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

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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 perilipin 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.
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 diptheria toxin-A (DTA) genes (29) as shown in Fig. 1A. Starting from a Δ-FIXII clone from a National Institutes of Health Swiss mouse genomic library (Stratagene) containing the complete mouse HSL gene, a 3-kb SpeI-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 SpeI-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 line derived from 129/ola mice (30), and the PCR product was cloned into the SpeI 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 SpeI site. Finally, the long arm of the targeting construct was also amplified from homologous DNA and inserted into the XbaI site at the 3′ end of the targeting construct. Addition of DTA sequences on the ends of this construction was achieved by inserting the DNA into the ClaI and NotI restriction sites of plasmid pUC-2Dta. pUC-2Dta was prepared by excising two Dta cassettes from the plasmid pDta (29) by EcoRV-KpnI and KpnI-XbaI digestion, respectively, and ligation together into pUC-19 (Molecular Biochemicals) cut with XbaI and XcoI. The final targeting construct was named pHSL-flox.

**ES Cell Culture and Generation of HSL-deficient Mice**—HM1 ES cells were cultured on gelatin-coated dishes with 1000 units/ml recombinant murine LIF (ESGRO™ Invitrogen). After linearization of 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 SpeI site. Finally, the long arm of the targeting construct was also amplified from homologous DNA and inserted into the XbaI site at the 3′ end of the targeting construct. Addition of DTA sequences on the ends of this construction was achieved by inserting the DNA into the ClaI and NotI restriction sites of plasmid pUC-2Dta. pUC-2Dta was prepared by excising two Dta cassettes from the plasmid pDta (29) by EcoRV-KpnI and KpnI-XbaI digestion, respectively, and ligation together into pUC-19 (Molecular Biochemicals) cut with XbaI and XcoI. The final targeting construct was named pHSL-flox.

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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, PstI, EcoRV, SspI, 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. D, PCR analysis used for genotyping of mice. The PCR was performed using three primers. +/+ DNA from a wt mouse (340 bp); +/- DNA from heterozygous HSL-ko mice (340 and 250 bp); –/– DNA from a homozygous HSL-ko mouse (250 bp). S, 100-bp DNA ladder.

Inc.) to generate a glutathione S-transferase-HSL fusion gene. The primers used in the reaction are as follows: I, 5′-GGATCCACAGCCT-CAGGTTTCTCA-3′; II, 5′-GATATCGGCGACTAAACGGGAG-3′. The glutathione S-transferase-HSL construct was expressed in yeast strain S.l. 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 mol 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 mol 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 antiserum 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′-CATGACATAGTGGCCATCTT-3′; II, 5′-CTACGAGGCTGCTGCTGCTTCG-3′; and III, 5′-CTACGAGGCTGCTGCTGCTTCG-3′. PCR conditions in a reaction volume of 50 μl are as follows: 200–900 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 1 min, 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/acetone/acetic acid (96:4:1) as solvent. The lipids were visualized with phosphomolybdic acid 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).
Analysis of DG and TG Mass Distribution by ESI Mass Spectrometry—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 (IKAR, 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 into 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, Southern 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 oligosperma. 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.

The mixture was vortexed vigorously three times over a period of 15 min. After centrifugation (4,000 × g, 10 min), 0.4 ml of the upper phase was collected and evaporated under nitrogen. The lipid pellet was dissolved in chloroform and loaded onto a TLC plate (Merck Silica Gel 60). The TLC was developed with chloroform/acetic acid (96:4:1) as solvent. The lipids and FFA were visualized with iodine vapor, and the bands corresponding to mono-, di-, triolein, and oleic acid were cut out. The comigrating radioactivity was determined by liquid scintillation counting, and the molar concentrations of the products were calculated.

The specific TG hydrolase activity (TG to DG conversion) was calculated from the total acylglyceride lipolytic activity and the DG formed during the reaction (considering that MG do not accumulate) by Equation 1.

\[
\text{FFA}_{\text{mg tissue}} \times 3 \times \frac{1}{3} - \text{DG}_{\text{mg tissue}} = 0.030
\]

In Vitro Lipolysis of Isolated Subepidermal and Epididymal WAT—Subepidermal and epididymal fat pads were surgically removed and washed several times with phosphate-buffered saline. Fat pads were incubated in a final volume of 1 ml of Dulbecco’s modified Eagle’s medium (Invitrogen) containing 2% fatty acid-free bovine serum albumin (Sigma) with or without 10 μM isoproterenol (Sigma), at 37°C. Aliquots of the medium were collected hourly and investigated for the FFA and glycerol content by using commercial kits (Wako Pure Chemicals, Germany; Roche Molecular Biochemicals). After an incubation period of 6 h, pieces from the epididymal fat pads were weighed and subjected to lipid extraction (CHCl3/MeOH, 2:1) and TLC analysis. TG and DG corresponding bands were scraped from the plates and quantitated as described above.
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Equal amounts of total acylglycerols (based on determination of glycerol) were analyzed. As shown in Fig. 4, a strong signal was obtained at Rₚ 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 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 species 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).

TABLE I

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</table>

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.
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 (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 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 phenotype through expression of the selection marker gene (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 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 (equal to total FFA release), the formation of DG, and the specific TG hydrolyase 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
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FIG. 9. Tissue lipid analysis. Tissues were taken from 12- to 14-week-old fasted male animals after perfusion with a 0.9% NaCl solution. Tissue lipids were extracted by the method of Folch et al. (34), and concentrations of acylglycerides (TG and DG) were measured enzymatically. All values represent means ± S.D. of four animals. The abbreviations used are as follows: SM, skeletal muscle; CM, cardiac muscle. *, p < 0.05; **, p < 0.01 compared with HSL-wt mice.
Alternatively, it is conceivable that other DG hydrolases that the FFA/DG ratio might be more affected than in other tissues. and the fat content are high and where reusage of lipolyzed cellular DG accumulation. In all tissues the FFA/DG ratio is an important DG hydrolase that, when absent, provokes intra-

brain. The physiological role of HSL in both the liver and the we conclude that HSL is present and functionally active in the bilatization of liver TG stores. From the observed absence of deficiency.

In the liver, HSL deficiency caused reduced acylglyceride hydrolase activity with a concomitant accumulation of DG, indicating that HSL is present and functional in liver. This finding is in contrast to previous studies (2, 39) that assumed TG hydrolase activity in the brain of HSL-knockout mice is not affected, again arguing for the existence of an alternative TG hydrolase that can compensate for HSL deficiency.

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