Effects of estrogen on gene expression profiles in mouse hypothalamus and white adipose tissue: target genes include glutathione peroxidase 3 and cell death-inducing DNA fragmentation factor, α -subunit-like effector A

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Abstract

Obesity has become a major health problem in many parts of the world. Estrogens are known to reduce adipose tissue mass in both humans and animals but the molecular mechanisms are not well characterized. We used gene expression profiling to study long-term effects of estrogen on gene expression in mouse white adipose tissue and hypothalamus. Overall, the effects of estrogen on hypothalamic gene expression were much smaller than the corresponding effects on white adipose tissue gene expression. We characterize in detail estrogenic regulation of glutathione peroxidase 3 (GPX3). Our studies suggest that GPX3 is a direct estrogen receptor α target gene in white adipose tissue. Since obesity is correlated with

oxidative stress, and GPX3 has been demonstrated to be lower in obesity and higher after weight loss, we hypothesize that GPX3 is one important mediator of effects of estrogen in relation to fat mass. Additional genes that were affected by estrogen in adipose tissue include cell death-inducing DNA fragmentation factor, α -subunit-like effector A (*CIDEA*), a gene shown to be related to body fat in mice. We conclude that estrogen has large effects on gene expression in white adipose tissue and hypothesize that GPX3 and *CIDEA* could be important mediators of the effects of estrogen on fat mass.

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Introduction

Obesity has become a major health problem in many parts of the world. Overweight and obesity are associated with an increased risk for diseases such as type 2 diabetes, hypertension, coronary heart disease, and certain cancers (Khaodhiar *et al.* 1999). The disease incidence is primarily related to a central, android fat distribution, typical for males (Blaak 2001).

Epidemiological and experimental studies link estrogen to the maintenance and distribution of body fat. This includes observations that women usually increase their body fat mass after menopause when estrogen levels decrease, and also shift to an android fat distribution (Lovejoy 2003) and that estrogen administration reduces adipose tissue mass in both humans and animals (Wade & Gray 1979, Mattiasson *et al.* 2002). The molecular mechanisms for these effects are, however, not well characterized.

Estrogens exert their effects via two nuclear receptors, estrogen receptors (ER) α and β (Kuiper *et al.* 1996, Nilsson *et al.* 2001). The receptors function as ligand-dependent

transcription factors that bind to estrogen-response elements (EREs) or, for example, in association with fos and jun to activator protein 1 (AP-1) sites in target gene promoters (Nilsson *et al.* 2001). ER α is the main ER mRNA expressed in mouse adipose tissue (Lundholm *et al.* 2004). Both ER α and β mRNAs are expressed in mouse hypothalamus (Couse & Korach 1999).

Knockout mouse models have shed light on the role of estrogen and its receptors in rodent obesity. Mice that lack aromatase (ArKO mice) are unable to synthesize endogenous estrogen and display an obese phenotype (Jones *et al.* 2000). A similar phenotype is observed in mice lacking ER α (ERKO) but not ER β (BERKO), indicating that ER α is the major mediator of the effects of estrogen to reduce fat mass in mice (Heine *et al.* 2000, Ohlsson *et al.* 2000). This is further supported by the decreased body weight seen in rats after treatment with an ER α -selective agonist propyl pyrazole triol (PPT) but not an ER β -selective agonist diarylpropionitrile (DPN; Roesch 2006).

Estrogen could influence adipose tissue mass by central and peripheral effects. Regulation via the central nervous system

could include behavioral regulation of physical activity and feeding, where the hypothalamus has a central role in regulating appetite and satiety. There are reports on increased running wheel activity in estrogen-treated mice, primarily mediated through ER α (Ogawa *et al.* 2003). Estrogen has been shown to reduce food intake, postulated to occur partly via increased cholecystokinin signaling and negative feedback on meal size (Geary 2001). However, it has been shown that changes in food intake are not sufficient to explain the effects of estrogen on body weight and fat mass (Roy & Wade 1977). Finally, ERKO mice develop obesity despite equal food intake as wild-type (WT) mice, demonstrating that signaling via ER α affects fat mass via mechanisms that are distinct from effects on food intake (Heine *et al.* 2000).

Studies of changes in adipose gene expression in mice in response to estrogen administration support a direct role of the adipose tissue in control of fat mass by demonstrating estrogen regulation of key mediators of lipid synthesis (Lundholm *et al.* 2004, D'Eon *et al.* 2005).

In this study, we determine estrogen-induced changes in gene expression profiles for white adipose tissue (WAT) and hypothalamus, two tissues that could be targets for the effects of estrogen on fat mass.

Materials and Methods

Animals

For the 3-week estrogen treatment study, female C57BL/6 mice were ovariectomized at 11 weeks of age. After recovery for 1 week on normal diet, the animals were kept on soy-free diet for 1 week prior to initiation of treatment and further on during the treatment. Mice were injected daily (s.c.) with 100 μ g/kg 17 β -estradiol (E₂) for 3 weeks and killed at 16 weeks of age ($n=8$). Control mice received injections of vehicle oil (olive oil, Apoteksbolaget, Göteborg, Sweden; $n=9$). Gonadal WAT, interscapular brown adipose tissue (BAT), and hypothalamus were collected and kept at -80°C . Hypothalamus was dissected as in Allan *et al.* (2000), retaining the entire hypothalamus including the arcuate, ventromedial, dorsomedial, and paraventricular nuclei. Hearts were collected from mice exposed to the same experimental conditions.

For the 2-, 4-, and 6-h treatment study, female C57BL/6 mice were ovariectomized at 10 weeks of age, and treated and killed at 14 weeks of age. Mice were injected (s.c.) with 100 μ g/kg E₂ (Sigma), 5 mg/kg ER α -selective ligand PPT; (Tocris Cookson Inc., Ellisville, MO, USA), or 5 mg/kg ER β -selective ligand DPN; (Tocris Cookson Inc.) respectively ($n=5$ per treatment group). Control mice received injections of vehicle oil. Gonadal WAT was collected and kept at -80°C .

Female C57BL/6 mice were sham-operated or ovariectomized ($n=5$) at 11 weeks of age. After recovery for 1 week on normal diet, the animals were put on a soy-free diet for 1 week before they were killed at 13 weeks of age.

Gonadal WAT and interscapular BAT were collected and kept at -80°C . Gonadal WAT was also collected and kept at -80°C from female ($n=4$) and male ($n=6$) C57BL/6 mice killed at 4 months of age, and from female knockout mice of mixed background (with no ovariectomy or treatment). ERKO mice ($n=5$) were killed at 11–13 weeks of age and BERKO mice ($n=3$) were killed at 13 weeks of age, together with their respective WT controls. All studies were approved by local ethical committees for animal experiments.

Microarray analysis and bioinformatics

Total RNA was prepared from gonadal WAT and hypothalamus, from individual animals, using Trizol Reagent (Invitrogen) and further purified using RNeasy Mini Kit (Qiagen). RNA was reverse transcribed into cDNA, *in vitro* transcribed into cRNA, and prepared for DNA microarray analysis according to the Affymetrix Gene Chip Expression Analysis manual (Affymetrix, Santa Clara, CA, USA). Affymetrix Mouse Expression Array 430A was used to profile WAT mRNA and Mouse Genome 430A 2.0 arrays to profile hypothalamus mRNA. These two types of arrays include identical probe pairs and are only distinguished by different feature formats. Three estrogen treatment samples and three vehicle samples from individual mice were analyzed for each tissue. The scanned output files were analyzed using Affymetrix Microarray Suite Version 5.0 software.

Each estrogen-treated sample was compared with each vehicle-treated sample, resulting in nine pairwise comparisons. Genes were regarded as differentially expressed when producing concordant calls (increased or decreased call) in at least seven of nine comparisons (Affymetrix Data Mining Tool Version 3.0.). In addition, genes designated as absent on all chips and genes with mixed probe sets ($\_x\_at$, the probe sets contain at least one probe that cross-hybridizes with other sequences) were excluded from further analysis. For duplicate probe sets, only one set is reported in Table 3 to reduce the complexity of this table. In this case, probe sets are included based on the following criteria: 1. unique identifiers ($\_at$, according to Affymetrix nomenclature), 2. highest number of present calls, 3. highest concordance, and 4. highest average fold change. In Table 3, a higher stringency was applied, only including genes regulated in all comparisons (100% concordance). Using pairwise comparisons with a high concordance level as a filter for identifying differentially expressed genes identifies, from our experience, genes that regularly can be confirmed using an independent approach.

Real-time PCR analysis

An amount of 1 μ g total RNA from individual animals was reverse transcribed into cDNA using reverse transcription reagents with random hexamer primers (Applied Biosystems, Foster City, CA, USA). Analysis was performed using the ABI Prism 7700 Sequence Detection System and 7500 Real-Time PCR System (Applied Biosystems). Primer and probe

sequences are shown in Table 1. An SYBR Green-based protocol was applied for most assays, and PCR products were further analyzed by melting curve analysis to confirm a single product. A TaqMan probe-based protocol was used for glutathione peroxidase 3 (GPX3), uncoupling protein 1 (UCP1), ER α , and β (ER sequences from Lundholm *et al.* 2004). mRNA levels were calculated using the standard curve method (user bulletin no. 2, Applied Biosystems) and normalized to 18S rRNA (Applied Biosystems) or hypoxanthine ribosyltransferase (HPRT; Lengacher *et al.* 2004, de Kok *et al.* 2005).

Plasmid constructs

The mouse GPX3 promoter was amplified from mouse genomic DNA using Taq polymerase (Promega Corp.). Three promoter fragments, $-3600/-123$, $-3600/-1900$, and $-2000/-123$, were cloned into the pGL3 promoter vector (Promega Corp.). The sequences of the inserted promoter fragments were verified by DNA sequencing. Expression vectors containing mouse ER α and β cloned into the pSG5 vector (Stratagene, La Jolla, CA, USA) were used for cotransfections.

Transient transfections

Mouse preadipocyte cells (3T3-L1) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose (Invitrogen) supplemented with 10% calf serum, 2 mM L-glutamine, 100 U/penicillin per ml, and 100 μ g/streptomycin per ml. Twenty thousand cells per well were seeded in 24-well plates 16–20 h before transfection in phenol red-free DMEM (Invitrogen) supplemented with glucose corresponding to 4.5 g/l, 10% dextran-coated charcoal-treated FBS, 2 mM L-glutamine, 100 U/penicillin per ml, and 100 μ g streptomycin/ml. An amount of 0.8 μ g mGPX3 promoter constructs (expressing firefly luciferase as a reporter gene) was cotransfected with 0.2 μ g ER α , ER β , or empty pSG5 vector using Lipofectamine 2000 in Opti-Mem I Reduced Serum Medium according to the standard protocol (Invitrogen). A plasmid expressing *Renilla* luciferase under control of the thymidine kinase promoter (pRL-TK,

Promega Corp.) was used for normalization. Medium was changed after 5 h to charcoal-treated FBS-containing media with 10 nM E₂, 100 nM 4-OH-tamoxifen (Tam), or vehicle (99.5% EtOH). Cells were harvested 24 h after transfection, and firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega Corp.) according to the manufacturer's instructions.

Statistical analysis

All values represent mean $\pm$ S.D. When significant differences are discussed, a Student's *t*-test was used (two-tailed, two-sample unequal variance).

Results

Gonadal WAT is decreased in E₂-treated mice compared with vehicle-treated mice

As previously described by Lindberg *et al.* (2002), mice treated with E₂ for 3 weeks displayed decreased adipose tissue mass, which was not related to a general loss of body weight. Gonadal WAT weighed 297 ± 137 and 156 ± 40 mg in the vehicle- and E₂-treated mice respectively ($P < 0.05$). There was no difference in body weight between vehicle- and E₂-treated mice (24.2 ± 2.0 and 25.7 ± 0.9 g, $P > 0.05$ respectively).

Gene expression profiling reveals limited effects of chronic estrogen exposure on mRNA levels in hypothalamus

Gene expression profiles were determined for hypothalamus of mice treated with vehicle and E₂ respectively. A limited number of genes were found to be regulated in this part of the brain. At 77% concordance (see Materials and Methods), only six genes were scored as increased and eight genes as decreased. Two and three genes were increased and decreased at 100% concordance respectively (Table 2). Downregulation of nuclear receptor subfamily 4, group A, member 1 (NR4A1), nerve growth factor induced clone B (NGFI-B), and Nur77 was confirmed by real-time PCR (Fig. 1).

Table 1 Primers and probes for real-time PCR

	Forward primer (5'–3')	Reverse primer (5'–3')	Probe (5'–3')
mRNA			
NR4A1	CGAAAGTTGGGGGAGTGTGCTA	GGCTCGTTGCTGGTGTCCATA	–
GPX3	CTACAAGAAGAACTTGGGCCATTT	TCTCGCTGGCTCCTGTTT	TTCTGGGCTTCCCTTCCAACCAATTTG
C3	GTCCCAGCTACTAGTGCTACTG	GCTCTCCAGCCGTAGGACATTG	–
ApoC1	AGGAGTTTGGGAACACTTTGGA	TTCAACTTCTCCTTCACTTTGC	–
IRS1	GCAACTATGCCAGCATCAGCTT	ATGAGTTAAAAGGAGGATTTGCT	–
UCP1	CGTACCAAGCTGTGCGATGT	CCCGAGTCGCAGAAAAGAA	ACAAACCCTTTGAAAAAGGCCGTCGG
CIDEA	GTACTCGGTGTCTACGACATC	TCATCTGTGCAGCATAGGACATA	–

Nuclear receptor subfamily 4, group A, member 1 (NR4A1), glutathione peroxidase 3 (GPX3), complement component 3 (C3), apolipoprotein C1 (ApoC1), insulin receptor substrate 1 (IRS1), uncoupling protein (UCP1) and cell death-inducing DNA fragmentation factor, α -subunit-like effector A (CIDEA).

Table 2 Genes regulated by estrogen in hypothalamus at 77–100% concordance

	FC	%
Name		
Increased genes		
α2-macroglobulin	2.1	100
Synaptotagmin-like 4	1.8	100
Prolactin receptor	2.2	88
Fatty acid-binding protein 7, brain	1.4	88
Von Willebrand factor homolog	1.8	77
Folate receptor 1 (adult)	1.7	77
Decreased genes		
Heat shock protein 1B	−3.0	100
Nuclear receptor subfamily 4, group A, member 1	−1.6	100
FBJ osteosarcoma oncogene	−1.4	100
Dual specificity phosphatase 1	−1.3	88
Dnaj (Hsp40) homolog, subfamily B, member 1	−1.2	88
Crystallin, αB	−1.3	77
Early growth response 1	−1.3	77
CDC-like kinase 1	−1.2	77

FC, fold change.

Effects of chronic estrogen exposure on WAT gene expression profiles

A large number of genes were regulated in WAT after chronic estrogen exposure; 240 genes were increased and 113 genes were decreased according to our selection criteria with a concordance level of 77% (see Materials and Methods). Seventy-nine and forty-seven genes were increased or decreased at 100% concordance respectively (displayed in

Figure 1 Confirmation of microarray data by real-time PCR, using cDNA from the same individual mice ($n=3$) as in the microarray experiment. Real-time PCR data from hypothalamus and adipose tissue are shown in bars. Average fold changes (FC) from real-time PCR and microarray data respectively are presented as numbers below the bars. The hypothalamus gene was normalized to HPRT, and the WAT genes were normalized to 18S. NR4A1, nuclear receptor subfamily 4, group A, member 1; GPX3, glutathione peroxidase 3; C3, complement component 3; ApoC1, apolipoprotein C1; IRS1, insulin receptor substrate 1; and UCP1, uncoupling protein. * $P<0.05$ and ** $P<0.01$.

Table 3). (The complete list of genes changed using a filter of 77% concordance is provided as Supplementary Table 1, see Supplementary Table 1 in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol196/issue3/>). A subset of genes identified as regulated by estrogen in the microarray experiments were assayed using real-time PCR (Fig. 1). The estrogen regulation observed for these genes in the microarray experiment could be confirmed for all tested genes.

Additionally, several genes previously identified as being regulated by estrogen in various tissues were found to be regulated in this study. These genes included complement component C3 (Fan *et al.* 1996), ceruloplasmin (Middleton & Linder 1993), and angiotensinogen (Gordon *et al.* 1992; Table 3).

By comparing Tables 2 and 3, it can be concluded that the effects of chronic estrogen exposure were much greater on WAT than on hypothalamus as determined using a gene expression profiling assay. Another analysis method, significance analysis of microarrays (SAMs), also gave a clear difference in number of regulated genes between hypothalamus and WAT (data not shown). SAM assigns a score on the basis of its change in gene expression relative to the s.d. for that gene (Tusher *et al.* 2001). We performed this analysis using default analysis settings ($\Delta=1.2$). This analysis ranked 7 highly regulated genes for hypothalamus compared with 150 for WAT.

Total ER mRNA levels are higher in WAT than in hypothalamus

We next investigated whether the observation that E_2 exerts greater effect on the gene expression profiles of WAT than of hypothalamus was correlated with expression levels of ER mRNAs. Studies of ER mRNA expression in these tissues revealed that ER α is expressed at five to ten times higher levels in WAT than in hypothalamus (Fig. 2A). ER β is expressed at about eight times higher levels (vehicle) in hypothalamus than in WAT (Fig. 2B). However, overall, ER α expression is highest in both tissues. The ER α/β ratio (calculated using known amounts of ER α and β plasmids) is ~ 6 in hypothalamus and several hundreds in WAT (data not shown; the ratio in WAT is in consonance with our previous report (Lundholm *et al.* 2004)). Importantly, the total ER mRNA levels are higher in WAT than in hypothalamus which might contribute to the quantitative differences in the effects of estrogen on gene expression profiles observed between these tissues. Figure 2 also shows that E_2 treatment significantly decreased ER α expression in WAT and ER β expression in hypothalamus.

Genes coordinately regulated by acute and chronic estrogen treatments in WAT

We compared the genes in WAT identified in this study as regulated by chronic administration of estrogen with genes

that we have previously identified as regulated after 10 h of estrogen treatment (Lundholm *et al.* 2004). Thirteen genes that displayed increased expression and five genes that displayed decreased expression in this study were similarly regulated in the previous study (Table 4). Many of these genes represent novel estrogen-regulated genes.

Estrogen regulates mRNA levels of GPX3, a gene involved in the response to oxidative stress

One of the genes regulated after 10 h and 3 weeks of estrogen exposure was *GPX3* (Table 4). *GPX3* was regulated at numerous time points after E_2 treatment in WAT, in the liver, and in the heart (Table 5), implicating that this gene is subject to a general regulation by estrogen.

To investigate whether *GPX3* is a direct estrogen target gene *in vivo*, mice were treated with estrogen for 2, 4, and 6 h. As can be seen in Fig. 3A, mRNA for *GPX3* was increased *in vivo* already after 2 h of estrogen treatment which is strongly indicative of direct regulation.

To determine ER specificity for *GPX3* mRNA regulation *in vivo*, mice were treated with the $ER\alpha$ - and β -selective ligands, PPT and DPN respectively for 2, 4, and 6 h. As can be seen in Fig. 3B, *GPX3* mRNA was specifically increased by the $ER\alpha$ -selective ligand PPT. This regulation was significant at 4 and 6 h, and there was a clear indication of regulation already at 2 h although it did not reach statistical significance ($P=0.07$). In addition, the levels of *GPX3* mRNA in WAT were lower in ERKO mice compared with WT mice (Fig. 3C), further supporting a critical role of $ER\alpha$ in *GPX3* expression in WAT. The expression of *GPX3* was similar in BERKO and WT mice (Fig. 3C).

To characterize the regulation of *GPX3* transcription at the promoter level, we cloned the mouse *GPX3* promoter (−3600 to −123). This promoter fragment responded to both $ER\alpha$ and β (Fig. 4A). However, the response to $ER\alpha$ was not ligand dependent. Division of the promoter fragment into a −3600/−1900 fragment and a −2000/−123 fragment showed that the ER response is maintained in the −2000/−123 fragment. This fragment contains ERE half sites and an AP-1 site (Fig. 4B). However, mutation of these sites individually or in pair in the context of the −2000/−123 fragment did not reduce the estrogen response (data not shown). Attempts to map the estrogen response within the −2000/−123 fragment by deletion constructs corresponding to −2000/−1400, −1400/−761, and −761/−123 respectively were unsuccessful as all fragments retained some estrogen response (data not shown). This would indicate that the *GPX3* promoter is subject to a complex regulation by estrogen and in light of this it is not surprising that mutation of single or pairs of response elements did not eliminate estrogen responsiveness in the context of the −2000/−123 fragment. Using less stringent criteria for prediction of EREs and AP-1 sites, sites in addition to those indicated in Fig. 4 were identified. Interestingly, 4-OH-tamoxifen activated the $ER\alpha$ response in the

−2000/−123 construct, while it was without effect on the $ER\beta$ response.

Cell death-inducing DNA fragmentation factor, α -subunit-like effector A (CIDEA) mRNA expression is decreased by estrogen treatment in WAT

CIDEA, involved in lipid metabolism and apoptosis, was decreased by estrogen treatment (Table 3). We confirmed decreased expression of CIDEA by real-time PCR (Fig. 5A). A role of estrogen in the suppression of CIDEA was supported by an observed increase in CIDEA expression in estrogen-deficient OVX mice versus sham-operated mice in WAT (Fig. 5B). Further studies of CIDEA expression in WAT from animals treated for 2, 4, and 6 h with estrogen and ER-selective ligands did not provide evidence for CIDEA being a direct estrogen target gene (data not shown). However, the variation between different animals at these short treatment times was extensive, thus not permitting us to exclude an effect.

CIDEA and UCP1, both regarded as brown fat-specific proteins in mice, have much higher mRNA levels in female, which are the focus of this study, than in male mice WAT (Fig. 5C and D). UCP1 mRNA is also decreased after estrogen treatment in WAT (Table 3; Fig. 1).

Discussion

In this study, we show that estrogen regulates a large number of genes in WAT, which is consistent with an important effect of estrogen on this organ. In hypothalamus, on the other hand, few genes responded to estrogen treatment according to our criteria. The number of regulated genes might be related to the total ER expression in the tissues. $ER\alpha$ is the major ER expressed in both these tissues and it is expressed at higher levels in WAT than in hypothalamus. Another reason for detecting fewer changed genes in hypothalamus could be due to its more complex structure. Different regions could respond differently and changes in specific regions could be attenuated using this assay.

In WAT, there were very few genes that were equally regulated after short- and long-term estrogen treatment respectively. This is perhaps not so surprising, since time-course studies often show that genes have different temporal regulations with distinct primary, secondary, and tertiary regulation (Sismondi *et al.* 2007). A significant number of cytoskeletal/motility-related mRNAs were regulated in WAT. Regulation of these could be relevant for the preadipocyte to adipocyte conversion (Takenouchi *et al.* 2004).

The identification of *GPX3*, a gene involved in the cellular response to oxidative stress, as an estrogen target gene in WAT is interesting since obesity (body mass index, BMI) is highly associated with systemic oxidative stress (Keaney *et al.* 2003). Furthermore, it has been demonstrated that *GPX3* enzyme activity is negatively correlated to BMI; (Olusi 2002), and

Table 3 Genes regulated by estrogen in white adipose tissue (WAT) at 100% concordance

Name	FC
Increased genes	
RIKEN cDNA 9030611N15 gene	26·8
Spondin 1, (f-spondin) extracellular matrix protein	25·6
Proprotein convertase subtilisin/kexin type 5	9·8
Glutathione peroxidase 3	7·3
Leucine-rich α 2-glycoprotein	6·9
Major urinary protein 1	6·8
Angiotensinogen	6·6
Chemokine (C–C motif) ligand 8	6·4
Haptoglobin	4·9
Oxytocin receptor	4·9
Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	4·6
Growth arrest-specific 6	4·6
Complement component 4 (within H-2S)	3·8
Early growth response 2	3·7
RIKEN cDNA D330035F22 gene	3·7
RIKEN cDNA 2210414H16 gene	3·5
Cadherin 11	3·4
RIKEN cDNA 1300017C10 gene	3·3
Osteoglycin	3·3
RIKEN cDNA 4921521F21 gene	3·1
RIKEN cDNA 3300001H21 gene	2·9
Lumican	2·9
Coagulation factor XIII, α -subunit	2·8
Phospholipase A1 member A	2·8
Complement component 3	2·7
Neurotrophic tyrosine kinase, receptor, type 2	2·6
CD163 antigen	2·6
Gap junction membrane channel protein α -1	2·5
Macrophage galactose N-acetyl-galactosamine-specific lectin 1	2·5
UDP-Gal: β GlcNAc β -1,3-galactosyltransferase, polypeptide 2	2·5
Contactin 1	2·4
Protease, serine, 11 (Igf binding)	2·3
RAS, dexamethasone-induced 1	2·3
Fc receptor, IgG, low affinity IIb	2·3
Complement component 1, q subcomponent, β -polypeptide	2·2
Macrophage galactose N-acetyl galactosamine specific lectin 2	2·2
Extracellular link domain-containing 1	2·2
Expressed sequence AI132321	2·2
Mannose receptor, C type 1	2·2
Complement component 1, q subcomponent, γ -polypeptide	2·1
Transglutaminase 2, C polypeptide	2·1
Ficolin A	2·1
Glucose-6-phosphate dehydrogenase 2	2·1
Odd Oz/ten-m homolog 4 (<i>Drosophila</i>)	2·1
Insulin-like growth factor-binding protein 6	2·0
Growth hormone receptor	2·0
Folate receptor 2 (fetal)	1·9
Cathepsin C	1·9
RIKEN cDNA 4921526G09 gene	1·9
Epidermal growth factor receptor	1·9
CD53 antigen	1·9
Carbonic anhydrase 5b, mitochondrial	1·9

(continued)

Table 3 Continued

	FC
ATP-binding cassette, subfamily A (ABC1), member 1	1·9
SMAF1	1·9
Glycerol-3-phosphate acyltransferase, mitochondrial	1·9
Amyloid β (A4)-precursor protein	1·8
Glial cell line derived neurotrophic factor family receptor α -1	1·8
Glucose-6-phosphate dehydrogenase X-linked	1·8
Phosphogluconate dehydrogenase	1·8
Glycoprotein m6b	1·8
Purinergic receptor P2X, ligand-gated ion channel 4	1·7
Acetyl coenzyme A carboxylase	1·7
RIKEN cDNA 1110032E23 gene	1·7
Nuclear receptor-binding factor 1	1·7
Ceruloplasmin	1·7
Lysophospholipase 3	1·7
Melanoma antigen, family D, 1	1·7
Lysyl oxidase-like	1·6
<i>Mus musculus</i> , clone IMAGE:5028619, mRNA	1·6
Carboxypeptidase X 1 (M14 family)	1·6
<i>Mus musculus</i> , Similar to RIKEN cDNA 1700066C05 gene, clone MGC:28125 IMAGE:3980327, mRNA, complete cds	1·6
Expressed sequence AI195443	1·6
Peptidylprolyl isomerase C-associated protein	1·6
Cytochrome P450, family 4, subfamily v, polypeptide 3	1·6
RIKEN cDNA 2810418J22 gene	1·6
X-box-binding protein 1	1·5
Hypothetical protein LOC214597	1·5
Microtubule-associated protein 1 light chain 3	1·4
Pyruvate carboxylase	1·4
Decreased genes	
Troponin I, skeletal, fast 2	–572·9
Troponin C, fast skeletal	–369·4
Myosin, heavy polypeptide 4, skeletal muscle	–207·8
Actinin- α -3	–190·2
Myosin light chain, phosphorylatable, fast skeletal muscle	–156·1
Titin	–131·6
Troponin T3, skeletal, fast	–81·6
Myoglobin	–80·6
Actin, α -1, skeletal muscle	–67·3
ATPase, Ca ⁺⁺ transporting, cardiac muscle, fast twitch 1	–61·3
Myosin, heavy polypeptide 2, skeletal muscle, adult	–45·4
Uncoupling protein 1, mitochondrial	–43·5
Creatine kinase, muscle	–36·4
Parvalbumin	–34·0
Ryanodine receptor 1, skeletal muscle	–27·6
Muscle glycogen phosphorylase	–24·4
Ankyrin 1, erythroid	–20·5
Adenylosuccinate synthetase 1, muscle	–20·4
Apolipoprotein B editing complex 2	–17·8
Enolase 3, β -muscle	–13·3
Tropomyosin 1, α	–13·1
RIKEN cDNA 2310076E16 gene	–11·6
Fatty acid-binding protein 3, muscle, and heart	–8·9
Titin-cap	–8·7
Myozenin 1	–8·3
Myosin light chain, alkali, fast skeletal muscle	–7·7
Myomesin 1	–7·1

(continued)

Table 3 Continued

	FC
Tropomyosin 2, β	-6.6
Desmin	-5.1
Four and a half LIM domains 1	-5.0
Cytochrome c oxidase, subunit VIIa 1	-4.4
Glutamate oxaloacetate transaminase 1, soluble	-4.4
Upregulated during skeletal muscle growth 4	-4.3
RIKEN cDNA 1810015C04 gene	-4.3
Cell death-inducing DNA fragmentation factor, α -subunit-like effector A	-3.8
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-3.8
Cytochrome c oxidase, subunit VIIIb	-3.3
LIM and cysteine-rich domains 1	-3.1
RIKEN cDNA 1110003B01 gene	-2.9
SPARC-related modular calcium binding 2	-2.5
3-Ketoacyl-CoA thiolase B	-2.5
Lactate dehydrogenase 2, B chain	-2.3
Hypothetical protein MGC18894	-2.1
Regulator of G-protein signaling 5	-1.9
Suppressor of initiator codon mutations, related sequence 1 (<i>S. cerevisiae</i>)	-1.7
Cyclin D1	-1.6
RIKEN cDNA 4930542G03 gene	-1.4

FC, fold change

GPX3 mRNA was shown to be increased in obese women after a period of energy-restricted diet (Dahlman *et al.* 2005). GPX3 mRNA is present in many cell types (Bierl *et al.* 2004), but both earlier and array data indicate a high expression of GPX3 mRNA in WAT (Maeda *et al.* 1997; data not shown). Interestingly, two additional genes involved in the response to oxidative stress, catalase and isocitrate dehydrogenase 1 (NADP+, soluble), were upregulated by 3 weeks of estrogen exposure (1.6- and 1.9-fold at 88 and 77% concordance respectively; Supplemental Table 1), further supporting a role of estrogen in the protection against oxidative stress. However, our additional experiments did not support that these represent direct estrogen target genes and they were not investigated further (data not shown).

We show here that GPX3 mRNA is regulated as early as 2 h after estrogen treatment *in vivo*, indicating a direct transcriptional regulation. Furthermore, GPX3 was shown to be regulated specifically via ER α in adipose tissue, since activation was achieved using the ER α -selective ligand PPT but not the ER β -selective ligand DPN. However, this most likely reflects the relative levels of the two ERs in this tissue rather than selective promoter regulation. We were unable to detect the GPX3 protein in WAT by western blot analysis to confirm its regulation at the protein level. However, the kidney is known to express high levels of GPX3 and in this tissue we could detect a robust increase in GPX3 following 3 weeks of estrogen exposure (data not shown).

In transient transfection assays, the GPX3 promoter was regulated by both ERs. However, ER α regulation of the GPX3 promoter was ligand independent in this system.

Figure 2 mRNA levels of (A) ER α and (B) ER β in WAT and hypothalamus, relative to the WAT vehicle level for the respective receptor mRNA ($n=7-9$). mRNA was normalized to HPRT. There was a significant decrease in ER α mRNA in WAT, and ER β mRNA in hypothalamus, after long-term estrogen treatment. Observe that the ER α / β ratio is several hundred fold for WAT (data not shown; see Results). * $P<0.05$.

Decreasing the amounts of transfected ER α did not result in estrogen dependency (data not shown). The reason for the ligand-independent activation is unclear. Interestingly, ligand activation of ER α was observed with 4-OH-tamoxifen (Fig. 4A). However, we have observed that both E₂ and 4-OH-tamoxifen enhance activation, in the presence of transfected ER α , of the GPX3 promoter in the mouse hepatoma Hepa cell line (data not shown). The observed activation by 4-OH-tamoxifen suggests that activation is mediated via AP-1 sites as tamoxifen has been shown to act as an agonist via such sites (Paech *et al.* 1997). Tamoxifen has also previously been shown to induce a stronger response than E₂ in the presence of ERs on an electrophile (or antioxidant)-response element (EpRE or ARE) in the human quinone reductase promoter (Montano *et al.* 2005). The EpRE is nearly identical to our reported AP-1-site, only differing by one base (mouse GPX3 -1159 bp: 5'-TCTGAGTCA-3', the underlined T is a G in the human EpRE sequence). However, we did not observe reduced activation of the GPX3 promoter after mutation of this EpRE site (data not shown; Rushmore *et al.* 1991).

Regulation of GPX3 enzyme activity by estrogen has been described by L'Abbe *et al.* (1992). Upregulation of GPX3 mRNA in response to estrogen has been reported in uterus

Table 4 Genes regulated after 10 h (experimental setup from Lundholm *et al.* (2004) and 3 weeks (this study) of estrogen treatment in white adipose tissue (WAT)

	GO biological process description	Previously shown E ₂ regulation
Increased genes		
Glucose-regulated protein/phospholipase C, α	Electron transport	Yes
Glutathione peroxidase 3	Response to oxidative stress	Yes
Growth arrest specific 6	Regulation of cell growth, cell adhesion	Yes
Haptoglobin	Proteolysis and peptidolysis, acute-phase response	No
Osteoglycin/mimecan	–	No
Prostaglandin I ₂ (prostacyclin) synthase	Prostaglandin biosynthesis, electron transport	Yes
Protease, serine, 11 (Igf binding)	Regulation of cell growth, proteolysis, and peptidolysis, negative regulation of transforming growth factor β -receptor signaling pathway, negative regulation of BMP signaling pathway	No
Purine-nucleoside phosphorylase	–	No
Leucine-rich α -2-glycoprotein/ <i>RIKEN cDNA 1300008B03</i> gene	–	No
Myoinositol 1-phosphate synthase A1/ <i>RIKEN cDNA 1300017C10</i> gene	Myoinositol biosynthesis, phospholipid biosynthesis	No
<i>RIKEN cDNA 1810009A15</i> gene	–	No
Sec61 α -1 subunit (<i>S. cerevisiae</i>)	Protein targeting, transport, protein secretion, protein transport	No
Thrombospondin 1	Cell adhesion, negative regulation of angiogenesis	Yes
Decreased genes		
Aquaporin 1	Transport, water transport	Yes
DNA segment, human D4S114	Regulation of transforming growth factor β -receptor signaling pathway	No
Manic fringe homolog (<i>Drosophila</i>)/ β -1,3- <i>N</i> -acetylglucosaminyltransferase	Development	No
Myocilin	–	No
Regulator of G-protein signaling 5	Signal transduction, G-protein-coupled receptor protein signaling pathway	No

LocusLink numbers were used to compare gene lists since the experiments were performed on different arrays. The column heading ‘previously shown E₂ regulation’ refers to the fact that these genes have previously been reported to be regulated by estrogen in various cells and tissues.

and ovary after 3 days of treatment (Waters *et al.* 2001), and GPX3 mRNA expression was decreased in adipose tissue of ovariectomized compared with control mice (Ye *et al.* 2005). However, to our knowledge, this represents the first report demonstrating GPX3 as a direct target gene for estrogen. Our data are consistent with a causative effect of this gene on fat mass as GPX3 regulation occurs prior to the decrease in adipose tissue mass.

CIDEA knockout mice show a lean phenotype and are resistant to obesity (Zhou *et al.* 2003). The mice have markedly less WAT, as a result of reduced lipid accumulation, and increased lipolysis in BAT (Zhou *et al.* 2003). Similarly there is increased lipolysis in humans, in preadipocytes treated with

siRNA against CIDEA (Nordstrom *et al.* 2005). Previous publications have shown high expression of CIDEA in mouse BAT and expression in human s.c. WAT, but indicated that CIDEA is not expressed or expressed only at low levels in mouse WAT (Zhou *et al.* 2003, Nordstrom *et al.* 2005). However, we do observe expression in mouse WAT as determined by the Affymetrix analysis and real-time PCR. This discrepancy could be explained by a difference in CIDEA expression levels between female and male mice. Only male mice were studied in Nordstrom *et al.* (2005), and gender was not indicated in Zhou *et al.* (2003). We were unable to detect CIDEA protein in any tissue where the mRNA was regulated to confirm its regulation at the protein level.

Table 5 Average fold changes for glutathione peroxidase 3 (GPX3) mRNA assayed by microarray analysis in 17 β -estradiol (E₂)- versus vehicle-treated mice

	WAT				Liver	Heart
GPX3 fold change on microarray	10 h	24 h	48 h	3 weeks	3 weeks	3 weeks
	1.9	1.7	2.3	7.3	8.8	1.6

Data from different tissues of ovariectomized mice were used, and E₂ treatment was performed according to the indicated time periods. The 10- to 48-h data are from the experiment described in Lundholm *et al.* (2004), and the liver data were previously published as supplemental data in Lindberg *et al.* (2003).

Figure 3 mRNA expression of GPX3 is significantly increased by (A) E₂ after 2, 4, and 6 h and by the (B) ER α -selective ligand PPT after 4 and 6 h in WAT. Mice were treated with E₂, PPT, or DPN and killed at the indicated time points ($n=5$, except 6 h E₂ where $n=4$). (C) mRNA expression of GPX3 is significantly decreased in ERKO mice but not in BERKO mice compared with WT in WAT. GPX3 mRNA expression was determined using a real-time PCR assay. mRNA was normalized to HPRT. * $P<0.05$ and ** $P<0.01$.

Figure 4 (A) The mouse GPX3 promoter (the $-2000/-123$ region) is responsive to estrogen in a transient transfection assay. Cells were transfected with expression vectors (ER α and β , or empty expression vectors) and reporter vectors and treated as indicated. (B) Several possible ER-binding sites are present in the mouse GPX3 promoter. The marked EREs start at -3226 , -1211 , and -914 and the AP-1 site at -1159 .

Figure 5 mRNA expression of CIDEA in WAT is significantly (A) decreased by E₂ after 3 weeks of treatment ($n=8-9$), and similarly (B) increased in OVX mice compared with sham-operated mice ($n=5$). mRNA expression of both (C) CIDEA and (D) UCP1 in WAT is lower in male than in female mice (UCP1 not significantly changed; $n=4-6$). CIDEA and UCP1 mRNA expressions were determined using a real-time PCR assay. mRNA was normalized to (A and B) HPRT and (C and D) 18S. * $P<0.05$.

Additional genes that were identified as increased after estrogen treatment in this study include apolipoprotein CI (ApoCI) and AE-binding protein 1 (2.1- and 1.6-fold respectively at 88% concordance; Supplementary Table 1). These genes might be implicated in the reduced adiposity seen after estrogen treatment, ApoCI is involved in reducing triglyceride uptake and AE-binding protein 1 is a down-regulator of adipogenesis. Mice overexpressing ApoCI have reduced adipose tissue weight, probably due to a reduction in uptake of fatty acids into adipocytes (Jong *et al.* 2001). When crossbred on the obese ob/ob mouse background, the transgenic mice were fully protected from development of obesity and had improved insulin sensitivity compared with normal ob/ob mice (Jong *et al.* 2001).

Several genes involved in facilitating cholesterol and lipid efflux were increased by estrogen, including ATP-binding cassette subfamily A member 1 (ABCA1; Table 3), apolipoprotein E, and low-density lipoprotein receptor-related protein 1 (1.7- and 1.5-fold respectively at 77% concordance; Supplementary Table 1). Interestingly, in our previous short-term study (Lundholm *et al.* 2004), the liver X receptor (LXR)- α pathway, including ABCA1 and apolipoprotein E, was identified as decreased by E₂. The long-term effects of E₂ are apparently opposite for these two genes, but expression of LXR α was not changed after 3 weeks of E₂ treatment.

During these studies, the paper by D'Eon *et al.* was published. In our study, we do not observe decreased lipogenic genes in WAT as described in D'Eon *et al.* (2005). The studies differ, however, both in treatment time and mode, and access to and type of food. D'Eon *et al.* used pair-fed mice on standard rodent chow (not soy-free) that were treated (with E₂ pellets) for about twice as long time (40 days) as in our study, and fasted before killing. In addition, Penza *et al.* (2006) showed that, unlike in our study, lipogenic and adipogenic genes were decreased and UCP1 was increased after E₂ treatment. However, they study 4-week-old male mice compared with our studies of older female mice. Comparing their gene expression data with ours, there are about 20 genes that are similarly regulated in both studies.

Recently, ER α was specifically silenced in the ventromedial nucleus of the hypothalamus, with obesity as a consequence (Musatov *et al.* 2007). This was preceded by increased food intake but also to a decline in energy expenditure (Musatov *et al.* 2007). The relative role of ER α in central versus peripheral tissues remains to be elucidated. Still, no obvious appetite- or satiety-related genes were found to be regulated in hypothalamus in our study, although we do see a decreased adipose tissue mass.

In conclusion, we have shown that estrogen has large effects on gene expression in WAT. We have in detail characterized estrogen regulation of GPX3, a gene involved in the cellular response to oxidative stress. We hypothesize that estrogen regulation of GPX3 could be involved in protection against oxidative stress and associated phenotypes such as obesity.

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