Expression of human hormone-sensitive lipase in white adipose tissue of transgenic mice increases lipase activity but does not enhance in vitro lipolysis

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Abstract **Hormone-sensitive lipase (HSL) catalyzes the hydrolysis of acylglycerols and cholesteryl esters (CEs). The enzyme is highly expressed in adipose tissues (ATs), where it is thought to play an important role in fat mobilization. The purpose of the present work was to study the effect of a physiological increase of HSL expression in vivo. Transgenic mice were produced with a 21 kb human genomic fragment encompassing the exons encoding the adipocyte form of HSL. hHSL mRNA was expressed at 3-fold higher levels than murine HSL mRNA in white adipocytes. Transgene expression was also observed in brown adipose tissue (BAT) and skeletal muscle. The human protein was detected in ATs of transgenic (Tg) mice. The hydrolytic activities against triacylglycerol (TG), diacylglycerol (DG) analog, and CE were increased in transgenic mouse AT. However, cAMP-inducible adipocyte lipolysis was lower in transgenic animals. In the B6CBA genetic background, transgenic mice up to 14 weeks of age showed lower body weight and fat mass. The phenotype was not observed in older animals and in mice fed a high-fat diet (HFD). In the OF1 genetic background, there was no difference in fat mass of mice fed ad libitum. However, transgenic mice became leaner than their wild-type (WT) littermates after a 4 day calorie restriction. The data show that overexpression of HSL, despite increased lipase activity, does not lead to enhanced lipolysis.**—Lucas, S., G. Tavernier, C. Tiraby, A. Mairal, and D. Langin. **Expression of human hormone-sensitive lipase in white adipose tissue of transgenic mice increases lipase activity but does not enhance in vitro lipolysis.** ***J. Lipid Res.*** **2003.** 44: **154–163.**

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In adipose tissue (AT), hormone-sensitive lipase (HSL) catalyzes the hydrolysis of triacylglycerol (TG) and diacylglycerol (DG) (1). The enzyme is also responsible for the hydrolysis of cholesteryl esters (CEs) and retinyl esters. Unlike other known mammalian TG lipases, HSL phosphorylation by protein kinase A leads to an activation of the enzyme. Through modulation of cAMP levels, catecholamines and insulin therefore control HSL activity (2). Two phosphorylation sites, Ser^{659} and Ser^{660}, have been shown to be responsible for in vitro activation of HSL (3). Activation of the extracellular signal-regulated kinase pathway is also able to activate lipolysis by phosphorylating HSL on Ser^{600} (4). In vivo, an important step in lipolysis activation seems to be the translocation of HSL from a cytosolic compartment to the surface of the lipid droplet (5).

HSL is classically considered to be the enzyme catalyzing the rate-limiting step of AT lipolysis (6). Indeed, a strong linear correlation was found between HSL protein levels and the maximum lipolytic capacity of human subcutaneous adipocytes stimulated by a β-adrenergic agonist (7). However, in HSL-null mice, catecholamine-induced lipolysis is blunted but unstimulated lipolysis is not altered in isolated adipocytes (8, 9). Accumulation of DG in AT shows that HSL is the rate-limiting enzyme for the catabolism of DG but not TG (10). The data therefore suggest the existence of a TG lipase different from HSL.

Access to the lipid droplet constitutes another potential mechanism for the control of lipolysis (2). Perilipins are proteins covering the large lipid droplets in adipocytes. They shield stored TG from cytosolic lipases. Upon phosphorylation, perilipins allow access of lipases to the lipid droplet. Whereas mice lacking HSL are not obese (8, 9), ablation of perilipin results in decreased fat mass

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Abbreviations: ALBP, adipocyte lipid binding protein; AT, adipose tissue; BAT, brown adipose tissue; CE, cholesteryl ester; C/EBPα, CCAAT/enhancer binding protein α; HFD, high-fat diet; mHSL, murine HSL; MOME, 1(3)-mono-oleoyl-2-*O*-mono-oleylglycerol; PPARγ_2, peroxisome proliferator-activated receptor γ_2; SREBP, sterol response element binding protein; Tg, transgenic; WAT, white adipose tissue; WT, wild type.

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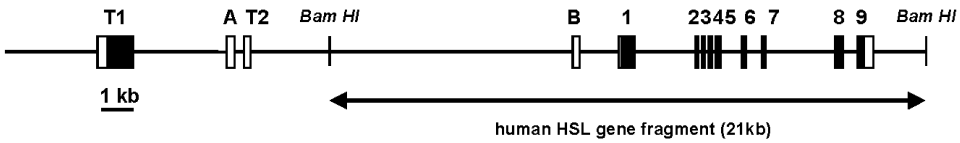

Fig. 1. Genomic organization of the hHSL gene coding sequences (filled boxes) and untranslated regions (open boxes). Exons T1 and T2 are used in testis. Exons A and B are used in the colon adenocarcinoma cell line HT29 and adipose tissue (AT), respectively. Exons 1 to 9 are used in all tissues expressing HSL. The two *Bam*HI enzymatic restriction sites used to generate the 21 kb transgene fragment are shown in italic.

and increased lean body mass (11, 12). The mice are resistant to genetic and diet-induced obesity. Basal lipolysis is increased in perilipin-deficient adipocytes, which is in line with a role of perilipin as a suppressor of lipolysis in quiescent cells.

Several forms of HSL transcripts have been characterized in humans. Adipocyte HSL is an 88 kDa protein translated from a 2.8 kb mRNA (13, 14). The transcription start site was mapped in a short noncoding exon called exon B (**Fig. 1**). Nine exons encode the 775 amino acid protein. In the adenocarcinoma cell line HT29, two mRNA species are found, the adipocyte HSL mRNA and an mRNA with a different 5′ end transcribed from exon A. Two testicular forms of HSL have been characterized (15, 16). The 3.9 kb mRNA encodes a 1,076 amino acid protein that contains a unique NH_2-terminal region encoded by exon T1. The first 95 bp of the 5′-flanking sequence upstream of exon T1 confers expression of a reporter gene exclusively in testis of transgenic (Tg) mice (17). The 3.3 kb mRNA encodes a protein that is identical to the adipocyte HSL form. However, the mRNA species differ in their 5′ ends. Exon usage is mutually exclusive; exon T2 is only transcribed in testis, and exon B is only transcribed in AT.

The classical view places HSL as the rate-limiting enzyme of the lipolytic pathway (6). In various physiological and pathological situations, correlationships have been shown between HSL levels and lipolysis data (2). However, the phenotype of HSL null mice challenges the pivotal role of HSL (8). To study the impact of a physiological increase in HSL expression, we produced Tg mice with a genomic region that contains 7.6 kb of 5′-flanking sequence, exon B, exons encoding adipocyte HSL, and 1.5 kb of 3′-flanking sequence.

MATERIALS AND METHODS

Transgenic mice

Animal studies followed the INSERM and Louis Bugnard Institute Animal Facility guidelines. Cosmid 10819 from the 19 q13.1-13.2 chromosomal region was linearized with *Bam*HI to purify a 21 kb human genomic fragment that contains the HSL gene (Fig. 1). Tg mice were generated by microinjection of the transgene into one-cell embryos of B6D2/F_1 female mice (IFFA CREDO) (18). To identify founders, genomic DNA from mouse tails was digested with *Bam*HI and subjected to Southern blotting. The membranes were hybridized with the transgene fragment. Two Tg founders were obtained and backcrossed for at least six generations to two mouse strains, B6CBA/F_1 (B6CBA) and OF1 (IFFA CREDO). In the different studies, Tg mice from 6th to 8th generations were compared with wild-type (WT) littermates. Presence of the transgene in offsprings was determined by PCR analysis. Animals were raised with alternating 12 h light/dark cycles (7:00 AM–7:00 PM) and fed a chow diet (A03, Unité d'Alimentation Rationnelle). For high-fat diet (HFD) experiments, mice were weaned at the age of 3 weeks and fed for 14 weeks with HFD (Unité d'Alimentation Rationnelle) containing, in percentage of energy, 45% fat, 35% carbohydrate, and 20% protein. For calorie restriction experiments, mice were fed a 50% calorie restricted chow diet at 7 PM for four nights. Visceral (i.e., perigonadic, perirenal, and retroperitoneal) white adipose tissue (WAT), subcutaneous (i.e., inguinal and gluteal) WAT, interscapular brown adipose tissue (BAT), and liver were rapidly dissected out, weighed, put into liquid nitrogen, and stored at −80°C until extraction. Plasma was obtained after centrifugation with heparin and kept frozen at −80°C until analysis.

Analysis of mRNA expression by reverse transcription real-time quantitative PCR

Total RNA from various tissues was isolated using RNeasy kit (Qiagen) for AT or RNASTAT-60 (AMS Biotechnology) for other

TABLE 1. Primers used in real time quantitative PCR

mRNA	Accession Number	Sense Primer	Antisense Primer
ALBP/aP2	M13385	5′-TTCGATGAAATCACCGCAGA-3′	5′-GGTCGACTTTCCATCCCACTT-3′
C/EBPα	NM_007678	5′-ATAGACATCAGCGCCTACATCGA-3′	5′-CTGTCGGCTGTGCTGGAA-3′
FAS	AF127033	5′-TGGTGAATTGTCTCCGAAAAGAG-3′	5′-CACGTTCATCACGAGGTCATG-3′
hHSL	L11706	5′-GTGCAAAGACGGAGGACCACTCCA-3′	5′-GACGTCTCGGAGTTTCCCCTCAG-3′
mHSL	NM_010719	5′-GGCTTACTGGGCACAGATACCT-3′	5′-CTGAAGGCTCTGAGTTGCTCAA-3′
hmHSL		5′-CCACGAGCCCTACCTCAAGA-3′	5′-CCAGGGAGTAGTCGATGGAGA-3′
LPL	NM_008509	5′-TTATCCCAATGGAGGCACTTTC-3′	5′-CACGTCTCCGAGTCCTCTCTCT-3′
Perilipin	XM_133574	5′-CTCCGAAGCGCCAGGAA-3′	5′-CCATCCCCAAGGCAAGCT-3′
$PPAR_{\gamma 2}$	U09138	5′-CTGTTTTATGCTGTTATGGGTGAAA-3′	5′-GCACCATGCTCTGGGTCAA-3′
UCP1	NM_009463	5′-CCTGCCTCTCTCGGAAACAA-3′	5′-TGTAGGCTGCCCAATGAACA-3′

ALBP, adipocyte lipid binding protein; C/EBPα, CCAAT/enhancer binding protein α; FAS, fatty acid synthase; hHSL and mHSL, human and murine hormone-sensitive lipase (HSL), respectively; hmHSL, primers for quantitation of human (hHSL) and murine HSL (mHSL); LPL, lipoprotein lipase; PPAR γ_2, peroxisome proliferator-activated receptor γ_2; UCP1, uncoupling protein 1.

tissues. Total RNA (1 μg) was treated with DNase I (DNase I amplification grade, Invitrogen), then retrotranscribed using random hexamers (Amersham Biosciences) and SuperScript II reverse transcriptase (Invitrogen) to quantitate HSL mRNA levels, or Thermoscript reverse transcriptase (Invitrogen) to measure other gene expression, according to the manufacturers' recommendations. Real-time quantitative PCR was performed on GeneAmp 7000 Sequence Detection System using SYBR green chemistry (Applied Biosystems). Primers were designed using software Primer Express 1.5 (Applied Biosystems) (**Table 1**). 18S rRNA was used as control to normalize gene expression using the rRNA Control Taqman Assay kit (Applied Biosystems).

Preparation of cytosolic proteins from AT

AT samples were homogenized in 4 vols of homogeneization buffer [0.25 M sucrose, 1 mM EDTA (pH 7.0), 1 mM dithioerythritol, 20 μg/ml leupeptin, 20 μg/ml antipain] and centrifuged at 110,000 *g* at 4°C for 45 min to obtain fat-free infranatants. Protein concentrations were determined with Bio-Rad Protein Assay using BSA as standard.

Western blot analysis of HSL

Samples of 50 μg of proteins from fat-free infranatants were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membrane (Hybond ECL, Amersham), and probed with specific polyclonal anti-human HSL (hHSL) or anti-rat HSL antibodies raised in rabbit and hen, respectively (gift from Dr. Cecilia Holm, University of Lund, Sweden). Immunoreactive protein was determined by enhanced chemiluminescence reagent (Amersham) and visualized by exposure to Fujifilm.

Enzyme activity assays

In vitro enzymatic activities were performed on fat-free infranatants (19). Briefly, triolein, 1(3)-mono-oleoyl-2-*O*-mono-oleylglycerol (MOME), and cholesterol oleate were emulsified with phospholipids by sonication. Fat-depleted BSA was used as a fatty acid acceptor. AT infranatants were incubated for 30 min at 37°C with the different subtrates. Hydrolysis was stopped, and released radiolabeled oleic acid was measured using a Tri-Carb 2100TR scintillation counter (Packard). One unit of hydrolase activity is equivalent to 1 μmol of fatty acid released per min at 37°C.

Preparation of isolated adipocytes and stroma vascular fraction from WAT

Adipocytes were isolated by collagenase digestion of visceral WAT. Digestion was performed for 30 min at 37°C in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.5 mM $CaCl_2$, 238 mg/100 ml HEPES, 108 mg/100 ml glucose, 3.5% BSA, and 1 mg/ml collagenase (C-6885, Sigma). At the end of digestion, the fat cell suspension was filtered and rinsed three times. Fat cell diameter was determined from ~200 adipocytes for each mouse by examination with a microscope equipped with an image analysis program (Visiolab). The stroma vascular fraction was obtained after filtration and centrifugation at 2,000 rpm for 10 min of B6CBA 4-week-old male visceral WAT digested with collagenase.

In vitro lipolysis experiments

Isolated packed adipocytes were diluted to 1/20th and incubated in Krebs-Ringer bicarbonate buffer containing 1.0 units/ml adenosine deaminase and 100 nM N^6-phenylisopropyladenosine for basal lipolysis determination and 100 μM isoproterenol or 5 mM dibutyryl-cAMP for maximal lipolysis determination. Incubations were carried out for 90 min. Glycerol and NEFA were measured by a radiometric assay (20) and the NEFA C kit (Wako), respectively. Total lipid content was determined gravimetrically after organic extraction. The number of fat cells was estimated by dividing the total lipid weight by the mean cell weight.

In vivo lipolysis experiments

After a 7 h fast, mice were injected ip with saline (0.9% NaCl) and blood was collected from the orbital plexus 15 min later. Two days later, after a 7 h fast, mice were injected ip with isoproterenol (10 mg/kg body weight) and blood was collected 15 min later. Plasma glycerol and NEFA concentrations were measured as decribed above.

Statistical analysis

Data are presented as means ± SEM. Values in Tg and WT mice were compared using the Mann-Whitney nonparametric test (Stat View software, Abacus Concepts).

RESULTS

Generation of Tg mice

To generate Tg mice with overexpression of HSL in AT, a 21 kb hHSL genomic fragment (Fig. 1) was microinjected into B6D2F1 zygotes. Two Tg founders, 51 and 56, were obtained and bred with mice from two strains, B6CBA and OF1, to establish lines in different genetic backgrounds. Lines 51 and 56 had 10–20 and 5–10 transgene copies, respectively. Transmission of the transgene to offsprings followed Mendelian rules. All the results presented here were obtained from Tg and WT littermates backcrossed for at least six generations.

TABLE 2. mRNA levels for human (hHSL) and murine (mHSL) hormone-sensitive lipase in different tissues of 6-week-old mice from two transgenic lines

	Line 51		Line 56
Background	B6CBA	OF1	OF1
Visceral WAT			
hHSL	17.4 ± 3.6	5.7 ± 0.5	1.5 ± 0.3
mHSL	4.2 ± 0.6	1.8 ± 0.1	3.5 ± 0.3
Subcutaneous WAT			
hHSL	21.6 ± 1.4	10.6 ± 0.7	0.5 ± 0.1
mHSL	5.7 ± 1.3	2.5 ± 0.3	2.1 ± 0.1
BAT			
hHSL	36.7 ± 13.3	9.5 ± 2.1	2.1 ± 0.4
mHSL	4.5 ± 0.3	3.3 ± 0.0	4.5 ± 0.3
Diaphragm			
hHSL	0.3 ± 0.1	0.2 ± 0.2	0
mHSL	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Soleus			
hHSL	0.02 ± 0.01	0.03 ± 0.01	0
mHSL	0.10 ± 0.02	0.05 ± 0.01	0.07 ± 0.02

WAT, white adipose tissue; BAT, brown adipose tissue; ND, not determined. Data (×1000) for each gene were normalized with ribosomal 18S RNA. Values are means ± SEM from 6-week-old male mice (n = 3).

TABLE 3. mRNA levels in isolated adipocytes of 14-week-old B6CBA mice

Gene	WT (6)	Tg (4)	Ratio Tg-WT
mHSL	10.9 ± 1.1	11.5 ± 2.2	1.0
hmHSL	7.3 ± 0.5	29.1 ± 4.3	4.0[a]
LPL	1132 ± 236	1025 ± 554	0.9
FAS	16.6 ± 3.5	10.2 ± 2.1	0.6
UCP1	1.9 ± 0.4	1.8 ± 0.8	0.9
ALBP/aP2	850 ± 145	788 ± 108	0.9
Perilipin	176 ± 22	191 ± 39	1.1
PPAR γ_2	1.1 ± 0.1	1.4 ± 0.2	1.3
C/EBPα	6.9 ± 0.6	7.5 ± 1.5	1.1

Data (×1000) for each gene were normalized with ribosomal 18S RNA. Values are means ± SEM from independent determinations indicated in parentheses.

[a] $P < 0.05$, Tg versus wild type WT mice.

Expression profile of human hormone-sensitive lipase in Tg mice

Real-time quantitative PCR, with specific primers for the hHSL form, was used to determine the expression level of hHSL mRNA in different tissues from 6-week-old Tg male mice. Young mice were analyzed to avoid excessive AT development and fat contamination of other tissues (**Table 2**). hHSL mRNA could be detected, for the two Tg lines, in visceral and subcutaneous WAT and in BAT. Using PCR primers for quantitation of hHSL and murine HSL (mHSL) (Table 1), we found that total HSL mRNA levels were 4.1- and 2.4-fold higher in visceral WAT of line 51 Tg mice from B6CBA and OF1 backgrounds compared with WT mice. For line 56, there was only a 1.2-fold difference. There was no PCR amplification in WT WAT. hHSL mRNA could be detected in line 51 diaphragm and soleus muscles. For the two Tg lines, we did not detect hHSL mRNA in other HSL-expressing tissues such as testis, heart, adrenals, and peritoneal macrophages. hHSL mRNA was also absent in liver, spleen, and kidney (data not shown). Measurements were also performed on isolated visceral adipocytes from 14-week-old line 51 B6CBA mice (**Table 3**). Transgene expression did not modify mRNA levels of mHSL. Total HSL mRNA levels were 4-fold higher in Tg mice, suggesting that hHSL mRNA levels were 3-fold higher than mHSL mRNA levels. Total HSL mRNA levels were also increased 4.5-fold in BAT of Tg mice (data not shown). RT-PCR with primers located in exon 5 (sense primer) and exon 7 (antisense primer) was performed to determine if the hHSL form resulting from exon 6 alternative splicing was present in WAT (21). We could not detect the short HSL mRNA in any fat pad from Tg lines (data not shown).

To detect hHSL protein, Western blot analyses with infranatants from the three fat pads were performed and revealed with antibodies against human and rat HSL (**Fig. 2**). The 88 kDa hHSL protein was specifically detected in visceral and subcutaneous WAT and BAT from line 51 and 56 Tg mice. Only the 82 kDa mHSL protein was present in WT visceral WAT (Fig. 2A). There was no difference in mHSL protein amount between Tg and WT mice (Fig. 2C). Identical results were obtained with line 51 mice in the OF1 genetic background (data not shown).

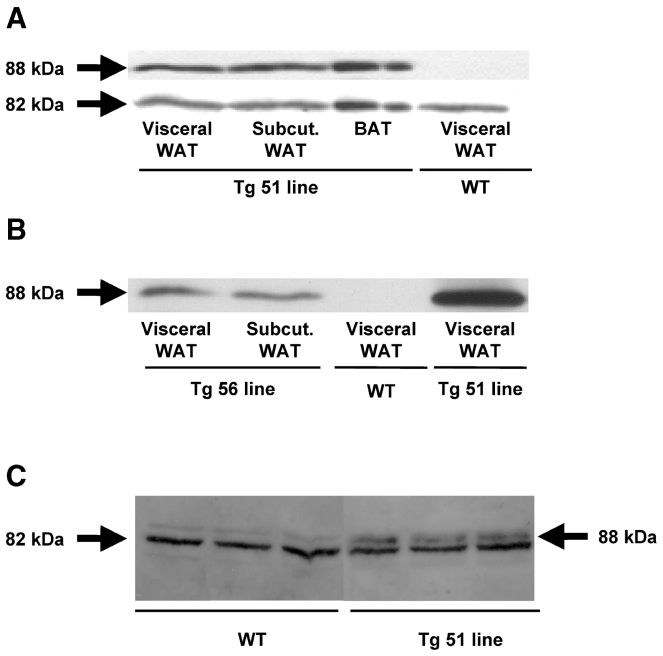

Fig. 2. Western blot analysis of adipose HSL infranatants of visceral white adipose tissue (WAT), subcutaneous (Subcut.) WAT, and brown adipose tissue (BAT) prepared as described in Materials and Methods. Each lane was loaded with 50 μg protein and subjected to SDS-PAGE and Western blot with specific anti-human (A, upper panel; B), and anti-rat (A, lower panel; C), HSL antibodies. Arrows show human (88 kDa) and murine (82 kDa) HSL protein. A: ATs from 14-week-old transgenic (Tg) and wild-type (WT) males from line 51 in the B6CBA genetic background. B: ATs from 10-week-old males from line 56 in the OF1 genetic background and 14-week-old B6CBA males from line 51. C: ATs from 14-week-old Tg and WT B6CBA males from line 51.

In vitro hydrolase activities

The use of different antibodies directed against human and rat HSL precludes a quantitation of total HSL. Therefore, we decided to measure three in vitro activities related to the different hydrolytic properties of HSL. Hydrolase activity against a TG (triolein) substrate measures TG, DG, and monoacylglycerol lipase activities. Using a diglyceride analog (MOME), in which only the first ester bond can be hydrolysed, we can determine DG lipase activity. Cholesterol esterase activity can be measured with cholesterol oleate. As shown in **Table 4**, hydrolase activities against triolein, MOME, and cholesterol oleate were higher in line 51 Tg mice visceral WAT and BAT. Data were similar for the two genetic backgrounds. The ratio between Tg and WT mice differed according to the substrate. In visceral WAT from B6CBA mice, it increased from 2.3 with triolein to 4.6 with cholesterol oleate. An intermediate ratio was obtained with MOME. The same profile was observed in visceral WAT and BAT from OF1 mice. The measurement of DG lipase activity in different fat pads of OF1 mice showed that transgene expression

TABLE 4. Hydrolytic activities against a triglyceride (triolein), a diglyceride analogue [1(3)-mono-oleoyl-2-*O*-mono-oleylglycerol, (MOME)], and a cholesterol ester (cholesterol oleate) substrate in visceral WAT and BAT

	Triolein	MOME	Cholesterol Oleate
Line 51 WAT (B6CBA background)			
WT (7)	7.4 17 ± 1.0	61 ± 6	7.6 ± 1.0
Tg (7)	2.3 17 ± 1[b]	221 ± 7[b]	35 ± 2[b]
Ratio Tg / WT		3.6	4.6
Line 51 WAT (OF1 background)			
WT (4)	9.7 ± 1.8	65 ± 8	9.7 ± 1.5
Tg (4)	18 ± 3[a]	195 ± 32[a]	32 ± 7[a]
Ratio Tg / WT	1.8	3.0	3.3
Line 51 BAT (OF1 background)			
WT (7)	3.1 ± 0.5	41 ± 3	5.2 ± 0.6
Tg (7)	5.1 ± 0.7[a]	80 ± 7[b]	12 ± 2[b]
Ratio Tg / WT	1.7	1.9	2.3
Line 56 WAT (OF1 background)			
WT (7)	7.5 ± 0.6	78 ± 6	10 ± 1
Tg (7)	8.1 ± 0.5	86 ± 6	12 ± 1
Ratio Tg / WT	1.1	1.1	1.2

In vitro activities, expressed as nmol fatty acid/min/mg protein, were determined in tissue lysates from 17-week-old mice. Values are means ± SEM from independent determinations indicated in parentheses.

[a] $P < 0.05$, Tg versus wild type WT mice.

[b] $P < 0.01$, Tg versus wild type WT mice.

led to a higher increase in visceral WAT (129 ± 32 nmol FFA/min/mg prot) than in subcutaneous WAT (43 ± 8 nmol FFA/min/mg protein) and BAT (38 ± 7 nmol FFA/min/mg protein). No increase of hydrolase activities was observed in line 56 Tg mice.

Phenotype of male mice from line 51 in the B6CBA genetic background

Genetic background is known to modify transgene-induced phenotypes. We therefore decided to breed the founder of line 51 on two different strains. The B6CBA strain is a hybrid strain from C57BL/6 and CBA. The OF1 strain is a fast-growing inbred strain that shows higher visceral fat mass than B6CBA mice at similar ages (data not shown).

As shown in **Table 5**, 12-week-old Tg B6CBA male mice had lower body weight (14%) and visceral WAT (45%) than WT mice. Median diameter of Tg visceral adipocytes was significantly lower, suggesting adipocyte hypotrophy. Lower body weight and fat mass were maintained until at least 14 weeks of age. Compared with WT, Tg mice had significant reduction of body weight (14%), visceral WAT (43%), and subcutaneous WAT (32%). At the age of 27 weeks, differences between Tg and WT mice tended to disappear. Only subcutaneous WAT and BAT mass were significantly reduced in Tg mice. In fact, Tg visceral WAT weight became heterogeneous. Considering the percentage of visceral WAT/body weight, 5/13 Tg males and 6/8 WT males had a visceral adiposity percentage higher than 5.5% (7.0 ± 0.3% and 7.1 ± 0.1%, respectively), 8/13 Tg males and only 2/6 WT males had a visceral adiposity percentage lower than 5.5% (4.0 ± 0.5% and 3.5 ± 0.8%, respectively). When males were weaned at the age of 3 weeks and fed with a HFD until the age of 17 weeks, no alteration (except for BAT mass, 39% lower in Tg mice) could be measured for body weight curves, tissue weights, or adipocyte diameter. Plasma glycerol and NEFA were measured in fed animals at either 10 AM (12-week-old, and half-cohort fed a HFD) or 3.00 PM (other animal groups). Whatever the group, plasma glycerol and NEFA concentrations were not significantly different between Tg and WT mice.

Phenotype of male mice in the OF1 genetic background

No phenotypic difference was observed between Tg mice in the OF1 genetic background and their WT littermates. As shown in **Table 6**, food intake, body weight (as well as weight curves; data not shown), fat pads, adipocyte median diameter, and plasma parameters from 13-week-old males were nearly identical. Fifteen-week-old OF1 mice were fed with 50% calorie restricted chow diet for 4 days and killed at 9:00 AM. There was no difference in initial body weights between the two groups of mice. After 4 days with restricted food access, body weight decreased about 11% for Tg mice and 8% for WT mice. Compared

TABLE 5. Phenotype of B6CBA male mice from transgenic line 51

	12-Week-Old		14-Week-Old		27-Week-Old		17-Week-Old (High-Fat Diet)	
	WT (6)	Tg (6)	WT (5)	Tg (4)	WT (9)	Tg (13)	WT (13)	Tg (13)
Body weight (g)	32.4 ± 1.4	28.0 ± 1.0[a]	38.1 ± 0.5	32.7 ± 2.2[a]	41.0 ± 1.15	39.3 ± 0.9	42.1 ± 1.2	39.6 ± 0.75
Tissue weight (mg)								
Visceral WAT	1392 ± 170	772 ± 80[b]	2050 ± 317	1175 ± 218[a]	2662 ± 287	2077 ± 243	3320 ± 215	3223 ± 205
Subcut. WAT	ND	ND	1098 ± 146	750 ± 81[a]	1609 ± 166	1031 ± 77[a]	2084 ± 124	1642 ± 96
BAT	ND	ND	140 ± 34	82 ± 8	173 ± 18	118 ± 12[a]	211 ± 20	129 ± 14[b]
Liver	ND	ND	1586 ± 42	1480 ± 147	1744 ± 92	1925 ± 70	1749 ± 130	1544 ± 134
Visceral adipocytes								
Median diameter (μm)	76 ± 5	61 ± 2[a]	ND	ND	ND	ND	94 ± 6	98 ± 4
Plasma								
Glycerol (μM)	545 ± 33	481 ± 30	584 ± 29	577 ± 30	702 ± 41	623 ± 39	522 ± 45	528 ± 28
NEFA (μM)	992 ± 75	834 ± 135	562 ± 77	586 ± 64	917 ± 101	755 ± 61	1360 ± 211	1247 ± 99

Mice fed a chow diet were killed at the age of 12, 14, or 27 weeks. Mice fed a high-fat diet (HFD) during 14 weeks were killed at the age of 17 weeks. Values are means ± SEM from independent determinations indicated in parentheses.

[a] $P < 0.05$, Tg versus wild type WT mice.

[b] $P < 0.01$, Tg versus wild type WT mice.

TABLE 6. Phenotype of OF1 male mice from transgenic line 51

	Ad Libitum		Calorie Restriction (96 h)	
	WT (6)	Tg (8)	WT (7)	Tg (7)
Food intake (g)	6.7 ± 0.2	6.6 ± 0.2	3.4 ± 0.1[c]	3.3 ± 0.1[c]
Body weight (g)	42.1 ± 1.3	41.2 ± 1.5	38.9 ± 1.6	36.8 ± 1.7[a]
Tissue weight (mg)				
Visceral WAT	1960 ± 300	1761 ± 235	1004 ± 210[b]	461 ± 210[a,c]
Subcutaneous WAT	533 ± 85	567 ± 70	653 ± 176	318 ± 90[a,b]
BAT	147 ± 12	115 ± 11	97 ± 14[b]	70 ± 15[b]
Visceral adipocytes				
Median diameter (μm)	74 ± 3	74 ± 3	69 ± 5	52 ± 6[a,b]
Plasma				
Glycerol (μM)	494 ± 44	540 ± 14	394 ± 21[b]	324 ± 27[b]
NEFA (μM)	931 ± 64	987 ± 47	561 ± 35[b]	475 ± 43[b]

WAT, white adipose tissue; BAT, brown adipose tissue. Mice fed a chow diet ad libitum or a 50% calorie-restricted diet for 96 h were killed at the age of 13 weeks (Ad Libitum) or 15 weeks (Calorie Restriction). Values are mean ± SEM from independent determinations indicated in parentheses.

[a] $P < 0.05$, Tg versus wild type WT mice.

[b] $P < 0.05$, Ad Libitum versus Calorie-Restricted mice.

[c] $P < 0.01$, Ad Libitum versus Calorie-Restricted mice.

with Tg mice fed ad libitum, calorie-restricted Tg mouse fat mass was significantly lower (74%, 44%, and 35% less for visceral and subcutaneous WAT and BAT, respectively). Adipocyte median diameter was reduced by 30%. For WT mice, the effect of calorie restriction was less important. Only visceral WAT and BAT were significantly diminished (respectively, 49% and 34%). During calorie restriction, visceral and subcutaneous WAT were lower in TG than in WT mice (54% and 51%, respectively). Median diameter of visceral adipocytes was also significantly lower. Plasma glycerol and NEFA were significantly decreased after calorie restriction but were not different between the two groups.

Lipolysis of male mice from line 51

In vitro lipolysis studies were realized with isolated adipocytes, and results were normalized to cell number. A nonselective β-adrenergic agonist, isoproterenol, and a stable analog of cAMP, dibutyryl-cAMP, were used to stimulate lipolysis. In **Fig. 3**, in vitro lipolysis from visceral WAT of 12-week-old and 17-week-old B6CBA males fed a HFD is shown. In 12-week-old mice, stimulated lipolysis with isoproterenol and dibutyryl-cAMP was decreased in Tg mice. In the HFD group, basal and stimulated lipolysis was lower in Tg mice compared with WT mice. Dose-response curves revealed that isoproterenol EC_{50} was not modified in Tg HFD ($2.7\ 10^{-7} \pm 0.6^{-7}$ M) compared with WT HFD ($3.9\ 10^{-7} \pm 0.8^{-7}$ M).

A similar in vitro lipolysis profile was observed in WAT of OF1 male mice. In mice fed ad libitum, basal activity (data not shown) was unchanged, whereas isoproterenol and dibutyryl cAMP-stimulated lipolysis expressed as fold induction over basal lipolysis was lower in Tg mice (**Fig. 4**). Using fat-depleted BSA, NEFA release during in vitro lipolysis was measured. Variations of NEFA levels in the medium were comparable to those of glycerol (data not shown). The ratios of NEFA to glycerol were not significantly different between Tg (2.0 ± 0.2) and WT (2.3 ± 0.2) mice. From dose-response curves, we could deter-

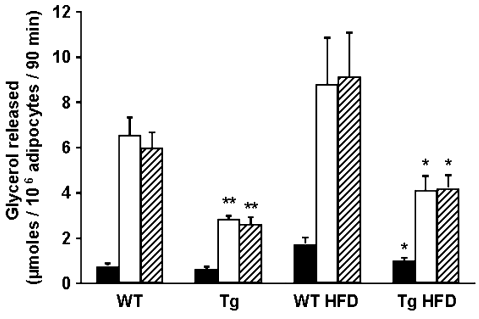

Fig. 3. In vitro lipolysis in adipocytes isolated from visceral WAT from B6CBA male mice. Mice fed a chow diet were killed at the age of 12 weeks (Tg, n = 6 and WT, n = 6). Mice fed a high-fat diet (HFD) for 14 weeks were killed at the age of 17 weeks (Tg HFD, n = 7 and WT HFD, n = 7). Glycerol release was measured from adipocytes either unstimulated (black bars) or incubated with isoproterenol (100 μM, white bars) or dibutyryl-cAMP (5 mM, striped bars). Values are means ± SEM. * $P < 0.05$ and ** $P < 0.01$; transgenic (Tg) versus wild type (WT) in each group.

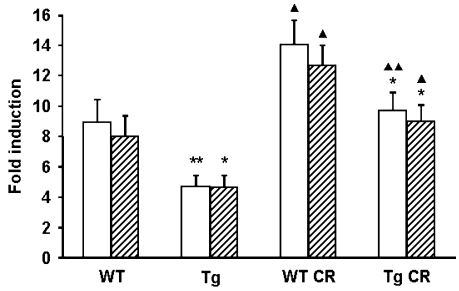

Fig. 4. In vitro lipolysis in adipocytes isolated from visceral WAT of OF1 male mice. Mice fed a chow diet ad libitum or a 50% restricted diet for 96 h (CR) were killed at the age of 13 weeks [transgenic (Tg), n = 8 and wild type (WT), n = 6] or at the age of 15 weeks (Tg CR, n = 7 and WT CR, n = 7). Fold increase over basal lipolysis of glycerol release from adipocytes incubated with isoproterenol (100 μM, open bars) or dibutyryl-cAMP (5 mM, striped bars). * $P < 0.05$ and ** $P < 0.01$; Tg versus WT mice; single triangle, $P < 0.05$; and double triangle, $P < 0.01$ ad libitum versus 50% calorie restricted diet mice.

mine that isoproterenol EC_{50} was not modified in Tg mice compared with WT mice (data not shown). Calorie-restricted diet resulted in a significantly enhanced lipolytic capacity in Tg and WT mice (Fig. 4). During calorie restriction, lipolysis rates were lower in Tg mice compared with WT mice when adipocytes were stimulated by isoproterenol and dibutyryl-cAMP.

We next calculated correlations between fat cell volume and lipolysis in WT and Tg mice from the various groups (n = 26). For basal lipolysis, correlations were observed in WT ($r = 0.87$, $P < 0.0001$) and Tg ($r = 0.83$, $P < 0.0001$) mice. For isoproterenol and dibutyryl cAMP-induced lipolysis, correlations were stronger in WT mice ($r = 0.71$, $P < 0.0001$ and $r = 0.79$, $P < 0.0001$) than in Tg mice ($r = 0.46$, $P < 0.05$ and $r = 0.55$, $P < 0.01$).

In vivo lipolysis was studied after ip isoproterenol injection in body weight pair-matched OF1 mice (**Table 7**). Lipolytic agent administration led to a 2.3-fold-increase in plasma glycerol and NEFA concentrations. There was no difference between Tg and WT plasma glycerol and NEFA measured in basal (saline injection) and stimulated (isoproterenol injection) conditions.

Gene expression of male mice from line 51

Gene expression studies were performed using real-time RT-PCR on isolated adipocytes from 14-week-old B6CBA Tg and WT mice (Table 3). There was no statistical difference for lipoprotein lipase (LPL) and fatty acid synthase, which are involved in triglyceridogenesis. Perilipin and adipocyte lipid binding protein (ALBP), two proteins involved in the lipolytic process, showed no changes in mRNA expression. Adipogenesis did not seem to be altered because CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated protein γ_2 ($PPAR\gamma_2$) mRNA levels were not modified. In WAT and BAT (data not shown), uncoupling protein 1 mRNA levels were not different in Tg mice. In WAT from 27-week-old mice, there was also no difference in the pattern of gene expression (data not shown). Similarly, we did not detect any significant modification in gene expression from 16-week-old Tg visceral WAT and BAT from the OF1 background.

To determine whether adipocyte differentiation could be altered, cells from visceral WAT stroma vascular fraction were prepared and gene expression was measured. hHSL in Tg stroma vascular fraction was detectable, but 100-fold lower than in mature adipocytes. There was no modification in mRNA levels of mHSL, LPL, ALBP, perilipin, adipose differentiation-related protein, $PPAR\gamma_2$, C/EBPα, retinoid X receptor, sterol response element binding protein (SREBP)1, and SREBP2 (data not shown).

TABLE 7. In vivo lipolysis in 15-week-old OF1 male mice

	Glycerol		NEFA	
	WT	Tg	WT	Tg
	μM		μM	
Saline	465 ± 41	480 ± 60	614 ± 58	609 ± 34
Isoproterenol	1038 ± 56	1002 ± 63	1415 ± 80	1381 ± 82
Fold increase	2.3 ± 0.3	2.2 ± 0.2	2.4 ± 0.3	2.3 ± 0.2

After a 7 h fast, plasma glycerol and NEFA levels were measured 15 min after saline and isoproterenol (10 mg/kg) ip injection, as described in Materials and Methods. Values are means ± SEM from six transgenic (Tg) and seven wild-type mice (WT).

DISCUSSION

To date, there is scarce information on the mechanisms controlling regulation of HSL gene expression in AT. The transcription start site used in the adipocytes was mapped in the short 5′ noncoding exon B (Fig. 1). The 5′-flanking region contains an active promoter and several DNase I hypersensitive sites (14). Recently, we showed that an E-box and two GC-boxes are critical *cis*-acting elements in the proximal promoter (22). The E-box mediates glucose induction of HSL gene expression. However, the sequences responsible for the tissue-specific expression of HSL are not present in this region, since the pattern of promoter activity up to −2.4 kb was similar in adipocytes and in HeLa cells that do not express HSL (14). Adipose-specific enhancer can be located far upstream of the transcriptional start site, as shown for the ALBP fat-specific enhancer located at −5.4 kb (23). Here, we show that the 21 kb genomic fragment encompassing 7.6 kb of the 5′ flanking region and intronic sequences confers strong mRNA expression in BAT and WAT. Several lines of evidence suggest that HSL is expressed in skeletal muscle. The protein has been detected in muscle preparations from young rats that were devoid of intramuscular AT and in isolated muscle fibers (24). The apparent molecular mass was identical to that observed in AT. Disruption of the HSL gene leads to the disappearance of the HSL immunoreactive band in skeletal muscle of HSL-null mice (10). hHSL mRNA was detected in different skeletal muscles of young Tg mice. This suggests that the 21 kb genomic fragment also contains the regulatory elements necessary for HSL expression in skeletal muscle. It also shows that the coding exons are similar for adipocyte and skeletal muscle HSL, in agreement with the identical molecular mass of the protein in the two tissues. The role of HSL in testis has recently been revealed by the phenotype of HSL-deficient male mice, which are sterile because of a defect in spermatogenesis (8, 25). We did not detect hHSL mRNA in Tg testes. The transgene expression profile is in agreement with the organization of the HSL gene (13, 15, 16). The 5′ ends of the two human testicular mRNAs are transcribed from exons that were not contained in the 21 kb transgene (Fig. 1). Moreover, we have shown that the human exon T1 5′ flanking region conferred expression of a reporter gene only in testes of Tg mice (17). Therefore, it seems clear that the regulatory elements responsible for HSL expression in testis are distinct from those involved in AT. Whereas the expression of HSL in murine macrophages has repeatedly been demonstrated, there is a controversy regarding its expression in human monocytes/macrophages (26–29). The nature of the enzyme responsible for CE hydrolysis has attracted much interest because CE accumulation in arterial macrophages is gen-

erally believed to be a proatherogenic event. Tg mice did not express hHSL mRNA in peritoneal macrophages. The lack of transgene HSL transcripts may be interpreted either as a confirmation that hHSL is not expressed in macrophages or as a demonstration that regulatory elements necessary for macrophage expression lie outside the 21 kb region. Altogether, our data pave the way for future characterization of the *cis*-acting elements conferring human expression in AT and skeletal muscle.

In WAT and BAT, HSL enzymatic activities were increased, indicating that we obtained a Tg model of HSL overexpression. The increase in lipase activities was comparable to the variations observed in clinical studies (7, 30, 31). The ratio of hydrolytic activities between Tg and WT mice was highest for cholesterol oleate, intermediate for MOME, and lowest for triolein. The data suggest that increased expression of HSL preferentially leads to a rise in enzymatic activities in the following rank order: cholesterol esterase>DG lipase>TG lipase. The simplest explanation for this observation is that HSL is the only cholesterol esterase expressed in AT, whereas it accounts for only part of the TG lipase activity. Consistent with this hypothesis, the hydrolytic capacity toward CE is totally abrogated in HSL-null mice, but the TG hydrolysis activity is only partially blunted (8, 10). It was also shown that DG accumulates in HSL knock-out mice and that HSL is rate limiting for DG hydrolysis (10). Altogether, the data suggest the putative involvement of another TG lipase in WAT. A possible candidate is the recently cloned neutral CE and TG hydrolase, which is expressed in various tissues, including WAT (32, 33). However, this enzyme is a microsomal carboxylesterase. It is therefore unlikely that it contributes to the TG but not the CE hydrolase activity of the adipocyte cytosolic fraction. The identification and characterization of the TG lipase is critical for a complete understanding of the lipolytic process.

The most unexpected aspect of the phenotype is the decrease of in vitro lipolysis despite the increased expression of HSL. Such a paradoxical effect is reminiscent of the results obtained in HSL-overexpressing macrophages. HSL overexpression in RAW264.7 macrophage cells resulted in decreased CE accumulation (34). However, Tg mice with targeted overexpression of HSL in macrophages, despite increased CE hydrolysis activities, develop thicker aortic fatty lesions than WT mice (35). Similarly, HSL overexpression in AT despite increased lipase activity results in decreased lipolytic capacity of isolated fat cells. It is well known that the lipolytic rate is related to fat cell size (36). As expected, a strong correlation between stimulated lipolysis and fat cell volume was found in WT mice. However, the relationship was much lower in Tg mice. These data suggest that fat cell size is not a primary determinant of the changes in lipolytic capacity resulting from HSL overexpression. In HSL knock-out mice, β-adrenergic-stimulated lipolysis was totally blunted, suggesting that the other TG lipase is not regulated by cAMP levels (8, 9). In our model, the decrease in lipolysis is observed on stimulated adipocytes when hydrolytic activity against a stable emulsion of TG is increased. It seems, therefore, that a decreased expression of a TG lipase does not explain the decreased lipolytic capacity. There is now ample evidence that lipolysis is much more than the mere interaction between TG and a lipase. Perilipin seems to play a very important role in the control of lipolysis. In agreement with data obtained in perilipin-null mice (11, 12), adenoviral-mediated expression of HSL and perilipin in NIH 3T3 fibroblasts genetically modified to accumulate TG reveals that perilipin reduces the lipolytic action of HSL (37). In this cellular model, protein kinase A-mediated perilipin phosphorylation abrogates the inhibitory effect of the coating protein on lipolysis. The exact mechanism of perilipin modulation of lipolysis is not known. Phosphorylation of perilipin may facilitate lipase access to the lipid droplet through a change in perilipin conformation and/or via a translocation off the lipid droplet. HSL phosphorylation induces translocation of the enzyme from a cytosolic compartment to the surface of the lipid droplet (5). Overexpression of HSL may alter these mechanisms and result in decreased lipolysis.

The effect of transgene expression on fat mass was dependent on age in the B6CBA background but not in the OF1 background. The decrease in AT mass in B6CBA mice was seen at 12 and 14 weeks of age. No difference was seen between Tg and WT animals for older mice or 17-week-old mice fed a HFD. Fed the same chow diet, OF1 mice have a larger amount of WAT than B6CBA mice of the same age. The data suggest that the effect of HSL overexpression on fat mass is not seen in animals with a high capacity to gain fat mass, either because of genetic predisposition (OF1 vs. B6CBA mice) or because of the diet (high fat vs. chow diet). Interestingly, the effect of HSL overexpression can be unmasked in OF1 mice during calorie restriction. There are now numerous examples of Tg modification of fat mass influenced by genes and/or environment (38). Mice with a knock-out of the gene encoding ALBP, an intracellular fatty acid binding protein that interacts with the HSL N-terminal region, do not become obese in the C57BL6 background but show increased fat mass compared with WT littermates in the leptin-deficient ob/ob background (39). Conversely, lack of LPL expression in AT results in impaired storage of lipid in AT of ob/ob mice but not in AT of mice in a standard genetic background (40). Interaction between genes and diet was recently illustrated in mice lacking β_3-adrenoceptors and expressing human α_2-adrenoceptors in AT (41). HFD but not chow diet induced obesity in these mice. In our model, a more pronounced phenotype may be revealed in obesity-resistant strains and/or in situations of chronic negative energy balance, such as prolonged calorie restriction.

A decreased fat mass was observed in Tg mice that showed a concomitant increase in enzymatic activity and a decrease in in vitro lipolysis. There were no changes in the expression of key genes of adipogenesis and fat metabolism in WAT and BAT. These data suggest that the phenotype results from an adaptation of metabolic fluxes. The regulation of the fine balance between lipolysis and reesterification in WAT may explain the moderate variations in fat mass. In isolated cells, the reuptake of fatty acids was low, with a ratio of NEFA to glycerol above 2, as previously shown (42). Therefore, the reesterification pro-

cess was not assessed in our lipolysis experiments. However, up to 60% of fatty acids resulting from AT lipolysis are reesterified in fasted rats (43). HSL could be more prone to hydrolyze the newly reesterified acylglycerols, especially DG. Consistently, depletion of newly synthesized DG is observed when lipolysis is stimulated in human adipocytes (44), and HSL null mice show an accumulation of DG hydrolysis in WAT (10). Moreover, early work suggests that adipocytes may contain several lipid-metabolizing compartments: a small cytosolic pool with a high turnover rate and a large pool associated with the lipid droplet exhibiting a slow turnover (45). One can speculate that access to the acylglycerols of the small pool may be less tightly controlled by perilipins, allowing HSL to exert its hydrolase activity. Further studies will be necessary to evaluate in our model the contribution of HSL to TG and DG hydrolysis according to the metabolic origin of acylglycerols.

To conclude, our Tg model reveals that the 21 kb genomic region contains the regulatory elements necessary for human adipocyte HSL expression in WAT and BAT. The increase in lipase and esterase activities is not paralleled by an increase in in vitro lipolysis. The data strengthen the view that hydrolysis of TG stored in the lipid droplet relies on both lipase and nonenzymatic components. Further studies are required to determine whether increased HSL overexpression influences AT reesterification and hydrolysis of newly synthesized acylglycerols.

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