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Molecular mechanisms of antidiabetic effects of estrogen

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To my family

ABSTRACT

The role of estrogen in the development and regulation of female reproduction is well established. However, recent studies demonstrate that estrogen has a multiple effects on body functions. At present, it is becoming well recognized that one of the important roles of estrogen is the maintenance of glucose homeostasis.

The overall objective of this study was to investigate the molecular mechanisms that mediate the beneficial effects of estrogen on glucose tolerance (GT) and insulin sensitivity. Using estrogen receptor (ER) α knockout (ERKO) and ERβ knockout (BERKO) mice we have shown that a lack of ERα, but not of ERβ resulted in the development of glucose intolerance and insulin resistance in both female and male mice. Euglycaemic-hyperinsulinemic clamp with the determination of glucose turnover revealed that in ERKO mice impaired insulin sensitivity is due to pronounced hepatic insulin resistance. Gene expression profiling in ERKO mice demonstrated an increased expression of genes, involved in lipid biosynthesis in the liver, and the decreased expression of hepatic leptin receptor (*Lepr*), that could be potential mechanisms of hepatic insulin resistance in ERKO mice (**Paper I**). To further develop this hypothesis we treated ob/ob mice with 17β-estradiol (E2) for 30 days and found a significant improvement in GT and insulin sensitivity. Concordant with above findings these effects of E2 were accompanied by the downregulation of lipogenic genes and the increased hepatic expression of *Lepr* in liver. Microarray analyses revealed an increased hepatic mRNA expression of signal transducer and activator of transcription 3 (*Stat3*) after E2 treatment; we also showed that *Stat3* is direct target gene of E2 in liver. Hence, the improvement in GT and insulin sensitivity in ob/ob mice after E2 treatment may result from stimulation of the hepatic expression of Stat3, leading to the downregulation of hepatic lipogenic genes (**Paper II**). In **Paper III**, we demonstrated that similar to E2, the ERα-selective agonist propyl pyrazol triol (PPT) improved GT and insulin sensitivity in ob/ob mice. Thus, we confirmed that the antidiabetic effects of E2 are mediated via ERα-signaling. The lack of any effect of PPT treatment on glucose uptake by muscle and adipocytes indicates that its antidiabetic properties are mainly due to the improvement of hepatic insulin sensitivity. Increased hepatic expression of *Stat3* and decreased hepatic expression of glucose-6-phosphatase (*G6pc*) could constitute one important mechanism behind the antidiabetic effects of E2 and PPT. In **Paper IV** we have used high fat diet (HFD) fed mice, a model of obesity, glucose intolerance and insulin resistance to further elucidate the molecular mechanisms underlying the antidiabetic and weight-lowering effects of E2. C57BL mice were given HFD for up to 12 months and treated with E2 during last month of feeding. E2 administration resulted in decreased body weight and abdominal fat mass, and improvements in GT and insulin sensitivity. Mechanisms underlying these effects of E2 are linked to the downregulation of expression of sterol regulatory element binding protein 1c (*Srebp1c*) and its target lipogenic genes in white adipose tissue (WAT) and of stearoyl-CoA desaturase (*Scd1*) in the liver. Supression of hepatic *G6pc* could also play a crucial role in this context.

In conclusion, our data provide a deeper understanding of the molecular mechanisms behind the antidiabetic effects of E2. E2, acting via ERα exerts its beneficial effects on GT and insulin sensitivity mainly by regulating the expression of genes involved in the control of fatty acid synthesis and of *G6pc* expression, and also by modulating of adipokine signaling.

Key words*: estrogen, glucose tolerance, insulin sensitivity, lipogenic genes*, *Lepr, Stat3, G6pc, Srebp1c,*

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LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. **Bryzgalova G***, Gao H*, Ahren B, Zierath JR, Galuska D, Steiler TL, Dahlman-Wright K, Nilsson S, Gustafsson J-Å, Efendic S, Khan A. Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia* 2006 Mar;49(3):588-97.
*Contributed equally

II. Gao H*, **Bryzgalova G*,** Hedman E, Khan A, Efendic S, Gustafsson J-Å, Dahlman-Wright K. Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol Endocrinol* 2006 Jun; 20(6):1287-99.
*Contributed equally

III. Lundholm L*, **Bryzgalova G*,** Gao H, Portwood N, Fält S, Berndt KD, Dicker A, Galuska D, Zierath JR, Gustafsson J-Å, Efendic S, Dahlman-Wright K, Khan A. The estrogen receptor alpha-selective agonist PPT improves glucose tolerance in ob/ob mice; potential molecular mechanisms. Submitted manuscript.
*Contributed equally

IV. **Bryzgalova G**, Lundholm L, Portwood N, Gustafsson J-Å, Khan A, Efendic S, Dahlman-Wright K. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high fat diet-fed mice. *Am J Physiol* In press.

OTHER PUBLICATIONS BY THE SAME AUTHOR

I. Davani B, Portwood N, Bryzgalova G, Reimer MK, Heiden T, Ostenson CG, Okret S, Ahren B, Efendic S, Khan A. Aged transgenic mice with increased glucocorticoid sensitivity in pancreatic beta-cells develop diabetes. *Diabetes* 2004 Feb;53 Suppl 1:S51-9.

II. Dun XP, Wang GH, Chen L, Lu J, Li FF, Zhao YY, Cederlund E, Bryzgalova G, Efendic S, Jörnvall H, Chen ZW, Bergman T. Activity of the plant peptide aglycin in mammalian systems. *FEBS J* 2007 Feb;274(3):751-9.

III. Bryzgalova G, Efendic S, Khan A, Rehnmark S, Barbounis P, Boulet J, Dong G, Singh R, Shapses S, Malm J, Webb P, Baxter JD, Grover GJ. Anti-obesity, anti-diabetic, and lipid lowering effects of the thyroid receptor β subtype selective agonist KB-141. *J Steroid Biochem Mol Biol* 2008 In press.

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LIST OF ABBREVIATIONS

Acc	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AF	activation function
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BERKO	estrogen receptor β knockout mice
CoA	coenzyme A
Cpt1	carnitine palmitoyl transferase 1
DBD	DNA binding domain
EGP	endogenous glucose production
ER	estrogen receptor
ERE	estrogen receptor element
ERKO	estrogen receptor α knockout mice
E2	17β-estradiol
Fas	fatty acid synthase
FFA	free fatty acid
G6pc	glucose-6-phosphatase, catalytic
GT	glucose tolerance
HFD	high fat diet
IL	interleukin
i.p.	intraperitoneal
KHB	Krebs-Henseleit bicarbonate buffer
KRB	Krebs-Ringer bicarbonate buffer
LBD	ligand binding domain
Lce	long-chain elongase
LXRα	liver X receptor
PPAR	peroxisome proliferator activated receptor
PPT	propyl pyrazole triol
s.c.	subcutaneous
SERM	selective estrogen receptor modulator
Scd1	stearoyl-CoA desaturase 1
Srebp1c	sterol regulatory element binding protein 1c
Stat3	signal transducer and activator of transcription 3
TG	triglyceride
TNF-α	tumor necrosis factor-α
T2D	type 2 diabetes
WAT	white adipose tissue

1 INTRODUCTION

1.1 Estrogen physiology and biosynthesis

Estrogens are a steroid compounds that function as the primary female sex hormone.They are synthesized in the ovary, testis, adrenal cortex and other tissues. Estrogens induce the development and maturation of female secondary sexual characteristics, the pubertal growth spurt, skeletal maturation, and play a central role in the control of reproductive function and sexual behavior both in females and males (1). At present, it is becoming recognized that the impact of estrogens in physiology is even wider, since estrogens exert profound regulatory effects on differentiation, growth and function in a variety of tissues including the brain, adipose tissue and cardiovascular system (1-5).

Estrogens were the first isolated steroid hormones and like other steroids, are derived from cholesterol (6). Side chain cleavage of cholesterol leads to synthesis of pregnenolone, which is precursor for C^{19} and C^{18} steroids. Androstenedione is the key intermediary in the synthesis of sex steroids. A fraction of androstenedione is converted to testosterone, which in turn undergoes conversion to 17β-estradiol (E2). Alternatively, androstenedione is converted to estrone, which is subsequently converted to E2 (Figure 1).

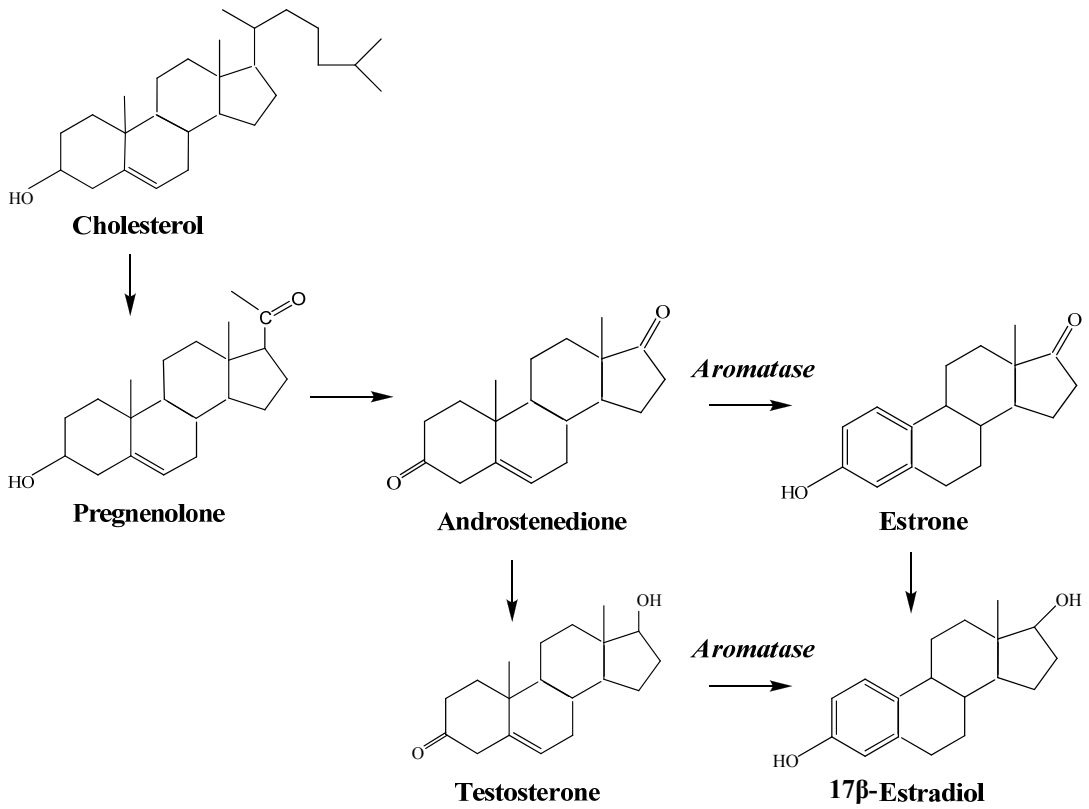

Figure 1. ***Summary of the pathway of estrogen biosynthesis***

The final step in the biosynthesis of estrogens from C19 steroids is catalyzed by aromatase cytochrome P450, which is located in the endoplasmic reticulum of estrogen-producing cells and is the product of the CYP19 gene (7). E2 is the major ovarian estrogen in premenopausal women and functions as a circulating hormone to act on distal target tissues. However, in postmenopausal women estrone is the major estrogenic form, which can be readily converted to E2 for actual use. Estriol is the most abundant estrogen during pregnancy. When the ovaries cease to produce estrogens, a number of extragonadal sites synthesize estrogens, which act predominantly at the local tissue level in a paracrine or intracrine fashion (8). These extragonadal sites include adipose tissue (9), bone (10), the vascular system (11) and the brain (12).

1.2 Estrogen receptors

Jensen and Jacobson (13) were the first who demonstrated in 1962 that E2 exerts its effect by binding to a receptor. Later Green *et al*. (14) in 1986 and Greene *et al*. (15) in 1986 cloned the estrogen receptor (ER) protein. However, it was assumed that only one form of ER existed, until Kuiper *et al*. (16) discovered in 1996 a second ER which was termed ERβ; the classical ER was referred to as ERα. Now it is clear that ERα and ERβ (595 and 530 amino acids, respectively) are encoded by different genes located on different chromosomes (locus 6q and locus 14q, respectively) and do not represent splice variants (17, 18).

1.2.1 Structure of estrogen receptors

ERα and ERβ belong to the nuclear receptor superfamily of ligand-activated transcription factors. The structure of nuclear receptors is typical for all members of this family. ERs consist of five distinct domains: A/B, C, D, E and F. The N-terminal A/B domain contains the ligand-independent activation function (AF-1) which activates the transcription of target genes. The C domain, the DNA-binding domain (DBD), is highly conserved and responsible for DNA-binding specificity and receptor dimerization. The D domain is a hinge, providing flexibility between the C and E domains. The E domain is called the ligand-binding domain (LBD) and is responsible for ligand binding, receptor dimerization and transcriptional activation, and possesses a ligand-dependent activation function (AF-2). The function of the F domain is still unclear (19) (Figure 2). Transcriptional activity of ERα is mediated by two transcription activation functions: AF-1 and AF-2 (20), while ERβ seems to have a less pronounced AF-1 activity, acting mostly through the ligand-dependent AF-2 (21).

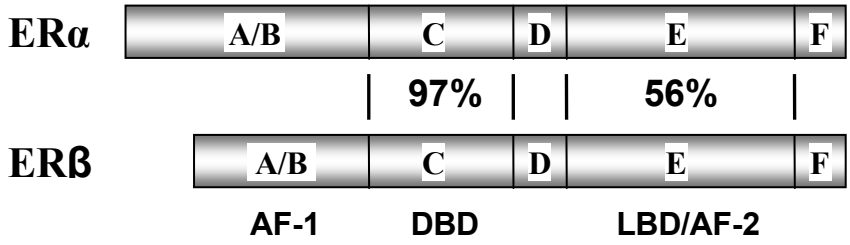

Figure 2. ***Schematic organization of the structure of the human estrogen receptors***

1.2.2 Tissue distribution of estrogen receptors

ERα and ERβ are expressed in a wide spectrum of tissues in both males and females. However, the average ratio of ERα to ERβ in the different tissues varies, and some tissues contain both ERs, but with isoform-specific localization to different regions or cell types. For example, ERα is mainly expressed in the uterus, ovary (theca cells), prostate (stroma), testes (Ledig cells), pituitary, hypothalamus, bone, breast, skeletal muscle, liver and adipose tissue, whereas ERβ is expressed in ovary (granulosa cells), the prostate (epithelium), testis, bone marrow, vascular endothelium and some brain regions including the limbic system, cerebellum and cerebral cortex (22, 23, 24).

1.3 Mechanisms of estrogen actions

1.3.1 Genomic mechanism

The classical genomic mechanism of E2 signaling states that in the absence of ligand the ER is bound to heat shock proteins, which maintain the receptor in an inactive condition. Binding of E2 to its receptor induces its dissociation from heat-shock proteins, followed by dimerization and translocation to the nucleus. There, the ERs bind to the estrogen receptor element (ERE) in the promoters of target genes (25). The subsequent activation or inhibition of gene transcription depends on the E2/ER complex-dependent recruitment of specific co-regulatory proteins which may be coactivators or corepressors (26).

1.3.2 Non-genomic mechanism

In addition to the classic genomic mechanism, E2 induces cellular effects independently of ER transcriptional activity. These non-genomic mechanisms become activated quickly, within seconds to minutes (27). These effects do not involve the synthesis of mRNA or protein and cannot be blocked by transcription and translation inhibitors (28). E2 induces

rapid signals involving the activation of several signal transduction pathways (29, 30) in E2-sensitive cells, and some of these signaling pathways could be cell-type specific.

1.4 Current approaches for studying E2/ERs signaling

Accumulating data about ER structure and the mechanisms of ER action enable various approaches for investigating of the respective roles of ERα and ERβ in the control of the physiology of E2 target organs and tissues in both the normal state and in different pathological conditions.

1.4.1 Genetically modified mice

A powerful tool for the examination of E2 signaling via ERα and/or ERβ has been provided by the generation of mice with disruption of one or both ERs. ERα knockout (ERKO) mice were created by Lubahn *et al.* (31) in 1993. Both female and male mice survived to adulthood and had a normal external phenotype, but were infertile. ERβ knockout (BERKO) mice were generated in 1998 by Krege *et al*. (32). Both sexes of BERKO mice had normal sexual behavior and were fertile. Further study demonstrated that in ERKO mice, but not in BERKO mice, body mass, fat deposition and cholesterol levels were increased (33) and both female and male ERKO mice had impaired glucose tolerance (GT), (34).

In 1998, another knockout mouse model was created by targeted disruption of the aromatase gene (ARKO mice) (35). These mice express both ERs, but cannot synthesize estrogens. Estrogen insufficiency in ARKO mice led to the development of adiposity with simultaneous decreases in lean mass in both sexes, in association with hyperleptinemia, hyperinsulinemia and hypercholesterolemia (36).

1.4.2 Selective estrogen receptor modulators

Another approach to study ER signaling came from the design of selective estrogen receptor modulators (SERMs), agonists and antagonists that work by virtue of their interaction with the ER.

The molecular basis of SERM activity involves binding of the SERM ligand to the ER, causing conformational changes which facilitate interactions with coactivator or corepressor proteins, leading to the activation or inhibition of transcription of target genes. SERM activity is intrinsic to each ER ligand, which accomplishes its unique profile by specific interactions in the target cell, leading to tissue selective actions (37, 38, 39).

The SERM tamoxifen emerged more than 30 years ago as the first antiestrogenic ligand that is clinically applicable to breast cancer, and turned out to act as an antagonist in the breast and an agonist in the uterus and bone (40). Raloxifen is one of a second generation of SERMs which possess improved tissue selectivity, and which are used for the prevention of osteoporosis in postmenopausal women (41).

Propyl pyrazole triol (PPT) represents the first ERα-specific agonist and has a 400-fold binding affinity preference for ERα over ERβ (42). In ovariectomized rats, PPT was as efficacious as E2 in stimulating uterine weight gain and prevented ovariectomy-induced body weight gain and loss of bone mineral density (43).

1.5 Insulin secretion and action

1.5.1 Insulin secretion

Two hormones, insulin and glucagon, produced by the pancreatic islets are responsible for controlling blood glucose concentrations. Insulin decreases blood glucose levels and glucagon increases glucose concentrations in the blood. The majority of the cells in pancreatic islets are insulin-secreting β-cells. The remaining cell types are glucagon-secreting α-cells, delta-cells which produce somatostatin, and PP cells which release pancreatic polypeptide. Insulin secretion from pancreatic β-cells is stimulated by elevated blood glucose levels. Glucose is transported into the β-cell by facilitated diffusion through the glucose transporter GLUT2, and undergoes glycolytic metabolism. This leads to the increased production of ATP in the mitochondria. The cytoplasmic ATP/ADP ratio acts as an intracellular messenger coupling nutrient metabolism to electrical activity in β-cells. An increase in the cytoplasmic ATP/ADP ratio leads to the closure of K_{ATP} channels, resulting in depolarization of plasma membrane. This in turn leads to the opening of voltage-gated L-type Ca^{2+} channels and an influx of extracellular Ca^{2+}. Elevations in cytoplasmic levels of Ca^{2+} trigger exocytosis of insulin granules and insulin release (44), (Figure 3).

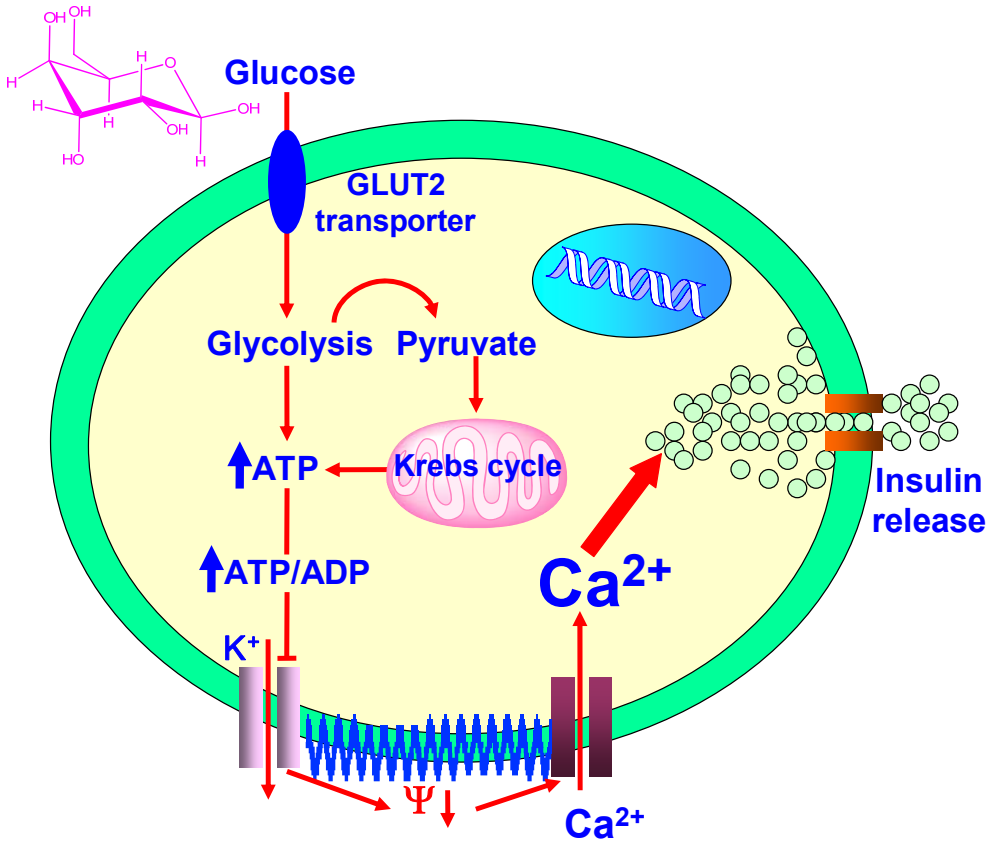

Figure 3. ***Insulin secretion from β-cells***

1.5.2 Insulin action

Insulin, secreted in response to high levels of circulating glucose, stimulates the uptake of glucose into the liver, muscle and fat. In both liver and muscle, insulin stimulates glycogen synthesis, and suppress gluconeogenesis and glycogenolysis in liver (45), resulting in decreased hepatic glucose production. If glucose intake continues after muscle and liver glycogen storage are saturated, glucose is converted to triglyceride (TG) which is then transported to the adipose tissue for storage. In adipose tissue insulin increases TG synthesis (46) and exerts an antilipolytic effect (47).

1.6 Role of liver and white adipose tissue in the regulation of glucose homeostasis

1.6.1 Liver

Liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose via glycogenesis and the release glucose via glycogenolysis and gluconeogenesis. The production of glucose from other metabolites such as lactate, certain amino acids or glycerol, a process called gluconeogenesis, is

increased during starvation and is necessary in providing a fuel source for the brain, testis, erythrocytes and kidney medulla, in which glucose is the sole energy source. Phosphoenolpyruvate carboxykinase and gucose-6-phosphatase (*G6pc*) are key enzymes in gluconeogenesis. *G6pc* catalyzes the terminal step in both the gluconeogenic and glycogenolytic pathways (45). Glucose excess in the liver can be converted to fatty acids and consequently to TG. The synthesis of fatty acids from carbohydrates occurs in both liver and adipose tissue (48). Glucose is converted to pyruvate by glycolysis. The pyruvate is converted to acetyl-CoA, which is the starting material for the synthesis of fatty acids and subsequently TG (Figure 4).

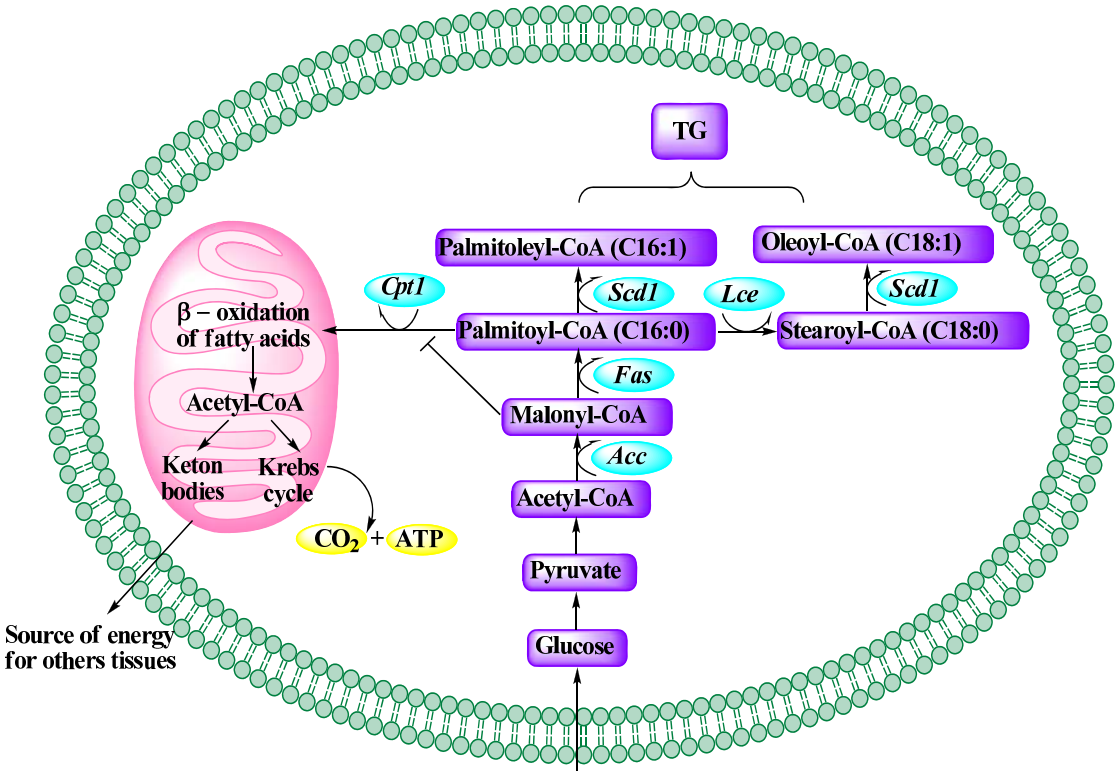

Figure 4 ***Main pathways of fatty acid metabolism. Fatty acid synthesis:*** *Acc – acetyl-CoA carboxylase, Fas – fatty acid synthase,* ***Scd1*** *– stearoyl CoA desaturase, Lce – long chain elongase.* ***Fatty acid oxidation:*** *Cpt1 – carnitine palmitoyl transferase 1.*

1.6.2 White adipose tissue

In white adipose tissue (WAT), glucose is converted to TG. The most important function of WAT is the storage of energy for the body's need. Approximately 60 to 85% of the weight of WAT is lipid, with 90-99% being TG. Adipocytes in WAT contain only a single large fat droplet, which is almost entirely TG. The main function of these cells is the synthesis and storage of TG and the release of free fatty acids (FFA) into the blood stream when required. TG undergo lipolysis, catalyzed by hormone sensitive lipase and are broken down into glycerol and fatty acids. Once released into the blood, the relatively hydrophobic FFA bind to serum albumin for transport to tissues that require energy. Thus, the size of the adipose tissue storage depot increases in periods of positive energy balance and declines when energy expenditure is in excess of intake (49).

However, WAT is no longer viewed as a passive site for the storage of TG and the source of fatty acids, but also plays an active role in diverse metabolic processes including the regulation of energy balance, food intake, insulin action, lipid and glucose metabolism (50, 51).

1.7 Type 2 diabetes

Type 2 diabetes (T2D), also called non-insulin-dependent diabetes mellitus, is a metabolic disorder that is primarily characterized by hyperglycemia, insulin resistance and β-cell dysfunction. The incidence of T2D is rapidly increasing worldwide and is considered as one of the main threats to human health in the 21st century (52). The amount of people with diabetes is estimated to be 220 million in 2010, and will exceed 300 million in 2025 (53), among whom almost 90% are accounted for by T2D. The prevalence of T2D among the Swedish population is about 4%, which corresponds to about 300 000 people (54). There is a great deal of evidence that both genetic and environmental factors are of importance in the pathogenesis of T2D. Whereas the genetic factors are still poorly understood, numerous studies have shown that obesity, sedentary lifestyle and high fat diet (HFD) are the major risk factors for developing T2D (55, 56, 57). T2D increases the risk for cardiovascular diseases, which constitutes about 75% of all mortality in diabetic patients (58).

Both impaired insulin secretion and insulin resistance contribute to the hyperglycemic state and subsequently to the development of T2D. The interplay between insulin secretion and insulin action constitute the framework within which glycemia is controlled.

1.7.1 β-cell dysfunction

It is well known that there is a progressive deterioration in β-cell function over time in T2D subjects (59). Pancreatic islet function was found to be about 50% of normal at the time of diagnosis, independent of the degree of insulin resistance, with the reductions in function probably commencing 10-12 years before diagnosis (60). These changes were accounted for by the reduction in β-cell mass by apoptosis, wheareas new islet formation and β-cell replication remained normal or increased (61, 62). When insulin action decreases (as occurs in obesity), the system tries to compensate by increasing β-cell function (63). Importantly, even mild elevated glucose levels over a long period of time cause damage of β-cells, because of glucotoxicity (64). Furthermore, increased concentrations of FFA, which result from enhanced adipocyte lipolysis, lead to lipotoxicity and β-cell dysfunction (47).

1.7.2 Insulin resistance

Insulin resistance is the condition in which responsivenes to insulin is decreased in fat, muscle and liver cells. Initially, resistance can be compensated for by an increase in insulin secretion, and blood glucose remains under control. However, as insulin resistance worsens, insulin secretion progressively decreases and hyperglycemia of a greater magnitude ensue (65). Insulin resistance in muscle cells reduces glucose uptake, whereas insulin resistance in liver cells reduces storage of glycogen and increases gluconeogenesis. In the fasting state, endogenous glucose production (EGP) by liver is responsible for all glucose entering the blood circulation. EGP is accelerated in patients with T2D or in people with impaired fasting glucose levels (65, 66). Insulin resistance in fat cells reduces the effects of insulin and results in elevated hydrolysis of stored TG, that in turn leads to increased FFA levels in the blood circulation.

1.7.3 Obesity and insulin resistance

The prevalence of obesity is increasing world-wide, not only in industrialized countries but also in developing countries, including the Far East, the Middle East and South America (67). Excess body weight and an elevated body mass index have been clearly identified as risk factors for insulin resistance and T2D (62). Body fat distribution is itself a critical determinant in the development of insulin resistance. An increased mass of stored TG, especially in visceral and deep abdominal subcutaneous depots, leads to large adipocytes, that are less sensitive to the antilipolytic effects of insulin. These two depots possess high lipolytic activities, compared to superficial subcutaneous adipocytes (68, 69). As a result, these adipocytes contribute to high FFA levels in the circulation, and promote ectopic

storage of fat in non-adipose cells such as hepatocytes and myocytes (70, 71), which results in an aggravation of insulin resistance (72). Obesity is also associated with low-grade inflammation in WAT, with altered levels of pro-inflammatory adipokines, such as TNF-α, IL-6 and IL-8 (73) which are implicated in the development of insulin resistance. WAT also synthesizes and releases adipokines, such as leptin, adiponectin and resistin which affect insulin action, glucose and lipid metabolism and consequently, contribute to the regulation of insulin sensitivity (74).

1.8 Adipokines

1.8.1 Leptin

Leptin is produced mainly in adipocytes, but is also synthesized at low levels in other tissues including the stomach, intestine, skeletal muscle, placenta, mammary glands and brain (75, 76). The net of leptin action is to regulate appetite, thermogenesis, fatty acid oxidation, lipid and glucose homeostasis, body weight and fat mass (77-80). Obesity is associated with high plasma leptin concentrations (81), due to central and/or peripheral leptin resistance (82, 83, 84). Both leptin-deficient and leptin-resistant mice demonstrate severe insulin resistance. Leptin treatment rapidly ameliorates this condition in leptin deficient mice, even before reductions in body weight (85). The beneficial effects of leptin on insulin action may be largly mediated through its promotion of fatty acid oxidation and supression of lipid synthesis, and its regulation of the AMP-activated protein kinase (AMPK) and hepatic *Scd1* pathways, respectively (86).

1.8.2 Adiponectin

Adiponectin is synthesized and secreted by mature adipocytes (87). Adiponectin levels are reduced in obese, insulin resistant, diabetic or dyslipidemic subjects (88-90) and increased in response to severe weight loss (87). Adiponectin-deficient mice develop insulin resistance, glucose intolerance, and hyperlipidemia when fed on a HFD, whereas adiponectin replacement reverses these abnormalities (91, 92). However, one study has demonstrated that adiponectin knockout mice do not show aggravated insulin resistance when fed on HFD, compared to wild type mice (93). The insulin-sensitizing effect of adiponectin is partly mediated by increased fatty acid oxidation in the muscle and liver, through activation of AMPK (94, 95, 96). In contrast to leptin, injection of adiponectin into the lateral cerebral ventrical increased energy expenditure, but did not affect feeding (97).

1.8.3 Resistin

Resistin is produced by adipocytes in rodents (98) and macrophages in humans (99). In rodents, hyperresistinemia leads to insulin resistance and glucose intolerance due to increased hepatic glucose production (100, 101). Conversely, resistin deficiency created by deletion of the gene incoding resistin, or by antisense therapy improves insulin sensitivity and glucose metabolism (102, 103). The role of resistin in hepatic insulin resistance has been well established in rodents, but in humans its role is less clear. Thus, in patients with nonalcoholic fatty liver disease, levels of resistin postively correlate with the histological score of inflammation, but did not support a link between resistin and insulin resistance or BMI (104). However, overexpression of resistin in human hepatocytes impaired insulin-stimulated glucose uptake and glycogen synthesis in HepG2 cells (105).

1.9 Transcriptional factors related to insulin resistance

Signal transducer and activator of transcription 3 (*Stat3*) is a transcription factor which improves insulin sensitivity by regulating glucose homeostasis. Mice with liver-specific ablation of *Stat3* show insulin resistance which is associated with increases in the hepatic expression of gluconeogenic genes, whereas restoration of hepatic *Stat3* expression corrected these abnormalities (106). *Stat3* also contributes to sensitizing insulin signalling by negatively regulating glycogen synthase kinase-3β (107).

Sterol regulatory element binding protein 1c (*Srebp1c)*, which promotes fatty acid synthesis and lipid deposition, could also be responsible for insulin resistance (108). Thus, liver overexpression of *Srebp1c* has been described in several models of insulin resistance, such as ob/ob mice (109) and insulin receptor substrate-2 knockout mice (110). While overexpression of *Srebp1c* in mouse liver increased expression of lipogenic genes, *Srebp1c* knockout mice exhibit a reduction in the expression of these genes (111).

Liver X receptor α (*LXRα*) plays an important role in lipogenesis and cholesterol clearance (112), and *Srebp1c* is a direct *LXR* target gene (113). Expression levels of *Srebp1c* and its target lipogenic genes such as *Fas*, *Acc*, and *Scd1*, were reduced in *LXRα*-deficient mice, and conversely, admistration of a synthetic LXR agonist to C57BL mice increased the expression of fatty acid biosynthetic genes (114)

Peroxisome proliferator-activated receptors α and γ (*PPAR*α and γ) also play pivotal roles in both liver and WAT. *PPAR*α controls metabolism by regulating β-oxidation of fatty acids (115). *PPAR*α knockout mice demonstrate higher TG storage in liver, which

indicates the protective role of *PPARα* against steatosis (116). *PPARγ* is mostly involved in the regulation of adipocyte differentiation and energy storage (117). Thus, heterozygous *PPARγ*-deficient mice showed decreased lipogenesis and prevention of adiposity, thereby ameliorating HFD-induced obesity and insulin resistance (118). Liver *PPARγ* could be involved in the regulation of hepatic TG storage (119).

1.10 Regulation of glucose homeostasis by estrogen

There is continuously growing evidence that estrogen plays an important role in the control of glucose homeostasis (120). The onset of menopause is accompained by declining of estrogen levels, decreased lipid oxidation and reduced energy expenditure resulting in increases in visceral adipose tissue mass (121). In postmenopausal women, when estrogen deficiency is present, the development of visceral obesity, hyperinsulinemia and insulin resistance all lead to an increased risk for T2D (120). After menopause, obese women have increased leptin and resistin levels and decreased adiponectin levels, and BMI strongly correlates with insulin resistance as well as adipokine concentrations (122). Estrogen replacement therapy improves GT and insulin sensitivity, decreases visceral obesity, lowers TG and cholesterol levels, and consequently reduce the risk for development of T2D (123, 124). Duration and route of E2 administration also impact on the beneficial effects of estrogen (125, 126). According to the Women's Health Initiative study, postmenopausal therapy with E2 alone or in combination with progesterone may have a protective effect on the incidence of T2D (127, 128). However there are studies which have shown either no effect (129) or an impairment of insulin sensitivity upon E2 treatment (130).

Studies on rodent models of estrogen insufficiency or estrogen resistance confirm the effects of estrogen on glucose homeostasis. Thus, ovariectomy leads to the development of obesity, insulin resistance and impaired insulin secretion in mice and rats and E2 treatment reverses these abnormalities (131, 132, 133). The production of IL-6 and IL-8 in WAT was also increased in ovariectomized, estrogen-deficient rats and this could be attenuated by E2 replacement (134). Estrogen deficiency in ARKO mice is associated with obesity, severe hepatic steatosis and the development of glucose intolerance and insulin resistance in an age-dependent manner, all of which were ameliorated by E2 treatment (135, 136). Depletion of ERα in ERKO mice results in impaired GT, insulin resistance and obesity (34). All these observations together demonstrate that E2 is an important molecule in maintanance of glucose homeostasis.

2 AIMS OF THE STUDY

The overall aim of this study was to investigate the molecular mechanisms underlying the antidiabetic effects of estrogen.

The specific aims of the present work were:

1. To study mechanisms of estrogen action on glucose homeostasis using ERKO and BERKO mice

2. To investigate the effect of long-term E2 treatment on GT and insulin sensitivty in ob/ob mice and to explore the molecular mechanisms that underlie the antidiabetic effects of E2 in the mouse liver

3. To validate the role of ERα signaling in the regulation of glucose metabolism and to compare the molecular events upon treatment with either E2 or estrogen receptor alpha-selective agonist PPT in ob/ob mice

4. To elucidate the molecular mechanisms underlying the antidiabetic and weight-lowering effects of E2 in mice fed on a HFD

3 MATERIALS AND METHODS

3.1 Animals

Female and male ERKO mice, female BERKO mice and their respective controls were purchased from Taconic M&B (Ejby, Denmark). Ob/ob mice were bred in our own colony, Karolinska Institute, and in Umeå University, Sweden. Ovariectomized C57BL mice were obtained from Taconic M&B (Ejby, Denmark) and intact C57BL mice were purchased from Scanbur B&K (Sollentuna, Sweden). All animals were maintained on 12 h light-dark cycle, with food and water available *ad libitum,* in a temperature-controlled room (22 - 23˚C).

3.2 HFD fed mice

One week after arrival, eight week old female C57BL/6 mice were randomly housed on the following diets: a regular chow diet containing 4.5 g% fat, 14.5 g% protein and 69 g% carbohydrate (Lactamin AB, Kimstad, Sweden), (lean mice) or a HFD containing 34.9 g% fat, 26.2 g% protein and 26.3 g% carbohydrate (Research Diet, New Brunswick, NJ, USA), (HFD mice) until 12 months of age. At 11 months of age mice were divided into 4 groups: lean mice; lean mice, treated with E2; HFD mice; HFD, treated with E2.

3.3 Animal treatment procedures

To investigate the antidiabetic effects of E2 and PPT we performed the following treatments:

- ob/ob mice were administrated with E2 subcutaneously (s.c.) for 30 days at a dose 0.1 mg/kg body weight/day (Paper 2).
- ob/ob mice were treated s.c. with either E2 or PPT for 7 or 30 days at a dose of 0.1 mg/kg body weight/day (Paper 3).
- ob/ob mice were treated s.c. with either E2 (0.005, 0.05 and 0.1 mg/kg body weight/day) or PPT (0.1, 1.0 and 2.5 mg/kg body weight/day) for 30 days (Paper 3).
- C57BL/6 mice were ovariectomized at 10 weeks of age. At 14 weeks of age mice were injected s.c. with either E2 (0.1 mg/kg body weight) and killed 2 or 4 h after injection (Paper 2), or PPT (5 mg/kg/body weight) and euthanized 2, 4 or 6 h after injection (Paper 3).
- ERKO mice and matched controls were administrated with PPT at a dose of 1.0 mg/kg body weight/day for 7 days (Paper 3)

- HFD and age-matched lean mice at 11-months of age were administered s.c. with E2 at a dose of 0.05 mg/kg/day for 30 days. (Paper 4).

E2 and PPT were dissolved in 90% sesam oil/10% ethanol. Control animals in each experiment received equal volumes of solvent. All experiments were performed on 5 hours (ERKO and BERKO) or overnight (ob/ob and HFD) fasted mice. At the end of the experiments all animals were decapitated and blood was collected in heparinized tubes, centrifuged, and plasma was stored at -20ºC. Livers and abdominal adipose tissue were removed, weighed and stored at -80ºC. The regional ethical committee has approved all animal experiments.

3.4 Intraperitoneal glucose tolerance test and insulin response

Intraperitoneal glucose tolerance test and insulin response to a glucose load was carried out in fasted mice. Blood glucose concentrations and plasma insulin levels were measured at the basal state (0 min) and then at 10, 30, 60 and 120 min after an intraperitoneal (i.p.) injection of glucose (2 g/kg body weight). Blood glucose concentrations were assayed with a blood glucose sensor (Abbott Scandinavia AB, Solna, Sweden). For insulin assay blood was collected from tail tip into heparinized tubes, centrifuged, and plasma was stored at -20ºC. Plasma insulin levels were measured by radioimmunoassay with rat insulin as standard.

3.5 Intraperitoneal insulin tolerance test

For intraperitoneal insulin tolerance test, blood glucose levels were measured in fasted animals and insulin was injected at a dose of 0.25 U/kg body weight, i.p. Ten minutes later, glucose was injected at a dose of 1 g/kg body weight, i.p. and blood glucose concentrations were measured at 15, 30, 60, 90 and 120 min after the glucose load.

3.6 Insulin secretion *in vitro*

Pancreatic islets were isolated by collagenase digestion (137). Groups of five islets were preincubated at 37°C for 1h in Krebs-Ringer bicarbonate buffer (KRB), containing 115 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.6 mM $CaCl_2$ 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16 mM HEPES and 2 mg/ml BSA (pH 7.4), with 3.3 mM glucose. The islets were then incubated in KRB for 1 h at 37°C with 3.3 or 16.7 mM glucose, or with 3.3 mM glucose and 20 mM arginine. After incubation, the supernatants were stored at −20°C prior

to insulin assay. Insulin was measured by radioimmunoassay, employing rat insulin as standard.

3.7 Glucose uptake in skeletal muscle

Mice were anesthetized by i.p. injection of 2.5% avertin (0.02 ml/g body weight), and the extensor digitorum longus and soleus muscles were removed for *in vitro* incubation. Isolated muscles were incubated for glucose uptake as described for the rat epitrochlearis muscle (138). Incubation medium was prepared from a stock solution of Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5mM HEPES and 0.1% bovine serum albumin and continuously gassed with 95% O_2 : 5% CO_2. Isolated muscles were incubated in KHB in the absence or presence of insulin (0.18 nM or 12 nM) for 30 min. Glucose transport was assessed using 2-deoxyglucose (139). Muscles were transferred to vials containing glucose-free KHB supplemented with 20 mM mannitol for 10 min. Thereafter, muscles were incubated in KHB containing 1 mmol/l [^{3}H]-2-deoxyglucose (3.7 GBq/ml) (American Radiolabeled Chemicals, St Louis, MO, USA) and 19 mmol/l [^{14}C]-mannitol (2.59 GBq/ml) (Moravec Biochemicals, Brea, CA, USA) for 20 min and then immediately frozen in liquid nitrogen. The extracellular space and intracellular 2-deoxyglucose concentrations were determined as previously described (138, 139).

3.8 Glucose uptake in adipose tissue

Adipocytes were isolated from adipose tissue by collagenase digestion (140). Isolated adipocytes were incubated at a concentration of 3.5% (vol/vol) in Krebs-Ringer phosphate buffer (pH 7.4) containing albumin (40 mg/ml), [3-^{3}H]glucose (5×10^6 cpm/ml), unlabelled glucose (1 μmol/l), and various concentrations of insulin (0, 10^{-15}-10^{-6} M) for 2 h at 37°C using air as the gas phase. Incubations were stopped by rapidly chilling the incubation vials to 4°C and adding 50 μl 6M H_2SO_4. Incorporation of radiolabelled glucose into adipocyte lipids was determined.

3.9 Euglycaemic-hyperinsulinemic clamp

Mice were anesthetized with midazolam (0.25 mg/mouse; Dormicum, Hoffman-LaRoche, Basel, Switzerland) and a combination of fluanisone (0.5 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm, Janssen, Beers, Belgium). Thereafter, the right jugular vein and the left carotid artery were catheterized. Thirty minutes after introduction of the catheters ($t = -100$ min), a bolus injection of [3-^{3}H]glucose (3 MBq; Amersham Pharmacia

Biotech, Amersham, UK) was given, followed by a continuous infusion of 0.056 MBq kg^{-1} min^{-1} [3-^{3}H]glucose. This infusion was continued throughout the 190 min study period, at which point a steady state was reached. At t=0 min, a blood sample was taken for the determination of insulin and [3-^{3}H]glucose concentrations, followed by an insulin infusion at the rate of 20 mU kg^{-1} min^{-1} (Actrapid, Novo Nordisk, Denmark). Blood glucose concentrations were then determined at 5 min intervals and were maintained at a concentration of 6.5 mmol/l by infusion of a solution of 2.2 mol/l glucose at a variable rate. Blood samples were taken at 60 and 90 min for the determination of insulin, and at 90 min for the determination of [3-^{3}H]glucose. Whole-body insulin sensitivity was calculated as the 60–90 min glucose infusion rate divided by the mean of the 60-90 min insulin levels. The blood samples (100 µl) taken at 0 and 90 min were deproteinized, evaporated, and resuspended in deionized water for the determination of radioactivity and glucose levels. Basal endogenous glucose production (EGP) was calculated by dividing the rate of infusion of [3-^{3}H]glucose by the plasma glucose specific activity (i.e. dpm per min divided by dpm per mg glucose). The glucose appearance at 90 min was measured by dividing the infusion rate in dpm by the plasma glucose specific activity at this time point. EGP at this time was calculated by subtracting the glucose infusion rate from the glucose appearance rate. Finally, the glucose disposal rate was calculated as the glucose appearance rate divided by the glucose concentration.

3.10 RNA preparation

Total RNA was prepared from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy Mini Kits (QIAGEN, Valencia, CA, USA). RNA quality was assayed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

3.11 Microarray experiment

For microarray analysis labelled cRNA was synthesised from total RNA according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA, USA) and hybridized to Mouse 430 A or 430 2.0 gene chips, which were washed and scanned.

Scanned data files were analysed using MAS 5.0 software from Affymetrix (Paper 1 and 2). In paper 3 output files from microarray analyses were analyzed using Affymetrix GeneChip Operating Software (GCOS).

3.12 Real-time RT-PCR

Total RNA from each individual animal was reverse-transcribed into cDNA using Superscript II (Invitrogen) or Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with random hexamer primers. Analyses were performed using a 7500 Fast Real-Time PCR system and the Power SYBR Green reagent (Applied Biosystems). PCR products were analyzed by melting curve analysis to confirm the presence of single products. mRNA levels were normalized to 18S or to hypoxanthine ribosyltransferase mRNA.

3.13 Western blot

Liver protein extracts were prepared by homogenizing tissue in RIPA buffer [20 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride] including 1x protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 1x Halt phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Total cell lysates were centrifuged at 12000 x g for 10 min. Protein concentrations of extracts were determined using a protein assay dye reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by electrophoresis. Proteins were transferred to Hybond-C membranes (GE Healthcare, Buckinghamshire, UK). Membranes were probed using either phospho-Stat3 (Tyr705), Stat3 (Cell Signaling Technology, Danvers, MA), Fasn (NB 400-114; Novus Biologicals, Littleton, CO), G6P (C-14, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (AC-74, Sigma-Aldrich, Stockholm, Sweden) antibodies. Protein-antibody complexes were detected using ECL chemiluminescence system (Pierce or GE Healthcare Buckinghamshire, UK). The bands were scanned and quantified by Scion image software (Scion Corp., Frederick, MD) or ImageJ (free software from NIH).

4 RESULTS AND DISCUSSION

4.1 Evidence that estrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver (Paper 1)

In this paper we investigated the mechanism(s) underlying the effects of estrogen on glucose homeostasis using ERKO and BERKO mice. We found, that irrespective of gender, ERKO mice developed fasting hyperglycemia, hyperinsulinaemia and impaired GT. In contrast, BERKO mice had normal fasting blood glucose levels and GT. These data clearly demonstrate the difference in functions of ERs with respect to the regulation of glucose metabolism, and suggest that ERα is the major player. Because impaired insulin secretion from pancreatic β-cells can contribute to hyperglycemia we studied the glucose- and arginine-stimulated insulin release from isolated pancreatic islets in ERKO mice. We did not find differences in basal and stimulated insulin release between ERKO and wild-type mice, which suggests that hyperglycemia in ERKO mice is mainly due to impaired insulin sensitivity in target tissues. On the other hand, the absence of an increased insulin response in the presence of insulin resistance might suggest islets dysfunction in ERKO mice.

Insulin resistance could be caused by either increased hepatic EGP, and/or decreased glucose uptake by peripheral tissues. Using the euglycaemic-hyperinsulinemic clamp technique we measured glucose turnover with [3-^{3}H]glucose during basal and hyperinsulinaemic conditions in ERKO mice. During the clamp, the whole body insulin sensitivity was decreased. Importantly, ERKO mice showed impaired suppression of EGP by increased insulinaemia, while glucose clearance, indicating glucose uptake, was normal. Since non-hepatic tissues contribute only slightly to EGP (141, 142), impaired insulin sensitivity in ERKO mice is most likely due to insulin resistance in the liver. However, we also found that insulin-mediated glucose uptake in isolated skeletal muscles was moderately decreased in ERKO mice, which suggests the presence of at least minor extrahepatic insulin resistance.

ERKO mice had increased plasma leptin leves and decreased adiponectin concentrations, whereas resistin levels were unchanged.

In order to study the molecular mechanisms behind the hepatic insulin resistance in ERKO mice we analyzed gene expression profile from ERKO and wild-type mouse livers, using the Affymetrix microarrays. This experiment revealed that in the livers of ERKO mice the expression of genes involved in lipid biosynthesis was increased, while the expression of genes regulating lipid transport was decreased. Among lipogenic genes we found increased expression of *Scd1*, the key enzyme in the biosynthesis of unsaturated fatty acids. Increased expression of *Scd1* leads to the development of the metabolic syndrome (143), whilst targeted disruption of *Scd1* demonstrated increased insulin sensitivity and fatty acid metabolism (144). ERKO mice also had decreased expression of *Lepr* in liver. These reciprocal expression for *Scd1* and *Lepr* may be one of the mechanisms providing the basis for hepatic insulin resistance in ERKO mice.

In conclusion, the results of this study demonstrates that estrogens acting via ERα regulate glucose homeostasis mainly by modulating hepatic insulin sensitivity. We suggest that hepatic insulin resistance in ERKO mice could be result from the upregulation of lipogenic genes via the suppression of *Lepr* expression.

4.2 Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3 (Paper 2)

We used ob/ob mice as a model of severe insulin resistance to elucidate the effect of long-term treatment with E2 on insulin sensitivity and to further explore the molecular mechanisms that underlie the antidiabetic effects of estrogen. Administration of E2 during 30 days resulted in significantly decreased basal glucose and insulin levels, and markedly improved GT and insulin sensitivity. We found, that after long-term treatment with E2 hepatic TG content was significantly reduced in ob/ob mice which may contribute to the improvement in hepatic insulin sensitivity (145). Moreover, microarray analysis revealed that E2 treatment resulted both in the decreased expression of genes involved in lipid biosynthesis and in increased *Lepr* expression supporting our previous observation in ERKO mice that reciprocal expression of *Lepr* and lipogenic genes may be an important mechanism regulating hepatic insulin sensitivity. Further searches for possible direct target genes mediating antidiabetic effects of E2 in the mouse liver revealed that E2 treatment increased the hepatic expression of *Stat3* and of its several target genes. Thus, *Stat3* has

been implicated in the regulation of lipid synthesis in the liver (146). Liver-specific deficiency of *Stat3* leads to insulin resistance, whereas hepatic overexpression of *Stat3* ameliorates glucose tolerance and insulin sensitivity (106). We also showed that the level of phosphorylated *Stat3* was increased after E2 treatment, indicating an increase in *Stat3* signalling pathway activity in the liver. Furthermore ChiP assays demonstrated a direct recruitment of ERα to the promoter of the *Stat3* gene in vivo. These lines of evidence together suggest that S*tat*3 is a direct target gene for E2. We conclude that E2 treatment improves glucose tolerance and insulin sensitivity in ob/ob mice by increasing hepatic insulin sensitivity and this is accompained by the decreased expression of lipogenic genes. The antidiabetic effect of E2 could result from increased expression of S*tat*3 and *Lepr* which modulates lipid metabolism.

4.3 The estrogen receptor alpha-selective agonist PPT improves glucose tolerance in ob/ob mice; potential molecular mechanism (Paper 3)

In our previous studies we have shown, that estrogen acting via ERα regulates glucose homeostasis by modulating insulin sensitivity. ERKO mice developed glucose intolerance and insulin resistance which was mainly due to profound hepatic insulin resistance and which was accompained by the upregulation of genes, involved in fatty acid biosynthesis (Paper 1). Using ob/ob mice we then demonstrated that long-term treatment with E2 resulted in improvements in insulin sensitivity, in association with the decreased expression of lipogenic genes and decreased TG content in the liver (Paper 2). In this study we firstly aimed to validate the role of ERα signaling in the regulation of glucose homeostasis and secondly, we sought to further investigate the molecular mechanisms, which underlie the antidiabetic effects of E2. For these purposes, we treated female ob/ob mice with either the ERα-selective agonist PPT or with E2.

Both 7 and 30 days of treatment with PPT and E2 improved GT and insulin sensitivty in ob/ob mice. However, only long-term treatment resulted in significantly decreased fasting blood glucose levels. These data are consistent with previous reports, suggesting that ERα plays a critical role in glucose homeostasis (34). To confirm that the effects of PPT on glucose homeostasis were the result of ERα-specific signaling, ERKO mice were treated with PPT for 7 days. We found that PPT had no effect on either glucose or insulin tolerance in ERKO mice, indicating that PPT acts as ERα agonist.

To investigate whether improvements in insulin sensitivity in ob/ob mice could be of exprahepatic origin we measured *in vitro* glucose uptake in muscle and adipose tissues. Both basal and insulin-stimulated glucose uptake in skeletal muscles and adipose tissue of PPT treated mice were similar to those of vehicle-treated animals. Therefore, the lack of any effect of PPT treatment on peripheral tissues *in vitro* indicates that its antidiabetic effects in ob/ob mice are mainly due to improvement in insulin sensitivity in the liver. In addition, PPT treatment did not affect insulin release from isolated pancreatic islets. To validate the target tissues responsible for the antidiabetic effects of PPT, it will be necessary to perform *in vivo* studies, including euglycaemic-hyperinsulinemic clamps in combination with studies of glucose turnover.

Although, PPT and E2 had similar effect on glucose metabolism, hepatic lipid content was decreased in E2-treated mice, but was unchanged after PPT treatment. Furthermore, treatment with PPT did not alter the expression levels of *Scd1* and *Fasn* whereas E2 reduced expression of these genes (Paper 2). This suggests that the regulation of hepatic lipid metabolism is not crucial for the positive effects of PPT on GT and insulin sensitivity.

To further investigte the molecular effects of PPT in ob/ob mice, we performed gene expression profiling experiments with livers from PPT- and E2-treated animals. We focused on genes that were co-regulated by both E2 and PPT treatment in ob/ob mice. Importantly, the expression levels of *Stat3* and *G6pc* were increased and decreased, respectively, in both PPT and E2 treated animals. These genes were regulated already after 4 hours of PPT treatment and are primary target genes for ERα. We conclude that selective activation of the ERα signaling pathway improves GT and insulin sensitivity. *Stat3* and *G6pc* were identified as possible primary mediators of antidiabetic actions of E2 and PPT.

4.4 Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high fat diet-fed mice (Paper 4)

Obesity and/or lack of physical activity are two of the main determining factors in the development of insuln resistance that precede the diagnosis of T2D (147). Estrogen deficiency, which accompanies menopause in woman and results from ovariectomy in rodents is also strongly associated with obesity and insulin resistance. Estrogen treatment decreases weight gain and improves insulin sensitivity in both conditions. To further elucidate the molecular mechanisms underlying the antidiabetic and weight-lowering

effects of estrogen, we used a mouse model of obesity, impaired GT and insulin resistance, and focused on both adipokine levels as well as the changes in the expression of genes involved in fatty acid metabolism in the liver and adipose tissue.

C57BL mice, maintainined on a high fat diet for up to 12 months developed obesity, fasting hyperglycemia and hyperinsulinemia. These mice also displayed impairments of GT and insulin sensitivity. During the last month of feeding mice were treated with E2 and this resulted in normalized GT, increased insulin sensitivity and decreases in body weights and abdominal fat mass. HFD mice exhibited low plasma E2 levels, but after E2 treatment plasma E2 levels reached those of young adult mice.

Obesity and insulin resistance are associated with altered adipokines levels (74). In HFD mice we found that resistin levels were increased compared to lean mice, but after E2 treatment resistin levels were significantly reduced. This finding is consistent with observations that decrease in resistin level leads to improvement in GT and insulin sensitivity in HFD mice (148).

Plasma leptin concentrations were elevated in HFD mice in association with obesity and insulin resistance. The liver weights of HFD mice were similar to those of lean mice and showed only moderate steatosis (data not shown). The protective role of leptin in TG accumulation in the liver has been shown in Sprague-Dawley rats, receiving high-fat meals (149). E2 treatment of HFD mice resulted in reduction in body weight, plasma leptin concentration and hepatic TG contents, which could also contribute to the improvement of insulin sensitivity. The expression levels of leptin and resistin in WAT were decreased after E2 treatment, in concordance with the reductions in plasma levels of these hormones.

Unexpectedly, adiponectin levels, which are commonly decreased by obesity, were similar in HFD and lean mice. Although, E2 treatment of HFD mice reduced body weights and hepatic TG storage, plasma adiponectin levels was also decreased. Of relevance to our data there are observations in ovariectomized mice that E2 treatment decreased adiponectin levels in association with a reduction in adiposity (150). The expression of adiponectin mRNA in adipose tisue was unchanged.

In our previous studies (Paper 1 and 2) we have suggested that in ERKO and ob/ob mice, insulin resistance could be due to altered lipid metabolism in the liver. In HFD mice we have shown that treatment with E2 resulted in significant downregulation of hepatic *Scd1* expression, although it did not alter the expression of *Fas* and *Acc1*. It is known that the

expression of these lipogenic genes is under the control of *Srebp1* (151), which is in turn regulated by *LXRα* (113). However we did not find any changes in the expression of *Srebp1* or *LXRα* in the liver of HFD mice. Therefore, it appears that alternative mechanism exists by which E2 can regulate the expression of *Scd1* in the liver of HFD mice.

Following E2 treatment we did not detect any alterations in the hepatic expression of *PPARα*, a transcription factor, which promotes fatty acid oxidation (115). Therefore it is possible, that this expression level of *PPARα* is sufficient to maintain oxidative capacity in the liver. Treatment with E2 resulted in reduction of in *G6pc* expression in HFD mice, which may play a crucial role in the improvement of hepatic insulin sensitivity.

We also demonstrated that E2 treatment of HFD mice resulted in the downregulation of both *Srebp1c* and of its target lipogenic genes *Fas* and *Scd1* in WAT, although the expression of *LXRα* was unchanged. It has been previously shown that in the WAT of ovariectomized mice, E2 treatment lead to reductions in expression of *LXRα, Srebp1c* and their downstream regulated genes such as *Fas* and *Scd1* (150, 152). Decreased expression of genes, involved in the regulation of lipogenesis was accompained by a decreased WAT weights and improvements in insulin sensitivity in HFD mice. We also showed that E2 can directly regulate the activities of *Srebp1c* and *Scd1* promoters in mouse preadipocyte cells.

Finally, the expression of *PPARγ*, which is involved in adipogenesis, was reduced in WAT of HFD mice after E2 treatment. *PPARγ*-deficient mice demonstrate decreased lipogenesis and prevention of adiposity and thereby ameliorating HFD-induced obesity and insulin resistance (153). Therefore E2 could also reduce adiposity and insulin resistance by regulating the expression of *PPARγ*.

In conclusion, our data demonstrate that E2 treatment exerts antidiabetic and antiobesity effects in HFD mice. The important mechanisms behind these effects include both the downregulation of lipogenic genes in WAT and liver and suppresson of the hepatic expression of *G6pc*. Decreased plasma resistin levels also play an important role in this context.

5 CONCLUSIONS

This study has attempted to define the molecular mechanisms by which estrogen promotes beneficial effects on glucose homeostasis and insulin sensitivity. The following conclusions can be made from our results:

- Estrogen exerts its antidiabetic effects via ERα. Lack of ERα, but not ERβ, caused glucose intolerance and insulin resistance in both female and male mice. Insulin resistance in ERKO mice is mainly due to profound hepatic insulin resistance, which results from the upregulation of lipogenic genes via the suppression of *Lepr* expression.

- E2 improves GT and insulin sensitivity in ob/ob mice. This effect may be mediated via stimulation of expression of *Stat3* and *Lepr* leading to decreased expression of hepatic lipogenic genes. Our data suggest that *Stat3* is a direct target gene for E2.

- Study with the ERα agonist PPT in ob/ob mice strongly confirmed that the antidiabetic effect of E2 is mediated via ERα signaling. Increases in the expression levels of *Stat3* and decreases in the expression levels of *G6pc* were identified as being involved in the potential mechanism behind the effects of E2 and PPT on glucose homeostasis.

- E2 treatment reduces obesity, improves GT and insulin sensitivity in HFD mice. These effects of E2 are mediated by downregulation of *Srebp1c* and its down-stream regulated lipogenic genes, such as *Fas* and *Scd1* in WAT and *Scd1* in liver. Suppression of hepatic *G6pc* expression and decrease level of resistin upon E2 treatment could play a crucial role in this context.

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