Fatty acids released from adipose triacylglycerol stores by lipolysis provide veebrates with an important source of energy. We investigated the role of microsomal triacylglycerol hydrolase (TGH) in the mobilization of adipocyte triacylglycerols through inactivation of the TGH activity by RNA interference or chemical inhibition. Attenuation of TGH activity resulted in decreased basal but not isoproterenol-stimulated efflux of fatty acids from 3T3-L1 adipocytes. Lack of TGH activity was accompanied by accumulation of cellular triacylglycerols and cholesterol esters without any changes in the expression of enzymes catalyzing triacylglycerol synthesis (diacylglycerol acyltransferases 1 and 2) or degradation (adipose triglyceride lipase and hormone-sensitive lipase). Inhibition of TGH-mediated lipolysis also did not affect insulin-stimulated Glut4 translocation from intracellular compartments to the plasma membrane or glucose uptake into adipocytes. These data suggest that TGH plays a role in adipose tissue triacylglycerol metabolism and may be a suitable pharmacological target for lowering fatty acid efflux from adipose tissue without altering glucose import.

Obesity is a chronic disease with increasing prevalence in most of the world but particularly in Western countries (1). It is estimated that obesity-related illnesses cause about 300,000 deaths per year in the United States alone (2). Obese individuals are at risk for a number of medical conditions including cardiovascular disease, hypertension, dyslipidemias, and type 2 diabetes. Obese insulin-resistant subjects have higher levels of circulating fatty acids due to increased flux of free fatty acids (FA) released from adipose tissue triacylglycerol (TG) stores. As a consequence, accumulation of excess lipid and lipid precursors in non-adipose tissues ensues and culminates in cell dysfunction and/or cell death, a condition known as lipotoxicity (3). One of the strategies that may lead to decreased circulating fatty acid levels and increased insulin sensitivity is through pharmacological inhibition of lipolysis of adipose tissue TG. The enzymes that have been postulated to mediate hydrolysis of adipose tissue TG and, thus, provide an energy source (FA) to other tissues are hormone-sensitive lipase (HSL) (4) and the recently discovered adipose triglyceride lipase (ATGL) (5), also termed desnutrin (6) or calcium-independent phospholipase A2 (7). About 40% of TG lipolytic activity is retained in the white adipose tissue of HSL null mice (8, 9), and inhibition of ATGL in cytosols prepared from HSL null adipocytes decreases total lipase activity by 80% (5). Therefore, it can be concluded that ATGL together with HSL accounts for the majority of isoproterenol-stimulated cytosolic activity in the adipose tissue.

We have characterized another neutral intracellular TG hydrolase (TGH) in hepatic microsomes (10). TGH (also termed Ces3) associates with hepatic lipid droplets (11) and hydrolyzes stored hepatic TG (12–15). TGH is also expressed in 3T3-L1 adipocytes (16, 17) and adipose tissue (18–20) where it has been also shown to associate with adipocyte lipid droplets (19). Therefore, we hypothesized that the hydrolase may play a similar role in the mobilization of adipocyte TG as it does in the liver.

It has been reported that TGH (named p62/carboxylesterase in that report) co-fractionated with glucose transporter 4 (Glut4)-containing membranes isolated from rat adipocytes (21). In addition, introduction of neutralizing anti-TGH antibodies into permeabilized adipocytes abolished the insulin-induced Glut4 recruitment to plasma membrane-enriched fractions. Therefore, it was concluded that TGH plays a role in Glut4 recruitment, although the precise role of the enzyme in this process remained uncharacterized. Recent data suggest that ablation of HSL expression leads to decreased deoxy-d-glucose uptake (22). HSL deficiency does not prevent adipose TG lipolysis but affects diacylglycerol (DG) breakdown, which results in the accumulation of DG (9). DGs activate the protein kinase C signaling pathway, which results in phosphorylation and inactivation of the insulin receptor tyrosine kinase activity (for review, see Ref. 23). Thus, the decrease of glucose uptake in HSL null adipocyte appears to be the result of alteration in insulin signaling.

Here we demonstrate that inhibition of basal lipolysis by TGH attenuates FA efflux but does not lead to accumulation of

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The abbreviations used are: FA, fatty acid; ATGL, adipose triglyceride lipase; DG, diacylglycerol; DMEM, Dulbecco’s modified Eagle’s medium; E600, diethyl-p-nitrophenylphosphate; ER, endoplasmic reticulum; Glut4, glucose transporter 4; HSL, hormone-sensitive lipase; PDI, protein disulfide isomerase; TG, triacylglycerol; TGH, TG hydrolase; BSA, bovine serum albumin; TGH, 4,4,4-trifluoro-2-((2-(3-methylphenyl)hydrazono)-1-(2-thienyl)butane-1,3-dione; shRNA, short-hairpin RNA; PBS, phosphate-buffered saline.
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DG in mature 3T3-L1 adipocytes. Furthermore, we show that TGH does not co-localize with Glut4 in 3T3-L1 adipocytes in either basal or insulin-stimulated conditions and that inhibition of TGH-catalyzed TG lipolysis in 3T3-L1 adipocytes does not prevent insulin-stimulated Glut4 translocation from intracellular compartment to plasma membrane or insulin-stimulated glucose uptake in intact cells.

EXPERIMENTAL PROCEDURES

Materials—Dexamethasone, 3-isobutyl-1-methylxanthine, bovine insulin, horseradish peroxidase-labeled goat anti-rabbit IgG, diethyl-p-nitrophenylphosphate (E600), 4-methylumbelliferyl heptanoyl, sodium taurodoxycholate, Triton X-100, egg yolk phosphatidylcholine, oleic acid, dioleoylglycerol, trioleoylglycerol, cholereyst oleate, essentially fatty acid-free bovine serum albumin (BSA), and isoproterenol were purchased from Sigma. A TGH-specific inhibitor, 4,4,4-trifluoro-2-[2-(3-methylphenyl)hydrazono]-1-(2-thienyl)butane-1,3-dione (TGHI), was provided by GlaxoSmithKline. Nicotinucleo membranes, SDS-PAGE reagents, and molecular mass markers were purchased from Bio-Rad. Anti-calnexin rabbit polyclonal antibodies and anti-protein disulfide isomerase (PDI) mouse monoclonal antibodies were obtained from Stressgen Biotechnologies, Victoria, BC, Canada. The enhanced chemiluminescent (ECL) substrate kit, 3-O-methyl-D-[1-3H]glucose (5 Ci/mmole) and [9,10(n)-3H]oleic acid (8 Ci/mmole), were from Amersham Biosciences. Goat anti-Glut4 polyclonal antibodies were obtained from Santa Cruz Biotechnology, CA. Fluorescein (fluorescein isothiocyanate)-conjugated donkey anti-goat IgG and Texas Red-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories. ProLong Antifade kit was purchased from Molecular Probes. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Invitrogen.

All other reagents were of analytical or higher grade.

Differentiation of 3T3-L1 Fibroblasts—3T3-L1 fibroblasts obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM containing 10% fetal bovine serum. Cells were grown in 6-well or 60-mm culture dishes except for confocal studies, where the cells were grown on the coverslips. Cells were cultured in growth media till confluent. Two days after reaching confluency, cells were induced to differentiate by the addition of 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 3 μg/ml insulin for 3 days. Cells were then washed and incubated in normal growth media till differentiated. The media were changed every 2 or 3 days. Unless otherwise noted, experiments were performed with adipocytes at 6 days post-differentiation.

Construction of Plasmid Expressing a Short-hairpin RNA for Mouse TGH—The pSilencer™ 4.1-CMV hygro vector (Ambion, Austin, TX) was used to produce TGH short-hairpin RNA (shRNA) expression vector encoding the sense and antisense template of mouse TGH from nucleotides 840 – 860 bp of the coding sequence. The following oligonucleotides were used: shRNA sense template, 5'-GAT CCA GCC CAT TGC TGG TCT GGT TTC AAG AGA ACC AGA CCA GCA ATG GGC TTT A-3' and shRNA antisense template, 5'-AGC TTA AAG CCC ATT GCT GTG CTT TTC TTG AAA CCA GAC CAG CAA TGG GCT G-3'. The annealed shRNA templates were ligated into pSilencer™ 4.1-CMV hygro vector, which was digested with BamHI and Hind III.

Stable Transfection of 3T3-L1 Fibroblasts and Characterization of Stable Clones—3T3-L1 fibroblasts at 50% confluency in 100-mm dishes were transfected with 10 μg of pSilencer™ 4.1-CMV hygro or shRNA construct using the calcium phosphate precipitation method. Cells were grown for 48 h after transfection and then passaged 1:3 into media containing 0.4 mg/ml hygromycin B. To characterize the stable cell lines, the selected single colonies were induced to differentiate, and microsomes were isolated at 6 days post-differentiation to analyze TGH protein levels by immunoblotting and enzyme activity using 4-methylumbelliferyl heptanoyl as a substrate.

RNA Isolation and Reverse Transcription PCR Analysis—Total RNA was isolated from differentiated 3T3-L1 stable cell lines using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis from 2 μg of total RNA was performed using SuperScript™ II reverse transcriptase (Invitrogen) primed by oligo(dT)12–18 primers. The following primers were used: ap2 (adipocyte lipid-binding protein), 5'-GAA CCT GGA AGC TTG TCT TCG-3' (forward) and 5'-ACC AGC TTC GTA CCA CCA TCG-3' (reverse); cyclophilin, 5'-TCC AAA GAC AGC AGA AAA CTT TCG-3' (F) and 5'-TCT TCG TCT TGG TCG TCT CAT TCC-3' (R); ATGL, 5'-TGG AAC ATC TCA TCT TCT GGT GG-3' (F) and 5'-AAT GGC GCC ATC CAC GAT A-3' (R); adiponutrin, 5'-GGG AGG TGT GGC CCG CCT TCT CC-3' (F) and 5'-GCC AGA TGT CAT GCT CAC CC-3' (R); perilipin-activated receptor γ, 5'-ATG TCT CAT AAT GCC ACC ATC-3' (F) and 5'-CTA GTA CAA GTC CTT GTA-3' (R); diacylglycerol acyltransferase 1, 5'-ATT CAC GGA TCA TTG AGC G-3' (F) and 5'-CTG CCA TGT CTG CGG TTG-3' (R); diacylglycerol acyltransferase 2, 5'-CTA GTG TCA ACT TCC-3' (F) and 5'-AAC CAG ATC AGC TCC ATG G-3' (R). The PCR program included denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 40 s (25 cycles). The PCR products were separated by electrophoresis on 1% (w/v) agarose gels.

Subcellular Fractionation—Differentiated 3T3-L1 adipocytes were harvested in 1 ml of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 1 mM EDTA) and homogenized by a Potter Elvijhem homogenizer. Unbroken cells were pelleted by brief centrifugation, and the resulting supernatants were centrifuged at 99,000 rpm (TL100.2 rotor) for 45 min to yield soluble (cytosolic containing cytosolic lipid droplets) and membrane fractions. Membranes were washed with Tris-buffered saline (TBS) and re-suspended in 1 ml of TBS by sonicating. Lipid droplets were isolated from differentiated 3T3-L1 adipocytes that were incubated with DMEM media containing 2% fatty acid-free BSA in the presence or absence of 10 μM isoproterenol for 1 h. Adipocytes were harvested in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 1 mM EDTA), and the homogenates were overlaid with equal amounts of Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and centrifuged at 83,000 × g (SW60 rotor) for 1.5 h to yield cytosolic lipid droplets, cytosol, and membrane pellets.
**Immunoblotting**—Equivalent amounts of protein present in cell homogenates or subcellular fractions were resolved in 10% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and immunoblotted with polyclonal anti-TGH, anti-HSL, or anti-calnexin antibodies followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG as secondary antibody. Proteins were visualized by enhanced chemiluminescence.

**Lipase Assay**—Differentiated 3T3-L1 adipocytes were incubated in the presence or absence of lipase inhibitors for 4 h. The concentration of the inhibitors used in the experiments was previously determined to be effective in inhibition of TGH activity both in vitro and in intact cells (13). Cells were then washed with Tris-buffered saline and harvested in an ice-cold homogenization buffer, and cytosolic and membrane fractions were prepared as described above. Lipolytic activities in cytosols or membranes were assessed by mixing 20 μl of 250 μM 4-methylumbelliferyl heptanoate in reaction buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 300 μM taurodeoxycholate), 20 μl of enzyme preparation (cytosol or membranes), and 60 μl of the reaction buffer in 96-well black plates and incubating at room temperature with shaking (24). The fluorescence of the reaction product was continuously recorded over a 5-min period using FluoroScan Ascent FL (Thermo Labsystems) at 355 and 460 nm as excitation and emission wavelengths, respectively.

**Lipid Analysis**—Cells were harvested in phosphate-buffered saline (PBS), sonicated on ice, and centrifuged at 1500 rpm for 5 min in a microcentrifuge to pellet cell debris. Supernatant (cell homogenate) was collected and used in analyses. Media were centrifuged at 1500 rpm for 5 min in a microcentrifuge to remove cell debris. Lipids were extracted from cell homogenates and media with chloroform/methanol (2:1, v/v) in the presence of nonradioactive lipid carriers (phosphatidylcholine, oleic acid, dioleoylglycerol, trioleoylglycerol, and cholesteryl oleate) as described previously (12, 13). The chloroform phase containing lipids was dried under nitrogen, re-dissolved in a small volume of chloroform, and applied to silica gel H thin-layer chromatography plates. The plates were developed in heptane/isopropyl ether/acetic acid 60:40:4 (by volume) to separate neutral lipids (FA, TG, and cholesteryl esters) (12, 13). Lipid classes were visualized by exposure to iodine vapor, bands were scraped, and the associated radioactivity was determined by scintillation counting. In lipids, mass determinations extractions were carried out in the absence of lipid carriers. For lipid mass analysis, cell homogenates were prepared from control or inhibitor-treated or shRNA-expressing 3T3-L1 cells at various stages of differentiation. Lipids were extracted in the presence of known amounts of tridecanoylglycerol (internal standard). Neutral lipids (TG, DG, monoacylglycerols, FA, cholesterol, cholesteryl ester, and tridecanoylglycerol) were resolved from glycerophospholipids by thin-layer chromatography using the chloroform/methanol/acetic acid/water (25:15:4:2) solvent system, and neutral lipids migrating with the solvent front were extracted, derivatized, and analyzed by gas chromatography as previously described (25).

**FA Release from Differentiated 3T3-L1 Adipocytes**—3T3-L1 adipocytes were washed with DMEM and incubated in DMEM containing 5 μCi of [9,10(14)H]oleic acid or 0.5 μCi of [1,14C]oleic acid per dish for 3 h. The adipocytes were then washed with PBS and incubated for 4 h with 1 ml of 0.05% BSA in PBS in the presence or absence of lipase inhibitors, 200 nM insulin, or 10 μM isoproterenol as indicated in individual experiments. Media lipids were extracted and resolved by thin-layer chromatography, and radioactivity in FA was determined by scintillation counting.

To measure mass of FA and glycerol release from differentiated 3T3-L1 adipocytes, cells were incubated in DMEM containing 2% fatty acid-free BSA in the presence or absence of 10 μM isoproterenol for up to 4 h. Media were collected at specified time points, and the mass of fatty acids was determined with NEFA kit (Wako Co.), and glycerol mass was measured with triglyceride (GPO-Trinder) kit (Sigma).

**FA Composition**—For analysis of released FA and TG-associated FA in 3T3-L1 adipocytes, the differentiated 3T3-L1 adipocytes were incubated in DMEM containing 2% fatty acid-free BSA for 4 h. Cell and media lipids were extracted with chloroform/methanol (2:1, v/v). The organic (chloroform) phases were dried under nitrogen and dissolved in chloroform, and lipids were separated by TLC (hexane/diisopropyl ether/acetic acid, 60:40:4). Cell TG and media FA were recovered from the TLC plates and converted to FA methyl esters by incubation with 1.5 ml of 6% H2SO4 in methanol for 2 h at 80 °C. Resulting fatty acid methyl esters were analyzed by gas chromatography. Results are shown as the percentage of specific fatty acid species.

**Glucose Uptake**—3T3-L1 adipocytes were incubated in normal growth media in the presence or absence of lipase inhibitors for 12 h. The cells were then washed twice with DMEM without glucose and incubated in DMEM without glucose with or without lipase inhibitors for 2 h. Cells were incubated in the presence or absence of 200 nM insulin for an additional 1 h followed by incubation with 100 μM 3-O-methyl-d-[-1,3H]glucose for 10 min. Cells were then washed twice with PBS, harvested in 1 ml of PBS, and sonicated. The glucose uptake was measured by counting the radioactivity in cell homogenates.

**Glut4 Translocation**—For confocal immunofluorescence studies, differentiated 3T3-L1 adipocytes were treated as above for glucose uptake. After incubations in the presence or absence of insulin, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 1% Triton X-100 in PBS for 2 min, then probed with anti-PDI, anti-Glut4, and anti-TGH antibodies in the presence of 3% BSA. Glut4 was visualized by incubation with fluorescein-conjugated donkey anti-goat IgG, and TGH was visualized by incubation with Texas Red-conjugated donkey anti-rabbit IgG. PDI was visualized with either fluorescein-conjugated or Texas Red-conjugated anti-mouse IgGs as shown in the figures. All incubations were performed at 37 °C and washed at room temperature. After immunostaining, coverslips were mounted onto glass slides using ProLong Antifade kit. The images were captured with a Zeiss laser scanning confocal imaging system.

**Statistical Analysis**—Values are expressed as the means ± S.D. Statistical significance was evaluated by the two-tailed Student’s t test using Excel software.
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FIGURE 1. Subcellular localization of lipolytic enzymes in differentiated 3T3-L1 adipocytes. After various treatments, cells were harvested and homogenized, and cell homogenates were centrifuged to prepare soluble/lipid droplet (supernatant) and microsomal (membrane) fractions as described under “Experimental Procedures.” Equivalent volumes of the fractions were resolved on SDS-PAGE and immunoblotted with polyclonal anti-TGH, anti-HSL, and anti-calnexin (Caln) antibodies, and immunoreactive proteins were detected by chemiluminescence.

RESULTS

Subcellular Localization of TGH in Differentiated 3T3-L1 Adipocytes—TGH is primarily associated with the ER (15, 26) and is also found to co-fractionate with lipid droplets in the liver (10, 11, 15) and mouse and 3T3-F442A adipocytes (19). Cellular localization of TGH and HSL after treatment of adipocytes with insulin, with a lipase inhibitor (E600), or with both insulin and E600 were essentially the same as in no treatment conditions (Fig. 1). The supernatants contained the majority of immunoreactive HSL. A small amount of HSL also co-fractionated with the membranes. The association of HSL with the ER membranes has been also observed by others (27). As expected, TGH was mainly associated with membrane fractions. However, a significant amount of TGH was found in the high speed supernatant representing TGH associated with the cytoplasmic lipid droplets (19), which may be in continuum with the ER as indicated by the presence of an ER integral membrane protein calnexin. Treatments with a general lipase inhibitor or insulin resulted in a decrease in the cytosolic/droplet-associated lipase activity (20% decrease, p < 0.05, Table 1) but not TGH levels (Fig. 1). The insulin-mediated inhibition of the cytosolic/droplet lipase activity may be due to insulin-stimulated dephosphorylation of HSL since phosphorylated HSL was described to be the more active form of the lipase (29), although insulin may also affect the activity of ATGL since no accumulation of DG was observed upon the treatment (results not shown). TGH activity and TGHi-mediated TG turnover can be specifically inhibited by TGHi (13). Because this inhibitor is non-covalent and would be diluted out during cell homogenization and fractionation, the protocol employed in Table 1 would not be expected to show any inhibition. However, when the inhibitor is present in the lipase assay mixture cytosolic/droplet-associated lipase activity is decreased by 24% and membrane lipase activity is inhibited by 73%, confirming that TGH represents the majority of the membrane-associated lipase activity in 3T3-L1 adipocytes.

Inhibition of Lipase Activity—The in vitro lipolytic activity of 3T3-L1 adipocyte homogenates was 21.0 ± 2.13 nmol/mg of protein/min using 4-methylumbelliferyl heptanoate as substrate. Approximately 70% of the total lipase activity was in the soluble (cytosolic) fractions, with the rest of the activity (30%) associated with microsomes. TGH accounts for the majority of microsomal lipase activity because both HSL and ATGL are present in the cytosol or on cytosolic lipid droplets. Table 1 shows soluble (cytosolic and lipid droplet-associated) and membrane lipase activities in differentiated 3T3-L1 adipocytes after treatment with insulin in the presence or absence of the lipase inhibitor E600. This hydrophobic inhibitor was previously demonstrated to enter the cells and inhibit TGH activity through covalent modification of the catalytic serine residue (10, 11, 13). E600 treatment decreased the membrane-associated lipase activity by 75%, showing that adipose TGH activity is sensitive to treatments with E600 to a similar extent as in hepatocytes (13). Preincubation of the cells with insulin had no significant effect on the membrane-associated lipase activity, suggesting that insulin signaling is not involved in the regulation of TGH in the adipose tissue, a result similar to that obtained in hepatocytes (28). E600 also decreased cytosol/droplet-associated lipolytic activity by 60% (Table 1). Preincubation with insulin resulted in a decrease in the cytosolic/droplet-associated lipase activity (20% decrease, p < 0.05, Table 1) but not TGH levels (Fig. 1). The insulin-mediated inhibition of the cytosolic/droplet lipase activity may be due to insulin-stimulated dephosphorylation of HSL since phosphorylated HSL was described to be the more active form of the lipase (29), although insulin may also affect the activity of ATGL since no accumulation of DG was observed upon the treatment (results not shown). TGH activity and TGH-mediated TG turnover can be specifically inhibited by TGHi (13). Because this inhibitor is non-covalent and would be diluted out during cell homogenization and fractionation, the protocol employed in Table 1 would not be expected to show any inhibition. However, when the inhibitor is present in the lipase assay mixture cytosolic/droplet-associated lipase activity is decreased by 24% and membrane lipase activity is inhibited by 73%, confirming that TGH represents the majority of the membrane-associated lipase activity in 3T3-L1 adipocytes.

Inhibition of TGH Decreases FA Efflux from Differentiated 3T3-L1 Adipocytes—To evaluate the effect of TGH inhibition on FA release from adipocytes, we treated the cells with inhibitors under basal (non-HSL- and non-ATGL-mediated lipolysis) and isoproterenol-stimulated (HSL- and ATGL-mediated lipolysis) conditions (Table 2). Both E600 (100 μM) and TGHi (10 μM) treatments resulted in a 40–46% decrease of FA release from differentiated 3T3-L1 adipocytes in control and insulin-treated conditions. These results suggest that the basal lipolysis is not dependent on the presence of HSL, supporting recent studies with HSL inhibitors (30). Isoproterenol treatment aug-

TABLE 1
Inhibition of lipase activity in cytosol and membranes from 3T3-L1 adipocytes

3T3-L1 adipocytes were treated for 4 h as indicated, cells were collected and homogenized, subcellular fractions (cytosol and membranes) were prepared, and lipase activities were determined.

| Treatment | % Activity remaining compared to control (100%) |
|-----------|-----------------------------------------------|
|           | Cytosol                                       | Membranes                                   |
| E600 (100 μM) | 37.1 ± 2.8a                                | 24.3 ± 1.1a                                 |
| Insulin (200 nM) | 78.9 ± 10.9a                              | 88.2 ± 4.9                                  |
| Insulin (200 nM) + E600 (100 μM) | 35.9 ± 5.2a                             | 22.7 ± 8.5a                                 |

*p < 0.0001, with respect to the appropriate control incubations.
*a p < 0.05.

TABLE 2
Inhibition of TGH decreases basal fatty acid efflux from 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated with radiolabeled oleic acid, washed, and incubated for 4 h as indicated in the individual experiments. Media were collected and centrifuged, lipids were extracted and resolved by thin-layer chromatography, and radioactivity in fatty acids was determined by scintillation counting as described under “Experimental Procedures.” Results are expressed as the means ± S.D.

| Treatment | % Fatty acid release compared to control (100%) |
|-----------|-----------------------------------------------|
| E600 (100 μM) | 57.5 ± 2.7a                                 |
| TGHi (10 μM) | 59.0 ± 3.1a                                 |
| Insulin (200 nM) | 104 ± 19.6                                  |
| Insulin (200 nM) + E600 (100 μM) | 61.5 ± 10.4a                              |
| Insulin (200 nM) + TGHi (10 μM) | 65.9 ± 9.1a                                |
| Isoproterenol (10 μM) | 220.2 ± 8.7a                               |
| Isoproterenol (10 μM) + E600 (100 μM) | 59.7 ± 11.7a                              |
| Isoproterenol (10 μM) + TGHi (10 μM) | 164.8 ± 10.3a                              |

*p < 0.001, with respect to the appropriate control incubations.
a p < 0.05, with respect to the appropriate control incubations.
Reduced TGH expression by RNA interference decreases basal fatty acid efflux from adipocytes—To further assess the role of TGH in adipocyte TG mobilization, we knocked down TGH expression in 3T3-L1 adipocytes. Stable clones expressing shRNA targeting TGH were isolated and examined for TGH protein levels and TGH activity (Fig. 2A). Expression of shRNA resulted in a significant decrease of TGH expression and up to an 80% decrease of TGH activity in microsomal fractions. Total in vitro lipolytic activity in cell homogenates was decreased by 30–40%. Knockdown of TGH expression was not accompanied with changes in the expression of either HSL or ATGL, indicating that the loss of lipase activity is due to lower TGH levels (Fig. 2B). The expression of adiponutrin, a protein with in vitro lipase activity (33), was also not altered. Knockdown of TGH also did not lead to decreased expression of genes involved in TG synthesis (diacylglycerol acyltransferase 1 and 2) and of aP2, a protein that interacts with HSL and prevents product inhibition of the lipase by removing fatty acids released by lipolysis (34, 35). The low expression of TGH is accompanied with a 5-fold increase in triglyceride storage (Fig. 2C), which suggests that neither HSL nor ATGL can compensate for the loss of TGH activity under normal growth conditions. TGH functions as an sn-1,3-lipase since no differences were observed in cellular DG levels between control and TGH knockdown adipocytes. Interestingly, the attenuation of TGH activity also led to a 10-fold increase in cellular cholesteryl ester (CE) levels, supporting the role of TGH in CE hydrolysis (19, 36–38). The concomitant 2-fold increase in phospholipids levels most likely represents the need for providing the augmented neutral lipid storage with a surface phospholipid coat. In basal conditions 44.6 ± 8 nmol of fatty acid/mg of protein/h and 32.9 ± 1.4 μg of glycerol/mg of cell TG/h were released from control adipocytes. Attenuated expression of TGH also decreased 50% of the mass of released glycerol by adipocytes (Fig. 3D).

Reduction of TGH Expression by RNA Interference Changes the FA Composition of Adipocyte TG and Released FA from 3T3-L1 Adipocytes—FA composition of the adipocyte TG fraction was determined by gas chromatography analysis (Fig. 4A). TG was composed mainly of saturated palmitic acid (16:0) and stearic acid (18:0), which together accounted for 70–80% of total TG-FA. TGH knockdown cells had increased levels of 16:0 and decreased levels of 18:0 and eicosanoic acid (20:0) with a concomitant increase in unsaturated linoleic acid (18:2) and docosahexanoic acid (22:6) in TG. Media FA composition largely correlated with that of cellular TG. The majority of fatty acid composition found in the media were those comprising cellular TG. However, 16:0 content was increased in TGH knockdown cell TG compared with control cell TG and decreased in the media of TGH knockdown cells (Fig. 4B), which may suggest specificity of TGH for TG containing palmitic acid in the sn-1 (-3) position.
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reported the presence of TGH in Glut4-containing organelles. In addition, introduction of inhibitory anti-TGH antibodies to permeabilized adipocytes inhibited insulin-stimulated translocation of Glut4 (21). Therefore, it was concluded that TGH plays a direct role in Glut4 transport. TGH is localized to the ER in hepatocytes (10, 11, 15, 26). Because Glut4 has been shown to be primarily recycling between endosomes and trans-Golgi network (39, 40), we have re-assessed the localization of TGH in 3T3-L1 adipocytes by confocal immunofluorescence. TGH extensively co-localized with the ER-resident protein PDI in both basal and insulin-stimulated conditions in the presence or absence of lipase inhibitor E600 (Fig. 5A), whereas Glut4 did not show any significant ER localization (Fig. 5B). In unstimulated 3T3-L1 adipocytes (basal conditions), Glut4 was localized predominantly in an intracellular compartment (Fig. 5C), in agreement with previous reports (39). As expected, upon treatment with insulin, translocation of Glut4 to the plasma membrane was observed (Fig. 5C). However, TGH did not show any significant co-localization with Glut4 in either basal or insulin-stimulated states. In addition, inhibition of TGH by E600 did not appear to interfere with insulin-dependent translocation of Glut4 from intracellular storage compartments to the plasma membrane.

Inhibition of TGH-mediated Lipolysis Does Not Affect Insulin-dependent Glucose Uptake into Differentiated 3T3-L1 Adipocytes—Ablation of the murine HSL gene results in impaired adipose tissue glucose uptake and metabolism (22). This has been explained to be due to substantially elevated levels of intracellular DG because of the preferred DG lipase activity of HSL and ensuing metabolic block through up-regulation of DG signaling pathways (9). We assessed whether inhibition of TGH-mediated affects insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes. Although E600 inhibits TGH-mediated lipolysis and fatty acid release from adipocytes incubated with insulin (Tables 1 and 2), the treatment did not appear to influence either insulin-stimulated Glut4 translocation (Fig. 5, B and C) or insulin-stimulated glucose uptake (Fig. 6A). To address more specifically the potential role of TGH in insulin-dependent glucose uptake, we performed the studies in TGH knockdown adipocytes. Similarly to E600-treated cells, adipocytes with dramatically decreased TGH expression did not show impaired insulin-stimulated glucose uptake (Fig. 6B). Taken together the data indicate that TGH does not play a role in glucose uptake but does mediate basal triacylglycerol hydrolysis.

TGH Does Not Co-localize with Glut4-containing Compartments—By performing subcellular fractionation of adipocytes and immunoaffinity purification, Lee et al. (21)
Adipose tissue has the largest capacity of TG storage in the body. During metabolic excess, insulin action mediates TG deposition (fatty acid and glucose uptake as well as TG synthesis), and when energy is required (during fasting), free fatty acids are liberated from TG stores by lipolysis and carried by serum albumin in the plasma for delivery to tissues for utilization (41, 42). The enzyme historically recognized to be responsible for all lipolysis in adipose tissue TG is HSL. However, targeted deletion of the murine $Hsl$ gene eliminated only about half of the basal TG lipase activity of the adipose tissue, revealing that another TG lipase activity exists in adipocytes (8, 9, 43). Currently, only two other intracellular neutral TG lipases expressed in adipocytes in addition to HSL are known, TGH (18, 19) and ATGL (5). HSL and ATGL are soluble cytosolic proteins, whereas TGH is localized to particulate (microsomal) fractions (10). Initially, TGH was shown to play a role in hepatic TG metabolism (12–16). Overexpression of TGH in hepatocytes resulted in increased hydrolysis of preformed TG storage pool and increased delivery of the lipolytic products for re-esterification into very low density lipoprotein TG (12, 15). Conversely, chemical inhibition of TGH resulted in decreased mobilization of stored TG for very low density lipoprotein assembly (13). Because TGH is also expressed in the adipose tissue (17–19), we hypothesized that TGH may also be involved in the mobilization of fat depots and may contribute to plasma free fatty acid levels. Chronically increased concentrations of

**DISCUSSION**

Adipose tissue has the largest capacity of TG storage in the body. During metabolic excess, insulin action mediates TG deposition (fatty acid and glucose uptake as well as TG synthesis), and when energy is required (during fasting), free fatty acids are liberated from TG stores by lipolysis and carried by serum albumin in the plasma for delivery to tissues for utilization (41, 42). The enzyme historically recognized to be responsible for all lipolysis in adipose tissue TG is HSL. However, targeted deletion of the murine $Hsl$ gene eliminated only about half of the basal TG lipase activity of the adipose tissue, revealing that another TG lipase activity exists in adipocytes (8, 9, 43). Currently, only two other intracellular neutral TG lipases expressed in adipocytes in addition to HSL are known, TGH (18, 19) and ATGL (5). HSL and ATGL are soluble cytosolic proteins, whereas TGH is localized to particulate (microsomal) fractions (10). Initially, TGH was shown to play a role in hepatic TG metabolism (12–16). Overexpression of TGH in hepatocytes resulted in increased hydrolysis of preformed TG storage pool and increased delivery of the lipolytic products for re-esterification into very low density lipoprotein TG (12, 15). Conversely, chemical inhibition of TGH resulted in decreased mobilization of stored TG for very low density lipoprotein assembly (13). Because TGH is also expressed in the adipose tissue (17–19), we hypothesized that TGH may also be involved in the mobilization of fat depots and may contribute to plasma free fatty acid levels. Chronically increased concentrations of
circulating fatty acids have been implicated in the genesis of muscle and hepatic insulin resistance and in impairment of insulin secretion. Therefore, inhibition of adipose tissue lipolysis and, consequently, decreased release of fatty acids from the fat depots presents an attractive pharmacological target. However, recent results suggest that ablation of HSL-mediated lipolysis may lead to the accumulation of intracellular DG levels (9) that could potentially adversely affect insulin-stimulated glucose uptake into the adipose tissue, a condition that could result in worsening of hyperglycemia. We wished to determine whether inhibition of the basal lipase (TGH) activity would result in decreased efflux of fatty acids from adipocytes and if inhibition of TGH affected glucose uptake into the fat cell.

HSL is characterized as a cytosolic protein, and its translocation to the lipid droplet is regulated by a process that depends on production of cAMP and protein kinase A-dependent phosphorylation by hormones such as catecholamines, glucagon, corticotropin, and others (4, 31, 32). On the other hand the activity of TGH appears to be insensitive to the above stimuli and may represent the basal lipase activity in the adipose tissue. Recent data suggested that the majority of the basal lipase activity in HSL null adipocytes could be attributed to TGH (19), and therefore, TGH-dependent lipolysis may significantly contribute to the plasma free fatty acid levels. ATGL was also suggested to catalyze fatty acid release in basal conditions (5, 44). Simultaneous knockdown of HSL (83%) and ATGL (96%) expression in 3T3-L1 adipocytes resulted in an 85% decrease of fatty acid efflux from isoproteoln-stimulated adipocytes, but the combined attenuation of HSL and ATGL expression decreased basal fatty acid release by only 50% (44). Recent studies in ATGL null mice, however, suggest that ATGL does not play a role in basal lipolysis, as fatty acid and glycerol release from white adipose tissue were similar in the knock-out and wild type mice under basal conditions (45). Inhibition of hepatic TGH resulted in markedly attenuated lipolysis of cytoplasmic TG (13). Our data show that inhibition of TGH in adipocytes results in a significant decrease of basal fatty acid and glycerol release despite the presence of unaltered expression of ATGL and HSL. In addition, whereas ablation of HSL activity (through targeted deletion of the Hsl gene) leads up to an 8-fold increase of intracellular DG levels (9), inhibition of TGH-mediated triacylglycerol lipolysis in adipocytes does not appear to elevate DG levels. Palmitic and stearic acids are the predominant fatty acids found in 3T3-L1 adipocyte TG. Attenuation of TGH expression led to an increase of palmitic acid content in cellular TG and decrease palmitic acid content in the media, suggesting some specificity for the primary (sn-1 or sn-3) positions in TG since these positions are generally occupied by palmitic acid.

Our data show extensive co-localization of TGH with a known ER resident protein PDI, a result that is consistent with our observation in hepatic TGH distribution (11, 15). We have not been able to observe any TGH localization with the Golgi compartment by immunogold electron microscopy (15). Although we observed extensive colocalization of TGH with an ER marker in differentiated 3T3-L1 adipocytes, Glut4 localization was clearly in a compartment distinct from the ER. Subsequently, TGH and Glut4 also appeared to be present in different subcellular regions in both basal and insulin-stimulated conditions. Inhibition of TGH activity not only did not affect the ability of Glut4 to translocate from an intracellular compartment to plasma membrane upon insulin treatment but also did not affect insulin-dependent glucose uptake into the adipocytes. These findings are important because selective inhibition of the predominantly DG-hydrolyzing lipase, HSL, appears to interfere with glucose metabolism in the fat cell (22).

Together our data suggest that TGH may represent a suitable pharmacological target for alleviation the lipotoxicity associated with the pathogenesis of peripheral insulin resistance. Inhibition of TGH in adipose would decrease the flux of fatty acids to the liver, and concurrent inhibition of TGH in the liver would be expected to attenuate the secretion of very low density lipoprotein without being accompanied by liver steatosis.

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