Hormone-sensitive Lipase Deficiency in Mice Causes Diglyceride Accumulation in Adipose Tissue, Muscle, and Testis*

Guenter Haemmerle‡, Robert Zimmermann‡, Marianne Hayn‡, Christian Theussl‡, Georg Waeg‡, Elke Wagner‡, Wolfgang Sattler‡, Thomas M. Magin‡, Erwin F. Wagner‡, and Rudolf Zechner‡‡

From the ‡Institute of Molecular Biology, Biochemistry, and Microbiology, University of Graz, A-8010, Austria, §Research Institute of Molecular Pathology, Vienna A-1030, Austria, ¶Institute of Genetics and Bonner Forum Biomedizin, University of Bonn, D-53117, Germany, and ‡Institute of Medical Biochemistry and Medical Molecular Biology, University of Graz, A-8010, Austria

Hormone-sensitive lipase (HSL) is expressed predominantly in white and brown adipose tissue where it is believed to play a crucial role in the lipolysis of stored triglycerides (TG), thereby providing the body with energy substrate in the form of free fatty acids (FFA). From in vitro assays, HSL is known to hydrolyze TG, diglycerides (DG), cholesteryl esters, and retinyl esters. In the current study we have generated HSL knock-out mice and demonstrate three lines of evidence that HSL is instrumental in the catabolism of DG in vivo. First, HSL deficiency in mice causes the accumulation of DG in white adipose tissue, brown adipose tissue, skeletal muscle, cardiac muscle, and testis. Second, when tissue extracts were used in an in vitro lipase assay, a reduced FFA release and the accumulation of DG was observed in HSL knock-out mice which did not occur when tissue extracts from control mice were used. Third, in vitro lipolysis experiments with HSL-deficient fat pads demonstrated that the isoproterenol-stimulated release of FFA was decreased and DG accumulated intracellularly resulting in the essential absence of the isoproterenol-stimulated glycerol formation typically observed in control fat pads. Additionally, the absence of HSL in white adipose tissue caused a shift of the fatty acid composition of the TG moiety toward increased long chain fatty acids implying a substrate specificity of the enzyme in vivo. From these in vivo results we conclude that HSL is the rate-limiting enzyme for the cellular catabolism of DG in adipose tissue and muscle.

Hormone-sensitive lipase (HSL) is thought to be a key enzyme for the mobilization of triglycerides (TG) deposited in adipose tissue. Human HSL is composed of 775 amino acids that are encoded by a 2.9-kb mRNA transcribed from a single gene composed of 9 exons (1, 2). The mouse and the human genes are similar in size and share a high degree of sequence homology. A tissue-specific size variation has been observed in testis where an additional exon 13 kb upstream of exon 1 in adipose tissue gives rise to a 3.9-kb mRNA and a 1076-amino acid protein (3). The molecular basis of size variations in HSL mRNA in muscle, macrophages, and ovaries is unknown.

HSL-mediated lipolysis is strictly controlled by hormones. The enzyme is activated by catecholamines and other lipolytic hormones upon phosphorylation by the cAMP-dependent protein kinase A and the lipotransin-mediated translocation of the enzyme from the cytoplasm to the lipid droplet (4–6). Insulin, the major antilipolytic hormone, inhibits HSL through phosphodiesterase-3-dependent cAMP degradation and interference with the lipotransin-mediated enzyme translocation. Accordingly, mice with elevated protein kinase A activity exhibit increased lipolysis and a lean phenotype (7). Absence of peripherin in mice also resulted in leanness through constitutive activation of HSL (8). Conversely, mice that lack the insulin receptor substrate 2 become obese (9). These results imply that imbalances between lipid accumulation and fat mobilization in adipose tissue due to the dysregulation of HSL might contribute to the development of obesity and related disorders (10–13).

Alterations in HSL activity levels in response to various physiological conditions such as feeding/fasting and exercise directly affect plasma concentrations of FFA (14–16) which in turn are known to determine carbohydrate and lipid utilization, storage, and synthesis in liver and muscle. These effects are explained by the ability of FFA and their derivatives to regulate lipogenic genes by the activation of nuclear receptors including peroxisome proliferator-activated receptors and sterol-responsive element-binding protein-1/adipocyte differentiation-dependent factor (17–21). Thus, HSL is considered to be an important enzyme in the maintenance of energy homeostasis.

A particular feature of HSL is its multifunctional ability to hydrolyze TG, diglycerides (DG), monoglycerides (MG), cholesteryl esters (CE), and retinyl esters in various tissues (22–25). The enzyme exhibits an ~10-fold higher specific activity for DG compared with TG, MG, or CE (22) when analyzed in in vitro assay systems, suggesting a specific role of the enzyme in DG catabolism. The view that HSL is the rate-limiting enzyme for TG hydrolysis, however, has recently been challenged by the demonstration that HSL-deficient mice exhibited normal body weight and were still able to catabolize adipose tissue fat stores (26). These observations suggested that at least one additional TG hydrolase must exist in adipose tissue to compensate for the lack of HSL.

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‡ To whom correspondence should be addressed: Institute of Molecular Biology, Biochemistry, and Microbiology, University of Graz, Heinrichstrasse 31a, A-8010 Graz, Austria. Tel: 43-316-380-1900; Fax: 43-316-380-9016; E-mail: rudolf.zechner@kfunigraz.ac.at.

1 The abbreviations used are: HSL, hormone-sensitive lipase; ES cells, embryonic stem cells; NEO, neomycin resistance; HSV-TK, herpes simplex virus thymidine kinase; DIA, diphertheria toxin-A; ko, knock-out; wt, wild type; TG, triglycerides; DG, diglycerides; MG, monoglycerides; CE, cholesteryl esters; FFA, free fatty acids; FA, fatty acid; WAT, white adipose tissue; BAT, brown adipose tissue; GC, gas chromatography; ESI, electrospray ionization.
To define further the role of HSL in the lipolytic breakdown of TG, DG, and MG, we now demonstrate that HSL-ko mice accumulate DG in various tissues including adipose tissue. This block of diglyceride hydrolysis results in the absence of the isoproterenol-stimulated glycerol release from adipose tissue. Additionally, we demonstrate a preferred substrate specificity of HSL for long chain fatty acids within acylglyceride substrates.

EXPERIMENTAL PROCEDURES

Construction of the HSL Targeting Vector—A replacement vector was designed containing three loxP recombination sites (27), a neomycin resistance (NEO) gene fused with the gene of herpes simplex virus thymidine kinase (HSV-TK) (28), and two dihydrothiazone toxin (Dta) genes (29) as shown in Fig. 1A. Starting from a A-FIXII clone from a National Institutes of Health Swiss mouse genomic library (Stratagene) containing the complete mouse HSL gene, a 3-kb spa-BamHI fragment was excised and cloned into pBluescript. This clone contained exons 2–7 of the HSL gene. Adjacent to exon 7, a double-stranded oligonucleotide containing a loxP recombination sequence was inserted into the BamHI site. From this clone the spa-XbaI fragment (containing the loxP site) was subcloned into pBluescript. To maximize the recombination frequency, the short arm of the targeting construct was amplified by PCR from the HM1-embryonic stem (ES) cell derived from 129/ola mice (30), and the PCR product was cloned into the SpaI site 5′ of exon 2. The DNA cassette containing the NEO gene and the HSV-TK gene flanked by two loxP sequences (from plasmid pGH-4A kindly provided by K. Rajewsky, Cologne, Germany) (28) was then inserted into the same SpaI site. Finally, the long arm of the targeting construct was also amplified from isogenic DNA and inserted into the XbaI site at the 3′ end of the targeting construct. Addition of Dta site sequences on the ends of this construction was achieved by inserting the DNA into the CiaI and NotI restriction sites of plasmid pUC-2DtaA. pUC-2DtaA was prepared by excising two DtaA cassettes from the plasmid pDtaA (29) by EcoRV-KpnI and KpnI-XbaI digestion, respectively, and ligation together into pUC vectors. The Molecular Biochemicals cut with SpaI and XbaI. The final targeting DNA construction was named pHSL-flox.

ES Cell Culture and Generation of HSL-deficient Mice—HM-1 ES cells were cultured on gelatin-coated dishes with 1000 units/ml recombinant murine LIF (ESGRO™ Invitrogen). After linearization of the pHSL-flox targeting vector with NdeI, 200 μg of DNA were electroporated (Bio-Rad gene pulser) into 20 × 10⁶ ES cells at 800 V and 3 microfarads.Neo−/− selection was initiated by G418 (Invitrogen) treatment at a concentration of 400 μg/ml. Next day selection was initiated by G418 (Invitrogen) treatment at a concentration of 1000 μg/ml. The PCR product was cloned into the SpaI site 5′ of exon 2. The DNA cassette containing the NEO gene and HSV-TK gene flanked by two loxP sequences (from plasmid pGH-4A kindly provided by K. Rajewsky, Cologne, Germany) (28) was then inserted into the same SpaI site. Finally, the long arm of the targeting construct was also amplified from isogenic DNA and inserted into the XbaI site at the 3′ end of the targeting construct. Addition of Dta site sequences on the ends of this construction was achieved by inserting the DNA into the CiaI and NotI restriction sites of plasmid pUC-2DtaA. pUC-2DtaA was prepared by excising two DtaA cassettes from the plasmid pDtaA (29) by EcoRV-KpnI and KpnI-XbaI digestion, respectively, and ligation together into pUC vectors. The Molecular Biochemicals cut with SpaI and XbaI. The final targeting DNA construction was named pHSL-flox.

FIG. 1. The HSL gene, targeting vector, and Southern blot analysis after homologous recombination. A exhibits a partial restriction map of the HSL gene (exons 1–9, black boxes), the pHSL-flox targeting vector, and the predicted structure of the recombinant allele. The targeting vector contains 8.4-kb homologous HSL genomic DNA, with 17 kb HSV-TK cassette flanked by two loxP sites (Fig. 2A). The recombinant allele would be deleted but the HSL gene would remain intact with 2 loxP sites flanking exons 2 and 7 (“floxed” gene). Third, if the loxP sites 2 and 3 would be utilized for recombination (Type III deletion, not shown), a knock-out construction containing the marker cassette would be the result. This recombination type can be detected by neomycin treatment. In this study, cells containing the complete knock-out allele were expanded and injected into C57BL/6J host blastocysts, and 10–20 embryos were transferred into the uterine horn of (C57BL/6J × CBA/J) F1 surrogate mothers (32). All animals with a high degree of coat color chimeraism were back-crossed to C57BL/6J mice and germ line transmission was observed by coat pigment. Heterozygous mutants were confirmed by genomic Southern blotting of tail tip DNA (Fig. 2C) and interbred to generate homozygous HSL-ko mice.

DNA and RNA Analysis—Genomic ES cell DNA was isolated by digesting cells in lysis buffer (100 mM Tris-Cl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml proteinase K) for 5 h at 37 °C and spooling genomic DNA after adding an equal volume of isopropyl alcohol. Tail tip DNA was prepared with the same lysis buffer with 400 μg/ml proteinase K and incubation overnight at 55 °C. The DNA was analyzed using standard Southern blotting techniques (33). Total RNA was isolated from epidydimal WAT using the TRI-reagent procedure according to the manufacturer’s protocol (Molecular Research Center, Karlsruhe, Germany). HSL mRNA was detected using standard Northern blotting techniques with 10 μg of total RNA (33). Murine HSL-specific probe was generated using random priming from a 800-bp HSL PacI-cDNA restriction fragment. Specific hybridization of Southern or Northern blot was visualized by exposure to a Phosphorlmager Screen (Aptiotech, Freiburg, Germany) and analyzed using ImageQuant Software.

Generation of an HSL Polyclonal Antiserum—Full-length mouse HSL cDNA was generated by reverse transcription of 3T3-L1 adipocytes total RNA using Advantage RT-for-PCR Kit (CLONTECH) according to the manufacturer’s protocol. A 2.4-kb PCR product encompassing the complete HSL cDNA including the initiation codon and stop codon of translation was subcloned into pYEX 4T-1 (CLONTECH Lab.
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Inc.) to generate a glutathione S-transferase-HSL fusion gene. The primers used in the reaction are as follows: I, 5'-GATCCAGCGTCTCATCACCTGAGTTTCTCA-3' and II, 5'-GATCCAGCGTCTCATACAGAGGAG-3'. The glutathione S-transferase-HSL construct was expressed in yeast strain S. C. strain DX150 using the YEpXpress system (CLONTECH) according to the manufacturer's instructions. Glutathione S-transferase-HSL fusion protein was purified by size exclusion chromatography of the yeast extracts under denaturing conditions (1% SDS, 1 mM dithiothreitol). Purified glutathione S-transferase-HSL fusion protein (0.5 mg, 0.5 ml) was emulsified with 0.6 ml of complete Freund's adjuvant (Sigma), and two rabbits were immunized by subcutaneous injection of 0.5 ml of glutathione S-transferase-HSL construct was expressed in yeast strain S. C. strain DX150 using the YEpXpress system (CLONTECH) according to the manufacturer's instructions. Glutathione S-transferase-HSL fusion protein was purified by size exclusion chromatography of the yeast extracts under denaturing conditions (1% SDS, 1 mM dithiothreitol). Purified glutathione S-transferase-HSL fusion protein (0.5 mg, 0.5 ml) was emulsified with 0.6 ml of complete Freund's adjuvant (Sigma), and two rabbits were immunized by subcutaneous injection of 0.5 ml of suspension per animal. Two weeks after the second injection of the glutathione S-transferase-HSL fusion protein, blood was collected from the ear vein of the rabbits, and serum was obtained by centrifugation.

Western Blotting—For the preparation of protein extracts, epididymal WAT was homogenized in a buffer containing 2% SDS, 2 mM dithiothreitol, 2 mM EDTA, and 50 mM Tris-HCl, pH 6.8. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell). Blots were incubated with 1:1000 diluted polyclonal antisemur against HSL. Bound immunoglobulins were detected with a horseradish peroxidase anti-rabbit IgG conjugate (Vector Laboratories) and visualized by ECL detection (Amersham Biosciences) according to the manufacturer's instructions.

Genetic Analysis—Mice were maintained on a regular light-dark cycle (14 h light, 10 h dark) and kept on a standard laboratory chow diet (4.5% w/w fat). For breeding experiments heterozygous HSL-ko mice were used to generate homozygous ko mice. Genotyping of HSL-ko mice was performed by a single step PCR using three primers. The primers used in the reaction are as follows: I, 5'-CATGACGGAGCCACCTCCCTC-3'; II, 5'-CTCACTGGGCGCTGTCTCTG-3'; and III, 5'-TACGGCGCATCCTGTAAG-3'. PCR conditions in a reaction volume of 50 µl are as follows: 200-300 ng of tail tipped DNA, 250 ng of each primer, 0.02 mM dNTPs, 1 unit of DynaZyme II polymerase, denaturation at 94 °C for 1 min, annealing at 56 °C for 90 s, and extension at 72 °C for 2 min for a total of 40 cycles. Primers I and II produced an amplification product of 340 bp for the wild type (wt) allele, whereas primers I and III produced an amplification product of 250 bp for the knock-out allele.

**Tissue Lipid Analysis**—From anesthetized mice blood was removed from the left ventricle, and mice were perfused with 0.9% NaCl solution. Tissues were excised, weighed, and frozen. Total lipids were extracted from organs by the method of Folch et al. (34). Total acylglycerides were quantitated on the basis of total glycerol released from acylglycerides by the action of a yeast lipase. Lipid extracts were incubated in a buffer containing 4 units/ml Candida rugosa lipase (Sigma), 50 mM Tris-HCl, pH 7.4, 5% bovine serum albumin for 3 h at 37 °C to achieve complete acylglyceride hydrolysis. Subsequently the released glycerol was quantitated with a commercially available TG-kit (GPO-Trinder 20) obtained from Sigma. For TLC analysis of lipid extracts, equimolar amounts of total acylglycerides were loaded on the plates after complete hydrolysis (0.34 µmol equivalent to 300 µg of TG based on the measurement of total glycerol). Tissue lipids were separated with chloroform/methanol/acetic acid (96:4:1) as solvent. The lipids were visualized with phosphomolybdenum vapor.

**FA Composition**—For analysis of FFA and TG-associated FA in epididymal fat pads, tissue specimens were weighed, and the lipids were extracted with 2 ml of H2O and 4 ml of chloroform/methanol (2:1) for 40 min at room temperature. After centrifugation, the organic phase was collected, and the aqueous phase was extracted again. The organic phases were pooled, dried under nitrogen, and dissolved with 400 µl of toluene. Aliquots of 100 µl were separated by TLC (hexane/diethyl ether/acetic acid, 70:30:1), and the TG bands were scraped from the plates and dissolved with 500 µl of toluene including an internal standard (50 µg of 15:0 fatty acid). Transesterification and gas chromatographic (GC) analysis was performed according to the method of Sattler et al. (35).

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**FIG. 2. Cre-mediated recombinations and DNA analysis of ES cell and mouse tail tip genomic DNA.** 
A, recombinant allele after the first homologous recombination and deletion events after transient expression of Cre recombinase. loxP sites are indicated as triangles. Type I deletion is a result of recombination between the 5' loxP site of the selection cassette (white box) and the most 3' loxP site. Type II deletion is a result of recombination between the loxP sites flanking the selection cassette (B). BamHI, EcoRV, S, SpeI X, XbaI. 
B, Southern blot analysis of ES cell genomic DNA. Total DNA was digested with XbaI, separated by agarose gel electrophoresis, blotted on nylon paper, and hybridized with a 32P-labeled probe which is indicated in A. WT DNA from wt ES cells; lanes 1 and 2, ES cell DNA that underwent type I deletion (ko-allele); lane 3, ES cell DNA that underwent type II deletion (178 floxed allele 178). C, Southern blot analysis of XbaI-digested mouse tail tip DNA hybridized with the probe indicated in A. +/+ DNA from a wt mouse; +/− DNA from a heterozygous HSL-ko mouse; −/− DNA from a homozygous HSL-ko mouse. 
C, PCR analysis used for genotyping of mice. The PCR was performed using three primers. +/+ DNA from a wt mouse (340 bp); +/− DNA from a heterozygous HSL-ko mouse (340 and 250 bp); −/− DNA from a homozygous HSL-ko mouse (250 bp).

**FIG. 3. HSL mRNA and protein analysis of WAT.** 
A, Northern blot analysis of total RNA from WAT of wt-mice (+/+ ) and homozygous HSL-ko mice (−/− ). Total RNA from WAT was isolated and separated by formaldehyde/agarose gel electrophoresis. After blotting on nylon paper, specific signals were visualized by hybridization with a 32P-labeled 800-bp PstI HSL cDNA probe and autoradiography. The ethidium bromide-stained gels are also shown to compare the amounts of total RNA loaded on the gels. 
B, Western blot analysis of total WAT protein of wt-mice (+/+ ) and homozygous HSL-ko mice (−/− ). Protein extracts were subjected to SDS-PAGE and electrophoresed on nitrocellulose. HSL protein (84 kDa) was detected with a polyclonal rabbit anti-HSL antiserum and a horseradish peroxidase anti-rabbit IgG as secondary antibody.
Analysis of DG and TG Mass Distribution by ESI Mass Spectroscopy—WAT lipids were extracted from 200 mg of epididymal WAT according to the method of Folch et al. (34). Mass spectrometric analysis was performed with a Finnigan-Mat (San Jose, CA) model TSQ 7000 triple stage quadrupole instrument equipped with a nanoelectrospray source (Protana). Econotips (New Objective, Cambridge, MA) operating at a flow rate of 20–80 nl/min were used. Samples were dissolved in 90% chloroform, 10% methanol containing 10 mM ammonium acetate. The spray was started by applying 600–900 V to the capillary. Acylglycerols were measured as ammonium adducts in the positive mode. Spectra were recorded from 600 to 1000 m/z. The intensities of TG and DG were corrected for isotope effects.

Analysis for Lipolytic Activities and Lipolysis Products—Total acylglyceride lipase activity (measured as total FFA release) was measured with triolein as substrate containing [9,10-3H]triolein (PerkinElmer Life Sciences) as radioactive tracer. The substrate was prepared by sonication (Virsonic 475) exactly as described by Doolittle et al. (36). The tissues were surgically removed and washed in phosphate-buffered saline containing 1 mM EDTA. Homogenization was performed on ice in lysis buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin) using a ultraturax (IKA®, Janke & Kunkel). The infranatants were obtained after centrifugation at 20,000 × g, at 4 °C for 90 min. The reaction was performed in a water bath at 37 °C for 60 min with 0.1 ml of substrate and 0.1 ml of infranatant. The reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After centrifugation (800 × g, 20 min) the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting, and the total release of FFA (total acylglyceride hydrolase activity) was calculated.

The rate of DG formation was determined after termination of the lipolysis reaction by adding 25 μl of 1 M HCl and 1 ml of hexane/isopropyl alcohol (3:2) containing oleic acid (10 μg/ml) and standards for mono- and diolein (sn-1.2 and sn-1.3; Sigma) and triolein (10 μg/ml).

**FIG. 4.** Lipid analysis of WAT and BAT by TLC. Lipids from WAT and BAT of wt mice (+/+) and HSL-ko mice (−/−) mice were extracted, and equimolar amounts of total acylglycerides (0.34 μmol, equivalent to 300 μg of TG) were analyzed by TLC. A mixture of TG, sn-1.2/1.3 DG, MG, and FC (free cholesterol) was used as standard (S). Lipids were visualized with phosphomolybdate. Age of fasted male animals at the time of analysis was 14–16 weeks.

**FIG. 5.** Lipid and fatty acid analysis by mass spectrometry. Mass spectra of lipid extracts from WAT of wt mice and HSL-ko mice. The insets in the figure indicate the relative proportion (n = 4) of DG content compared with total WAT TG. The TG and DG numbers indicate the sum of carbons in the fatty acid side chains.
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RESULTS

Targeted Disruption of HSL in ES Cells—HSL-ko mouse lines were generated by the insertion of a mutated HSL gene into the genome of 129/J mice by homologous recombination of ES cells. In the first step, a replacement vector was designed (Fig. 1A) which contained a selection cassette (NEO HSV-TK) within intron 1 of the HSL gene. This selection marker was flanked by two loxP recombination sites. Additionally, a single loxP site was placed within intron 7 of the HSL gene. The linearized targeting vector was electroporated in HM-1 ES cells (30), and homologous recombination events were confirmed by antibiotic resistance screening in cell cultures and Southern blot analysis of ES cell genomic DNA. Three independent cell clones were identified that underwent homologous recombination at the appropriate genomic location (Fig. 1B). Two positive cell clones were expanded and transfected with circular pCre-Pac (31) for the transient expression of the Cre recombinase (Fig. 2A). After pulse treatment with puromycin, three types of cell clones could be identified. Depending on the usage of loxP recombination sites, the HSL gene locus lacked the following: (i) both the DNA region between exons 2 and 7 and the selection cassette (HSL-ko allele), (ii) only the selection cassette (floxed allele), or (iii) only the DNA region between exons 2 and 7. Fig. 2B exhibits the Southern blotting analysis of DNA isolated from ES cell clones that underwent the type I deletion, which resulted in two bands of 10.4 and 3.1 kb, or the type II deletion, which resulted in a 10.4-kb band and a 6.2-kb band in comparison to control ES DNA (only one 10.4-kb band).

Four clones positive for the type I deletion (HSL-ko allele) were expanded and injected into 3.5-day-old C57BL/6J blastocysts that were transferred into female pseudopregnant recipient mice (32). Several chimeric mice were obtained that exhibited a greater than 90% coat color chimerism. Back-crossing of C57BL/6J females revealed 100% germ line transmission of the ES cell-derived genome. Males heterozygous for the HSL knock-out mutation were mated to heterozygous females, and the expected frequency of homozygous animals was obtained for all independent lines. Mice were genotyped by Southern blot analysis (Fig. 2C) or PCR analysis (Fig. 2D). Southern blot analysis revealed a single band at 10.4 kb in size for wt mice and a single band of 3.1 kb for homozygous knock-out mice. Heterozygous animals exhibited both bands at about equal intensities. For PCR analysis, three primers were used in a single reaction that resulted in a 340-bp product for wt and a 250-bp product for the knock-out allele.

Absence of HSL mRNA and HSL Protein in HSL-ko Mice—To analyze HSL mRNA expression, Northern blotting was performed from total RNA isolated from various mouse tissues. Results obtained with RNA isolated from the epididymal fat pad of wt and HSL-ko mice are shown in Fig. 3A. As expected, homozygous HSL-ko mice lacked HSL mRNA. The absence of HSL protein in knock-out mice was also confirmed by Western blotting of protein extracts from WAT (epididymal fat pad). Although wt mice exhibited an 84-kDa band when a specific polyclonal rabbit anti-HSL antiserum was used, no signal specific for HSL protein (84 kDa) could be detected in WAT of knock-out mice (Fig. 3B). The absence of HSL mRNA and protein was also confirmed in cardiac muscle, skeletal muscle, testis, and liver (not shown). In accordance with earlier observations (26), male mice were infertile due to oligospermia. Otherwise, homozygous HSL-ko mice appeared superficially normal and exhibited normal body weight.

Accumulation of DG in Adipose Tissue of HSL-ko Mice—To investigate whether HSL deficiency affects the lipid composition in adipose tissue in vivo, lipid extracts from WAT and BAT from fasted control and HSL-ko mice were analyzed by TLC.
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Tissues were taken from 10- to 12-week-old male animals after an overnight fast. Assays were performed with fat-free infranatants from HSL-wt (+/+) and HSL-ko (−/−) mice as described under “Experimental Procedures.” All values represent means ± S.D. The abbreviations used are: SM, skeletal muscle; CM, cardiac muscle; ND, not determined.

TABLE I

| Tissue | Genotype | n | FFA release | DG concentration | FFA/DG molar ratio | TG hydrolysis activity |
|--------|----------|---|-------------|------------------|-------------------|-----------------------|
|        |          |   | (nmol/h · mg protein) | (nmol/mg protein) |                   | (nmol/mg protein)     |
| WAT    | +/+      | 4 | 402.3 ± 55  | 9.6 ± 2.2        | 42.8 ± 4         | 140.5 ± 19.8         |
|        | −/−      | 4 | 117.9 ± 40\(^a\) | 49.2 ± 5.2\(^b\) | 2.3 ± 0.6\(^b\) | 72.1 ± 16.8\(^b\) |
| BAT    | +/+      | 4 | 250.0 ± 4.5 | 2.34 ± 0.4       | 109.7 ± 20       | 84.9 ± 1.4\(^b\)    |
|        | −/−      | 4 | 149.2 ± 38.34\(^c\) | 49.4 ± 11.3\(^c\) | 3.0 ± 0.1\(^c\)  | 82.6 ± 20.3         |
| Testis | +/+      | 4 | 20.8 ± 4.2  | 1.9 ± 0.3        | 10.8 ± 1.2       | 8.2 ± 1.6            |
|        | −/−      | 4 | 7.8 ± 1.3\(^c\) | 4.0 ± 0.6\(^c\)  | 2.0 ± 0.5\(^c\)  | 5.3 ± 0.5 \(p = 0.07\) |
| SM     | +/+      | 4 | 5.3 ± 1.2   | 0.4 ± 0.07       | 12.9 ± 0.7       | 2.0 ± 0.4            |
|        | −/−      | 4 | 2.6 ± 0.3\(^c\) | 1.5 ± 0.07\(^c\) | 1.8 ± 0.2\(^c\)  | 1.8 ± 0.2            |
| CM     | +/+      | 4 | 26.5 ± 5.8  | 3.3 ± 0.3        | 8.0 ± 2.4        | 11.1 ± 1.8           |
|        | −/−      | 4 | 19.3 ± 3.9  | 6.6 ± 0.9\(^b\)  | 2.9 ± 0.3\(^b\)  | 10.9 ± 1.9           |
| Liver  | +/+      | 4 | 3.2 ± 0.1   | 0.3 ± 1.3        | 10.1 ± 1.7       | 1.3 ± 0.1            |
|        | −/−      | 4 | 1.9 ± 0.2\(^c\) | 0.5 ± 1.8\(^c\)  | 3.7 ± 0.3\(^c\)  | 1.0 ± 0.2            |
| Brain  | +/+      | 4 | 0.21 ± 0.04 | ND               | ND                | ND                    |
|        | −/−      | 4 | 0.07 ± 0.014\(^c\) | ND            | ND                | ND                    |

\(^{a}\) \(p < 0.01\) compared with HSL-wt mice.
\(^{b}\) \(p < 0.001\) compared with HSL-wt mice.
\(^{c}\) \(p < 0.05\) compared with HSL-wt mice.

from HSL-ko mice exhibited strong signals in the mass range of DG 34 and DG 36 which consists of 8% of the total fat mass (Fig. 5, inset). Within the TG moiety, marked changes in the relative mass distribution were observed. The proportion of TG subtypes with longer chain fatty acids increased in HSL-ko WAT (+40% for TG-54, \(p < 0.05\), and +100% for TG-56, \(p < 0.01\)), whereas the TG fraction with shorter chain fatty acids, TG-48, TG-50, and TG-52, decreased compared with HSL-wt WAT (Fig. 5).

Altered Composition of WAT-TG Fatty Acids and Released FFA from WAT in HSL-ko Mice—On the basis of the observed changes in the mass distribution of TG by mass spectroscopy, the FA composition of the adipose tissue TG fraction was determined by GC analysis (Fig. 6A). As predicted, a significant shift from reduced short chain FFA (−41% for 14:0 and −34% for 16:0) to elevated long chain FFA (−19% for 18:1 and +12% for 18:2) was found in HSL-deficient WAT compared with WAT from control mice. Additionally, lipolysis experiments with isoproterenol-stimulated fat pads from HSL-ko and control mice revealed the preferential release of short chain FFA in HSL-deficient fat pads (Fig. 6B) suggesting an altered substrate specificity of the lipolytic system in the absence of HSL.

Reduced Aci glyceride Hydrolase Activity and DG Hydrolysis in HSL-ko Tissue Extracts—The observed accumulation of DG in adipose tissue of HSL-ko mice prompted the assumption that HSL deficiency leads to incomplete hydrolysis of TG with an interruption of the lipolytic cascade at the stage of DG hydrolysis. To evaluate this hypothesis, lipase activity assays with extracts from various tissues from control and HSL-ko mice were performed, and the formation of FFA (as a measure of total lipolytic activity), DG, and MG was determined (Table I). HSL deficiency in the extracts from tissues of HSL-ko mice caused decreased formation of FFA in WAT (−70%), BAT (−40%), and testis (−62%). Reduced FFA formation was also found in skeletal muscle (−50%), cardiac muscle (−27%), liver (−41%), and brain (−67%) extracts suggesting that HSL is normally expressed in these tissues and involved in the degradation of acylglycerides. The accumulation of DG was markedly increased during lipolysis when extracts from HSL-deficient tissues were used. The effect was most pronounced when HSL-ko BAT extracts (21-fold DG increase) and WAT extracts (5-fold DG increase) were used. The accumulation of DG and the decreased FFA formation resulted in a drastically altered molar FFA:DG ratio that can be considered as a measure of the

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**Fig. 7. In vitro lipolysis from isolated epididymal WAT of wt mice (wt) and HSL-ko (ko) mice.** Pieces of WAT from 12- to 14-week-old male mice were incubated in the presence (solid squares and circles) or absence (open squares and circles) of 10 μM isoproterenol (iso). The amounts of glycerol and FFA released into the medium were determined over a time course of 3 h. Data are expressed as means ± S.D. of three mice.

Equal amounts of total acylglycerols (based on determination of glycerol) were analyzed. As shown in Fig. 4, a strong signal was obtained at \(R_p\) values typical for sn-1,2-DG and sn-1,3-DG in HSL-deficient WAT and BAT that was not present in WAT and BAT of wt mice. DG accumulation was also observed in lipid extracts of testis, skeletal muscle, and cardiac muscle (data not shown).

**ESI Mass Spectroscopy of Lipid Extracts from WAT—Experimental support for DG accumulation in HSL-deficient adipose tissue was also provided from the analysis of WAT lipid extracts by ESI mass spectroscopy (Fig. 5).** Compared with WAT from control mice that essentially lacked DG in relation to the TG content (less than 1% of TG mass, see inset in Fig. 5), WAT...
tissue DG hydrolysis. A molar ratio of 1 would indicate that virtually all FFA are derived from the hydrolysis of tri- to diglycerides. From the FFA/DG ratio we calculated that the DG hydrolysis capacity in control WAT and BAT was 20–36-fold higher than in HSL-deficient WAT and BAT. The DG hydrolysis capacity was also markedly reduced in non-adipose tissues but to a lesser extent. MG did not accumulate in the assay mixture independent of the tissue extracts used in the assay (data not shown). The determination of total FFA release and DG formation also permitted the calculation of the specific TG hydrolase activity (enzymatic conversion of TG to DG) in these tissues. Reduced specific TG hydrolase activities were observed in WAT (−50%) and testis (−40%) of HSL-ko mice. In all other tissues, the capacity to hydrolyze TG to DG was essentially unchanged, arguing for the existence of compensatory enzymes in these tissues.

**Lack of Glycerol Release and the Accumulation of DG during in Vitro Lipolysis in Isolated Fat Pads**—To specify further the defect in DG hydrolysis in HSL-deficient adipose tissue, in vitro lipolysis experiments were performed using pieces of epididymal and subepidermal WAT from control and HSL-deficient mice. Fig. 7 demonstrates that 3 h after stimulation with isoproterenol, the release of glycerol from epididymal wt WAT was increased 4.3-fold. In contrast, the release of glycerol from isoproterenol-stimulated HSL-ko WAT was only marginally enhanced (1.2-fold). The release of FFA from HSL-wt and HSL-ko WAT was enhanced by isoproterenol 7.6- and 4.4-fold, respectively. Compared with HSL-wt mice, the release of FFA was decreased by 35%. Essentially identical results were obtained when subepidermal fat pads were used for the experiments (data not shown). Analysis of the lipid composition during in vitro lipolysis experiments in the presence or absence of isoproterenol is summarized in Fig. 8. Although the DG content in HSL-deficient fat pads was already 4-fold increased previous to the experiment, hormone stimulation caused an additional 88% increase in the tissue DG content (Fig. 8A). The tissue TG content did not significantly change during the experiment (Fig. 8B).

**Tissue TG Stores**—When various tissues were analyzed for their TG content, marked differences were observed in fasted HSL-ko mice compared with controls as shown in Fig. 9. In BAT, the TG content was increased by 30% which was also evident by the observed hypertrophy of brown adipocytes in HSL-ko mice (26). In WAT, testis, and skeletal muscle, the amount of tissue TG concentrations were identical in control and HSL-ko mice. In contrast, HSL-deficient cardiac muscle and liver exhibited drastically reduced tissue TG levels (−70% and −90%, respectively) when compared with control tissues.

**DISCUSSION**

The functional role of HSL in adipocytes and other cell types and tissues is insufficiently understood. In *in vitro* studies have shown that in addition to TG, HSL catalyzes the hydrolysis of DG, MG (37, 38), CE (39, 40), and retinyl esters (25). It was previously believed that the enzyme catalyzes the rate-limiting reaction in the catabolism of adipose tissue TG depots, namely the hydrolytic cleavage of the primary ester bonds of TG. This essential role of HSL, however, was questioned when HSL-ko mice were shown to have normal body weight and white adipose tissue mass (26). These findings suggested the existence of other TG-mobilizing enzymes in adipose tissue.

To define the functional role of HSL in various tissues, HSL-ko mice were generated, and the effects of HSL deficiency on the intracellular lipid metabolism were studied in adipose tissue, muscle, testis, liver, and brain. In a first step, a targeting vector was assembled containing a *loxP* flanked selection cassette within intron 1 and a single *loxP* site within intron 7. After homologous recombination in an embryonic stem cell line, the bacteriophage-derived Cre-*loxP* recombination system (27, 41, 42) was utilized to eliminate the selection marker gene and exons 2–7 of the endogenous HSL gene. This strategy was utilized to avoid possible perturbations of the HSL-ko pheno-type through expression of the selection marker (43, 44). HSL-deficient mice appeared grossly normal; however, male animals were sterile due to gonadal hypotrophy and oligospermia as reported by previously Osuga *et al.* (26).

The disruption of the HSL gene in mice caused a defect in the catabolism of cellular DG. TLC analysis of tissue lipid extracts revealed a massive accumulation of DG in various tissues which was verified by ESI mass spectrometry. Defective DG hydrolysis in the absence of HSL was further substantiated by two experimental approaches. First, in *in vitro* TG-lipase assays were performed employing an artificial TG substrate and extracts from HSL-ko and control tissues. This assay permitted the determination of the total acylglyceride lipase activity of the extract (equals total FFA release), the formation of DG, and the specific TG hydrolase activity (conversion of TG to DG) in every tissue analyzed. Second, lipolysis experiments were performed with isoproterenol-stimulated HSL-ko fat pads to determine the hormone-induced release of FFA and glycerol in WAT in the presence and absence of HSL.

From these experiments it became evident that in HSL-deficient WAT both the hydrolysis of TG and the hydrolysis of...
DG are impaired. Decreased FFA and increased DG accumulation led to a drastic (20-fold) decrease in the FFA/DG ratio when WAT extracts from HSL-ko mice were used in in vitro lipase assays. However, this defect in the lipolytic cascade was not associated with increased WAT TG content nor increased fat mass in HSL-ko animals. Similar observations were reported by Osuga et al. (26, 45). Interestingly, lipolysis experiments with isoproterenol-stimulated pieces of HSL-ko fat pads also revealed that the net release of FFA was reduced relative to controls. A specific inhibition of DG hydrolysis resulted in intracellular DG accumulation and drastically reduced glycerol formation in HSL-ko fat pads. The molar FFA/glycerol ratio after isoproterenol stimulation was comparatively low in both HSL-ko WAT and control WAT providing evidence for the efficient reesterification of FFA within a futile cycle. Efficient reusage of lipolyzed FFA for reesterification and lipid deposition has been reported previously (46) in studies with WAT fragments. As a consequence of the defect in the lipolytic cascade in HSL-ko mice, the decreased release of FFA into the vascular system causes reduced plasma levels of FFA and glycerol (26). Taken together these results indicate that the lipolytic cascade is impaired in WAT in the absence of HSL. However, at least one alternative TG hydrolase must exist in addition to HSL to compensate for the breakdown of stored TG in the absence of HSL. A possible candidate is a recently cloned neutral cholesteryl ester hydrolase/TG hydrolase (47, 48) that was found to be expressed in various tissues including WAT. The fact that HSL-ko mice are not obese despite their apparent defect in TG and DG hydrolysis is unexpected and suggests a putative compensatory down-regulation of TG synthesis in HSL-deficient WAT.

In addition to the increased DG levels observed in HSL-deficient WAT, ESI mass spectroscopy and GC analysis of WAT lipid extracts revealed a relative increase of long chain unsaturated fatty acids (18:1 and 18:2) in DG and TG of HSL-ko WAT. Conversely, when lipolysis experiments were performed with isoproterenol-stimulated fat pads, the release of 18:1 and 18:2 fatty acids was decreased. From these in vivo results we conclude that HSL exhibits substrate specificity for long chain fatty acids. Evidence for substrate specificity of HSL in vitro has been reported recently (49). The substrate specificity of HSL might be associated with the polarity of TG in adipose tissue (19, 50, 51).

Similarly to the situation observed in WAT, the BAT DG content is also increased due to the decreased hydrolysis of DG. In contrast to WAT, however, BAT exhibited normal TG hydrolase activity suggesting that in the absence of HSL the conversion of TG to DG is facilitated by an alternative pathway that entirely compensates for HSL. Expectedly, the impaired lipolytic breakdown of DG to glycerol and FFA led to decreased total FFA release in HSL-deficient adipose tissue extracts when in vitro lipolysis experiments were performed. This defect might be responsible for the morphological changes observed in BAT, which resulted in increased BAT lipid content and BAT hypertrophy (26).

In skeletal muscle, HSL deficiency also caused DG accumulation, whereas specific TG hydrolase activity was normal. This finding argues for a functional role of HSL in the mobilization of DG.
Alternatively, it is conceivable that other DG hydrolases that and the fat content are high and where reusage of lipolyzed cific differences in the FFA/DG ratio are likely the result of a cellular DG accumulation. In all tissues the FFA/DG ratio is acylglyceride hydrolase activity in the brain of HSL-ko mice, bilization of liver TG stores. From the observed absence of finding is in contrast to previous studies (2, 39) that assumed indicating that HSL is present and functional in liver. This deficiency. TG to DG is not affected, again arguing for the existence of an diac muscle TG breakdown is under acute hormonal control, extremely high lipolytic turnover of myocardial TG (54). Car-

were not altered in HSL-ko mice. In contrast to skeletal muscle, the metabolic requirements of cardiac muscle are met by an extremely high lipolytic turnover of myocardial TG (54). Car
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