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Impact of estradiol, estrogen receptor subtype-selective agonists and genistein on energy homeostasis

Einfluss von Estradiol, Estrogenrezeptor-Subtyp-selektiven Agonisten und Genistein auf die Energiehomöostase

DISSERTATION

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Eidesstattliche Erklärung

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Abbreviations

αERKO	ER alpha knockout
αMSH	alpha melanocyte stimulating hormone
βERKO	ER beta knockout
AgRP	agouti-related protein
Alpha	ER alpha-selective agonist, 16alpha-LE2
ATP	adenosine triphosphate
Beta	ER beta-selective agonist, 8beta-VE2
BMI	body mass index
CART	cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
E2	17beta-estradiol
ER	estrogen receptor
ERE	estrogen response element
ERKO	estrogen receptor knockout
FAS	fatty acid synthase
FFA	free fatty acids
Gen	genistein
GLUT4	glucose transporter 4
HDL	high-density lipoprotein
HF	high fat
HOMA	homeostasis assessment model
HRT	hormone replacement therapy
Igf1	insulin-like growth factor 1
IPGGT	intraperitoneal glucose tolerance test
IRMS	isotope-ratio mass spectrometry
LDL	low-density lipoprotein

LF	low fat
NFκB	nuclear factor kappa B
NPY	neuropeptid Y
NR	nuclear receptor
OVX	ovariectomized
Pax7	paired box 7
POMC	proopiomelanocortin
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR responsive element
RER	respiratory exchange ratio
SERM	selective estrogen receptor modulator
SREBP-1c	sterol regulatory element binding protein 1c
T2DM	type 2 diabetes mellitus
TC	total cholesterol;
TG	triglyceride
VLDL	very-low-density lipoprotein
VPDB	Vienna Pee Dee Belemnite
WHO	World Health Organization
ZDF	Zucker diabetic fatty

Abstract

The prevalence of obesity is dramatically increasing and thus constitutes a major risk factor for developing chronic diseases such as type 2 diabetes, dyslipidemia, cardiovascular diseases, and certain forms of cancer. High-caloric nutrition and a lack of physical activity are the main contributing factors for this global epidemic. Estrogen receptors (ERs) are recognized to be involved in many processes related to the control of energy homeostasis.

In my studies, I investigated the impact of estrogens (17beta-estradiol (E2)) on energy homeostasis. Special emphasis was given to the effects of two synthetic ER subtype-selective agonists, 16alpha-LE2 (Alpha) and 8beta-VE2 (Beta), to determine to what extend the two distinct ER subtypes are involved in the underlying molecular mechanisms. Because of its estrogenic activity and also its widespread use as a nutritional supplement the influence of the isoflavone genistein (Gen) was examined. For this purpose two different female rat models were used: Wistar rats with nutrition-induced obesity and leptin resistant Zucker diabetic fatty (ZDF) rats. In both experiments, the animals were ovariectomized (OVX) and treated with vehicle (untreated controls) or the estrogenic compounds.

The most important finding was that treatment of OVX animals with Beta enlarges soleus muscle fiber sizes in both animal models compared to untreated OVX animals. This anabolic effect may in turn improve the muscle/fat ratio of the body that enhances muscular uptake and utilization of fuels. By contrast, in the gastrocnemius muscle of OVX ZDF rats substitution with Alpha increased expression and distribution of the insulin-dependent glucose transporter 4 (GLUT4). Consequently, systemic insulin sensitivity in both animal models was improved by treatment with estrogenic compounds compared to untreated OVX animals. The strongest effect was observed in E2-treated rats that indicate an additive effect through activation of both pathways.

In all OVX rats, treatment with either ER subtype-selective agonist showed an anti-lipogenic effect in adipose tissue, liver, and skeletal muscle of nutrition-induced obese Wistar rats in comparison to OVX animals without treatment. Decreased visceral fat mass, adipocyte sizes, serum leptin levels, triglyceride accumulation in liver and muscle as well as mRNA expression of genes that are involved in lipo-/adipogenesis reflected this. Therefore, the lower visceral fat mass as well as decreased accumulation of triglycerides in non-adipose tissues such as liver and skeletal muscle most likely contributes to the improved insulin sensitivity in such treated animals.

Gen exerted effects similar to those of the ER beta-selective agonist (except on adipose tissue in Wistar rats). Especially, the similar ability to induce anabolic activity in the soleus muscle might be highly relevant. Gen-treated animals might have a more effective utilization of fuels compared to untreated OVX animals because they showed a lower TG content in muscle and liver as well as improved glucose metabolism.

In conclusion, because of my studies and the fact that ER beta signaling is not involved in proliferation of uterus and mammary gland, an effective way to treat obesity and co-morbidities in postmenopausal women might be substances that only activate ER beta. A combination with physical activity may support the therapy of obesity and co-morbidities. The isoflavone Gen is able to activate both ER-subtypes. This compound is already placed on the market for treatment of postmenopausal complaints, although adverse effects of Gen cannot be excluded so far (e.g., increased risk of breast cancer). However, Gen might be a natural alternative – not only to the conventional hormone replacement therapy, but also as a strategy for treatment of obesity and co-morbidities – that deserves further research with respect to these new data.

Zusammenfassung

Die dramatisch zunehmende Prävalenz der Adipositas und das damit verbundene Risiko für Folgeerkrankungen wie Diabetes mellitus, Hypertonie, Dyslipidämie und koronare Herzkrankheiten stellt eine große Herausforderung für das Gesundheitswesen dar. Als Hauptursache wird ein chronisches Missverhältnis der Energiehomöostase aufgrund permanenter Überernährung und Bewegungsmangel postuliert. Estrogene beeinflussen den Glukose- und Lipidstoffwechsel und sind somit in die Regulation des Energiehaushaltes involviert. Estrogene vermitteln ihre Effekte über zwei Estrogenrezeptor (ER)-Subtypen, den ER alpha und den ER beta.

Ziel der vorliegenden Arbeit war es mittels tierexperimentellen Studien den Einfluss von Estrogenen, speziell 17beta-Estradiol, auf den Energiehaushalt zu untersuchen. Um einen tieferen Einblick in die zugrundeliegenden molekularen Mechanismen zu erhalten, wurden zwei Subtyp-selektive ER-Agonisten, 16alpha-LE2 (Alpha) and 8beta-VE2 (Beta), synthetischer Herkunft eingesetzt. Aufgrund der estrogenen Aktivität und der Verfügbarkeit als Nahrungsergänzungsmittel wurde des Weiteren der Einfluss des Isoflavons Genistein untersucht. Für die Studien wurden zwei Tiermodelle genutzt: zum einen weibliche Wistar-Ratten mit ernährungsinduzierter Adipositas und zum anderen weibliche leptinresistente „Zucker diabetic fatty“ (ZDF)-Ratten. Die Tiere wurden ovarektomiert (OVX) und entweder mit einem Vehikel (unbehandelte Kontrolltiere) oder mit der entsprechenden estrogenen Substanz behandelt.

Die interessanteste Erkenntnis war, dass im Vergleich zu unbehandelten OVX-Tieren beider Tiermodelle die Behandlung mit Beta zur Vergrößerung der Faserquerschnitte im Soleusmuskel führte. Dieser anabole Effekt könnte die muskuläre Aufnahme und Verwertung von Brennstoffmolekülen verbessern und sich insgesamt positiv auf die Körperzusammensetzung auswirken. Den stärksten Effekt hinsichtlich einer erhöhten Expression und Translokation des insulinabhängigen Glukosetransporters 4 (GLUT4) in die Zellmembran des Gastrocnemiusmuskels zeigte sich dagegen durch die Behandlung

von OVX ZDF-Ratten mit Alpha. Im Endergebnis zeigten die Tiere beider Modelle durch die Behandlung mit estrogenen Substanzen eine verbesserte systemische Insulinsensitivität im Vergleich zu unbehandelten Kontrolltieren. E2-behandelte Tiere tolerierten die Glukose am besten und lassen einen additiven Effekt aufgrund der Aktivierung beider Signalwege vermuten.

Im Vergleich zu unbehandelten OVX Wistar-Ratten führte die Behandlung mit E2 oder mit jeweils einem der beiden ER-Subtyp-selektiven Agonisten zu einer geringeren viszeralen Fettmasse, kleineren Fettzellen, niedrigeren Leptinspiegeln im Serum und geringeren Triglyzeridwerten in Leber und Muskel. Auf der Ebene der Genexpression waren zudem geringere mRNA-Spiegel von lipo- und adipogenen Genen messbar. Somit scheinen beide ER-Subtypen in die antilipogene Wirkung von E2 involviert zu sein. Sowohl die reduzierte viszerale Fettmasse als auch die geringere Anreicherung von Triglyzeriden in Leber und Muskel tragen sehr wahrscheinlich ebenfalls zur verbesserten Insulinsensitivität bei.

Die Behandlung von OVX Tieren mit Gen führte zu ähnlichen Ergebnissen wie die Behandlung mit Beta. Eine alleinige Ausnahme stellte das Fettgewebe dar, da hier eine Gen-Behandlung keine antilipogenen/-adipogenen Effekte zeigte. Speziell die Fähigkeit von Gen ebenfalls anabol zu wirken, könnte die molekulare Grundlage sein, weshalb Gen-behandelte Tiere im Vergleich zu unbehandelten Tiere eine verbesserte Toleranz gegenüber Glukose und eine geringere Anreicherung von Triglyzeriden in Muskel und Leber zeigten.

Der ER beta ist nicht in die estrogenvermittelte Proliferation von Uterus und Brustdrüse involviert. Vor diesem Hintergrund lassen meine Ergebnisse vermuten, dass eine Behandlung mit ER beta-selektiven Substanzen eine effektive Möglichkeit darstellt, um Adipositas und deren Folgeerkrankungen in postmenopausalen Frauen zu behandeln, ohne deren Risiko für estrogenabhängige Krebsformen zu erhöhen. Eine Kombination mit regelmäßiger körperlicher Aktivität könnte die Erfolge bei der Behandlung von Adipositas und deren Folgeerkrankungen noch maximieren bzw. eine geringere Dosierung der verwendeten Substanz bei gleichbleibendem Behandlungserfolg ermöglichen. Das Isoflavon Gen mit seiner Fähigkeit beide ERs zu aktivieren ist eine bereits auf dem Markt befindliche Substanz und wird zur Behandlung von postmenopausalen Beschwerden eingesetzt, obwohl mögliche negative Effekte (z.B. ein erhöhtes Brustkrebsrisiko) noch nicht abschließend geklärt sind. Falls diese Risiken von Gen ausgeräumt werden können, könnte diese Substanz eventuell eine kostengünstige Alternative darstellen, um sowohl postmenopausale Beschwerden als auch Adipositas und deren Folgekrankheiten zu behandeln.

Chapter One

General introduction

Prevalence of obesity and co-morbidities

Obesity is defined as excessive fat enrichment that is mainly accumulated inside the abdominal cavity. Its prevalence has nearly doubled between 1980 and 2008 worldwide; in some countries of Europe even tripled. According to the WHO, in 2008, nearly 1.5 billion adults around the world were overweight (BMI greater than 25 kg/m^2) – at least 500 million of them were obese (BMI greater than 30 kg/m^2) (WHO, 2012). Since 1990, the incidence of overweight individuals among children has steadily increased too. Globally in 2010, approximately 43 million children under five years were overweight (WHO, 2010).

Excessive body fat accumulation is now recognized as a major risk factor for serious health problems. These include insulin resistance and type 2 diabetes mellitus (T2DM), dyslipidemia, and hypertension (a well-documented risk factor for cardiovascular diseases like stroke and heart complaints) (Chan and Woo, 2010; Mokdad et al., 2003; Newbold et al., 2009; Swinburn et al., 2004). In combination, these disorders are called the metabolic syndrome (Eckel, 2011; Miranda et al., 2005). Further, obesity has been identified to increase the likelihood for developing certain forms of cancer such as cancers of the colon and rectum, breast (postmenopausal), and prostate (Mokdad et al., 2003; Ronco et al., 2012). Especially in the western world, obesity is stigmatized and thus further results in serious social and psychological problems.

Obesity is attributable mainly to a chronic imbalance of energy homeostasis meaning that energy intake exceeds the expenditure caused by high-caloric nutrition combined with a more and more sedentary lifestyle (Low et al., 2009; Newbold et al., 2009; Wasan and Looije, 2005). However, obesity is often multifactorial in origin, besides physical activity and dietary habits genetic and environmental factors contribute to its prevalence (Comuzzie et al., 2001; Goulart et al., 2009).

Meanwhile, obesity and co-morbidities affect all ages, sexes, races, and socio-economic groups and are therefore responsible for constantly rising health costs and premature death. Therefore, it is not exaggerated to say that prevention and therapy of obesity is one of the greatest public health challenges of our century. Thus, more scientific

knowledge about the underlying physiological causes of obesity is of fundamental importance.

Central regulation of energy homeostasis

Energy homeostasis means a well-balanced intake and expenditure of energy that is strictly controlled via the hypothalamus by hormonal signaling. There are varieties of peripheral signals that inform the central nervous system (CNS) about the energy state of the body. “Immediate” signals are released from the gastrointestinal tract in response to the presence of food (e.g., ghrelin as a hunger-stimulating hormone and peptide YY or Cholecystokinin (CCK) as mediators of satiation). As a “longer-term” signal, leptin is secreted by adipose cells and thus also referred to as “adipositiy” signal. After crossing the blood-brain barrier, these hormones act on several hypothalamic areas including the arcuate nucleus, the paraventricular nucleus, the lateral hypothalamus, the dorsomedial hypothalamus, and the ventromedial hypothalamus. In particular, the arcuate nucleus houses two types of neurons - AgRP/NPY and POMC/CART neurons – that play a key role in regulation of energy intake and expenditure. The first subpopulation of neurons releases the neuropeptids agouti-related protein (AgRP) and neuropeptide Y (NPY). The second population secretes alpha melanocyte stimulating hormone (α-MSH), a posttranslational derivative of proopiomelanocortin (POMC), and cocaine- and amphetamine regulated transcript (CART). Neuronal projections from these two types of neurons enable communication with other hypothalamic areas. Agrp and Npy are referred to as orexigenic hormones, because they stimulate food intake and decrease energy expenditure. α-MSH and CART in turn inhibit food intake and increase energy expenditure and referred to as anorexigenic hormones (Barsh and Schwartz, 2002; Gale et al., 2004; Morton et al., 2006; Schwartz, 2001; Simpson et al., 2009).

Leptin appears to be crucial to maintain the individual body weight/fat within certain limits through negative feedback regulation between adipose tissue and the brain. This hormone serves as an index of body energy storage, because circulating leptin levels correlate strongly to the whole body fat stores. In normal physiology, increased fat mass leads to an elevated leptin level, which in turn restricts appetite and increases energy expenditure, and vice versa (Gale et al., 2004; Morton et al., 2006; Schwartz, 2001; Simpson et al., 2009).

Insulin, synthesized by pancreatic beta cells, has been shown to have properties similar to leptin in the brain. Like leptin, it also crosses the blood-brain barrier, where it inhibits food intake and stimulates energy expenditure by acting on the hypothalamus.

Unlike leptin, insulin is secreted acutely in response to food intake, but it is believed that prolonged high levels of insulin may induce leptin secretion (Gale et al., 2004; Schwartz, 2001).

Whereas normal-weight individuals are protected against excessive body weight gain/loss, homeostatic control is impaired in obesity. The vast majority of obese individuals do not respond to their extremely high serum levels of leptin. This physiological phenomenon is referred to as leptin resistance. Moreover, obesity is associated with both leptin and insulin resistance (Gale et al., 2004; Morton et al., 2006).

Peripheral regulation of fuel molecules

Liver, skeletal muscle and adipose tissue (in particular the visceral fat) are highly metabolically active tissues.

The liver plays a central role in supplying the brain, the muscles, and other peripheral organs with fuels. The majority of all nutrients that are absorbed from the gastrointestinal tract enter the liver through the blood stream (triglycerides packaged in chylomicrons, glucose, and amino acids). Here they undergo various chemical processes triggered by several hormones that enable their immediately utilization (energy demand of the liver), storage (for later use during food scarcity) or export (fuel supply) (Stryer, 1994).

The skeletal muscle supports the skeleton enabling movement of the body by muscle contraction. For all forms of muscle work, but also at rest, adenosine triphosphate (ATP) needs to be provided. Storage of this energy-rich compound is limited. Therefore, main types of fuel to produce ATP are free fatty acids (FFA) and glucose that can be stored in significant amounts within the muscle as triglycerides and glycogen, respectively (Atalay and Hänninen, 2009).

An important function of adipose tissue is the storage of energy as lipids. During periods of food abundance adipocytes ingest excess fuel molecules, convert them into triglycerides, and stores them in intracellular lipid droplets. During periods of food scarcity stored triglycerides are broken down into FFA and glycerol, which then are released into the blood. In muscle, FFA serve as fuel to produce ATP. In liver, FFA and glycerol are used for synthesis of ketone bodies and glucose or they participate in hepatic TG production (Anghel and Wahli, 2007).

With respect to the metabolism of lipids, the liver is the key tissue in manufacturing (*de novo* lipogenesis) or importing FFA from the blood. Within the liver, FFA are synthesized to triglycerides that can be stored as lipid droplets or incorporated into VLDL for transport

via blood. In the blood stream, triglyceride content of these particles is progressively reduced by the lipoprotein lipase, an enzyme that is attached at the endothelial surface in capillaries. With this process the composition of the molecule changes – the triglyceride content decreases relatively to cholesterol – and VLDL becomes IDL and later on LDL. Released lipids can enter the cell for storage (adipose tissue) or energy production (muscle) (Anghel and Wahli, 2007).

With respect to the metabolism of glucose, the central organ is also the liver that ensures adequate blood glucose supply. The liver is the only organ that – depending on requirements – can both store and release glucose into the blood for use by other tissues. This is of great importance for certain tissues like brain and red blood cells that exclusively use glucose as energy source. The liver uses three metabolic processes to manage glucose:

1. glycogenesis - excess glucose is converted to glycogen for storage within the liver,
2. glycogenolysis - to raise blood glucose levels the liver breaks down stored glycogen, and
3. gluconeogenesis - when glucose is low and the deposit of glycogen is exhausted the liver is able to synthesize glucose from lactate and alanine (derived from muscle) or glycerol (derived from adipose tissue).

Skeletal muscle is the major source of stored glycogen. Depending on muscle mass and nutrative state up to three quarter of body glycogen is stored in myocytes. Especially during moderate and intensive exercise, glucose (derived from glycogen by glycogenolysis) is the major supply of energy. In contrast to hepatocytes, myocytes lack the enzyme glucose-6-phosphatase that is necessary to release glucose into the blood. Therefore, glucose that once entered into myocytes must be used ore stored within the cells.

Also the adipose tissue participates in the regulation of glucose homeostasis. Via the glycolytic pathway, adipocytes are involved in glucose disposal like liver and muscle. Within the tissue, glucose provides the substrate for *de novo* synthesis of FFA and glycerol. Important, released FFA by adipocytes into the circulation influence the sensitivity of liver and muscle to glucose (Stryer, 1994).

Hormonal signaling is essential in the peripheral regulation of fuel metabolism. Insulin but also glucagon, adrenaline, and noradrenaline play an important role in mobilization and storage of fuels.

Insulin is released in response to enhanced levels of glucose and hormones of the gastrointestinal tract (gastrin, secretin, and incretins). Like in the brain, insulin serves as

an index of satiety to peripheral tissues. After binding to its receptor several signaling pathways are activated resulting in physiological effects as follows:

1. Insulin promotes glucose uptake by translocation of glucose transporters to the cell membrane. Two isoforms, which are regulated by insulin, are important in this context – GLUT2 (expressed in liver and pancreas) and GLUT4 (expressed in skeletal muscle and adipose tissue). By contrast, glucose uptake occurs also insulin-independent (e.g., via GLUT1 in the brain).
2. Insulin accelerates glycolysis in liver, muscle, and adipose tissue, which in turn promotes enhanced fatty acid synthesis.
3. Insulin increases glycogen synthesis and decreases glycogenolysis in liver, skeletal muscle, and adipose tissue.
4. Insulin decreases gluconeogenesis in liver.
5. Insulin enhances uptake of blood lipids into muscle and fat tissue and induces fatty acid and triglyceride synthesis.
6. Insulin inhibits lipolysis in adipose cells, which results in lowered serum FFA levels.
7. Insulin inhibits fatty acid oxidation in liver and skeletal muscle.
8. Insulin promotes amino acid uptake into tissues, which in turn increases the rate of protein synthesis.
9. Insulin inhibits protein degradation at least in muscle.

In general, low levels of insulin reverse the above-described effects. Thus, insulin promotes anabolic processes by inhibiting catabolic ones (Dimitriadis et al., 2011; Stryer, 1994).

Glucagon, secreted by pancreatic alpha cells, has opposite effects compared to insulin. Glucagon stimulates glycogenolysis and gluconeogenesis, inhibits glycogenesis and glycolysis, decreases synthesis of fatty acids, and promotes the mobilization of triglycerides in adipose cells (Stryer, 1994).

Deregulation of lipid metabolism is associated with excessive fat enrichment in adipose tissue but also elevated accumulation of triglycerides in liver and muscle and strongly correlates with obesity and peripheral insulin resistance. Further, increased hepatic triglyceride accumulation is accompanied by over production of VLDL and is believed to be one key metabolic disturbance that results in a dyslipidemic profile (characterized by increased serum VLDL, LDL, and triglycerides combined with decreased HDL), which represents in turn a major risk factor of developing several cardiovascular diseases (Fon Tacer and Rozman, 2011).

Deregulation of glucose metabolism is caused primarily through disturbed insulin production/secretion (T1DM) or impaired insulin sensitivity of the tissues (T2DM). In both cases insulin-dependent glucose uptake in tissues is ineffective. Consequently, glycolysis is inhibited in all tissues, gluconeogenesis is stimulated in liver, lipolysis is increased in adipose cells, and protein degradation is enhanced in muscle. Overall, this means that primarily lipids and proteins serve as energy source, while glucose remains in the blood stream triggering pathological changes in blood vessels (Stryer, 1994). Obesity is strongly associated with T2DM.

A combination of disorders like insulin resistance, dyslipidemia, obesity, cardiovascular diseases are called the metabolic syndrome (Miranda et al., 2005).

Estrogen signaling

In both males and females, estrogens are crucial for normal development and maintenance of sexual and reproductive function. The three major naturally occurring estrogens are estrone (E1), estradiol (E2), and estriol (E3), whereby estradiol is the biologically most active form (Heldring et al., 2007; Kuiper et al., 1997). Estradiol is the predominant estrogen during the reproductive phase of females and the developing follicles in the ovaries are the primary source. The estrogen biosynthesis starts in the theca interna cells of the ovaries with the synthesis of pregnenolone and progesterone from cholesterol. These substances serve as precursors for the synthesis of androgens, which requires several enzymatic steps. The final step is catalyzed by the enzyme aromatase that converts androgens into estrogens (Löffler and Petrides, 2003). Estriol is produced in large amounts by the placenta during pregnancy, whereby estrone dominates in postmenopausal females. In males and postmenopausal females the principles sites of estrogen secretion are the adrenal cortex and adipose tissue. Extragonadal estrogen biosynthesis occurs further in bone, brain, and vasculature. In males, also the Leydig cells of the testes produce small amounts of estrogens. However, the adipose, brain, and bone are able to convert androgens into estrogens by aromatase, but they do not synthesize androgenic precursors from cholesterol. Hence, estrogenic production in adipose, brain, and bone depends on circulating androgens (Simpson et al., 1999; Simpson et al., 2005).

Cellular signaling of estrogens is mediated via two distinct estrogen receptors (ERs), ER alpha and ER beta, which belong to the nuclear receptor (NR) family of transcription factors. Both receptors are located at different chromosomal segments as well as have tissue and gender specific expression patterns. Whereas the ER alpha is expressed primarily in the uterus, kidney, heart, and liver, the ER beta expression is predominant in

the ovary, prostate, gastrointestinal tract, bladder, lung, and hematopoietic and central nervous systems. Some tissues express both ER subtypes, i.e., the mammary gland, epididymis, adrenal, skeletal muscle, adipose tissue, thyroid, bone, and certain regions of the brain. Coexpression of ER alpha and beta in the same cell type is described for several neurons in the brain and thymocytes as well as in skeletal muscle and adipose tissue (Barros et al., 2009; Enmark et al., 1997; Hillisch et al., 2004; Matthews and Gustafsson, 2003; Nilsson et al., 2001).

Like many other members of the NR family, ERs are composed of functionally distinct domains. The N-terminal domain contains the ligand-independent activation function 1 (AF1) which enables the modulation of target gene expression through direct interaction with coactivators/corepressors or other components of the transcription machinery. A second ligand-dependent activation function (AF2) is harbored in the ligand-binding domain (LBD) at the C-terminus. This domain is involved in ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression by interaction with coregulatory proteins. Unlike the N-terminal regions of ER alpha and ER beta, which vary strongly in sequence and length, the LBDs are relatively high conserved. Although the AF1 revealed transcriptional activity in the absence of AF2, the full ER activity is believed to be reached when the two AFs act in a synergistic manner. Further, the AF1 of ER alpha is more active compared to the activity of the AF1 domain of ER beta and the transcriptional activity of each AF depends on cell type and promoter characteristics. Between the two domains at the N- and C-terminus, there are two other regions, which are referred to as the DNA-binding domain (DBD) and the hinge-domain. The DBD contains two zinc finger motifs, which is essential for receptor dimerization and specific binding to estrogen responsive elements (ERE) in the regulatory regions of estrogen target genes. This domain represents the highest degree of sequence homology and therefore the specificity and affinity to bind to various EREs is similar for both the ER alpha and beta. Finally, the hinge domain of the two ER subtypes shows low conservation, harbors a nuclear localization signal, and contributes to flexibility between receptor and DNA (Enmark and Gustafsson, 1999; Heldring et al., 2007; Nilsson et al., 2001; Zhao et al., 2008).

ERs exert their large variety of actions by using different molecular pathways. There are the direct (classical) and indirect pathways which start with ligand-dependent ER activation followed by homo- or hetero-dimerization of those receptors. ER dimers then bind either to EREs on DNA (direct pathway) or interact with other transcription factors such as SP1, AP1, and NFκB (indirect pathway), but both variants finally result in modulation of gene expression. Another genomic pathway is ligand-independent. In this case, ERs interact with other signaling pathways (i.e., several growth factors and

neurotransmitters), whereby ERs become phosphorylated by activated kinases that subsequently lead to ER activation and dimerization, DNA-binding, and gene regulation. In addition, activated ERs are also able to mediate non-genomic effects, which occur rapidly within seconds or minutes. These rapid effects involve the activation of several signaling cascades such as protein kinase A and C, and activated mitogen-activated protein kinase, which in turn affect ion channel fluxes or lead to other cellular responses. Apart from the classical ER alpha and beta, also a membrane-associated receptor (G protein-coupled receptor) may be involved in the rapid pathway. This receptor was identified in the last few years and might be able to mediate a response to E2 too (Cenni and Picard, 1999; Heldring et al., 2007; Nilsson et al., 2001).

Although the overall homology of the LBDs between the ER alpha and beta is relatively high, the binding cavities of the two ER subtypes differ clearly in size and flexibility. The endogenous estrogens show overall a quite similar binding affinity for both ERs, but there is a range of other ligands of natural or synthetic origin for which the two ER subtypes exhibit different affinities. Further, the interaction of such estrogenic compounds with the binding cavities of the ERs may result in an agonistic or antagonistic effect, whereby in turn such agonistic/antagonistic properties can be restricted to specific tissues or cell types apparently dependent on the co-factors that are expressed and recruited in the respective tissues. In these cases, the estrogenic compounds are referred to as selective estrogen receptor modulators (SERMs) (Heldring et al., 2007; Hillisch et al., 2004; Kuiper et al., 1997). Examples of compounds with tissue specific estrogenic activity are Tamoxifen and Raloxifen. Both substances act as an ER agonist in uterus and bone, but show ER antagonist activity in the breast, therefore these synthetic SERMs have been used for therapy of breast cancer and osteoporosis for several years (Diel, 2002; Ohmichi et al., 2005; Wade and Heller, 1993). Therefore, it is difficult to predict the magnitude and direction of an estrogenic response, because both the binding affinity of a ligand but also the cellular/nuclear context in which the ligand acts enable a wide range of combinations to modulate a physiological response to E2 or other ligands.

Another interesting compound with affinity to ERs is the isoflavone genistein (Gen). This compound is also referred to as a phytoestrogen. Based on its chemical structure Gen can activate both ER subtypes, but has a higher binding preference to ER beta than to ER alpha (Kuiper et al., 1998). Genistin, the glycoside of Gen, and Gen itself are natural compounds of various plants and are primarily absorbed from food. Soybeans and soy-derived food items or supplements constitute the main source for humans. In the intestine, both microbial enzymes and endogenous phase II enzymes metabolize soy isoflavones, whereby the individual microbial flora influences the quality and quantity of metabolites. Compared to Western countries people in Asia are highly exposed to Gen,

because the traditional diet is rich in soy and soy products (Orgaard and Jensen, 2008). However, nowadays genistin and its aglycone Gen can be found in dietary supplements. Therefore, also people in Western countries may be highly exposed to these isoflavones. Beside its ability to bind on ERs, Gen at higher doses is also known to produce non-hormonal effects (e.g., inhibition of several tyrosine kinases (Akiyama et al., 1987; Elmarakby et al., 2011), AMPK activation (Arunkumar and Anuradha, 2012), epigenetic changes (Li et al., 2009; Molinie and Georgel, 2009), and inhibition of inflammation (Valsecchi et al., 2011).

To discriminate whether an E2-induced physiological process is ER alpha-mediated, ER beta-mediated or both, ligands that selectively bind to only one of the two ER subtypes as well as ERKO animals that only express either subtype (αERKO or βERKO) are available. Examples of ER subtype-selective agonists and important for the presented work are the ER alpha-selective agonist 16alpha-LE2 (Alpha) and the ER beta-selective agonist 8beta-VE2 (Beta). Both substances are steroidal in origin and were designed based on the protein structure and the existing differences in size and flexibility of both ER ligand-binding cavities (Hillisch et al., 2004). In addition, such substances with high affinity to only one of both ER subtypes provide new therapeutic concepts for several diseases.

Impact of estrogen receptors on energy homeostasis

Beside their enormous impact on growth and development of the reproductive tract and maintenance of reproductive function, the two ER subtypes are involved in the regulation of a wide range of biological processes in the whole body. For example, these processes include the function of the cardiovascular, the immune, and the central nervous system, but also diverse processes that are related to energy homeostasis. At present, particularly the influences of estrogens on energy intake and expenditure as well as lipid and glucose metabolism are being intensively investigated in humans and rodents. For example, the enormous decline of endogenous estrogens in women during menopausal transition is associated with body weight gain. Additionally, body weight gain is combined with an unfavorable change of the body composition (higher fat/muscle ratio) and leads in turn to a higher incidence of visceral obesity, insulin resistance, and T2DM (Curtis and Wilson, 2005; Kristensen et al., 1999; Ley et al., 1992; Louet et al., 2004; Rolland et al., 2007; Toth et al., 2000a). Numerous clinical studies have investigated the effect of hormone replacement therapy (HRT) on postmenopausal women. The outcomes have shown a decrease of central obesity, lower incidence of T2DM, increased sensitivity towards glucose and insulin, and an improvement in lipid metabolism (Andersson et al., 1997;

Curtis and Wilson, 2005; Kanaya et al., 2003; Pentti et al., 2009; Samaras et al., 1999; Santen et al., 2010). Also men display a higher prevalence of visceral obesity and impaired glucose metabolism, when compared with premenopausal women (Kuhl et al., 2005). Similar effects were observed in several animal models. Ovariectomy in rodents led to body weight gain and the development of obesity (Hertrampf et al., 2006a; Hertrampf et al., 2008b; Naaz et al., 2002) as well as to dyslipidemia and impaired glucose and insulin tolerance (Riant et al., 2009; Saengsirisuwan et al., 2009). Treatment with E2 antagonized these effects. Moreover, also aromatase-knockout mice were described to develop glucose intolerance and insulin resistance (Simpson et al., 2005; Takeda et al., 2003).

Beside the impact of estrogens receptors on energy homeostasis alone, the interaction of estrogens and physical activity regarding this issue are of great importance, because obesity mainly results from chronic energy imbalance caused by both increased intake and decreased expenditure of energy (Low et al., 2009; Newbold et al., 2009; Wasan and Looije, 2005). Therefore, physical activity as a key determinant to increase energy expenditure is obvious. Consequently, it is not surprising that the beneficial effect of exercise in prevention or therapy of obesity and co-morbidities has been proven before many times (Eriksson and Lindgarde, 1991; Hamman et al., 2006; Knowler et al., 2002; Lindstrom et al., 2006; Pan et al., 1997; Wing, 2010). The described beneficial effects of exercise includes weight loss by catabolising body fat that results in a more favorable body composition and a lower risk of developing insulin resistance and cardiovascular diseases. However, studies that focus on the interaction of estrogens and physical activity regarding energy metabolism in females are limited. This issue is not only relevant for young individuals but also for women after menopause with HRT. Recently, two animal studies using female wistar rats on a high fat diet were conducted in our laboratory, which evaluated the effect of E2 in combination with physical activity on both prevention and therapy of obesity (Zoth et al., 2010; Zoth et al., 2012)[1]. The outcomes of the studies showed that regular exercise training, performed in OVX rats for six weeks, results in effects that were similar to those of E2 treatment alone. The observed effects covered body weight and body fat reduction as well as improved glucose metabolism (at lest in tendancy compared to sedentary OVX rats without E2 treatment). A combination of exercise training and E2 treatment showed the strongest effect in prevention and therapy of obesity.

Both, selective ER subtype-selective agonists and ER knockout animals (as mentioned above) were used to determine the impact of the two distinct ER subtypes in

[1] Both studies were performed with my colleague Dr. Nora Zoth at the German Sports University. This work has already been used for her dissertation and is therefore not subject to the work presented here.

the beneficial role of estrogens on metabolism. In general, to date the metabolic role of ER alpha is better investigated than the role of ER beta, because most studies so far focused on the ER alpha subtype. For example, several animal experiments revealed that the ER alpha is responsible for diminished body weight gain in both sexes (Bryzgalova et al., 2006; Cooke et al., 2001; Heine et al., 2000; Hertrampf et al., 2008a; Hertrampf et al., 2008b). Improved tolerance towards glucose and insulin was shown by using ERKO mice and wild type counterparts. αERKO mice revealed increased hepatic lipogenesis (Bryzgalova et al., 2006), decreased GLUT4 expression in skeletal muscle and white adipose tissue (Barros et al., 2009; Barros et al., 2006) as well as a higher rate of pancreatic beta-cell apoptosis (Le May et al., 2006) when compared to wild types. However, the role of ER beta on E2 regulation of energy homeostasis is less clear and somewhat controversial. For example, there are studies showing that the lack of ER beta result in an obese phenotype (Foryst-Ludwig et al., 2008; Seidlova-Wuttke et al., 2012), whereas other studies do not show this effect (Cooke et al., 2001; Faulds et al., 2012; Nilsson and Gustafsson, 2011). Similar inconsistent results exist regarding glucose homeostasis. βERKO mice were described to show significant improved glucose tolerance (Barros et al., 2009; Foryst-Ludwig et al., 2008) but also to tolerate glucose on a similar level as wild types (Bryzgalova et al., 2006). Therefore, further investigations are needed to understand the metabolic role of ER beta. In general, the molecular pathways that are activated by the two distinct ER subtypes are not completely resolved.

Objectives of the work

The overall aim of my studies was to evaluate the role of E2 on energy metabolism, especially what occurs when the primary function of the ovaries has ceased. Special emphasis was given to the effects of the ER subtype-selective agonists Alpha and Beta. Additionally, the impact of the phytoestrogen Gen was investigated because of its estrogenic activity and the controversial discussion concerning its impact on energy homeostasis. Finally, the effect of E2 in combination with physical activity on prevention and therapy of obesity was evaluated.

For this purpose two animal experiments using female rats were performed. In general, the rats were ovariectomized (OVX) to stop the primary endogenous estrogen production or SHAM-operated. Subsets of OVX rats were treated with E2, Alpha, Beta, or Gen.

The thesis addresses the following research questions that will be discussed in detail in the later chapters.

CHAPTER TWO

- How does E2 treatment influence parameters that are related to energy homeostasis in healthy Wistar rats with nutrition-induced obesity?
- To what extent are the two distinct ER subtypes, ER alpha and ER beta, involved in the action of E2?
- Can the application of Gen induce effects similar to those of E2?

CHAPTER THREE

- What are the underlying molecular mechanisms responsible for the observed effects of treatment with Alpha, Beta, or Gen in chapter two?

CHAPTER FOUR

- To what extent are the both ER subtypes responsible for the existing gender dimorphism regarding glucose metabolism in obese leptin resistant Zucker diabetic fatty (ZDF *fa/fa*) rats?

Chapter Two

Impact of estradiol, ER subtype-selective agonists and genistein on energy homeostasis in a rat model of nutrition-induced obesity

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Abstract

Estrogens are known to be involved in the control of energy homeostasis. Here, the role of ER alpha and ER beta in a model of nutrition-induced obesity was investigated. Ovariectomized Wistar rats were fed with a high fat diet and received either vehicle, E2, ER subtype-selective agonists (Alpha and Beta), or Genistein. After 10 weeks, body weight, visceral fat, serum levels of leptin and lipids, and in the skeletal muscle anabolic markers were determined. Treatment with E2 and Alpha decreased body weight, total cholesterol, and VLDL. In contrast, visceral fat mass, adipocyte size, and serum leptin were reduced by E2, Alpha, and Beta. In the soleus muscle, treatment with E2 and Beta modulated Igf1 and Pax7 gene expression and resulted in larger muscle fibers.

The data indicate that blood lipids are affected via ER alpha, whereas activation of ER beta results in an increase of soleus muscle mass. Adipose tissue homeostasis seems to be affected via both ERs.

Introduction

Obesity, defined as a BMI greater than 30 kg/m^2 in adults, has been identified as a major risk factor for chronic diseases, including type 2 diabetes, insulin resistance, dyslipidemia, hypertension, cardiovascular disease, coronary heart disease, liver and gall bladder diseases and certain forms of cancer (Chan and Woo, 2010; Mokdad et al., 2003; Newbold et al., 2009; Swinburn et al., 2004). Already in 1997, the WHO formally recognized obesity as a global epidemic (Caballero, 2007). In 2005, approximately 1.6 billion adults (age 15+) around the world were overweight (BMI greater than 25 kg/m^2) - at least 400 million of them were obese, and these numbers will continue to rise (Chan and Woo, 2010; Low et al., 2009).

Obesity is most likely caused by a combination of genetic, behavioral, and environmental factors (Comuzzie et al., 2001; Goulart et al., 2009). However, the main reason of this epidemic rapid growth of obesity and overweight over the last several decades is thought to be a chronic imbalance of energy homeostasis. This chronic imbalance is attributed to increased consumption of higher energy-dense and nutrient-poor foods with high levels of saturated fats and sugar combined with reduced opportunities to use physical energy (Low et al., 2009; Newbold et al., 2009; Wasan and Looije, 2005).

Meanwhile, obesity affects virtually all ages, sexes, races and socioeconomic groups, and therefore poses a serious social and psychological problem. The loss of circulating estrogen in postmenopausal women is associated with body weight gain and increased abdominal fat mass (Kristensen et al., 1999; Ley et al., 1992; Toth et al., 2000a). Similar observations were done in different animal models. Ovariectomy in mice, rats, and Syrian hamster resulted in body weight gain and the development of obesity, whereas treatment with E2 antagonized these effects (Hertrampf et al., 2006a; Hertrampf et al., 2008b; Naaz et al., 2002; Wade and Powers, 1993). In the meantime, it is well known, that estrogens directly influence energy homeostasis through modulation of glucose and lipid metabolism (Ropero et al., 2008).

Cellular signaling of estrogens is mediated via two distinct ERs, ER alpha and ER beta, which belong to the nuclear receptor family of transcription factors. Both receptors are located at different chromosomal segments and have tissue as well as gender specific expression patterns. Like many other members of the nuclear receptor family, ERs are consisted of functionally distinct domains. ER alpha and ER beta differ considerably in their three-dimensional structure of the ligand-binding domain, representing the key requirement for SERMs (Heldring et al., 2007; Kuiper et al., 1997). Therefore, the binding affinity of the ligand as well as the cellular/nuclear context in which the ligand acts, enable a wide range of combinations to modulate a physiological response to E2 or other potential ligands (Diel, 2002; Ohmichi et al., 2005).

To evaluate the role of the ERs in both disease and normal physiology, especially to answer the question of which physiological process is ER alpha-mediated, ER beta-mediated or both, subtype-selective ER agonists offer an important tool. Highly subtype-selective and steroidal ER agonists 16alpha-LE2 (Alpha) and 8beta-VE2 (Beta) were designed based on the protein structure and the existing differences in size and flexibility of both ligand-binding cavities of ER alpha and ER beta. *In vitro* studies using radio ligand competition and transactivation assays showed that 16alpha-LE2 binds to ER alpha and 8beta-VE2 binds to ER beta selectively (~200 fold), but with similar potency to E2 (Hillisch et al., 2004). Animal experiments using these compounds confirmed the selective stimulation of either ER alpha or ER beta. ER alpha mediated effects of E2 include uterus proliferation, inhibition of cardiac hyperthrophy, protection of bone mineral density, increased movement drive and body weight control (Hertrampf et al., 2007; Hertrampf et al., 2008a; Hertrampf et al., 2008b; Hillisch et al., 2004; Pelzer et al., 2005). Metabolic studies using αERKO animals revealed that ER alpha absence caused increased white adipose tissue content, insulin resistance, and impaired glucose tolerance in both sexes (Heine et al., 2000). The role of ER beta in E2 regulation of energy homeostasis is less clear and somewhat controversial. Other interesting compounds with affinity to ERs are plant derived. For example, the isoflavones Gen and Daidzein can activate both ER subtypes based on their chemical structure, but have greater binding affinity to ER beta than for ER alpha (Kuiper et al., 1998).

Because tissues involved in the control of energy homeostasis such as adipose tissue, pancreatic tissue, skeletal muscle, and brain express both ER alpha and ER beta, it is of interest to determine to what extent ERs are involved in the underlying molecular mechanisms to develop more specific treatment strategies for obesity and diabetes in the future.

Thus, the aim of this study was to investigate the impact of E2, the ER subtype-selective agonists Alpha and Beta, and Gen on energy homeostasis in a rat model of

nutrition-induced obesity. For this purpose, juvenile OVX Wistar rats on a HF diet were treated with E2, Alpha, Beta, and Gen for 10 weeks. The SHAM and untreated OVX animals as well as the LF diet served as controls. After the experimental period, the effects of the different diet and substances on energy intake, body weight, visceral body fat mass, serum levels of leptin and blood lipids as well as on the soleus muscle were investigated.

Materials and methods

Animals

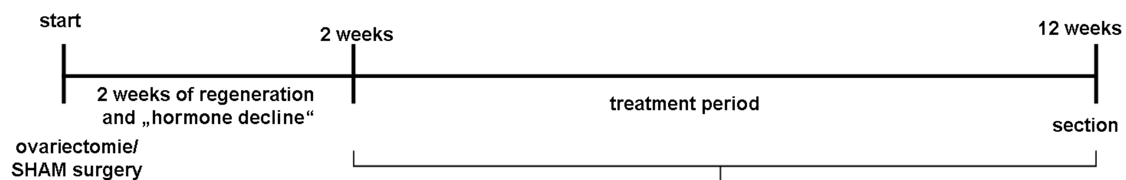
Juvenile female Wistar rats (6-weeks old, 120–150g) were obtained from Janvier (Janvier, Le Genest St Isle, France) and kept at constant room temperature (20 °C ± 1), relative humidity (50–80%) and illumination (12-h light/dark cycles). The animals were housed 3–4 in each cage, with food (SSniff GmbH, Soest, Germany) and water provided ad libitum. All animal procedures were approved by the Committee on Animal Care and compliant with accepted veterinary medical practice.

Animal treatment and diet

Prior to experimental exposure procedures, all animals were ovariectomized (OVX) or SHAM operated via the dorsal route. Following two weeks of regeneration and endogenous hormonal decline, the animals were randomly allocated to different treatment and diet groups (n=7). Two different diets were used, a phytoestrogen free LF diet (ssniff® EF D12450B* (I) mod. *LS** containing a metabolizable energy of 15.0 MJ/kg) and a phytoestrogen free HF diet (ssniff® EF R/M acc. D12451 (I) mod.* containing a metabolizable energy of 19.2 MJ/kg). All SHAM animals and a subset of OVX animals were fed with the LF diet or the HF diet. The remaining OVX animals received the HF diet and were treated with E2, Alpha, Beta or Gen (composition of diets and animal grouping see table 1 and Fig. 1). E2 (4µg/kg b. wt per day), Alpha (10 µg/kg b. wt per day), and Beta (100 µg/kg b. wt per day) were applied via ALZET® osmotic mini pumps, whereby the substances were dissolved in dimethylsulfoxide and water according to the manufacturer’s instructions for filling. Gen was administered via Gen-enriched food (700 mg/kg HF diet resulting in approx. 42 mg/kg b. wt per day). Ssniff Spezialdiäten GmbH, Soest, Germany, did the food enrichment with Gen. The treatment doses of the respective substances were chosen based on previous experiments (Diel et al., 2004; Hegele-Hartung et al., 2004; Hertrampf et al., 2009a; Hertrampf et al., 2005; Hertrampf et al.,

2008b). E2 was provided by Sigma-Aldrich (Deisenhofen, Germany), ER subtype-selective substances Alpha and Beta were obtained from Bayer Schering Pharma AG, Berlin and Gen was obtained from LC Laboratories (Woburn, MA 01801 USA). Throughout the treatment period, body weight and food intakes were monitored once a week.

After 10 weeks of treatment, the animals were sacrificed by decapitation after light anaesthesia with CO_2 inhalation and the blood was collected. The whole abdominal body fat (periovarian, perirenal, mesenteric/omental) and the uteri were prepared and the wet weights were determined. Periovarian fat pads were immediately fixed in 4% neutrally buffered formalin for morphometric analysis. To measure the subcutaneous fat content, a piece of 4 cm^2 from the back of each animal was dissected and the wet weight was determined. The soleus muscles were removed and directly frozen in liquid nitrogen for analysis of molecular markers or embedded in TissueTek® (Sakura, Staufen, Germany), cooled in isopentane, and frozen in liquid nitrogen for histological analysis.



groups	hormone	diets and treatment					
(n = 7)	state	LF	HF	E2	ALPHA	BETA	Gen
1	SHAM	X					
2	OVX	X					
3	SHAM		X				
4	OVX		X				
5	OVX		X	X			
6	OVX		X		X		
7	OVX		X			X	
8	OVX		X				X

Fig. 1. Experimental design.
SHAM = sham operated, OVX =ovariectomized; LF = low fat diet, HF = high fat diet, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein.

Table 1. Composition of the diets.
HF = high fat; LF = low fat.

Ingredients	HF diet	LF diet
Dry matter [%]	96.2	96.1
Crude fat [%]	23.1	5.1
Fatty acids [%]		
C 12:0	0.02	/
C 14:0	0.29	0.02
C 16:0	5.15	0.55
C 16:1	0.62	0.03
C 18:0	2.83	0.24
C 18:1	8.99	1.32
C 18:2	3.19	2.65
C 18:3	0.37	0.32
C 20:0	0.01	0.03
C 20:4	0.35	/
Cholesterol [mg/kg]	175	/
Crude protein [%]	22.5	18.1
Crude fibre [%]	4.0	6.1
Crude ash [%]	5.9	6.2
N free extracts [%]	40.7	60.6
Starch [%]	6.7	40.7
Sugar [%]	20.1	8.6
Dextrine [%]	11.6	7.9

Determination of leptin

The serum concentration of leptin was measured in duplicate using ELISA kits for rats according to the manufacturer's instruction (ALPCO Diagnostics, Salem, NH 03079, USA).

Determination of serum lipids

Serum was obtained by centrifugation at 4 °C and 3000g and stored at –20 °C. Serum levels of triglyceride were analyzed by colorimetry using ABX Pentra reagent (ABX Diagnostics Montpellier, France). The determination of TC, HDL and LDL occurred via photometry using reagents from DIALAB (Wiener Neudorf, Austria). To measure the serum lipids a chemistry analyzer (Roche Hitachi Cobas Mira Plus) was used. Concentrations of VLDL were calculated by subtraction of the subunits LDL and HDL from TC.

H&E staining and determination of adipocyte areas

Sections from paraffin embedded periovarian fat tissue were cut at 7 µm thickness. Following dewaxing, rehydration, and H&E staining sections were photographed by using a light microscope (KS 300, Zeiss, Jena, Germany) and the corresponding Image

software 3.0. The areas of adipocytes were measured in captured images using the ImageJ program, developed by the National Institutes of Health.

Immunohistochemical staining and determination of soleus muscle fiber areas

Cross-sections (7 µm) from frozen and Tissue Tek® embedded soleus muscles were fixed in ice-cold acetone for 10 minutes. After drying, sections were incubated in 5% BSA/TBS for 1h at room temperature to block non-specific binding. Sections were incubated with anti-Laminin antibody (L9393, Sigma-Aldrich, Deisenhofen, Germany) at a dilution of 1:400 in 1% BSA/TBS overnight at 4°C. Then the sections were incubated first with the corresponding secondary antibody (E0432, Dako, Glostrup, Denmark) and then with the Streptavidin-biotinylated horseradish peroxidase complex (RPN1051V, GE Healthcare, Munich, Germany) each 1:400 diluted in TBS for 1h at room temperature. To visualize the antigen-antibody complexes 3,3´-Diaminobenzidine-tetrahydrochloride (DAB, Sigma-Aldrich, Deisenhofen, Germany) was used as substrate. Between the individual incubation steps sections were washed several times with TBS. Muscle fiber areas were measured with an inverse microscope (Axiovert200, Zeiss, Jena, Germany) and the corresponding software AxioVision Rel. 4.6.3.

Real-time RT-PCR experiments

Total RNA was extracted from frozen soleus muscle tissue using the standard TRIzol® method (Life Technologies GmbH, Darmstadt, Germany). RNA was quantified by spectrophotometry (NanoDrop™ 1000, Thermo Scientific, Wilmington, DE 19810, USA) and cDNA was synthesized from 1 µg RNA using a Reverse Transcription System (QuantiTect®, Qiagen). Real-time RT-PCR was performed with Taq DNA polymerase (Invitrogen, Germany) in the presence of a fluorescent dye (SYBR Green, BioRad) on an Mx3005P™ qPCR System (Stratagene). All reactions were run in triplicate in a 50 µl total volume. The PCR program was as follows: 95 °C for 3 min for 1 cycle, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and 1 cycle for 1 min at 72 °C. Fluorescence was quantified during the 58 °C annealing step and product formation was confirmed by melting curve analysis (58–95 °C). Relative mRNA amounts of target genes were calculated after normalization to an endogenous reference gene (Cytochrom c Oxidase, subunit 1A) following the $\Delta\Delta C_T$ method (Pfaffl, 2001). Specific primer pairs were designed based on the genomic sequences available at the UCSC Genome Bioinformatics database using the software Primer3 (Rozen and Skaletsky, 2000) and confirmed by the sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). Life Technologies GmbH (Darmstadt, Germany) synthesized all the following primers: 1A_fwd 5'-CGTC ACAGCCCATGCATTCG-3', 1A_rev 5'-CTGTTCATCCTGTTCCAGCTC-3',

Igf1_fwd 5'-CCGCTGAAGCCTACAAAGTC-3', Igf1_rev 5'-TGTTTTGCAGGTTGCTC AAG-3', Pax7_fwd 5'-ACTCTGCCTCCTCCATCT CAG-3', and Pax7_rev 5'-TGTGTA GACAGGCTCACGTTTT-3'.

Statistical analysis

All data are expressed as arithmetic means ± SD. Statistical significance of differences was calculated using Kruskal–Wallis one-way analysis of variance with a subsequent Mann–Whitney U test. Differences in soleus muscle gene expression were assessed by one-way ANOVA followed by Tukey's HSD post hoc test (SPSS Statistical Analysis System, Version 17.0). Statistical significance was established at $p<0.05$.

Results

Diet, energy intake and body weights

Measurement of uterine wet weight is the most reliable biomarker to determine estrogenic activity of administrated compounds (Owens and Ashby, 2002). To ascertain the estrogenic response of the experimental treatment and the adequate delivery of compounds, the uterine wet weight of all animals was measured. As shown in Fig. 2, OVX resulted in a dramatic atrophy of the uterus. The uterine wet weights of those animals were 6 to 7-fold lower than the wet weights of the SHAM animals. As expected, treatment of OVX rats with E2 and Alpha led to a strong stimulation of uterine wet weight (similar to that of SHAM groups), whereas the administration of Beta and Gen revealed no such uterotrophic effect. According to earlier studies this result clearly shows that the stimulatory effect of E2 on the uterine wet weight is ER alpha mediated (Hertrampf et al., 2006a; Hertrampf et al., 2008a).

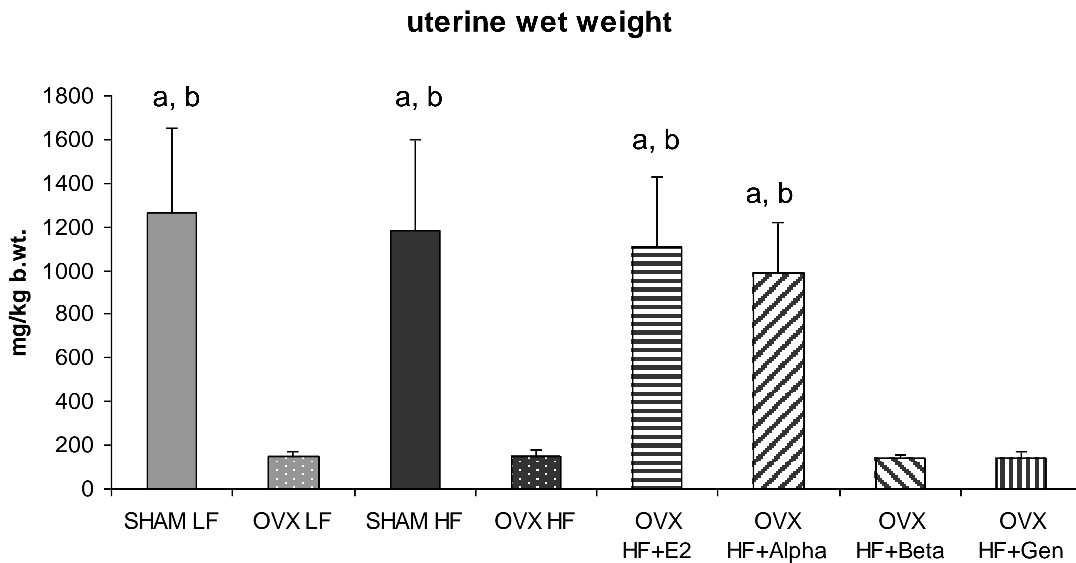


Fig. 2. Effects of E2, ER subtype-selective agonists and Gen on uterine wet weights after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD. Mean values were significantly different for the following comparisons: (a) $p \leq 0.05$ vs. OVX LF, (b) $p \leq 0.05$ vs. OVX HF.

SHAM and untreated OVX animals fed with the LF diet had a significantly higher food intake (1.2 fold) than the respective animal groups on a HF diet (Fig. 3A). However, compared to the energy content of the different diets there was no significant difference on energy intake among the animal groups (3B). Nevertheless, after 10 weeks of treatment and independent from the diets, body weight gain in the SHAM-, E2- and Alpha-treated groups was significantly lower than in the untreated OVX animals. Treatment of OVX rats on HF diet with Beta or Gen did not significantly counteract the body weight gain (Fig. 3C).

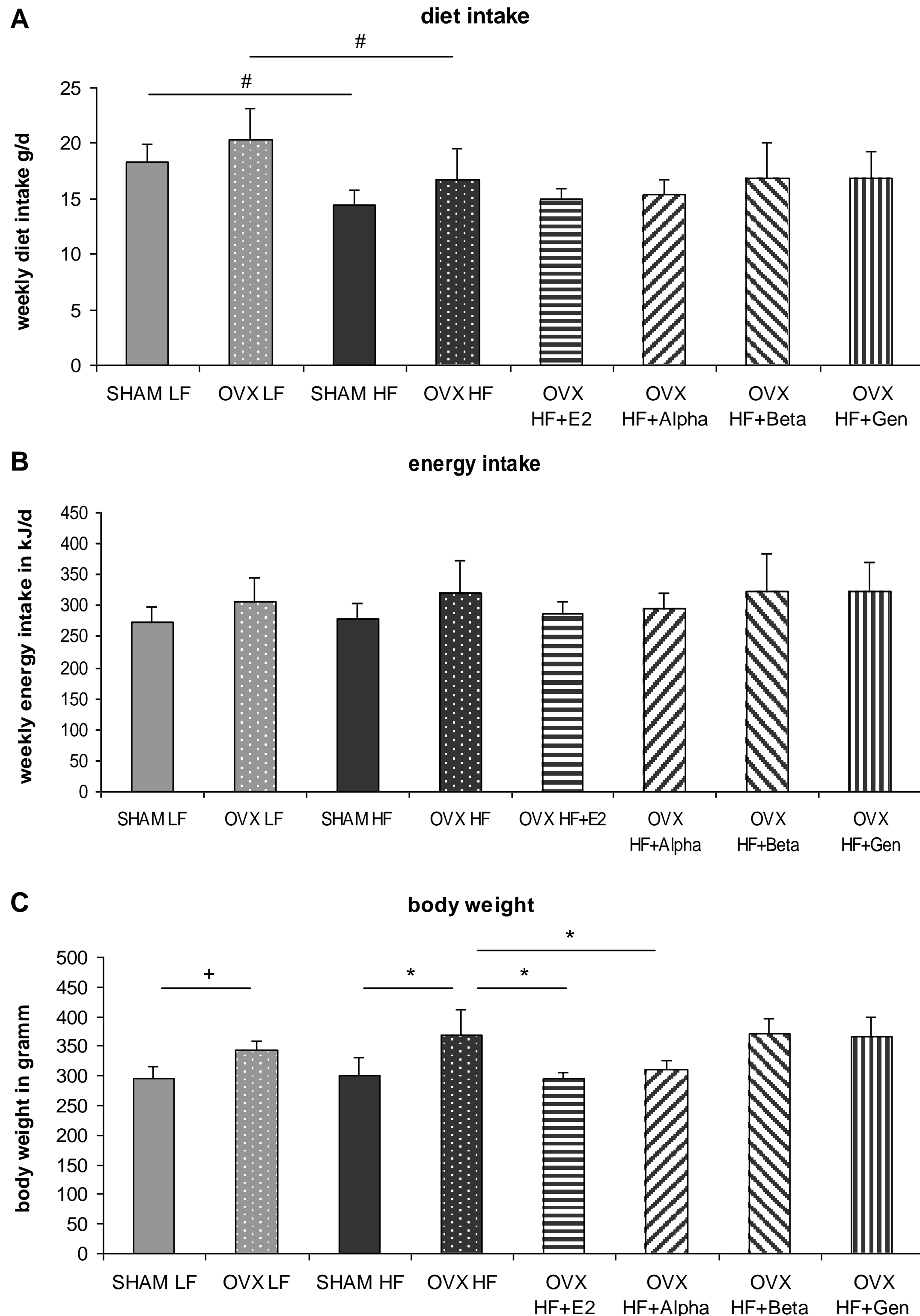


Fig. 3. Effects of E2, ER subtype-selective agonists and Gen on (A) diet intake, (B) energy intake, and (C) body weight after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD. Statistical significance was established at $p \leq 0.05$ and is indicated by lines for the following comparisons: + marks a significant difference between OVX and SHAM on LF diet, * marks significant differences between OVX HF and other groups on HF diet, # marks significant differences between the two different diets.

Visceral body fat content, adipocyte size, serum levels of leptin

Menopause is associated with an increase in body weight, in particular fat mass with a simultaneous alteration in fat distribution that seems to be correlated with a massive decline of ovarian steroids (Cooke and Naaz, 2004; Heine et al., 2000; Ley et al., 1992). In this study, visceral body fat content (periovarian, perirenal, mesenteric omental) and adipocyte size (periovarian) were determined. The highest visceral body fat content (Fig. 4A) as well as the largest adipose cells (Fig. 4B) was observed in untreated OVX HF animals. SHAM-, E2- and Alpha-treated animals and interestingly also the Beta-treated animals on the same diet revealed significant lower abdominal fat mass as well as smaller fat cells. Administration of Gen to OVX HF rats did not influence the abdominal fat mass and had only a small effect on adipocyte size. The same OVX induced effect on abdominal fat mass and adipocytes was observed in LF diet animal groups, although only the adipocyte size was statistically significant. Moreover, comparing the data of SHAM LF and HF shows that significant larger adipose cells were measured in the SHAM HF group. Furthermore, neither hormone treatment nor diet had a significant effect on subcutaneous fat depots (data not shown). Leptin, an adipose tissue derived hormone, is well known as a key regulator for energy intake and energy expenditure (Rosen and Spiegelman, 2006). The data show that in comparison to the SHAM animals the leptin levels were significantly increased in both untreated OVX groups (Fig. 4C). The combinatory effect of ovariectomy and HF diet seems to have an additive effect and led to even higher leptin values. Serum leptin levels of OVX rats were significantly lowered following the application of E2, Alpha and Beta but not by administration of Gen.

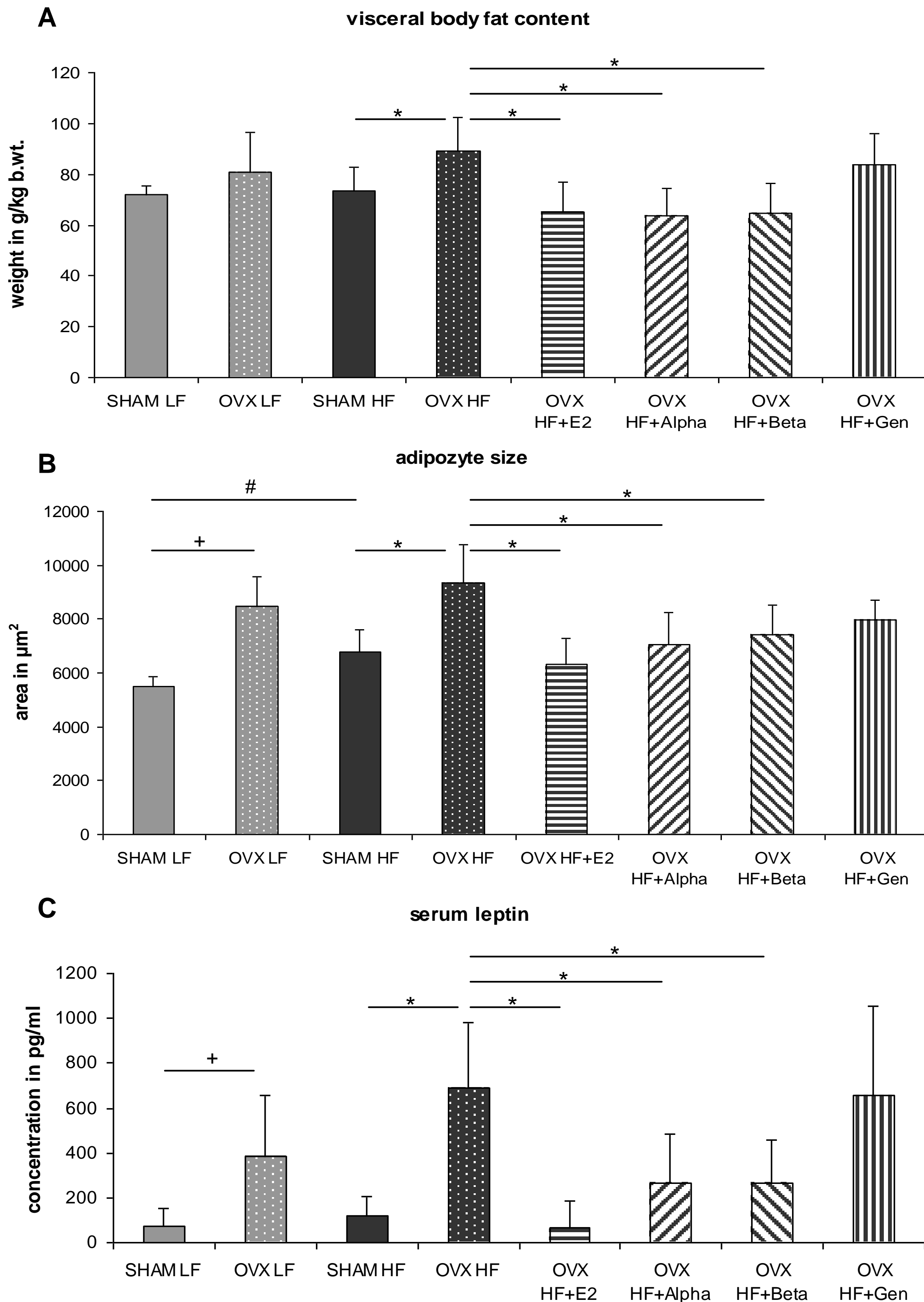


Fig. 4. Effects of E2, ER subtype-selective agonists and Gen on (A) visceral body fat content, (B) adipocyte size, and (C) serum leptin after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD. Statistical significance was established at $p \leq 0.05$ and is indicated by lines for the following comparisons: + marks a significant difference between OVX and SHAM on LF diet, * marks significant differences between OVX HF and other groups on HF diet, # marks significant differences between the two different diets.

Serum levels of lipids

The serum lipid levels after 10 weeks of treatment are shown in Fig. 5. Compared to SHAM animals, OVX resulted in significantly higher serum levels of TC and their subunits HDL, LDL, and VLDL. The LF diet seems to increase this effect, although this effect was statistically significant only for the LDL level. Concentrations of TC and VLDL were reduced by treatment with E2 and Alpha. In the same animal groups, a mild reduction of LDL was observed, while the HDL concentration remained unchanged. The application of Beta or Gen showed no significant impact on TC or their subunits. Serum triglyceride levels were not influenced by treatment or different diet (data not shown).

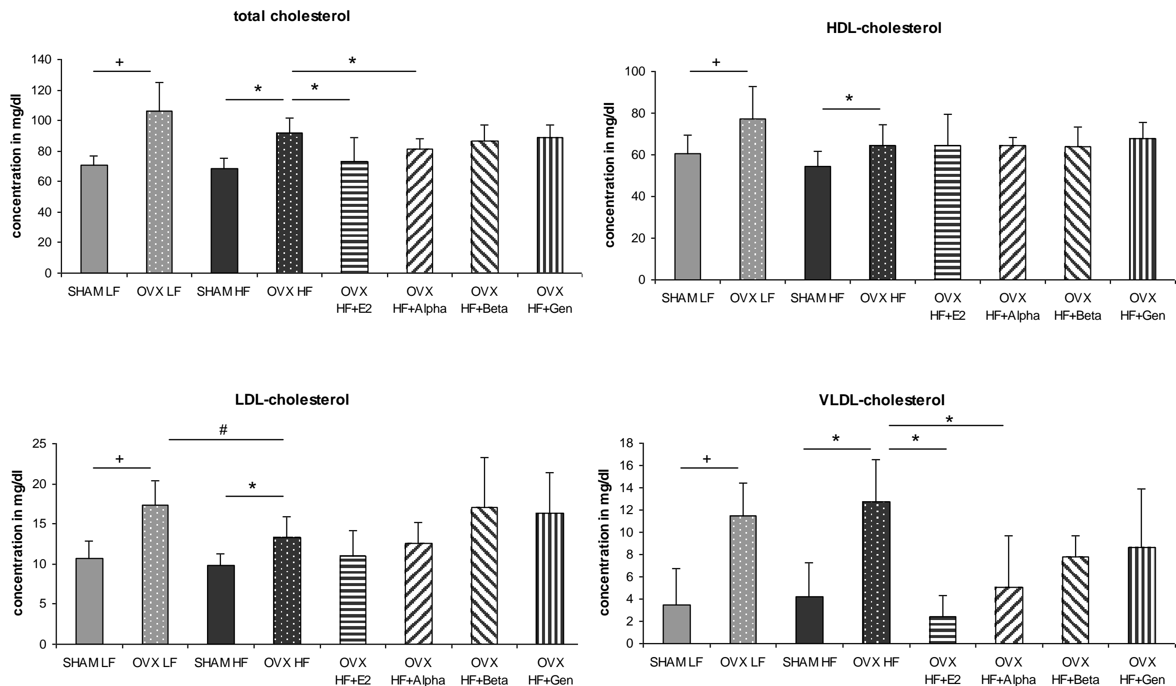


Fig. 5. Effects of E2, ER subtype-selective agonists and Gen on serum lipids after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD. Statistical significance was established at $p \leq 0.05$ and is indicated by lines for the following comparisons: + marks a significant difference between OVX and SHAM on LF diet, * marks significant differences between OVX HF and other groups on HF diet, # marks significant differences between the two different diets.

mRNA expression of Igf1 and Pax7 in the soleus muscle

Our results regarding body weight and visceral body fat content clearly indicate a negative correlation in the Beta-treated animals. These animals have a relative high body weight (similar to untreated OVX HF animals), but a relative low visceral body fat content (similar to E2- and Alpha-treated OVX HF animals). Based on the observation, it can be

hypothesized that Beta-treated animals have a higher fat-free body mass. Therefore, anabolic effects of the Beta selective agonist on the soleus muscle were measured. mRNA expression of Igf1 as an indicator for anabolic activity (Barton-Davis et al., 1998; Barton-Davis et al., 1999; Mavalli et al., 2010; Philippou et al., 2007; Weber, 2002) and of Pax7 as a marker for the specification/expansion of the satellite cell population (Charge and Rudnicki, 2004) were measured. Satellite cells, the so called myogenic stem cells, are required for post-natal growth, repair and maintenance of skeletal muscles (Chen and Goldhamer, 2003). OVX rats on the HF diet showed significantly higher mRNA level of Igf1 in the soleus muscle than SHAM animals. Application of E2, Beta, and Gen but not Alpha antagonized this OVX effect. Furthermore compared to OVX animals on the LF diet, the HF diet in OVX rats led to even higher Igf1 expression levels (Fig. 6A). Independent of the diet, ovariectomy resulted in a significant down-regulation of Pax7 gene expression compared with SHAM animals. Treatment with E2 and Beta completely antagonized the OVX induced effect, whereas the application of Alpha and Gen revealed only a tendency (Fig. 6B).

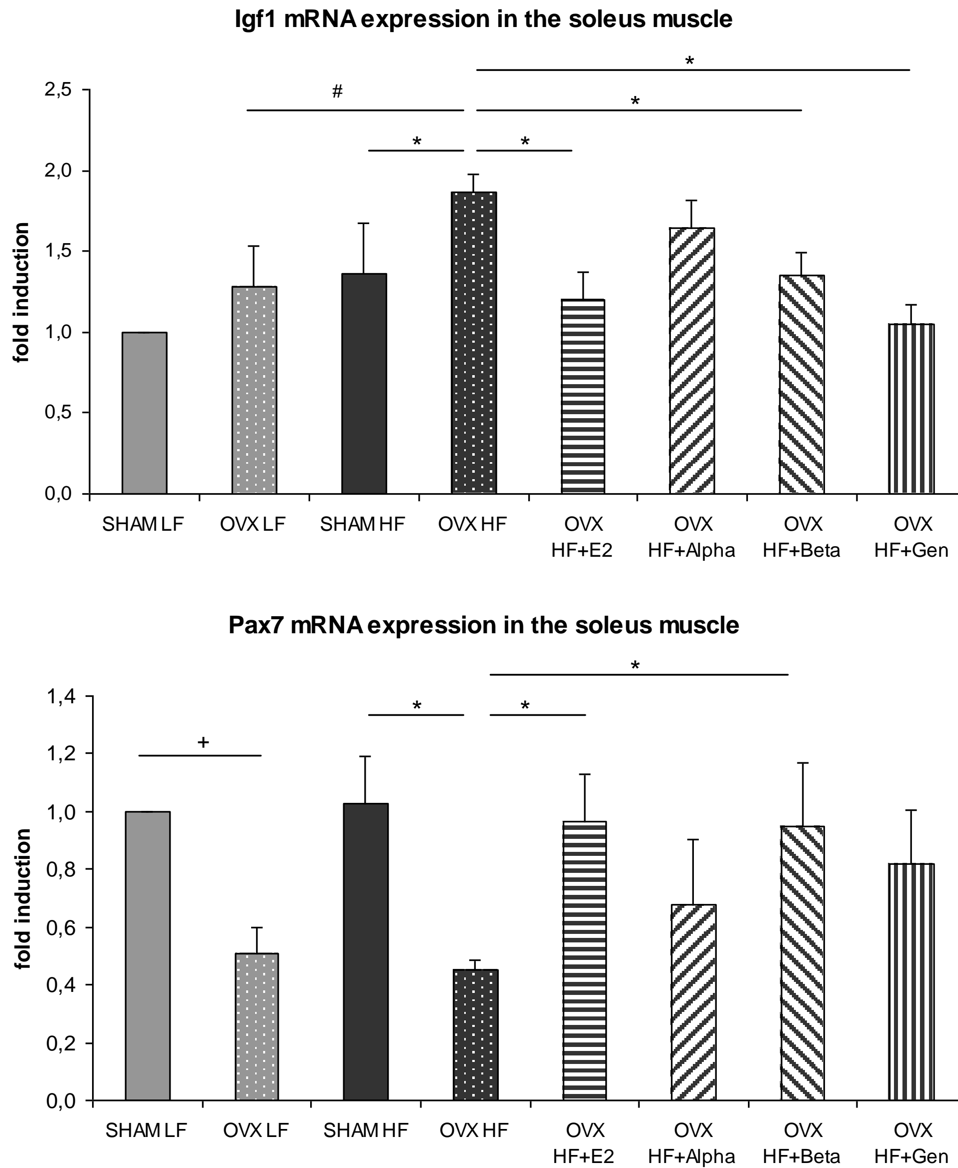


Fig. 6. Effects of E2, ER subtype-selective agonists and Gen on mRNA expression of (A) Igf1 and (B) Pax7 in the soleus muscle after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD. Statistical significance was established at $p \leq 0.05$ and is indicated by lines for the following comparisons: + marks a significant difference between OVX and SHAM on LF diet, * marks significant differences between OVX HF and other groups on HF diet, # marks significant differences between the two different diets.

Cross-sectional areas of soleus muscle fibers

Gene expression patterns of Pax7 and Igf1 indicate an anabolic activity of Beta in the soleus muscle. To confirm further these results, the soleus muscle fiber cross-sectional areas in histological sections were measured. Treatment of OVX HF rats with E2 and Beta led to significantly larger muscle fibers compared with untreated OVX HF animals, whereby the largest muscle fibers were measured in the Beta-treated animals (Fig. 7).

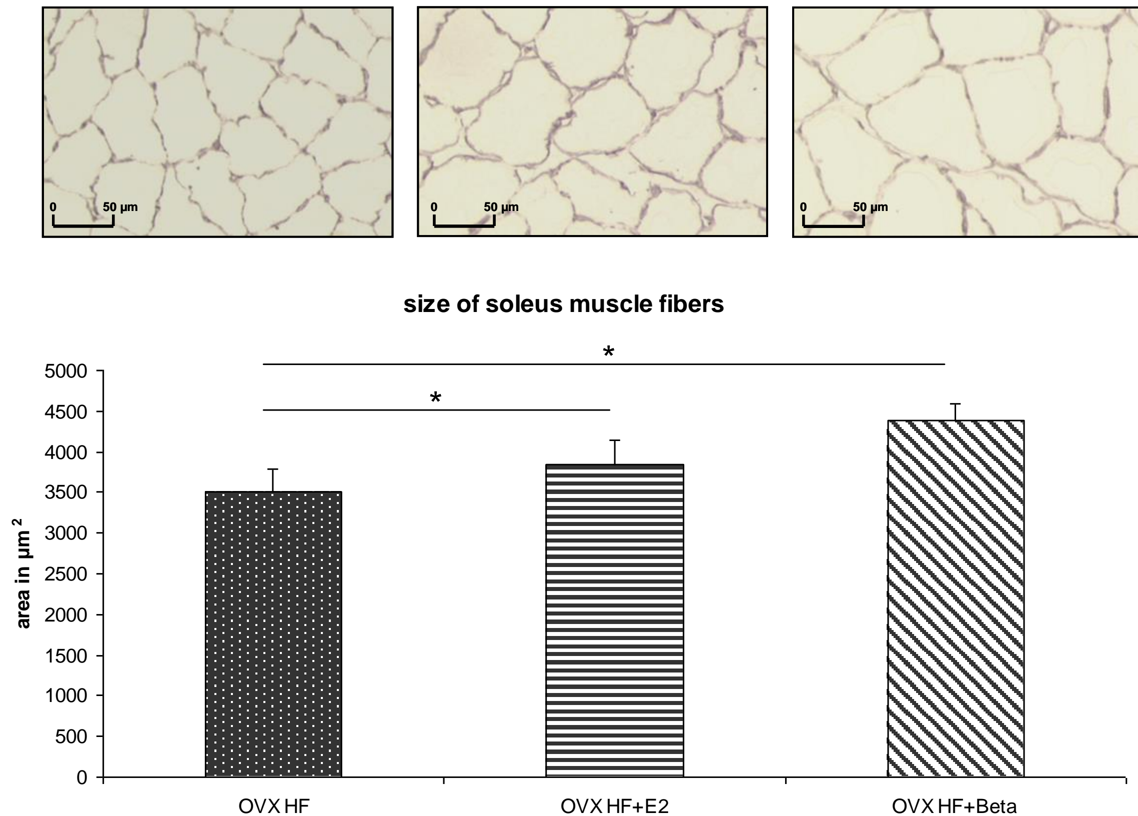


Fig. 7. Effects of E2 and Beta on soleus muscle fiber sizes after 10 weeks of treatment.
OVX = ovariectomized, E2 = treated with 17beta-estradiol, Beta = treated with ER beta-selective agonist, HF = high fat diet. Data shown are means ± SD. * marks significant differences as indicated by lines vs. OVX HF. Statistical significance was established at $p \leq 0.05$.

Discussion

The goal of the study was to obtain more detailed insights into the action of E2 on energy intake, body weight, lipid metabolism and fat to muscle mass ratio in a rat model of nutrition-induced obesity. Special emphasis was given to the effects of synthetic ER subtype-selective agonists Alpha and Beta and the phytoestrogen Gen.

In line with already published data (Hertrampf et al., 2007; Hertrampf et al., 2008b; Hillisch et al., 2004) it is clearly visible that uterine growth response is ER alpha mediated (Fig. 2). Moreover, the response of the uterus to administration of ER ligands is a clear indication for the adequate delivery of the estrogenic compounds by osmotic mini pumps or enriched food.

A major focus of this study was to investigate effects of the different ER agonists on the development of body weight and fat mass. Previous studies demonstrated that E2 supplementation in OVX animals significantly decreases body weight via ER alpha mediated mechanisms (Hertrampf et al., 2007; Hertrampf et al., 2008b). The data obtained from the present study confirm this result and suggest that the HF diet given to OVX animals has an additive, although non-significant stimulatory effect on body weight gain compared to OVX animals on LF diet (Fig 3C). An important finding of the present study is the observation that the modulation of weight gain by the different ER ligands is not caused by a modulation of energy intake. In fact, the influence of E2 on food intake has been discussed controversially. Meli and colleagues reported a significantly lower food intake in E2 supplemented OVX rats and SHAM animals compared to untreated OVX rats (Meli et al., 2004). Naaz et al. assumed that the anorectic action of E2 is ER alpha mediated (Naaz et al., 2002). In contrast, another group, using OVX female Wistar rats postulated that ER beta in the central nervous system is involved in the inhibitory effect of estrogens on food intake (Liang et al., 2002). In the present study, only the groups fed with the LF diet showed significantly higher food intake following 10 weeks of treatment. This effect could be due to the lower content of metabolizable energy in the diet, since no significant differences in terms of energy intake between all animal groups was measured (Fig 3A,B). This finding is in line with previous results of our group and others (Heine et al., 2000; Zoth et al., 2010) and demonstrates that differences in weight gain are caused by alterations in the metabolic activity by the estrogenic compounds and not by a different intake of energy.

Over the last decades, numerous studies have shown that adipose tissue plays a major role in the regulation of energy balance (Barsh and Schwartz, 2002; Rosen and Spiegelman, 2006). Obesity, caused by an excessive enrichment of adipose tissue, especially intra-abdominal fat, constitutes a massive risk for metabolic abnormalities

(Chan and Woo, 2010; Mokdad et al., 2003; Swinburn et al., 2004; Wasan and Looije, 2005). Men tend to have more visceral fat enrichment whereas perimenopausal women accumulate more fat subcutaneously. After menopause, fat distribution in women becomes resembles that in men due to a decline of endogenous estrogens, clearly indicating that estrogens are involved in adipose tissue biology (Ley et al., 1992; Toth et al., 2000b). Previous reports indicate a direct anti-lipogenic and pro-lipolytic action of estrogens in adipose tissue (D'Eon et al., 2005; Rogers et al., 2009), but the underlying molecular mechanisms and whether these actions are mediated via ER alpha and/or ER beta have been not fully elucidated so far. For example, a study undertaken by Heine and colleagues revealed an increase of adipose tissue in male and female αERKO mice (Heine et al., 2000). Another experiment, using the stable ER alpha transfected 3T3-L1 pre-/adipocyte cell line showed reduced lipoprotein lipase mRNA and decreased triglyceride accumulation (Homma et al., 2000). In contrast, a recently published research paper suggests an anti-lipogenic action of ER beta in adipose tissue mediated via a negative cross talk of ER beta with PPAR gamma (Foryst-Ludwig et al., 2008). The present study using highly subtype-selective ER agonists clearly demonstrate that activation of the ER alpha and the ER beta results in a reduction of adipose tissue mass. The visceral body fat mass was significantly lower and the adipocyte size was significantly smaller in E2-, Alpha-, and Beta-treated animals in comparison to untreated OVX HF animals (Fig. 4A,B). This result suggests a beneficial role of E2 preventing metabolic abnormalities because of the existing link between large increases of adipose tissue and impaired glucose tolerance and insulin sensitivity (Chan and Woo, 2010; Heine et al., 2000).

A further indication that body fat mass was affected by E2, Alpha, and Beta comes from data concerning Leptin modulation. Leptin, a hormone almost exclusively secreted by adipocytes, circulates in the blood directly in proportion to the amount of adipose tissue (Friedman, 2002; Maffei et al., 1995). Thus, leptin serves as an indicator of body energy storage. The hormone acts on specific regions of the brain (primarily the hypothalamus) to reduce food intake and to enhance energy expenditure by modulating the expression of several neuropeptides (Barsh and Schwartz, 2002; Rosen and Spiegelman, 2006; Schwartz, 2001; Schwartz, 2006; Schwartz et al., 1996). Additionally, various reports have described anti-hyperglycaemic actions of leptin through improvement of insulin sensitivity in muscles and liver as well as the ability of leptin to prevent the accumulation of lipids in non-adipose tissues (Kamohara et al., 1997; Minokoshi et al., 2002). Leptin data presented in this study confirm the well-documented correlation between body fat mass and circulating leptin levels in the blood. The E2-, Alpha- and Beta-treated animals as well as the SHAM-treated animals, which showed significantly lower visceral fat mass than the

untreated OVX HF animals also exhibited significantly lower leptin levels (Fig 4C). Different studies have shown that obesity is accompanied by high serum leptin levels, which is generally attributed to central leptin insensitivity (Furuhata et al., 2000; Morrison, 2008; Morton et al., 2006).

In the study presented here, the Beta-treated animal group shows a discrepancy between the body weights on the one hand and the visceral body fat content and the serum leptin level on the other. Similar to the E2- and Alpha-treated groups the Beta-treated animals had relative low visceral body fat and serum leptin, but a relative high body weight comparable to untreated OVX and Gen-treated animals (Fig. 3C, 4). An important role of the ER beta for skeletal muscle growth and regeneration was established in our laboratory using subtype selective agonists or βERKO mice in a model of toxin induced skeletal muscle injury. ER beta-selective agonist accelerated the regeneration of damaged skeletal muscles in female rats and revealed anabolic potency in a classical Hershberger assay using healthy male Wistar rats (Velders, 2011). Based on these observations, it can be speculated that treatment with the selective ER beta agonist may also increase the skeletal muscle mass in female Wistar rats in a model of nutrition-induced obesity. The mRNA data of Igf1 and Pax7 in the soleus muscle, presented in this study, support this hypothesis, since both genes were significantly modulated through the substitution of OVX HF animals with E2, Beta, and partly by Gen but not by Alpha compared to untreated OVX HF animals (Fig. 6). Pax7, a paired-box transcription factor, is specifically expressed in satellite cells and is essential for satellite cell function. Muscle satellite cells are myogenic progenitors in adult skeletal muscle and are responsible for growth, repair and maintenance of skeletal muscle (Chen and Goldhamer, 2003; Hawke and Garry, 2001; Kuang et al., 2006). Igf1 as a potent anabolic marker is involved in the regulation of muscle growth via activation of satellite cells and stimulation of protein synthesis (Barton-Davis et al., 1999; Mavalli et al., 2010; Philippou et al., 2007; Svensson et al., 2010). Recent studies have shown that E2 substitution of gonadectomized rodents enhanced skeletal muscle regeneration and maintenance by increasing the number of total, activated, and proliferating satellite cells and by affecting the Igf1 signalling cascade (Enns and Tiidus, 2008; Svensson et al., 2010; Tiidus et al., 2005). The results obtained in this study suggest that signaling through ER beta is responsible for these effects and seems to be the reason for the larger soleus muscle fiber sizes in Beta- and E2-treated rats compared with untreated OVX animals (Fig. 7). This findings strongly indicate that skeletal muscle mass, at least in the soleus muscle, is enhanced in Beta- and E2-treated groups compared to untreated OVX animals. The slow-twitch fiber type soleus, rich in mitochondria, and located in the back part of the lower leg generates energy (ATP) predominantly by means of oxidative metabolism, whereby besides glucose mainly fatty

acids serve as substrates (Baker et al., 2010; Delp and Duan, 1996; Janovska et al., 2010; Scott et al., 2001). Previous studies have shown that in oxidative muscles the insulin binding capacity, the kinase activity of the insulin receptor, the content of glucose transporter 4, and the insulin-stimulated glucose uptake is higher than in glycolytic muscles (Bonen and Tan, 1981; James et al., 1986; Kern et al., 1990). Against this background, the present results suggest that the ER beta improves energy homeostasis in the soleus muscle through enhanced utilization of glucose and lipids.

One important risk factor for the development of cardiovascular diseases is an unfavourable change in the lipoprotein profile. Hypercholesteremia coupled with low HDL and high LDL and VLDL levels are well documented. Obesity, type 2 diabetes mellitus, insulin resistance, but also the loss of ovarian function after the onset of post-menopause is associated with this kind of dyslipidemia (Baxter et al., 2003; Bierman, 1992; Chan and Woo, 2010; Kushwaha et al., 1990; Sbarouni et al., 1998; Schaefer, 2002; van Beek et al., 1999). Therefore, serum lipid profiles were determined. Figure 5 shows that ovariectomy raises the concentrations of TC, HDL, LDL, and VLDL independent from different diets compared to SHAM animals. Treatment with E2 and ER alpha selective agonist led to a significant reduction of TC and VLDL in comparison to OVX HF rats. The same animal groups also showed a slight decrease of LDL, whereas HDL levels were not altered. Thus, the LDL/HDL ratio as well as the (VLDL+LDL)/HDL ratio is more favourable concerning the development of cardiovascular diseases in the SHAM-, E2- and Alpha-treated groups compared to untreated OVX animals. This beneficial impact of E2 on blood lipids is in line with other studies and explains the relative cardiovascular protection of premenopausal women in comparison to women after menopause and men (Dubey et al., 2005; Ling et al., 2006). This study demonstrates that the positive effect of E2 is mediated via ER alpha and agrees with the observation that the liver, as the most important tissue in the modulation of blood lipid composition, primarily expresses the ER alpha (Matthews and Gustafsson, 2003; Taylor and Al-Azzawi, 2000).

Taken together, the results clearly show that E2, Alpha and Beta significantly modulate various metabolic parameters investigated in this study. In contrast, the phytoestrogen Gen, orally administrated, only showed an effect on Igf1 and Pax7 mRNA expression in the soleus muscle (Fig. 6). It has been reported that the isoflavone Gen decreases food intake, body weight and adipose tissue mass (Kim et al., 2006; Lee et al., 2006; Michael McClain et al., 2006; Naaz et al., 2003; Penza et al., 2006; Yang et al., 2006). However, the administrated doses of Gen (subcutaneous and/or dietary) in these animal experiments exceeded the dose used in our study. The rats in our study received Gen in the range from 60 to 25 mg/kg b. wt per day (changes in intake must be attributed to an increase of body weight with a decrease of food intake during the whole

experimental period, observed among all animal groups). Such high dosages can only be obtained with dietary supplements but not with dietary uptake in humans (Reagan-Shaw et al., 2008). A human equivalent dose can be reached by a daily dose which is two to four times higher than the maximum dose commonly used for postmenopausal treatment (Andres and Lampen, 2012; Hooper et al., 2010; Mahady, 2005; Messina and Wood, 2008; Steinberg et al., 2011; Wei et al., 2012). The data indicate that such a dose has no influence on energy intake, body weight, visceral fat mass or adipocyte size (Fig. 3 and 4A,B). This interpretation is in agreement with other studies using similar Gen doses (Kim et al., 2006; Naaz et al., 2003; Penza et al., 2006). However, Gen-treated animals display a similar mRNA modulation of Igf1 and Pax7 as compared with Beta and E2 treatment (Fig. 6). Therefore, based on the knowledge that Gen has a pronounced binding affinity to ER beta (Kuiper et al., 1997), its role in skeletal muscle homeostasis needs further investigations. There are also controversial reports about the effects of Gen on serum lipid levels (Kirk et al., 1998). In the study presented here, Gen treatment did not affect the serum lipid profile (Fig. 5).

In general, the HF diet, fed to animals in this study, was used to induce obesity. This approach made is possible to examine the impact of hormonal treatment on obese OVX rats. The LF diet used in the study served as control and was only fed to SHAM and untreated OVX animals. The results shows that the HF diet led to higher body weight gain, visceral fat mass, adipocyte sizes, as well as serum levels of leptin in SHAM and untreated OVX animals compared to the same groups fed with the LF diet. However, the differences were statistically not significant (except adipocyte sizes in SHAM animals). Interestingly, serum levels of TC and LDL in OVX animals on LF diet were higher than in OVX animals receiving the HF diet (statistifically significant only on LDL results). Therefore, after an experimental period of 10 weeks the loss of ovarian function resulted in effects that were more adverse regarding body weight and visceral fat mass than the fat-enriched diet.

Conclusion

In summary, our results show that E2, the ER selective agonists and Gen had no significant effect on energy uptake. The lower body weight gain observed in E2-treated as compared with untreated OVX rats can be attributed to ER alpha mediated effects. Visceral fat content, adipocyte size as well as serum leptin concentration were significantly reduced by substitution of OVX rats with E2, ER alpha and ER beta agonists. These results can be taken as a strong indication that the positive effects of E2 on

parameters involved in regulation of fat metabolisms are mediated via both the ER alpha and the ER beta. Serum lipid levels reveal that the E2 induced decrease of TC and VLDL is predominantly mediated via the ER alpha. The administration of Gen (when provided in the form and dose used in this study) to OVX HF animals has no beneficial influence on the investigated parameters in adipose tissue and blood. The soleus muscle mRNA expression of Igf1 and Pax7, genes involved in growth, repair and maintenance of skeletal muscle, were affected by treatment with E2, Beta, and partly by administration of Gen. Soleus muscle fiber cross-sectional areas were significantly larger in E2- and Beta-treated animals compared to untreated OVX animals. Thus, the ER beta seems to have the potency to increase the skeletal muscle mass which may result in an improved glucose uptake, insulin sensitivity and an enhanced utilization of glucose and lipids. Therefore, our data suggest that the ER beta has a specific role in the regulation of skeletal muscle homeostasis whereas ER alpha may dominate in regulating effects in adipose tissue. To acquire deeper knowledge about the underlying molecular mechanisms of estrogenic effects on metabolic pathways, future mechanistic studies in adipose tissue but also in the liver, pancreatic tissue and skeletal muscle are warranted.

Acknowledgments

The authors thank Sebastian Gehlert (German Sports University, Germany) for providing the anti-Laminin antibody and helpful tips for its use and Lena Andersch for technical assistance.

Chapter Three

Molecular effects of ER alpha- and beta-selective agonists on regulation of energy homeostasis in obese female wistar rats

This study has been submitted and is under the review as:

Carmen Weigt, Torsten Hertrampf, Felix M. Kluxen, Ulrich Flenker, Frank Hülsemann, Karl Heinrich Fritzemeier, Patrick Diel.

Resubmitted to Molecular and Cellular Endocrinology in May 2012.

Abstract

The molecular mechanisms underlying the effects of selective ER subtype activation on lipogenesis, adipogenesis, lipid utilization and storage as well as glucose metabolism are currently largely unknown and were analyzed in female OVX Wistar rats on a high-fat diet. Rats received 17beta-estradiol (E2), ER subtype-selective agonists (Alpha and Beta), and genistein (Gen) for 10 weeks. In adipose tissue, E2, Alpha, and Beta significantly decreased lipogenic (SREBP-1c, FAS) and adipogenic genes (LPL, PPAR gamma). In liver and skeletal muscle of E2-, Alpha-, Beta-, and Gen-treated animals, lipogenesis and triglyceride accumulation were significantly reduced. Increased hepatic and muscular PPAR gamma mRNA expression was observed in untreated, Beta- and Gen-treated animals, which correlates with increased hepatic glucose uptake. However, only untreated animals showed impaired insulin sensitivity compared to all other groups. Therefore, PPAR gamma up-regulation in untreated animals suggests a compensatory mechanism, probably due to increased triglyceride accumulation in non-adipose tissues. Beta- and Gen-treated animals may benefit from the anabolic potency of ER beta that ameliorates lipid and glucose utilization in muscle. Activation of either ER subtype reduces fat enrichment and improves systemic insulin sensitivity. Depending on the investigated tissue, different molecular pathways seem to be involved.

Introduction

Obesity, defined as excessive fat accumulation, is one of the major public health challenges of our time. Between 1980 and 2008, its prevalence has nearly doubled worldwide, with tripling rates being observed in many European countries. In 2008, 1.5 billion adults were overweight (BMI greater than 25 kg/m^2) worldwide. Of those affected more than 200 million men and around 300 million women can be classified as obese (BMI greater than 30 kg/m^2) (WHO, 2012). In addition, the number of overweight children increased steadily since 1990. In 2010, nearly 43 million children under five were overweight worldwide (WHO, 2010). These numbers are expected to continue to rise, particularly among children. Excessive body weight is now recognized to drastically increase the risk of developing a number of chronic diseases. These include cardiovascular diseases (mainly heart disease and stroke), T2DM, musculoskeletal disorders, dyslipidemia, hypertension, and certain forms of cancer. Consequently, obesity and its co-morbidities are responsible for constantly rising health costs and premature death. Obesity is most likely caused by combination of genetic, behavioural, and environmental factors (Chen et al., 2009; Comuzzie et al., 2001; Goulart et al., 2009). However, the most common reason for the epidemic growth of obesity rates over the last several decades is thought to be a chronic imbalance of energy homeostasis due to increased caloric intake combined with a sedentary lifestyle (Chen et al., 2009; Low et al., 2009; Newbold et al., 2009; Wasan and Looije, 2005).

Estrogens are well known to regulate energy homeostasis through the modulation of glucose and fat metabolism (Ropero et al., 2008). For example, systemic loss of endogenous estrogens in women following menopause is associated with body weight gain combined with the development of visceral obesity, insulin resistance and T2DM (Curtis and Wilson, 2005; Ley et al., 1992; Louet et al., 2004; Toth et al., 2000a). Numerous clinical studies investigating the effect of HRT on postmenopausal women have shown a decrease of central obesity, lower incidence of T2DM, increased sensitivity towards glucose and insulin, and an improvement in lipid metabolism (Andersson et al.,

1997; Curtis and Wilson, 2005; Kanaya et al., 2003; Pentti et al., 2009; Samaras et al., 1999; Santen et al., 2010). Similar observations have been made in several animal experiments. Ovariectomy in rodents leads to body weight gain and the development of obesity, whereas E2 supplementation antagonized these effects (Hertrampf et al., 2006a; Hertrampf et al., 2008b; Naaz et al., 2002; Zoth et al., 2010).

Estrogens exerts their physiological effects via two ER subtypes, ER alpha and ER beta. How these subtypes, especially ER alpha, affect metabolism pathways has been intensively investigated in the last decade. The suppressive role of ER alpha on body weight gain has been convincingly shown in a number of studies (Bryzgalova et al., 2006; Heine et al., 2000; Hertrampf et al., 2007; Hertrampf et al., 2008a; Hertrampf et al., 2008b). The influence of ER alpha and ER beta on energy intake is still a matter of debate. Both the activation of ER alpha (Naaz et al., 2002) and the activation of ER beta (Liang et al., 2002) have been described to reduce food intake.

Regarding glucose metabolism, the loss of ER alpha was shown to impair systemic glucose tolerance and insulin sensitivity in mice (Barros et al., 2009; Barros et al., 2006; Bryzgalova et al., 2006; Heine et al., 2000). By contrast, in ER beta-deficient mice glucose tolerance was similar to their wild type counterparts (Barros et al., 2009; Barros et al., 2006; Bryzgalova et al., 2006). Conversely, the activation of ER alpha using the ER alpha selective agonist PPT (propyl pyrazole triol) improved glucose tolerance and insulin sensitivity in mice compared to vehicle-treated animals (Lundholm et al., 2008). The role of ER beta was not investigated in this study. In skeletal muscle and white adipose tissue, ER alpha deficiency was shown to reduce GLUT4 expression when compared to wild types, while the loss of ER beta revealed the opposite effect, suggesting a pro-diabetic effect of ER beta (Barros et al., 2009; Barros et al., 2006). Furthermore, ER alpha-deficient mice displayed also hepatic insulin resistance compared to wild types that was associated with increased expression of lipogenic genes in liver (Bryzgalova et al., 2006). The role of ER beta in hepatic lipogenesis was not investigated in this study. Moreover, in comparison to wild type mice, the loss of ER alpha in mice of both sexes was described to increase white adipose tissue mass (Heine et al., 2000). Another study using the stable ER alpha-transfected 3T3-L1 pre-/adipocyte cell line displayed reduced lipoprotein lipase mRNA and decreased TG accumulation (Homma et al., 2000). These results suggest an important role of ER alpha in adipose tissue biology, but again, the impact of ER beta was not investigated in these two studies. ER beta has been shown to reduce PPAR gamma expression in adipocytes (Foryst-Ludwig et al., 2008). Overexpression of ER beta, but not ER alpha, resulted in a decreased PPAR gamma transcriptional activity and inhibited adipocyte differentiation in 3T3-L1 preadipocytes. A subsequent in vivo experiment revealed that ER beta deficiency increased PPAR gamma activity in adipose tissue and

led to increased body weight and fat mass in the presence of improved insulin sensitivity compared to wild type mice (Foryst-Ludwig et al., 2008). Taken together, activation of ER alpha has been shown to improve glucose and lipid metabolism, while the role of ER beta on energy homeostasis needs clarification.

Recently, the impact of the ER subtype-selective agonists, 16alpha-LE2 (Alpha) and 8beta-VE2 (Beta), and the phytoestrogen genistein (Gen) on energy homeostasis in a rat model of nutrition-induced obesity was investigated. The results clearly demonstrated that neither Alpha nor Beta affected food uptake. Treatment with Alpha reduced the body weight gain, visceral fat mass, adipocyte size, and serum levels of leptin and lipids compared to untreated rats. Beta treatment did not decrease blood lipids or body weight but reduced fat mass and adipocyte size. In addition, selective activation of ER beta increased skeletal muscle mass (Weigt et al., 2011) which could explain the higher body weight of these animals. By contrast, application of Gen, orally administered in a daily dose, which was two to four times higher than an equivalent dose commonly used for postmenopausal treatment (Andres and Lampen, 2012; Hooper et al., 2010; Messina and Wood, 2008; Steinberg et al., 2011; Wei et al., 2012), had no significant influence on the investigated parameters in adipose tissue or blood. However, it may affect skeletal muscle homeostasis similar to the ER beta subtype-selective agonist (Table 2) (Weigt et al., 2012).

The aim of the current study was to further investigate the underlying molecular mechanisms behind the effects of ER subtype-selective agonists and Gen on energy homeostasis. Therefore, the impact of Alpha-, Beta- and Gen-treatment on the expression of genes involved in the regulation of glucose and fatty acid metabolism in adipose tissue (SREBP-1c, FAS, LPL, and PPAR gamma), liver (SREBP-1c, FAS, PPAR alpha, and PPAR gamma), and soleus muscle (SREBP-1c, FAS, PPAR alpha, PPAR gamma, and PPAR delta) was investigated. TG content in muscle and liver was determined. Furthermore, to investigate glucose tolerance in the animals, the systemic insulin level and the hepatic glucose uptake by using isotope-ratio mass spectrometry (IRMS) were measured.

Table 2. Overview of the results of the previous and present study conducted in female OVX Wistar rats on a high fat diet and treated with ER subtype-selective agonists and genistein in comparison to untreated OVX animals. ↓ decrease, ↑ increase, ⇔ no influence, ↘ tendency towards decrease, ? not determined.

Tissue / Fluid	physiological action/effect	Impact of ER alpha selective agonist (16α-LE2) on OVX Wistar rats	Impact of ER beta selective agonist (8β-VE2) on OVX Wistar rats	Impact of Genistein (nutrativ) (25–60mg/kg/day) on OVX Wistar rats	published in
Uterus	**uterine wet weight**	↓	⇔	⇔	Weigt et al. 2012
CNS	**energy intake**	⇔	⇔	⇔	Weigt et al. 2012
Visceral fat	**fat mass**	↓	↓	⇔	Weigt et al. 2012
	adipocyte size	↓	↓	⇔	Weigt et al. 2012
	lipogenesis	↓	↓	⇔	in this paper
	(mRNA expression of SREBP-1c. FAS, and ACC-1)				
	adipogenesis	↓	↓	⇔	in this paper
	(mRNA expression of PPAR gamma and LPL)				
Liver	**lipogenesis**	↓	↓	↓	in this paper
	(mRNA expression of SREBP-1c and FAS)				
	fat oxidation	⇔	⇔	⇔	in this paper
	(mRNA expression of PPAR alpha)				
	TG accumulation	↓	↓	↘	
	glucose uptake	↓	⇔	⇔	in this paper
	insulin sensitivity and lipid accumulation	↓	⇔	⇔	
	(mRNA expression of PPAR gamma)				in this paper
Skeletal Muscle (Soleus)	**soleus muscle fiber size**	?	↑	?	Weigt et al. 2012
	skeletal muscle growth and maintenance	⇔	↓	⇔	Weigt et al. 2012
	(mRNA expression of PAX7 and IGF-1)				
	lipogenesis	↓	↓	↓	in this paper
	(mRNA expression of SREBP-1c and FAS)				
	fat oxidation	⇔	⇔	⇔	in this paper
	(mRNA expression of PPAR alpha and PPAR delta)				
	TG accumulation	↘	↘	↘	in this paper
	insulin sensitivity and lipid accumulation	↓	⇔	⇔	in this paper
	(mRNA expression of PPAR gamma)				
Serum	**leptin**	↓	↓	⇔	Weigt et al. 2012
	insulin	↘	↘	↘	in this paper
	triglycerides	⇔	⇔	⇔	Weigt et al. 2012
	TC	↓	⇔	⇔	Weigt et al. 2012
	HDL-C	⇔	⇔	⇔	Weigt et al. 2012
	LDL-C	⇔	⇔	⇔	Weigt et al. 2012
	VLDL-C	↓	⇔	⇔	Weigt et al. 2012

Materials and methods

Animals

Juvenile female Wistar rats (6-weeks old, 120–150g) were obtained from Janvier (Janvier, Le Genest St Isle, France) and kept at constant room temperature (20 °C ± 1), relative humidity (50–80%) and illumination (12 h light/dark cycles). The animals were housed 3–4 in each cage, with food (SSniff GmbH, Soest, Germany) and water provided ad libitum. All animal procedures were approved by the Committee on Animal Care and compliant with accepted veterinary medical practice.

Animal diet

Two different diets were used. First, a phytoestrogen-free LF diet (ssniff® EF D12450B* (I) mod. *LS** containing a metabolizable energy of 15.0 MJ/kg) was used that contains soy oil as the sole source of fat (2.5%). Thus, this diet is rich in unsaturated fatty acids (approx. 25 % C18:1 n9, > 50 % 18:2 n6) and served as control diet. As a second diet, a phytoestrogen-free HF diet (ssniff® EF R/M acc. D12451 (I) mod.* containing a metabolizable energy of 19.2 MJ/kg) was used. The main source of fat (20.5 %) in this diet originates from animals (lard) and only 5 % from soy oil (to cover the needs of essential fatty acids), resulting in a high level of saturated fatty acids. The HF diet was designed to induce obesity and secondary diseases. The protein source in both diets was casein (detailed composition of diets was described before (Weigt et al., 2012)). A portion of the HF diet was enriched with genistein (700 mg/kg diet), enabling an oral route of exposure. Gen was obtained from LC Laboratories (Woburn, MA 01801 USA). Ssniff Spezialdiäten GmbH, Soest, Germany, provided the food enrichment with Gen.

Animal treatment

Prior to experimental exposure procedures, all animals were ovariectomized (OVX) or SHAM operated (SHAM) via the dorsal route. Following 2 weeks of regeneration and endogenous hormonal decline the animals were randomly allocated to different treatment and diet groups (n=7, animal grouping was described before (Weigt et al., 2012)). All SHAM animals and a subset of OVX animals received either the LF diet or the HF diet. The remaining OVX animals were fed with the HF diet and treated with E2, Alpha (the ER alpha-selective agonist 16alpha-LE2 (3,17-dihydroxy-19-nor-17alpha-pregna-1,3,5(10)-triene-21,16alpha-lactone)), Beta (the ER beta-selective agonist 8beta-VE2 (8-vinylestra-1,3,5(10)-triene-3,17beta-diol)) or Gen. E2 (4 µg/kg b.wt per day), Alpha (10 µg/kg b.wt per day), and Beta (100 µg/kg b.wt per day) were applied via ALZET® osmotic mini

pumps, whereby the substances were dissolved in dimethylsulfoxide and water according to the manufacturer's instructions for filling. Gen was administered via Gen-enriched food (about 42 mg/kg b.wt per day. The treatment doses of the respective substances were chosen based on previous experiments (Diel et al., 2004; Hegele-Hartung et al., 2004; Hertrampf et al., 2009a; Hertrampf et al., 2005; Hertrampf et al., 2008b). E2 was provided by Sigma-Aldrich (Deisenhofen, Germany) and the ER subtype-selective substances Alpha and Beta were obtained from Bayer Schering Pharma AG, Berlin. These two steroidal and highly subtype-selective agonists Alpha and Beta were synthesized to specifically fit differences in the three-dimensional structure of the ligand-binding domain of the two ERs. *In vitro* studies using radio-ligand competition assays showed for 16alpha-VE2 a 70-fold higher affinity to ER alpha compared to ER beta (tested for rERs and hERs). 8beta-VE2 binds preferentially to ER beta (93-fold preference for rER beta and 180-fold preference for hER beta). The estrogenic potency was also documented in transactivation experiments, where 16alpha-LE2 exhibited a 250-fold ER alpha selectivity and 8beta-VE2 183-fold ER beta selectivity. Subsequent animal experiments in female Wistar rats confirmed the selective stimulation of either ER alpha or ER beta. More detailed information about the structure-based design, the synthesis strategy, and the characterization of both ligands (*in vitro* and *in vivo)* can be found elsewhere (Hillisch et al., 2004). Selectivity of this two ER subtype agonists was also shown by the analysis of the uterine wet weights in our uterotrophic assay (Weigt et al., 2012). According to earlier studies, only the ER alpha agonist mediated uterine growth, while the ER beta agonist did not (Hertrampf et al., 2007; Hertrampf et al., 2008a). Throughout the treatment period, body weight and food intakes were monitored once a week. On the day before section, the food was removed at 8 p.m. for an overnight fast.

After 10 weeks of treatment, the animals were sacrificed by decapitation after light anaesthesia with CO_2 inhalation and blood was collected. Periovarian fat pads, liver, and soleus muscle were collected and directly frozen in liquid nitrogen for analysis of molecular markers. Tissue wet weights, energy intake, histological analysis of the soleus muscle, as well as serum levels of leptin and lipids were investigated and published before (Weigt et al., 2012).

Real-time RT-PCR experiments

Total RNA was extracted from frozen tissues (liver, periovarian fat, soleus muscle) using the standard TRIzol® method (Life Technologies GmbH, Darmstadt, Germany). RNA was quantified by spectrophotometry (NanoDrop™ 1000, Thermo Scientific, Wilmington, DE 19810, USA) and cDNA was synthesized from 1 µg RNA using a Reverse Transcription System (QuantiTect®, Qiagen). Real-time RT-PCR was performed with Taq DNA

polymerase (Life Technologies GmbH, Darmstadt, Germany) in the presence of a fluorescent dye (SYBR Green, BioRad) on an Mx3005P™ qPCR System (Stratagene). All reactions were run in triplicate in a total volume of 50 µl. The PCR program was as follows: 95 °C for 3 min for 1 cycle, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C (60°C for amplification of ER alpha and ER beta mRNA), 30 s at 72 °C, and 1 cycle at 72 °C for 1 min. Fluorescence was quantified during the annealing step and product formation was confirmed by melting curve analysis. Relative mRNA amounts of target genes were calculated after normalization to an endogenous reference gene (cytochrom c oxidase, subunit 1A) following the delta delta C_T method (Pfaffl, 2001) with exception of ER alpha and ER beta mRNA amplification. Here, the PCR products were separated on 2 % agarose gels and visualized by ethidium bromide staining under UV illumination. Specific primer pairs were published before (Höfer et al., 2009; Möller et al., 2009) or designed based on the genomic sequences available at the UCSC Genome Bioinformatics database using the software Primer3 (Rozen and Skaletsky, 2000) and confirmed by the sequences in the NCBI database (http://www.ncbi.nlm.nih.gov). The primers were synthesized by Life technologies GmBH (Darmstadt, Germany) and are depicted in Table 3. The metabolic role of the analyzed genes involved in lipid and glucose metabolism is described in the supplementary material Table 4.

Table 3. Primers used in Real-time RT-PCR experiments.

Gene	Direction	Primer sequenz (5' - 3')	published in
1A	fwd	CGTCACAGCCCATGCATTCG	Höfer et al., 2009
	rev	CTGTTCATCCTGTTCCAGCTC	
SREBP-1c	fwd	GTACCTGCGGGACAGCTTAG	
	rev	CAGGTCATGTTGGAAACCAC	
FAS	fwd	GGCTAGAGACCTTGGCACTG	
	rev	TAGCCCTCTGCTCTGGTCAC	
LPL	fwd	TGCCATGACAAGTCTCTGAAG	
	rev	TGCTATCGGCCATTAGGTTC	
PPAR alpha	fwd	GGCTGCTATAATTTGCTGTGG	
	rev	TTCTTGATGACCTGCACGAG	
PPAR gamma	fwd	GCCCTTTGGTGACTTTATGG	
	rev	CATCTTCTGGAGCACCTTGG	
PPAR delta	fwd	TCATTGAGCCCAAGTTCGAG	
	rev	GGAAGAGGTACTGGCTGTCG	
ER alpha	fwd	GGAAGCACAAGCGTCAGAGAGAT	Möller et al., 2009
	rev	AGACCAGACCAATCATCAGGAT	
ER beta	fwd	CTACAGAGAGATGGTCAAAAGTGGA	Möller et al., 2009
	rev	GGGCAAGGAGACAGAAAGTAAGT	

Western Blotting

Frozen liver tissue was powdered and homogenized in lysis buffer (50 mM Tris pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 1 % Triton-X 100) containing protease inhibitor PMSF (10 mM, phenylmethanesulfonylfluoride). Protein concentration was measured by the method of Lowry using Bio-Rad (DC Protein Assay, Bio-Rad, Munich, Germany) reagents with BSA as standard. Equal amounts of sample (25 µg) were loaded onto 4–12 % Bis-Tris NuPAGE® gels (Life Technologies GmbH, Darmstadt, Germany). Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Hartenstein, Würzburg) and blocked with 5 % non-fat milk in Tris buffered saline solution containing 0.1 % Tween 20 (TBST, 100 mM; pH 7.4) at room temperature for 1 h. The immobilized proteins were detected using specific antibodies against ER beta (sc-8974, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), diluted 1:400 in TBST or FAS (ab22759, abcam, Cambridge, United Kingdom), and diluted 1:400 in TBST. As a loading control beta-actin (Millipore GmbH, Schwalbach/Ts, Germany), diluted 1:15.000 in TBST was used. Membranes were incubated with primary antibody solution overnight at 4 °C and subsequently rinsed with TBST (four times for 5 min each). As secondary antibodies a polyclonal swine anti-rabbit antibody (P0217, Dako, Glostrup, Denmark) or a polyclonal rabbit anti-mouse antibody (P0260, Dako), in each case HRP-conjugated and diluted 1:1000 in TBST, was used. After four more washing steps with TBST blot signals were visualized with the Lumi-Light Plus Western Blotting Substrate (Roche Diagnostics, Mannheim, Germany) and a FluorChem digital imaging system (Alpha Innotech, CA, USA). FAS protein bands were quantified by densitometric analysis using ImageJ 1.38 sofware (National Institute of Health, USA, http://rsb.info.nih.gov/ij/). Expression levels of the reference protein beta-actin were calculated to account for non-homogenous protein loading.

As a positive control of ER beta detection, HCEC cells (SV40 large T antigen-immortalized human colon epithelial cells) were used that were kindly provided by Prof. Pablo Steinberg of the University of Veterinary Medicine Hannover, Foundation.

Hepatic and soleus muscle TG content

Frozen tissues of liver and soleus muscle (~100 mg) were pulverized in liquid nitrogen and lysed in lysis buffer (50 mM Tris pH 8.0, 2 mM CaCl2, 80 mM NaCl, 1% Triton-X 100, 1 mM PMSF). After homogenization and incubation on ice for 30 min, the lysates were centrifuged (15 min, 4 °C, 13000 rpm) and the TG content was analyzed in the supernatants by colorimetry using ABX Pentra reagent (ABX Diagnostics Montpellier, France). In the same supernatants, the total amount of protein was measured using the Lowry method (DC Protein Assay, Bio-Rad, Munich, Germany).

Stable isotope ratio analysis

2.5 h before decapitation the rats received an oral application of ^{13}C labeled glucose (2 g/kg b.wt.), enriched to +140 ‰ $\delta^{13}C_{VPDB}$. 50 mg of the frozen liver was homogenized and dehydrated. All samples were enclosed in tin capsules and analysed by EA-IRMS using an elemental analyser (Eurovektor EA 3000, Hekatech, Wegberg, Germany) coupled to a continuous-flow isotope ratio mass spectrometer (Delta C, Finnigan, Bremen, Germany). Carbon isotope ratios are expressed in per mill relative to VPDB: $\delta^{13}C$ [‰] = $[(R_{Sample}/R_{Standard}) - 1] \cdot 10^3$ with R is the $^{13}C/^{12}C$ ratios (Coplen, 2011). Reference gases (CO_2, purity 5.3 from Linde, Munich, Germany) were calibrated using the reference materials IAEA-CH-6 and IAEA-CH-7. Within the measurements, creatine-monohydrate from one batch was used as a working standard. The standard deviation of repeated measurements of working standard or samples was 0.1 ‰. Isotope ratios were calculated using the ISODAT NT software (version 2.0, Finnigan, Bremen, Germany).

Determination of insulin, serum glucose, and calculation of the HOMA-Index

Serum was obtained by centrifugation at 4 °C and 3000 x g and stored at – 20 °C. The serum concentration of insulin was measured in duplicates using ELISA kits for rats according to the manufacturer's instruction (80-INSRTU, ALPCO Diagnostics, Salem, NH 03079, USA). Serum levels of glucose were analyzed by colorimetry using reagents from DIALAB (Wiener Neudorf, Austria). To measure the serum glucose a chemistry analyzer (Roche Hitachi Cobas Mira Plus) was used. Homeostasis Assessment Model (HOMA)–Index was calculated using the following formula: HOMA–Index = Insulin (fasting, µU/ml) x blood glucose (fasting, mg/dl) / 405.

Statistical analysis

Except IRMS data, statistical significance of differences was calculated using Kruskal–Wallis one-way analysis of variance with a subsequent Mann–Whitney U-test (SPSS Statistical Analysis System, Version 20.0).

IRMS data were analysed by means of LME models (Pinheiro, 2000). The software was R in the latest version (R Development Core Team, 2011). LME analysis required the nlme library (Pinheiro, 2011). Treatment (OVX, SHAM) and substitution, respectively, represented the fixed effects. Random effects were assumed for the different individuals. This was required because duplicate analyses were performed. The magnitude of random variation was allowed to vary between different treatment levels. The significance of this effect was investigated by means of likelihood statistics.

Statistical significance was established at $p \leq 0.05$.

Results

mRNA expression of SREBP-1c, FAS, LPL, and PPAR gamma in adipose tissue

Several genes are involved in the regulation of lipogenesis, adipocyte differentiation, and lipid storage in adipose tissue. The gene FAS encodes a multi-domain enzyme, which is important for *de-novo* lipogenesis, whereas the transcription factor SREBP-1c regulates the expression of FAS in response to feeding and insulin (Horton et al., 2002). In adipose tissue, the nuclear receptor and transcription factor PPAR gamma is highly expressed and induces fatty acid uptake, adipogenesis, and leads to lipid repartitioning into adipose tissue from skeletal muscle and liver (Anghel et al., 2007; Vacca et al., 2011). Further, LPL as a target gene of PPAR gamma promotes TG formation in adipocytes through uptake of circulating lipids (Wong and Schotz, 2002). Substitution of OVX HF rats with E2, Alpha or Beta significantly decreased the expression of SREBP-1c, FAS (Fig. 8A and B), PPAR gamma, and LPL (Fig. 9A and B) as compared with untreated OVX HF animals. The administration of Gen did not show a significant effect on these investigated genes. While animals on HF diet showed a general decrease in the mRNA expression of FAS and LPL compared to animals on LF diet this effect was not observed regarding SREBP-1c and PPAR gamma expression.

mRNA expression of SREBP-1c, FAS, and PPARs in liver and soleus muscle

E2, ER subtype-selective agonists, and genistein also affected the expression of SREBP-1c and FAS in liver and soleus muscle. In agreement to the observed results in the adipose tissue, ovariectomy led to a general increase of SREBP-1c mRNA expression compared to SHAM animals in both tissues. Treatment with E2, Alpha, and Beta markedly antagonized the OVX induced increase of SREBP-1c mRNA (Fig. 8C and E). The down-regulation of SREBP-1c mRNA was again associated with a decreased mRNA expression of its target gene FAS in the appropriate animal groups (Fig. 8D and F). Interestingly, the expression of both lipogenic genes was also reduced by administration of Gen, although the effect on the SREBP-1c level in the soleus muscle was statistically not significant. Similar to expression in adipose tissue, FAS mRNA expression in liver was lower in animals on HF diet compared to rats on LF diet. Conversely, mRNA expression of FAS in muscle and SREBP-1c in both tissues was higher in animals fed with the HF diet than with the LF diet.

The mRNA expression of PPAR alpha in both tissues and PPAR delta in the soleus muscle were measured as markers for fatty acid utilization. In contrast to the lipogenic genes SREBP-1c and FAS, no significant changes on hepatic and muscular mRNA expression of PPAR alpha and PPAR delta were detected (data not shown).

Because of the improved insulin sensitivity by PPAR gamma activation, indicated by a reduced gluconeogenesis in the liver and an increased glucose uptake into the muscle (Vacca et al., 2011), the mRNA expression of PPAR gamma in liver and soleus muscle was measured. In both tissues, untreated OVX rats on the LF as well as the HF diet showed significantly higher mRNA levels of PPAR gamma than the appropriate SHAM groups. Application of E2 and Alpha to OVX HF rats antagonized this OVX effect. Treatment with Beta and Gen had no significant influence on the mRNA level of PPAR gamma as compared to OVX HF animals. Long-term exposure to the fat-enriched diet had an additive effect on PPAR gamma expression in the liver of SHAM and untreated OVX animals compared to SHAM and OVX animals on LF diet (Fig 9C). In the soleus muscle, the additive effect of the HF diet was only observed in SHAM animals, whereas untreated OVX animals on the HF diet revealed significantly lower PPAR gamma mRNA levels than OVX animals on the LF diet (Fig. 9E).

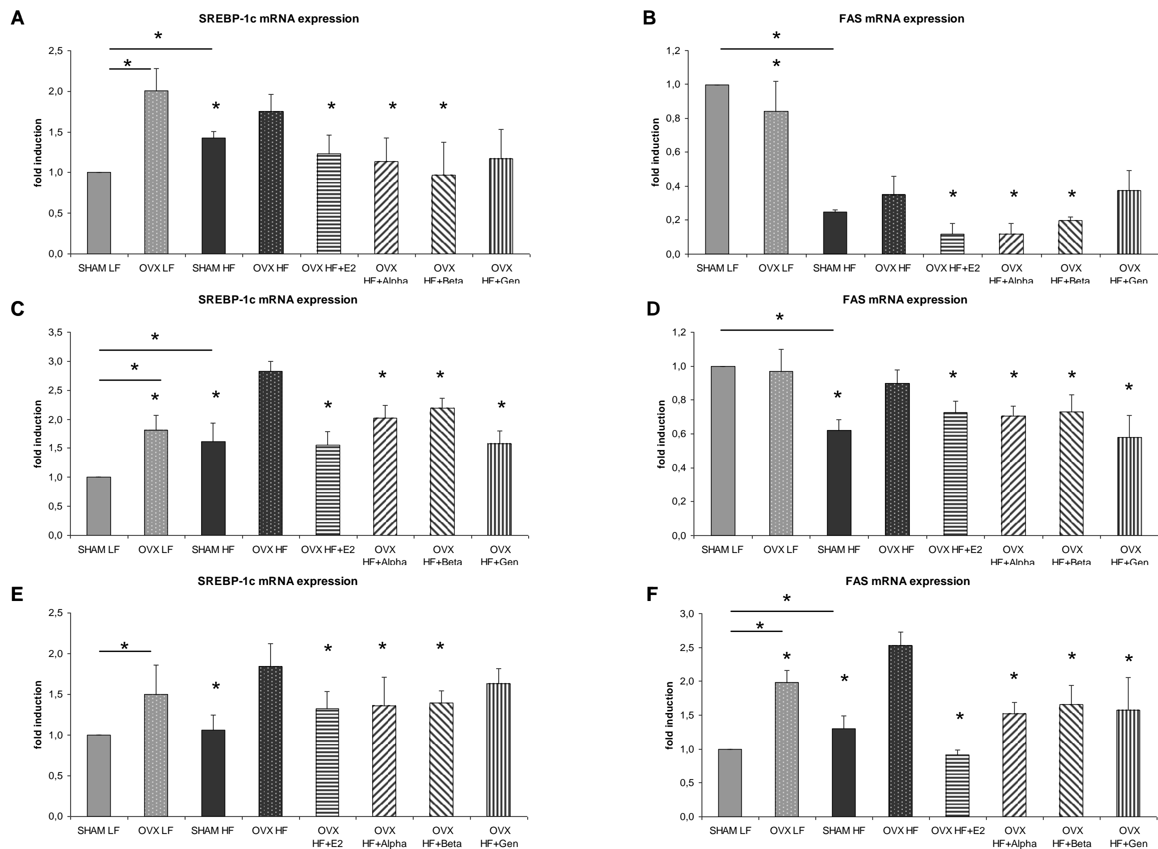


Fig. 8. Effects of E2, ER subtype-selective agonists and Gen on the relative mRNA expression of SREBP-1c and FAS in (A and B) adipose tissue, (C and D) liver, and (E and F) soleus muscle after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD for n=7. asterisk (*) marks values significantly different from OVX HF or as indicated by lines. Statistical significance was established at $p \leq 0.05$.

Stable isotope ratio analysis in the liver

To measure the glucose tolerance of the liver in the animals on a HF diet, IRMS was performed. Relative to SHAM animals, ovariectomy resulted in a significant increase of the mean $\delta^{13}C$. In comparison to untreated OVX animals, treatment with E2 or Alpha resulted in significant ^{13}C depletion. Gen treatment resulted in noteworthy depletion, but this effect was not statistically significant (p=0.083). The effect of Beta treatment was hardly detectable and statistically not significant (Fig. 9D).

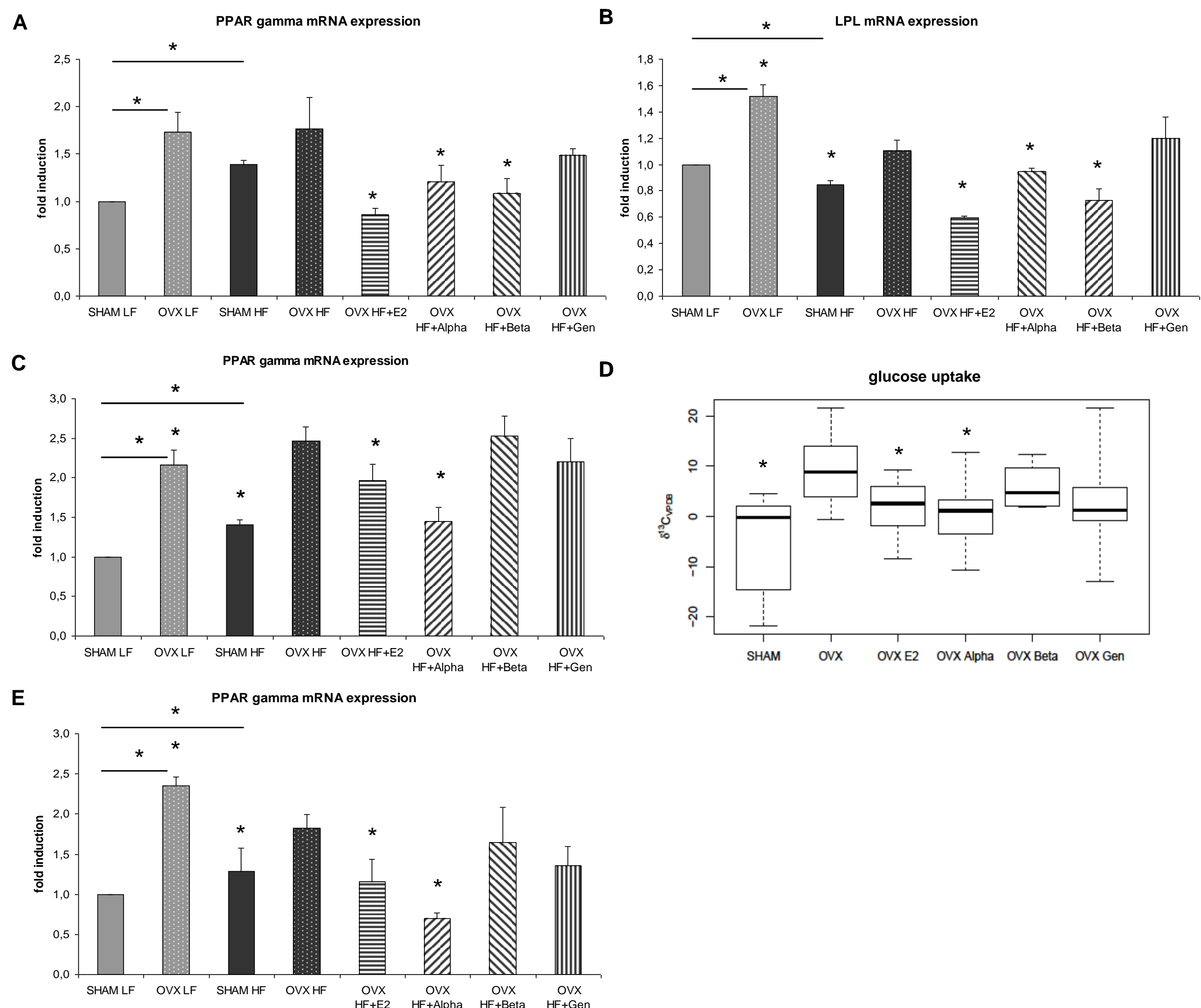


Fig. 9. Effects of E2, ER subtype-selective agonists and Gen on the relative mRNA expression of (A) PPAR gamma and (B) LPL in the adipose tissue as well as (C) PPAR gamma expression in liver, and (E) muscle after 10 weeks of treatment. (D) Effects on hepatic glucose uptake in animals on HF diet are shown.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, LF = low fat diet, HF = high fat diet. Data shown are means ± SD in the case of mRNA expression levels. Boxplots (E) are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution (n=7). Asterisk (*) marks values significantly different from OVX HF or as indicated by lines. Statistical significance was established at $p \leq 0.05$.

mRNA expression of ER alpha and ER beta in liver, adipose tissue, and soleus muscle

To investigate ER subtype expression in liver, adipose tissue, and soleus muscle of the animals, RT-PCRs were performed. Specific amplicons for ER alpha (382 bp) and ER beta (215 bp) were detected in all three tissues and in all animal groups (Fig. 10A, C, and D, depicted are representative results of ER alpha and ER beta mRNA expression (untreated OVX and E2-treated OVX animals in each case). The mRNA expression of ER beta was lower in all three tissues when compared to ER alpha expression (indicated by weaker signals on the gel). Especially in the liver, the expression level of ER beta was low, but detectable in each case.

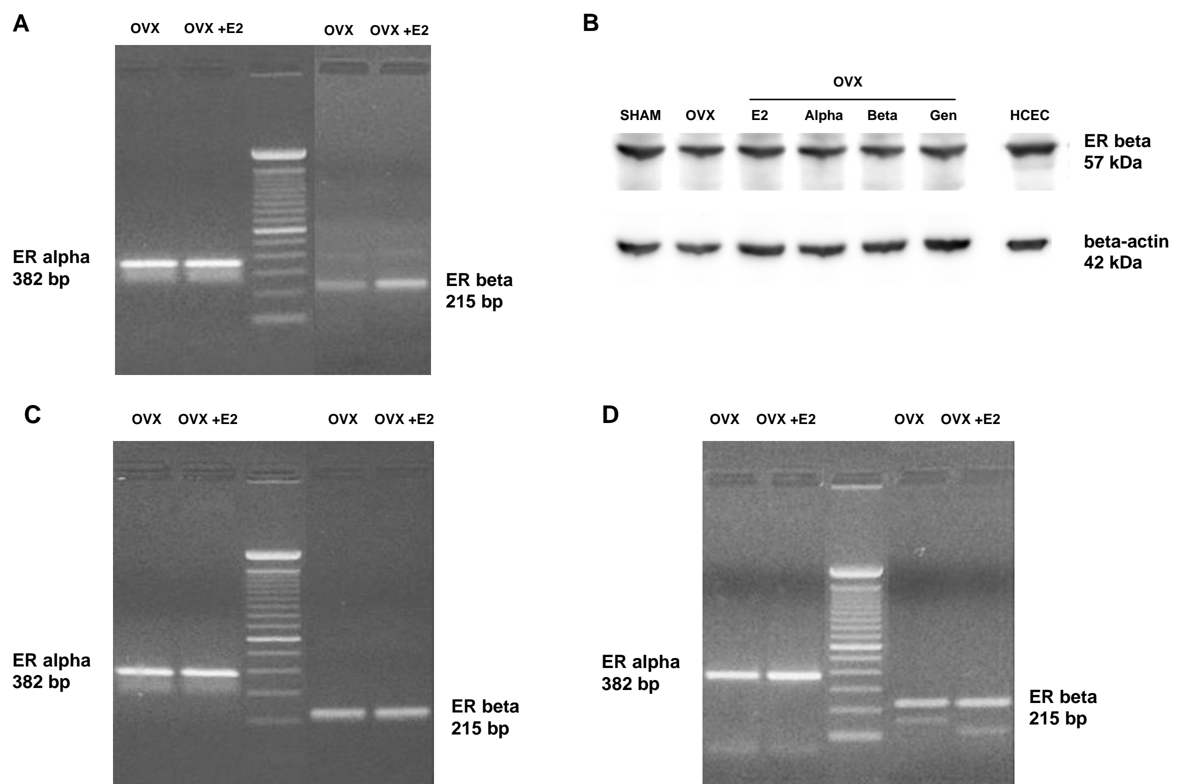


Fig. 10. mRNA expression of ER alpha and ER beta in (A) liver, (C) adipose tissue, and (D) soleus muscle. Shown are expression in untreated OVX and E2-treated animals on HF diet. (B) Protein expression of ER beta in liver is shown in all animals on HF diet (n=7).
HCEC cell line was used as a positive control for ER beta protein expression. SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, HF = high fat diet, HCEC = SV40 large T antigen-immortalized human colon epithelial cells that express ER beta.

Protein expression of FAS and ER beta in liver

Western blotting with specific antibodies against FAS and ER beta confirmed the mRNA results on the respective mRNA expression in the liver. FAS protein expression pattern was down-regulated by treatment with the estrogenic compounds compared to untreated OVX animals on HF diet (Fig. 11). Further, the expression level of ER beta was similar in all animal groups on a HF diet (Fig. 10B). The specificity of the ER beta antibody used was previously tested by using different cell lines in our laboratory (http://docserv.uni-duesseldorf.de/servlets/DocumentServlet?id=24043).

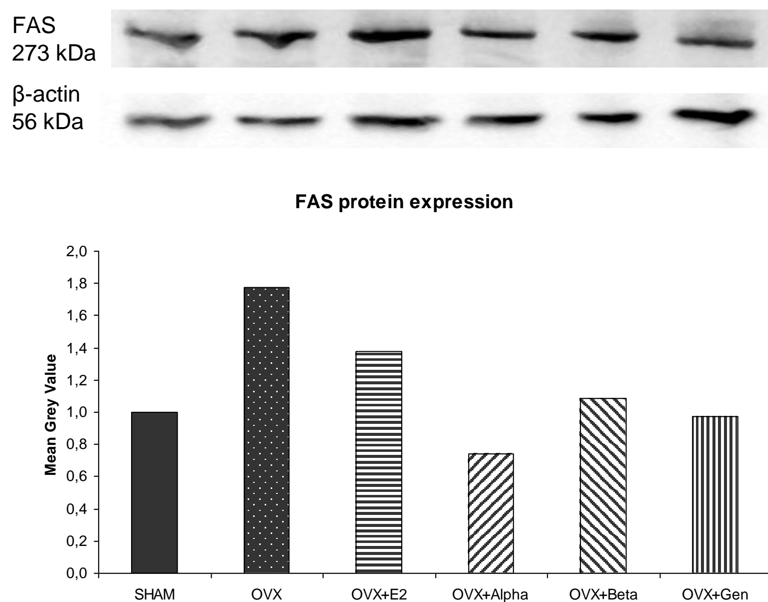


Fig. 11. Effects of E2, ER subtype-selective agonists and Gen on hepatic FAS protein expression in animals on HF diet after 10 weeks of treatment (n=7).
Shown is one representative blot from four consecutive blots with the associated densiometric analysis. SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, HF = high fat diet. Statistical significance was not established.

TG content in liver and soleus muscle

Untreated OVX rats revealed higher hepatic triglyceride values compared to the SHAM-operated groups. A combination of ovariectomy and HF diet showed the highest level of hepatic triglycerides. Treatment with E2, Alpha, and Beta significantly antagonized this increase, whereas treatment with genistein had only a small effect (Fig. 12A). In the soleus muscle, the TG content showed a similar pattern. The highest TG content was measured in untreated OVX rats on the HF diet. SHAM animals and OVX animals substituted with E2, Alpha, Beta, or Gen showed lower TG concentrations within the soleus muscles, although the differences were not significant (Fig. 12B).

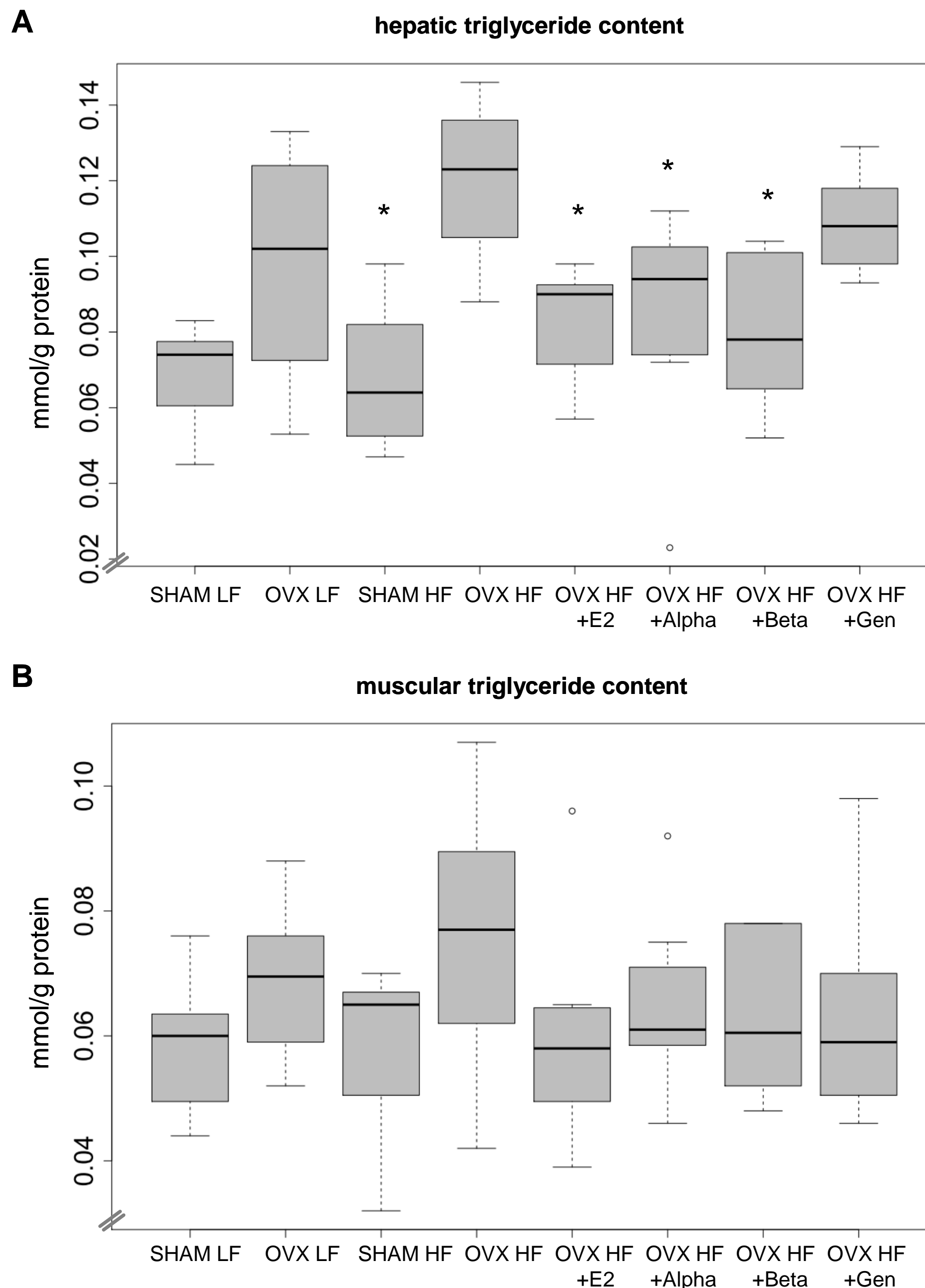


Fig. 12. Effects of E2, ER subtype-selective agonists and Gen on the TG content in (A) liver and (B) soleus muscle after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, LF = low fat diet, HF = high fat diet. Data are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution, empty circles display outliers (n=7 in liver and n=6 for OVX HF+Beta and n=7 for remaining groups in soleus muscle). Asterisk (*) marks values significantly different from OVX HF or as indicated by lines. Statistical significance was established at $p \leq 0.05$.

Serum levels of insulin and glucose as well as calculation of the HOMA-Index

Serum levels of glucose and insulin were measured as well as the HOMA-Index was calculated, because obesity is associated with an impaired systemic sensitivity to glucose and insulin. In spite of different hormonal treatments and diets, all animals were

normoglycemic after the experimental period (data not shown). In contrast, measurement of serum insulin showed the highest concentration in the untreated OVX animals (OVX LF, OVX HF), whereas in all other groups the insulin level was at least 30 % lower (Fig. 13A). The HOMA-Index, a mathematical model of the glucose-insulin feedback system, revealed also the highest values in the untreated OVX animals (Fig. 13B).

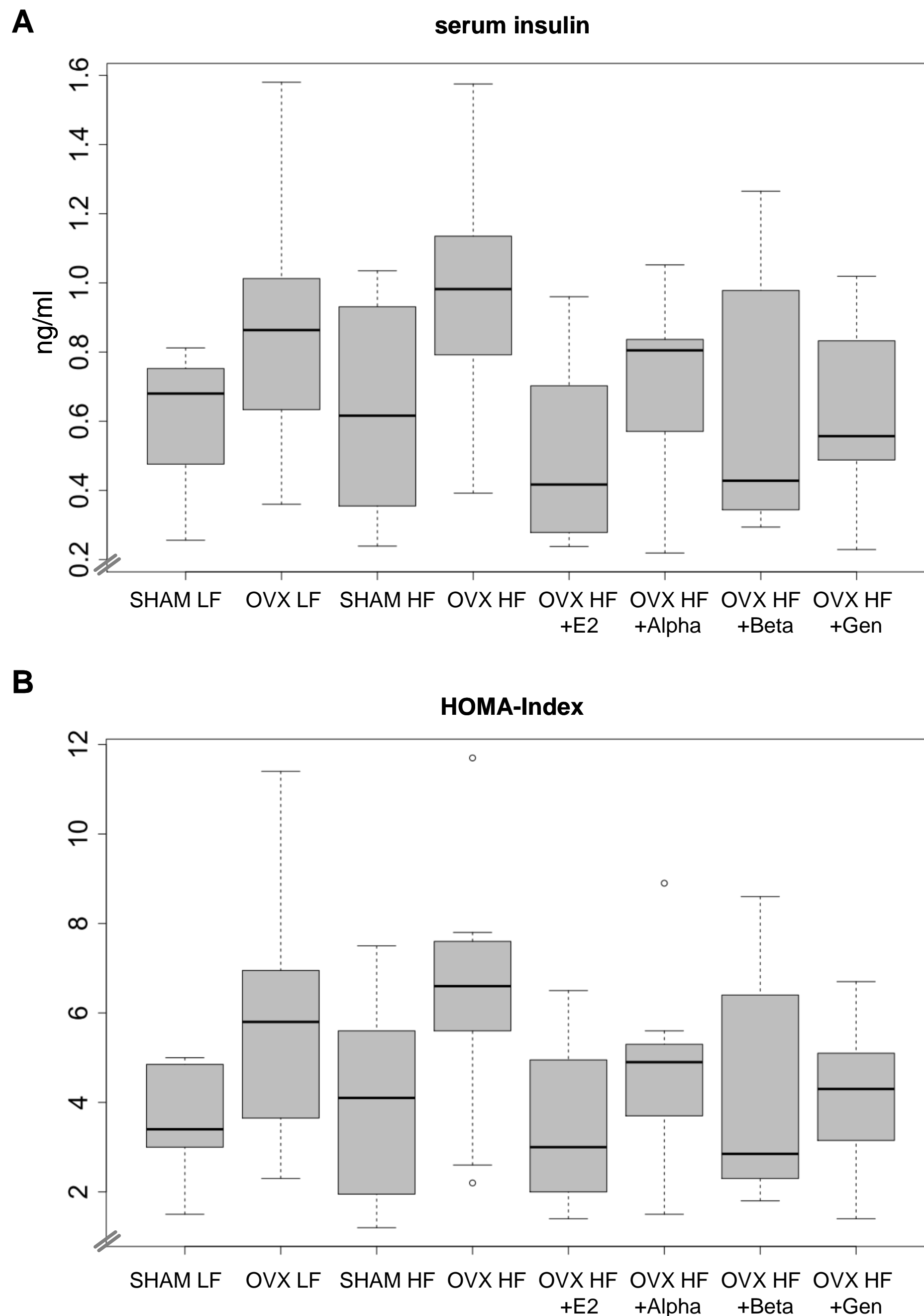


Fig. 13. Effects of E2, ER subtype-selective agonists and Gen on (A) serum level on insulin and (B) on the HOMA-Index after 10 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein, LF = low fat diet, HF = high fat diet. Data are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution for n=6 (OVX HF+Beta) and n=7 (remaining groups). Empty circles display outliers. No significant differences in serum insulin or HOMA-Index were found between the groups.

Discussion

In the present study, the molecular mechanisms of ER alpha- and ER beta-mediated effects on fat and glucose metabolism in adipose tissue, liver, and skeletal muscle were investigated. Obese OVX Wistar rats were treated with ER subtype-selective agonists. In addition, a subset of OVX animals received a diet enriched with the phytoestrogen Gen in a relatively high dosage. Here, the aim was to find out whether Gen is able to induce effects similar to those of estrogens.

Three major results are presented in this study: treatment with either ER subtype-selective agonist resulted in a decrease of lipogenesis and adipogenesis in adipose tissue. Alpha as well as Beta treatment decreased lipogenesis in liver and soleus muscle and lowered TG accumulation in both tissues. Systemic insulin sensitivity was improved by activation of ER alpha but also by ER beta, most likely because of the anabolic activity of ER beta.

Lipogenesis and adipogenesis is decreased via either ER subtype-selective agonist in adipose tissue

In adipose tissue, the expression of lipogenic (SREBP-1c and FAS) and adipogenic genes (PPAR gamma and LPL) was significantly decreased in E2-, Alpha-, and Beta-treated OVX HF animals compared to untreated OVX HF rats (Fig. 8A and B and Fig. 9A and B). These findings are in line with previous results of our group and others demonstrating that estrogens (e.g., E2) reduce the development of adiposity (Bryzgalova et al., 2008; D'Eon et al., 2005; Weigt et al., 2012; Zoth et al., 2010; Zoth et al., 2012). Further, these results are consistent with observations in postmenopausal women who tend to accumulate more fat in total than perimenopausal women do with a simultaneous alteration in fat distribution from the lower body segment to the upper body (Cooke and Naaz, 2004; Ley et al., 1992).

The previous study demonstrated that activation of either ER subtype decreases visceral fat mass, adipocyte size, and circulating leptin concentration. Interestingly, the amount of energy intake was not significantly affected by any treatment (Weigt et al., 2012). The present study shows for the first time that expression of genes associated with lipogenesis (FAS and SREBP-1c) and adipogenesis (PPAR gamma and LPL) were inhibited by treatment with both ER subtype-selective agonists. These results confirmed the observed inhibition of lipogenic genes (SREBP-1c and FAS) by treatment of OVX animals with E2, which was described before in two other animal studies (Bryzgalova et al., 2008; D'Eon et al., 2005). The role of ER alpha and ER beta in this context has been controversially discussed. Several studies have shown that either ER subtype is able to modulate fat homeostasis. As described in detail in the introduction, deficiency of ER

alpha results in increased visceral fat mass suggesting that ER alpha is critical in adipose tissue biology (Heine et al., 2000). However, an anti-lipogenic action of ER beta was demonstrated in a study conducted with βERKO mice, based on negative cross talk with PPAR gamma in adipocytes (Foryst-Ludwig et al., 2008). The expression of LPL, a target gene of PPAR gamma (Wong and Schotz, 2002), was suppressed by ER beta (Foryst-Ludwig et al., 2008) and also by ER alpha (Homma et al., 2000). Thus, taken together, both ER subtypes may be involved in adipose tissue biology (recently reviewed also by Faulds and colleagues (Faulds et al., 2012)) and by (at least partly) similar molecular mechanisms.

The higher body weight and fat content in αERKO mice compared to wild types was attributed to increased food intake and lipogenesis in adipose tissue and liver (Bryzgalova et al., 2006; Homma et al., 2000) as well as impaired insulin sensitivity and glucose tolerance in liver, skeletal muscle, and adipose tissue (Barros et al., 2009; Barros et al., 2006; Bryzgalova et al., 2006). An increased body weight and fat mass compared to wild types was also observed for βERKO mice. Here an up-regulation of PPAR gamma in the presence of improved insulin sensitivity was discussed as the relevant molecular mechanism (Foryst-Ludwig et al., 2008). In light of the results presented here, a further explanation for the increased body weight in βERKO mice could be an altered body composition. In the previous study, anabolic activity of ER beta was demonstrated. ER beta activation resulted in significantly larger soleus muscle fiber sizes (Weigt et al., 2012). The specific role of ER beta in muscle growth and repair was also shown in an animal study by using the ER subtype-selective agonists and βERKO mice in a model of toxin-induced skeletal muscle damage (Velders et al., 2012). Here the ER beta-selective agonist accelerated the regeneration of damaged skeletal muscles in female rats and displayed anabolic potency in a classical Hershberger assay. In addition, serum creatine kinase activity after notexin treatment was highest in βERKO mice compared to αERKO and wild types while markers such as embryonic myosin heavy chain (muscle cell regeneration) were lowest in βERKO animals. Therefore, it can be hypothesized that βERKO mice utilize lipids and glucose less efficient than wild type counterparts due to a lower muscle mass that subsequently result in an increased body weight and fat mass.

Lipogenesis is decreased via both ER subtype-selective agonists and Gen in liver and soleus muscle

In this study, treatment with E2, Alpha, Beta, and less pronounced with Gen decreased the expression of lipogenic genes (SREBP-1c and FAS) in liver and soleus muscle compared to untreated HF controls (Fig. 8C-F). The same pattern was observed for FAS protein expression in the liver (Fig. 11). This is in agreement with the observed down-

regulation of these genes by E2 (Bryzgalova et al., 2008; D'Eon et al., 2005). In αERKO mice, expression of lipogenic genes were increased compared to wild types, suggesting a functional involvement of ER alpha is responsible for the effect (Bryzgalova et al., 2006). However, the role of ER beta was not investigated in their study. The study presented here, shows for the first time that an ER beta-selective agonist was able to mediate a down-regulation of these lipogenic genes also in the liver. This is confirmed by the observation that both ER subtype-selective agonists mediated a similar anti-lipogenic effect in the soleus muscle.

Several studies report that the liver primarily expresses the ER alpha, whereas the ER beta subtype is absent or expressed only at low levels (Matthews and Gustafsson, 2003; Taylor and Al-Azzawi, 2000). However, in the present study the analysis of hepatic mRNA expression of ER beta clearly demonstrated that this ER subtype is expressed in rat liver tissue (Fig. 10A) and confirms the work of Maruyama and colleagues that showed the expression of two ER beta isoforms in different rat tissues including the liver (Maruyama et al., 1998). The expression of ER beta in liver was also confirmed on protein level (Fig. 10B). Therefore, it can be suggested that the anti-lipogenic effects observed in the Beta-treated animals are mediated via ER beta.

To make the story even more complex: it has been shown that some membrane responses to estrogens require both ER alpha and ER beta to be initiated (Levin, 2011). This aspect has to be investigated in more detail in future studies. However, in the animal model used in this study, the E2-treated rats did not show an increased response of the investigated parameters compared to rats treated with the ER subtype-selective agonists.

Interestingly, mRNA expression levels of markers associated with fatty acid transport and utilization were not significantly affected between the respective treatment groups in liver and soleus muscle (data not shown). In both tissues, mRNA expression levels of PPAR alpha were analyzed. Additionally, PPAR delta mRNA expression was measured in the soleus muscle. The PPAR alpha has been identified to regulate fatty acid utilization and the first PPAR response element (PPRE) was found in the 5'-flanking sequence of the rat acyl-CoA oxidase (Tugwood et al., 1992). This transcription factor is predominantly expressed in the liver, but also in other tissues such as heart, kidney and skeletal muscle (Fruchart, 2007; Tobin and Freedman, 2006). In skeletal muscle, PPAR delta is highly expressed (10–50 fold higher than PPAR alpha or PPAR gamma) and also contributes to fatty acid transport and oxidation (Braissant et al., 1996).

Taken together, lipogenic genes are up-reglated in liver and soleus muscle of untreated OVX animals, while the utilization of fatty acids seems to be unaffected compared to SHAM and substituted OVX groups.

TG accumulation is decreased via either ER subtype-selective agonist in liver and soleus muscle

In this study, untreated OVX animals on HF diet showed the highest hepatic TG content. This is in line with hepatic up-regulation of lipogenic genes (SREBP-1c and FAS) and PPAR gamma expression (associated not only with improved insulin sensitivity (Vacca et al., 2011), but also with higher lipid accumulation (Way et al., 2001)). SHAM, E2-, Alpha-, and Beta-treated animals accumulated significantly less triglycerides in liver. Gen treatment had no significant effect (Fig. 12A). A similar pattern was observed in the soleus muscle, although the effect was not statistically significant (Fig.12B). Thus, untreated OVX animals revealed increased lipogenesis and adipogenesis in adipose tissue as well as enhanced lipogenesis and TG accumulation in liver and soleus muscle than the SHAM, E2-, Alpha-, and Beta-treated animals. Gen treatment showed at least the same tendency in liver and soleus muscle. These findings are of great importance, because fat accumulation within non-adipose tissues as well as obesity itself is associated with a high risk of developing insulin resistance and T2DM (Shimabukuro et al., 1997; Unger, 1995).

Insulin sensitivity

In this study, ovariectomy markedly increased PPAR gamma mRNA expression compared to SHAM animals in liver and soleus muscle. In animals on HF diet, this effect was antagonized by treatment with E2 and Alpha but not by Beta and Gen (Fig 9C and E) and thus appears to be mediated via ER alpha. This is in agreement with literature data showing that E2 down-regulates PPAR gamma expression (Bryzgalova et al., 2008). The rate of hepatic glucose uptake, determined by IRMS, in animals on a HF diet fits to the observed PPAR gamma expression pattern. A significantly higher glucose uptake in the untreated OVX HF animals was observed compared to SHAM, E2-, and Alpha-treated animals (Fig. 9D).

However, Beta decreased PPAR gamma expression in adipocytes (Fig. 9A). These divergent observations between tissue types may be explained by the different functions of PPAR gamma in adipose tissue compared to liver and skeletal muscle, as follows. PPAR gamma is highly expressed in white adipose tissue (Escher et al., 2001), and induces primarily adipogenesis and lipid repartition (Vacca et al., 2011). In liver and skeletal muscle PPAR gamma activation improves insulin sensitivity (Kim and Ahn, 2004; Vacca et al., 2011; Way et al., 2001). In liver cells, PPAR gamma activation increases insulin sensitivity through inhibition of genes involved in gluconeogenesis (i.e., PEPCK, pyruvat carboxylase, and glucose-6-phosphatase) (Davies et al., 1999; Way et al., 2001) and induces the hepatic expression of glucokinase (the rate limiting enzyme in glycolysis), and the hepatic glucose transporter (Kim and Ahn, 2004; Way et al., 2001). In skeletal

muscle, it has been demonstrated that stimulation of PPAR gamma expression increases glucose uptake and insulin sensitivity via stimulation of the AMP-activated protein-kinase (Vacca et al., 2011), a kinase that is also activated by E2 via non-genomic pathways (D'Eon et al., 2005). Taken together, PPAR gamma expression patterns in liver and soleus muscle and hepatic glucose uptake measurement suggest improved insulin sensitivity in untreated, Beta-, and Gen-treated OVX animals compared to SHAM, E2-, and Alpha-treated rats. However, according to the literature, insulin sensitivity is improved in the presence of E2 exclusively activating ER alpha (Barros et al., 2009; Barros et al., 2006; Bryzgalova et al., 2006; Heine et al., 2000; Ropero et al., 2008). Thus, by showing that PPAR gamma activation and glucose uptake in liver and soleus muscle is stimulated by ER beta, this study presents contradictory findings compared to other published data (as described above).

The contradicting results might be explained by comparing the PPAR gamma expression with the blood lipid pattern that was also assessed. Stimulation of PPAR gamma in liver and skeletal muscle is associated with increased lipogenesis as well as decreased fatty acid oxidation (Way et al., 2001). It therefore contributes to fatty acid production and storage.

The increased hepatic PPAR gamma expression in the untreated OVX HF group compared to SHAM, E2-, and Alpha-treated animals is in agreement with the effects on blood lipid levels that were observed in the previous study. The untreated OVX animals showed significantly higher levels of TC, LDL, and VLDL compared to SHAM animals and treatment with E2 and Alpha antagonized the OVX-induced effect in animals on HF diet. The levels of TC, LDL, and VLDL in the Beta- and Gen-treated animals were not significantly reduced (Weigt et al., 2012). These findings correspond with the PPAR gamma expression in this study. Increased PPAR gamma activation may also promote higher fat accumulation and VLDL assembly in the liver.

Therefore, it can be hypothesized that the up-regulation of PPAR gamma (at least) in untreated OVX rats could be a mechanism to compensate the high accumulation of lipids in non-adipose tissues that is associated with impaired insulin sensitivity. This hypothesis is underlined by the systemic insulin sensitivity in the animals, which was analyzed by measuring serum insulin levels and calculation of the HOMA-Index. Whereas serum glucose levels revealed no significant differences between the animal groups (data not shown), the concentration of serum insulin was higher in the untreated OVX animals than in the SHAM and substituted OVX animals (Fig. 13A). These results are in line with the calculated HOMA-Index, a model that is used to estimate the degree of insulin sensitivity (Fig. 13B). Hence, untreated OVX animals may need higher concentrations of insulin compared to the other groups to achieve similar serum glucose concentrations. This may

indicate that untreated OVX animals have decreased insulin sensitivity and that excessive accumulation of intracellular lipids is strongly associated with impaired insulin sensitivity.

In light of the rather complex regulation of glucose sensitivity and blood lipids, data obtained in this study indicate that PPAR gamma expression, blood lipids, and glucose uptake in Beta- and Gen-treated rats is similar to untreated animals. However, systemic insulin sensitivity in these animals was comparable to SHAM, E2- and Alpha-treated animals. Therefore, it can be hypothesized that Beta-treated animals, due to increased muscle mass, may have an improved utilization of lipids and glucose in the muscle. This contributes to a clearance of TG-rich lipoproteins from the bloodstream and thus to a lower deposition of lipids in adipose or non-adipose tissues. Untreated OVX rats may lack this ability and therefore tent to accumulate fat in liver and muscle despite PPAR gamma activation in adipocytes.

The results presented here suggest a specific role of ER beta in skeletal muscle homeostasis that results in a favorable body composition (higher muscle/fat ratio) with regard to utilization of lipids and glucose and insulin sensitivity. Compounds that selectively target ER beta might therefore provide new therapeutic concepts for diseases such as T2DM and the metabolic syndrome.

Genistein

In line with the previous results, treatment with Gen had no significant effects on the investigated parameters in adipose tissue. This may be due to the fact that Gen and its metabolites may be present only in low concentrations in this tissue, which needs to be verified by determination of tissue concentrations. In liver and soleus muscle, application of Gen to OVX HF rats led to less pronounced but similar effects as treatment with Beta. Gen-treated animals also showed reduced lipogenesis and TG content in liver and muscle, and a PPAR gamma mRNA expression, glucose uptake and blood lipid composition pattern that was similar to Beta-treated animals.

The dose of Gen used was relatively high - a human equivalent dose (Reagan-Shaw et al., 2008) can be reached by a daily dose which is two to four times higher than the maximum dose commonly used for postmenopausal treatment (Andres and Lampen, 2012; Hooper et al., 2010; Messina and Wood, 2008; Steinberg et al., 2011; Wei et al., 2012). Since Gen was given orally in the present study, Gen and resulting metabolites may act in a synergistic manner. Moreover, tissue concentrations of Gen and its metabolites may differ depending on tissue type. In contrast to Alpha and Beta – at the given doses – Gen binds to both receptors, although a preferential binding affinity to ER beta has been shown (Kuiper et al., 1998). Therefore dependent on the expression ratios of ER alpha and beta in a respective tissue it may act more as an ER alpha or ER beta

selective agonist. This has been demonstrated in a variety of previous studies from our group (Hertrampf et al., 2007; Schleipen et al., 2011; Velders et al., 2012).

The pronounced activity of Gen in these tissues may be due to pharmacokinetic aspects: e.g., the concentration of Gen in the liver may be higher than in other tissues. In the skeletal muscle, Gen's ability to activate ER beta-specific mechanisms such as anabolic effects may be relevant. In a recent publication of our group (Velders et al., 2012) it was demonstrated that Gen activates anabolic mechanisms in the skeletal muscle similar to Beta. Therefore, similar to treatment with Beta, Gen-treated animals may improve their energy homeostasis through enhanced utilization of glucose and lipids.

Impact of the HF diet

In general, the HF diet fed to the animals in this study was used to induce obesity. This approach made is possible to examine the impact of hormonal treatment on obese OVX rats. The LF diet used in the study served as control and was only fed to SHAM and untreated OVX animals. In the previous study, higher gain of body weight and visceral fat mass were observed comparing SHAM and OVX animals on HF diet with SHAM and OVX animals on LF diet respectively, but the differences were statistically not significant (Weigt et al., 2012). In this study, no significant differences were observed between the two diets in SHAM and untreated OVX animals on hepatic and muscular TG content as well as serum levels of insulin, although animals on the fat-enriched diet tended to higher values similar to the previous study. Therefore, after an experimental period of 10 weeks the loss of ovarian function resulted in effects that were more adverse regarding the above-mentioned parameters than the fat-enriched diet. However, on the molecular level diet-induced effects were more pronounced. In adipose tissue and liver, mRNA expression of FAS was significantly down-regulated in SHAM and untreated OVX animals (only in adipose tissue) on HF diet compared to the same animal groups fed with the LF diet. This effect can be most likely attributed to the high content of fat in the diet, which seems to result in a general down-regulation of *de novo* lipogenesis in both tissues. Significant effects between the two diets were also observed on mRNA expression levels of SREBP-1c (in adipose tissue and liver) and PPAR gamma (in adipose tissue, liver, and soleus muscle), whereby here the HF diet resulted in an higher mRNA expression levels compared to the mRNA levels in animals on LF diet. The higher expression level of PPAR gamma in animals on the fat-enriched diet could be again a mechanism to compensate the high accumulation of lipids and to increase insulin sensitivity.

Conclusion

In summary, the data presented here provide evidence that estrogens down-regulate lipogenic and adipogenic genes through either ER subtype in adipose tissue. Furthermore, in liver and soleus muscle, treatment with all estrogenic compounds decreased expression of lipogenic genes and lowered TG accumulation compared to untreated OVX HF animals. Hepatic and muscular insulin sensitivity appears to be increased in untreated, Beta-, and Gen-treated animals (similar patterns of PPAR gamma expression and glucose uptake). Because systemic insulin sensitivity was most restricted in untreated OVX animals on HF diet, these rats might compensate for increased levels of intracellular lipids by up-regulating PPAR gamma. This hypothesis is in line with the association of intracellular lipid accumulation in non-adipose tissues with T2DM. Further, it can be hypothesized that anabolic effects of ER beta may contribute to improved utilization of glucose and lipids in Beta- and Gen-treated animals. Thus, lipid and glucose metabolism is positively affected by E2 activating either ER subtype. The anti-diabetic effects of ER alpha and ER beta seem to be mediated via different molecular mechanisms in soleus muscle and liver.

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Supplementary material

Table 4. Description of genes that were analyzed by real-time RT-PCR.

Gene name	Description	Effects of gene activation regarding metabolic homeostasis in			Reference
		adipose tissue	liver	soleus muscle	
SREBP-1c	transcription factor	*de-novo*-lipogenesis ↑	*de-novo*-lipogenesis ↑	*de-novo*-lipogenesis ↑	Horton et al. 2002
FAS	target gene of SREBP-1c	*de-novo*-lipogenesis ↑	*de-novo*-lipogenesis ↑	*de-novo*-lipogenesis ↑	Horton et al. 2002
PPAR gamma	transcription factor	adipogenesis ↑ fatty acid uptake ↑ repartition of lipids from muscle and liver ↑	gluconeogenesis ↓ glucose uptake ↑ insulin sensitivity ↑ lipid accumulation ↑	glucose uptake ↑ insulin sensitivity ↑ lipid accumulation ↑	Anghel et al., 2007 Kim and Ahn, 2004 Way et al., 2001
LPL	target gene of PPAR gamma	fatty acid uptake from circulating lipids ↑			Wong and Schotz, 2002 Homma et al., 2000
PPAR alpha	transcription factor		fatty acid transport and oxidation ↑	fatty acid transport and oxidation ↑	Vacca et al., 2011
PPAR delta	transcription factor			fatty acid transport and oxidation ↑ mitochondrial respiration ↑	Vacca et al., 2011

Chapter Four

Impact of estradiol, ER subtype-selective agonists and genistein on food intake, body weight, and glucose metabolism in leptin resistant ovariectomized Zucker diabetic fatty rats

This study will be submitted soon as:

Carmen Weigt, Torsten Hertrampf, Ulrich Flenker, Frank Hülsemann, Karl Heinrich Fritzemeier, Pinar Kurnatz, Patrick Diel.

Abstract

The leptin resistant female Zucker diabetic fatty (ZDF *fa/fa*) rats are described to be hyperphagic and obese, but compared to their male counterparts they remain euglycaemic. Since estrogens are known to affect glucose metabolism, the long-term effect of treatment with estradiol (E2), the ER subtype-selective agonists (Alpha and Beta), and genistein (Gen) was investigated in ovariectomized ZDF rats. After 4 months, food intake, body weight, systemic glucose tolerance, and muscular GLUT4 expression as well as muscle fiber areas were determined. Additionally, the impact of E2 and Alpha on glucose uptake in liver, skeletal muscle, and adipose tissue as well as localization of muscular GLUT4 was analyzed. Food intake and body weight were markedly reduced by E2 and Alpha treatment. Glucose tolerance was improved by treatment with E2, Alpha, Beta, and Gen. Glucose absorbance in liver, skeletal muscle, and adipose tissue was accelerated in E2 and Alpha treated animals compared to untreated OVX rats (impact of Beta and Gen was not investigated). In skeletal muscle, treatment with Alpha enhanced the expression and translocation of GLUT4 to the cell membrane, whereas Beta substitution resulted in the largest muscle fiber sizes. Administration of Gen suggested affecting both GLUT4 expression and muscle fiber sizes.

Thus, the data demonstrate that ER alpha mediates decreased food intake and body weight in leptin resistant female ZDF rats. Activation of ER alpha and beta improves glucose tolerance, but at least in the skeletal muscle via different pathways. Further, the ER alpha accelerates the glucose absorbance in liver and adipose tissue.

Introduction

In recent years, estrogens have been shown to modulate glucose homeostasis in humans and rodents. For example, long-term administration of E2 to OVX rats on standard diet as well as on HF diet improved systemic glucose tolerance and insulin sensitivity and enhanced insulin signaling in the skeletal muscle (Riant et al., 2009; Saengsirisuwan et al., 2009). Further, E2 substitution improved glucose tolerance in a rat model of already established obesity, when compared to untreated OVX animals (Zoth et al., 2012). Moreover, also aromatase-knockout mice, which do not produce estrogens, were described to develop glucose intolerance and insulin resistance (Simpson et al., 2005; Takeda et al., 2003). In postmenopausal women, estrogen deficiency is linked to a higher risk of developing insulin resistance and T2DM. Hormonal therapies have been shown to lower this incidence (Crespo et al., 2002; Curtis and Wilson, 2005; Kanaya et al., 2003; Louet et al., 2004). Generally, men display a higher prevalence of impaired glucose metabolism, when compared with premenopausal women (Kuhl et al., 2005), which also points to the protective role of female sex hormones.

Estrogens mainly act through two distinct ER subtypes, ER alpha and ER beta that belong to the nuclear hormone receptor family. Activated ERs regulate the transcription of diverse ER target genes, resulting in a wide range of biological effects. Both ER subtypes show distinct tissue and gender specific expression patterns and show differences in the three-dimensional structure of their ligand-binding domain. Thus, a range of compounds, synthetic or natural origin, selectively activates either ER.

The role of the two distinct ER subtypes in the molecular mechanisms underlying the beneficial role of estrogens in glucose homeostasis is still not completely understood. Several studies in ERKO animals have shown that the loss of ER alpha in male and female mice lowers glucose tolerance and insulin sensitivity as compared to wild type counterparts (Cooke et al., 2001; Heine et al., 2000). This metabolic phenotype was attributed to a reduced expression level of GLUT4 in the skeletal muscle and the white adipose tissue (Barros et al., 2009; Barros et al., 2006), but also to impaired insulin

sensitivity in the liver (Bryzgalova et al., 2006). By contrast, for ER beta a pro-diabetic action through a negative cross talk with adipose PPAR gamma was postulated (Foryst-Ludwig et al., 2008). However, the potency of ER beta to accelerate the regeneration of damaged skeletal muscle as well as to increase the skeletal muscle mass was recently demonstrated in female rodents (Velders et al., 2012; Weigt et al., 2012).

The obese Zucker Diabetic Fatty (ZDF *fa/fa*) rat strain is characterized by hyperphagia, hyperleptineamia, and obesity, because of a mutation of the leptin receptor that causes an ineffective interaction between leptin and its receptor. Obese male animals become hyperglycaemic between 7 and 10 weeks and onset of diabetes is described at the age of 12 weeks. Interestingly, female ZDF rats also become obese but they remain normoglycaemic. Female ZDF rats only become diabetic when they receive a special fat enriched diet (http://www.criver.com/SiteCollectionDocuments/ZDF.pdf).

It was hypothesized that ovariectomized ZDF rats on normal diet also develop impaired glucose tolerance due to hormonal decline, whereas ovary intact females or E2-substituted OVX rats might be protected. In the present study, this hypothesis was tested by using female ZDF rats, which were ovariectomized or SHAM-operated. Groups of OVX animals were treated with either E2, ER subtype-selective agonists (Alpha or Beta), or genistein to achieve deeper knowledge in the protective role of estrogens on the existing gender dimorphism regarding glucose homeostasis in ZDF rats. The ER agonists 16alpha-LE2 (Alpha) and 8beta-VE2 (Beta) selectively activate ER alpha and ER beta respectively (Hillisch et al., 2004). The isoflavone Gen is plant-derived and can activate both ER subtypes. After the experimental period, the effects of the estrogenic compounds on energy intake, body weight, glucose metabolism, and on the soleus muscle fibers were investigated.

Materials and methods

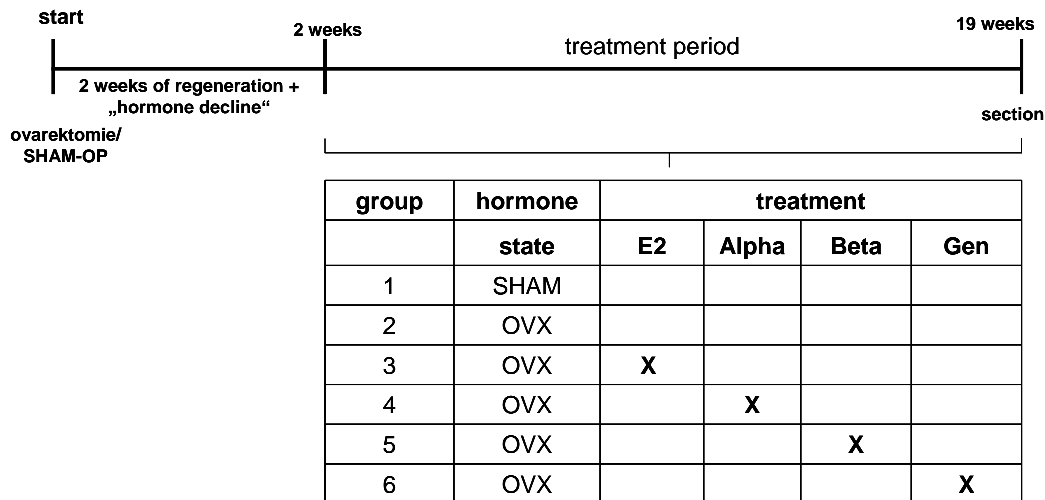
Animals

Juvenile female Zucker diabetic fatty (ZDF *fa/fa*) rats (6-weeks old, 180–240 g) were obtained from Charles River (Charles River Laboratories, Research Models and Services, Germany GmbH) and kept at constant room temperature (20 °C ± 1), relative humidity (50–80 %) and illumination (12 h light/dark cycles). The animals were housed 3–4 in each cage, with food (Purina 5008, SSniff GmbH, Soest, Germany) and water provided ad libitum. All animal procedures were approved by the Committee on Animal Care and compliant with accepted veterinary medical practice.

Animal diet and treatment

All animals were fed with a soybean free diet (PMI 5008, Ssniff®). Prior to onset of experimental procedures, all animals were ovariectomized (OVX) or SHAM-operated (SHAM) via the dorsal route. Following two weeks of regeneration and endogenous hormonal decline the OVX animals were randomly allocated to treatment and vehicle groups (untreated OVX rats). The treatment groups received either E2, Alpha (the ER alpha-selective agonist 16 alpha-LE2 (3,17-dihydroxy-19-nor-17alpha-pregna-1,3,5(10)-triene-21,16alpha-lactone)), Beta (the ER beta-selective agonist 8beta-VE2 (8-vinylestra-1,3,5(10)-triene-3,17beta-diol)) or Gen. E2 (4 µg/kg b.wt per day), Alpha (10 µg/kg b.wt per day), and Beta (100 µg/kg b.wt per day) were applied via ALZET® osmotic mini pumps, whereby the substances were dissolved in dimethylsulfoxide and water according to the manufacturer's instructions for filling. Gen was administered via Gen-enriched food (about 42 mg/kg b.wt per day). Composition of diets and animal grouping see Table 5 and Fig. 14. The treatment doses of the respective substances were chosen based on previous experiments (Diel et al., 2004; Hegele-Hartung et al., 2004; Hertrampf et al., 2009a; Hertrampf et al., 2005; Hertrampf et al., 2008b) and at a dose that ensures selectivity for the ER subtype-selective agonists. E2 was provided by Sigma-Aldrich (Deisenhofen, Germany) and Gen was obtained from LC Laboratories (Woburn, MA 01801 USA). Ssniff Spezialdiäten GmbH, Soest, Germany, provided the food enrichment with Gen. The two steroidal and highly subtype-selective agonists Alpha and Beta were obtained from Bayer Schering Pharma AG, (Berlin, Germany) and detailed information about the structure-based design, the synthesis strategy, and the characterization of both ligands *in vitro* and *in vivo* can be found elsewhere (Hillisch et al., 2004). Throughout the treatment period, body weight and food intakes were monitored twice a week.

After 17 weeks of treatment, the animals were sacrificed by decapitation after light anaesthesia with CO_2 inhalation and the complete blood was collected. The uteri were prepared free of fat and the wet weights were determined. Periovarian fat pads and livers were immediately frozen in liquid nitrogen. The soleus and gastrocnemius muscles were removed and directly frozen in liquid nitrogen for analysis of molecular markers or embedded in TissueTek® (Sakura, Staufen, Germany), cooled in 2-methylbutane, and frozen in liquid nitrogen for histological analysis.



group	hormone state	treatment E2	Alpha	Beta	Gen
1	SHAM				
2	OVX				
3	OVX	X			
4	OVX		X		
5	OVX			X	
6	OVX				X

Fig. 14. Experimental design.
SHAM = sham operated, OVX =ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with genistein.

Table 5. Composition of the diet.
PMI5008 without soybean products, sniff Spezialdiäten GmbH, Soest, Germany. 1) physiological fuel values

Product No.	% of the diet
Wheat	18.205
Corn, pretreated	22.000
Wheat bran	16.000
Oats, rolled	8.000
Potato protein	3.000
Corn gluten meal	5.000
Fish meal	9.000
Poultry protein hydrol. SD	5.000
Brewer's yeast	1.000
Whey powder	1.000
L-Lysine HCl	0.220
DL-Methionine	0.200
Choline chloride	0.360
Vitamin & trace element premix	1.100
NaCl, salt	0.500
MCP, calcium phosphate monobasic	0.800
Calcium carbonate	1.100
Ca propionate	0.400
Sorbic acid	0.100
BHT (Butylhydroxytoluol)	0.015
Lignocellulose	2.000
Sugar beetpulp	1.500
Pork lard	3.500

Product No.	% of the diet
Crude protein, CP	23.500
Crude fat	7.900
Crude fibre, CF	4.400
Crude ash	6.200
Starch	33.900
Sugar	2.900
Σ Carbohydrates	**46.600**
Energy (Atwater), MJ/kg 1)	14.900
kJ% Protein	26.000
kJ% Carbohydrates	54.000
kJ% Fat	**20.000**
Fatty acids	% of the diet
C16:0	1.520
C18:0	0.590
C20:0	0.020
C16:1	0.220
C18:1	2.460
C18:2 *n6*	1.980
C18:3 *n3*	0.140

Intraperitoneal glucose tolerance test (ipGTT)

After 15 weeks of treatment, animals were fasted overnight and blood glucose levels were determined. The animals were injected i.p. with glucose solution (1 g/kg b.wt), and blood glucose concentration was measured at 15, 30, 60, and 120 min after glucose load. Blood samples were analyzed using the ACCU CHECK® Compact Plus Meter Kit (Roche diagnostics GmbH, Mannheim, Germany).

Stable isotope ratio analysis

3.5 h before decapitation two rats of the SHAM, OVX, OVX+E2, and OVX+Alpha animal groups received an oral application of ^{13}C labeled glucose (2 g/kg b.wt)[2], enriched to +140 ‰ $\delta^{13}C_{VPDB}$. 50 mg of frozen liver, periovarian fat pads, gastrocnemius muscle, and soleus muscle of those glucose-substituted animals were homogenized and dehydrated. Same procedure was performed with two animals without glucose application from the respective groups, which served as negative controls. All samples were enclosed in tin capsules and analysed by EA-IRMS using an elemental analyser (Eurovektor EA 3000, Hekatech, Wegberg, Germany) coupled to a continuous-flow isotope ratio mass spectrometer (Delta C, Finnigan, Bremen, Germany). Carbon isotope ratios are expressed in per mill relative to VPDB: $\delta^{13}C$ [‰] = $[(R_{Sample}/R_{Standard}) - 1] \cdot 10^3$ (Coplen, 2011) with R is the $^{13}C/^{12}C$ ratio. Reference gases (CO_2, purity 5.3 from Linde, Munich, Germany) were calibrated using the reference materials IAEA-CH-6 and IAEA-CH-7. Within the measurements creatine-monohydrate from one batch was used as a working standard. The standard deviation of repeated measurements of working standard or samples was 0.1 ‰. Isotope ratios were calculated using the ISODAT NT software (version 2.0, Finnigan, Bremen, Germany).

Real-time RT-PCR experiments

Total RNA from the frozen gastrocnemius muscle was extracted using the standard TRIzol method (Life Technologies GmbH, Darmstadt, Germany). RNA was quantified by spectrophotometry (NanoDrop ™ 1000, Thermo Scientific, Wilmington, DE 19810, USA) and cDNA was synthesized from 1 µg RNA using a Reverse Transcription System (QuantiTect ®, Qiagen). Real-time RT-PCR was performed with Taq DNA polymerase (Life Technologies GmbH, Darmstadt, Germany) in the presence of a fluorescent dye (SYBR Green, BioRad) on an Mx3005P™ qPCR System (Stratagene). All reactions were run in triplicate in a total volume of 50 µl. The PCR program was as follows: 95 °C for 3 min for 1 cycle, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and 1

[2] Because of a pilot study, the stable isotope ratio analysis was not performed in all animal groups.

cycle at 72 °C for 1 min. Fluorescence was quantified during the 58 °C annealing step and product formation was confirmed by melting curve analysis (58–95 °C). Relative mRNA amounts of target genes were calculated after normalization to an endogenous reference gene (Cytochrom c Ocidase, subunit 1A) following the $\Delta\Delta C_T$ method (Pfaffl, 2001). Based on the genomic sequences available at the UCSC Genome Bioinformatics database the specific primer pair for GLUT4 was designed using the software Primer3 (Rozen and Skaletsky, 2000) and confirmed by the sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). The primer pair for the reference gene 1A was published elsewhere (Höfer et al., 2009). Primers were synthesized by Life Technologies GmbH, Darmstadt, Germany: GLUT4_fwd 5-TATTTGGCT TTGTGGCCTTC-3', GLUT4_rev 5'-CGGCAAATAGAAGGAAGACG-3', 1A_fwd 5'-CGTCACAGCCCATGCATTCG-3', 1A_rev 5'-CTGTTCATCCTGTTCCAGCTC-3'.

Immunohistochemical staining and visualization of GLUT4 in the gastrocnemius muscle

The same animals that received an oral application of ^{13}C labeled glucose were used to evaluate the distribution of the insulin-dependent GLUT4 in the gastrocnemius muscle. Cross-sections (7 µm) from frozen and Tissue Tek® embedded gastrocnemius muscles were fixed in ice-cold acetone for 10 minutes. After drying, sections were incubated in 5 % BSA/TBS for 1 h at room temperature to block non-specific binding. Sections were incubated with anti-GLUT4 antibody (ab 654-250, abcam, Cambridge, United Kingdom) at a dilution of 1:400 in 1 % BSA/TBS for 2 h at room temperature. After washing with TBS, the sections were incubated with the CY3 conjugated secondary antibody (111-165-144, Jackson ImmunoResearch, USA) which was diluted 1:1000 in TBS for 1 h at room temperature. The sections were subsequently washed (4-times) and counterstained with DAPI (D9542, Sigma-Aldrich, Germany) at a dilution of 1:600 in TBS for 10 min at room temperature. GLUT4 detection was conducted under an inverse microscope (Axiovert200, Zeiss, Germany) with the appropriate filters for fluorescence of CY3 (red) and DAPI (blue) and the corresponding software AxioVision Rel. 4.6.3.

Immunohistochemical staining and determination of soleus muscle fiber areas

Cross-section, fixation, and blocking of non-specific binding were performed as described above for the gastrocnemius muscle. Soleus sections were incubated with anti-Laminin antibody (L9393, Sigma-Aldrich, Germany) at a dilution of 1:400 in 1 % BSA/TBS overnight at 4 °C. Afterwards the sections were incubated first with the corresponding secondary antibody (E0432, Dako, Denmark) and after that with the Streptavidin-biotinylated horseradish peroxidase complex (RPN1051V, GE Healthcare, Germany) each 1:400 diluted in TBS for 1 h at room temperature. To visualize the antigen-antibody

complexes 3,3´-Diaminobenzidine-tetrahydrochloride (DAB, Sigma-Aldrich, Germany) as substrate was used. Between the individual incubation steps sections were washed several times with TBS. Muscle fiber areas were measured with an inverse microscope (Axiovert200, Zeiss, Germany) and the corresponding software AxioVision Rel. 4.6.3.

Statistical analysis

Statistical significance of differences was calculated using Kruskal-Wallis one-way analysis of variance with a following Mann-Whitney U-test (SPSS Statistical Analysis System, Version 20.0). Statistical significance was established at $p \leq 0.05$.

Results

Uterine wet weight, food intake and body weight

To ascertain the estrogenic response of the experimental treatment and adequate delivery of compounds via osmotic mini pumps or enriched food, the uterine wet weights of all animals as the most reliable biomarker for estrogenic activity were measured (Owens and Ashby 2002) (Fig. 15). Only treatment with E2 or Alpha prevented the uterine atrophy in OVX rats, whereas administration of Beta and Gen had no effect. These results are in line with previous studies that have shown that only ER alpha mediates the stimulatory effect of E2 on the uterine wet weight (Hertrampf et al., 2007; Hertrampf et al., 2008a; Hillisch et al., 2004; Weigt et al., 2012)

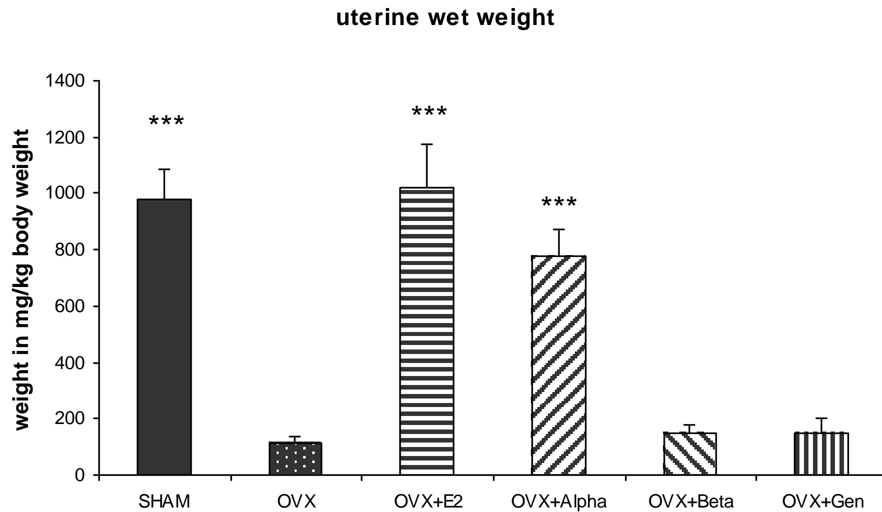


Fig. 15. Effects of E2, ER subtype-selective agonists and Gen on uterine wet weights after 17 weeks of treatment.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. Data shown are means ± SD. Mean values were significantly different for the following comparisons: *** vs. OVX ($p \leq 0.001$)

Throughout the experimental period, OVX rats revealed significantly higher food consumption than the SHAM-treated animals. The OVX effect was antagonized by administration of E2 and Alpha, but not by Beta or Gen (Fig. 16C).

The different feeding behavior of the animals was reflected by the body weight gain during the treatment period (Fig. 16B) and at the end of the experiment (Fig. 16A). All animal groups were obese at this time point, but the SHAM-, E2-, and Alpha-treated animals displayed significantly lower body weights than the untreated OVX animals. Substitution of OVX animals with Beta and Gen resulted in a body weight comparable to untreated OVX rats.

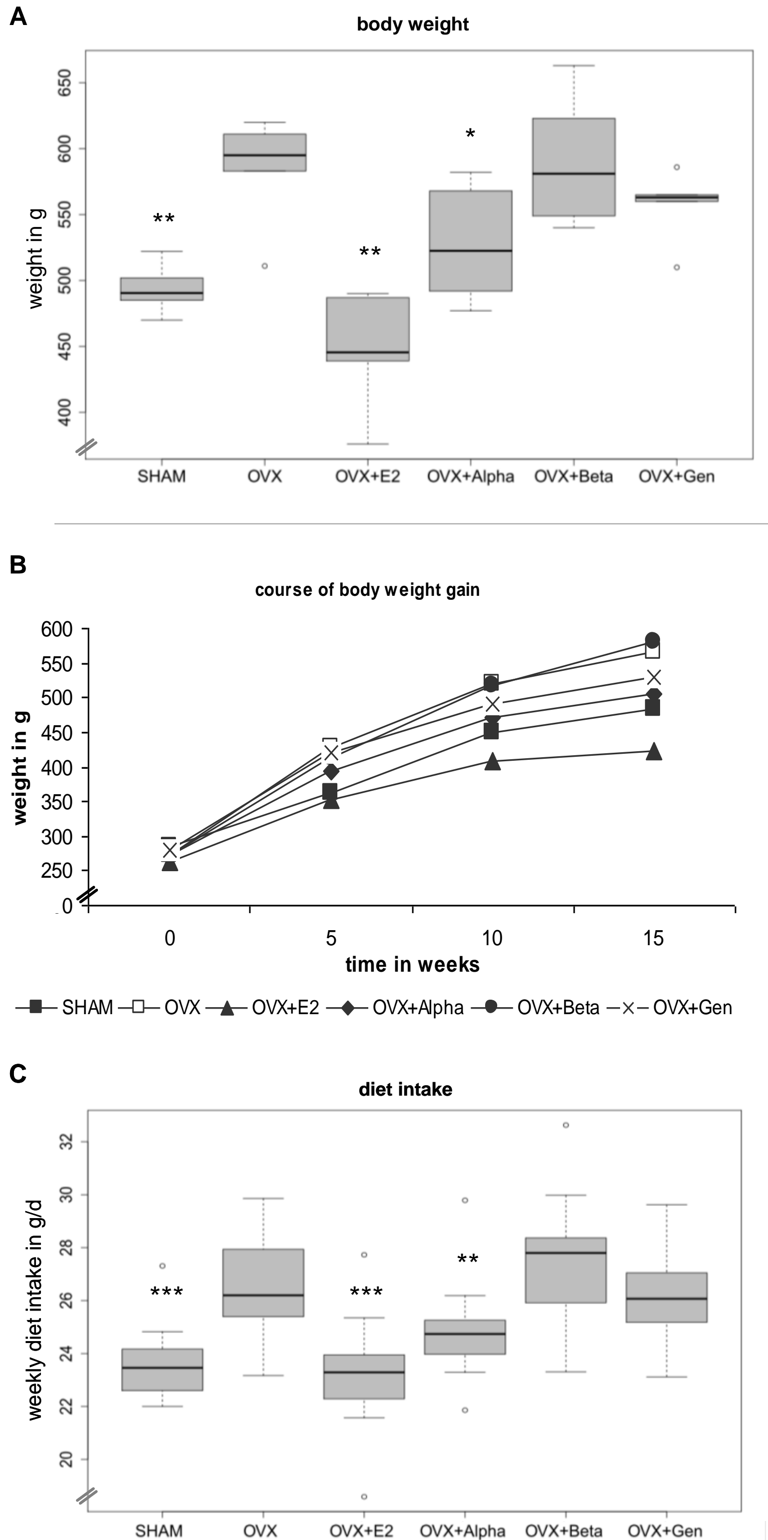


Fig. 16. Effects of E2, ER subtype-selective agonists and Gen on (A) body weight after 17 weeks of treatment, (B) course of body weight between week 0 and 15, and (C) weekly diet intake throughout the experimental period.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. Data shown in A and C are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution, empty circles dispay outliers. Data shown in B are means at the respective time points. Statistical significance for A and C was established as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs OVX.

Intra-peritoneal glucose tolerance test

After an over-night fast, at time-point 0, the untreated OVX animal group displayed the highest blood glucose levels. Significantly lower levels were measured in the SHAM- and E2-treated animals, whereas the Alpha-, Beta -and Gen-substituted rats showed at least the same tendency (Fig. 17). 15, 30, 60, and 120 minutes after a bolus of 1 g/kg b.w. glucose was given intraperitoneally the glucose concentration were determined. Compared with untreated OVX animals the treatment with E2 led to significantly lower glucose concentrations at all time-points. The SHAM, Alpha, and Beta treated animals revealed significantly lower glucose levels after 30 and 60 min relative to untreated OVX rats, whereas substitution with Gen lowered the glucose level at time point 60 and 120 min markedly. The slower glucose clearance in the untreated OVX rats was also reflected by the area under the plasma concentration/time curve (Fig. 18).

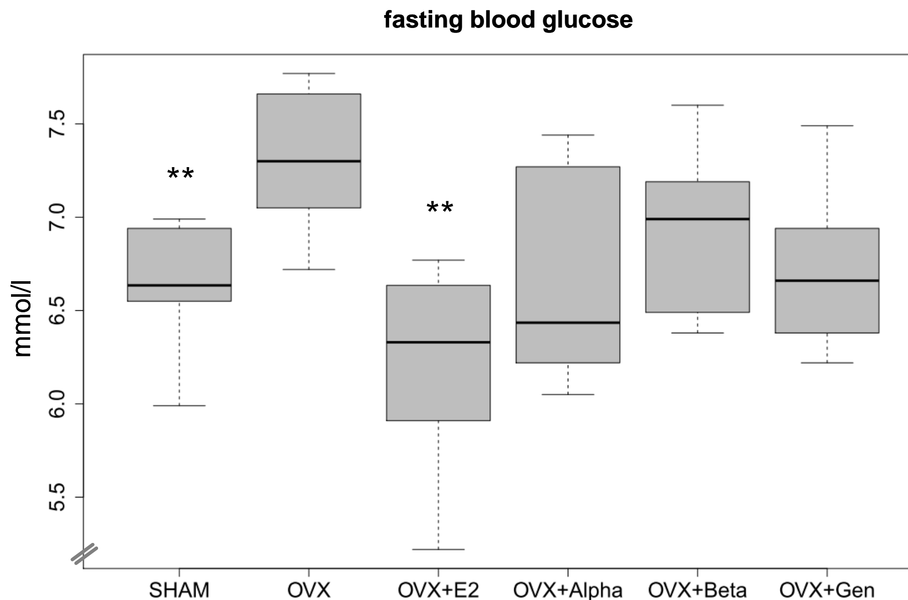


Fig. 17. Effects of E2, ER subtype-selective agonists and Gen on fasting blood glucose.
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. Data are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution. Statistical significance was established as follows: **$p \leq 0.01$ vs OVX.

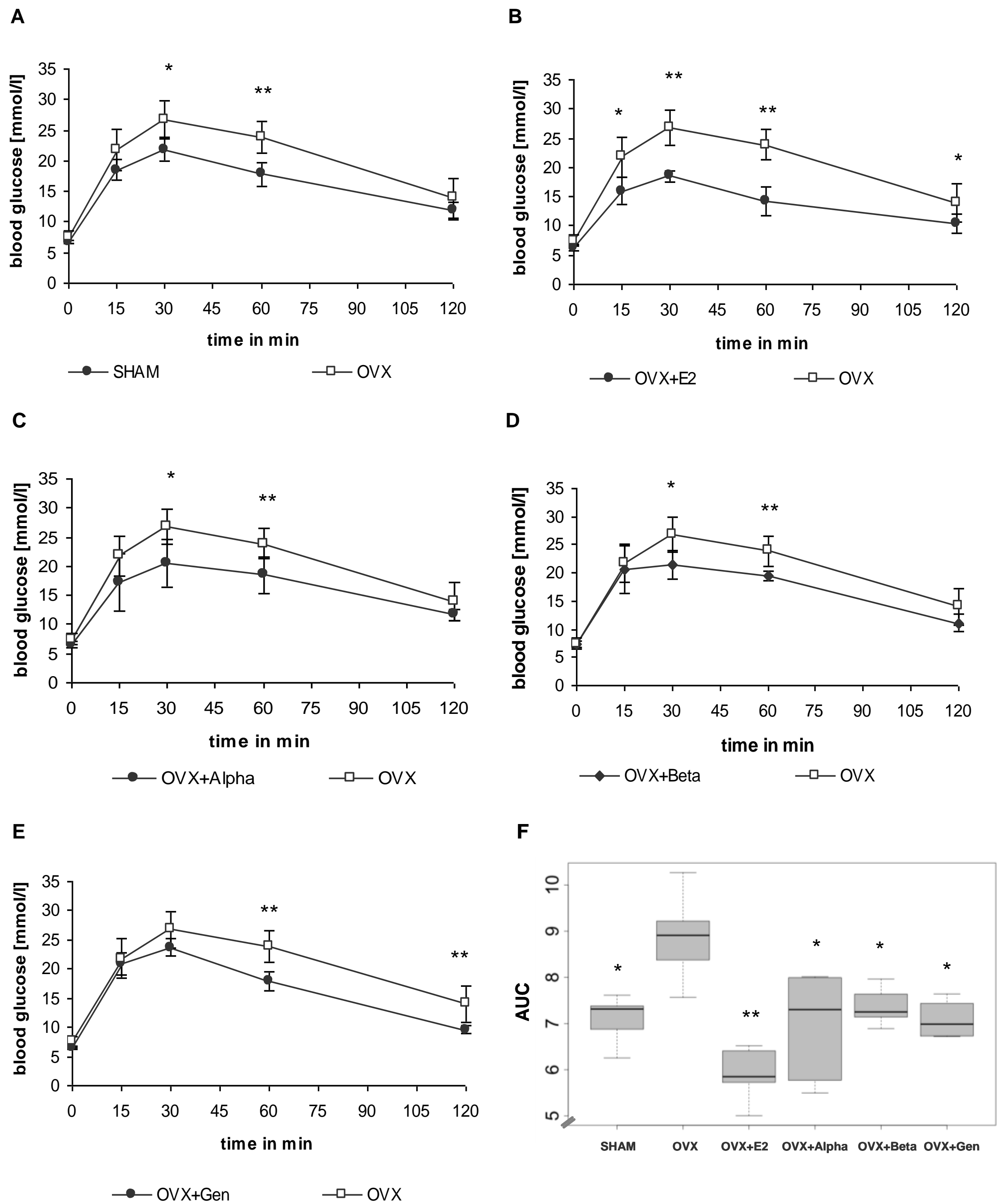


Fig. 18. Effects of E2, ER subtype-selective agonists and Gen on intraperitoneal glucose tolerance test (ipGTT).
A: comparison of SHAM and OVX, B: comparison of OVX+E2 and OVX, C: comparison of OVX+Alpha and OVX, D: comparison of OVX+Beta and OVX, E: comparison of OVX+Gen and OVX, F: measurement of AUC (area under the curve).
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. Data shown in A–E are means ± SD. Data shown in F are depicted as minimum, 25th, 50th (median), 75th percentile and maximum of the distribution. Statistical significance was established as follows: *p≤0.05, **p≤0.01 vs OVX.

Stable isotope ratio analysis in liver, skeletal muscle, and adipose tissue

To measure the glucose uptake in different tissues of the animals, the IRMS-analysis was used. In liver cells, the $\delta^{13}C$ values in the untreated OVX animals were decreased relative to SHAM, E2- and Alpha-treated animals. Similar results were analyzed in both the soleus and the gastrocnemius muscle and the white adipose tissue displayed at least the same tendency (Fig. 19). Because of a pilot study, the impact of Beta and Gen treatment on glucose uptake was not determined.

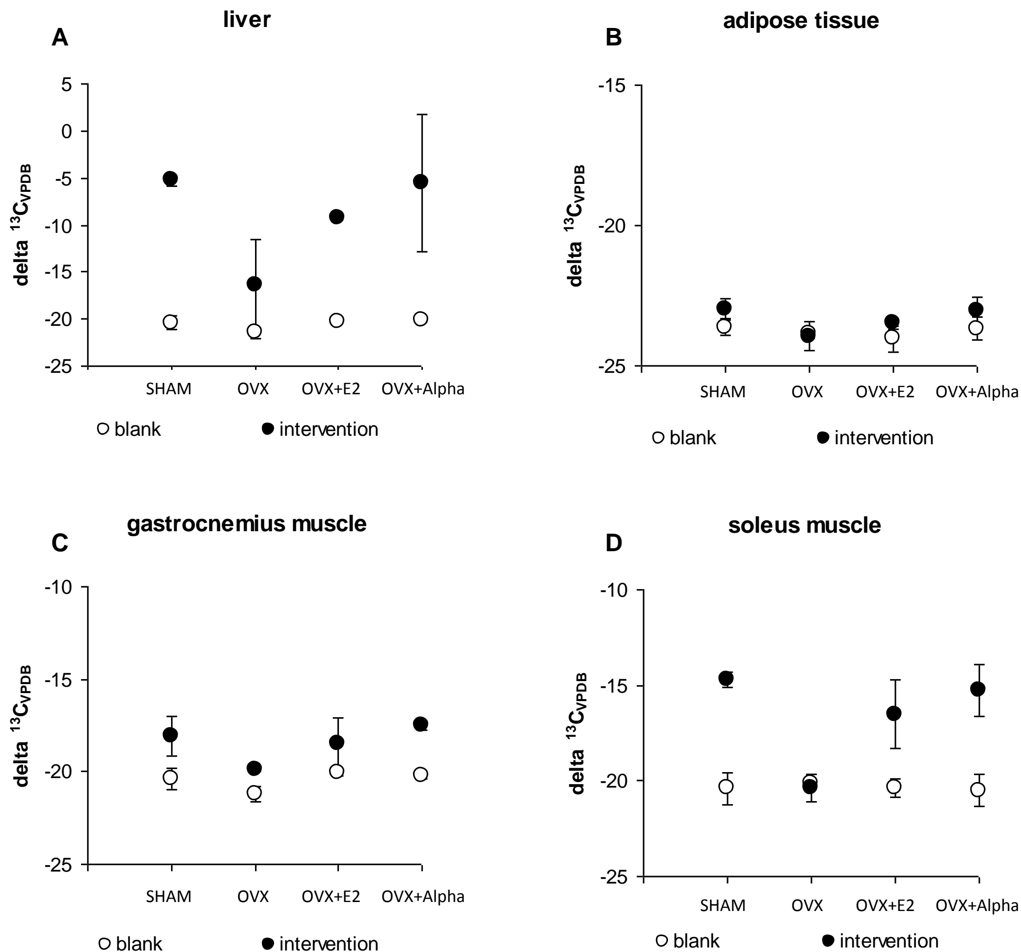


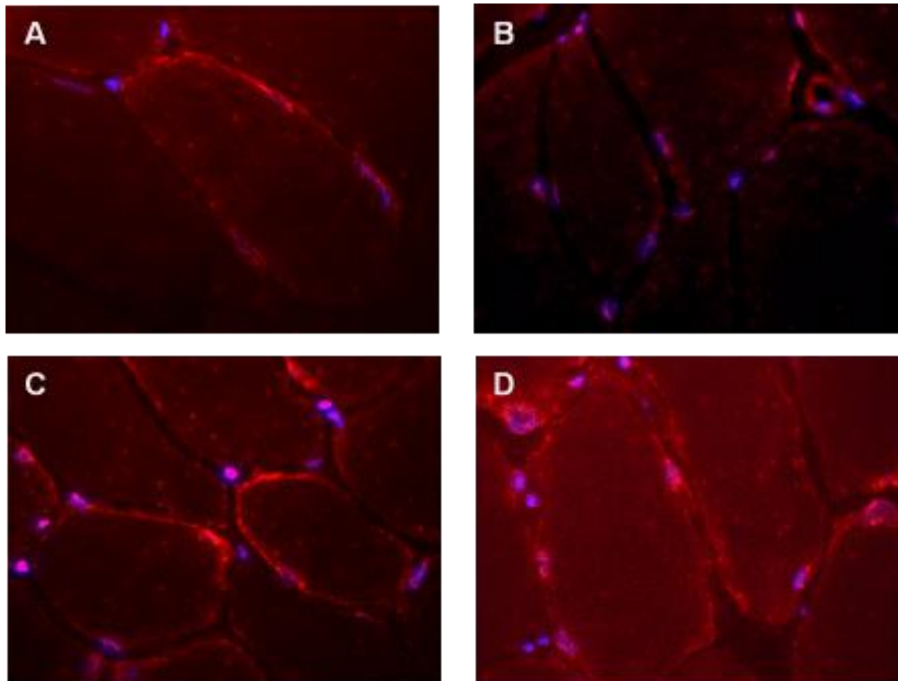
Fig. 19. Effects of E2 and Alpha on glucose uptake in liver (A), adipose tissue (B), gastrocnemius muscle (C), and soleus muscle (D).
SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, blank = animals without glucose application, intervention = animals received an oral application of ^{13}C labeled glucose 3.5 hours before decapitation. Data shown are means ± SD. Each measuring point represents two animals analyzed in duplicate. Statistical significance was not established.

GLUT4 expression in the gastrocnemius muscle

Gastrocnemius muscles from animals that received glucose for IRMS-Analysis 3.5 h before decapitation were also evaluated for distribution of the insulin-dependent GLUT4

by immunohistochemical staining. The presence of GLUT4 in the cell membrane of SHAM-, E2-, and Alpha-treated animals was substantially higher than in untreated OVX animals (Fig. 20A–D).

Real-time RT-PCR experiments, performed in all animals, revealed that the mRNA level of GLUT4 was lowest in untreated OVX rats. Significantly increased to OVX was the gene expression in Alpha- and Gen-treated animals, whereas the higher mRNA expression in SHAM-, E2-, and Beta-treated animals did not reach significance (Fig. 20E)



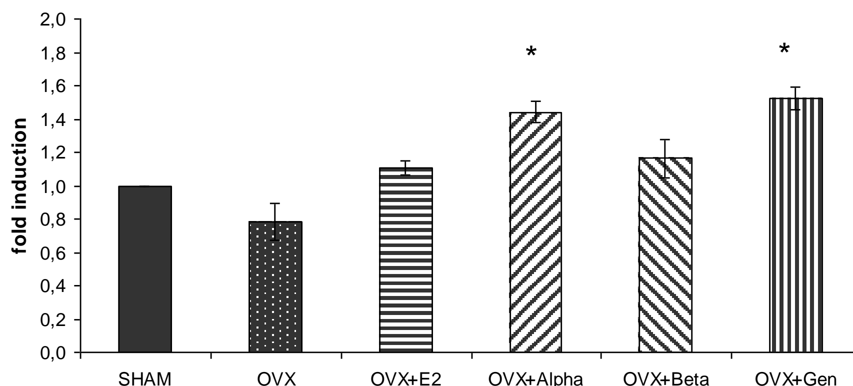


Fig. 20. (A-D) Effects of E2 and Alpha treatment on the GLUT4 expression and distribution in the gastrocnemius muscle. (E) Effects of treatment with E2, ER subtype-selective agonists and Gen on GLUT4 mRNA expression in the gastrocnemius muscle.
GLUT4 was stained with Cy3 (red) and nuclei with DAPI (blue). A: SHAM, B: OVX, C: OVX+E2, D: OVX+Alpha. SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. mRNA expression data are means ± SD. *p≤0.05 vs OVX.

Cross-sectional fiber areas of the soleus muscle

Recently, the ability of ER beta to increase the skeletal muscle mass in a rat model of nutrition-induced obesity was demonstrated (Weigt et al., 2012). To assess whether ER beta activation modulates skeletal muscle mass in leptin resistant ZDF rats the soleus muscle fiber areas in histological sections were measured. Treatment of OVX rats with estrogenic compounds led to significantly larger muscle fiber areas compared to untreated OVX HF animals, whereby muscle fiber sizes were largest in Beta- and Gen-treated animals (Fig. 21).

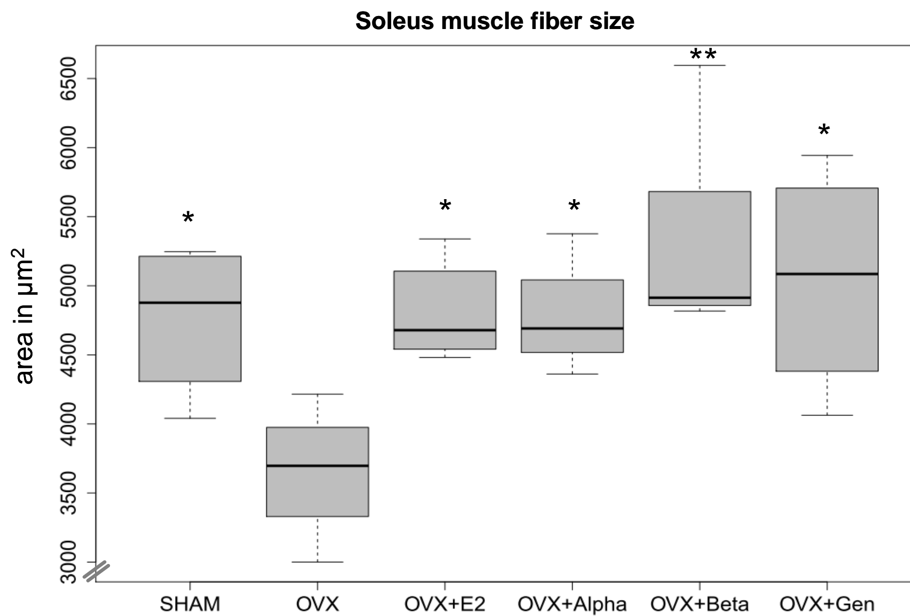


Fig. 21. Effects of E2, ER subtype-selective agonists and Gen on soleus muscle fiber sizes. SHAM = sham-operated, OVX = ovariectomized, E2 = treated with 17beta-estradiol, Alpha = treated with ER alpha-selective agonist, Beta = treated with ER beta-selective agonist, Gen = treated with Genistein. Data shown are means ± SD. *$p \leq 0.05$, **$p \leq 0.01$ vs. OVX.

Discussion

In the present study, it was investigated how estrogens modulate glucose metabolism in leptin resistant female ZDF rats. Those animals were treated with E2, or, to achieve deeper knowledge which ER subtype is involved, with ER subtype-selective agonists Alpha or Beta for 17 weeks. In addition, a subset of OVX animals received a diet enriched with Gen to evaluate whether this phytoestrogen is able to induce effects similar to those of estrogens.

The major finding in this study is that estrogens ameliorate disturbed glucose homeostasis via both ER subtypes.

Uterine wet weight and body weight gain

After 17 weeks of treatment uterine wet weight (Fig. 15) and body weight gain (Fig. 16A and B) in the animals were as expected and are in line with previous results. Only the ER alpha is responsible for the uterine growth response and the lower body weight gain compared to untreated OVX rats (Hertrampf et al., 2007; Hertrampf et al., 2008a; Hertrampf et al., 2008b; Hillisch et al., 2004; Weigt et al., 2012). These results confirm further the adequate delivery of all estrogenic compounds and the selectivity of the ER subtype-selective agonists.

Food intake

Although all animals were hyperphagic, ovariectomy led to a significantly higher food intake compared to SHAM animals. This OVX-induced effect was only antagonized by treatment with E2 and Alpha, but not by substitution with Beta or Gen (Fig. 16C). In previous results of our group this finding was only shown in tendency, where differences in food intake between untreated and with estrogenic compounds treated OVX animals or intact animals never reached significance (Hertrampf et al., 2006a; Hertrampf et al., 2007; Weigt et al., 2012; Zoth et al., 2010). Apart from our work, several research papers revealed a significant higher food intake in OVX animals compared to intact animals. However, this was often observed in short-term experiments (Ainslie et al., 2001; Chen and Heiman, 2001; Latour et al., 2001; Silva et al., 2010). In the present study, leptin resistance of the used rat strain might explain the more pronounced effect of E2- and Alpha-induced food intake reduction.

Leptin is mainly produced by adipocytes and plays a key role in regulating feeding behavior and energy homeostasis. Circulating leptin crosses the blood-brain barrier and binds to its receptor in the hypothalamus (Friedman, 2002; Maffei et al., 1995). The

hypothalamus contains a number of small, interconnected nuclei such as the arcuate nucleus, ventromedial nucleus, paraventricular nucleus, lateral hypothalamic area, and dorsomedial nucleus. All of them express the leptin receptor and are implicated in the regulation of feeding behavior and energy expenditure (Simpson et al., 2009). Especially in the arcuate nucleus the catabolic effect of leptin by inducing the anorexigenic neurons POMC and CART and inhibiting the orexigenic neurons AgRP and NPY resulting in decreased food consumption and increased energy expenditure is well described (Barsh and Schwartz, 2002; Morton et al., 2006; Simpson et al., 2009).

Both ER subtypes are expressed in the hypothalamus (Weiser et al., 2008) and literature data indicate that also estrogens interact with the neurobiological control of feeding behavior. For example, in cycling female rats the lowest food intake can be observed when the estrogen concentration is greatest (Blaustein and Wade, 1976; Butera, 2010; ter Haar, 1972). Similar results were described for other mammals including guinea pig (Czaja et al., 1983; Czaja and Goy, 1975), sheep (Tarttelin, 1968), rhesus monkey (Czaja, 1975; Czaja and Goy, 1975), and the human females (Buffenstein et al., 1995). However, to what extend the both ER subtypes are involved in estrogenic modulation of eating behavior is still unclear. Administration of E2 into the ventromedial nucleus (that exclusively express the ER alpha (Weiser et al., 2008)), and into the paraventricular nucleus (described to express only the ER beta (Weiser et al., 2008)) have shown to decrease food intake in female rats (Bonavera et al., 1994; Butera and Beikirch, 1989; Palmer and Gray, 1986; Wade and Zucker, 1970). Further, in the arcuate nucleus (where both ER subtypes are expressed) estrogen deficiency stimulated the expression of NPY mRNA, while the expression of POMC mRNA was decreased (Pelletier et al., 2007). Taken together, this study with leptin resistant ZDF rats shows that the E2-induced lower food intake is mediated via the ER alpha and seems to be independent of leptin signaling.

Systemic glucose tolerance

The hypothesis that ovariectomy of ZDF rats results in impaired glucose tolerance when compared to intact or E2-substituted animals has been confirmed. A significantly lower fasting glucose was found in SHAM and E2-treated animals compared to untreated OVX animals. Administration of Alpha, Beta, and Gen showed a similar tendency (Fig. 17). Regarding the ipGTT, all animal groups that were intact or received estrogenic compounds, tolerated the glucose load better than untreated OVX animals (as demonstrated by lower AUCs in Fig. 18), whereby the strongest effect was shown in E2-treated rats. However, the high glucose levels that are described for obese male ZDF rats were not reached. Apparently, the lower estrogen levels in males cannot be solely responsible for gender dimorphism of glucose homeostasis in ZDF rats.

Glucose homeostasis in liver, adipose tissue, and skeletal muscle

IRMS was used to elucidate the glucose uptake in metabolic relevant tissues further (in SHAM, OVX, OVX+E2, and OVX+Alpha animals). 3.5 h after an oral glucose load the fasted uptake was measured in liver (Fig. 19A): here, in SHAM, E2-, and Alpha-substituted animals the amount of absorbed glucose was substantially higher than in untreated animals. A similar pattern was observed in skeletal muscle (gastrocnemius and soleus) and adipose tissue, although the effect was weak in adipocytes (Fig. 19B–D). Thus, the IRMS results reflect the systemic glucose tolerance in the animals (at least in the SHAM, OVX, OVX+E2, and OVX+Alpha animals).

Clearance of high blood glucose levels is mostly promoted by insulin. In liver insulin regulates glucose metabolism by decreasing gluconeogenesis and inducing glycogenesis. In skeletal muscle (the major site of whole body glucose disposal) and white adipose tissue (the major site of energy storage) insulin initiates the translocation of vesicles containing GLUT4 to the plasma membrane, enabling the uptake of glucose (Gould and Holman, 1993; Magkos et al., 2010).

The metabolic role of ER alpha was investigated in several studies and is better understood than the impact of ER beta.

In liver, ER alpha deficiency was described to result in hepatic insulin resistance compared to wild types, mainly attributed to ineffective suppression of endogenous glucose production. Further, αERKO mice displayed increased hepatic lipogenesis compared to wild type mice, an additional indication of impaired insulin sensitivity due to enhanced lipid accumulation within the liver. βERKO mice were not investigated regarding hepatic insulin resistance and lipid accumulation in this study (Bryzgalova et al., 2006).

In skeletal muscle, the ER alpha was shown to increase GLUT4 expression, while the ER beta mediated its repression (Barros et al., 2009; Barros et al., 2006). The present study confirmed this effect of ER alpha in the gastrocnemius muscle, because GLUT4 mRNA expression was highest in Alpha-treated animal groups (Fig. 20E). A similar pattern was observed by treatment with Gen (effects of Gen will be discussed at the end of the discussion part). Further, the translocation of insulin-dependent GLUT4 in Alpha-treated OVX rats was similar to E2-substituted and SHAM animals and more effective than in untreated OVX rats (Fig. 20A–D and only analyzed in the animals that received a glucose load at the day of section).

The impact of both ER subtypes on glucose metabolism in adipose tissue is less clear but also here the ER alpha seems to increase GLUT 4 expression (Barros et al., 2009).

Taken together, the ipGTT and the IRMS confirmed the anti-diabetic role of ER alpha in leptin resistant ZDF rats. Unfortunately, IRMS data for Beta- or Gen-treated animals

cannot be provided, but the ipGTT of these both groups displayed a similar improved glucose tolerance like Alpha-treated animals (Fig. 18).

The ipGTT data in Beta-treated animals may be explained by the anabolic effect of ER beta that was previously demonstrated: activation of ER beta stimulated the muscle growth in a classical Hershberger assay, accelerated the regeneration of injured skeletal muscles and caused larger soleus muscle fiber areas in rats (Velders et al., 2012; Weigt et al., 2012). These findings are in line with the soleus muscle fiber sizes presented in this study, where the Beta-substituted OVX ZDF rats again displayed the largest cross-sectioned fiber areas in the soleus muscle (Fig. 21). This observation in Beta-treated rats might compensate the decreased GLUT4 expression, which was described for ER alpha deficient mice (Bryzgalova et al., 2006) and measured on gene expression level in this study. However, the increased muscle mass and the fact that the slow-twitch fiber type soleus generates energy predominantly by means of oxidative metabolism it can be hypothesized that at least Beta-treated animals possess an enhanced utilization of glucose, which results in an improved glucose tolerance.

The strongest effect regarding improved systemic glucose tolerance was observed in E2-treated animals and might be explained through activation of both signaling pathways.

Genistein

The isoflavone Gen in this study was given orally in a range from 55 to 30 mg/kg/day (changes in intake must be attribute to body weight gain during the experimental period, observed in all animal groups). Such a dosis is relatively high – an human equivalent dosage (Reagan-Shaw et al., 2008) can only be reached by dietary supplements using a dose, which is three to four times higher than the maximum dose commonly used for postmenopausal treatment (Andres and Lampen, 2012; Hooper et al., 2010; Messina and Wood, 2008; Steinberg et al., 2011; Wei et al., 2012). Treatment with Gen showed significant effects in skeletal muscle as follows. Compared to untreated OVX ZDF rats, Gen-treated animals revealed significantly larger soleus muscle fiber areas similar to that of Beta-treated animals (Fig. 21). However, Gen-treated rats also showed increased GLUT4 mRNA expression in the gastrocnemius muscle similar to Alpha-treated animals (Fig. 20E). Consequently, the systemic glucose tolerance in animals that were treated with Gen was improved compared to untreated OVX rats and similar to SHAM and substituted animals (Fig. 18). The ability of Gen to activate ER beta-selective mechanisms such as anabolic effects was already shown in a recent publication of our group: Gen activates anabolic mechanisms in the skeletal muscle similar to Beta (Velders et al., 2012). However, in contrast to Alpha and Beta – at the given doses – Gen binds to both receptors even. Therefore, it can be speculated that Gen-treated animals may improve

their glucose homeostasis through enhanced expression of GLUT4 as well as larger muscle fibers in skeletal muscle.

Conclusion

In summary, the results provide evidence that ER alpha affects the feeding behavior of female ZDF rats independent of leptin signaling. Substitution of OVX rats with E2, ER subtype-selective agonists Alpha and Beta, and Gen improved systemic glucose tolerance compared to untreated OVX rats. This result correlates to the accelerated glucose uptake in liver, adipocytes, and skeletal muscle in the appropriate animal groups (only measured in SHAM, OVX, E2, and Alpha-treated animals). In skeletal muscle, the ER alpha ameliorated glucose homeostasis through an increased expression of GLUT4 and translocation to the cell membrane. By contrast, the ER beta enlarges the muscle fibers that may result in enhanced glucose utilization. The isoflavone Gen showed an increased GLUT4 expression level (similar to Alpha) as well as larger soleus muscle fiber areas (similar to Beta). Therefore, Gen seems to affect both pathways (when provided in the form and dose used in this study). Taken together, ER alpha accelerated the glucose absorbance in liver and adipose tissue and decreased food intake. Activating either ER subtype improved the glucose metabolism in female ZDF rats, but at least in the skeletal muscle via two different molecular pathways.

Chapter Five

General discussion

In this final chapter, the key findings of the thesis are represented which subsequently will be discussed and compared with the current literature. Against this background, a conclusion and outlook are given finally.

As the main sex hormones in women, estrogens are essential for menstrual cycle control, reproduction, and development of female secondary sexual characteristics. Also in men, balanced levels of estrogens contribute to male fertility and a healthy libido. Beside their important function on the reproductive system, estrogens play an important regulatory role on the cardiovascular, immune, excretory, and central nervous system as well as in metabolic processes (Heldring et al., 2007). This far-reaching influence of estrogens on the whole body becomes obvious in pathophysiological conditions when estrogen production is disturbed as well as after menopause when the primary function of the ovaries has ceased. In both cases, of those affected have a higher risk of developing numerous diseases, e.g., cardiovascular disease, neurogenerative diseases, various types of cancer (breast, endometrium, colon, prostate), osteoporosis, and obesity (Deroo and Korach, 2006; Louet et al., 2004; Rolland et al., 2007; Toth et al., 2000b). Obesity is the result of a chronic imbalance of energy homeostasis, meaning that intake of energy continuously exceeds its expenditure. Particularly, due to the rapidly increasing prevalence of obesity and co-morbidities, the impact of estrogens on processes related to energy homeostasis became the research focus in the last years. Estrogens act through activation of two distinct ER subtypes, ER alpha and ER beta (Enmark and Gustafsson, 1999; Heldring et al., 2007; Nilsson et al., 2001). Since both receptors could serve as the basis for therapeutic interventions it is of great interest to discriminate between physiological processes regulated through ER alpha, ER beta or both. Substances that may play a role in therapy of estrogen-associated diseases could be of synthetic origin (SERMS) but also plant-derived (phytoestrogens). Here, the isoflavone genistein, which can activate both ER subtypes, are under intensive investigation.

Key findings of the presented work

The aim of the present work was to obtain more detailed insights into the action of E2 on energy intake, body weight, and metabolism of glucose and lipids in adipose tissue, liver, and skeletal muscle by means of animal experiments. Special emphasis was given to the effects of the two ER subtype-selective agonists Alpha (16alpha-LE2) and Beta (8beta-VE2) and the isoflavone genistein.

The major finding was:

- The ER beta-selective agonist results in an enhanced utilization of glucose and lipids by anabolic activity.

Other key findings were as follows:

- Activation of ER alpha affects feeding behavior in ZDF rats – an effect that might be relevant in leptin resistant individuals.
- Estradiol reduces body weight gain via the stimulation of ER alpha.
- Estradiol decreases lipogenesis via either ER subtype-selective agonist in adipose tissue, liver, and skeletal muscle.
- Estradiol improves glucose tolerance and insulin sensitivity via either ER subtype – at least in the skeletal muscle by two different molecular pathways.
- Genistein exert effects on energy homeostasis similar to those of the ER beta-selective agonist.

ER beta signaling results in an enhanced utilization of glucose and lipids by anabolic activity

Menopause is characterized by an increase of adipose tissue and a loss of muscle mass, resulting in a significant change of the body composition. In average, postmenopausal women have 20% more fat than premenopausal women (Ley et al., 1992). Further, studies have shown a muscle mass decline of 0.6% per year after menopause (Rolland et al., 2007).

In postmenopausal women, redistribution of fat pads can be observed as follows: the “gynoid” fat distribution pattern, which is typical for premenopausal women and characterized by fat accumulation around the hips and thighs, becomes similar to that in men (also referred to as “android” fat distribution pattern). In men, fat is mainly

accumulated around the trunk and upper body such as the abdomen, chest, neck and shoulder (Ley et al., 1992). Epidemiologic studies correlate the “android” fat pattern with an increased risk to develop cardiovascular disease and insulin resistance (Anghel et al., 2007).

In light of the above-mentioned findings, the anabolic potency of the ER beta-selective agonist that was observed in the presented work may be highly relevant. Rats treated with E2 and the ER beta-selective agonist showed a significant modulation of soleus muscle genes involved in muscle growth, repair and maintenance (Pax7 and Igf1) compared to untreated OVX animals (chapter two). Further, treatment with the ER beta-selective agonist resulted in the largest soleus muscle fibers in Wistar rats with nutrition-induced obesity (chapter two) as well as in obese leptin resistant ZDF rats (chapter four).

This specific role of ER beta in skeletal muscle homeostasis was previously shown in a model of toxin-induced skeletal muscle damage. Here, ER beta signaling accelerated the regeneration of injured skeletal muscles in female Wistar rats, whereas ER beta depletion in ERKO mice resulted in a reduced ability. Anabolic potency of the ER beta-selective agonist was also shown in a classical Hershberger assay with healthy male Wistar rats (Velders et al., 2012).

Thus, the ER beta seems to have the potency to increase skeletal muscle mass that in turn improves muscle/fat ratio of the body, and enhances skeletal muscle uptake and utilization of fuels. This important observation might explain the improved lipid and glucose metabolism in animals treated with the ER beta-selective agonist compared to untreated animals, which will be discussed in detail below.

Activation of ER alpha affects feeding behavior in ZDF rats – an effect that might be relevant in leptin resistant individuals

Increased daily food consumption with insufficient energy expenditure is considered as the main cause for the development of obesity. In the relevant literature, the influence of estrogens on central regulation of energy balance is still a matter of debate.

My studies indicate that in a long-term experiment of nutrition-induced obese female Wistar rats neither E2 nor other ER ligands had a significant effect of energy intake (chapter two). The higher food intake that was observed in animals on LF diet had to be attributed to its lower energy content compared to the HF diet (chapter two). This result is in line with previous findings from our laboratory (Hertrampf et al., 2006a; Hertrampf et al., 2007). In contrast, E2 treatment in leptin resistant female obese ZDF rats decreased

weekly energy intake markedly throughout the experimental period; an effect that was mediated via the ER alpha (chapter four).

In the literature, there are animal studies showing that ovariectomy increases daily food consumption. This effect was often described in short-term experiments (Ainslie et al., 2001; Chen and Heiman, 2001; Latour et al., 2001; Roesch, 2006; Saengsirisuwan et al., 2009; Santollo et al., 2007; Silva et al., 2010) and related to activation of ER alpha (Roesch, 2006; Santollo et al., 2007). Studies in αERKO rodents provided also different results: deletion of ER alpha showed both an increase of daily food consumption (Geary et al., 2001; Musatov et al., 2007; Naaz et al., 2002) and no effect on this parameter (Faulds et al., 2012; Heine et al., 2000).

Leptin is the most important protein that regulates energy intake and expenditure by acting on several neurons on the hypothalamus. Some of these neuronal subsets, which are involved in leptin signaling, have also been described to express the ER alpha (ventromedial nucleus), the ER beta (paraventicular nucleus) or both ER subtypes (arcuate nucleus). Several animal studies were carried out to elucidate the action of E2 in the central nervous system, but the outcomes were somewhat controversial. Both administration of E2 in the paraventricular nucleus (Bonavera et al., 1994; Palmer and Gray, 1986) and in the ventromedial nucleus (Jankowiak and Stern, 1974; Wade and Zucker, 1970) have shown to reduce food intake in OVX rats, indicating that both ER subtypes may be involved in eating behavior. A further study in OVX female rats utilizing intracerebroventricular infusions of E2 alone or in combination with antisense oligodeoxynucleotides for both ER subtypes postulated that the ER beta is responsible for the anorectic action of E2 (Liang et al., 2002).

Taken together, my studies indicate that activation of ER alpha has the potency to attenuate daily energy intake. However, this effect only becomes an issue when individuals are resistant to leptin. Whether and to what extent the ER beta may play a role in energy consumption remains to be clarified.

Estradiol reduces body weight gain via the stimulation of ER alpha

Obesity is defined as excessive fat enrichment with a BMI greater than 30 kg/m^2 in adults. Therefore, increased body weight is an initial predictor for generation of obesity. All animal experiments that were carried out in the presented work demonstrated a significantly lower body weight gain by treatment with E2 (chapter two and four) and the ER alpha selective agonist (chapter two and four) when compared to untreated OVX animals. These data agree with data from the literature (Roesch, 2006; Saengsirisuwan et al., 2009;

Santollo et al., 2007; Wegorzewska et al., 2008) and with previous results from our group (Hertrampf et al., 2007; Hertrampf et al., 2008a; Hertrampf et al., 2008b). To my knowledge, only one study in OVX rats showed a decreased body weight gain by activation of ER beta – an effect resulting from decreased food intake by the rats compared to OVX animals (Liang et al., 2002). Body weight data from ERKO mice are more controversial. Increased body weight was shown in αERKO mice (Bryzgalova et al., 2006; Geary et al., 2001; Musatov et al., 2007; Naaz et al., 2002), but also in βERKO mice (Foryst-Ludwig et al., 2008; Seidlova-Wuttke et al., 2012) when compared to wild type counterparts.

To date the cause of body weight gain following estrogen deficiency is not exactly known. Multiple factors may contribute to the E2-induced reduced rate of body weight gain including both central and peripheral effects. As mentioned above, the attenuated daily food intake mediated via ER alpha was discussed in animal models of E2 depletion (OVX) (Roesch, 2006; Santollo et al., 2007) and ERKO (Geary et al., 2001; Musatov et al., 2007; Naaz et al., 2002) as one important reason for the higher body weight gain. Further, decreased energy expenditure shown in αERKO mice compared to wild types was also discussed to cause increased body weight in these animals (Heine et al., 2000; Musatov et al., 2007). The latter observation is in line with a previous study in our laboratory. Here, ovariectomy of Wistar rats significantly decreased their movement drive compared to SHAM animals – an effect that was antagonized by treatment with E2 and Alpha but not by application of Beta (Hertrampf et al., 2007). Moreover, an older study using intact female rats showed that energy expenditure of rats is highest during proestrus when circulating levels of estrogens are highest (Anantharaman-Barr and Decombaz, 1987).

In light of the specific role of ER beta on skeletal muscle, a further explanation for the increased body weight in Beta-treated animals could be an altered body composition. In Beta-treated Wistar rats, visceral fat content and related parameters (adipocyte size, leptin levels, lipo-/adipogenesis) were lower than in OVX animals and similar to Alpha- and E2-treated animals. In contrast, the complete body weight of Beta-treated animals was comparable to OVX rats.

Taken together, the conducted studies demonstrate that activation of ER alpha is responsible for the reduced body weight gain in E2-treated rats. Increased movement drive by activation of ER alpha seems to be an important factor for this observation, while feeding behaviour does not appear to be essential. Based on the observed anabolic potency of ER beta the increased body weight by activation of ER beta might indicate that in these animals the body composition has changed.

Estradiol decreases lipogenesis via either ER subtype-selective agonist in adipose tissue, liver, and skeletal muscle

Obesity is characterized by excessive fat enrichment in the abdominal cavity. Moreover, an excess supply of fuel molecules leads to increased lipogenesis and further to fat accumulation in non-adipose tissues like liver and muscle. Hence, such affected tissues are implicated in an impaired insulin signaling (Anghel and Wahli, 2007). The liver is the key organ in composition of blood lipids. Here, FFA (*de novo*-synthesized or delivered by food) are processed to triglycerides that are then incorporated into VLDL for transport via blood to adipose tissue (storage) or muscle (energy production). A dyslipidemic profile in the blood is one important risk factor to develop cardiovascular diseases and is often associated with obesity, impaired insulin sensitivity but the loss of ovarian function also comes into play (Baxter et al., 2003; Chan and Woo, 2010; van Beek et al., 1999).

Influence on adipose tissue

The work presented here demonstrated that E2 treatment of obese Wistar rats results in lower visceral fat content, smaller adipose cells, and reduced circulating leptin levels compared to untreated OVX animals. Further, this effect was mediated via either ER subtype-selective agonist (chapter two). This was in line with the observation that both ER subtype-selective agonists decreased adipose mRNA levels of several genes that are involved in lipogenesis (SREBP1c and FAS) and adipogenesis (LPL and PPAR gamma) (chapter three).

The anti-lipogenic effect of E2 on adipose cells was described before by different authors (Bryzgalova et al., 2008; Cooke and Naaz, 2004; D'Eon et al., 2005; Rogers et al., 2009; Saengsirisuwan et al., 2009). Only few studies were conducted to evaluate whether these actions are mediated through ER alpha, ER beta or both receptors and the results from these studies are still controversially discussed. The key role on fat homeostasis is attributed to the action of ER alpha, because this ER subtype was shown to reduce the accumulation of triglycerides, expression of LPL, and adipose tissue mass (Bryzgalova et al., 2006; Heine et al., 2000; Homma et al., 2000). However, the impact of ER beta was not investigated in these studies. Another study using only βERKO mice revealed an anti-lipogenic action of ER beta through a negative cross talk with PPAR gamma (Foryst-Ludwig et al., 2008). In summary, these reports already indicate that both ER subtypes are involved in the anti-lipogenic action of estrogens in adipose tissue. This indication is supported by my studies: the observed effects are mediated by activation of either subtype.

Influence on liver and skeletal muscle

In the rat model of nutrition-induced obesity, the anti-lipogenic action of E2 was also shown in liver and soleus muscle. Treatment with E2 and both ER subtype-selective agonists reduced both TG accumulation and mRNA levels of lipogenic genes (SREBP-1c and FAS). By contrast, mRNA expression of markers involved in fatty acid utilization and transport showed no significant differences between the experimental animal groups (chapter three).
Similar to adipose tissue an E2-induced decrease of lipogenesis in liver and muscle was shown before (Bryzgalova et al., 2008; D'Eon et al., 2005; Gao et al., 2006) and related to activation of ER alpha (Bryzgalova et al., 2006). But again, the impact of ER beta was not investigated.

Influence on blood lipids

In obese Wistar rats ovariectomy resulted in significantly increased serum levels of TC and the lipoproteins HDL, LDL, and VLDL compared to SHAM. E2 treatment markedly reduced levels of TC and VLDL, whereas LDL levels were moderately decreased – an effect that was mediated by activation of ER alpha. HDL levels were not influenced by any treatment (chapter two). Thus, the LDL/HDL as well as (VLDL+LDL)/HDL ratios were more favourable with respect to the development of cardiovascular diseases in E2- and Alpha-treated animals. This observation confirms the relative cardiovascular protection of premenopausal women compared to women after menopause (Dubey et al., 2005; Ling et al., 2006). However, based on the supposed higher metabolic rate in Beta-treated animals the observed higher blood lipids could be an indication for enhanced transport of lipid molecules to the muscle for energy production.

Taken together, in the study with obese Wistar rats highly selective agonists for both ER subtypes were used in one experiment. To my knowledge, this is the first report demonstrating that stimulation of either ER subtype participates in the anti-lipogenic properties of E2 in adipose, liver, and skeletal muscle. Moreover, at least in part the underlying molecular mechanisms are similar. However, an additive effect by treatment with E2 including activation of both ER subtypes was not observed.

Estradiol improves glucose tolerance and insulin sensitivity via either ER subtype – at least in the skeletal muscle by two different molecular pathways

Insulin resistance is one obesity-associated risk factor and is characterized by an inadequate response of cells to the action of insulin.

Wistar rats with nutrition-induced obesity were normoglycemic and did not show significant differences on fasting glucose and insulin levels after 10 weeks (chapter three). However, insulin levels were highest in untreated OVX animals compared to animals that received estrogenic compounds or intact rats (chapter three). The calculated HOMA-Indices fit these data, indicating that untreated OVX animals need higher insulin levels for glucose disposal from the blood than the other groups. By contrast, ovariectomy increased hepatic rate of glucose uptake – an effect that was antagonized by treatment with E2 and Alpha but not by Beta and Gen. mRNA expression pattern of PPAR gamma (a marker for increased insulin sensitivity) correlated to hepatic glucose uptake, because highest mRNA level of PPAR gamma was found in untreated, Beta- and Gen-treated animals (chapter three). However, in light of increased TG accumulation in liver and muscle of untreated OVX animals at least in these animals the up-regulation of PPAR gamma mRNA could be an offset mechanism to improve insulin sensitivity.

In obese leptin resistant ZDF rats, estrogen deficiency led to a more disturbed glucose homeostasis compared to Wistar rats with nutrition-induced obesity. In female ZDF rats, ovariectomy already resulted in significant higher fasting glucose levels compared to SHAM and E2-treated rats (chapter four). Activation of either ER subtype showed at least the same tendency. The results obtained from the performed ipGTT were more obvious: treatment with all estrogenic compounds improved glucose tolerance compared to untreated OVX rats. The strongest effect was observed in E2-treated animals that significantly better tolerated the glucose load at each time point compared to OVX. Moreover, increased glucose uptake was also measured in liver, adipose tissue, and skeletal muscle in SHAM, E2-, and Alpha-treated animals compared to OVX. Further investigations in the skeletal muscle indicated that stimulation of the two ER subtypes activates two distinct molecular pathways: GLUT4 expression and translocation to the cell membrane in the gastrocnemius muscle was most effective by treatment with Alpha, while Beta treatment led to the largest soleus muscle fiber sizes compared to OVX rats.

In the literature, improved sensitivity towards glucose and insulin (systemic and of peripheral tissues) by E2 was shown in several studies using either animal models of ERKO or E2 replacement/application (Bryzgalova et al., 2008; Gao et al., 2006; Riant et al., 2009; Saengsirisuwan et al., 2009). This finding was related to activation of ER alpha (Barros et al., 2009; Barros et al., 2006; Bryzgalova et al., 2006; Heine et al., 2000;

Lundholm et al., 2008) Lundholm 2008, Barros 2006, 2009). Investigations with βERKO mice showed that the ER beta had either no effect (Bryzgalova et al., 2006) or an adverse impact (Barros et al., 2009; Foryst-Ludwig et al., 2008) on systemic glucose sensitivity compared to wild types. The pro-diabetogenic action of ER beta was described to result from decreased muscular GLUT4 expression and distribution (Barros et al., 2009; Barros et al., 2006) or a negative cross-talk with adipose PPAR gamma (Foryst-Ludwig et al., 2008). The finding from Barros and colleagues fit to my results so far that the ER alpha is responsible for GLUT4 modulation. The described PPAR gamma effect by Foryst-Ludwig and colleagues was in my studies also mediated through ER alpha stimulation.

Taken together, my studies demonstrate that either ER subtype improves glucose and insulin sensitivity. At least in the skeletal muscle, the underlying molecular pathways are different. Interestingly, systemic glucose tolerance was highest in E2-treated ZDF rats that might be an indication for an additive effect by activation of both pathways in skeletal muscle.

Genistein exerts effects on energy homeostasis similar to those of the ER beta-selective agonist

The influence of the isoflavone Gen on energy metabolism has been the focus of numerous studies. The results acquired regarding food intake, body weight, adipose tissue mass (reviewed in Orgaard and Jensen, 2008) and glucose metabolism (Behloul and Wu, 2013; Fu et al., 2010; Lee et al., 2009; Zhong et al., 2011) are very different. The reasons therefore are many variables, such as *in vitro* or *in vivo* studies, age and sexes of animals used, food composition, use of soy protein or isolated isoflavones, doses used and dosage form, individual intestinal microbiota, duration of the experimental period as well as differences in the applied methods.

In my studies, Gen was given orally by enriched food that corresponds to the usual route of exposure. In both animal experiments, the daily Gen intake has changed during the experimental period due to body weight gain and differences in food intake of the animals. However, the dose used was relatively high and can only be reached by dietary supplements in humans (Reagan-Shaw et al., 2008). A human equivalent dose can be obtained using a daily dose, which is approximately two to four times higher than the maximum dose commonly used for postmenopausal treatment (Andres and Lampen, 2012; Hooper et al., 2010; Mahady, 2005; Messina and Wood, 2008; Steinberg et al., 2011; Wei et al., 2012). Gen and Gen-metabolites may act in a synergistic manner,

whereby their concentrations may differ dependeng on tissue type. In general, in my studies administration of Gen resulted in effects very similar to those that were observed by treatment with Beta.

Comparable to Beta treatment, Gen administration to OVX animals showed no effect on uterine wet weight, energy intake, and body weight in both animal models compared to untreated OVX rats (chapter two and four). These observations confirm – at least in part – studies that used a similar dosage of genistein (Hertrampf et al., 2009b; Kim et al., 2006; Naaz et al., 2003; Penza et al., 2006).

Compared to untreated OVX animals, in liver and muscle of nutrition-induced obese Wistar rats Gen and Beta treatment led to a similar reduction of hepatic and muscular lipogenesis and TG content. Further, muscular mRNA expression of Igf1 and Pax7 was modulated in a similar manner. No influence of Gen and Beta treatment was observed regarding hepatic and muscular PPAR gamma mRNA expression, glucose uptake in liver, and blood lipid profile. Contrary to Beta treatment no influence of Gen was measured on adipose tissue (visceral fat mass, adipocyte size, circulating leptin levels, and lipo-/adipogenetic mRNA levels) when compared to untreated OVX rats (chapter two and three).

Also in obese ZDF rats, administration of Gen showed very similar effects to Beta treatment regarding fasting glucose, ipGTT curve, and soleus muscle fibers (chapter four). Especially the ability of Gen to act in an ER beta-specific manner on skeletal muscle was shown before (Velders et al., 2012).

The specific action of Gen on various tissues may depend on their ability to accumulate Gen or its metabolites. Such data are very limited, but there are reports showing that particularly endocrine-active tissues (thyroid gland, liver, brain, prostate, ovaries, uterus, and mammary gland) accumulate high levels of Gen, whereby its concentration often exceeds plasma levels (Chang et al., 2000; Hedlund et al., 2005). Interestingly, Gen concentration in livers of female Sprague-Dawley rats was approximately ten times higher than in male livers (Chang et al., 2000). This may explain the observed hepatic effects of Gen in my studies. To my knowledge, no data are available for Gen accumulation in adipose tissue and skeletal muscle so far.

Gen's preference to bind to ER beta was reported before by Kuiper and colleagues and my results confirm this observation (Kuiper et al., 1998). However, unlike to the ER subtype-selective agonist Gen can act via both ER subtypes. Gen's preference for one of the two ER subtypes seems also to depend on their expression ratio in the respective tissue. This was shown in previous studies of our group (Hertrampf et al., 2007; Schleipen et al., 2011). Coexpresion of ER alpha and beta in skeletal muscle cells (Barros et al., 2009) could explain the observed effects of Gen in the skeletal muscle of ZDF rats, where

Gen treatment seems to affect both pathways (GLUT4 expression via ER alpha and muscle fiber size via ER beta).

Finally, Gen also modulates non-hormonal effects. Therefore, the here observed effects do not have to be exclusively mediated via ERs.

Taken together, results obtained from this work indicate that Gen acts similarly to the ER beta-selective agonist (except on adipose tissue). Especially, the similar ability to induce anabolic activity in the soleus muscle might be highly relevant. A more effective utilization of fuels could explain the lower TG content in muscle and liver as well as improved glucose metabolism in the respective animals. Gen might offer an accessible and cheap treatment alternative to already existing therapies. In general, more research is warranted regarding tissue-specific accumulation and potential adverse effects of genistein such as cancer risk. With regard to my results, the effects of Gen observed in this work should be examined by using lower Gen doses (comparable to dietary supplements) to minimize potential adverse effects.

Conclusion and outlook

With the onset of menopause, the enourmous decline of endogenous estrogens results in far-reaching physiological changes that – in combination with physical inactivity – are the major reason for the development of obesity and co-morbidities. New strategies that focus on prevention and therapy of obesitiy in menopausal and postmenopausal women have to be found.

My studies clearly demonstrate that estrogens decrease lipogenesis and increase insulin sensitivity via either ER subtype. Especially the observed anabolic potency of ER beta in the skeletal muscle, which seems to be the underlying molecular mechanism for improved glucose and lipid metabolism in such treated animals, might be of great importance. ER beta signaling is not involved in regulation of proliferation of uterus and mammary gland (described before by (Curtis Hewitt et al., 2000; Diel et al., 2004; Molzberger et al., 2011) and shown in my studies by uterine wet weights). Therefore, compounds that only activate ER beta might be an excellent strategy for prevention and therapy of obesity and co-morbidities in postmenopausal women without increasing the risk for estrogen-sensitive forms of cancer.

Beside synthetic SERMs, the natural phytoestrogen Gen might be a further alternative, because Gen administration affects skeletal muscle homeostasis in a similar way to Beta treatment. Gen can activate both ERs and is already placed on the marked for treatment of postmenopausal complaints. Women, who suffer from climacteric

complaints, are recommended to replace hormones. HRT is based on the idea to treat such discomforts by supplementing estrogens and progestins. However, conventional HRT is strongly associated with a larger incidence of heart attacks, strokes, and breast cancer (most likely through overstimulation of proliferation by estrogens (Beral, 2003). Previous animal studies of our laboratory, and shown in the presented work, revealed that Gen, when given orally, does not, or only very weakly, induce uterus proliferation (Hertrampf et al., 2006b; Hertrampf et al., 2009a; Hertrampf et.al., 2009b). Therefore, Gen might be an excellent alternative – not only to the conventional HRT for treatment of climacteric complaints, but also as a strategy for prevention and therapy of obesity and co-morbidities. However, the role of Gen on the incidence of breast cancer in postmenopausal women – particularly in the Western world – is discussed controversially and needs further investigations (Andres and Lampen, 2012; Hertrampf et al., 2006b; Molzberger et al., 2012).

Therefore, based on my studies I conclude that an effective way to treat obesity and co-morbidities in postmenopausal women might be substances that only activate ER beta. A combination with physical activity may support the therapy of obesity and co-morbidities. For postmenopausal women suffering from climacteric complaints as well as obesity and co-morbidities, the isoflavone Gen might be a natural alternative.

To verify my results more research is warranted. This includes both *in vitro* and *in vivo* experiments. *In vitro* studies enable a more detailed insight in the underlying molecular mechanisms of which molecular pathway either ER subtype-selective agonist influences metabolism. Further, the question whether the observed effects of estrogens are ER-mediated can be addressed by using selective ER agonists and antagonists in combination. Animal studies using both ER subtype-selective agonists may offer deeper knowledge in the action of E2 and/or physical activity in liver, adipose tissue, brain, and skeletal muscle but also the endocrine pancreas. Foremost a combination of physical activity and Beta treatment and whether Gen might be used as an alternative should be investigated by using animal experiments. Moreover, human intervention studies should be performed. Here the IRMS analyses provide a very interesting tool to encourage stategies for prevention and therapy of obesity and its metabolic consequences particularly in postmenopausal women. Measurements of $^{15}N/^{14}N$ ratios enable to distinguish whether an organism is more in an anabolic or catabolic state (Huelsemann et al., 2009). Human urine and/or hair can serve as test material and can be taken easily. Moreover, based on the metabolic fluxes within a human body the appropriate level of physical activity as well as the influence of dietary isoflavones (e.g., soy milk) can be evaluated.

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List of Publications

Publications (in Books and Journals)

*P.R. Diel and **C. Weigt**.* Leptin and Obesity in Ovarian Dysfunction in Menopause (book chapter), in: C.J.H. Martin et al. (eds.), Nutrition and Diet in Menopause, Nutrition and Health, DOI 10.1007/978-1-62703-373-2_19, © Springer Science+Business Media New York 2013 – In press.

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under review

Carmen Weigt, *Torsten Hertrampf, Felix M. Kluxen, Ulrich Flenker, Frank Hülsemann, Karl Heinrich Fritzemeier, Patrick Diel.* Molecular effects of ER alpha- and beta-selective agonists on regulation of energy homeostasis in obese female wistar rats. Resubmitted for publication in Molecular and Cellular Endocrinology in May 2013.

Abstracts (for Poster and Talks)

C Weigt, *T Hertrampf, U Flenker, F Huelsemann, KH Fritzemeier, P Diel*. Impact of estradiol, estrogen receptor subtype specific agonists and genistein on food intake, body weight, and glucose metabolism in leptin resistant ovariectomized Zucker diabetic fatty rats. (Abstract for Poster P660; EuroPRevent, the EACPR Annual Meeting, April 18-20, 2013, Rom, Italy)

Weigt C*, Hertrampf T, Flenker U, Hülsemann F, Fritzemeier KH, Diel P.* Impact of estradiol, estrogen receptor subtype specific agonists and genistein on food intake, body weight, and glucose metabolism in leptin resistant ovariectomized Zucker diabetic fatty rats. (Abstract for talk OP4-25; 56. Symposium der Deutschen Gesellschaft für Endokrinologie, March 13-16, 2013, Düsseldorf)

Weigt C*, Hertrampf T, Zoth N, Fritzemeier KH, Diel P.* Impact of Estradiol, ER Subtype specific Agonists and Genistein on Energy Homeostasis in a Rat Model of Nutrition induced Obesity. (Abstract for Poster P2 5-4; 55. Symposium der Deutschen Gesellschaft für Endokrinologie, March 7-10, 2012, Heidelberg/Mannheim)

Weigt C*, Hertrampf T, Fritzemeier KH, Diel P*: Impact of Estradiol, ER Subtype specific Agonists and Genistein on Energy Homeostasis in a Rat Model of Nutrition induced Obesity. (Abstract for Poster; Spetses Summer School on Nuclear Receptor Signaling in Physiology and Disease, August 28-September 2, 2011, Greece)

Weigt C*, Hertrampf T, Laudenbach-Leschowsky U, Fritzemeier KH, Diel P*: Impact of Estradiol and Genistein on Energy Homeostasis in a Rat Model of Nutrition induced Obesity. (Abstract for Poster PS1-03-4; 54. Symposium der Deutschen Gesellschaft für Endokrinologie, March 30-April 2, 2011, Hamburg)

Weigt C*, Hertrampf T, Flenker U, Hülsemann F, Laudenbach-Leschowsky U, Fritzemeier KH, Diel P:* Effects of ER subtype specific ligands on energy homeostasis in a rat model of nutrition induced obesity – analysis of glucose uptake by IRMS. (Abstract for talk; GASIR; German Association of Stable Isotope Research, October 4-6, 2010, Köln)

Weigt C*, Zoth N, Hertrampf T, Laudenbach-Leschowsky U, Fritzemeier KH, Diel P*: Combinative impact of estradiol, genistein and physical activity on energy homeostasis. (Abstract for Poster PS1-05-4; 53. Symposium der Deutschen Gesellschaft für Endokrinologie, March 3-6, 2010, Leipzig)

Weigt C*, Zoth N, Hertrampf T, Laudenbach-Leschowsky U, Fritzemeier KH, Diel P*: Impact of Estradiol and Genistein on Energy Homeostasis. (Abstract for Poster; 4th International Symposium, October 7-8, 2009, Düsseldorf)