Gene expression profiling identifies liver X receptor alpha as an estrogen-regulated gene in mouse adipose tissue

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Abstract

Estrogens reduce adipose tissue mass in both humans and animals. The molecular mechanisms for this effect are, however, not well characterized. We took a gene expression profiling approach to study the direct effects of estrogen on mouse white adipose tissue (WAT). Female ovariectomized mice were treated for 10, 24 and 48 h with 17 β -estradiol or vehicle. RNA was extracted from gonadal fat and hybridized to Affymetrix MG-U74Av2 arrays. 17 β -Estradiol was shown to decrease mRNA expression of liver X receptor (LXR) α after 10 h of treatment compared with the vehicle control. The expression of several LXR α target genes, such as sterol regulatory element-binding protein 1c, apolipoprotein E, phospholipid transfer protein, ATP-binding cassette A1 and ATP-binding cassette G1, was similarly decreased. We furthermore identified a 1.5 kb LXR α promoter fragment that is negatively regulated by estrogen. Several genes involved in lipogenesis and lipolysis were identified as novel targets that could mediate estrogenic effects on adipose tissue. Finally, we show that ER α is the main estrogen receptor expressed in mouse white adipose tissue (WAT) with mRNA levels several hundred times higher than those of ER β mRNA.

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Introduction

The prevalence of obesity is rapidly increasing in the western world (Kuczmarski *et al.* 1994). Obesity is associated with an increased risk of several diseases, such as type 2 diabetes, cardiovascular disease and cancer in breast, prostate, endometrium, colon and gallbladder (Bray 2002). Obesity is the result of increases in adipocyte number and size (Faust *et al.* 1978). Adipocyte size is determined by the balance between lipid synthesis (lipogenesis) and lipid breakdown (lipolysis) (Prins & O'Rahilly 1997). Lipogenesis, which involves fatty acid synthesis and subsequent triglyceride (TG) synthesis, occurs in both adipose tissue and liver (Eckel 1989). Lipolysis is the hydrolysis of TG, stored in adipocytes, to free fatty acids and glycerol (Langin *et al.* 1996). When a

critical adipocyte size is reached, further fat accumulation causes an increase in adipocyte number (Faust *et al.* 1978).

Women generally have more body fat than men and a higher proportion of fat in the gluteal–femoral region, the so-called gynoid fat distribution (Blaak 2001). Men generally have more body fat in the abdominal (visceral or central) region, which is called an android fat distribution (Blaak 2001). After menopause, women accumulate more intra-abdominal fat and therefore obtain an increased central adiposity (Poehlman 2002). In particular, this central distribution of body fat is a powerful and independent predictor of disease (Poehlman 2002).

Estrogens have important effects on many tissues in the body, among them adipose tissue (Mueller & Korach 2001). Estrogen could influence adipose

tissue mass by both central and peripheral effects. Regulation via the central nervous system includes behavioral regulation of feeding and physical activity. Peripheral effects are exerted directly on the adipose tissue. A correlation between estrogen and adipose tissue mass has been seen in both humans and rodents (Heine *et al.* 2000). An increased adipose mass is seen in women after menopause when estrogen levels decrease, while estrogen replacement therapy decreases adipose mass (Tchernof *et al.* 1998). Ovariectomy and estrogen treatment in rodents have similar effects on adipose tissue to those seen in humans (Wade & Gray 1979). Estrogen reduces food intake, which is postulated to occur partly via increased cholecystokinin-signaling and negative feedback on meal size (Geary 2001). There are also reports on increased running-wheel activity in estrogen-treated mice, primarily mediated through estrogen receptor (ER) α (Ogawa *et al.* 2003). Estrogens are also known to decrease adipose tissue mass by increasing lipolysis (Lincova *et al.* 1984, Tomita *et al.* 1984), but the molecular mechanisms for this phenomenon are still unclear. Furthermore, postmenopausal women have reduced energy expenditure (Poehlman 2002).

Estrogen exerts its effects via two nuclear receptors, ER α and ER β (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 mRNA and protein expression (Price & O'Brien 1993, Mizutani *et al.* 1994) and ER β mRNA expression (Crandall *et al.* 1998, Pedersen *et al.* 2001) have been detected in human adipose tissue. In mouse and rat adipose tissue, estrogen receptors have been detected by estradiol-binding (Seiki *et al.* 1978, Wade & Gray 1978, Haslam & Shyamala 1981). However, to our knowledge, the relative levels of ER α and β have not been reported.

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 , but not in mice lacking ER β , showing that ER α is the major mediator of the down-sizing effects of estrogen on mouse fat mass (Heine *et al.* 2000,

Ohlsson *et al.* 2000). In addition, ER β has been shown to have anorectic effects mediated via the central nervous system (Liang *et al.* 2002b). However, in another report, ER β was indicated to have opposite effects to ER α , as shown by decreased fat mass in ER α knockout (ERKO) mice following ovariectomy (Naaz *et al.* 2002).

Earlier reports have shown some mechanisms by which estrogen affects metabolism. Estrogen increases hormone-sensitive lipase, which is the rate-limiting enzyme in lipolysis (Palin *et al.* 2003). Estrogen decreases lipoprotein lipase (LPL) enzyme activity in humans and animals, which is expected to lead ultimately to a decrease in TG deposition in fat cells (Homma *et al.* 2000, Yamaguchi *et al.* 2002). Estrogen also represses the activity of hepatic lipase, an enzyme that hydrolyzes TG and phospholipids in lipoproteins, resulting in an increase in high-density lipoprotein (HDL) cholesterol (Jones *et al.* 2002).

To understand the effects of estrogen in relation to metabolic disease, we have begun to characterize its effects on adipose tissue at the molecular level. In this study, we use gene expression profiling to determine estrogen-regulated genes in mouse white adipose tissue (WAT).

Materials and methods

Animals

For determination of ER α and ER β expression levels, 11-week-old female C57BL/6 wild-type mice were ovariectomized or sham-operated. The animals were kept on soy-free diet for 1 week before they were killed at 13 weeks of age. Gonadal WAT and interscapular brown adipose tissue (BAT) were collected and stored at -80°C .

For microarray experiments, 8-week-old female C57BL/6 mice were ovariectomized. After recovery for 2 weeks, the mice were injected (subcutaneously) with $2.3\text{ }\mu\text{g}/\text{mouse}$ of 17β -estradiol (E2) and killed 10, 24 or 48 h after the injection. Control mice received injections of vehicle oil (olive oil, Apoteksbolaget, Göteborg, Sweden). Gonadal WAT and liver were collected and kept at -80°C . Arterial blood was sampled and centrifuged to obtain serum, in which E2 levels were measured using a radioimmunoassay monitoring E2 (DiaSorin, Saluggia, Italy) with a sensitivity of $10\text{ pg}/\text{ml}$. All studies were approved by Stockholm

South Ethical Committee for animal experiments and the ethical committee for animal care at Göteborg University.

Microarray analysis

RNA preparation and experimental design

Total RNA was prepared from gonadal WAT ($n=7$ for all groups), using Trizol Reagent as previously described (Chomczynski & Sacchi 1987). RNA was further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were pooled into two pools ($n=3$ or 4 per pool) per treatment group. There were vehicle-treated and estradiol-treated groups for each time point, generating 12 pools in total. 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 (MG-U74Av2 array; Affymetrix, Santa Clara, CA, USA).

Bioinformatics

The scanned output files were analyzed with Affymetrix Microarray Suite Version 5.0 software. Comparisons were made between two estrogen-treated and two vehicle-treated samples, generating four comparisons for each time point, using Affymetrix Data Mining Tool Version 3.0. Filters used for the presented data were as follows. For supplemental data, genes were included if the Affymetrix change algorithm gave increased (I) or decreased (D) calls respectively for all comparisons. Genes absent (A) according to the Affymetrix detection algorithm on both arrays of a comparison have been excluded. Furthermore, the average fold change was ≥ 2 . For Table 1, average fold changes are given for genes increased (I) or decreased (D) respectively in at least three out of four comparisons.

Real-time PCR analysis

Total RNA was prepared as described above. An amount of 1 μ g of 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 with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primer sequences for apolipoprotein E (apoE), glucose-6-phosphate dehydrogenase (G6DH) and

glycerol-3-phosphate acyltransferase (GPAT) (Liang *et al.* 2002a) and sterol regulatory element-binding protein (SREBP) 1c (Stulnig *et al.* 2002) have been described previously. Additional primer and probe sequences are shown in Table 2. A SYBR Green-based protocol was used for apoE, G6PD and GPAT mRNA expression analysis. In this case, PCR products were further analyzed by melting curve analysis to confirm a single product. A TaqMan probe-based protocol was used for all other genes. mRNA levels were calculated by the 'Standard Curve Method' (multiplex reactions, according to instructions in User Bulletin no. 2, Applied Biosystems). mRNA expression levels were normalized to 18S rRNA in all studies except for the ER α and ER β expression study, where glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used (Applied Biosystems). In addition, known amounts of mouse ER α and ER β (Pettersson *et al.* 1997) and rat GAPDH (gift from Dr Christine Sadek, Department of Biosciences) plasmids were used as standards to calculate absolute amounts of the receptor mRNAs.

Plasmid constructs

The mouse liver X receptor (LXR) α -1513/+1 promoter fragment was obtained by PCR-amplification from a longer promoter fragment, using Taq Polymerase (Roche Diagnostics, Indianapolis, IN, USA) with primers 5'-TTAAT GACGCGTCATGGGAATTGGAGTTCACA-3' and 5'-GTCAGCCTCGAGTCCCCCCTCCTC CAAA-3' incorporating MluI and XhoI restriction sites (underlined) respectively. The PCR product was cleaved with the appropriate enzymes and cloned into the pGL3-Basic Vector (Promega, Madison, WI, USA). The sequence of the promoter fragment was verified by DNA sequencing. An expression vector containing mouse ER α cloned into the pSG5 vector (Stratagene, La Jolla, CA, USA) was used in the cotransfections (Pettersson *et al.* 1997). Empty pGL3-Basic and pSG5 vectors were used as controls.

Transient transfections

Mouse hepatoma cells (Hepa) were cultured in DMEM containing 4.5 g/l glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U penicillin/ml and 100 μ g streptomycin/ml. A total of 10 000 cells/well were seeded in 24-well

Table 1 Genes involved in metabolism. Average fold changes are given for genes increased or decreased respectively in at least three out of four comparisons. Genes are marked if not changed (NC) in at least three out of four comparisons. The fold change was calculated from the signal log ratio ($2^{\text{signal log ratio}} = \text{fold change}$) and the average fold change of all four comparisons was included in the table. Sorting into groups was done according to the references included in the table. The accession numbers were accessed from www.affymetrix.com, using the Netaffx Analysis Center (Liu *et al.* 2003). *Genes mentioned in the text. *Genes increased from absent to present or decreased from present to absent, which could give less accurate fold changes.

Name	Accession no.	Average fold change			Reference
		10 h	24 h	48 h	
Genes increased in adipogenesis/lipogenesis					
adipocyte complement related protein/adiponectin [#]	NM_009605		-1.2	NC	(Soukas <i>et al.</i> 2001)
adipsin	NM_013459		NC	NC	(Soukas <i>et al.</i> 2001)
adrenergic receptor, beta 3 [#]	X72862	-1.8	-1.9		(Collins <i>et al.</i> 2001, Nadler <i>et al.</i> 2000)
adrenergic receptor, beta 3 [#]	X72862	-1.9	-1.7		(Collins <i>et al.</i> 2001, Nadler <i>et al.</i> 2000)
adrenergic receptor, beta 3 [#]	X72862	-1.6	-1.5		(Collins <i>et al.</i> 2001, Nadler <i>et al.</i> 2000)
angiotensinogen	NM_007428		1.7	1.7	(Nadler <i>et al.</i> 2000)
ATP citrate lyase	AW121639	NC		NC	(Shimomura <i>et al.</i> 1999)
CCAAT/enhancer binding protein (C/EBP), alpha [#]	NM_007678	-1.4	NC	NC	(Soukas <i>et al.</i> 2001)
CCAAT/enhancer binding protein (C/EBP), beta	NM_009883	1.6	1.6		(Soukas <i>et al.</i> 2001)
CCAAT/enhancer binding protein (C/EBP), delta	NM_007679		NC	NC	(Soukas <i>et al.</i> 2001)
fatty acid binding protein 4, adipocyte/aP2	NM_024406	NC	NC	NC	(Soukas <i>et al.</i> 2001)
fatty acid synthase [#]	X13135	NC		NC	(Shimomura <i>et al.</i> 1999)
glucose-6-phosphate dehydrogenase X-linked [#]	NM_008062		2.4	1.6	(Shimomura <i>et al.</i> 1999)
glycerol phosphate dehydrogenase 1, mitochondrial	NM_010274	NC			(Nadler <i>et al.</i> 2000)
glycerol-3-phosphate acyltransferase, mitochondrial [#]	NM_008149		1.5	1.4	(Shimomura <i>et al.</i> 1999)
insulin I	NM_008386				(Kersten 2001)
insulin I	NM_008386	NC	NC	NC	(Kersten 2001)
insulin II	NM_008387				(Kersten 2001)
lipoprotein lipase [#]	NM_008509			NC	(Yamaguchi <i>et al.</i> 2002)
lactate dehydrogenase 2, B chain [#]	NM_008492	NC	-2.3		(Nadler <i>et al.</i> 2000)
liver X receptor alpha [#]	NM_013839	-1.6	-1.2		(Schultz <i>et al.</i> 2000)
glucocorticoid receptor	NM_008173		NC	NC	(Rosen & Spiegelman 2000)
peroxisome proliferator activated receptor gamma	NM_011146		NC		(Soukas <i>et al.</i> 2001)
phosphoenolpyruvate carboxykinase 1, cytosolic (PEPCK)	NM_011044		NC	NC	(Nadler <i>et al.</i> 2000)
pyruvate carboxylase [#]	NM_008797	-1.8	1.7		(Nadler <i>et al.</i> 2000)
pyruvate carboxylase [#]	NM_008797	-1.9	1.4		(Nadler <i>et al.</i> 2000)
pyruvate carboxylase [#]	NM_008797	-1.7			(Nadler <i>et al.</i> 2000)
retinol binding protein 4, plasma [#]	U63146	-1.5		NC	(Nadler <i>et al.</i> 2000)
signal transducer and activator of transcription 1 [#]	NM_009283	-2.0	NC	NC	(Soukas <i>et al.</i> 2001)
stearoyl-coenzyme A desaturase 1	NM_009127	NC	NC		(Soukas <i>et al.</i> 2001)
stearoyl-coenzyme A desaturase 2	NM_009128	NC	NC	NC	(Soukas <i>et al.</i> 2001)
sterol regulatory element binding factor 1 [#]	NM_011480	-1.6	-1.4	NC	(Soukas <i>et al.</i> 2001)

Table 1 Continued

		Average fold change			
	Accession no.	10 h	24 h	48 h	Reference
Genes involved in lipolysis					
adrenergic receptor, beta 1	NM_007419				(Jimenez <i>et al.</i> 2002)
adrenergic receptor, beta 2	X15643	NC	NC	NC	(Jimenez <i>et al.</i> 2002)
adrenergic receptor, beta 3 [#]	X72862	-1.8	-1.9		(Jimenez <i>et al.</i> 2002)
adrenergic receptor, beta 3 [#]	X72862	-1.9	-1.7		(Jimenez <i>et al.</i> 2002)
adrenergic receptor, beta 3 [#]	X72862	-1.6	-1.5		(Jimenez <i>et al.</i> 2002)
AE binding protein 1	NM_009636				(Soukas <i>et al.</i> 2001)
CHOP-10/GADD153 [#]	NM_007837	1.9*			(Mandrup & Lane 1997)
fibroblast growth factor 1	NM_010197		4.0*		(Navre & Ringold 1989)
growth hormone receptor [#]	NM_010284	-2.1	1.6	1.3	(Kersten 2001)
growth hormone receptor [#]	U15012	-1.7	1.4	1.4	(Kersten 2001)
high mobility group nucleosomal binding domain I	NM_008251	1.7		NC	(Soukas <i>et al.</i> 2001)
interleukin 1 alpha	NM_010554				(Rosen & Spiegelman 2000)
interleukin 1 beta	NM_008361				(Rosen & Spiegelman 2000)
interleukin 1 receptor, type I	NM_008362	NC	NC	NC	(Rosen & Spiegelman 2000)
interleukin 1 receptor, type II [#]	NM_010555	2.8*			(Rosen & Spiegelman 2000)
interleukin 6	NM_031168				(Langhans 2002)
interleukin 6 receptor	NM_010559				(Langhans 2002)
leptin	NM_008493	-1.9			(Collins <i>et al.</i> 2001)
leptin	NM_008493	-2.1			(Collins <i>et al.</i> 2001)
leptin receptor	NM_010704		-4.0*	-3.3	
lipase hormone sensitive	NM_010719			1.1	(Langin <i>et al.</i> 2000)
natriuretic peptide receptor 3	NM_008728	-3.1*		NC	(Langin <i>et al.</i> 2000)
procollagen, type I, alpha 1 [#]	U03419	NC	3.2	1.3	(Nadler <i>et al.</i> 2000)
procollagen, type VI, alpha 1	NM_009933	1.6*	2.2		
thyroid hormone receptor alpha	NM_011584	-1.6	-2.1		(Viguerie <i>et al.</i> 2002)
thyroid hormone receptor alpha	NM_011584	NC	NC	NC	(Viguerie <i>et al.</i> 2002)
tumour necrosis factor alpha	NM_013693				(Rosen & Spiegelman 2000)
uncoupling protein 1, mitochondrial [#]	NM_009463	4.6		-13.9	(Collins <i>et al.</i> 2001)
uncoupling protein 2, mitochondrial	NM_011671				(Collins <i>et al.</i> 2001)
uncoupling protein 3, mitochondrial	NM_009464	-1.6	-1.5	NC	(Collins <i>et al.</i> 2001)
Knockout mouse models of obesity resistance					
protein tyrosine phosphatase, receptor type, B [#]	NM_029928	-1.4	NC		(Chen & Farese 2001)
diacylglycerol O-acyltransferase 1 [#]	NM_010046	-1.5	-1.4		(Chen & Farese 2001)
Genes involved in insulin signaling					
cysteine rich protein 61/insulin-like growth factor binding protein 10	NM_010516	NC	1.8	-2.0	
ESTs, highly similar to IRS2_mouse insulin receptor substrate-2 (IRS-2)(4PS)[<i>M. musculus</i>]	AF090738				
insulin receptor	NM_010568				
insulin receptor substrate 1	NM_010570				
insulin-like growth factor 1	NM_010512	NC		NC	
insulin-like growth factor 1	NM_010512			1.4	
insulin-like growth factor 2	NM_010514				
insulin-like growth factor 2	NM_010514				
insulin-like growth factor 2 receptor [#]	NM_010515	-1.4	-1.7	NC	
insulin-like growth factor binding protein 1	NM_008341				
insulin-like growth factor binding protein 1	NM_008341				
insulin-like growth factor binding protein 2	NM_008342		NC		
insulin-like growth factor binding protein 3	NM_008343			-3.4*	
insulin-like growth factor binding protein 4	NM_010517				
insulin-like growth factor binding protein 4	NM_010517	-1.4	1.5	NC	

Table 1 *Continued*

	Accession no.	Average fold change			Reference
		10 h	24 h	48 h	
insulin-like growth factor binding protein 5	NM_010518	NC	1.9	NC	
insulin-like growth factor binding protein 6	NM_008344		2.4		
insulin-like growth factor I receptor [#]	AF056187		-3.0	-2.3	
Proteins related to lipoproteins					
apolipoprotein A-I	NM_009692				
apolipoprotein A-II	NM_013474				
apolipoprotein A-IV	NM_007468				
apolipoprotein C-I	NM_007469			NC	
apolipoprotein C-II	NM_009695				
apolipoprotein C-IV	NM_007385				
apolipoprotein D	NM_007470				
apolipoprotein E [#]	NM_009696	-1.7	NC	NC	
low density lipoprotein receptor [#]	NM_010700		1.7	NC	
low density lipoprotein receptor-related protein I	NM_008512	NC	1.7	NC	
nuclear receptor subfamily 2, group F, member 2/COUP-TF2/Apo (A1) regulatory protein 1 (ARP1)	NM_009697	NC	-1.5		
very low density lipoprotein receptor	NM_013703				
Other genes					
ATP-binding cassette, sub-family A, member 1 (ABCA1) [#]	NM_013454	-1.9			
ATP-binding cassette, sub-family A, member 1 (ABCA1) [#]	NM_013454	-1.8	NC		
ATP-binding cassette, sub-family G, member 1 (ABCG1) [#]	NM_009593	-1.2*		NC	
CCAAT/enhancer binding protein (C/EBP), gamma	AB012273		NC	NC	
cytochrome P450, 7a1	NM_007824				
fatty acid coenzyme A ligase, long chain 2 [#]	NM_007981	-1.5	-1.6	1.3	
glucose regulated protein/phospholipase C-alpha	NM_007952	2.0		1.4	
isocitrate dehydrogenase 1 (NADP+), soluble	NM_010497	NC	2.1		
lipase, hepatic	NM_008280				
nuclear receptor coactivator 1/SRC-1	NM_010881				
peroxisome proliferative activated receptor, gamma, coactivator 1 (PGC1)	NM_008904				
peroxisome proliferator activated receptor alpha	NM_011144				
phospholipid transfer protein [#]	NM_011125	-1.7	NC	NC	

plates 20 h before transfection in phenol red-free, high-glucose DMEM (Invitrogen) supplemented with 10% dextran-coated charcoal-treated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U penicillin/ml and 100 µg streptomycin/ml. Mouse preadipocyte cells (3T3-L1K) were cultured in DMEM containing 4.5 g/l glucose (Invitrogen) supplemented with 10% calf serum, 2 mM L-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml. A total of 20 000 cells/well were seeded in 24-well plates 16 h before transfection in the same charcoal-treated FBS-containing media as

described above for Hepa. The -1513/+1 LXRα promoter construct or empty pGL3-Basic vector (both expressing firefly luciferase as a reporter gene) was cotransfected with ERα 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) was included as a positive control to exclude a general effect on transcription and/or translation of estrogen in the transfected cells. The medium was changed after 5 h to

Table 2 Real-time PCR primers and probe sequences for mER α , mER β and mLXR α

mERα (NM_007956)	Forward primer	5'-GTGCCTGGCTGGAGATTCTG-3'
	Reverse primer	5'-GAGCTTCCCCGGGTGTTTC-3'
Over exon-exon boundary	Probe	5'-TGATTGGTCTCGTCTGGCGCTCC-3'
mERβ (NM_010157)	Forward primer	5'-CAGTCCGTCTGGCCAACCT-3'
	Reverse primer	5'-ACCACATTTTGCACCTCA-3'
Over exon-exon boundary	Probe	5'-TGTTCCATGCCCTTGTTACTGATGTGCCT-3'
mLXRα (AJ132599)	Forward primer	5'-TCGCAAATGCCGCCA-3'
	Reverse primer	5'-TCAAGCGGATCTGTTCTTCTGA-3'
Over exon-exon boundary	Probe	5'-AGCACACACTCCTCCCTCATGCCTG-3'

charcoal-treated FBS-containing media with 10 nM E2 or vehicle (99.5% EtOH). Cells were harvested 24 h after transfection, and firefly and *Renilla* luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Results

ER α and ER β mRNA expression in mouse adipose tissue

mRNA levels of ER α and ER β are shown in Fig. 1A and B respectively. ER α levels in WAT were approximately double those in BAT for both sham-operated and ovariectomized mice (Fig. 1A). Low levels of ER β mRNA could be detected in mouse adipose tissue by quantitative real-time PCR; however, ER α is the main ER expressed in both WAT and BAT of mouse (Fig. 1C).

Gene expression profiling identifies estrogen-regulated genes in mouse WAT

To identify estrogen-regulated signaling networks in mouse WAT, we treated mice with a single injection of E2. E2 levels were highest after 10 h and decreased with time (Fig. 2A). Gene expression profiles were analyzed after 10, 24 and 48 h treatment. An overview of the number of estrogen-regulated genes after the different treatment times is shown in Fig. 2B. The number of changed genes is highest after 24 h.

The microarray results were confirmed with real-time PCR (Fig. 3). Two genes with increased and two genes with decreased expression were selected for the confirmation. Genes involved in adipogenesis or lipogenesis and lipolysis were

identified from the literature and are presented in Table 1. Genes known to be increased in lipogenesis or adipogenesis, and that were decreased following estrogen treatment, are likely to play a role in the mechanism of estrogenic decrease of adipose tissue mass. CCAAT/enhancer-binding protein (C/EBP α), signal transducer and activator of transcription 1 (STAT1), retinol-binding protein (RBP) and pyruvate carboxylase were all decreased after 10 h treatment, as were lactate dehydrogenase-B and adiponectin after 24 h.

Genes known to be repressed in lipogenesis/adipogenesis or increased during lipolysis might also play a role in decreasing adipose tissue mass. CHOP-10/GADD153 and uncoupling protein 1 (UCP1) were increased after 10 h, and alpha-1 type I procollagen after 24 h. Some receptors for lipolytic hormones were also increased, interleukin 1 receptor type II after 10 h and growth hormone receptor after 24 and 48 h, although the growth hormone receptor was decreased first (after 10 h).

Several other metabolism-associated genes were found to be under estrogenic control, such as low-density lipoprotein receptor (LDLR), fatty acid coenzyme A ligase long chain 2, insulin-like growth factor I receptor and insulin-like growth factor 2 receptor.

Many knockout mouse models exist with a phenotype of leanness and obesity resistance (Chen & Farese 2001). Two of the genes knocked out in the leanness models, protein tyrosine phosphatase 1B and acyl-CoA:diacylglycerol acyltransferase (DGAT), decreased in expression after 10 h, and after 10 and 24 h of treatment respectively.

Several other regulated genes were found in this study. Many genes with increased expression following E2 administration are involved in cell adhesion, most of them encoding procollagens of

Figure 1 ER α expression is much higher than ER β expression in mouse WAT and BAT. ER mRNA expression in WAT and BAT in sham-operated and ovariectomized (OVX) 13-week-old female C57BL/6 mice ($n=4$) was analyzed with real-time PCR. (A) ER α mRNA levels, (B) ER β mRNA levels and (C) relative expression of ER α and ER β . Results presented are means $\pm$ standard deviation normalized to GAPDH, with the first sample set to 1. In (C), known amounts of plasmids were used as standards in the calculation of the absolute amounts of receptor mRNAs. Statistical significance relies on Student's t -test: ** $P<0.01$; *** $P<0.001$.

Figure 2 Serum estradiol levels and number of estrogen-regulated genes. (A) Serum estradiol levels were measured by a radioimmunoassay detecting estradiol with a sensitivity of 10 pg/ml ($n=7$). (B) Number of genes that were increased or decreased by E2 at each time point. Included genes were similarly regulated in all four comparisons (see Materials and methods) with average fold change ≥ 2 .

different types. Several genes with increased expression represent heat-shock proteins involved in heat-shock response. Many genes with decreased expression are involved in transcription regulation, including some SRY-box containing genes.

LXR α is a target gene for estrogen in WAT and liver

Interestingly, one gene found to be decreased 10 h after estrogen treatment was the nuclear receptor LXR α . LXRs have been shown to play important roles in lipid and cholesterol metabolism (Steffensen *et al.* 2002). Recently, LXR double-knockout mice

Figure 3 Confirmation of Affymetrix results by real-time PCR. Estrogen regulation of (A) apolipoprotein E (apoE), (B) glucose-6-phosphate dehydrogenase (G6PD), (C) glycerol-3-phosphate acyltransferase (GPAT) and (D) liver X receptor (LXR) α was confirmed at different time points. RNA from individual mice (the same mice as for the gene expression profiling) was used ($n=7$). Results presented are means $\pm$ standard deviation normalized to 18S, with the first sample set to 1. D=decreased, I=increased in three or four out of four comparisons (3/4 or 4/4, respectively); however, all comparisons were used for the calculation of average fold change. The numbers included are fold changes for the E2 vs vehicle treatments. Statistical significance relies on Student's t -test: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 3 Regulation of LXR α target genes in fat as determined by Affymetrix analysis. Regulation is indicated with the average fold change as in Table 1, and time point. Numbers in parenthesis are the numbers of comparisons. References for target gene regulation by LXR α are included in the reference column.

Name	Regulation	Function (Edwards <i>et al.</i> 2002)	Reference
SREBP-1c	-1.6 10 h (3/4); -1.4 24 h (4/4)	Fatty acid synthesis	(Repa <i>et al.</i> 2000)
Apolipoprotein E	-1.7 10 h (4/4)	Clearance of cholesterol	(Laffitte <i>et al.</i> 2001)
Phospholipid transfer protein	-1.7 10 h (4/4)	HDL metabolism	(Cao <i>et al.</i> 2002)
ABCA1	-1.9 10 h (4/4)	Efflux of phospholipid and/or cholesterol	(Costet <i>et al.</i> 2000)
ABCG1/ABC8	-1.2 10 h (4/4)	Efflux of phospholipid and/or cholesterol	(Venkateswaran <i>et al.</i> 2000)

were reported to have significantly decreased WAT and BAT (Juvet *et al.* 2003). A 10-h estrogen treatment also repressed the mRNA levels of several known target genes normally upregulated by LXR α , such as sterol regulatory element-binding protein 1c (SREBP-1c), apolipoprotein E

(apoE), phospholipid transfer protein (PLTP), ATP-binding cassette (ABC) A1 and ABCG1 (Table 3). The regulation of LXR α and SREBP-1c observed with the Affymetrix gene chips was confirmed using real-time PCR analysis (Fig. 4A and B). LXR β was not regulated by estrogen in this

Figure 4 LXR α and SREBP-1c, but not LXR β , mRNA levels were decreased after 10 h of estrogen treatment in adipose tissue. (A) LXR α mRNA levels, (B) SREBP-1c mRNA levels and (C) LXR β mRNA levels. RNA from individual mice (the same mice as for the gene expression profiling) was used ($n=7$). Results presented are means $\pm$ standard deviation normalized to 18S, with the first sample set to 1. Statistical significance relies on Student's t -test: * $P<0.05$.

Figure 5 LXR α mRNA is decreased after 24 h of estrogen treatment in liver. RNA from liver was used, and expression was analyzed by real-time PCR. RNA from individual mice (the same mice as for the gene expression profiling) was used ($n=7$). Results presented are means $\pm$ standard deviation normalized to 18S, with the first sample set to 1. Statistical significance relies on Student's t -test: * $P<0.05$.

system (Fig. 4C). Liver is another target organ for the metabolic effects of estrogen. Interestingly, estrogen decreases mRNA levels of LXR α also in liver after 24 h of treatment (Fig. 5).

The LXR α promoter is repressed by estrogen

To determine whether the LXR α promoter is a direct target for estrogen repression, an approximately 1.5 kb fragment of the 5'-flanking region of the mouse LXR α gene (positions -1513 to +1), was cloned, as described in Materials and methods (Fig. 6A). The construct was transiently transfected into Hepa cells and 3T3-L1K cells together with an ER α expression plasmid as indicated. Estrogen treatment, in the presence of ER α , decreases the activity of the firefly luciferase reporter gene by 60% in Hepa cells (Fig. 6B) and 77% in 3T3-L1K cells (Fig. 6C). A positive control was included to exclude a general effect on transcription and/or translation of estrogen in the transfected cells (Fig. 6B–C).

Discussion

In this paper, we describe the direct effects of estrogens on WAT gene expression. In particular, we demonstrate downregulation of LXR α and

several of its target genes after 10 h of estrogen treatment. Our findings are consistent with a recent report showing estrogen regulation of LXR α in primary macrophages and leukemic, monocytic THP-1 cells (Kramer & Wray 2002). However, our report is the first to demonstrate estrogen regulation of LXR α in metabolic tissues. Furthermore, we demonstrate direct downregulation of the LXR α promoter by estrogen. The studied fragment of the LXR α promoter contains one estrogen

response element (ERE) sequence with deviation from the consensus at three positions, and several AP-1 sites (Fig. 6A). Further studies will be needed to determine whether these sites contribute to the observed downregulation of LXR α by estrogen. Downregulation of LXR α expression was observed only after 10 h of estrogen treatment. We speculate that levels of estrogen in WAT after 24 h are insufficient to maintain suppression of the LXR α promoter.

Estrogen action on adipose tissue could be mediated via LXR α and its target genes. SREBP-1c activates genes involved in fatty acid synthesis (Repa *et al.* 2000). SREBP-1c-deficient mice have reduced plasma TGs and reduced mRNA levels of enzymes of fatty acid and TG synthesis (Liang *et al.* 2002a). Therefore, the decrease in SREBP-1c following estrogen could explain the decrease in amount of adipose tissue seen after estrogen treatment.

Phospholipid transfer protein (PLTP), apoE, ABCA1 and ABCG1 are other target genes of LXR α that were regulated by estrogen in this experiment. There has been one report of a decreased PLTP activity after 71 days of hormone replacement therapy in women (Ulloa *et al.* 2002). Serum concentration of apoE was decreased by estrogen in prostate cancer patients (Usui *et al.* 2002) but was also found to be increased by estrogen in brain (Levin-Allerhand *et al.* 2001). Only two LXR α target genes, fatty acid synthase (FAS) and lipoprotein lipase (LPL), were unchanged according to our criteria for assessing changed expression of genes. For LPL, however,

Figure 6 Estrogen decreases expression from the LXR α promoter in Hepa and 3T3-L1K cells. An amount of 0.2 μ g LXR α -1513/+1 promoter construct or empty pGL3-Basic vector (expressing firefly luciferase) was cotransfected with 0.05 μ g ER α or empty pSG5 vector. Amounts of 0.002 or 0.0005 μ g of a plasmid expressing *Renilla* luciferase under control of the thymidine kinase promoter were added to the transfections of Hepa and 3T3-L1K cells respectively to exclude a general effect on transcription and/or translation of estrogen in these cells. Cells were treated with vehicle (99.5% EtOH) or 10 nM E2. (A) LXR α promoter construct, (B) transfection of Hepa cells and (C) transfection of 3T3-L1K cells. The data presented are relative values based on average luciferase activities and are representative of at least three independent transfections. The first sample is set to 1. Statistical significance relies on Student's *t*-test: **P* < 0.05; ****P* < 0.001.

the regulation by LXR α was observed only in liver and macrophages, not in adipose tissue or muscle (Zhang *et al.* 2001). Direct regulation of FAS by LXR α has been shown before in HepG2 cells (Joseph *et al.* 2002).

Several other important genes involved in lipid metabolism were found to be regulated by estrogen in adipose tissue. CCAAT enhancer-binding protein α (C/EBP α) is required for differentiation of WAT and fat accumulation (Linhart *et al.* 2001). C/EBP α knockout mice die just after birth due to severe hypoglycemia, but they also fail to accumulate interscapular fat (Wang *et al.* 1995, Flodby *et al.* 1996). Since hepatic dysfunction was the major cause of lethality, mice expressing C/EBP α exclusively in the liver were generated. These transgenic C/EBP α knockout mice survived longer and lacked subcutaneous, perirenal and epididymal WAT already at 7 days of age (Linhart *et al.* 2001).

Downregulation of C/EBP α might play an important part in the mechanism of estrogen-induced decrease in adipose tissue, since, in this study, estrogen treatment decreased C/EBP α expression. C/EBP α is required for expression of the β 3-adrenergic receptor during adipogenesis (Dixon *et al.* 2001). The β 3-adrenergic receptor was also downregulated after 10 and 24 h of estrogen treatment. Interestingly, peroxisome proliferator activated receptor (PPAR) γ was not regulated in our experiment. This lack of PPAR γ regulation was confirmed by real-time PCR (data not shown). Therefore, PPAR γ does not appear to be a direct target for estrogen in mouse fat.

One of the genes knocked out in mouse models of obesity resistance, acyl coenzyme A:diacylglycerol transferase, is responsible for the final step in the glycerol phosphate pathway of TG synthesis (Bray 2002). These mice also show increased thermogenesis (Bray 2002). The other knockout gene mentioned which leads to obesity resistance, protein tyrosine phosphatase-1B (PTP-1B), encodes an enzyme that terminates the action of phosphorylated tyrosines in receptors (Bray 2002). The gene has been implicated in insulin resistance, since insulin resistance is reduced in this knockout (Bray 2002).

Several of the regulated genes (Table 1 and data not shown) can serve as positive controls for the identification of estrogen-regulated genes, since they are known to be regulated by estrogen. They

include the low-density lipoprotein receptor (LDLR) (Parini *et al.* 1997), creatine kinase (Somjen *et al.* 1997) and the SRY-box containing gene 4 (Hunt & Clarke 1999).

The identification of ER α as the major ER in adipose tissue is in good agreement with previous findings of obesity in ER α knockout mice, and supports the notion that direct ER α -mediated effects of estrogens in adipose tissue decrease adipose mass.

Estrogen is known to affect fat mass in humans. It is possible that SERMs (selective estrogen receptor modulators) specifically targeting adipose tissue might provide therapeutic opportunities for obesity.

In conclusion, we have discovered that LXR α represents an estrogen-regulated pathway in adipose tissue. Several metabolism-associated genes not previously known to be regulated by estrogens were identified as possible mediators of estrogen-induced reduction of adipose tissue, including SREBP-1c and C/EBP α . A large number of other estrogen-regulated genes were identified in mouse adipose tissue.

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