Absence of Hormone-sensitive Lipase Inhibits Obesity and Adipogenesis in Lep<sup>ob/ob</sup> Mice*

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Hormone-sensitive lipase (HSL) plays a crucial role in the hydrolysis of triacylglycerol and cholesteryl ester in various tissues including adipose tissues. To explore the role of HSL in the metabolism of fat and carbohydrate, we have generated mice lacking both leptin and HSL (Lep<sup>ob/ob</sup>/HSL<sup>−/−</sup>) by cross-breeding HSL<sup>−/−</sup> mice with genetically obese Lep<sup>ob/ob</sup> mice. Unexpectedly, Lep<sup>ob/ob</sup>/HSL<sup>−/−</sup> mice ate less food, gained less weight, and had lower adiposity than Lep<sup>ob/ob</sup>/HSL<sup>+/+</sup> mice. Lep<sup>ob/ob</sup>/HSL<sup>−/−</sup> mice had massive accumulation of preadipocytes in white adipose tissues with increased expression of preadipocyte-specific genes (CAAT/enhancer-binding protein β and adipose differentiation-related protein) and decreased expression of genes characteristic of mature adipocytes (CCAAT/enhancer-binding protein α, peroxisome proliferator activator receptor γ, and adipocyte determination and differentiation factor 1/sterol regulatory element-binding protein-1). Consistent with the reduced food intake, hypothalamic expression of neuropeptide Y and agouti-related peptide was decreased. Since HSL is expressed in hypothalamus, we speculate that defective generation of free fatty acids in the hypothalamus due to the absence of HSL mediates the altered expression of these orexigenic neuropeptides. Thus, deficiency of both leptin and HSL has unmasked novel roles of HSL in adipogenesis as well as in feeding behavior.

Excessive cellular accumulation of neutral lipids underlies many diseases such as obesity, type 2 diabetes, and atherosclerosis, all of which are epidemic in industrialized countries. Therefore, elucidating the metabolic pathways that degrade excessive neutral lipids is of extreme importance in the prevention of the diseases caused by lipotoxicity (1).

Hormone-sensitive lipase (HSL)<sup>2</sup> is an intracellular neutral lipase that catalyzes the hydrolysis of cellular triglycerols (TG), diglycerols (DG), monoacylglycerols, and cholesteryl esters as well as other lipids (2, 3). HSL is expressed in a wide variety of organs and cells, including adipose tissues, heart, skeletal muscle, adrenal glands, testes, ovaries, and pancreatic β-cells, and is under neural and hormonal control.

Several laboratories including ours have generated HSL-deficient (HSL<sup>−/−</sup>) mice by targeted gene disruption (4–6). Unexpectedly, these mice showed male sterility due to the failure of spermatogenesis (4, 7). Although HSL<sup>−/−</sup> mice had a decreased ability to release free fatty acids (FFA) from adipocytes in response to the β-adrenergic stimulation both in vivo and in vitro, they were neither obese nor cold-sensitive (4, 5). The attenuated development of adipocyte-associated phenotypes may result from the presence of a residual TG lipase activity in adipocytes, which is induced by both β-adrenergic agonist and tumor necrosis factor-α (8). However, it is unknown how HSL deficiency affects adiposity in the setting of obesity, which is commonly associated with type 2 diabetes.

Here we show that mice lacking both HSL and leptin (Lep<sup>ob/ob</sup>/HSL<sup>−/−</sup>) show impaired adipogenesis and paradoxical resistance to obesity primarily due to the reduced food intake. These observations in the leptin-deficient state have revealed new functions of HSL in adipogenesis and feeding behavior.

EXPERIMENTAL PROCEDURES

Animals—HSL<sup>−/−</sup> mice (4), which were backcrossed five times into the C57BL/6J background, were intercrossed with mice heterozygous for leptin deficiency (Lep<sup>ob/ob</sup>/C57BL/6J; Jackson Laboratories, Bar Harbor, ME) to generate double heterozygotes, which were then interbred to produce Lep<sup>ob/ob</sup>/HSL<sup>−/−</sup> mice. Genotyping was performed as described previously (4, 9). Mice were housed in a temperature-controlled environment with a 12-h light/dark cycle and allowed free access to water and a standard chow diet (Oriental MF, Oriental Yeast, Tokyo, Japan). Mice (16 weeks) were sacrificed after a 6-h fast unless otherwise stated. Mice were sacrificed at the end of the light cycle to isolate hypothalamic RNA. All experiments were performed in accord with institutional guidelines.

Biochemical Analyses—Blood was collected from the retro-orbital venous plexus after a 6-h fast. Cholesterol (Determinate TC; Kyowa Medex, Tokyo, Japan), TG and glycerol (TG LH; Wako Chemicals,

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The abbreviations used are: HSL, hormone-sensitive lipase; TG, triglyceride(s); DG, diglyceride(s); FFA, free fatty acid(s); WAT, white adipose tissue; ACTH, adrenocorticotropic hormone.
Tokyo, Japan), and FFA (NEFA C; Wako Chemicals) were measured enzymatically. Plasma glucose was measured by ANTSENSE II (Bayer Medical, Tokyo, Japan); plasma insulin was measured by the mouse insulin ELISA kit (Morinaga, Tokyo, Japan); and plasma corticosterone was measured by the mouse corticosterone kit (Pierc, Tokyo, Japan), and FFA (NEFA C; Wako Chemicals) were measured previously (4). Proliferating cells were detected by bromodeoxyuridine immunostaining. Two h before sacrifice, bromodeoxyuridine (Sigma) was administered intraperitoneally at 50 mg/kg body weight in phosphate-buffered saline. After fixation with 10% neutral buffered formalin, mouse epididymal fat was embedded in paraffin. Immunostaining was performed by a bromodeoxyuridine In-Situ Detection Kit (PharMingen, San Diego, CA) and counterstained with Mayer's hematoxylin (Dako, Carpinteria, CA). Loading was normalized by the expression of 36B4.

**Histology**—Sixteen-week-old mice were sacrificed by decapitation. Tissues were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin. Cell size was estimated by NIH Image. After blocking with 0.5% goat serum, sections of epididymal fat pads were incubated with anti-mouse S-100 antibody (Sigma) overnight and were incubated with an anti-rabbit secondary antibody followed by staining with the avidin-biotin complex complex-alkaline phosphatase method and counterstaining with Harris's hematoxylin. Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling using an in situ apoptosis detection kit (Takara Biomedicals Otsu, Japan) according to the manufacturer's instructions, with counterstaining with methyl green (Wako Chemicals). Proliferating cells were detected by bromodeoxyuridine immunostaining. Two h before sacrifice, bromodeoxyuridine (Sigma) was administered intraperitoneally at 50 mg/kg body weight in phosphate-buffered saline. After fixation with 10% neutral buffered formalin, mouse epididymal fat was embedded in paraffin. Immunostaining was performed by a bromodeoxyuridine In-Situ Detection Kit (PharMingen, San Diego, CA) and counterstained with Mayer's hematoxylin (Wako Chemicals).

**Detection of DNA Ladder**—Adipose tissue DNA was extracted as described previously (4). Five μg of DNA end-labeled with [α-32P]dCTP by Klenow was subjected to electrophoresis in a 1.5% agarose gel and then transferred to nylon membranes, as described previously (11). As a control, mouse thymocyte apoptotic DNA was loaded. In brief, mouse thymus was ground over a mesh, and the isolated thymocytes were induced to undergo apoptosis by culturing in RPMI containing 10% calf serum and 1 μM dexamethasone at 37°C for 6 h.

**Food Intake and Body Temperature**—Food intake and body weight were measured for 3 days after the mice were individually housed and adapted for 1 week. Feeding efficiency was defined as an increase in body weight divided by food intake during 3 days (n = 8–10). Body temperature was measured by an NK-YSI precision N550 thermometer and its probe (Nikkiso-YSI, Japan). These experiments were performed at the end of light cycle at the age of 14 weeks.

**Oxygen Consumption**—After a 1-day acclimation period, oxygen and bicarbonate expired by mice (14 weeks old) were measured every 7 min for 24 h by a calorimetric system (Alco System model, Chiba Japan). Oxygen consumption was normalized by body weight raised to the 0.7

FIG. 1. HSL−/− mice are resistant to genetic obesity. a, body weight of each mouse was measured weekly from 4 to 16 weeks (n = 10). ○, Lep−/−/HSL−/−; □, Lep+/−/HSL−/−; ◯, Lep+/+/HSL−/−; ●, Lep+/−/HSL+/−; ▪, Lep+/+/HSL+/−. b, tissue weights of each mouse were measured at 16 weeks of age after a 6-h fast (n = 10). L, liver; B, brown adipose tissue; H, heart; P, pancreas; Pg, paraganglal WAT; Pr, perirenal WAT; Sc, subcutaneous WAT; Q, quadriceps; EDL, extensor digitorum longus. Each value represents the mean ± S.E. * and **, significance at p < 0.05 and p < 0.01, respectively, versus HSL+/− mice in the same Lep background.
power, which is proportional to body surface area.

Statistics—Statistical differences between groups were analyzed by one-way analysis of variance and a post hoc Tukey-Kramer test, unless otherwise stated.

RESULTS

HSL Deficiency Ameliorates Obesity in Lepob/ob Background—In the Lep+/- background, there was no difference in body weight among HSL+/+, HSL-/-, and HSL-/-mice on a normal chow diet (Fig. 1a). In the Lepob/ob background, however, body weight of doubly homozygous mice was reduced by 26% compared with the Lepob/ob/HSL-/- mice at the age of 16 weeks (Fig. 1a). The difference was discernible at the age of 11 weeks in males and 9 weeks in females. Furthermore, the age-dependent weight gain was negligible in Lepob/ob/HSL-/- mice after 10 weeks of age.

At the age of 16 weeks, the weight of WAT in three different regions was selectively reduced in Lepob/ob mice compared with Lepob/ob/HSL-/- mice; paragonadal, perirenal, and subcutaneous WAT were reduced by 58, 51, and 44%, respectively (Fig. 1b).

HSL-/- Mice Have an Increased Number of Preadipocyte-like Cells in WAT—In the Lep+/- background, WAT from HSL-/-mice contained clusters of small cells devoid of lipids in addition to lipid-filled mature adipocytes (Fig. 2a), as we reported previously (4). In the Lepob/ob background, the small cells, which appeared slightly increased in Lepob/ob/HSL-/- mice, were robustly increased in Lepob/ob/HSL-/- mice (Fig. 2, a and b).

To determine the identity of these small cells, we performed immunohistochemistry for S-100 protein, which is used as a marker of adipocytes (12), particularly of preadipocytes, and neural cells (Fig. 2c). The small cells were strongly positive for S-100, indicating that they were preadipocytes. Upon Northern blot analyses of WAT (Fig. 3a), the expression of adipose differentiation-related protein (13) and CAAT/enhancer-binding protein β, which is dominant during the early stage of differentiation of adipocytes (14), was increased in HSL-/-WAT compared with HSL+/+WAT in both Lep+/- and Lepob/ob backgrounds. On the other hand, the expression of CAAT/enhancer-binding protein α, which is dominant during the late stage of differentiation (14), adipocyte determination and differentiation factor 1/sterol regulatory element-binding protein-1 (15), fatty acid synthase (16), and peroxisome proliferator activator receptor γ (17) was decreased in HSL-/- WAT compared with HSL+/+ WAT in both Lep+/- and Lepob/ob backgrounds. These results strongly support the results of immunohistochemistry and the conclusion that preadipocytes were increased in HSL-/- WAT. No significant differences were observed in the expression of lipoprotein lipase and uncoupling protein-2 between Lepob/ob/HSL-/- and Lepob/ob/HSL+/- mice.

To rule out the possibility that the small cells are apoptotic due to overaccumulation of intracellular TG, we performed terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling staining and DNA ladder detection. Although Lepob/ob WAT contained an increased number of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling-positive apoptotic cells and increased DNA ladder formation compared with Lep+/- WAT, there was no difference between HSL+/+ and HSL-/- WAT (Fig. 3b). Furthermore, we performed in vivo bromodeoxyuridine labeling to determine...
whether the small cells have increased proliferative activity. No increase in bromodeoxyuridine-positive cells was observed in HSL/H11002/WAT (data not shown).

Food Intake Is Reduced in Obese HSL/H11002/WAT Mice—To determine the causes of the lower adiposity in Lep(ob)/HSL(H11002)/ob mice, we examined food intake. In the Lep(Hsl/H11002)/ob background, there was no difference in food intake between HSL(H11001)/H11002 and HSL(H11002)/H11002 mice. In the Lepob/ob background, food intake, which was increased compared with the Lep(Hsl/H11002)/ob background, was reduced by 19% in HSL(H11002)/H11002 mice compared with HSL(H11001)/H11002 mice at the age of 14 weeks (Fig. 4a). The reduced food intake was apparent at least after 8 weeks of age (data not shown). Lepob/ob/HSL(H11002)/ob mice had significantly lower feeding efficiency than Lepob/ob/HSL(H11002)/ob mice (Fig. 4b), indicating that the reduced food intake was not the only mechanism contributing to the leanness of Lepob/ob/HSL(H11002)/ob mice.

Adrenalectomy reduces food intake (18, 19) in Lepob/ob mice, which are in hyper-ACTH status, and HSL deficiency reduces the corticosterone response to ACTH (20, 21). Therefore, we measured basal plasma corticosterone levels to examine whether adrenal insufficiency underlies the reduced food intake. There was no significant decrease in the plasma corticosterone levels at the age of 9 weeks (n = 10).
terone levels in \(\text{Lep}^{ob/ob}/\text{HSL}^{-/-}\) mice compared with \(\text{Lep}^{ob/ob}/\text{HSL}^{+/-}\) mice (Fig. 4c).

Hypothalamus expressed HSL protein in wild-type mice but not in \(\text{HSL}^{-/-}\) mice (Fig. 5a). The suppressed food intake in \(\text{Lep}^{ob/ob}\) mice led us to examine the expression of various neuropeptides that govern appetite and satiety in the hypothalamus (Fig. 5, b and c). The expression levels of NPY and AgRP, both of which were induced in \(\text{Lep}^{ob/ob}\) background, were decreased nearly to the level of lean \(\text{Lep}^{+/-}\) mice (Fig. 5, b and c).

The expression levels of pro-opiomelanocortin, which were suppressed in the \(\text{Lep}^{ob/ob}\) background, were not different between \(\text{HSL}^{+/-}\) and \(\text{HSL}^{-/-}\) mice (Fig. 5, b and c).

In addition to food intake, energy expenditure determines adiposity. Body temperature, which was reduced in the \(\text{Lep}^{ob/ob}\) background, was not significantly different between \(\text{HSL}^{+/-}\) and \(\text{HSL}^{-/-}\) mice (Fig. 6a). Consistently, the mean oxygen consumption during 24 h was not significantly different between \(\text{HSL}^{+/-}\) and \(\text{HSL}^{-/-}\) mice in the \(\text{Lep}^{ob/ob}\) background when normalized by body surface area (males, 0.120 ± 0.024 versus 0.136 ± 0.019 ml/min/g^0.7, \(p = 0.18\); females, 0.119 ± 0.021 versus 0.135 ± 0.025 ml/min/g^0.7, \(p = 0.18\)), although \(\text{Lep}^{ob/ob}/\text{HSL}^{-/-}\) mice appeared to have higher oxygen consumption than \(\text{Lep}^{ob/ob}/\text{HSL}^{+/-}\) mice (Fig. 6b).

**HSL Deficiency Decreases Plasma Levels of FFA and Glycerol—\(\text{Lep}^{ob/ob}/\text{HSL}^{-/-}\) mice showed significant reduction in plasma levels of FFA and glycerol compared with \(\text{Lep}^{ob/ob}/\text{HSL}^{+/-}\) mice (FFA, 663 ± 67 versus 1,174 ± 17 \(\mu\)M, \(p < 0.01\); glycerol, 10.0 ± 2.8 versus 43.4 ± 6.0 mg/dl, \(p < 0.01\)). In the lean \(\text{Lep}^{+/-}/\text{HSL}^{-/-}\) mice, plasma cholesterol levels were increased, whereas plasma TG levels were decreased compared with those in \(\text{Lep}^{+/-}/\text{HSL}^{+/-}\) mice, which was due to an increase in high density lipoproteins and a decrease in very low density lipoproteins, respectively (data not shown), as reported previously (22). In the \(\text{Lep}^{ob/ob}\) background, no significant difference was observed between \(\text{HSL}^{+/-}\) and \(\text{HSL}^{-/-}\) mice.

Leanness and low plasma FFA levels are commonly associated with those in \(\text{Lep}^{+/-}\) mice. The suppressed food intake in \(\text{Lep}^{ob/ob}\) mice increased nearly to the level of lean \(\text{Lep}^{+/-}\) mice (Fig. 5, b and c). The expression levels of pro-opiomelanocortin, which were suppressed in the \(\text{Lep}^{ob/ob}\) background, were not different between \(\text{HSL}^{+/-}\) and \(\text{HSL}^{-/-}\) mice (Fig. 5, b and c).

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Hormone-sensitive Lipase Deficiency in Lepob/ob Mice

Ated with increased insulin sensitivity, whereas impaired adipogenesis is associated with insulin resistance (23). To estimate insulin sensitivity, we measured blood levels of glucose and insulin. There were no differences in the blood glucose and plasma insulin levels between Lepob/ob/HSL+/− and Lepob/ob/HSL−/− mice when fasted (data not shown), indicating that insulin sensitivity was not significantly different between them.

DISCUSSION

In the present studies, we use Lepob/ob/HSL−/− mice to show that the absence of HSL inhibits adipogenesis and feeding behavior, thereby decreasing adiposity in leptin-deficient obesity. Resistance to obesity was also observed in a high fat diet-induced obesity model (24).

Adipocytes in WAT of Lepob/ob/HSL−/− mice displayed exaggerated size heterogeneity (Fig. 2, a and b). In particular, small cells were remarkably increased. Although adipocytes from Lepob/ob/HSL+/− mice are prone to apoptosis upon insulin depletion (25) and indeed Lepob/ob/HSL−/− WAT contained a substantial number of apoptotic cells, HSL deficiency did not further increase the number of apoptotic cells in either the Lep+/+ or Lepob/ob settings (Fig. 3c). Since the majority of the small cells were positive for S-100 protein (Fig. 2c), we consider the small cells to be preadipocytes. This view is consistent with the gene expression profiles of WAT; adipose differentiation-related protein and CAAT/enhancer-binding protein β, which characterize the early phase of adipocyte differentiation, were up-regulated, and CAAT/enhancer-binding protein α, peroxisome proliferator activator receptor γ, adipocyte determination and differentiation factor 1/sterol regulatory element-binding protein-1, and fatty acid synthase, which characterize the mature stage of adipocyte differentiation, were down-regulated (Fig. 3c). Based on these results, we speculate that HSL deficiency inhibits adipogenesis in a certain population of preadipocytes, thereby stimulating their accumulation. Substances released from adipocytes of Lepob/ob/HSL−/− mice may mediate the inhibition of adipocyte differentiation. In this context, it is of note that FFA, which characterize the early phase of adipocyte differentiation, were up-regulated, whereas impaired adipogenesis and feeding behavior, thereby decreasing adiposity in leptin-deficient obesity. Resistance to obesity was also observed in a high fat diet-induced obesity model (24).

Indeed, it was reported that exposure to methyl-isobutylxanthine, which lack expression of phosphoenolpyruvate carboxykinase, which lack expression of phosphoenolpyruvate carboxykinase activity in WAT, are similar to Lepob/ob/HSL−/− mice in that their WAT also has accumulation of lipodystrophy-like small cells dispersed among normal large fat cells (46). The significance of this similarity is currently unknown.

In conclusion, Lepob/ob/HSL−/− mice have impaired adipogenesis and resistance to obesity at least partially due to the reduced food intake. These findings should provide the basis for understanding the pathophysiology of obesity and can be exploited to develop novel therapy for the endemic disease.

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**Hormone-sensitive Lipase Deficiency in Lepob/ob Mice**

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Absence of Hormone-sensitive Lipase Inhibits Obesity and Adipogenesis in Lep\textsuperscript{ob/ob} Mice

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