is amplified and labeled using *in vitro* transcription, fragmented and hybridized to arrays (113). The arrays used

in this thesis contain over 12 000 (Paper I), 22 000 (Paper II and III) or 45 000 transcripts (Paper IV).

Preprocessing of microarray data include background correction, normalization and processing of raw data into expression values (116). The main methods for the latter are Affymetrix MAS5, dChip and the robust multichip/array average (RMA) method (116). We have used the MAS5 for all data except in Table 2 in Paper IV where RMA was used. Filtering of genes can be performed on the basis of expression levels or the presence and absence calls according to Affymetrix algorithms (116) There is no general consensus on how, or if, one should filter data prior to analysis (116). We did not perform filtering prior to data analysis for Paper I, II and IV. For Paper III, we excluded genes with absence calls on all arrays.

3.1.1.2 Identification of differentially expressed genes

The identification of differentially expressed genes can be performed using a number of approaches. The *t*-test which is commonly used for many biological applications is not recommended for microarray analysis due to the problem of multiple testing that occur when studying tens of thousands of genes (117). If using a cutoff of $p < 0.05$, 5% (500 genes per 10 000 genes) correspond theoretically to false positives, or are wrongly identified as significantly changed genes (117). When using more sophisticated methods, the problem of multiple testing is commonly controlled for using the adjusted Bonferroni correction (117).

In Papers I and III we applied the concordance call, using pair-wise comparisons, to identify estrogen-regulated genes. Although this method is not statistically independent, in our experience, it reliably identifies differentially expressed genes in experimental designs with few biological replicates. In Paper II, a paired experimental design was used, with measurements of the same individual before and after treatment. In Paper IV we applied the affylmGUI analysis method which is a freely available graphical user interface with powerful statistical tools, also for dealing with small sample sizes (118).

3.2 REAL-TIME PCR ANALYSIS

Real-time PCR analysis is a very sensitive method for quantification of specific mRNAs. Instead of using end-point measurement as in conventional PCR, amplification start for each transcript is detected in “real time”. Total RNA from tissues or cells of interest is reversely transcribed into cDNA and analyzed using gene-specific primers.

Two methods are available for detection of amplified DNA, gene-specific TaqMan-probes or the general DNA-binding SYBR Green dye. TaqMan-probes allow a highly specific recognition of the target cDNA. The SYBR Green method reduces the costs for real-time PCR analysis but requires that the primers are carefully designed for optimal selectivity and that melting curves are run for new primers to confirm a single PCR product. The dyes used generate fluorescence, which is relative to the amount of PCR product produced. Both TaqMan and SYBR Green based assays were used in the work described in this thesis. A reference gene is used to control for differences in input

amount. We have normalized the data to 18S, hypoxanthine ribosyltransferase or beta-2 microglobulin.

4 RESULTS AND DISCUSSION

4.1 GENE EXPRESSION PROFILING IDENTIFIES LIVER X RECEPTOR ALPHA AS AN ESTROGEN-REGULATED GENE IN MOUSE ADIPOSE TISSUE (PAPER I)

At first, we characterized the relative levels of ERs in mouse WAT, since it was not previously described. We found that *ERα* is the main ER expressed in mouse WAT with mRNA levels several hundred times higher than those of *ERβ*. The relatively high expression level of *ERα* in WAT should enable estrogen to act directly on adipose tissue.

We aimed initially at characterizing the more direct effects of estrogen on adipose tissue gene expression. Thus, gene expression profiles were determined after short-term estrogen exposure. Female ovariectomized mice were treated with estrogen for 10, 24 or 48 h and RNA from WAT was analyzed using gene expression profiling (Affymetrix microarrays). This single treatment dose produced high E2-levels after 10 h, which decreased by time and were almost back to basal levels after 48 h. The number of regulated genes was highest at 24 h. In this study, we report data for about 90 genes involved in different aspects of lipid metabolism.

One of the regulated genes was *LXRα*, a nuclear receptor involved in cholesterol and fatty acid metabolism (7). Estrogen decreased the mRNA levels of *LXRα* after 10 h of treatment compared to vehicle control. Further studies showed that a 1.5 kb *LXRα* promoter fragment was negatively regulated by estrogen via *ERα*, suggesting that *LXRα* is a direct estrogen target gene. Evidence for regulation of the *LXRα* pathway was provided by decreased expression of several *LXRα* target genes, such as *Srebp1c*, apolipoprotein E, phospholipid transfer protein, ATP-binding cassette A1 and ATP-binding cassette G1 after 10 h of estrogen treatment. The regulation of *Srebp1c* was of particular interest, since it is a key regulator of the fatty acid synthesis pathway. We hypothesize that the observed changes in gene expression could be important for the effects of estrogen on adiposity.

In this experiment we used two pools of RNA (n=3/4) for each treatment group. The number of microarrays was restricted by their high cost at the time. However, the direction of regulation and approximate fold change could be confirmed by real-time PCR of tested genes, using individual samples. In later microarray studies, we analyzed individual samples to facilitate the identification of outliers and most importantly to allow proper statistical analysis.

To determine if regulation of *LXRα* was critical for the estrogen-mediated effects, we treated wild type and *LXRα*-knockout mice with estrogen for 3 weeks and analyzed adipose tissue weight (Figure 4). The reduction in gonadal WAT weight upon estrogen treatment was larger in wild type than in *LXRα*-knockout mice, which implicates a function for *LXRα* in estrogen-induced fat loss. However, the WAT weight for vehicle-treated mice was lower in *LXRα*-knockout mice than in wild type mice. Since the

experiment was difficult to interpret, we did not include it in the paper. In retrospect, perhaps the *LXRαβ* double knockout mice would have given more conclusive results since the two *LXRs* can compensate for each other.

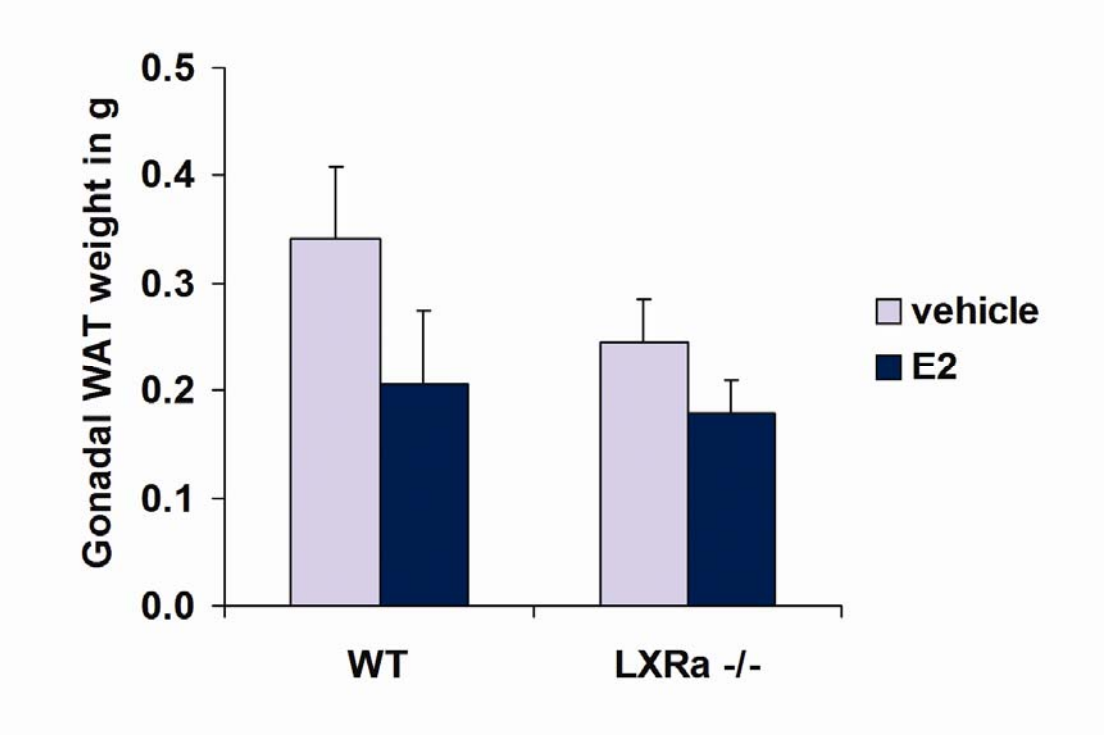

Figure 4. Gonadal WAT weight in wild type (WT) and *LXRα*-knockout mice (LXRa -/-) treated with 17β-estradiol (E2) and vehicle.

4.2 KEY LIPOGENIC GENE EXPRESSION CAN BE DECREASED BY ESTROGEN IN HUMAN ADIPOSE TISSUE (PAPER II)

In this paper, we studied changes in gene expression in subcutaneous abdominal adipose tissue biopsies from ten naturally postmenopausal women before and after 3 months of treatment with estradiol valerate. RNA was prepared and analyzed using gene expression profiling (Affymetrix microarrays). Genes involved in fatty acid synthesis, such as stearoyl-CoA desaturase (*Scd1*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase α (*Acc1*) and fatty acid desaturase 1, were decreased by estrogen treatment in a subgroup of women. Several of these genes are target genes for *LXRα* and *Srebp1c*, which were identified as estrogen-regulated in mouse adipose tissue in Paper I. Regulation of *Scd1*, *Fas* and *Acc1* have been reported in mice after long-term estrogen treatment (95, 97). The decreased expression of *Scd1*, *Fas* and *Acc1* after estrogen treatment was also verified in a human cell line. This indicates that regulation is not due to adaptive alterations in the subjects. This is the first report showing estrogen regulation of these genes in human abdominal adipose tissue.

This study clearly demonstrated that individuals respond differently with regard to estrogen-regulated changes in gene expression in human subcutaneous adipose tissue. Changes in the expression of key lipogenic genes were restricted to a subgroup of women. The changes were inversely correlated to baseline values of in particular

plasma TGs. This might indicate that the response to estrogens is determined by the individual metabolic status. We speculate that estrogen is more effective in decreasing lipogenic genes when the individual has a higher burden of TGs. Furthermore, changes in the expression of key lipogenic genes were positively correlated to changes in clinical parameters, in particular plasma TGs.

PPARγ, involved in adipogenesis, was decreased in a subgroup of individuals. *PPARγ* is required to coordinate the expression of adipogenic genes that characterize the terminal differentiation of adipocytes (38). Its down-regulation by estrogen would suggest that not only the lipid content but also the number of adipocytes could be decreased by estrogen administration.

4.3 EFFECTS OF ESTROGEN ON GENE EXPRESSION PROFILES IN MOUSE HYPOTHALAMUS AND WHITE ADIPOSE TISSUE; TARGET GENES INCLUDE GPX3 AND CIDEA (PAPER III)

The relative tissue contribution for the effects of estrogens on adiposity remains unclear. We approached this issue by investigating the effects of estrogen on gene expression profiles for two possible target tissues, hypothalamus and WAT. We studied long-term effects (3 weeks) of estrogen treatment on hypothalamus and WAT gene expression profiles in mice using Affymetrix microarrays. The number of regulated genes was small in hypothalamus compared to WAT, which could be indicative of the relative roles of these tissues with regard to the effects of estrogen on adipose tissue mass. Consistent with this, the total mRNA levels for ERs were also higher in WAT than in hypothalamus.

No genes regulating appetite or satiety were found to be regulated after 3 weeks of estrogen treatment in hypothalamus. Surprisingly, we did not identify *LXRα*, *Srebp1c*, *Scd1, Fas* or *Acc1* as regulated after long term treatment in mice. As the adipose tissue weight was nevertheless decreased, this implies that there are additional mechanisms mediating the effects of estrogen on fat mass. One such gene could be glutathione peroxidase 3 (*Gpx3*), encoding an enzyme that protects against oxidative stress.

In WAT, *Gpx3* was induced by 3 weeks of estrogen treatment. Its regulation by estrogen was characterized in detail. *Gpx3* was regulated by E2 treatment already after 2 h of treatment in WAT, suggesting that it is a direct estrogen target gene. It was also demonstrated to be regulated in other tissues, thus its regulation by estrogen is not confined to WAT. A 2 kb *Gpx3* promoter construct was induced by *ERα* and *ERβ* in the mouse preadipocyte 3T3L1K cell line. This suggests that *Gpx3* can be regulated by both *ERα* and *ERβ*.

Obesity is positively correlated with oxidative stress, which is true also for diabetes, cardiovascular disease and smoking (119). *Gpx3* expression is low in obesity and high after weight loss (120, 121), and we hypothesize that the observed changes in *Gpx3* expression could be important for the effects of estrogen on adiposity.

Another gene, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*), which is related to body fat in mice, was identified as decreased by estrogen treatment. *Cidea*-deficient mice are resistant to diet-induced obesity and diabetes (122). *Cidea* might have a role in regulation of energy expenditure, perhaps via inhibition of uncoupling protein 1 (123). We hypothesize that *Cidea* could be a possible mediator of the effects of estrogen on fat mass.

4.4 THE ESTROGEN RECEPTOR ALPHA-SELECTIVE AGONIST PPT IMPROVES GLUCOSE TOLERANCE IN OB/OB MICE; POTENTIAL MOLECULAR MECHANISMS (PAPER IV)

Previous studies by our group showed that glucose intolerance in ERKO mice is mainly due to hepatic insulin resistance (109), and that clear anti-diabetic effects of estrogen are demonstrated in ob/ob mice after treatment with E2 for one month (97). The glucose intolerance observed in ERKO mice would suggest that signaling via *ERα* is responsible for the anti-diabetic effects of estrogen. To directly validate the role of ERα signaling in regulation of glucose metabolism, mice were treated with the ERα-selective agonist PPT. In addition, we compared the molecular events occurring upon treatment with PPT and E2, respectively, in female ob/ob mice. PPT and E2 treatment improved glucose tolerance, insulin sensitivity and the insulin response to glucose *in vivo*. Fasting blood glucose levels and basal insulin levels were decreased, while there was no effect on insulin secretion from isolated islets. Basal and insulin-stimulated glucose uptake in skeletal muscle and adipose tissue was similar between PPT and vehicle-treated mice. The lack of effects of estrogen on non-hepatic tissues supports the notion that the anti-diabetic effects of estrogen and selective *ERα* modulators are due to improved hepatic insulin sensitivity in these animals.

We conclude that the effects of E2 on glucose metabolism are mediated via *ERα*, since PPT and E2 have similar effects on glucose metabolism. However, hepatic lipids were decreased only after E2 treatment. This was unexpected, since data from earlier studies showed an up-regulation of lipogenic genes in ERKO mice (109) and conversely a down-regulation of lipogenic genes and a reduction in liver TG levels after E2 treatment of ob/ob mice (97). Furthermore, the lipogenic genes *Scd1* and *Fas* were not significantly decreased in PPT-treated ob/ob mouse liver. This suggests that regulation of hepatic lipid metabolism is not critical for PPT-mediated improvements in glucose tolerance and insulin sensitivity.

We performed microarray experiments (Affymetrix) on RNA from liver samples and compared the genes changed by PPT and E2 treatment in ob/ob mice. Genes that are critical to the observed phenotype of improved glucose tolerance were supposed to be similarly regulated by these compounds. We identified the transcription factor signal transducer and activator of transcription 3 (*Stat3*) (increased) and the enzyme *G6pc* (decreased) as genes regulated by both compounds and thus candidate genes for mediating the effects of estrogen in relation to glucose tolerance. *Stat3* and *G6pc* are candidates for being primary *ERα*-target genes in the liver, as they are regulated already after 4-6 h of PPT treatment. Both genes are potentially interesting as mediators

of the effects of estrogen agonists on glucose metabolism. Liver-specific knockout and reexpression of *Stat3* provide strong evidence for its role in improving glucose tolerance and insulin resistance (124). Humans with mutations in *G6pc* display hypoglycemia. *G6pc* regulates crucial steps in glucose production and inhibitors of *G6pc* are potential targets for treatment of type 2 diabetes (75).

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

During my thesis studies I have extensively applied microarray technology for gene expression profiling. The technology and analysis methods have naturally developed during these years but it was clear that it was going to become a very powerful technology already when I started my thesis work. The microarray technology has its limitations; one always has to remember that the results represent a snapshot of the processes occurring at a given time. After for example long-term treatments, the timing since the last dose might be important. Important aspects in relation to long-term treatments are that changes can occur as either a cause or a consequence. In addition, a major challenge is the interpretation of the data. Usually it is not easy to judge the relative importance of one gene within a long list of regulated genes. Pathway analysis can sometimes assist in focusing on important altered pathways. Still, published literature provides most of the background information about previously known functions.

Nevertheless, we have gained significant new knowledge about estrogen receptor signaling in metabolic tissues during the course of this thesis. Prior to these studies, relatively few investigations on the metabolic effects of estrogens, especially in adipose tissue, had been reported.

The genes changed in our studies are generally different from those affected by either activation or knockout of other nuclear receptors than estrogen receptors or coregulators with clear metabolic functions. For example, *ERRα*, *PPARβ* and *PPARγ* coactivator (*PGC*) 1α all typically regulate genes involved in mitochondrial biogenesis, adaptive thermogenesis and fatty acid oxidation (125-127).

One interesting observation in our studies is that estrogenic effects on gene expression, and potentially other parameters, are more pronounced when physiologically "needed". Our studies in ob/ob mice (Paper IV) and ongoing studies in mice on high fat diet (unpublished results) suggest a stronger effect of estrogen on gene expression in mouse models of obesity and diabetes than in lean mice. In addition, in the human study (Paper II), individuals with higher TG levels had more decreased expression of lipogenic genes in adipose tissue than those with lower TG levels. This is supported by published data on HRT, where clinical trials revealed a larger reduction in insulin resistance in women with diabetes than without diabetes (93) and in women with coronary heart disease (predisposed to oxidative stress) (128). In addition, HRT had more pronounced effects on decreasing central adiposity in women with a waist circumference > 88 cm than < 88 cm (89).

Tissue-specific *ERα* knockouts will in the future aid in the determination of the roles of different tissues for the development of obesity and glucose intolerance.

Hopefully, this thesis will provide novel ideas for treatment strategies for metabolic disease. For example some of the target genes identified in this study might provide

novel therapeutic strategies for this indication. Furthermore, specific *ERα*-agonists acting only in relevant tissues could be pharmaceutically attractive. The crucial part includes avoiding stimulation of growth in breast and uterus. Novel strategies might include local hormone patches for treatment of abdominal obesity or use of liver-specific drugs.

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