Lipolysis in the Absence of Hormone-Sensitive Lipase

Evidence for a Common Mechanism Regulating Distinct Lipases

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Hormone-sensitive lipase (HSL) is presumed to be essential for lipolysis, which is defined as the mobilization of free fatty acids from adipocytes. In the present study, we investigated the effects of various lipolytic hormones on the lipolysis in adipocytes derived from mouse embryonic fibroblasts (MEF adipocytes) prepared from HSL-deficient mice (HSL−/−). HSL−/− MEF differentiated into mature adipocytes in a manner indistinguishable from that of wild-type mice. Both isoproterenol (ISO) and tumor necrosis factor (TNF)-α stimulated the rate of lipolysis in HSL−/− MEF adipocytes, although to a lesser extent than in wild-type cells, and these lipolytic activities were inhibited by H-89, a cAMP-dependent protein kinase inhibitor, and troglitazone, respectively. Thus, the responses of the residual lipolytic activity to lipolytic hormones and TNF-α were well conserved in the absence of HSL. Extracts from HSL−/− MEF adipocytes hydrolyzed triacylglycerol (TG) but not cholesterol ester, indicating that the residual lipolytic activity was mediated by another TG-specific lipase. The TG lipase activity, which was decreased in cytosolic fraction in response to ISO, was increased in fat cake fraction. Therefore, translocation of the TG lipase may explain, at least partially, the ISO-stimulated lipolysis in HSL−/− adipocytes. In conclusion, lipolysis is mediated not only by HSL but also by the non-HSL TG lipase, whose responses to lipolytic hormones are similar to those of HSL. We propose that both lipases are regulated by common mechanism of lipolysis. *Diabetes* 51:3368–3375, 2002

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aP2, adipocytes fatty acid–binding protein; BCA, bicinchoninic acid; CE, cholesterol ester; C/EBP, CAAT enhancer-binding protein; DEX, dexamethasone; FFA, free fatty acid; HODE, hydroxyoctadecadienoic acid; HSL, hormone-sensitive lipase; IBMX, 3-isobutyl-2-methylxanthine; ISO, isoproterenol; MEF, mouse embryonic fibroblasts; NCEH, neutral cholesterol ester hydrolase; PIA, (−)-N^6-(2-phenylisopropyl)-adenosine; PKA, protein kinase A; PPAR-γ, peroxisome proliferator–activated receptor-γ; TG, triacylglycerol; TNF, tumor necrosis factor; TZD, thiazolidinedione; WAT, white adipose tissue.

Free fatty acids (FFAs) influence diverse aspects of lipid and carbohydrate metabolism as well as energy balance. In heart and muscle, FFAs are primarily utilized as a source of energy for ATP synthesis through β-oxidation. Uncoupling of this reaction leads to adaptive thermogenesis in brown adipose tissue. Moreover, FFAs may influence both insulin sensitivity in muscle (1) and insulin secretion in pancreas (2). Products of fatty acid metabolism can function as ligands for nuclear receptors, thereby controlling the transcription of genes involved in adipogenesis and lipid metabolism (3,4). Thus, the regulatory mechanisms underlying lipolysis as defined as the mobilization of FFAs from triacylglycerol (TG) stored in adipocytes are important for understanding the pathophysiology of various metabolic disorders such as diabetes, obesity, hyperlipidemia, hypertension, and atherosclerosis.

Lipolysis is under the control of various hormones and cytokines in adipocytes. Lipolytic hormones such as catecholamines and ACTH stimulate cAMP-dependent protein kinase (PKA), which in turn phosphorylates hormone-sensitive lipase (HSL) (5,6) and perilipin (7) in adipocytes. Upon lipolytic stimulation, HSL is translocated from cytosol to fat droplets (8–10); conversely, perilipin is moved from fat droplets to cytosol (9,11).

HSL catalyzes the hydrolysis of cellular tri- and diacylglycerol in adipocytes, heart, skeletal muscles, and pancreatic β-cells (see ref. 12,13 for review), producing monoacylglycerols, which are subsequently hydrolyzed by monoglyceride lipase (14). In addition, HSL hydrolyzes cholesterol esters (CEs) in testes, ovaries, adrenals, and possibly macrophages. In contrast to lipolytic hormones, cytokines such as tumor necrosis factor-α (TNF-α) stimulate lipolysis more slowly (15,16). TNF-α downregulates not only Gi (17) but also perilipin (18), thereby leading to the activation of lipolysis. All of these findings strongly support the belief that lipolysis is primarily catalyzed by HSL.

We (19) and Wang et al. (20) recently generated HSL-deficient (HSL−/−) mice by targeted gene disruption. Unexpectedly, these mice were neither obese nor cold sensitive. Adipocytes isolated from epididymal fat pads of HSL−/− mice consistently showed a substantial increase in lipolysis in response to isoproterenol (ISO), although

the response was lower than that of wild-type adipocytes. These results indicate that HSL is not the only enzyme that catalyzes lipolysis in adipocytes (see ref. 21 for discussion).

To characterize lipolysis further in the absence of HSL, we used mouse embryonic fibroblasts (MEF), which are differentiated into adherent adipocytes (MEF adipocytes) (22), producing a more stable condition than can be obtained using fat pads or isolated adipocytes. Even in the absence of HSL, MEF differentiated to mature adipocytes in which the rate of lipolysis was stimulated in response to both ISO and TNF-α. Furthermore, most of the characteristics of the lipolytic activities in adipocytes were conserved in HSL−/− MEF adipocytes: ISO-stimulated lipolysis was suppressed by H-89 and PKA inhibitor, and TNF-α–stimulated lipolysis was almost completely inhibited by troglitazone, a thiazolidinedione (TZD). HSL−/− MEF adipocytes lacked neutral CE hydrolase (NCEH) activity but retained substantial TG lipase activity. ISO stimulated TG lipase activity in fat cake from HSL−/− adipocytes in a manner indistinguishable from that of wild-type adipocytes. Therefore, common regulatory mechanism may control the activity of distinct lipases.

RESEARCH DESIGN AND METHODS

Materials. Dexamethasone (DEX), 3-isobutyl-2-methylxanthine (IBMX), fatty acid–free BSA, and ISO were purchased from WAKO Pure Chemicals. Insulin, dibutyryl-cAMP, forskolin, triolein, lecithin, sodium taurocholate, adenosine, (−)-N^6-(2-phenylisopropyl)-adenosine (PIA), adenosine deaminase, and collagenase were purchased from Sigma Chemical. β1–24 ACTH (Cortrosyn) was purchased from Daiichi Pharmaceutical. H-89 was purchased from Calbiochem. TNF-α was purchased from Genzyme. BRL35135A and troglitazone were provided by Smith-Kline Beecham and Sankyo Pharmaceutical, respectively. Tri[^{3}H]oleoylglycerol and cholesterol [1-^{14}C]oleate were purchased from NEN Life Science Products.

Preparation of primary MEF and induction of adipocyte differentiation. Primary MEF were harvested from 14.5-day post coitum embryos of wild-type and HSL−/− mice as described (22). Cells were cultured at 37°C in high-glucose Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% (vol/vol) heat-inactivated FCS (JRH Biosciences). Upon reaching confluence, the cells were split into 6-well, 24-well, or 100-mm dishes (IWAKI) and cultured in α-modified Eagle's medium (Gibco/BRL) supplemented with 10% (vol/vol) calf serum (HyClone) to confluence. Two days later, medium was replaced with the standard differentiation induction medium containing 0.5 mmol/l IBMX, 1 μmol/l DEX, 5 μg/ml insulin, and 10% (vol/vol) calf serum. This medium was renewed every other day. For preparation of proteins for Western blot analysis and measurement of enzyme activity and of RNA for Northern blot analysis, cells were harvested at days 0, 2, 5, and 8 of differentiation. For analyses of cellular TG contents and lipolysis, cells were harvested at day 8 of differentiation.

Northern blot analysis. Fifteen micrograms of total RNA isolated from cultured cells plated to a 100-mm dish by TRIZOL reagent (Gibco/BRL) was used for Northern blot analysis as described (19). Probes were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from mouse white adipose tissue (WAT) as a template with the following sets of primers: murine adipocytes fatty acid–binding protein (aP2) sense, 5′-TGTGTGATGCCTTTGTGGGAAC-3′, antisense, 5′-TGGAAGTCACGCCTTTCATAAC-3′; murine CAAT enhancer-binding protein (C/EBP)-α sense, 5′-GAGCACGAGACGTCTATAGACA-3′, antisense, 5′-AGTGCGCGATCTGGAACTGCAA-3′; murine C/EBP-δ sense, 5′-CATCGACTTCAGCGCCTACATT-3′, antisense, 5′-GCTTCTGCTGCATCTCCTGGTT-3′; murine peroxisome proliferator–activated receptor-γ (PPAR-γ) sense, 5′-TTACCATGGTTGACACAGAG-3′, antisense, 5′-AAATTCGGATGGCCACCTCT-3′; and HSL (exon 8 probe) (19).

Western blot analysis. Cells were homogenized in buffer A (50 mmol/l Tris HCl [pH 7.0], 250 mmol/l sucrose, 1 mmol/l EDTA, 2 μg/ml leupeptin) and centrifuged at 100,000*g* for 45 min at 4°C. The supernatant was used for Western blot analysis as previously described (19). Anti-HSL antibody was raised by immunizing rabbits with glutathione S-transferase-fusion protein containing amino acid sequence 399–599 of mouse HSL.

Lipolysis assays. MEF adipocytes cultured in 24-well plates were incubated in α-modified Eagle's medium with 2% (wt/vol) fatty acid–free BSA in the absence of serum. After 12 h, cells were treated with various agents to induce lipolysis, and concentration of glycerol and FFA in the media were determined by the Triglyceride-G kit and the NEFA-C kit, respectively (WAKO Pure Chemicals). Cellular protein contents were measured by bicinchoninic acid (BCA) protein assay (Pierce) after solubilization in 0.1 N NaOH. Cellular TG contents were measured by the Triglyceride-G kit (WAKO Pure Chemicals), after extraction by hexane/isopropanol.

Assays for NCEH and TG lipase activity. MEF adipocytes were homogenized in buffer A and centrifuged at 100,000*g* for 45 min at 4°C. The supernatant was used for the enzyme assay. TG lipase activity was measured as described previously (19). In brief, the samples were incubated at 37°C for 30 min in a final volume of 200 μl of a reaction mixture containing 105 μmol/l tri[^{3}H]oleoylglycerol (99.4 μCi/μmol), 23.7 μmol/l lecithin, 12.5 μmol/l sodium taurocholate, 1 mol/l NaCl, and 85 mmol/l potassium phosphate (pH 7.0). The high concentration of NaCl was included to inactivate lipoprotein lipase, which is the major TG lipase expressed by adipocytes. NCEH activity was measured essentially as described previously (19), using a reaction mixture containing 6.14 μmol/l cholesterol [1-^{14}C]oleate (48.8 μCi/μmol).

Preparation of fat cake from isolated adipocytes. Adipocytes were isolated from parametrial fat pads of female mice (20–28 weeks old) by using collagenase digestion as described previously (19). Manipulation of adipocytes was performed in KRBH buffer (129.4 mmol/l NaCl, 5.2 mmol/l KCl, 1.3 mmol/l KH_2PO_4, 2.7 mmol/l $CaCl_2$, 1.3 mmol/l $MgSO_4$, 24.8 mmol/l $NaHCO_3$, 10 mmol/l HEPES [pH 7.4]), supplemented with 3% (wt/vol) BSA, 2 mmol/l glucose, 200 nmol/l adenosine, and 1 mg/ml collagenase. After digestion, adipocytes were washed in KRBH buffer supplemented with 2 mmol/l glucose and 200 nmol/l adenosine, but without BSA. Aliquots (400 μl) of adipocytes at ~25% packed cell volume were incubated in KRBH buffer containing 2 mmol/l glucose, 10 μmol/l PIA, 1 unit/ml adenosine deaminase, with or without 1 μmol/l ISO for 15 min at 37°C. The incubation medium was removed from below the floating cells to determine the concentration of glycerol by the Triglyceride-G kit. The remaining cells were lysed in 200 μl of ice-cold buffer A supplemented with 2 μg/ml aprotinin, 0.4 mmol/l benzamidine, 25 μg/ml soybean trypsin inhibitor, and 50 mmol/l NaF (buffer A^+). Cells were homogenized and centrifuged at 2,700*g* at 4°C for 40 min, as described previously with minor modifications (9).

Cytosolic fraction was harvested from below the fat cake fraction and centrifuged at 2,700*g* at 4°C for 40 min to remove any contamination of fat cake fraction. Cytosolic fractions were used for TG lipase activity assay and for the determination of protein concentration by BCA protein assay.

The remaining fat cake fraction was washed in 400 μl of buffer A^+ and centrifuged at 2,700*g* at 4°C for 40 min, and any contaminating cytosolic fraction was aspirated and discarded. The remaining fat cake fraction was suspended in 1.6 ml of ice-cold acetone and incubated at −20°C overnight, and insoluble fraction was collected as a precipitate by centrifugation at 1,700*g* for 15 min at 4°C. The precipitate was washed again in 0.8 ml of ice-cold acetone and centrifuged at 1,700*g* for 15 min at 4°C. The precipitate was then washed in 0.8 ml of ice-cold diethyl ether and centrifuged at 1,700*g* for 15 min at 4°C. The precipitate was dissolved and sonicated in buffer A^+, and insoluble residues were removed by centrifugation at 5,000*g* for 10 min at 4°C. The supernatant was designated as fat cake fraction and used for TG lipase activity assay and for the determination of protein concentration by BCA protein assay.

Statistical analysis. Means were compared by Student's t test, unless otherwise stated. Differences were considered significant at $P < 0.05$. Results are expressed as the means ± SE. All calculations were performed with STAT view version 5.0 for Macintosh (SAS Institute).

RESULTS

To determine the role of HSL in adipocyte differentiation, we prepared primary MEF from wild-type (+/+) and HSL−/− mice and treated them with standard differentiation induction medium when they reached confluence. Upon microscopic examination, accumulation of numerous lipid droplets was observed 5 days after differentiation in both wild-type and HSL−/− MEF adipocytes. At day 8 of differentiation, the cellular TG content was not different between wild-type and HSL−/− MEF adipocytes (Fig. 1*A*). We performed Northern blot analyses to examine the expression of adipocyte specific genes (aP2, PPAR-γ, C/EBP-α, C/EBP-δ, and HSL) during the course of differentiation (Fig. 1*B*). At days 0 and 2, the expression levels of aP2, PPAR-γ, and C/EBP-α were barely detectable. At

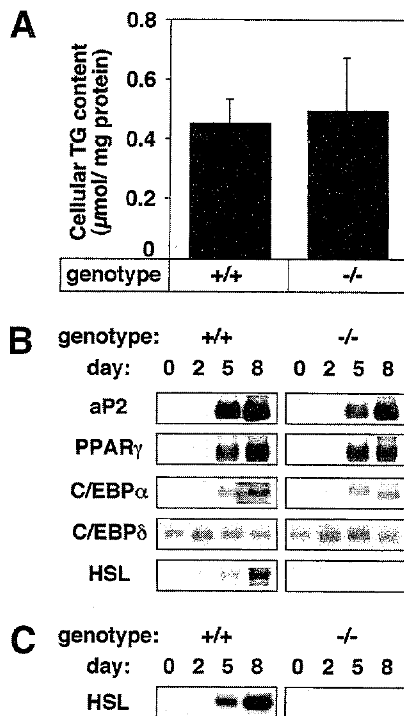

FIG. 1. Adipocyte differentiation in primary MEF. MEF were prepared from wild-type (+/+) and HSL−/− mice. Lipids, RNA, and proteins were extracted from MEF 0, 2, 5, and 8 days after the incubation with the differentiation induction medium. *A*: Cellular triglyceride content at day 8 of differentiation. Data are expressed as means ± SE (n = 5). +/+ versus −/−, $P > 0.05$. *B*: Northern blot analysis for the expression of adipocyte-specific genes. Fifteen micrograms of total RNA was subjected to Northern blot analyses using probes as indicated. *C*: Western blot analysis for HSL.

days 5 and 8, the expression of aP2, PPAR-γ, and C/EBP-α were increased in a time-dependent manner. This indicates a completely mature state of MEF adipocytes at day 8 of differentiation. C/EBP-δ was expressed earlier than aP2, PPAR-γ, and C/EBP-α, which is consistent with a previous report (23). There was no difference in the expression levels of these genes between wild-type and HSL−/− MEF adipocytes. However, HSL was expressed only in wild-type cells at days 5 and 8, confirming that HSL−/− MEF adipocytes lacked HSL gene expression. We also confirmed the lack of HSL protein in HSL−/− MEF adipocytes by Western blot analysis (Fig. 1*C*). Therefore, the absence of HSL does not alter the differentiation property of MEF adipocytes.

To estimate the rate of lipolysis, we measured glycerol and FFA release. Glycerol release was increased linearly up to 60 min after the addition of 100 nmol/l ISO in both wild-type and HSL−/− MEF adipocytes (Fig. 2*A* and *B*). Therefore, we measured the rate of lipolysis during the initial 60 min, with and without 100 nmol/l ISO, and compared these values between wild-type and HSL−/− MEF adipocytes (Fig. 2*C* and *D*). The basal rate of lipolysis was low in both wild-type and HSL−/− MEF adipocytes. ISO markedly increased both glycerol and FFA release in wild-type and HSL−/− cells above nonstimulated basal levels (glycerol 6.5-fold vs. 8.0-fold; FFA 22.7-fold vs. 21.4-fold). The absolute rate of glycerol release stimulated by ISO in HSL−/− MEF adipocytes was 2.2-fold lower than that in wild-type cells ($P < 0.05$). Similar stimulation of the rate of lipolysis was observed when 1 mmol/l dibutyryl-cAMP (cAMP analogue), 100 µmol/l forskolin (AC activator), 100 nmol/l BRL35135A (β_3-adrenergic receptor selective agonist), or 1 µmol/l ACTH was added to the incubation media in both wild-type and HSL−/− MEF adipocytes, although HSL−/− MEF adipocytes exhibited reduced responses to these agents compared with wild-type MEF adipocytes (data not shown).

To determine whether ISO-stimulated lipolysis occurs via PKA activation, we measured glycerol and FFA release

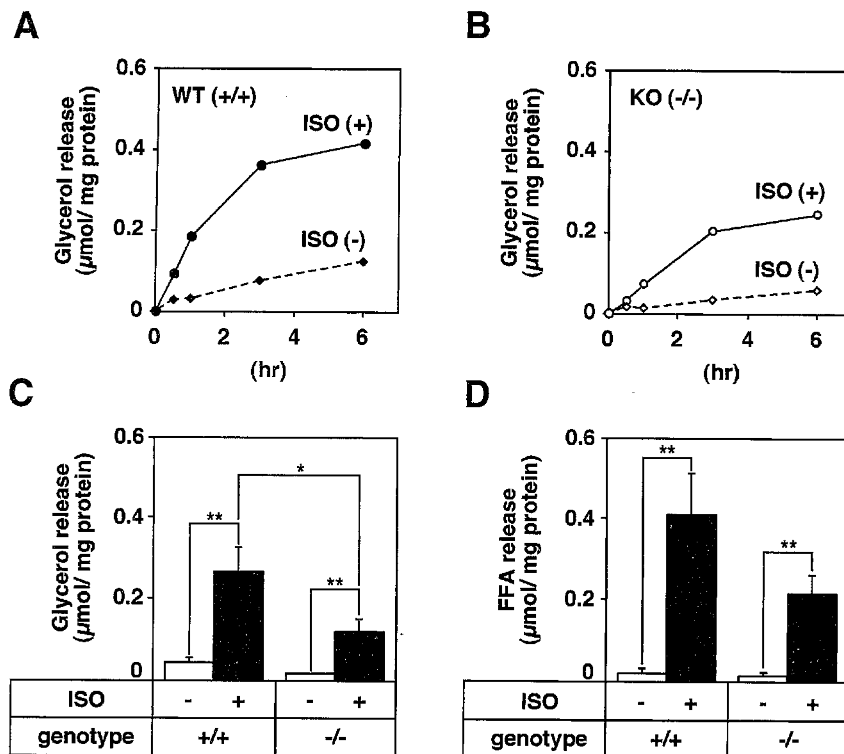

FIG. 2. ISO-stimulated lipolysis in MEF adipocytes. *A* and *B*: Time course of glycerol release from wild-type (*A*) and HSL−/− (*B*) MEF adipocytes incubated with or without 100 nmol/l ISO. Data are means of littermates (n = 2). *C* and *D*: Effect of ISO on the lipolysis in wild-type (+/+) and HSL−/− MEF adipocytes (−/−). MEF adipocytes were treated with or without 100 nmol/l ISO for 1 h, and glycerol (*C*) and FFA (*D*) in the media were measured. Data are expressed as means ± SE (n = 6–7). *$P < 0.05$; **$P < 0.01$.

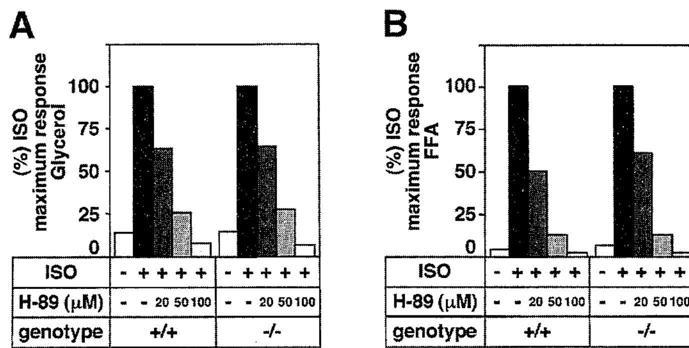

FIG. 3. Effect of H-89 on ISO-stimulated lipolysis. MEF adipocytes were pretreated with 0, 20, 50, and 100 μmol/l of H-89 for 1 h. Thereafter, 100 nmol/l ISO was added to the medium and incubated for an additional 1 h. Medium was collected and assayed for glycerol (*A*) and FFA (*B*) content. Data are means of littermates (n = 2) and are represented as the percentage of the values of ISO-treated, non–H-89–treated cells.

in the incubation medium with and without the addition of H-89, a specific cell-permeable inhibitor of PKA. H-89 inhibited the ISO-stimulated lipolysis in both wild-type and HSL−/− MEF adipocytes in a concentration-dependent manner (Fig. 3). Mild inhibition was observed at a concentration of 20 μmol/l, whereas near complete inhibition was observed at a concentration of 100 μmol/l. However, troglitazone did not suppress ISO-stimulated lipolysis even at 100 μmol/l (data not shown), which is consistent with a previous report (18).

Glycerol release was also increased linearly up to 24 h after the addition of 10 ng/ml TNF-α in both wild-type and HSL−/− MEF adipocytes (Fig. 4*A* and *B*). This stimulation was much slower than that by ISO, taking at least 12 h to have detectable effects. Therefore, we measured the rate of lipolysis during the initial 24 h, with and without 10 ng/ml TNF-α, and compared these values between wild-type and HSL−/− MEF adipocytes (Fig. 4*C* and *D*). TNF-α moderately increased glycerol release in both wild-type and HSL−/− MEF adipocytes by 29 and 66%, respectively ($P < 0.05$), whereas it increased FFA release more profoundly in both wild-type (3.7-fold; $P < 0.05$) and HSL−/− MEF adipocytes (5.9-fold; $P < 0.01$).

Troglitazone blocked TNF-α–stimulated lipolysis in both wild-type and HSL−/− MEF adipocytes in a concentration-dependent manner (Fig. 5). Mild inhibition was observed at a concentration of 1 μmol/l, whereas near complete inhibition was observed at a concentration of 100 μmol/l. In contrast, H-89 did not significantly inhibit the TNF-α–stimulated lipolysis (data not shown).

The lower rate of lipolysis in HSL−/− MEF adipocytes compared with wild-type MEF adipocytes was considered to be attributed to the difference in the amounts of intracellular TG lipase activity. To test this idea, we measured NCEH and TG lipase activity directly in wild-type and HSL−/− MEF adipocytes during differentiation (Fig. 6*A* and *B*). In wild-type cells, both NCEH and TG lipase activity was increased upon differentiation. In HSL−/− MEF adipocytes, however, only TG lipase activity was increased without any detectable change in NCEH activity. At day 8 after the differentiation, HSL−/− MEF adipocytes expressed negligible NCEH activity, suggesting that HSL was completely disrupted ($P < 0.01$; Fig. 6*C*). Treatment with 100 nmol/l ISO did not change NCEH activity in wild-type MEF adipocytes (Fig. 6*C*). However, HSL−/− MEF adipocytes expressed substantial TG lipase activity, which was significantly lower than that of wild-type cells (2.2-fold; $P < 0.01$; Fig. 6*D*). Treatment with 100

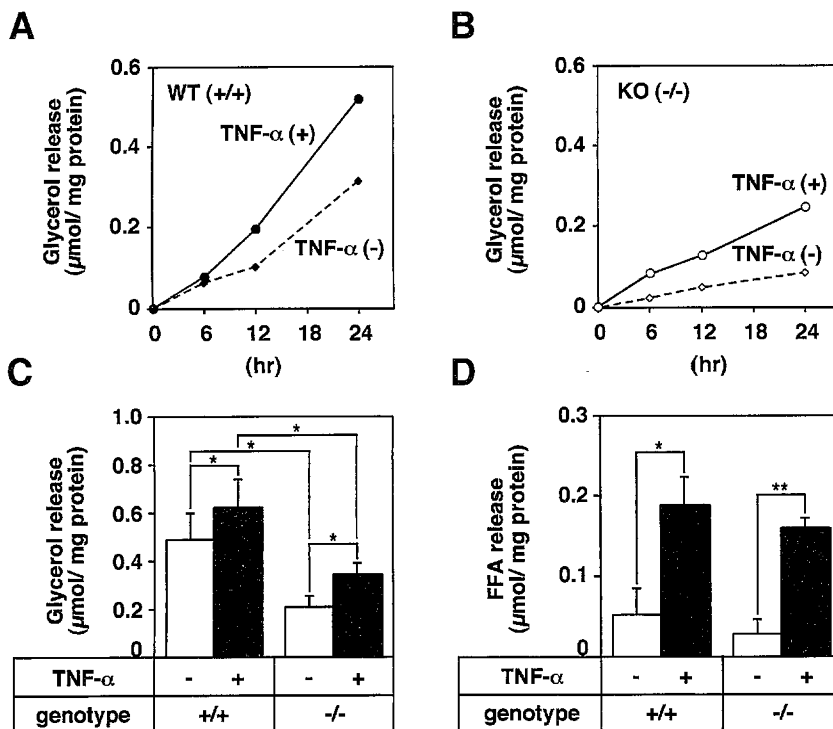

FIG. 4. TNF-α-stimulated lipolysis in MEF adipocytes. *A* and *B*: Time course of glycerol release from wild-type (*A*) and HSL−/− MEF adipocytes (*B*) incubated with or without 10 ng/ml TNF-α. *C* and *D*: Effect of TNF-α on the lipolysis in wild-type (+/+) and HSL−/− MEF adipocytes (−/−). MEF adipocytes were treated with or without 10 ng/ml TNF-α for 24 h, after which glycerol (*C*) and FFA (*D*) in the medium were measured. Data are expressed as means ± SE (n = 6–7). *$P < 0.05$; **$P < 0.01$.

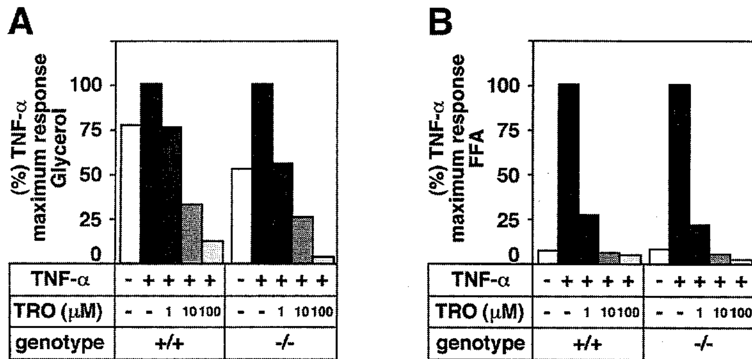

FIG. 5. Effect of troglitazone on TNF-α–stimulated lipolysis. MEF adipocytes were pretreated with 0, 1, 10, or 100 μmol/l troglitazone for 1 h. Thereafter, 10 ng/ml TNF-α was added to the medium and incubated for an additional 24 h. Medium was collected and assayed for glycerol (*A*) and FFA (*B*) content. Data are means of littermates (n = 2) and are represented as the percentage of the values of TNF-α–stimulated, non–troglitazone-treated cells.

nmol/l ISO decreased TG lipase activity in wild-type and HSL−/− MEF adipocytes by 45 and 78%, respectively ($P <$ 0.01; Fig. 6*D*), suggesting that both HSL and the non-HSL TG lipase were translocated from cytosol to fat cake.

Because fat cakes of MEF adipocytes were too small to recover detectable TG lipase activity, we used isolated adipocytes to further determine whether the TG lipase activity was increased in fat cake and decreased in cytosol in response to lipolytic stimulation. We measured TG lipase activity associated with fat cake and cytosolic fraction of adipocytes with or without stimulation by 1 μmol/l ISO (Fig. 7). Fat cake from HSL−/− adipocytes contained substantial amounts of TG lipase activity, which was 82% of that of wild-type fat cake (Fig. 7*B*). Treatment with ISO increased the TG lipase activity in wild-type and HSL−/− fat cakes by 30.1 and 17.0%, respectively (wild-type $P < 0.05$ [n = 7]; HSL−/− $P = 0.11$ [n = 10]). These values for the magnitude of the stimulation were smaller than that of the glycerol release observed in isolated adipocytes from both wild-type (sixfold) and HSL−/− mice (twofold; Fig. 7*A*). To further confirm the translocation of both HSL and the non-HSL TG lipase from cytosol to fat cake, we measured TG lipase activity in cytosolic fraction of adipocytes with or without stimulation by 1 μmol/l ISO (Fig. 7*C*). The cytosolic TG lipase activity was significantly decreased both in wild-type and HSL−/− adipocytes by 11.1 and 15.7%, respectively (wild-type $P <$ 0.05 [n = 7]; HSL−/− $P < 0.05$ [n = 10]).

DISCUSSION

In the present study, we have shown that the absence of HSL did not have profound effects on the ability of MEF to differentiate into mature adipocytes in response to the differentiation induction medium. We have further confirmed that lipolytic hormones markedly stimulated the rate of lipolysis via PKA-dependent pathway even in the absence of HSL. Furthermore, HSL−/− MEF adipocytes retained substantial basal and TNF-α–stimulated activity of lipolysis that was markedly inhibited by troglitazone. Because extracts from HSL−/− MEF adipocytes hydrolyzed TG but not CE, lipolysis is conceivably mediated by the non-HSL TG-specific lipase in these cells. The non-HSL TG lipase activity was increased in fat cake in response to

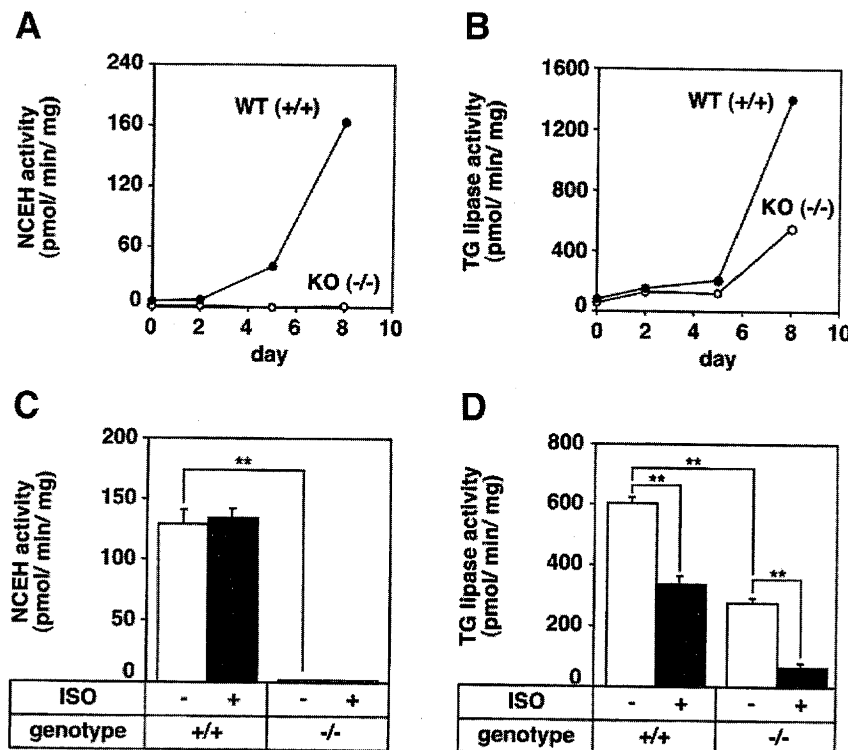

FIG. 6. NCEH and TG lipase activity in HSL−/− MEF adipocytes. NCEH (*A*) and TG lipase activity (*B*) were measured in wild-type (+/+) and HSL−/− (−/−) MEF adipocytes on 0, 2, 5, and 8 days during differentiation. NCEH (*C*) and TG lipase activity (*D*) in wild-type (+/+) and HSL−/− MEF adipocytes (−/−), incubated with or without 100 nmol/l ISO, at day 8 of differentiation. Data are expressed as means ± SE (n = 3–4). $^{**}P < 0.01$.

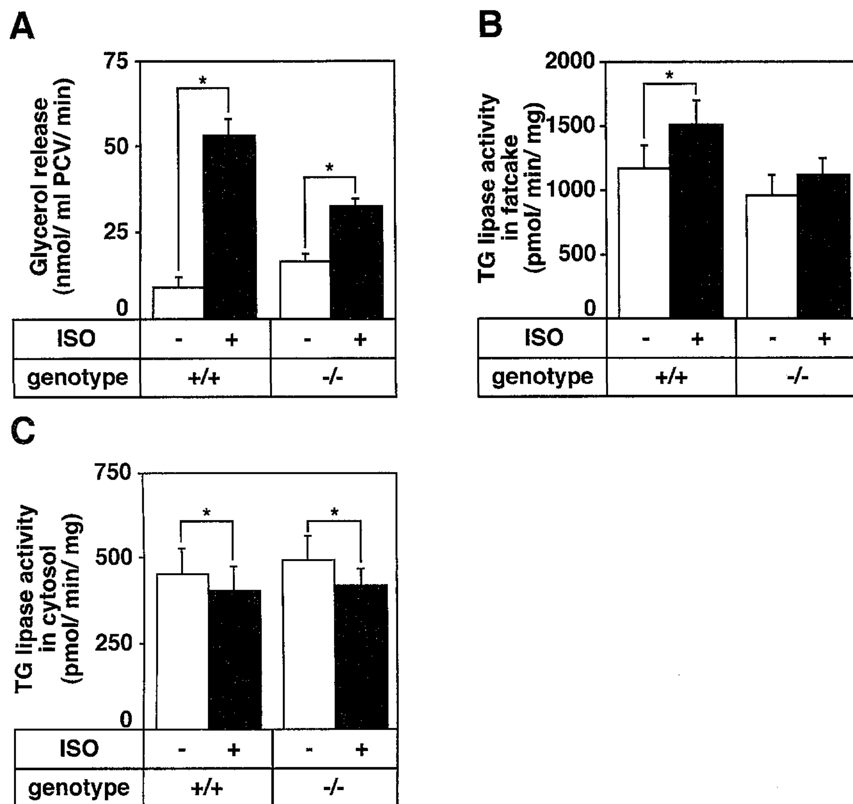

FIG. 7. Effects of ISO on lipolysis and TG lipase activity in fat cake and cytosol of adipocytes isolated from wild-type and HSL−/− mice. *A*: Glycerol release from isolated adipocytes of wild-type (+/+; n = 7) and HSL−/− mice (−/−; n = 10) incubated with or without stimulation by 1 μmol/l ISO for 15 min. *P < 0.05. *B* and *C*: TG lipase activity in fat cake (*B*) and cytosol (*C*) of isolated adipocytes of wild-type (+/+; n = 7) and HSL−/− mice (−/−; n = 10) with or without stimulation by 1 μmol/l ISO for 15 min. *P < 0.05 (paired t test).

ISO. These results strongly suggest that HSL and the non-HSL TG lipase share a common regulatory mechanism of lipolysis.

Adipocyte differentiation is a complex process involving a cascade of expression of many transcription factors and adipocyte-specific genes: C/EBP-α, -β, -δ; PPAR-γ; adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1; and others (23,24). It is known that PPAR-γ is a nuclear receptor that binds a variety of metabolites of fatty acids (25). For example, 15-deoxy prostaglandin J2, a product of arachidonic acid, and 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE, products of linoleic acid (26), have been shown to strongly transactivate PPAR-γ. Because HSL mediates the mobilization of fatty acids by the hydrolysis of tri-, di-, and monoacylglycerol as well as CE, its absence might be expected to affect adipocyte differentiation; however, our studies demonstrate that HSL is not essential for adipocyte differentiation (Fig. 1).

We have previously shown that adipocytes in both WAT and brown adipose tissue of HSL−/− are hypertrophic as a result of increased TG accumulation (19). In contrast, the cellular TG content of HSL−/− MEF adipocytes was not different from that of wild-type cells (Fig. 1*A*). One potential explanation for this discrepancy is that unphysiological concentration of insulin and DEX in the differentiation induction media may have masked subtle metabolic differences in MEF adipocytes. It is also possible that it takes a longer time to develop adipocyte hypertrophy in mice than in in vitro cell culture experiments. In this context, it is noteworthy that 3T3-F442A cells, which overexpressed HSL, are depleted of fats and are defective in differentiation as indicated by reduced expression of the late markers of adipocyte differentiation, aP2, and glycerol-3-phosphate dehydrogenase (27).

HSL−/− MEF adipocytes retained significant lipolytic activities that were stimulated by ISO (Fig. 2). This observation is in agreement with our previous findings that adipocytes isolated from WAT of HSL−/− mice showed release of glycerol and FFA in response to ISO (19). Furthermore, these HSL−/− MEF adipocytes expressed TG lipase activity but not NCEH activity (Fig. 6). Aside from HSL, monoglyceride lipase is the only known intracellular lipase in adipocytes, and this enzyme is not considered to be hormone sensitive (28). These results strongly indicate that another unknown intracellular TG specific lipase(s) is responsible for the residual hormone-sensitive lipolysis.

Catecholamines and other lipolytic hormones stimulate lipolysis by activating HSL catalytic activities, at least in part, through increasing its reversible phosphorylation (29). Indeed, PKA can phosphorylate HSL on serines 563, 659, and 660 (30,31). Site-directed mutagenesis of Ser 563 resulted in loss of activation of HSL, as measured by the hydrolysis of CE and tri- and diacylglycerol (32). However, activation of HSL by phosphorylation cannot explain all of the lipolytic activity stimulated by catecholamine in intact cells (33–36). In agreement with these previous reports, treatment with ISO paradoxically decreased the TG lipase activity in the supernatant fraction prepared by ultracentrifugation from MEF adipocytes (Fig. 6*D*) and isolated adipocytes (Fig. 7*C*), supporting the notion that the lipolytic enzymes were redistributed from cytosol to lipid droplets as originally proposed by Egan et al. (8). Consistently, TG lipase activity was increased in fat cake from wild-type and HSL−/− adipocytes upon stimulation by

ISO, although the increase was not statistically significant in HSL−/− adipocytes (Fig. 7*B*). It is surprising, however, that the increases in the TG lipase activity in fat cake were not as remarkable as these observed in lipolysis in both genotypes; the 1.2- to 1.3-fold increases in TG lipase activity was not sufficient to explain the 2- to 6-fold increases in lipolysis upon stimulation with ISO. One possible explanation for the difference is that our procedure to isolate fat cake was so mild that the lipases that were not tightly associated with fat droplets were recovered in fat cake fraction, thus mitigating the difference. In this context, it is of note that Clifford et al. (9), who used the same procedure as ours, found significant translocation of HSL to fat cake in young rats (180–220 g) but not in aged rats (230–280 g). Our mice (20–28 weeks old) might not be as young as the rats of 180–220 g in weight. Alternatively, the discrepancy between the lipolytic stimulation and translocation can be accounted for by the involvement of other regulatory mechanisms. For example, redistribution of perilipin contributes to the hormonally stimulated lipolysis (9), and changes in association of the lipolytic enzymes with novel docking molecules such as lipotransin (37) or adipocyte lipid binding protein (38) may play another contributory role. These considerations are largely compatible with the "hormone-sensitive substrate theory" of lipolysis as proposed by Okuda et al. (39): hormones do not act on the lipase but on the endogenous lipid substrate.

Finally, troglitazone completely suppressed the TNF-α–stimulated lipolysis irrespective of the presence of HSL (Fig. 5), indicating that troglitazone inhibits both HSL-mediated and the non-HSL TG lipase-mediated lipolysis. TNF-α is, at least partly, responsible for insulin resistance and impaired glucose tolerance (40). TZDs alleviate insulin resistance by decreasing the expression of TNF-α (41) as well as by reversing the inhibition of insulin signaling by TNF-α (42). In adipocytes, TNF-α causes net depletion of the TG store by decreasing the activity of lipoprotein lipase as well as by stimulating lipolysis (15), which is directly inhibited by TZDs (18). Thus, the non-HSL intracellular TG lipase(s) may be a key enzyme through which TNF-α stimulates lipolysis.

In conclusion, both ISO and TNF-α stimulated lipolysis even in HSL−/− MEF adipocytes. The residual lipolytic activity was mediated by another lipase that specifically hydrolyzed TG. ISO-stimulated lipolytic activation may involve, at least partly, translocation of the TG lipase from cytosol to fat cake. Thus, the TG lipase shares a common regulatory mechanism with HSL. This novel finding has a potentially significant impact on the view of the mechanism of lipolysis. Additional biochemical and molecular characterization of this TG lipase is needed for understanding the physiology of lipolysis and its relevance to various diseases such as diabetes and obesity.

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