

Response of the adipose tissue transcriptome to dihydrotestosterone in mice

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Zhang Y, Calvo E, Martel C, Luu-The V, Labrie F, Tchernof A. Response of the adipose tissue transcriptome to dihydrotestosterone in mice. *Physiol Genomics* 35: 254–261, 2008. First published August 26, 2008; doi:10.1152/physiolgenomics.00257.2007.—Androgens have been postulated to be important modulators of adipose tissue metabolism and fat cell function. In the present study, we investigated the response of male and female mice retroperitoneal adipose tissue to the nonaromatizable androgen dihydrotestosterone (DHT). Adipose tissue samples were obtained in gonadectomized animals treated with vehicle (control group), or injected with 0.1 mg DHT 1, 3, 6, 12, 18, and 24 h prior to necropsy. Fourteen animals were pooled at each time point (total 196 animals). Transcripts that were significantly modulated were considered as androgen-responsive genes. Quantitative real-time RT-PCR was used to confirm results from the microarray analysis in a subset of 46 probe sets in male mice and 98 probe sets in female mice. Considering peak time vs. control, we confirmed 74.0 and 63.3% of the modulated genes by PCR in males and females, respectively. Four genes were significantly stimulated in a similar manner by DHT in both sexes, namely metallothionein 1, growth arrest and DNA-damage-inducible 45 gamma, cyclin-dependent kinase inhibitor 1A, and fk506-binding protein 5. All these genes appear to be involved in the regulation of adipocyte differentiation/proliferation and adipogenesis. In conclusion, this study, which evaluated the acute transcriptome response of adipose tissue to DHT in male and female mice, suggests that DHT consistently modulates genes involved in the regulation of adipogenesis in retroperitoneal adipose tissue of both male and female animals.

retroperitoneal; microarray; adipogenesis

SEVERAL STUDIES SUGGEST THAT sex hormones modulate adipose tissue deposition at the level of preadipocyte differentiation and/or proliferation as well as adipocyte lipogenesis and/or lipolysis (2, 36, 40). Cross-sectional (35) and longitudinal (18, 22) studies found that circulating androgen levels are negatively associated with abdominal obesity and visceral fat accumulation in men. A number of studies also reported negative associations between circulating androgens and abdominal adiposity in women (3, 10, 47). On the other hand, female-to-male transsexuals treated with high doses of androgens preferentially accumulate intra-abdominal fat and progressively lose gluteal-femoral fat (15). In women with the polycystic ovary syndrome, a positive association between abdominal obesity and plasma androgen levels is found (13). Triglycerides in retroperitoneal and mesenteric adipose tissue increase after castration in rats but decrease when animals are treated with testosterone (25). Additionally, androgen receptor knock-

out mice develop late onset obesity through reduced adipose tissue lipolysis in animals of both sexes (16, 42). These findings suggest that androgens may be key steroids in the modulation of fat accumulation and distribution in both humans and rodent models.

The impact of androgens on gene transcription in adipose tissue from male mice has been previously examined by serial analysis of gene expression in one study (7). Although very informative, that approach did not consider temporal expression regulation in response to the hormone. In the present study, using DNA microarrays, we examined the acute response of the adipose tissue transcriptome to dihydrotestosterone (DHT) in gonadectomized male and female mice. We tested the general hypothesis that transcriptional profiling of androgen-responsive genes in both male and female mice would bring new insights on possible signaling pathways linking fat distribution and sex hormones.

METHODS

Mice and RNA sample preparation. Male and female, 14- to 15-wk-old, C57BL/6 mice were obtained from Charles River Canada (St-Constant, Québec, Canada). Fourteen animals were pooled for one replica at each time point (1, 3, 6, 12, 18, and 24 h), and fourteen mice were pooled to generate two replica of the control group in each sex. A total of 196 animals were used in our study. Mice were housed (1 per cage) in plastic cages under 12 h light-dark cycles; the animals had access to Lab Rodent Diet no. 5002 and were given tap water ad libitum. Bilateral gonadectomy (castration for male mice and ovariectomy for female mice) were performed under isoflurane-induced anesthesia 7 days prior to death and organ collection for the seven gonadectomized groups ($n = 14$ mice per group). DHT (0.1 mg) was injected subcutaneously 1, 3, 6, 12, 18, and 24 h prior to death in DHT-treated groups. One group was retained as control and received a vehicle solution [0.4% (wt/vol) Methocel A15LV Premium; 5% ethanol]. Retroperitoneal adipose tissue samples from all mice of the same group were collected, rapidly trimmed, snap-frozen in liquid nitrogen and stored at -80°C prior to mRNA extraction. These fat samples were pooled to eliminate interindividual variation and to extract a sufficient amount of mRNA. For blood DHT measurements, gonadectomized C57BL/6 male and female mice were injected with 0.1 mg DHT 1, 3, 6, 12, 18, or 24 h prior to blood sampling ($n = 12$ mice/time point/sex). Control mice ($n = 24$ mice/sex) were injected with the vehicle alone (5% ethanol-0.4% methylcellulose) 24 h prior to necropsy. Mice under isoflurane anesthesia were exsanguinated by cardiac venipuncture. Blood samples were processed for serum preparation and kept frozen at -80°C until use for the determination of serum DHT concentrations by gas chromatography mass spectrometry as previously described (23) with the exception of the serum volume used (0.15–0.3 ml in mice instead of 0.75 ml in humans). Serum

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samples from two mice of the same group were pooled for each determination of DHT levels ($n = 6$ for DHT groups; $n = 12$ for the control group). The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care. The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals. Institutional approval was obtained.

Target preparation. Total mRNA was isolated with TRIzol (Invitrogen, Burlington, Ontario) following the manufacturer's protocol. Total mRNA (20 μg) was converted to cDNA by incubation with 400 U SuperScript II reverse transcriptase (Invitrogen) using a T7-oligo-(dt)₂₄ primer [5'-GGCCAGTGAATTGTAATACGACTCATATAGGGAGGCGG-(dt)₂₄-3'] 1 \times first-strand buffer (50 mM Tris·HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT) and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed using 40 U DNA polymerase I (Invitrogen), 10 U *Escherichia coli* DNA ligase (Invitrogen), 2 U RNase H (Invitrogen), 1 \times reaction buffer [18.8 mM Tris·HCl pH 8.3, 90.6 mM KCl, 4.6 mM MgCl₂, 3.8 mM DTT, 0.15 mM NAD, 10 mM (NH₄)₂SO₄] and 0.2 mM dNTPs at 16°C for 2 h. cDNAs were blunt-ended with 10 U of T4 DNA polymerase (Invitrogen) incubated 5 min at 16°C. cDNA was extracted with phenol-chloroform using phase lock gels (Brinkman, Mississauga, Ontario, Canada), ethanol precipitated, and resuspended in 10 μl of DEPC-treated H₂O. cDNA was in vitro transcribed using a T7 BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) to produce biotinylated cRNA. The mixture (20 μl final volume) was incubated at 37°C for 5 h, with gentle mixing every 30 min. Labeled cRNA was purified with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Purified cRNA was fragmented to 30–200 mer using a fragmentation buffer (100 mM potassium acetate, 30 mM magnesium acetate, 40 mM Tris-acetate pH 8.1), for 20 min at 94°C. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by microcapillary electrophoresis (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA).

Microarray hybridization and scanning. Microarray analysis was performed using Murine GeneChip U74Av2 GeneChips (Affymetrix, Santa Clara, CA), which allow the analysis of the expression level of >12,400 mouse genes and expressed sequence tags (ESTs) on each array in the present study. Fragmented cRNA (15 μg) was incubated with 1 \times hybridization buffer (0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 5 nM control oligonucleotide B2) and 1 \times eukaryotic hybridization control solution (1.5 pM *BioB*, 5 pM *BioC*, 25 pM *BioD*, and 100 pM *cre*), for 16 h at 45°C with constant rotation (60 rpm). Microarrays were processed using an Affymetrix GeneChip Fluidic Station 400 (protocol EukGE-WS2Av4). Staining was performed with streptavidin-conjugated phycoerythrin (SAPE) followed by amplification with a biotinylated anti-streptavidin antibody and by a second round of SAPE solution. GeneChips were scanned using an Agilent GeneArray Scanner (Agilent Technologies). Signal intensities for β -actin and GAPDH genes were used as internal quality controls. The ratio of fluorescent intensities for the 5' and 3' ends of these housekeeping genes was <2. Scanned images were analyzed with Microarray Suite 5.0 (MAS, Affymetrix). Tissues from the 14 animals at each time point were pooled, and the cRNA probe corresponding to each time point was hybridized once on separate microarrays. Tissues from the 14 animals of the control group were pooled, and the cRNA probe from the control group was hybridized on two microarrays (duplicate). A total of 16 microarrays were used in our experiments. The microarray data have been deposited in the Gene Expression Omnibus repository (series number GSE9631).

Real-time RT-PCR. First-strand cDNA synthesis was performed using 5 μg of RNA extracted from each time pool in a reaction containing 200 units of Superscript II RNase H-reverse transcriptase (Invitrogen), 300 ng of oligo dT18, 500 μM dNTP, 10 mM DTT, and 34 units of human RNase inhibitor (Amersham Pharmacia) in a final

volume of 50 μl . The resulting products were then treated with 1 μg of RNase A for 30 min at 37°C and purified thereafter with Qiaquick PCR purification kits (Qiagen). For quantitative PCR analyses, a Light-Cycler PCR (Roche Diagnostics) was used to confirm the expression level of cDNAs measured in the microarray method. The FastStart DNA Master SYBR green kit (Roche Diagnostics) was used in a final reaction volume of 20 μl containing 3 mM MgCl₂ and cDNA corresponding to 20 ng total RNA. The PCR was carried out according to the following conditions: 95°C/10 min, 50 cycles of (95°C/10 s, 55–65°C/5 s, 72°C/11 s) with a temperature transition of 3°C/s. PCR results were calculated and normalized using the second-derivative and double-correction method as described previously (29), and with use of the housekeeping gene ATP synthase O subunit (*Atp5o*). This gene was found to display remarkably stable expression levels from embryonic life through adulthood in various tissues (48). Messenger RNA levels were expressed as the number of copies per microgram of total RNA using a standard curve of crossing points vs. logarithm of quantity. The standard curve was established using known cDNA amounts of 10, 10², 10³, 10⁴, 10⁵, and 10⁶ copies of *Atp5o*. The Light Cycler 3.5 program was provided by the manufacturer (Roche). Expression of the housekeeping gene was constant at all time points and not significantly influenced by DHT treatment.

Data processing and statistical analysis. Raw data of microarrays were collected and analyzed using the Affymetrix MAS 5.0 software. To control for variability of the measurement, which is greater at low expression level (50), we established a relationship between fold changes and expression levels as described previously (31). To select differentially expressed genes, we used the following criteria: 1) the fold change (FC) had to be higher than the limit fold change (LFC) over the time course; and 2) at least two "present calls" (P value <0.04) had to be obtained across all arrays. The LFC was defined as $\text{LFC} = a + b/X$, where X is the minimum average difference intensity (ADI, i.e., absolute expression level) for a given gene. The parameters a and b were estimated based on the distribution of ratios calculated from replicated chips (1.9 and 60, respectively, in the present experiment). The FC was calculated with the following equation: $\text{FC} = \text{Max ADI}/\text{Min ADI}$ over the time course and control. Average ADI of two technique replica of control were used in data analysis. To identify genes to be validated by quantitative real-time RT-PCR, genes were further filtered following additional criteria: 1) ADI of a given gene >400 on >60% of arrays; 2) absolute FC on peak time (peak time ADI/control ADI) ≥ 2.0 . Annotations of interesting probe sets were collected from different available databases using the NetAffx online tool (<http://www.affymetrix.com/analysis/index.affx>), and their identity was verified with BLAST (<http://www.ncbi.nlm.nih.gov>). The Ingenuity Pathway Analysis System (Ingenuity Systems, www.ingenuity.com) was also used to visualize gene expression data in the context of biological pathways.

Validation analyses were performed using peak time vs. control comparisons to examine the direction of fold changes with the microarray and RT-PCR methods. Since data for all time points were also compared with those of real-time RT-PCR for the genes selected, Spearman rank nonparametric correlation analysis was also used to compare time courses obtained with microarrays and real time RT-PCR. Time points from the microarray analysis were correlated to time points from the real-time RT-PCR analysis and DHT responses that generated correlation coefficients with a $P \leq 0.1$ were considered similar. This permissive cutoff was chosen to avoid underestimation of time course reproducibility.

RESULTS

In both male and female mice, serum DHT reached the maximal level 1 h after treatment and decreased thereafter to reach baseline at 24 h (Fig. 1). The overall objective of the data mining approach was the identification of candidate genes within adipose tissue that were sensitive to DHT treatment.

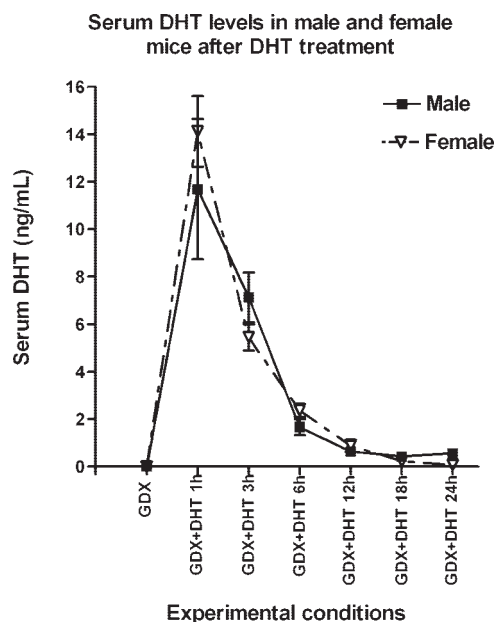


Fig. 1. Serum dihydrotestosterone (DHT) was measured at each time point after subcutaneous injection with 0.1 mg DHT in each male and female mouse as well as each of control gonadectomized (GDX) animals. Data are presented as means \pm SE.

Globally, based on the criteria mentioned in METHODS, we found that 94 probe sets were significantly modulated by DHT on chip arrays from male mice representing 93 genes (Supplemental Table S1).¹ A total of 116 probe sets were significantly modulated by DHT on chip arrays from female mice representing 112 genes (Supplemental Table S2). These genes exhibited gene expression changes that were ≥ 2.0 -fold comparing peak time point to control.

The distributions of peaking times for significantly modulated probe sets were examined (Fig. 2). In male mice, 38 (40%) probe sets presented peak times at 1 h. A total of 49 (42.2%) and 20 (17.2%) probe sets in female mice presented peak times at 18 and 24 h, respectively. In male mice, 33 (35.1%) upregulated probe sets and 61 (64.9%) downregulated probe sets were identified. In female mice, 51 (44%) probe sets were upregulated and 65 (56%) probe sets were downregulated.

Among the microarray data, a total of 21 genes were found to be significantly modulated in adipose tissue samples from both male and female mice. Sixteen of them changed in a similar direction in response to DHT. Five genes showed opposite regulation trends in males and females, including LOC677213, Tnnc2, Ddx6, Mgea5, and Myh1. Four of the latter genes were downregulated in male mice and upregulated in female mice. Only the Mgea5 gene was upregulated in male mice and downregulated in female mice (data not shown).

To confirm gene expression responses by an independent method, quantitative real-time RT-PCR validation was conducted. We validated subsets of 46 and 98 transcripts in male and female mice, respectively (Supplemental Tables S1 and S2). Using peak time vs. control comparisons, we confirmed 74.0 and 63.3% of modulated genes by PCR in male and

female mice, respectively. We also used a more stringent approach to precisely compare time courses between the two methods. Using $P \leq 0.1$ as a cut-off for significance, we performed Spearman nonparametric correlation analysis, and 17 genes (37.0%) showed reproducible time courses in PCR validation in male mice (Table 1). In female mice, 26 genes (26.5%) showed a Spearman rank correlation coefficient with a P value < 0.10 , indicating identical time courses in PCR validation (Table 2). Among upregulated genes with positive PCR validations in male mice, five were related to cell cycle; four to DNA replication, recombination, and repair; 10 to cell death; seven to cellular development; and eight to cellular growth and proliferation. Among downregulated genes, five were related to carbohydrate metabolism, three to cellular compromise, two to protein degradation, four to cell death, and three to cell-to-cell signaling and interaction. In female mice, among upregulated genes with positive PCR validations, nine were related to cellular development, eight to cellular growth and proliferation, eight to cell death, seven to cellular assembly and organization, and four to cellular compromise. Among downregulated genes, seven were related to lipid metabolism, nine to molecular transport, three to nucleic acid metabolism, 12 to small molecule biochemistry, and six to cell cycle.

Table 3 shows that four genes generated similar responses to the DHT treatment in both sexes, namely FK506 binding protein 5 (Fkbp5), cyclin-dependent kinase inhibitor 1A (p21 or Cdkn1a), growth arrest and DNA-damage-inducible 45 gamma (Gadd45g), and metallothionein 1 (Mt1). The DHT response of all these genes was validated by quantitative real-time RT-PCR. They all showed highly significant Spear-

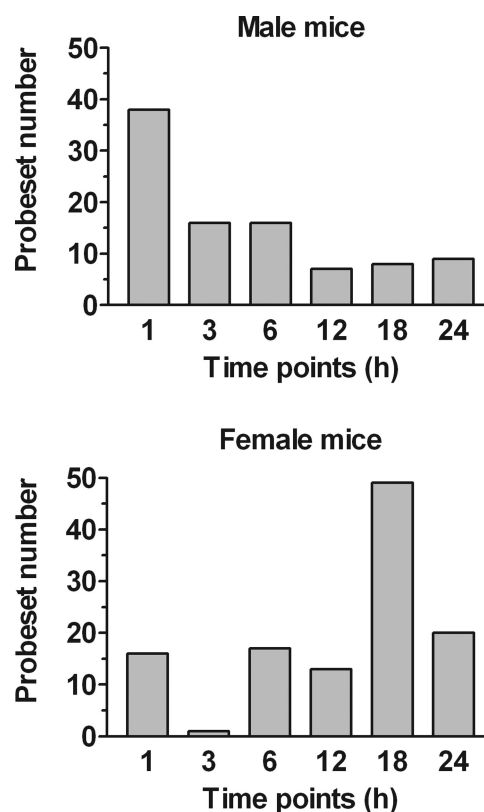


Fig. 2. Distribution of peaking times for probe sets that were significantly modulated in male and female retroperitoneal adipose tissue (AT) samples.

¹ The online version of this article contains supplemental material.

Table 1. In male mice, 17 genes were considered as responsive to DHT treatment

Probe Set	Gene Symbol	Description	Accession	P Value
98308_at	<i>Myh1</i>	myosin, heavy polypeptide 1, skeletal muscle, adult	AJ002522	0.000
102736_at	<i>Ccl2</i>	chemokine (C-C motif) ligand 2	M19681	0.000
93482_at	<i>Mylk</i>	myosin, light polypeptide kinase	AI117835	0.000
94297_at	<i>Fkbp5</i>	FK506 binding protein 5	U16959	0.000
101979_at	<i>Gadd45 g</i>	growth arrest and DNA-damage-inducible 45 gamma	AF055638	0.003
98092_at	<i>Plac8</i>	placenta-specific 8	AA790307	0.003
96344_at	<i>Eno3</i>	enolase 3, beta muscle	X61600	0.007
160894_at	<i>Cebpd</i>	CCAAT/enhancer binding protein (C/EBP), delta	X61800	0.014
94429_at	<i>Eef1a2</i>	eukaryotic translation elongation factor 1 alpha 2	L26479	0.014
94881_AT	<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A	AW048937	0.014
93573_at	<i>Mt1</i>	metallothionein 1	V00835	0.023
102906_at	<i>Tgtp</i>	T cell-specific GTPase	L38444	0.036
92599_at	<i>Pgam2</i>	phosphoglycerate mutase 2	AF029843	0.036
160754_at	<i>Pygm</i>	muscle glycogen phosphorylase	AI850363	0.052
100323_at	<i>Amd2</i>	S-adenosylmethionine decarboxylase 2	Z23077	0.071
94438_at	<i>Pfkm</i>	phosphofructokinase, muscle	AI852672	0.094
99667_at	<i>Cox6a2</i>	cytochrome c oxidase, subunit VI a, polypeptide 2	U08439	0.094

Time course responses of these genes were reproducible in the microarray and quantitative real-time RT-PCR experiments. DHT, dihydrotestosterone.

man rank correlation coefficients with *P* values of 0.0001, 0.014, 0.003, and 0.023 in fat samples from male mice and 0.0001, 0.01, 0.07, and 0.0001 in fat samples from female mice. These genes were all upregulated by DHT in both sexes (Table 3 and Fig. 3).

DISCUSSION

This work represents the first microarray investigation on the effects of DHT treatment in retroperitoneal adipose tissue from a rodent model. Serum DHT measures obtained over the course of the experiment suggest that our study should be considered as an acute response experiment. A significant modulation of the adipose tissue transcriptome was observed in response to

DHT in both male and female mice. A number of genes involved in distinct cellular functions were modulated in a sex-specific manner. A total of 21 genes were found to be simultaneously modulated in fat samples from both male and female mice. Sixteen of them showed similar regulatory patterns, and five genes showed opposite regulatory patterns.

Bolduc et al. (7) previously examined the effects of DHT on adipose tissue in male mice using serial analysis of gene expression at two time points (3 and 24 h). They found that various pathways were regulated by DHT in retroperitoneal adipose tissue. Of note, DHT treatment indicated a stimulation of genes involved in fatty acid and triacylglycerol production as well as lipolysis in fat samples. They concluded that almost

Table 2. In female mice, 26 genes were considered as responsive to DHT treatment

Probe Set	Gene Symbol	Description	Accession	P Value
94297_at	<i>Fkbp5</i>	FK506 binding protein 5	U16959	0.00
93573_at	<i>Mt1</i>	metallothionein 1	V00835	0.00
93783_at	<i>Ela1</i>	elastase 1, pancreatic	M27347	0.00
98488_at	<i>Myh4</i>	myosin, heavy polypeptide 4, skeletal muscle	AJ223361	0.00
94418_at	<i>Elov16</i>	ELOVL family member 6, elongation of long chain fatty acids (yeast)	AI839004	0.00
96588_at	<i>Ptger3</i>	prostaglandin E receptor 3 (subtype EP3)	D10204	0.00
97402_at	<i>Inmt</i>	indolethylamine N-methyltransferase	M88694	0.00
97524_f_at	<i>Amy2</i>	amylase 2, pancreatic	X02578	0.01
99571_at	<i>Acaa1b</i>	acetyl-coenzyme A acyltransferase 1B	AW012588	0.01
94881_AT	<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A	AW048937	0.01
100022_at	<i>Cish</i>	cytokine inducible SH2-containing protein	D89613	0.02
101922_at	<i>Kdelr2</i>	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	AW123408	0.04
92885_at	<i>Tnnt3</i>	troponin T3, skeletal, fast	L48989	0.04
100605_at	<i>Tpm2</i>	tropomyosin 2, beta	M81086	0.04
99939_at	<i>Cel</i>	carboxyl ester lipase	U37386	0.04
98308_at	<i>Myh1</i>	myosin, heavy polypeptide 1, skeletal muscle, adult	AJ002522	0.05
93028_at	<i>H19</i>	H19 fetal liver mRNA	X58196	0.05
94326_r_at	<i>Mrps18a</i>	mitochondrial ribosomal protein S18A	AV339603	0.05
103606_r_at	<i>Rgs19</i>	regulator of G protein signaling 19	AW121438	0.05
101979_at	<i>Gadd45 g</i>	growth arrest and DNA-damage-inducible 45 gamma	AF055638	0.07
92592_at	<i>Gpd1</i>	glycerol-3-phosphate dehydrogenase 1 (soluble)	M25558	0.07
97867_at	<i>Hsd11b1</i>	hydroxysteroid 11-beta dehydrogenase 1	X83202	0.07
101578_f_at	<i>Actb</i>	actin, beta, cytoplasmic	M12481	0.09
160711_at	<i>Decr1</i>	2,4-dienoyl CoA reductase 1, mitochondrial	AI844846	0.09
100566_at	<i>Igfbp5</i>	insulin-like growth factor binding protein 5	L12447	0.09
98441_at	<i>Fmr1</i>	fragile X mental retardation syndrome 1 homolog	L23971	0.09

Time course responses of these genes were reproducible in the microarray and quantitative real-time RT-PCR experiments.

Table 3. In both male and female mice, 4 genes were modulated in an identical manner

Gene Symbol	Description	Accession	Male		Female	
			Peaking Time, h	Fold Change	Peaking Time, h	Fold Change
<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A	AW048937	6	2.80	6	2.69
<i>Fkbp5</i>	FK506 binding protein 5	U16959	12	3.86	12	6.19
<i>Gadd45 g</i>	growth arrest and DNA-damage-inducible 45 gamma	AF055638	6	4.55	6	3.53
<i>Mt1</i>	metallothionein 1	V00835	12	2.70	6	3.44

Time course responses of these genes were reproducible in the microarray and quantitative real-time RT-PCR experiments. The fold change (FC) is peak time absolute FC = peak time ADI/average control ADI. ADI, average difference intensity. All data are from microarray analysis.

all aspects of cell function were affected by DHT through the modulation of gene expression. Much like the latter study, several cell functions were affected by DHT in the present analysis. For example, in male mice, we found that some genes related to cell cycle, DNA replication and recombination, as well as repair, molecular transport, and cell death were upregulated. Other genes involved in cellular function and maintenance were downregulated. In female mice, some genes related to connective tissue development and function or tissue morphology were upregulated, and some genes involved in cell cycle and cell death were downregulated.

Only four genes showed identical regulatory patterns in both male and female mice. These genes were *Fkbp5*, *Cdkn1a*, *Gadd45g*, and *Mt1*. All these genes, when upregulated, appear to be related to a negative regulation of adipocyte differentiation and adipogenesis. *Fkbp5* is considered as a glucocorticoid receptor heat shock protein 90-associated cochaperone and plays a central role in steroid hormone signaling (33). *Fkbp51* overexpression induces glucocorticoid resistance in squirrel monkeys by reducing hormone-binding affinity (38). Our finding indirectly suggests that upregulated expression of *Fkbp5* in response to DHT treatment may mediate inhibitory effects of DHT on glucocorticoid-induced fat cell differentiation and lipogenesis. Androgens stimulate lipolysis in adipose tissue and induce an antiadipogenic effect, at least in primary cultured preadipocytes, in adipocyte precursor cells and in 3T3-L1 preadipocytes (11, 43, 44).

The *Gadd45* family of genes is involved in cell cycle and programmed cell death. Specifically, proteins of the *Gadd45* family play a role in the inhibition of cellular growth (17, 49) and promote apoptotic cell death (19, 21, 28, 45). They are powerful suppressors of cell growth (20). Testosterone and DHT are thought to inhibit adipocyte differentiation through an AR-mediated nuclear translocation of β -catenin and activation of downstream Wnt signaling (43). Studies in a different model have shown that *Gadd45a* plays a role in the negative regulation of cell development through the suppression of β -catenin (20). Elevated expression of the *Gadd45g* gene in the present study may relate to reduced adipogenesis in response to DHT through this pathway.

Regarding *MT1*, obesity is induced in older *MT*-null transgenic mice (5), which are characterized by increases in body weight and white adipose tissue that are similar to that of obese diabetic (*db/db*) mice. Under cold exposure and induction by catecholamines, the *MT1* gene is abundantly detected in brown adipose tissue of rats (4, 6). Secretion or expression of *MT* protein is also observed in white adipose tissue from mice (46), dogs (14), and humans (12). All these observations indicate that expression of *MT* genes is linked to the regulation of

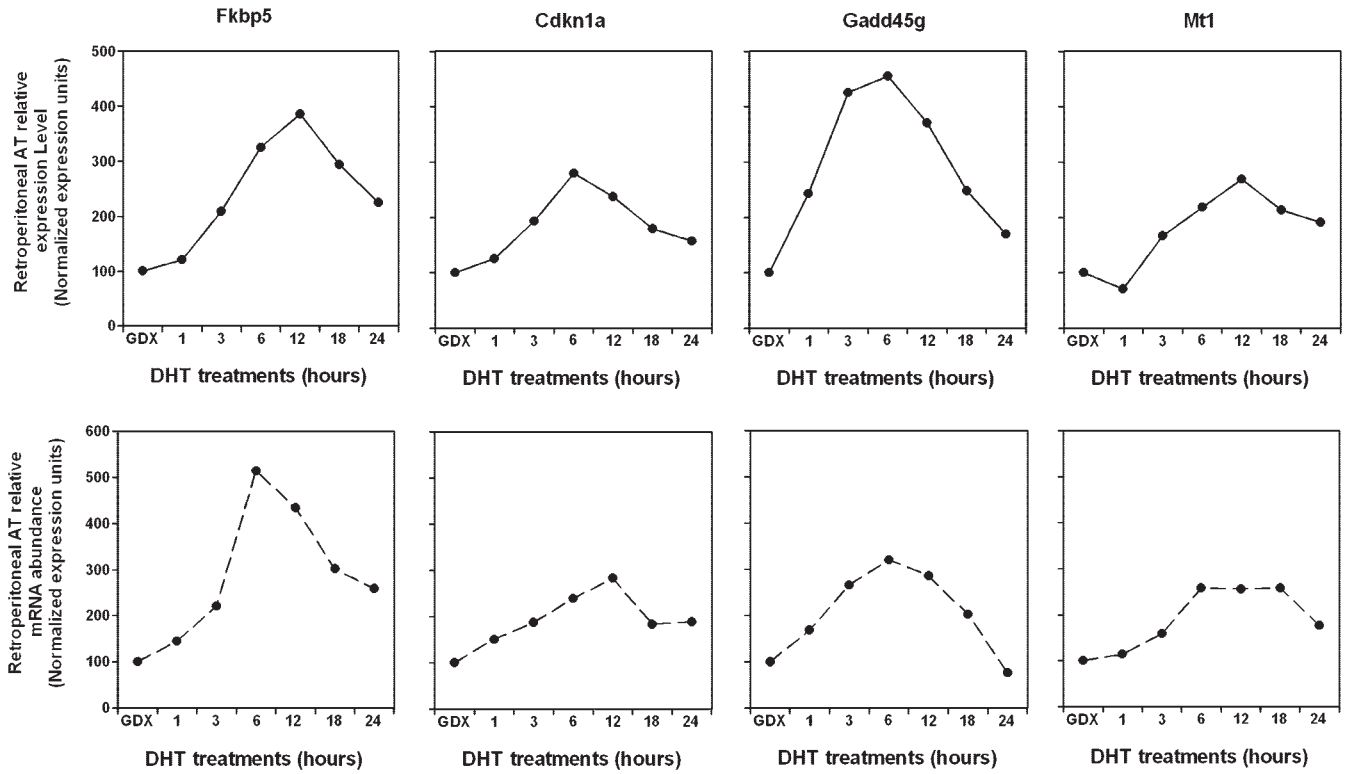
energy balance. Our data on increased *MT1* in response to DHT relate androgens to adipose tissue metabolism and energy homeostasis.

As an adipogenic transcription factor, $\text{PPAR}\gamma$ induces growth arrest and is involved in adipocyte differentiation through cyclin-dependent kinase inhibitors p18 and p21 (30). *p27/p21* double knockout mice become obese and show adipocyte hyperplasia and related metabolic consequences (32). Significantly upregulated *Cdkn1a* expression was detected in the present study, which suggests that DHT treatment may decrease adipocyte number and lead to reductions in retroperitoneal adiposity in male and female mice. We hereby provide evidence indicating that DHT signaling involves cross talks between adipocyte proliferation, apoptosis, and differentiation pathways as well as energy homeostasis in retroperitoneal fat. These phenomena appear to be independent of the sex of the animal.

We identified five genes that were simultaneously and significantly modulated in fat samples from both male and female mice but showed opposite regulatory patterns. *LOC677213*, *Tnnc2*, *Ddx6*, and *Myh1* were downregulated in male mice and upregulated in female mice. These genes are involved in the differentiation of skeletal muscle cells (9, 34) or immunity (1). *Mgea5* was upregulated in male mice and downregulated in female mice. The meningioma-expressed antigen 5 (hyaluronidase) gene (*Mgea5*) encodes a β -O-linked N-acetylglucosaminidase (O-GlcNAcase) and is involved in Type 2 diabetes as a biological and positional candidate gene (8).

Several other genes that were significantly modulated in the microarray analysis and positively validated by quantitative real-time RT-PCR in both male and female retroperitoneal fat samples were myogenic in nature. These genes included myosin light polypeptide kinase (*Mylk*), myosin heavy polypeptide 4 (*Myh4*), enolase 3 (*Eno3*), phosphofructokinase (*Pfkm*), and myosin heavy polypeptide 1 (*Myh1*). The procedure for fat sampling was carefully reviewed to ascertain that the sampling included only retroperitoneal adipose tissue and that the tissues were carefully trimmed. Similar findings of myogenic genes in fat were also reported in other studies (24, 41, 51). Adipose tissue apparently shows mesenchymal plasticity for myogenic differentiation (24), and adipocyte precursors and skeletal myoblasts are capable of differentiating along similar developmental lineages (39). The use of whole tissue samples prevents us from reaching conclusions on which cell type is responsible for the androgenic response observed and should be acknowledged. Nevertheless, Lin et al. (26) found that fat pad weight and total lipid content of myostatin-null mice were significantly lowered compared with wild-type mice and suggested that increased muscle development in myostatin knock-

Male mice



Female mice

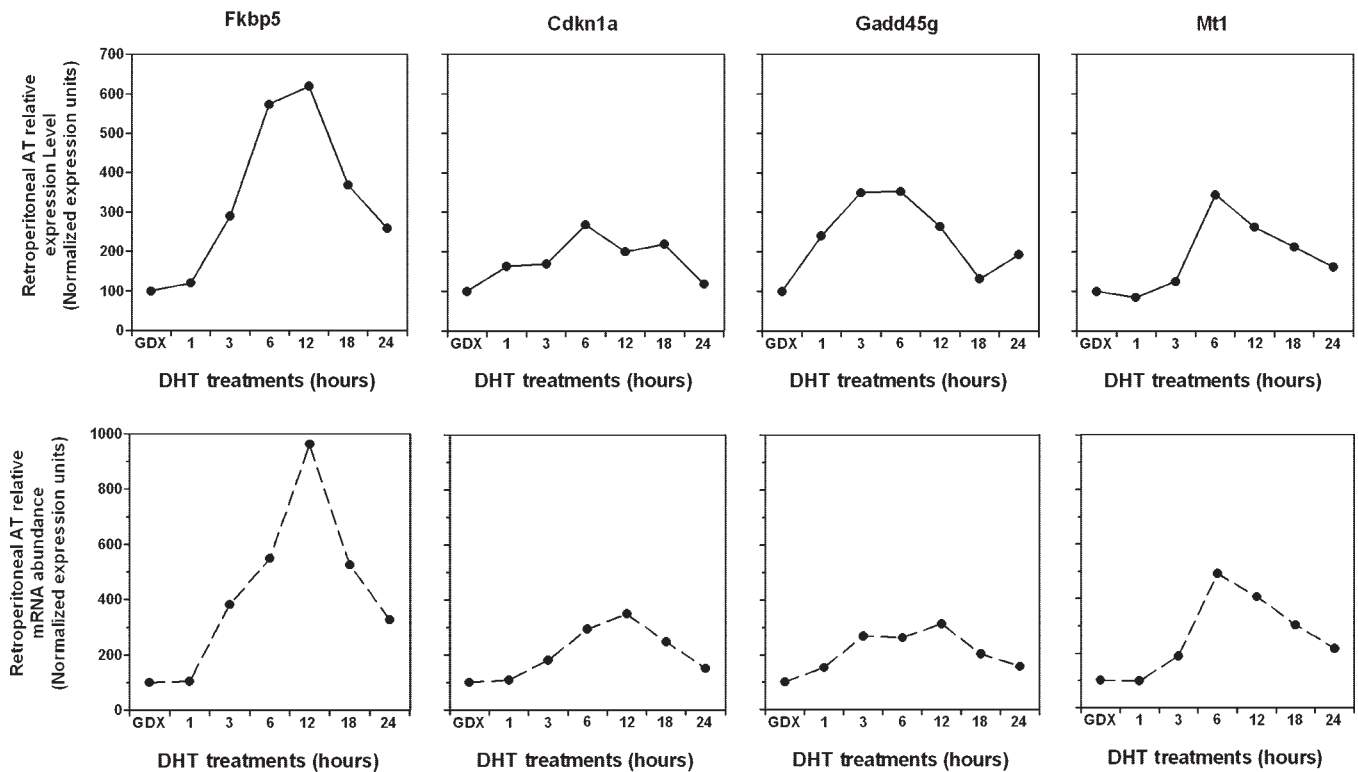


Fig. 3. Effect of DHT after 1, 3, 6, 12, 18, or 24 h of treatment or vehicle (GDX) on mRNA expression levels of 4 androgen-responsive genes in retroperitoneal AT from both male and female mice. Microarray results (solid line) were validated by quantitative real-time RT-PCR (dotted line). Data are shown in relative expression units for the results from the microarray analysis and in relative mRNA abundance for the results of RT-PCR validation.

out mice resulted in reduced adipogenesis and leptin secretion. Rebbapragada et al. (37) also found that BMP7-induced adipogenesis is restrained by myostatin in both mesenchymal precursor cells and preadipocytes. These previous reports and the present findings support the notion that a strong relationship between myogenic and adipose genes may exist in adipose tissue of both male and female mice.

In this study, we used six time points to examine trends of gene modulation in mice from both sexes. Significant modulations were observed with microarray data using criteria described in METHODS. With respect to validation by RT-PCR, most previous studies used only two time points on a limited number of genes. In the present study, a very large number of genes were validated by RT-PCR on all six time points and in the vehicle group. Using a rather stringent validation procedure, our analysis indicates that microarray results are moderately reproducible compared with quantitative real-time RT-PCR over several time points. Indeed, only 37.0 and 26.5% of transcripts in male and female mice respectively showed identical time courses. Using only peak time vs. control comparisons to validate the direction of fold changes in gene expression, valid findings increased to 74.0 and 63.3% of modulated genes in male and female mice, respectively. Because we used the pooled samples, our analysis may have contributed to yield lower than expected reproducibility rates. The use of several arrays would have allowed us to use repeated-measures ANOVA to identify significantly modulated genes. This may have led to higher reproducibility rates when comparing with RT-PCR results. We suggest, however, that the use of multiple time points and a rather stringent filtering procedure decreased the likelihood of identified false positives. Our results nevertheless suggest that careful validation on a different platform remains an absolute necessity (27).

In conclusion, our study indicates that microarray technology can be used to identify androgen-responsive genes in retroperitoneal adipose tissue from male and female mice. We found that DHT consistently stimulates genes that are associated with the regulation of adipogenesis in adipose tissue. This study also provides evidence to support the hypothesis that a myogenic response may be triggered by DHT in fat.

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