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The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells

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Kraemer FB, Shen WJ, Patel S, Osuga J, Ishibashi S, Azhar S. The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells. *Am J Physiol Endocrinol Metab* 292: E408–E412, 2007. First published September 19, 2006; doi:10.1152/ajpendo.00428.2006.—Steroid hormones are synthesized using cholesterol as precursor. To determine the functional importance of the low density lipoprotein (LDL) receptor and hormone-sensitive lipase (HSL) in adrenal steroidogenesis, adrenal cells were isolated from control, *HSL*$^{-/-}$, *LDLR*$^{-/-}$, and double *LDLR/HSL*$^{-/-}$ mice. The endocytic and selective uptake of apolipoprotein E-free human high density lipoprotein (HDL)-derived cholesteryl esters did not differ among the mice, with selective uptake accounting for >97% of uptake. In contrast, endocytic uptake of either human LDL- or rat HDL-derived cholesteryl esters was reduced 80–85% in *LDLR*$^{-/-}$ and double-*LDLR/HSL*$^{-/-}$ mice. There were no differences in the selective uptake of either human LDL- or rat HDL-derived cholesteryl esters among the mice. Maximum corticosterone production induced by ACTH or dibutyryl cyclic AMP and lipoproteins was not altered in *LDLR*$^{-/-}$ mice but was reduced 80–90% in *HSL*$^{-/-}$ mice. Maximum corticosterone production was identical in *HSL*$^{-/-}$ and double-*LDLR/HSL*$^{-/-}$ mice. These findings suggest that, although the LDL receptor is responsible for endocytic delivery of cholesteryl esters from LDL and rat HDL to mouse adrenal cells, it appears to play a negligible role in the delivery of cholesterol for acute adrenal steroidogenesis in the mouse. In contrast, HSL occupies a vital role in adrenal steroidogenesis because of its link to utilization of selectively delivered cholesteryl esters from lipoproteins.

neutral cholesteryl ester hydrolase; corticosterone; cholesterol; receptor low density lipoprotein; selective pathway; hormone-sensitive lipase

ADRENAL STEROIDOGENESIS REQUIRES a constant supply of cholesterol as a precursor for conversion to steroid hormones. This cholesterol can potentially be derived from several different sources as follows (3, 14): *1*) de novo cellular cholesterol synthesis, *2*) circulating lipoprotein-derived cholesteryl esters obtained by either receptor-mediated endocytic or "selective" cellular uptake, and *3*) the mobilization of stored cholesteryl esters via the actions of neutral cholesteryl ester hydrolase, which we have shown to be the result of the activity of hormone-sensitive lipase (HSL; see Ref. 18). Neither endogenous cellular cholesterol synthesis nor mobilization of stored cholesteryl esters is sufficient to support maximal steroid production, since hormone-stimulated steroid production is markedly augmented in the presence of lipoproteins (31). Lipoprotein-derived cholesteryl esters can be delivered by receptor-mediated endocytic uptake in which low density lipoprotein (LDL) or other apolipoprotein B- or apolipoprotein E-containing lipoproteins are processed via the LDL receptor (5) or can be delivered by selective uptake in which a variety of cholesterol-rich lipoproteins [high density lipoprotein (HDL) or LDL, regardless of apolipoprotein composition] bind to scavenger receptor class B type I (SR-BI) and release cholesteryl esters directly in cells without internalizing the lipoprotein particle itself (10, 19, 27, 32). Selective cholesteryl ester uptake appears to quantitatively deliver more cholesterol than endocytic uptake in hormone-stimulated rodent adrenal (4, 10, 19, 24, 26, 27, 32).

Once "selectively" delivered to the cell, cholesteryl esters must be hydrolyzed by nonlysosomal cholesteryl ester hydrolases before the free cholesterol can be transferred to mitochondria for steroid production (19). We have recently shown that HSL is functionally linked to the selective pathway and is critically involved in the intracellular processing and availability of cholesterol for adrenal steroidogenesis (17). Using *HSL*$^{-/-}$ mice, we showed that basal (50%) and hormone-stimulated (75–85%) corticosterone production in the absence or presence of LDL or HDL was markedly reduced in isolated adrenocortical cells. Moreover, we showed that HSL was critical for the conversion of HDL cholesteryl esters into corticosterone by demonstrating that the conversion of HDL cholesteryl esters into corticosterone was reduced 97% in adrenal cells from *HSL*$^{-/-}$ mice. Interestingly, the severity of the defect in hormone-stimulated corticosterone production observed in vitro in isolated adrenocortical cells was attenuated in vivo, although a defect was observed (17, 18). This attenuation was probably because of compensatory changes in the multiple pathways that can supply cholesterol requirements to the cell under physiological conditions. In particular, a threefold increase in the adrenal expression of LDL receptors was observed (17), suggesting a potential increase in the supply of cholesterol via receptor-mediated endocytic uptake.

The current studies were undertaken to test whether the absence of LDL receptors would exacerbate the defects observed in adrenal steroid production in *HSL*$^{-/-}$ mice by eliminating this potential compensatory pathway. In addition, because we are not aware of any prior studies examining acute adrenal steroidogenesis in *LDLR*$^{-/-}$ mice, we also examined the role of LDL receptors on lipoprotein-derived cholesteryl esters for corticosterone production in the mouse.

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MATERIALS AND METHODS

Chemicals. Reagents were obtained from the following sources: BSA (fraction V; Intergen, Purchase, NY); cholesterol oleate (Sigma Chemical, St. Louis, MO); [^{3}H]cholesteryl oleate, Na^{125}I, and [^{3}H]cholesteryl oleoyl ether (Perkin-Elmer Life Sciences, Boston, MA); and chloroform, methanol, and heptane (J. T. Baker, Phillipsburg, NJ). All other chemicals were obtained from standard commercial sources.

Animals and isolation and culture of mouse adrenocortical cells. *HSL*$^{-/-}$ mice were generated as described previously and were backcrossed for at least six generations in a C57BL/6J genetic background (21). *LDLR*$^{-/-}$ mice in a C57BL/6J genetic background were purchased from Jackson Laboratories (JAX mice, Bar Harbor, ME). *HSL*$^{-/-}$ and *LDR*$^{-/-}$ mice were interbred to yield *HSL*+/-/*LDLR*$^{-/-}$ mice, which were then mated to yield *HSL*+/+/*LDLR*$^{-/-}$ and *HSL*$^{-/-}$/*LDLR*$^{-/-}$ for studies. All animal experimentation was conducted in accord with accepted standards of humane animal care and was approved by the VA Palo Alto institutional committee on animal care. Mouse adrenocortical cells were isolated from control (*HSL*+/+/*LDLR*+/+), *HSL*$^{-/-}$, *LDL*$^{-/-}$, and double-*HSL*/*LDLR*$^{-/-}$ mice by a procedure described previously (17). In brief, mice were killed by cervical dislocation, and the adrenal glands were aseptically removed and dissected free of fat. A group of 20 adrenals (from 10 mice) was finely minced with scissors, and tissue fragments were suspended in sterilized medium 199 containing 40 mg/ml of BSA, 3.7 mg/ml of collagenase (type 1), and 5 μg/ml of DNase and incubated with shaking for 50 min in an atmosphere of 95% O_2-5% CO_2. The tissue suspension was then dissociated by repeated pipetting with a tuberculin syringe, the resulting suspension was filtered through a nylon mesh and washed by centrifugation, and the final pellet was resuspended in DMEM-F-12 (1:1). These cell preparations were either used immediately for various measurements or cultured overnight in serum-free culture medium (DMEM-F-12 containing 1 mg/ml BSA, 2 μg/ml insulin, 5 μg/ml transferrin, and 2 μg/cm^2 fibronectin) plus other test substances, as specified under Figs. 1–5.

Preparation of lipoproteins. Human LDL, human apolipoprotein E-free HDL (HDL_3), and rat HDL were isolated and characterized as previously described (25). For uptake and internalization studies, lipoproteins were modified with two nonreleasable labels, i.e., ^{125}I-labeled dilactitol tyramine to mark lipoprotein proteins and [^{3}H]cholesteryl oleolyl ether to mark lipoprotein cholesteryl esters, as described previously (4).

Measurement of corticosterone secretion. To assay steroidogenesis, triplicate samples of freshly isolated cells were incubated without (basal) or with ACTH-(1–24) (10 ng/ml) or Bt_2cAMP (2.5 mM) for 3 h, and subsequently samples of incubation medium were frozen and stored until assayed for corticosterone. To examine lipoprotein-supported corticosterone production, isolated adrenocortical cells were cultured for 24 h ± Bt_2cAMP (2.5 mM), ± human HDL_3 (500 μg protein/ml), ± human LDL (100 μg protein/ml), or ± rat HDL (130 μg protein/ml), and after incubation the incubation media were collected, frozen, and stored frozen until analyzed. Results are expressed as nanograms corticosterone produced per milligram cell protein and represent the means ± SE of duplicate determination of three different samples.

Analytic procedures. Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). The procedure of Markwell et al. (20) was used to quantify protein content of human HDL_3, human LDL, rat HDL, and doubly-labeled lipoprotein preparations. Cholesterol content of human HDL_3, human LDL, rat HDL, and doubly-labeled lipoproteins was determined according to the procedure of Tercyak (30).

Statistics. Data are expressed as means ± SE. Statistical analyses were performed by ANOVA and comparisons among groups by Fisher's Protected *t*-tests using SPSS software (SPSS, Chicago, IL) on a Power Macintosh computer.

RESULTS

To characterize the phenotype of the mice on the basis of the uptake of lipoprotein-derived cholesteryl esters, we measured total, LDL receptor-mediated (endocytic), and selective uptake using double-labeled lipoproteins (^{125}I-labeled dilactitol-[^{3}H]cholesteryl oleoyl ether) in isolated adrenal cells. As shown in Fig. 1*A*, endocytic uptake of human HDL_3-derived cholesteryl esters was extremely low (<3%) compared with selective uptake, consistent with the vast majority of HDL_3 cholesteryl ester delivery occurring via SR-BI; however, there were no differences in either total, receptor-mediated "endocytic," or selective uptake of HDL cholesteryl esters among control, *LDLR*$^{-/-}$, *HSL*$^{-/-}$, or double-*LDLR*/*HSL*$^{-/-}$ mice. For comparison (Fig. 1*B*), we measured total, receptor-mediated, and selective uptake of double-labeled human LDL (^{125}I-labeled dilactitol-[^{3}H]cholesteryl oleoyl ether-human LDL). As opposed to HDL_3, substantial amounts of LDL cholesteryl esters were derived from endocytic uptake (42% of total uptake in controls); yet selective uptake still accounted for the majority of cholesteryl ester delivery. There were no detectable differences in either total, receptor-mediated endocytic, or selective uptake of LDL cholesteryl esters between control and *HSL*$^{-/-}$ mice. In contrast, as would be expected from the absence of LDL receptors, total uptake of LDL cholesteryl esters was diminished equivalently in both *LDLR*$^{-/-}$ and double-*LDLR*/*HSL*$^{-/-}$ mice (34%). The decrease in total uptake of LDL cholesteryl esters reflected a 77–82% reduction ($P < 0.01$) in receptor-mediated endocytic uptake in both *LDLR*$^{-/-}$ and double-*LDLR*/*HSL*$^{-/-}$ mice, without any changes in selective uptake. Because the above studies of uptake of lipoprotein-derived cholesteryl esters were performed using human lipoproteins, for further comparison we measured total, receptor-mediated, and selective uptake of double-labeled rat HDL (^{125}I-dilactitol-[^{3}H]cholesteryl oleoyl ether HDL), which contains substantial amounts of apolipoprotein E. As shown in Fig. 1*C*, endocytic uptake of rat HDL-derived cholesteryl esters was greater than observed with human HDL_3 (24% of total); however, selective uptake still accounted for the vast majority of HDL cholesteryl ester delivery. There were no detectable differences in either total, receptor-mediated endocytic, or selective uptake of HDL cholesteryl esters between control and *HSL*$^{-/-}$ mice. However, similar to the observations with LDL, receptor-mediated endocytic uptake was decreased 82–87% ($P < 0.01$) in both *LDLR*$^{-/-}$ and double-*LDLR*/*HSL*$^{-/-}$ mice, most likely reflecting apolipoprotein E-mediated uptake. Thus the genotypes of the mice resulted in the expected phenotypes for the mechanisms of lipoprotein-derived cholesteryl ester delivery.

Although LDL receptor-mediated endocytic uptake of cholesterol is known to support adrenal steroidogenesis (9), we are not aware of any prior studies examining adrenal steroidogenesis in LDL receptor knockout mice. Therefore, we compared steroid hormone production from adrenal cells isolated from *LDLR*$^{-/-}$ mice with adrenal cells from control and *HSL*$^{-/-}$ mice (Fig. 2). Basal (absence of ACTH) corticosterone production from adrenal cells was similar in control and *LDLR*$^{-/-}$ mice but, as seen previously, was reduced ~50% in *HSL*$^{-/-}$ mice ($P < 0.02$). Exposure of adrenal cells to ACTH (10 ng/ml) or Bt_2cAMP (2.5 mM) increased corticosterone production ~10-fold in both control and *LDLR*$^{-/-}$ mice, but only ~3-

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to 4-fold in $HSL^{-/-}$ mice. Therefore, corticosterone production induced by ACTH or Bt_2cAMP was ~85–90% lower in adrenal cells from $HSL^{-/-}$ mice compared with either control or $LDLR^{-/-}$ mice ($P < 0.02$). Exposure of adrenal cells to

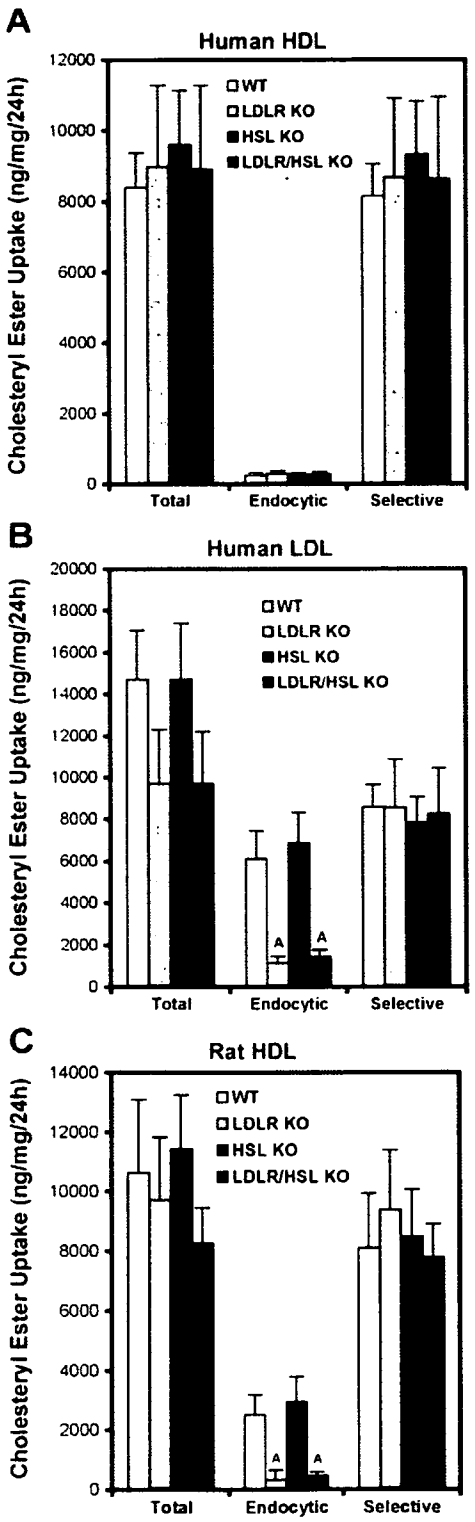

Fig. 1. Total, endocytic, and selective uptake of lipoprotein-derived cholesteryl esters by mouse adrenal cells from human apolipoprotein E-free high density lipoprotein (HDL_3; *A*), human low density lipoprotein (LDL; *B*), and rat high density lipoprotein (HDL; *C*). Adrenocortical cells were isolated from control, wild type (WT), LDL receptor (LDLR) knockout (KO), hormone-sensitive lipase (HSL) KO, and HSL/LDLR KO mice as described in MATERIALS AND METHODS. Cells were incubated with ^{125}I-labeled dilactitol-[^{3}H]cholesteryl oleolyl ether-human HDL_3 (*A*), ^{125}I-labeled dilactitol-[^{3}H]cholesteryl oleolyl ether-human LDL (*B*), or ^{125}I-labeled dilactitol-[^{3}H]cholesteryl oleolyl ether-rat HDL (*C*) for 24 h at 37°C. At the end of incubation, the cell samples were washed and then solubilized in 2 ml of 0.1 N NaOH. Aliquots (1 ml) were precipitated with an equal volume of 20% TCA to determine insoluble and soluble ^{125}I radioactivity or extracted with organic solvents to determine ^{3}H radioactivity. Endocytic uptake is calculated from TCA-soluble ^{125}I label only. The difference between total and TCA-soluble activity is taken as the surface-associated ^{125}I radioactivity. Because both ^{125}I and ^{3}H labels are on the same particle, the surface-bound ^{125}I is also equal to the surface-bound ^{3}H. Thus total ^{3}H minus surface-bound ^{3}H equals the total amount of ^{3}H internalized. To calculate "selective" uptake of cholesteryl ester, soluble ^{125}I radioactivity is subtracted from soluble ^{3}H radioactivity. Results are means ± SE of $n = 4$ in each group. $^AP < 0.01$ compared with control mice.

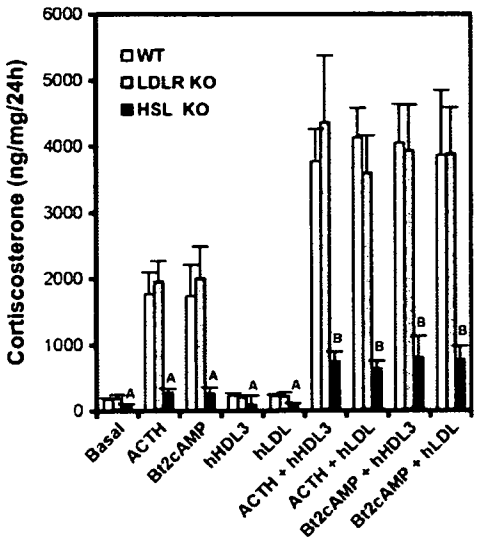

Fig. 2. Comparison of corticosterone secretion by cultured mouse adrenocortical cells. Adrenocortical cells were isolated from control, wild-type (WT), LDLR KO, and HSL KO mice as described in MATERIALS AND METHODS. Cells were incubated in the absence or presence of ACTH (10 ng/ml), Bt_2cAMP (2.5 mM), HDL_3 (500 μg protein/ml), LDL (100 μg protein/ml), or both ACTH (10 ng/ml) or Bt_2cAMP (2.5 mM) and HDL_3 (500 μg protein/ml) or LDL (100 μg protein/ml) for 24 h, and the amount of corticosterone secreted in the media was measured. Results are means ± SE of 3 independent experiments. $^AP < 0.02$ compared with control mice. $^BP < 0.005$ compared with control mice.

HDL or LDL had no effect on corticosterone production in either control, $LDLR^{-/-}$, or $HSL^{-/-}$ mice, but the combination of either ACTH or Bt_2cAMP and HDL or LDL increased corticosterone production 18- to 25-fold in both control and $LDLR^{-/-}$ mice, but only ~7- to 10-fold in $HSL^{-/-}$ mice. Therefore, maximum corticosterone production induced by ACTH or Bt_2cAMP and HDL or LDL was similar in control and $LDLR^{-/-}$ mice, but was ~80% lower in adrenal cells from $HSL^{-/-}$ mice ($P < 0.005$). Thus the absence of LDL receptors does not appear to impair lipoprotein-cholesteryl ester-supported murine adrenal steroidogenesis, whereas the absence of HSL severely interferes with steroid hormone production.

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Because our previous studies with *HSL*$^{-/-}$ mice showed a threefold increase in adrenal LDL receptor expression, which could provide a potential compensatory mechanism for maintaining corticosterone production in adrenal cells from *HSL*$^{-/-}$ mice, we generated double-*LDLR/HSL*$^{-/-}$ mice for study. Corticosterone production following Bt_2cAMP in the presence or absence of lipoproteins was then examined in adrenal cells isolated from control, *LDLR*$^{-/-}$, *HSL*$^{-/-}$, or double-*LDLR/HSL*$^{-/-}$ mice. As shown in Fig. 3, corticosterone production was identical between *HSL*$^{-/-}$ and double-*LDLR/HSL*$^{-/-}$ mice, whether stimulated with Bt_2cAMP in the absence or presence of human HDL_3, human LDL, or rat HDL, and was ~80% lower than control. Moreover, there were no differences in corticosterone production between *LDLR*$^{-/-}$ and control mice under any conditions. Thus LDL receptor deficiency alone had no effect on steroid production, and the addition of LDL receptor deficiency to HSL deficiency did not impact the marked defect in steroid production observed with HSL deficiency alone.

DISCUSSION

Faust et al. (9) originally reported that LDL cholesterol was used by mouse Y-1 adrenal cells for steroid hormone production via a receptor-mediated pathway and that HDL did not provide cholesterol for steroid synthesis in these cells, thus first documenting the involvement of the LDL receptor in adrenal steroidogenesis. In contrast, other investigators reported that cholesterol was transferred into and converted into steroids in rat adrenals from HDL at a higher rate than from LDL (11–13). Thus it was apparent that LDL and HDL cholesterol uptake in the rat (and the mouse) adrenal occurred by separate mechanisms but that HDL cholesterol appeared to be the major substrate based on quantitative data of the kinetics (2). The importance of the LDL receptor for delivery of cholesterol in adrenal steroid production has been supported by studies in rabbits and humans. First, studies in Watanabe heritable hyperlipidemic rabbits, which lack LDL receptors, showed that ACTH-stimulated corticosterone production was reduced compared with normal rabbits, although basal corticosterone concentrations were normal (15). Studies of human fetal adrenals documented the importance of LDL receptors in mediating cholesterol delivery to the adrenal for steroidogenesis; in fact, the impact of adrenal LDL receptors is such that adrenal LDL receptor-mediated uptake of LDL for steroid production is a major determinant of circulating LDL concentrations in the fetus (6). Nonetheless, subjects with homozygous familial hypercholesterolemia who lack LDL receptors were found to have a normal cortisol response to an acute injection of ACTH (1). In addition, plasma cortisol concentrations were also normal during prolonged infusions of ACTH in homozygous familial hypercholesterolemic subjects, although urine free cortisol excretion was reduced (16). Therefore, the accumulated evidence supports a role for the LDL receptor pathway in delivering cholesterol for adrenal steroidogenesis and, particularly, a role in adrenal steroidogenesis in humans; however, it is important to note that the primacy of LDL receptor-mediated endocytic delivery of cholesterol for adrenal steroidogenesis is not clearly established. In the current studies, we have examined the impact of the LDL receptor on steroidogenesis in isolated mouse adrenocortical cells. After documenting that isolated adrenocortical cells from *LDLR*$^{-/-}$ mice have a marked reduction in receptor-mediated endocytic uptake of cholesteryl esters from both LDL and rat HDL, we show that the absence of the LDL receptor has no measurable impact on adrenal steroid production under basal or hormone stimulated conditions in the absence or presence of lipoproteins. Thus the LDL receptor does not appear to contribute to the delivery of cholesteryl esters for adrenal steroid hormone production in the mouse under the current experimental conditions, at least when other pathways for cholesterol delivery (cellular cholesterol synthesis, selective cellular uptake of lipoprotein-derived cholesteryl esters, or the mobilization of stored cholesteryl esters) are intact. This conclusion is further supported by a recent report where chronic stimulation of adrenal steroidogenesis with daily injections of ACTH in vivo observed no differences in serum corticosterone levels between control and *LDLR*$^{-/-}$ mice until 5 days of stimulation (8). Thus the overall evidence suggests that the LDL receptor does contribute to the delivery of cholesteryl esters for adrenal steroid hormone production in the mouse, but this function only appears to become apparent following chronic stress.

SR-BI is responsible for mediating the selective uptake of cholesteryl esters from HDL (22, 29) and LDL (28) into adrenal cells. The absence of SR-BI results in an 85–90% reduction in cholesteryl ester uptake from HDL (22). This is similar to the effect observed with apolipoprotein AI deficiency, where adrenal cholesteryl esters are ~95% depleted along with a reduction in basal and ACTH-stimulated corticosteroids (23). Moreover, SR-B1-mediated selective cholesteryl ester uptake appears to quantitatively deliver more cholesterol than endocytic uptake in hormone-stimulated rodent adrenals (4, 10, 19, 24, 26, 27, 32). The cholesteryl esters that are selectively delivered to adrenocortical cells are hydrolyzed by HSL to unesterified cholesterol before transfer to mitochondria for steroid production (7, 18). Using

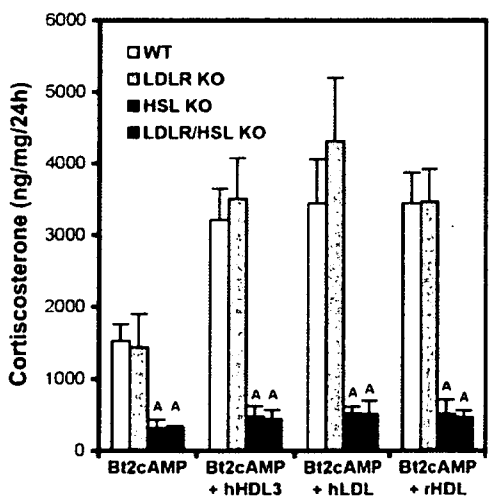

Fig. 3. Effects of cAMP and lipoproteins on corticosterone secretion by cultured mouse adrenocortical cells. Adrenocortical cells were isolated from control, WT, LDLR KO, HSL KO, and double-LDLR/HSL KO mice as described in MATERIALS AND METHODS. Cells were incubated in the absence or presence of Bt_2cAMP (2.5 mM) or both Bt_2cAMP (2.5 mM) and human HDL_3 (500 μg protein/ml), LDL (100 μg protein/ml), or rat HDL (130 μg protein/ml) for 24 h, and the amount of corticosterone secreted in the media was measured. Results are means ± SE of 3 independent experiments. $^{A}P < 0.005$ compared with control mice.

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isolated adrenocortical cells from $HSL^{-/-}$ mice, we previously showed that HSL is critically involved in the intracellular processing and availability of cholesterol derived from intracellular stores of cholesteryl esters and from lipoprotein-derived cholesteryl esters for adrenal steroidogenesis by demonstrating both a marked defect in corticosterone production under basal and hormone-stimulated conditions and a marked defect in the conversion of HDL cholesteryl esters into corticosterone (17). The severity of this defect in steroid production in vitro was attenuated in vivo and appeared to be due, at least in part, to a threefold upregulation of LDL receptor expression in the adrenals of $HSL^{-/-}$ mice (17). Thus we reasoned that the addition of LDL receptor deficiency to HSL deficiency would create a scenario whereby adrenal steroid production would be severely impaired, because of disruption of the supply of free cholesterol through loss of the following three sources of cholesterol: *1*) receptor-mediated endocytic uptake, *2*) utilization from selective uptake, and *3*) mobilization of stored cholesteryl esters. Surprisingly, the addition of LDL receptor deficiency to HSL deficiency did not impact the marked defect in steroid production observed with HSL deficiency alone.

In conclusion, although the LDL receptor is responsible for receptor-mediated endocytic delivery of cholesteryl esters from apolipoprotein B (LDL)- and apolipoprotein E-containing lipoproteins (rat HDL) to mouse adrenal cells, it appears to play a negligible role in the delivery of cholesteryl esters for acute adrenal steroid hormone production in the mouse. In contrast, HSL occupies a vital role in adrenal steroidogenesis because of the requirement for its action on cholesteryl esters selectively delivered from lipoproteins via SR-BI to be efficiently utilized. (11–13)

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Differential effects of apolipoprotein E isoforms on lipolysis of very low-density lipoprotein triglycerides

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Abstract

Apolipoprotein (apo) E plays a key role in lipoprotein metabolism and has been proposed to modulate triglyceride (TG) lipolysis. However, no systematic investigation on lipolysis using all 3 isoforms of apoE has been performed. To clarify the role of common human apoE isoforms in the lipolysis of very low-density lipoprotein (VLDL) TGs, we overexpressed human apoE isoforms in apoE and low-density lipoprotein receptor–deficient mice using adenoviral-mediated gene transfer and used VLDL particles obtained from these mice for in vitro lipolysis assay. Overexpression of apoE, regardless of its isoforms, increased the TG content of VLDL in mice in vivo. In vitro analysis of the effect of apoE on lipolysis revealed that irrespective of its isoforms, apoE did inhibit TG lipolysis at every concentration of apoE examined, and this inhibitory effect became more pronounced as the apoE content of VLDL increased. No difference was observed in TG lipolysis activity among isoforms at low apoE/TG ratio; however, intermediate ratios of apoE/TG, which reflect physiologic VLDL apoE/TG ratios, demonstrated a significantly greater level of lipolysis inhibition in apoE2, but less so in apoE4 compared with other isoforms. This differential effect by apoE isoforms on lipolysis was attenuated at higher apoE/TG ratios; nevertheless, apoE2 still inhibited lipolysis significantly more than did apoE4. Enrichment of VLDL with apoE decreased both the apoC contents and apoC-II/C-III ratios of VLDL, contributing, at least in part, to the inhibitory function of apoE on lipolysis. The present study clarifies the differential lipolysis-modulating effect of apoE isoforms, which would help explain the difference in pre- and postprandial TG levels among humans carrying different apoE isoforms.
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1. Introduction

Dyslipidemia is a major risk factor for atherosclerotic diseases. Epidemiologic and interventional studies have shown that low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels have strong positive and negative respective associations with coronary heart disease [1-3]. Several studies have suggested that increased plasma triglycerides (TGs) level is also a risk factor for coronary heart disease [4]. Furthermore, recent studies have proposed that postprandial dyslipidemia or hypertriglyceridemia accelerates atherosclerosis through several mechanisms including the accumulation of atherogenic remnant lipoprotein particles and an increase in the number of small dense low-density lipoprotein (LDL) particles [5].

Lipoprotein lipase (LPL), anchored on heparan sulfate proteoglycan (HSPG) of the vessel walls, plays a key role in the catabolism of lipoprotein core TGs using apolipoprotein (apo) C-II as an activator or a coenzyme [6-8]. A deficiency in these proteins causes type I hyperlipoproteinemia, where the plasma TG level usually exceeds 1000 mg/dL.

Apolipoprotein E is a ligand for several lipoprotein receptors and plays a key role in the receptor-mediated uptake of apoB-containing lipoproteins [9]. In addition to its well-established function, apoE is proposed to modulate TG lipolysis [10]. Several lines of evidence suggest that apoE inhibits the lipolysis of very low-density lipoprotein (VLDL) TGs [11-13], whereas other investigators have shown an increased lipolytic activity by apoE [14,15].

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There are 3 major apoE isoforms in humans: apoE2, E3, and E4. Epidemiologic studies have shown that this polymorphism is associated with interindividual variations in plasma lipid levels as well as susceptibility to atherosclerosis [10,16]. Apolipoprotein E4 is associated with higher plasma total cholesterol (TC) and LDL-C levels and higher apoB levels compared with apoE3. In addition, apoE4 is proposed to be an independent risk factor for coronary heart disease regardless of its effect on plasma cholesterol levels [17-19]. On the other hand, homozygotes for apoE2, insofar as they do not develop type III hyperlipoproteinemia, show lower plasma TC and LDL-C levels, lower apoB levels, and higher plasma TG levels compared with those of apoE3. The increase in postprandial plasma TG levels is prominent in homozygotes of apoE2 compared with those of apoE3 and apoE4 [20,21]. Furthermore, apoE2 is proposed to reduce the risk of atherosclerotic diseases. However, homozygotes for apoE2 who develop type III hyperlipidemia due to secondary precipitating factors are susceptible to atherosclerotic diseases [22,23].

The plasma TG level is determined by 3 steps in the lipoprotein metabolic pathway: (1) production of VLDL particles from the liver, (2) lipolysis of lipoprotein core TGs by lipase in the systemic circulation, and (3) uptake of TG-rich lipoprotein particles through the lipoprotein receptors. Previous studies have extensively examined the differential roles of apoE isoforms in the lipoprotein production in the liver [24,25] and their binding capacity to lipoprotein receptors [9,26]; however, no systematic investigation on lipolysis using all 3 isoforms has been performed. To clarify the role of apoE isoforms in the lipolysis of VLDL TGs, we overexpressed human apoE isoforms in mice deficient in both apoE and LDL receptor (LDLR) genes using adenoviral-mediated gene transfer and thus used VLDL particles obtained for the in vitro lipolysis assay. The present study provides the first comprehensive data demonstrating the inhibitory effect of all 3 apoE isoforms in the lipolysis of VLDL TGs and the isoforms' differential lipolysis-modulating effects.

2. Experimental design and methods

2.1. Materials

ApoE/LDLR double knockout mice were raised in the Animal Center for Biomedical Research at the University of Tokyo. They were fed with a normal chow. Second-generation adenoviral vectors AdE2, AdE3, and AdE4 [25], encoding apoE2, E3, and E4 isoform complementary DNA, respectively, were used to express human apoE isoforms. Bovine milk LPL was purchased from Sigma-Aldrich (St Louis, MO).

2.2. Preparation of plasma VLDL

Three groups of 12- to 16-week-old apoE/LDLR double knockout mice were injected intravenously with 6×10^9 plaque-forming units of AdE2, AdE3, or AdE4 through the tail vein; each group contained 2 mice. Three days after the injection of adenoviruses, the mice were placed on a lipid-free diet for 4 hours to minimize the contamination of chylomicron, and their blood was obtained from the retro-orbital plexus at a volume of 0.5 to 0.7 mL per mouse. Blood sampling was also performed on mice not injected with an adenovirus to extract VLDL free of apoE protein (apoE-null VLDL). Plasma was immediately separated by centrifugation and diluted with saline (d = 1.006). Diluted plasma samples were subjected to ultracentrifugation at 40000 rpm for 3 hours using RPL-42T rotor (Hitachi, Tokyo, Japan) [27], a rotor equivalent to the LP 42 Ti rotor (Beckman Coulter, Fullerton, CA) [28,29], after which the upper white layer was collected as VLDL. Extracted VLDLs from 2 mice were pooled together for each isoform, and TC and TG concentrations were measured enzymatically using Cholesterol C-test Wako kit and Triglyceride G-test Wako kit (Wako Pure Chemicals Industries, Osaka, Japan), respectively. Apolipoprotein E concentration in VLDL was measured using the immunoturbidimetric method with ApoE auto N (Daiichi) reagents (Daiichi Pure Chemicals, Tokyo, Japan).

2.3. In vitro lipolysis assay

Very low-density lipoprotein containing each apoE isoform was gently mixed with apoE-null VLDL and incubated for 2 hours at 37°C to equalize the concentration ratio of apoE/TG among VLDLs carrying each apoE isoform. Very low-density lipoprotein apoE was adjusted at 10, 20, 30, 50, and 70 μg/mg TG, respectively. Adjusted VLDL was dissolved in a buffer containing 2% bovine serum albumin (BSA) and 100 mmol/L Tris, pH 8.5, to set final TG concentrations of each sample at 30 mg/dL.

Lipolysis reaction was initiated by the addition of 1.25 U of bovine LPL. Fifteen minutes later, the reaction was terminated by adding stop solution containing 50 mmol/L KH_2PO_4 and 0.1% Triton X-100. As a control reaction, LPL and stop solution were added simultaneously to the samples. Nonesterified fatty acid concentrations of all samples were measured with NEFA-C kit WAKO (Wako Pure Chemicals Industries). Fatty acid release by lipolysis reaction was calculated by dividing the increment of fatty acid concentrations in each sample by reaction time. Each reaction was performed in sextuplets, and the lipolysis rate was expressed as a percentage, setting the fatty acid release of apoE-null VLDL sample at 100%.

2.4. Isoelectric focusing of apoC-II and apoC-III

After equalization of the VLDL-apoE/TG ratio, VLDL samples were diluted in d = 1.006 saline and ultracentrifuged again at 40000 rpm for 2 hours. The upper layer was collected as VLDL and delipidated by adding an ethanol-ether (3:1) mixture. Equal amounts of VLDL (corresponding to 18 μg TG) was dissolved in 100 μL of isoelectric focusing (IEF) sample buffer (100 mmol/L Tris, 6 mol/L urea, 1% wt/vol decyl sodium sulfate, 5% vol/vol 2-mercaptoethanol, and

1%wt/vol glycerol). Twenty microliters of each sample was electrophoresed on a 13% polyacrylamide IEF gel (pH 4-6.5) at 250 V overnight at 4°C and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 1 hour at room temperature and then incubated with antirabbit anti-apoC antibody (Biodesign International, Saco, ME) for 16 hours at 4°C, followed by incubation with horseradish peroxidase–conjugated goat antirabbit immunoglobulin G as a secondary antibody. An enhanced chemiluminescence ECL Plus kit (Amersham Biosciences, Piscataway, NJ) was used for the detection method. Images of the membrane were captured using computerized CCD camera LAS-1000 system (Fuji Photo Film, Tokyo, Japan) and analyzed by NIH Image software.

2.5. Statistical analysis

All data are presented as mean ± SEM. Statistical comparisons were made by 1-way analysis of variance using Prism Version 4 software (GraphPad Software, San Diego, CA). A 95% confidence level was deemed statistically significant.

3. Results

3.1. Lipid composition of VLDL after expression of each apoE isoform

Table 1 shows the lipids and apoE composition of VLDL purified and pooled from the apoE/LDLR double knockout mice injected with apoE adenoviruses. The VLDL TG/TC ratio increased with the expression of all 3 apoE isoforms. The apoE/TG ratio of VLDL was similar among all 3 isoforms (E2, 111.1 μg/mg TG; E3, 104.1 μg/mg TG; E4, 70.4 μg/mg TG).

3.2. Effect of apoE isoforms on VLDL lipolysis

The VLDL apoE concentration was adjusted to 10, 20, 30, 50, and 70 μg/mg TG by incubation with apoE-null VLDL and subjected to in vitro lipolysis assay. The VLDL TC/TG ratios after adjusting the apoE/TG ratio are shown in Table 2. The VLDL TC/TG ratio decreased in all isoforms as the apoE/TG ratio increased. Comparing all 3 apoE isoforms at equal apoE/TG ratios revealed that the VLDL TC/TG ratio was highest in apoE2-VLDL and lowest in apoE4-VLDL.

Table 1
Composition of VLDL purified from apoE/LDLR double knockout mice injected with apoE adenoviruses

Component	Adenovirus			
	(−)	AdE2	AdE3	AdE4
apoE	0	67.5	69.5	115.4
TG	257.8	607.6	667.6	1639.6
TC	984.5	781.8	589.7	891.1
TC/TG ratio	3.82	1.29	0.88	0.54
ApoE/TG ratio	0	0.111	0.104	0.070

Data are expressed in mg/dL. (−), without injection of adenovirus.

Table 2
VLDL TC/TG ratios after adjustment of apoE/TG ratios

	apoE/TG (μg/mg)				
	10	20	30	50	70
apoE2-VLDL	3.59	3.36	3.13	2.68	2.22
apoE3-VLDL	3.54	3.25	2.97	2.41	1.84
apoE4-VLDL	3.35	2.89	2.42	1.49	0.56

Very low-density lipoprotein containing each apoE isoform was gently mixed with apoE-null VLDL and incubated for 2 hours at 37°C to adjust the final apoE concentrations to 10, 20, 30, 50, and 70 μg/mg TG. Total cholesterol and triglyceride concentrations were measured in all samples for confirmation, followed by calculation of the TC/TG ratio.

The lipolysis rates, calculated by dividing released NEFA content by reaction time, are shown in Fig. 1. Because the rate of NEFA release was linear for at least 20 minutes, the lipolysis rate was determined from NEFA released in the initial 15 minutes. The lipolysis rates analyzed using apoE-null VLDL as a substrate were defined as 100%. As shown in Fig. 1A, lipolysis rates decreased significantly in all 3 apoE isoforms as the VLDL apoE/TG ratio increased. However, there was a characteristic difference in the pattern of decline among apoE isoforms. When the apoE/TG ratio was as low as 10, there was no difference in lipolysis rates among the 3 isoforms (E2, 91.8% ± 1.5%; E3, 95.5% ± 2.7%; E4, 89.6% ± 0.9%). When the apoE/TG ratios were increased to 20 (E2, 75.6% ± 0.2%; E3, 81.3% ± 1.8%; E4, 95.9% ± 0.5%) and 30 (E2, 64.4% ± 1.1%; E3, 76.1% ± 1.3%; E4, 90.6% ± 0.8%), the inhibition of lipolysis was greatest in apoE2 ($P < .01$ vs apoE3, $P < .001$ vs apoE4) and least in apoE4 ($P < .01$ vs apoE3); as for apoE4, there was no difference in lipolysis rates while apoE/TG ratios ranged between 10 and 30. However, when apoE/TG ratios were increased to 50 (E2, 55.1% ± 1.5%; E3, 58.3% ± 3.3%; E4, 61.0% ± 1.2%) and as high as 70 (E2, 48.1% ± 0.3%; E3, 50.6% ± 0.2%; E4, 55.0% ± 0.3%), the isoform-dependent differential effect on lipolysis was reduced. Nevertheless, apoE2 still demonstrated significantly greater inhibitory activity than apoE4 ($P < .05$).

3.3. Effect of apoE isoforms on VLDL apoC-II and apoC-III

We next examined whether an increase in apoE content within VLDL modified the apoC-II and/or apoC-III contents of VLDL particles. Proteins were purified from VLDL samples whose apoE/TG ratios were adjusted to 0, 30, and 70, and were subjected to IEF gel electrophoresis followed by Western blot analysis. As shown in Fig. 2A, in all 3 isoforms, both apoC-II and apoC-III contents decreased as the apoE/TG ratios increased. The apoC-II/C-III ratio, which was analyzed using a densitometer, also decreased as the apoE/TG ratios increased in all 3 isoforms (Fig. 2B). The apoC-II/C-III ratio tended to be less in apoE4-VLDL compared with apoE2- and apoE3-VLDL.

4. Discussion

Apolipoprotein E exists on the surface of several lipoprotein classes, especially on TG-rich lipoproteins such as chylomicron and VLDL. Therefore, besides serving as a ligand for lipoprotein receptors and facilitating VLDL secretion from the liver, apoE is proposed to play an important role on the lipolysis of pre- and postprandial plasma TGs. Several studies have examined the role of apoE on TG lipolysis; however, their results were not conclusive [11-15]. In addition, it has been difficult to compare the effect on lipolysis rate of one apoE isoform to that of another using purified VLDL extracted from humans because the lipids and apoE composition of VLDL are different among apoE isoforms. Some investigators used artificial lipid emulsion to modify the lipid and apoE content [11,30]; however, their results need to be interpreted carefully because artificial VLDL is different from the native VLDL in several respects. Apolipoprotein E is an apolipoprotein that can exchange between lipoprotein particles. As reported previously [31], the dissociation constant of apoE bound to microemulsion becomes greater at 37°C compared with one at 4°C. Another report [30] showed that apoE exchanged from plasma lipoprotein particles to lipid emulsion particles by mixing these particles at 37°C. Thus, to minimize the effect caused by the discrepancies in VLDL composition, in the present study, we adjusted the ratio of apoE to TGs by mixing at 37°C VLDL particles containing apoE with apoE-null VLDL, which lacks only the apoE protein while possessing all other components of natural VLDL.

As shown in Table 1, overexpression of apoE, regardless of its isoforms, increased the TG content of VLDL in apoE/

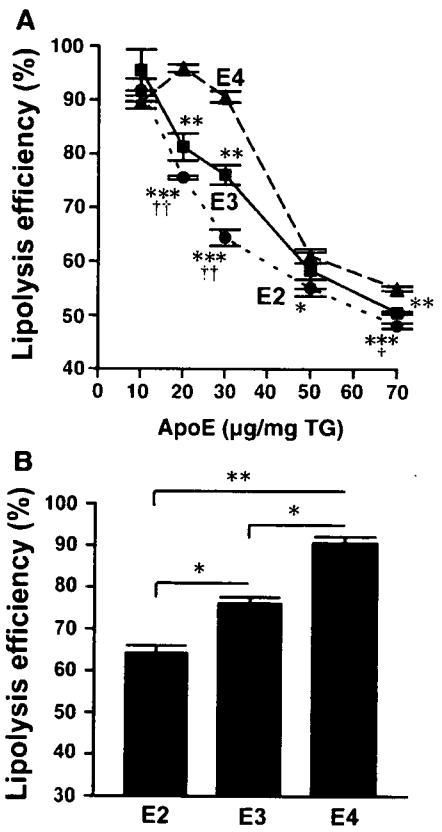

Fig. 1. A, Effect of apoE isoform on VLDL lipolysis. Lipolysis was initiated by adding 1.25 U of LPL to adjusted VLDL at a final triglyceride concentration of 30 mg/dL and terminated 15 minutes later by adding stop solution. Control reactions were performed by adding LPL and stop solution simultaneously. Nonesterified fatty acid concentrations of all samples were measured, and fatty acid release was determined by dividing the difference in fatty acid concentrations by reaction time. Lipolysis rate was calculated by setting the fatty acid release of apoE-null VLDL at 100%. The closed circles, closed squares, and closed triangles indicate apoE2, apoE3, and apoE4, respectively. Data are mean ± SEM (n = 6). $^{\dagger}P < .05$ vs apoE3; $^{\dagger\dagger}P < .01$ vs apoE3; $^{**}P < .01$ vs apoE4; $^{***}P < .001$ vs apoE4; $^{*}P < .05$ vs apoE4. B, Comparison of lipolysis rates among the 3 apoE isoforms at concentrations of apoE equivalent to physiologic plasma levels (apoE/TG ratio was 30 μg/mg). Data are mean ± SEM (n = 6). $^{*}P < .01$; $^{**}P < .001$.

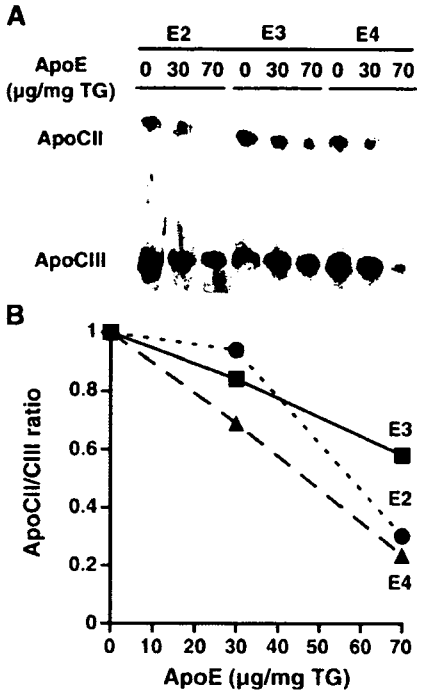

Fig. 2. A, Isoelectric focusing of mouse apoC-II and apoC-III. Very low-density lipoprotein samples containing 0, 30, and 70 μg/mg TG of apoE were dissolved in d = 1.006 saline and ultracentrifuged at 40000 rpm for 3 hours. Very low-density lipoprotein, manifested as the upper white layer, was collected and delipidated with an ethanol-ether (3:1) mixture. Equal amounts of VLDL (containing 3.6 μg TG) were electrophoresed on a 13% polyacrylamide IEF gel (pH 4-6.5) at 250 V overnight at 4°C, followed by Western blot analysis using rabbit anti-mouse apoC antibody and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G antibody as the primary and secondary antibodies, respectively. B, Very low-density lipoprotein apoC-II/C-III ratios for each apoE isoform. The image in A was analyzed with NIH Image software. ApoC-II/C-III ratio was calculated by dividing the intensity of the apoC-II signal by that of apoC-III, and the values were expressed with the apoC-II/C-III ratio of VLDL without apoE set at 1. The closed circles, closed squares, and closed triangles indicate apoE2, apoE3, and apoE4, respectively.

LDLR double knockout mice, implicating the inhibitory effect of apoE on VLDL TG lipolysis in vivo. A precise and systematic in vitro analysis of the effect of apoE on lipolysis using VLDL purified from these mice revealed that apoE inhibited lipolysis in TGs even at low concentrations, and this inhibitory effect became more pronounced as the apoE content increased (Fig. 1A). In addition, a comparison of this inhibitory effect among apoE isoforms revealed their differential inhibitory profiles. At low apoE/TG ratios, no difference was observed in the TG lipolysis activity among isoforms. However, intermediate ratios of apoE/TG reflecting the actual physiologic VLDL apoE/TG levels demonstrated a significantly greater level of lipolysis inhibition in apoE2, but less so in apoE4 compared with other isoforms (Fig. 1A and B).

One possible mechanism for the inhibitory effect of apoE on TG lipolysis is that an incremental change in apoE content within VLDL triggers displacement of the apoC-II and apoC-III—a potent activator and inhibitor of LPL, respectively—contents within VLDL. A previous study has confirmed that in apoC-III–overexpressing mice demonstrating a reduction in the lipolysis of TGs, levels of VLDL-apoC-II were reduced while those of VLDL–apoC-III increased [32]. As shown in Fig. 2, apoC-II, apoC-III, and apoC-II/C-III ratios decreased as apoE content increased in all 3 apoE isoforms. Therefore, alteration of apoE content on VLDL resulted in the changes in apoC contents of VLDL and affected, at least in part, the lipolysis of TGs.

However, these changes in apoC contents caused by the enrichment of VLDL with apoE do not completely explain the differential effect of apoE isoforms on lipolysis. Although the apoC-II content and apoC-II/C-III ratio tended to be less in apoE4-VLDL than in apoE2-VLDL, the inhibition of lipolysis by apoE2 was greater than that caused by apoE4, suggesting the existence of other mechanisms related to the isoform itself. One potential mechanism pertaining to the isoform itself may be the possibility that the interaction of apoE with apoB-containing lipoproteins might differ among 3 isoforms. Each apoE isoform is different from other isoforms by single arginine-cysteine interchanges at positions 112 and 158; apoE2 (112 Cys, 158 Cys), apoE3 (112 Cys, 158 Arg), and apoE4 (112 Arg, 158 Arg). Apolipoprotein E4, which possesses 2 arginines on both positions, is charged more positively than others. This property would reduce the affinity of the apoE4 protein to VLDL particles. Apolipoprotein E2, more rich in cysteine residues compared with the others, might bind tightly to VLDL particles through disulfide coupling, leading to a greater inhibitory effect on lipolysis. These mechanisms and hypotheses need to be clarified in future studies.

Some studies have proposed that as the TC content in VLDL increases, the lipolysis of TGs is inhibited [33]. As shown in Table 2, the TC/TG ratios in VLDL were higher in apoE2-VLDL than in apoE4-VLDL having the same apoE/TG ratios. These differences in TC/TG contents would, at least in part, result in the differential effects on lipolysis among the isoforms. However, in spite of decremental changes in TC/TG ratios with incremental changes in apoE/TG ratios in all 3 isoforms, the lipolysis rates decreased with every incremental change in apoE/TG ratios. Thus, we speculate that differences in TC content by itself do not explain the differential effects on lipolysis of the apoE isoforms.

de Man et al [34] proposed that the lipolysis of VLDL TGs is dependent on the apoE binding to HSPG. They demonstrated that apoE2 and apoE3-Leiden have less binding capability to HSPG compared with apoE3, leading to decreased lipolysis of VLDL carrying these apoE variants. We previously demonstrated that the binding ability of apoE2 to HSPG is reduced compared with that of apoE3 or apoE4 [35]. These evidences and our present study suggest that humans homozygous for apoE2 are susceptible to hypertriglyceridemia, a finding that correlates to actual clinical observations.

A possible limitation of our study is the utilization of bovine milk LPL. Previous studies have clarified that bovine milk LPL has almost similar molecular weight, lipase activity, and substrate specificity to human LPL, and is also activated by human apoC-II [36-38]. However, it remains to be seen whether soluble bovine milk lipase is similar in function to that of human LPL in the capillary endothelium, which is anchored on the HSPGs.

In summary, we demonstrated that apoE, regardless of its isoforms, inhibits VLDL-TG lipolysis even when its content within VLDL was below its physiologic counterpart. This inhibitory effect was at least partially due to the alteration of apoC contents within VLDL. In addition, we have for the first time demonstrated that apoE2 has the most and apoE4 the least inhibitory effect on lipolysis under physiologically sound apoE levels within VLDL, a finding that would, in part, explain the relevance of hypertriglyceridemia among those homozygous for apoE2.

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Original Article

Identification of a Novel Member of the Carboxylesterase Family That Hydrolyzes Triacylglycerol

A Potential Role in Adipocyte Lipolysis

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Molecular mechanisms underlying lipolysis, as defined by mobilization of fatty acids from adipose tissue, are not fully understood. A database search for enzymes with α/β hydrolase folds, the GXSXG motif for serine esterase and the His-Gly dipeptide motif, has provided a previously unannotated gene that is induced during 3T3-L1 adipocytic differentiation. Because of its remarkable structural resemblance to triacylglycerol hydrolase (TGH) with 70.4% identity, we have tentatively designated this enzyme as TGH-2 and the original TGH as TGH-1. TGH-2 is also similar to TGH-1 in terms of tissue distribution, subcellular localization, substrate specificity, and regulation. Both enzymes are predominantly expressed in liver, adipose tissue, and kidney. In adipocytes, they are localized in microsome and fatcake. Both enzymes hydrolyzed *p*-nitophenyl butyrate, triolein, and monoolein but not diolein, cholesteryl oleate, or phospholipids; hydrolysis of short-chain fatty acid ester was 30,000-fold more efficient than that of long-chain fatty acid triacylglycerol. Fasting increased the expression of both genes in white adipose tissue, whereas refeeding suppressed their expression. RNA silencing of TGH-2 reduced isoproterenol-stimulated glycerol release by 10% in 3T3-L1 adipocytes, while its overexpression increased the glycerol release by 20%. Thus, TGH-2 may make a contribution to adipocyte lipolysis during period of increased energy demand. *Diabetes* **55: 2091–2097, 2006**

Cellular accumulation of excessive 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) is an intracellular neutral lipase that catalyzes the hydrolysis of cellular triacylglycerol, diacylglycerol, monoacylglycerol, and cholesteryl ester, 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. To define the role of HSL in the hydrolysis of neutral lipids in vivo, several laboratories, including ours, have generated HSL-deficient ($HSL^{-/-}$) mice by targeted gene disruption (4–6). Although $HSL^{-/-}$ mice had a decreased ability to release free fatty acid (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 triacylglycerol lipase activity in adipocytes that mediates lipolysis stimulated by both β-adrenergic agonist and tumor necrosis factor-α (7). These findings resulted in the use of a bioinformatic approach to search for a protein that shares several motifs with HSL. Of 33 genes that have not been annotated, only 1 shows hydrolytic activity toward triacylglycerol and is expressed in adipose tissue.

Here we report the structure, substrate specificity, subcellular localization, tissue distribution, and regulation of expression in adipose tissue of this enzyme, which we have designated as triacylglycerol hydrolase (TGH)-2 because of its striking sequence similarity to microsomal liver/adipocyte TGH originally reported by Lehner and colleagues (8,9).

RESEARCH DESIGN AND METHODS

Triolein, lecithin, BSA fraction V (BSA), leupeptin, pentadecanoic acid, *p*-nitrophenyl butyrate (PNPB), 2-monoolein, dioleoylglycerol, dioleoyl-phos-

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ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; HSL, hormone-sensitive lipase; iPLA2, Ca^{2+}-independent PLA2; PNPB, *p*-nitrophenyl butyrate; TGH, triacylglycerol hydrolase; WAT, white adipose tissue.

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phatidylserine, dioleoyl-phosphatidylcholine, dioleoyl-phosphatidic acid, dioleoyl-phosphatidylethanolamine, and phosphatidylinositol (18:0, 24:0) were purchased from Sigma. Dexamethasone, 3-isobutyl-2-methylxanthine, fatty acid–free BSA, isoproterenol, and sodium taurocholate were purchased from Wako Pure Chemicals (Osaka). Pioglitazone was provided by Takeda Pharmaceutical (Osaka). Tri[^{3}H]oleoylglycerol and cholesteryl [1-^{14}C]oleate were purchased from Applied Biosystems (Foster City, CA). Adenovirus plasmid carrying β-galactosidase (pAd-LacZ) has been described previously (10).

Database search and amino acid sequence analysis. We used the MOTIF search program in the GENES protein database of Kyoto Encyclopedia of Genes and Genomes (11) to search for proteins containing lipase consensus motifs and α/β hydrolase fold. We predicted the secondary structures of proteins with the programs PSIPRED and PHDsec and searched for their orthologue or paralogue of TGH-2 by SSDB in the Kyoto Encyclopedia of Genes and Genomes database (11). Alignment of the deduced protein sequence to other lipases, and calculation of the percent identity, were performed using the CLUSTAL W program.

Mice. C57BL/6 male mice were purchased from Clea (Tokyo, Japan). Mice were caged separately with 12-h light/dark cycles and given free access to standard chow diet containing 0.075% cholesterol (MF; Oriental Yeast, Osaka, Japan) in accordance with the regulations of the animal care committee of the University of Tokyo. For refeeding, animals were fasted for 24 h (from 2200 to 2200) and then refed for 12 h (2200–1000). For fasting, animals were fasted for 24 h (1000–1000).

Cell culture. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum. 3T3-L1 cells were cultured in medium A (DMEM containing 10% calf serum, supplemented with Ca-pantothenate and biotin). To induce differentiation of 3T3-L1 cells into mature adipocytes, 2-day postconfluent preadipocytes (day 0) were incubated with 1 μmol/l dexamethasone and 0.5 mmol/l 3-isobutyl-2-methylxanthine, 5 μg/ml insulin, and 1 μmol/l pioglitazone for 48 h, followed by treatment with 5 μg/ml insulin and 1 μmol/l pioglitazone for 48 h. After the incubation period, cells were switched to medium A, and the medium was renewed every other day.

Northern blot analyses. Ten micrograms of total RNA, which was isolated by TRIzol reagent (Invitrogen), were used for Northern blot analysis as described (12). Probes for murine TGH-1 (mouse orthologue of TGH) and TGH-2 were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from mouse liver as a template. Because TGH-1 and -2 are highly homologous proteins, the region that is specific to each mRNA and shares no sequence homology with the other was chosen for the construction of cDNA probe, i.e., from 1,130 to 1,260 bp for TGH-1 and from 1,130 to 1,260 bp for TGH-2. The cDNA fragment of HSL encoded by exon 8 was amplified by RT-PCR using cDNA obtained from mouse adipose tissue as a template and used as a probe (12).

Preparation of polyclonal antibody. To prepare polyclonal anti-mouse TGH-1 and -2 antisera, amino acid residues containing the catalytic domain of TGH-1 (amino acids 196–332) and TGH-2 (amino acids 196–333) were expressed in *Escherichia coli* as a glutathione S-transferase fusion protein, which was subsequently purified by glutathione affinity chromatography and used for immunization of rabbits according to the standard protocols (7). Cross-reactivities of the antisera against TGH-1 and -2 were tested using cell lysates overexpressing TGH-1 or -2 by adenovirus-mediated gene delivery.

Western blot analysis. Cells or tissues were homogenized in buffer A (50 mmol/l Tris-HCl, pH 7.0, 250 mmol/l sucrose, 1 mmol/l EDTA, and 2 μg/ml leupeptin) and centrifuged at 100,000*g* for 45 min at 4°C. The supernatant was used as S-100 cytosolic fraction, and the precipitates were resuspended and used as microsomal fraction (12). For Western blot analysis of fatcake proteins, white adipose tissue (WAT) was homogenized in buffer A and centrifuged at 100,000*g* for 45 min at 4°C, followed by extraction of fatcake proteins from the floating fat layer by the acetone precipitation method, essentially as described (7). Ten micrograms of protein from each fraction were subjected to Western blot analysis, as previously described, using an anti–TGH-1, anti–TGH-2, anti-HSL, or anti-perilipin antibody that was raised according to the described method (7). NIH Image (http://rsb.info.nih.gov/nih-image/) was used to compare the intensity of the bands.

Expression in mammalian cells. The expression vector was constructed by ligating the cDNA fragments, which were amplified by RT-PCR from mouse liver, into the TA cloning site of pTARGET vector (Promega). DNA transfection was performed with HEK293 cells plated on a six-well plate using SuperFect reagent (Qiagen). Expression vector (1.5 μg) was simultaneously transfected with pSV-βgal (0.5 μg) to measure transfection efficiency. The total amount of DNA in each transfection was adjusted to 1.5 μg/well with the mock vector. After a 48-h incubation, the cells were harvested and assayed for enzyme activity.

Construction of recombinant adenoviruses. Recombinant adenoviruses that carried murine TGH-1 and -2 cDNA under the control of cytomegalovirus promoter, designated as Ad-TGH-1 and Ad-TGH-2, respectively, were constructed as previously described (12). The recombinant adenoviruses were expanded in HEK293 cells and purified by cesium chloride ultracentrifugation (13). The purified viruses were stored in 10% (vol/vol) glycerol/PBS at −80°C. In our preparations, 1 m.o.i. (multiplicity of infection) corresponded to 25 particles of adenovirus per cell, and cells were infected at 1 m.o.i. for HEK293 cells and 1,000 m.o.i. for 3T3-L1 adipocytes. Seventy-two hours after the infection, the cells were used for the experiments.

RNA silencing. The following 21-mer sense strands of RNA oligonucleotides were designed as described (14): scrambled, 5′-CAGUCGCGUUUGCGACUG GdTdT-3′; HSL, 5′-GCCAAAGAUGAAGUGAGACdTdT-3′; TGH-1, 5′-AGAACA GCAGAGACUACCAGUdTdT-3′; and TGH-2, 5-GAAUGUAGUAGGAGACCAU dTdT-3′. The sense and antisense strands were synthesized, mixed, and annealed. At 5 days of differentiation, 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation as described (15). After incubation in medium A for 48 h, the cells were used for the experiments.

Enzyme assays. Cells were sonicated in buffer A and centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was used for the enzyme assays. Neutral cholesteryl ester hydrolase or triacylglycerol lipase activities were measured using radiolabeled triolein or cholesterol oleate, respectively, as previously described (4). PNPB esterase activity was measured as previously described (16,17). Hydrolytic activities toward monoacylglycerol, diacylglycerol, and phospholipids were determined essentially as previously described (18,19). In brief, 2-monoolein, dioleoylglycerol, dioleoyl-phosphatidylserine, dioleoyl-phosphatidylcholine, dioleoyl-phosphatidic acid, and dioleoyl-phosphatidylethanolamine were incubated with cell extracts or tissue homogenates at 37°C for 1 h. Immediately thereafter, standard pentadecanoic acid (C15:0) was added to the reaction mixture and total lipids were extracted by Bligh and Dyer's method and reconstituted in chloroform/methanol (2:1 [vol/vol]). Mass spectrometry spectra were obtained by introducing the samples into a Quattro Micro tandem quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an ESI source at the flow rate of 30 μl/min. The eluting solvent used was acetonitrile/methanol/water (2:3:1 [vol/vol/vol]) containing 0.1% ammonium formate (pH 6.4). The mass spectrometer was operated in the negative ion scan mode. The nitrogen drying gas flow rate was 12 l/min and the temperature 80°C. The capillary voltage was set at 3.7 kV and cone voltage at 50 V. Amounts of oleic acid were deduced from its mass spectrometry spectra relative to that of standard pentadecanoic acid.

Lipolysis assay. The 3T3-L1 adipocytes either infected with the recombinant adenovirus or transfected with siRNA duplexes were cultured in 12-well plates. After incubation in medium A supplemented with 2% (wt/vol) fatty acid–free BSA in the absence of serum for 12 h, cells were treated with or without 10 mmol/l isoproterenol for the indicated amounts of time and the concentrations of glycerol and FFA in the media were determined by the Triglyceride-G kit and NEFA-C kit, respectively (Wako Pure Chemicals). Cellular protein contents were measured by bincinchoninic acid protein assay (Pierce) after solubulization in 0.1 N NaOH. Amounts of glycerol or FFA in the medium were normalized to cellular protein.

Statistical analyses. Results are presented as means ± SE. Student's *t* test was used to compare the means. All calculations were performed with StatView (version 5.0; SAS Institute) for Macintosh.

RESULTS

To search for enzymes with triacylglycerol lipase activity, we screened the gene database for murine and human proteins with structural homologies to known lipases, i.e., α/β hydrolase folds (20), the GXSXG active serine motif for serine esterases, and the His-Gly dipeptide motif that is present in 70–100 amino acids' NH_2-terminal of the catalytic site serine in many lipases (21), yielding 53 candidates that include HSL, lipoprotein lipase, hepatic lipase, endothelial lipase, pancreatic lipase, carboxyl ester lipase, gastric lipase, lysosomal acid lipase, monoacylglycerol lipase, and TGH. We selected 33 candidates that had not been previously annotated, and expressed them in HEK293 cells for measurement of triacylglycerol hydrolase activity. Northern blot analysis was used to verify the expression in mouse adipose tissue. Only one previously unannotated enzyme fulfilled these requirements, and we tentatively designated it as TGH-2 because it has sequence homology to TGH (22).

The murine gene for TGH-2 (National Center for Biotechnology Information nucleotide entry BC013479) en-

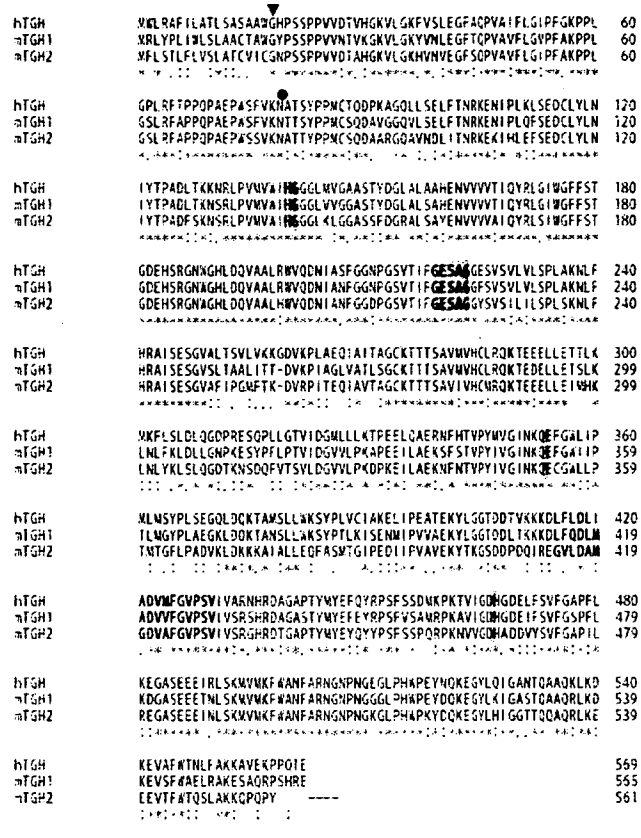

FIG. 1. Primary sequence of murine TGH-2 in comparison with human and murine TGH. The deduced amino acid sequence of murine TGH-1 (mTGH-1) and TGH-2 (mTGH-2) proteins are aligned and compared with human TGH (hTGH). The first Met residue is numbered as 1. The mature TGH proteins have been highly conserved in evolution, including *1*) conserved catalytic triad, Ser, Glu, His residues (shaded box); *2*) the His-Gly dipeptide motif (shaded box); *3*) the NH_2-glycosylation site (solid circle); and *4*) the signal peptide cleavage site (vertical arrow head), all of which are determined from the functional analysis of hTGH protein. Identical amino acids are shown by an asterisk (*). Amino acids considered strongly conserved are STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW and indicated by a colon (:). Amino acids considered weakly conserved are CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY and indicated by dots. The putative lipid binding domain is shown in boldface. Conserved cysteine residues that putatively form disulfide bonds are labeled with an open circle. A putative endoplasmic reticulum retrieval sequence (COOH-terminal HXEL) is indicated by outlined letters.

codes a 561–amino acid protein (NP_659179) with a calculated molecular mass of 61.6 kDa. Figure 1 shows alignments of amino acid sequences of murine TGH-2; its paralogue, TGH (22,23), which has also been designated as mouse carboxylesterase 3 and carboxylesterase MH1 (23); and its human orthologue (human carboxylesterase 1) (24,25). TGH-2 might be identical to carboxyesterase-ML1, based on the available partial amino acid sequence (26). TGH-2 shows 70.4% identity to murine TGH-1, which encodes a 565–amino acid protein. The human TGH gene encodes a 566–amino acid protein (NP_001257) with 77.9 and 67.0% identity to the murine TGH-1 and -2, respectively. The NH_2-terminal regions of 18 residues in both the murine and the human enzyme contain a predicted signal sequence that is cleaved in the mature human TGH protein (25). The Ser 221, Glu 353, and His 466 of the murine TGH-2 protein form a putative catalytic triad; this triad is highly conserved in human TGH, in which mutation of any of these triad residues to Ala abolishes TGH esterase activity (25). A potential NH_2-glycosylation site could be deduced at Asn 79 from the alignment with human TGH (25). The sequence contains four conserved Cys residues that are predicted to form disulfide bonds: Cys 87 to 116 and Cys 273 to Cys 284 (25). The protein sequence also contains a hydrophobic stretch of amino acids that may be involved in lipid binding: Gly 414 to Val 429. A COOH-terminal HXEL sequence possibly functions as an endoplasmic reticulum retrieval signal. In contrast to HSL, neither TGH-1 nor -2 contains putative phosphorylation site for protein kinase A.

A homology search revealed the presence of related proteins in a wide range of organisms, including animals, plants, and bacteria. In humans, two proteins, LOC390732 (XP_372639) and carboxylesterase 4–like (NP_057364), have a high degree of homology to human TGH (67.7 and 92.9%, respectively). These proteins appear to be distinct proteins and have been implicated as carboxylesterase. In mice, three other proteins, 2310039D24Rik (XP_134476), LOC244595 (XP_146438), and LOC382044 (XP_356117), have a high sequence similarity to murine TGH-2 (75.4, 67.5, and 63.8%, respectively). Homologous proteins are also found in *Drosophila melanogaster* and *Caenorhabditis elegans*. Interestingly, additional putative proteins sharing some degree of homology are present in *Arabidopsis thaliana*, *Cyanidioschyzon merolae*, and others in prokaryotes, including *Xanthomonas campestris*, *Bacillus subtilis*, and *Mycobacterium$_{avium}$ paratuberculosis*. The presence of TGH-1 and -2, as well as other related proteins, in such a wide variety of organisms suggests that TGH-1 and -2 comprise a subfamily of carboxyesterases mediating basic cellular functions that is not exclusive of higher organisms.

Northern blot analysis was performed to compare the mRNA expression of TGH-2 with that of HSL and TGH-1 in various tissues. The tissue distribution of TGH-2 mRNA expression is remarkably similar to that of TGH-1 except in intestine: they are expressed at high levels in WAT, brown adipose tissue (BAT), liver, and kidney but to a lesser degree in adrenal, ovary, and heart (Fig. 2). TGH-2, but not TGH-1, is highly expressed in intestine. To determine whether they are expressed in adipocytes in a differentiation-dependent manner, we performed Northern blot analysis of TGH-1, TGH-2, and HSL at 0, 2, 5, and 8 days postinduction of 3T3-L1 adipocyte differentiation (Fig. 2*B*). Like HSL, mRNA expression of both TGH-1 and -2 was induced during differentiation of 3T3-L1 adipocytes.

Because of the high sequence similarities between the peptides that were used for immunization, it is possible that the antibodies cross-react with TGH-1 and -2. To exclude this possibility, we performed Western blot analysis using the antibodies against TGH-1 or -2 (Fig. 3*A*). The antibody against TGH-1 detected TGH-1 but not TGH-2, which were both overexpressed in HEK293 cells over the background level. On the other hand, the antibody against TGH-2 detected TGH-2 but not TGH-1, which were both overexpressed in HEK293 cells over the background level. These results indicate that cross-reaction of the antibodies is negligible.

To determine subcellular localization of TGH-1, TGH-2, and HSL, we performed subcellular fractionation of WAT and BAT by ultracentrifugation. Substantial amounts of TGH-1, TGH-2, and HSL were present in fatcake fraction (Fig. 3*B*), corroborating the idea that TGH-1 and -2 mediate hydrolysis of neutral lipids in fat droplets where perilipin colocalizes. Unlike fatcake-specific perilipin, TGH-1, TGH-2, and HSL were also present in cytosolic and

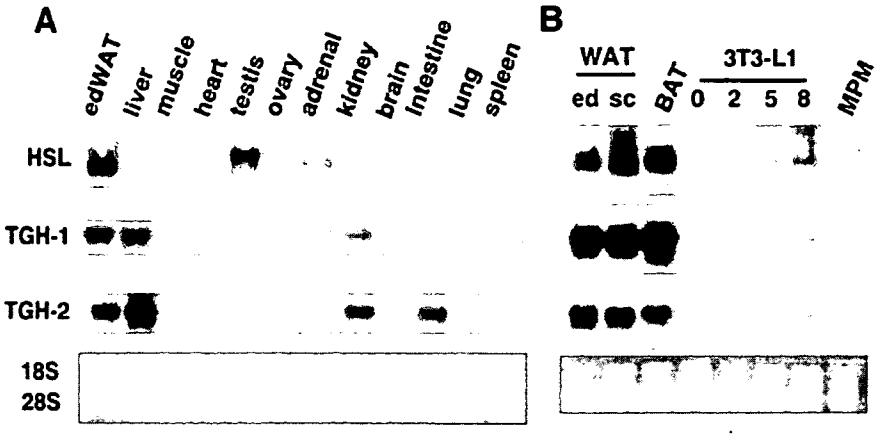

FIG. 2. Northern blot analysis of HSL, TGH-1, and TGH-2. Total RNA (10 μg) from tissues and mouse peritoneal macrophages (MPM) of C57BL/6J mice (*A* and *B*) and 3T3-L1 adipocytes at various stages of differentiation (*B*) were subjected to Northern blot analysis. Specific mRNAs were detected with a radiolabeled cDNA probes for HSL, TGH-1, or TGH-2. Ethidium bromide staining of gels is shown as a loading control. ed, epididymal; sc, subcutaneous.

microsomal fraction. There were no obvious differences in their distribution between subcutaneous and epididymal WAT and BAT (Fig. 3*C*).

To determine the types of lipids that TGH-2 hydrolyzes, we infected HEK293 cells with recombinant adenoviruses carrying expression cassettes of LacZ, HSL, TGH-1, or TGH-2. Whole-cell extracts of cells infected with Ad-TGH-1 or Ad-TGH-2 exhibited substantial amounts of hydrolytic activities toward PNPB, triacylglycerol, or monoacylglycerol (Table 1). Both TGH-1 and -2 hydrolyzed PNPB, a short-chain fatty acid ester, more preferentially than triolein, a long-chain fatty acid ester. Their preference was ~50- to 60-fold higher than that of HSL. However, neither TGH-1 nor TGH-2 showed detectable hydrolytic activities toward diacylglycerol, cholesteryl ester, or phospholipids. Thus, TGH-2 is indistinguishable from TGH-1 in their substrate specificity. The substrate specificity of HSL is different from TGH-1 and -2 in that it prefers triacylglycerol, diacylglycerol, and cholesteryl ester compared with TGH-1 and -2.

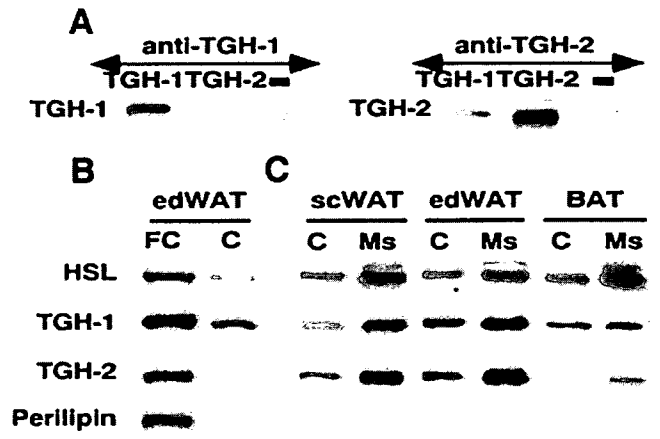

FIG. 3. Western blot analysis of HSL, TGH-1, and TGH-2. *A*: Cross-reaction of the antibodies. We overexpressed TGH-1 or -2 in HEK293 cells by plasmid transfection and used these cell lysate for Western blot analysis. Anti–TGH-1 and anti–TGH-2 detected the overexpressed TGH-1 and -2, respectively, but did not cross-react with TGH-2 and -1 over the background levels, respectively. *B* and *C*: Subcellular distribution of the lipases. Tissues of C57BL/6J mice were homogenized and centrifuged at 100,000*g*. Fatcake proteins (FC), which were extracted from floating fat layers, and supernatant (C) were subjected to Western blot analysis (*B*). Supernatant or microsomal (Ms) fractions were subjected to Western blot analysis (*C*). ed, epididymal; sc, subcutaneous.

To determine whether TGH-2 is a lipolytic enzyme that contributes to the lipolysis in adipocytes, we examined the effects of knockdown of TGH-2 by RNA silencing on the lipolysis in 3T3-L1 adiocytes (Fig. 4). Western blot analyses revealed that transfection of siRNA duplexes for HSL, TGH-1, or TGH-2 inhibited the expression of respective lipases by 82, 86, and 86%, respectively (Fig. 4*A*). When the cells were not stimulated with isoproterenol, the RNA interference for HSL, TGH-1, or TGH-2 did not significantly inhibit the amounts of either glycerol or FFA release at 3 h of the incubation ($n = 3$). When the cells were stimulated by isoproterenol, the RNA interference for HSL, TGH-1, or TGH-2 inhibited the amounts of glycerol release by 30% ($P < 0.05$), −2%, and 10% ($P < 0.05$), respectively (Fig. 4*B*), whereas it inhibited the amounts of FFA release by 36% ($P < 0.05$), 3%, and 6%, respectively, at 3 h after the stimulation with isoproterenol ($n = 3$).

To determine whether TGH-2 is involved in lipolysis in adipocytes, we infected 3T3-L1 adipocytes with Ad-TGH-2 and measured the amounts of FFA and glycerol released with or without isoproterenol (Fig. 5). In the absence of isoproterenol, we could not detect any differences in lipolysis. Isoproterenol stimulated basal lipolysis, as shown in the cells infected with Ad-LacZ. Overexpression of TGH-2 resulted in 3.6- and 2.0-fold increases in the protein expression in cytosolic and microsomal fractions, respectively. These changes were associated with further increases in the release of glycerol by 20% ($P < 0.05$) (Fig. 5*B*) and FFA by 26% (Fig. 5*C*) at 3 h after the stimulation with isoproterenol, supporting the idea that TGH-2 is involved in lipolysis.

To explore the possibility that mRNA expression of TGH-1 and -2 is regulated in response to feeding cycle, we examined changes in the mRNA expression of TGH-1 and -2 in various tissues, including WAT and BAT, after fasting or refeeding in comparison with the expression of HSL (Fig. 6). In contrast to HSL, whose expression was relatively stable during the fasting/refeeding cycle, mRNA levels of both TGH-1 and -2 were relatively low in a refed state and increased by fasting in WAT and BAT. In the liver, intestine, and kidney, TGH-2 was upregulated in the refed state, whereas this did not occur in WAT.

DISCUSSION

Here we report identification and characterization of a previously undescribed TGH that is predominantly expressed in adipose tissue. Because this enzyme has high

TABLE 1
Comparison of substrate specificity of HSL, TGH-1, and TGH-2

Substrates	LacZ	HSL	TGH-1	TGH-2
PNPB (nmol · min^{-1} · mg^{-1})	59	5,258	9,494 ± 1,268	14,349 ± 2,289
Triolein (pmol · min^{-1} · mg^{-1})	78 ± 20	9,581	429 ± 15	506 ± 35
Diolein (pmol · min^{-1} · mg^{-1})	10.3	113.3	10.5	10.0
Monoolein (pmol · min^{-1} · mg^{-1})	40.5	184.5	190.0	217.7
Cholesteryl oleate (pmol · min^{-1} · mg^{-1})	8.3 ± 4.5	1,184 ± 22	3.7 ± 0.5	2.6 ± 0.3
Phospholipid (pmol · min^{-1} · mg^{-1})	7.3	15.2	9.0	9.0

Data are means (duplicate measurements) or means ± SE (triplicate measurements). We overexpressed LacZ, HSL, TGH-1, or TGH-2 by adenovirus-mediated gene delivery and used whole-cell extracts for measurements of hydrolytic activities. Hydrolytic activities for PNPB were measured by colorimetric assay, hydrolytic activities for triolein and cholesteryl oleate were measured by determining the release of radiolabeled oleate, and hydrolytic activities for monoolein, diolein, and phospholipids were measured by determining the release of oleic acid by mass spectrometry. Since different buffers were used for different assays, care should be taken when making comparison between different methods.

sequence homology to TGH, we have designated the original TGH as TGH-1 and the newly identified one as TGH-2. These enzymes belong to a carboxylesterase family and have remarkable similarity in terms of tissue distribution, subcellular localization, substrate specificity, and regulation.

Recently, three laboratories have independently reported the cloning of a novel triacylglycerol lipase expressed in adipocytes (27–29). This protein, designated as desnutrin and adipose triglyceride lipase (ATGL) in mice and Ca^{2+}-independent PLA2 (iPLA2)ζ in humans according to each laboratory, contains α/β hydrolase fold and GXSXG active serine motif, is expressed in adipose tissue, is localized in both cytosol and lipid droplets, and mediates hydrolysis of triacylglycerol but not cholesteryl ester or diacylglycerol, thereby contributing to adipocyte lipolysis (28) and acylglycerol transacylation to synthesize diacylglycerol or triacylglycerol (29). The *Drosophila* orthologue Brummer lipase has also been reported (30). There are two other paralogues of ATGL: adiponutrin in mice (31), which is also called iPLA2ε in humans (29), and iPLA2η in humans (29). Desnutrin/ATGL/iPLA2ζ was not found in our database search because it does not contain an oxyanion His-Gly dipeptide motif. Instead, it contains a patatin domain that is conserved from plants to mammals (28). According to Zimmermann et al. (28) and Kershaw et al. (32), silencing ATGL gene by siRNA or antisense RNA markedly decreased lipolysis in stimulated and nonstimulated 3T3-L1 adipocytes, supporting the notion that desnutrin/ATGL/iPLA2ζ is the major non-HSL lipase mediating lipolysis in adipocytes.

Despite these substantial evidences for the involvement of desnutrin/ATGL/iPLA2ζ in the lipolysis, TGH-2 as well as TGH-1 may contribute to the residual triacylglycerol lipase activity in HSL-deficient adipocytes (4,6,7). First, the substrate specificity of TGH-1 and -2 is consistent with the characteristics of the residual lipase activity in HSL-deficient WAT: substantial triacylglycerol lipase activity with almost complete absence of neutral cholesteryl ester hydrolase and diacylglycerol lipase activity. When overexpressed in HEK293 cells, TGH-1 and -2 were both able to hydrolyze triacylglycerol and monoacylglycerol but not diacylglycerol, cholesteryl ester, or phospholipids (Table 1). Second, expression of TGH-1 and -2 was increased during the differentiation of 3T3-L1 adipocytes (Fig. 2*B*). Third, both fatcake and cytosolic fractions contain TGH-1 and -2 , which is consistent with the residual triacylglycerol lipase activity present in HSL-deficient WAT (Fig. 3) (7). Indeed, Soni et al. (17) have recently reported that TGH, carboxylesterase 3, is the major protein present in the second peak, with substantial PNPB esterase activity that was eluted from oleic acid–agarose chromatography of infranatant as well as fatcake fractions from mouse adipose tissue. However, our studies on the inhibition or overexpression of TGH-1 and -2 (Figs. 4 and 5) indicate that TGH-1 and -2 appear to make a relatively minor contribution to net triacylglycerol hydrolysis in adipose tissues compared with other adipocyte lipases such as desnutrin/ATGL/iPLA2ζ and HSL.

The robust induction of the expression of TGH-1 and -2 during fasting is intriguing from the point of view of energy homeostasis. Fasting also induces the expression of desnutrin/ATGL/iPLA2ζ (27,32). Induction of these multiple lipases is reasonable physiological response to fasting/starvation if these enzymes mediate lipolysis that may have evolved for adaptation to fasting/starvation. Desnu-

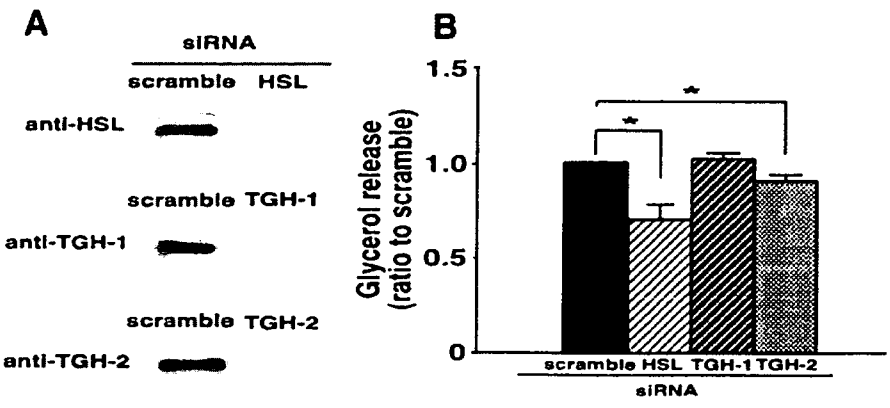

FIG. 4. Inhibition of TGH-2 by RNA silencing and its effect on lipolysis in 3T3-L1 adipocytes. At 5 days of differentiation, 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation. After 48 h, the cells were treated with isoproterenol for 3 h. Cell lysates were used for Western blot analysis (*A*), and the amounts of glycerol in the medium were measured and divided by the values for scramble siRNA in each experiment after normalizing to the cellular protein (*B*). Data are expressed as means ± SE of four independent experiments. *$P < 0.05$ vs. control scramble siRNA.

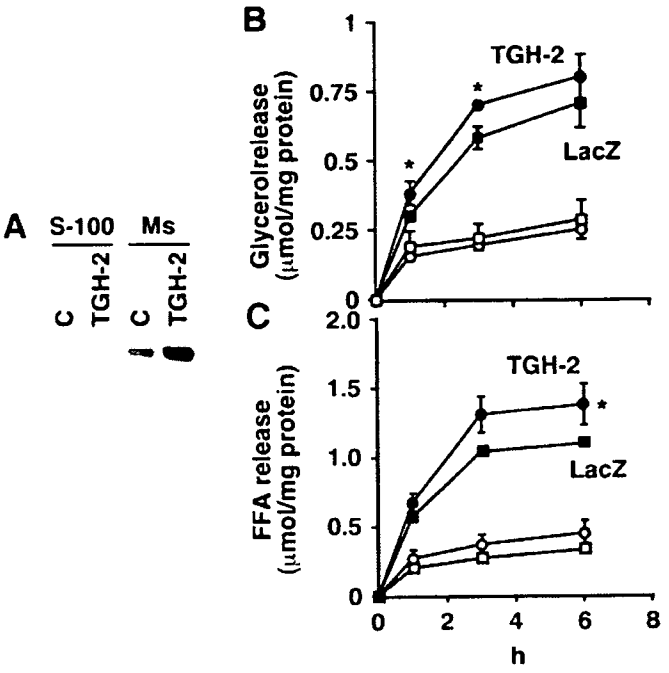

FIG. 5. Overexpression of TGH-2 by recombinant adenovirus and its effect on lipolysis in 3T3-L1 adipocytes. Recombinant adenoviruses carrying LacZ (Ad-LacZ) or TGH-2 (Ad-TGH2) were used to infect 3T3-L1 adipocytes on day 8 of differentiation, and experiments were performed 3 days after infection. *A*: Overexpression of TGH-2 protein was verified by Western blot analysis using cytosolic (S-100) and microsomal (Ms) fraction of 3T3-L1 adipocytes infected with Ad-TGH2 or Ad-LacZ for 72 h. *B* and *C*: Cells overexpressing TGH-2 (circle) or LacZ (square) were incubated in DMEM/2% fatty acid–free BSA in the absence (□ and ○) or presence (■ and ●) of 10 μmol/l isoproterenol for the indicated times, and glycerol (*B*) and FFA (*C*) released into the media were measured. Data are expressed as means ± SE of triplicate measurements. **P* < 0.05, vs. control Ad-LacZ–infected cells. C, control; Ms, microsomal fraction.

trin/ATGL/iPLA2ζ is primarily localized in cytosol and fatcake (28), while TGH-1 and -2 are mainly localized in fatcake and microsome (Fig. 3*B* and *C*). This difference in subcellular localization suggests that these lipases play distinct roles in response to fasting.

In addition to adipose tissues, TGH-1 and -2 are expressed in a wide variety of nonadipose tissues. Liver is the organ where both TGH-1 and -2 are most abundantly expressed. Lehner and colleagues (9,33,34) have previously assigned the function of hepatic TGH to VLDL assembly. This may be the case with TGH-2, since the expression of TGH-2 was increased in a refed state after VLDL production was stimulated. A similar role in lipoprotein assembly can be assigned to TGH-2, which is uniquely expressed in intestine where chylomicron is produced. Clearly, these intriguing possibilities await further investigation.

It is also noteworthy that TGH-1 and -2 have a much higher substrate specificity for short-chain carbonic acid esters than long-chain triacylglycerol (Table 1). Indeed, murine TGH is identical to carboxylesterase 3, also designated carboxylesterase MH1, which has been identified as a carboxylesterase isozyme induced by di-(2-ethylhexyl)phthalate, a peroxisome proliferator (23). Furthermore, based on the available partial amino acid sequence, TGH-2 itself might be identical to carboxylesterase-ML1 (26,35). From these considerations, both enzymes might be better designated by their original names, carboxylesterase-3 for TGH-1 and carboxylesterase-ML1 for TGH-2.

Our results in Table 1 do not appear to be consistent with the previous findings that HSL hydrolyzes diacylglycerol better than triacylglycerol (36,37). These contradictions might result from the difference in the sensitivity of two different methods used in our study: measurements of the end products, FFAs, by radioactivity versus mass spectrometry. The method that used mass spectrometry omitted phospholipids from the reaction mixture to avoid generation of FFAs from the hydrolysis of phospholipids; this could profoundly impair the sensitivity of the assay. When the triacylglycerol lipase activities of HSL were directly measured by the method that used mass spectrometry, its specific triacylglycerol lipase activity was calculated to be 6.9 pmol · min^{-1} · mg^{-1}. This value was 15-fold smaller than the diacylglycerol lipase activity mea-

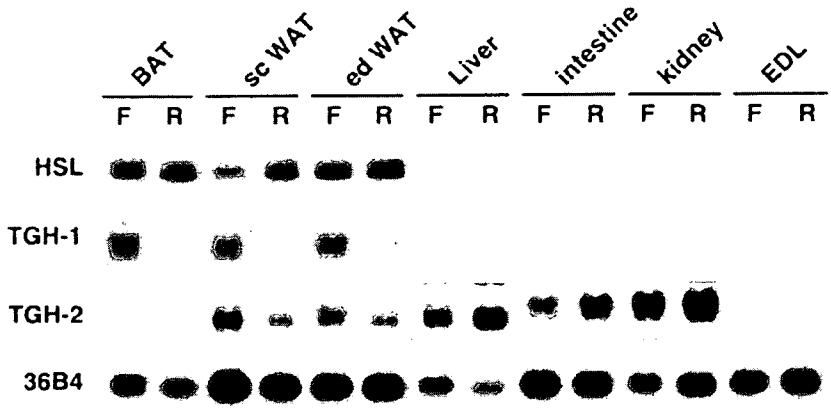

FIG. 6. Changes in the expressions of TGH-1 and -2 mRNA during fasting/refeeding. Total RNA (10 μg) from tissues of mice fasted for 24 h (F) or fasted and then refed for 12 h (R) were subjected to Northern blot analysis. Specific mRNAs were detected with a radiolabeled cDNA probes for HSL, TGH-1, TGH-2, or 36B4. ed, epididymal; EDL, extensor digitorum longus; sc, subcutaneous.

sured by the same method (103 pmol · min^{-1} · mg^{-1}) (Table 1), roughly corroborating the ratios in the literature (36,37).

In conclusion, we have identified a novel member of the carboxylesterase family, TGH-2, with structural, catalytic, and expressional similarity to TGH or TGH-1. TGH-1 and -2 are highly expressed in adipocytes and are transcriptionally regulated in response to changes in nutritional conditions, suggesting their potential role in adipocyte lipolysis. The elucidation of the true function of these "redundant" lipases awaits further in vivo studies of the gain or loss of function by pharmacologic and/or genetic manipulation.

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JOURNAL OF LIPID RESEARCH

Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver

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Abstract **Squalene synthase (SS) is the first committed enzyme for cholesterol biosynthesis, located at a branch point in the mevalonate pathway. To examine the role of SS in the overall cholesterol metabolism, we transiently overexpressed mouse SS in the livers of mice using adenovirus-mediated gene transfer. Overexpression of SS increased de novo cholesterol biosynthesis with increased 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase activity, in spite of the downregulation of its own mRNA expression. Furthermore, overexpression of SS increased plasma concentrations of LDL, irrespective of the presence of functional LDL receptor (LDLR). Thus, the hypercholesterolemia is primarily caused by increased hepatic production of cholesterol-rich VLDL, as demonstrated by the increases in plasma cholesterol levels after intravenous injection of Triton WR1339. mRNA expression of LDLR was decreased, suggesting that defective LDL clearance contributed to the development of hypercholesterolemia. Curiously, the liver was enlarged, with a larger number of Ki-67-positive cells. These results demonstrate that transient upregulation of SS stimulates cholesterol biosynthesis as well as lipoprotein production, providing the first in vivo evidence that SS plays a regulatory role in cholesterol metabolism through modulation of HMG-CoA reductase activity and cholesterol biosynthesis.**—Okazaki, H., F. Tazoe, S. Okazaki, N. Isoo, K. Tsukamoto, M. Sekiya, N. Yahagi, Y. Iizuka, K. Ohashi, T. Kitamine, R-i. Tozawa, T. Inaba, H. Yagyu, M. Okazaki, H. Shimano, N. Shibata, H. Arai, R-z. Nagai, T. Kadowaki, J-i. Osuga, and S. Ishibashi. **Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver.** ***J. Lipid Res.*** **2006. 47: 1950–1958.**

Supplementary key words lipoprotein • adenovirus • hyperlipoproteinemia • 3-hydroxy-3-methyglutaryl-CoA reductase • farnesyl diphosphate • mevalonate • hepatomegaly • cell proliferation • feedback regulation

Cholesterol biosynthesis is subject to tight regulation by a multivalent feedback mechanism at both the transcriptional and the posttranscriptional level (1). The transcriptional regulation is mediated through the action of sterol-regulatory element binding proteins (SREBPs), membrane-bound transcription factors that enhance transcription of genes encoding cholesterol biosynthetic enzymes and the LDL receptor (LDLR) (2). The translational regulation of 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase (EC1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis, is mediated by nonsterol mevalonate-derived isoprenoids, which act by an undefined mechanism (3). Its degradation is regulated by both sterols and nonsterol end products of mevalonate metabolism (4, 5). The sterol-regulated degradation of HMG-CoA reductase is mediated

Abbreviations: Ad-SS, recombinant adenovirus carrying SS cDNA under the control of cytomegalovirus promoter; apoB, apolipoprotein B; E, embryonic day; ER, endoplasmic reticulum; LDLR, LDL receptor; m.o.i., multiplicity of infection; SREBP, sterol-regulatory element binding protein; SS, squalene synthase; TC total cholesterol; TG, triglyceride; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling.

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by the binding of a pair of endoplasmic reticulum (ER) retention proteins called Insig-1 and Insig-2 to the sterol-sensing domain in membrane-spanning segments of HMG-CoA reductase (6, 7).

Inhibition of cholesterol biosynthesis in the liver by using inhibitors of HMG-CoA reductase, collectively called statins, is a widely used strategy for preventing heart attacks by lowering plasma cholesterol. However, statins have limited effects (8), partly because the deficiency of mevalonate-derived products following the inhibition of HMG-CoA reductase activity leads to a compensatory increase in HMG-CoA reductase protein, invoking the need for higher doses of statins (9), and to occasional severe adverse effects, such as rhabdomyolysis (10). Therefore, exploration of another target for the inhibition of cholesterol biosynthesis is needed.

Squalene synthase (SS, or farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC2.5.1.21) catalyzes the reductive head-to-head condensation of two molecules of farnesyl diphosphate to form squalene, the first specific intermediate in the cholesterol biosynthetic pathway (11, 12). SS is unique among other enzymes in cholesterol biosynthesis in that, in addition to the sterol-mediated transcriptional control through the action of SREBPs (13), expression of the *SS* gene is regulated by lipopolysaccharide, which potently inhibits SS, whereas it stimulates *HMG-CoA reductase* expression (14). SS is an attractive target for cholesterol-lowering therapy, because the inhibition of this step theoretically may not perturb the nonsterol pathway. Indeed, several potent SS inhibitors successfully lower plasma cholesterol levels without the potential limitations reported for statins (15–17).

To elucidate the role of SS in cholesterol metabolism, we have generated SS knockout mice. *SS* knockout mice ($SS^{-/-}$) were lethal between embryonic day (E) 9.5 and E 12.5 and exhibited severe retardation of development and defective neural tube closure, indicating that SS is essential for development, particularly of the central nervous system (18). Although $SS^{+/-}$ mice expressed only 50% SS activities in the liver compared with $SS^{+/+}$ mice, their plasma lipoprotein profiles and responses to dietary challenges were indistinguishable from those of $SS^{+/+}$ mice. These observations closely resemble those seen in *HMG-CoA reductase* knockout mice: *HMG-CoA reductase*$^{-/-}$ mice were embryonic lethal, and *HMG-CoA reductase*$^{+/-}$ mice showed no significant changes in lipoprotein profiles (19). However, the role of SS in cholesterol metabolism in adult liver remains largely unknown.

To better understand the role of SS in overall cholesterol metabolism in vivo in adult liver, we have used adenovirus-mediated gene delivery to overexpress SS in the liver and have examined changes in hepatic cholesterol biosynthesis and plasma lipoprotein profiles in vivo. We show here that increased SS activity in liver leads to increased cholesterol synthesis, as well as secretion of cholesterol-rich lipoproteins, providing the first in vivo evidence that SS plays a regulatory role in the development of hypercholesterolemia.

MATERIALS AND METHODS

Construction of recombinant adenoviruses

Recombinant adenovirus that carried murine SS cDNA under the control of the cytomegalovirus promoter, designated as Ad-SS, was constructed using the cDNA cloned by RT-PCR from mouse liver as described previously (20, 21). The recombinant adenoviruses were expanded in HEK293 cells and purified by cesium chloride ultracentrifugation. The purified viruses were stored in 10% (v/v) glycerol in PBS at −80°C. In our preparations, 1 multiplicity of infection (m.o.i.) corresponded to 25 particles of adenovirus per cell, and cells were infected at 300 m.o.i.

Animal studies

C57BL/6 male mice and LDLR knockout mice (22) that had been backcrossed into the C57BL/6 background were purchased from Charles River Japan, Inc. (Yokohama, Japan) and Jackson Laboratory (Bar Harbor, ME), respectively. Mice were maintained and cared for according to the regulations of the Animal Care Committees of the University of Tokyo and Jichi Medical University. Mice were caged separately, with 12 h light/dark cycles, and were given free access to standard chow diet containing 0.075% cholesterol (MF; Oriental Yeast Co., Ltd., Osaka, Japan). Mice were injected intravenously with 1.5×10^{11} particles (6×10^{9} plaque-forming units) of Ad-LacZ or Ad-SS. Each group contained six mice. Seven days after virus injection, food was withdrawn 4 h before collection of blood samples from the retroorbital plexus of anesthetized animals. Tissues were immediately collected, snap frozen in liquid nitrogen, and stored at −80°C.

Real-time RT-PCR

Total RNA was prepared from livers with TRIzol reagent (Life Technologies, Inc.). Two micrograms of total RNA was reverse transcribed (Taq Man Reverse Transcription Reagents; Applied Biosystems, Foster City, CA), and synthesized cDNA was quantified using Taq Man quantitative PCR analysis of each gene with the ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacture's protocol. The specific primer pairs for SS, HMG-CoA reductase, FAS, LDLR, apolipoprotein B (apoB), CYP7A, and SREBP-1a were designed as previously described (23); the others were purchased from Applied Biosystems. The relative amounts of all mRNAs were calculated by using the comparative $2^{-\Delta\Delta CT}$ method (User Bulletin, Applied Biosystems). β-actin mRNA was used as the invariant control.

Western blot analysis

Livers were homogenized in a buffer containing 15 mM nicotinamide, 2 mM $MgCl_2$, and 100 mM potassium phosphate, pH 7.4, and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants were centrifuged at 105,000 *g* for 1 h at 4°C, and the resultant pellets, a microsome fraction, were washed, resuspended in the same buffer, and stored in aliquots at −80°C. The supernatant was used for Western blot analysis as described previously (21), using an anti-SS antibody that was raised by immunizing rabbits with glutathione S-transferase fusion protein containing amino acid sequence 174–339 of mouse SS. For Western blot of HMG-CoA reductase, we prepared and solubilized the liver membrane fraction exactly as described by Sever et al. (7) and used antibodies from Upstate Cell Signaling Solutions (Charlottesville, VA) and from Dr. Y. K. Ho at University of Texas Southwestern Medical Center at Dallas.

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