Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis

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Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. Am J Physiol Endocrinol Metab 292: E1847–E1855, 2007. First published February 27, 2007; doi:10.1152/ajpendo.00040.2007.—Hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) regulate adipocyte lipolysis in rodents. The purpose of this study was to compare the roles of these lipases for lipolysis in human adipocytes. Subcutaneous adipose tissue was investigated. HSL and ATGL protein expression were related to lipolysis in isolated mature fat cells. ATGL or HSL were knocked down by RNA interference (RNAi) or selectively inhibited, and effects on lipolysis were studied in differentiated preadipocytes or adipocytes derived from human mesenchymal stem cells (hMSC). Subjects were all women. There were 12 lean controls, 8 lean with polycystic ovary syndrome (PCOS), and 27 otherwise healthy obese subjects. We found that norepinephrine-induced lipolysis was positively correlated with HSL protein levels (P < 0.0001) but not with ATGL protein. Women with PCOS or obesity had significantly decreased norepinephrine-induced lipolysis and HSL protein expression but no change in ATGL protein expression. HSL knock down by RNAi reduced basal and catecholamine-induced lipolysis. Knock down of ATGL decreased basal lipolysis but did not change catecholamine-stimulated lipolysis. Treatment of hMSC with a selective HSL inhibitor during and/or after differentiation in adipocytes reduced basal lipolysis by 50%, but stimulated lipolysis was inhibited completely. In contrast to findings in rodents, ATGL is of lower substrate affinity of HSL to TG, rather than the existence of a specific TG lipase with a different regulation than HSL. The pivotal role of HSL in adipocyte lipolysis was first questioned when data from HSL-deficient mice were reported. The animals showed normal weight, had markedly blunted stimulated fat cell lipolysis, but retained residual basal lipolysis (26, 33). Subsequent analysis demonstrated that knockout animals displayed an accumulation of DG in several tissues (12), suggesting that, although HSL may catalyze the rate-limiting step in TG hydrolysis, the major physiological substrates are DG and not TG. Recently, a novel TG-specific lipase has been isolated and termed adipose triglyceride lipase (ATGL—the term used in this work; see Ref. 34), desnutrin (32), and iPLA2α (15). The murine ATGL gene codes for a 486-amino-acid-long protein while the human homologue, displaying 86% identity, codes for a 506-amino-acid protein. Studies in rodents have shown that ATGL is predominantly expressed in white and brown adipose tissue, although significant levels are also expressed in cardiac and skeletal muscle. The enzyme displays substrate specificity for TG but, unlike HSL, activation of ATGL is not dependent on phosphorylation events. Further studies have shown that ATGL expression is increased by fasting (32) and decreased by insulin (16). The phenotype of mice homozygous for a null mutation in the ATGL gene was described (11). In these animals, both basal and catecholamine-stimulated lipolysis were markedly decreased, and this was true for both glycerol and FFA release (11). ATGL-deficient mice display a slightly larger white adipose tissue (WAT) mass but, more importantly, a massive TG accumulation in nonadipose tissue, in particular, heart muscle. This results in a rapidly developing heart failure and

MOBILIZATION OF LIPIDS through lipolysis in fat cells is a key event in energy homeostasis. Lipolysis is disturbed in many insulin-resistant disorders such as obesity and polycystic ovary syndrome (PCOS), which in turn are important risk factors for type 2 diabetes mellitus (1). Lipolysis in fat cells is under intense hormonal control. Hormone-sensitive lipase (HSL) has for decades been regarded as the main regulatory step in mammal lipolysis (14). It is stimulated by catecholamines and other prolipolytic hormones and inhibited by insulin.

Lipids are mainly stored in fat cells as triglycerides (TG). When lipolysis is stimulated, TG are usually completely hydrolyzed into glycerol and free fatty acids (FFA). In rodents and humans, there is also some partial lipolysis leading to the formation of diglycerides (DG) and FFA but not glycerol (2, 29). HSL hydrolyzes both TG and DG, although the affinity for the latter is 10-fold higher (9). Therefore, incomplete hydrolysis of TG to DG during lipolysis may be the result of the lower substrate affinity of HSL to TG, rather than the existence of a specific TG lipase with a different regulation than HSL. The pivotal role of HSL in adipocyte lipolysis was first questioned when data from HSL-deficient mice were reported. The animals showed normal weight, had markedly blunted stimulated fat cell lipolysis, but retained residual basal lipolysis (26, 33). Subsequent analysis demonstrated that knockout animals displayed an accumulation of DG in several tissues (12), suggesting that, although HSL may catalyze the rate-limiting step in TG hydrolysis, the major physiological substrates are DG and not TG. Recently, a novel TG-specific lipase has been isolated and termed adipose triglyceride lipase (ATGL—the term used in this work; see Ref. 34), desnutrin (32), and iPLA2α (15). The murine ATGL gene codes for a 486-amino-acid-long protein while the human homologue, displaying 86% identity, codes for a 506-amino-acid protein. Studies in rodents have shown that ATGL is predominantly expressed in white and brown adipose tissue, although significant levels are also expressed in cardiac and skeletal muscle. The enzyme displays substrate specificity for TG but, unlike HSL, activation of ATGL is not dependent on phosphorylation events. Further studies have shown that ATGL expression is increased by fasting (32) and decreased by insulin (16). The phenotype of mice homozygous for a null mutation in the ATGL gene was described (11). In these animals, both basal and catecholamine-stimulated lipolysis were markedly decreased, and this was true for both glycerol and FFA release (11). ATGL-deficient mice display a slightly larger white adipose tissue (WAT) mass but, more importantly, a massive TG accumulation in nonadipose tissue, in particular, heart muscle. This results in a rapidly developing heart failure and
reduced life span. Furthermore, the animals display a defective cold adaptation indicating that ATGL is essential to provide FFAs to fuel thermogenesis. A reduction in FFA availability results in an increase in glucose utilization that could explain the increase in glucose tolerance and insulin sensitivity observed in these animals. Finally, it was recently demonstrated in immortalized nonhuman cell lines that the lipid droplet-associated protein CGI-58 was essential for ATGL activity although the molecular mechanisms remain to be elucidated (20). In summary, data from animal studies suggest that ATGL is essential for basal and stimulated lipolysis and of major importance for energy homeostasis. Accordingly, in a mouse fat cell line, overexpression of ATGL resulted in increased basal and stimulated lipolysis, whereas inhibition by RNA interference (RNAi) had the opposite effect (16). Gene knockdown of HSL only decreased catecholamine-stimulated lipolysis. Similar results were obtained with FFA and glycerol measurements. These results suggest a critical role for ATGL as a regulator of both basal (i.e., spontaneous) and stimulated lipolysis, whereas HSL only determines stimulated lipolysis.

In contrast to these findings primarily obtained in rodents, the role of ATGL in humans is much less clear. An association between single nucleotide polymorphisms in the ATGL gene and circulating levels of FFA, TG, and risk for type 2 diabetes mellitus was recently demonstrated (27). Moreover, mutations in the CGI-58 gene are associated with Chanarin-Dorfman syndrome, a rare genetic disease characterized by excessive TG accumulation in nonadipose tissues. Recently, the importance of ATGL in human lipolysis was indirectly determined using a selective inhibitor of HSL in adipocytes from healthy donors (17). It was found that catecholamine or natriuretic peptide-stimulated lipolysis was completely inhibited, whereas basal lipolysis was only partially abrogated. This suggests that HSL is the major rate-limiting lipase in human lipolysis and that ATGL may above all play a role in the regulation of basal lipolysis. Other human studies demonstrated that ATGL mRNA, as opposed to HSL mRNA expression, is not regulated by obesity and that the in vitro TG hydrolysis activity of ATGL, in the absence of CGI-58, is substantially lower than that of HSL (22). Thus the enzymatic contributions of HSL and ATGL to TG hydrolysis and thereby lipolysis may differ between humans and rodents. However, the comparison between HSL and ATGL is incomplete so far in humans, since there is no information on ATGL protein levels or effects of genetic manipulation of lipase expression in relation to lipolysis in human fat cells.

In the present study, we have compared the physiological roles of HSL and ATGL in human fat cell lipolysis. This was done through parallel determinations of protein level, adipocyte lipolytic activity, and modulation of HSL and ATGL levels using RNAi technology. We also compared their expression in conditions with altered lipolysis, namely obesity and PCOS, which are two conditions with decreased expression and function of HSL (1).

**MATERIALS AND METHODS**

**Subjects.** One cohort consisted of obese women who underwent bariatric surgery or gallstone operations (n = 19) or were investigated as healthy volunteers (n = 8). Obesity was defined as a body mass index (BMI) >30 kg/m². The second cohort consisted of 12 healthy lean women with BMI <25 kg/m². The third cohort was composed of eight young lean women diagnosed with PCOS. The latter diagnosis was defined as infertility in combination with ovarian cysts detected by ultrasound investigation. The PCOS women were otherwise healthy. We chose to study young and lean PCOS women because we have previously shown that they have blunted catecholamine-induced lipolysis without being insulin resistant (8). These cohorts were investigated for lipolysis regulation and comparison of HSL and ATGL protein expression. Except for the 19 operated women, abdominal subcutaneous adipose tissue was obtained by biopsy under local anesthesia. For the operated women, abdominal subcutaneous adipose tissue was obtained from the surgical incision at the beginning of surgery, which was performed under general anesthesia. We have previously shown that lipolysis regulation in isolated adipocytes is identical whether the tissue is obtained during general surgery or by local biopsy (19). Clinical data for the subjects in cohorts 1–3 are summarized in Table 1. All subjects were examined at 8:00 A.M. after an overnight fast. Their height and weight were measured. Venous plasma samples were drawn for the analysis of insulin, glucose, S-testosterone, and sex hormone-binding globulin (SHBG) by the hospital’s accredited clinical chemistry laboratory. From these measures, the testosterone-to-SHBG ratio and homeostasis model assessment (HOMA) index ([plasma glucose (mmol/l) × plasma insulin (μU/l)/22.5 (7)] were calculated. HOMA is an indirect measure of in vivo insulin sensitivity. A fourth cohort consisted of six otherwise healthy women (age 43 ± 2.8 yr and BMI 25 ± 2.8 kg/m²) who underwent cosmetic abdominal subcutaneous liposuction. These subjects did not have clinical signs of diabetes or other diseases that may influence adipocyte function. The adipose tissue from these individuals was used to isolate preadipocytes and human mesenchymal stem cells (hMSC) for subsequent RNAi studies and investigations of HSL inhibition. Subjects from all four cohorts included in this study were healthy and free of medication. The studies were approved by the ethical committee at Karolinska Institutet and explained in detail to each of the subjects, from whom informed consent was obtained.

**Lipolysis assessment in mature fat cells and preadipocyte cultures.** Lipolysis experiments in mature fat cells were performed as described in detail previously (22). In brief, cells were incubated in an albumin-containing buffer (pH 7.4) for 2 h at 37°C with air as a gas phase

**Table 1. Antropometric data and relevant values on subjects from cohorts 1, 2, and 3**

<table>
<thead>
<tr>
<th></th>
<th>Obese (n = 27)</th>
<th>Lean (n = 12)</th>
<th>PCOS (n = 8)</th>
<th>P ANOVA</th>
<th>Lean vs. Obese</th>
<th>Lean vs. PCOS</th>
<th>PCOS vs. Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>38 ± 1.6</td>
<td>31 ± 1</td>
<td>30 ± 1.3</td>
<td>0.01</td>
<td>0.02</td>
<td>0.69</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>41.8 ± 1.2</td>
<td>22.6 ± 0.5</td>
<td>22.9 ± 1.0</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.89</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Testosterone/SHBG ratio</td>
<td>0.068 ± 0.009</td>
<td>0.019 ± 0.004</td>
<td>0.076 ± 0.010</td>
<td>0.01</td>
<td>0.008</td>
<td>0.008</td>
<td>0.64</td>
</tr>
<tr>
<td>P insulin, μU/l</td>
<td>19.6 ± 2.2</td>
<td>5.0 ± 0.58</td>
<td>8.1 ± 1.1</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.50</td>
<td>0.006</td>
</tr>
<tr>
<td>P glucose, mmol/l</td>
<td>5.9 ± 0.3</td>
<td>4.7 ± 0.11</td>
<td>4.8 ± 0.07</td>
<td>0.004</td>
<td>0.003</td>
<td>0.85</td>
<td>0.022</td>
</tr>
<tr>
<td>HOMA index</td>
<td>5.7 ± 1.0</td>
<td>1.1 ± 0.11</td>
<td>1.8 ± 0.3</td>
<td>0.0006</td>
<td>0.0004</td>
<td>0.73</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE; n, no. of subjects; PCOS, polycystic ovary syndrome; BMI, body mass index; SHBG, sex hormone-binding globulin; P, plasma; HOMA, homeostasis model assessment. Statistical significance between the three groups was calculated by ANOVA and by Student’s t-test for comparisons between two groups.
without (basal) or with increasing concentrations of the natural catecholamine 10^{-4} M norepinephrine or 10^{-5} M isoproterenol (a nonselective β-adrenoceptor agonist). Following incubation, medium was removed and kept at −20°C for subsequent measurement of glycerol concentration (an index of lipolysis) using a bioluminescence method (13). Lipolysis was then expressed as norepinephrine- or isoproterenol-induced glycerol release at maximum effective concentration divided by basal. When stated, lipolysis was also expressed as absolute values (μmol of glycerol·h^{-1}·10^{-7} cells or μmol of glycerol·h^{-1}·g lipids^{-1}). We preferred to use the relative expression of lipolysis because it correlates strongly with the protein expression of HSL in human fat cells (18). Lipolysis in human preadipocyte cultures was performed as described previously (4) with or without 10^{-5} M isoproterenol. Glycerol release was expressed per gram protein. Protein content was assayed spectrophotometrically using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) on 96-well microtiter plates with BSA as a standard. Glycerol was measured by bioluminescence (13) and fatty acid release by chemiluminescence (24).

Studies of hMSCs. hMSCs were obtained from adult adipose tissue and differentiated into the adipogenic lineage as described in detail elsewhere (3). We have previously shown that these cells display lipolytic activity comparable with differentiated human preadipocytes. hMSC derived from two different donors were used. Functional assessment of adipocyte differentiation was performed by determining glycereraldehyde-3-phosphate dehydrogenase (GAPDH) activity as described previously (31). Cells from two wells were washed with PBS (pH 7.4) and harvested in prechilled 25 mmol/l Tris-HCl buffer containing 1 mmol/l EDTA (pH 7.4) and 1 mmol/l 2-mercaptoethanol. After sonication, aliquots of the cell extracts were added to an assay mixture containing 100 mmol/l triethanolamine hydrochloride buffer (pH 7.5), 2.5 mmol/l EDTA, 0.12 mmol/l NADH, and 0.1 mmol/l β-mercaptoethanol, and GAPDH activity was measured spectrophotometrically, at 340 nm. The reactions were started by adding 0.2 mmol/l dihydroxyacetone phosphate. The GAPDH activity was related to the total protein content in each well and expressed as milliunits per microgram of total protein. Total protein lysates were obtained from cells in two wells using a lysis buffer that contained 1% Triton X-100, 50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), supplemented with protease inhibitors. Lipolysis experiments were performed on differentiated cells as described previously (31). In brief, cells were washed with DMEM-NUT·MIX·F-12 medium and then incubated in duplicate for 3 h at 37°C with DMEM-NUT·MIX·F-12 medium containing 20 mg/l BSA. The following concentrations were used for each agent: 10^{-8} M isoproterenol, 10^{-5} M norepinephrine, 10^{-4} M yohimbine, 10^{-3} M dibutyryl-cAMP (DbcAMP), and 10^{-4} M atrial natriuretic peptide (ANP). Incubation without drugs was made to determine basal lipolysis. The selective HSL inhibitor 4-isosopropyl-3-methyl-2-[1-3(S)-methyl-piperidin-1-yl]-methanoyl)-2H-isoxazol-5-1 (BAY; see Ref. 21) has previously been described in detail (17) and was used at the concentration of 1 μM. Following incubation, medium was removed and kept at −20°C for subsequent measurement of glycerol concentration (an index of lipolysis) using a bioluminescence method (13).

Protein expression of HSL and ATGL. Approximately 300 mg of WAT were crushed and lysed in protein lysis buffer (1% Triton X-100, Tris-HCl, pH 7.6, and 150 mmol/l NaCl, 4°C), supplemented with protease inhibitors [1 mmol/l PMSF and Complete (Boehringer Mannheim, Mannheim, Germany)], and homogenized using a microtome. The homogenate was centrifuged at 14,000 rpm for 30 min, and the infranatant was collected and saved. Protein content was assayed using the BCA Protein Assay Reagent Kit (Pierce) as under Lipolysis assessment in mature fat cells and preadipocyte cultures. To test if proteins remained in the fat cake following our protein extraction procedure, we performed the following control procedure. The fat cake was removed and subjected to methanol-CHCl₃ extraction (23), which effectively collects all proteins. These extracted proteins were dissolved in the same lysis buffer as above. Protein levels in fat cake extracts were below the detection limit of our assay. Thus there are insignificant amounts of lipases left in the fat cake. Total cellular protein (100 μg) was loaded on polyacrylamide gels and separated by standard 12% SDS-PAGE. Gels were transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). For HSL and β-actin detection, blots were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dried milk. This was followed by an overnight incubation at 4°C in the presence of antibodies directed against HSL or protein β-actin (Sigma, St. Louis, MO). The human-specific HSL antibody was a generous gift from C. Holm (Lund University) and has been characterized elsewhere (8). For the detection of ATGL, an affinity-purified polyclonal antibody was raised in rabbit against a 15-amino-acid peptide (amino acids 386–400, GRHLPSRLPEQOVERL) of human ATGL. To test antibody specificity, we expressed ATGL protein in COS cells by transfection with cDNA coding for human ATGL. For detection of ATGL, the following conditions were used. Blocking was performed in PBS supplemented with 4% BSA. This solution was used for antibody incubation after addition of 0.1% Tween 20, whereas PBS + 0.1% Tween 20 was used as wash solution. All incubations with the ATGL antibody were performed at room temperature. Secondary α-rabbit antibodies conjugated to horseradish peroxidase were from Sigma. Antigen-antibody complexes were detected by chemiluminescence using a kit of reagents form Pierce (Supersignal), and specific bands were detected using a Chemidoc XRS system (Bio-Rad). Images were analyzed using the Quantity One Software supplied by the manufacturer (Bio-Rad). To control for differences in loading, etc., β-actin was used as a control protein, and values for HSL and ATGL were expressed as the quotient to β-actin in relative units (RU). Furthermore, proteins from two subjects were ran on all gels to ensure similar exposure times, etc., to further improve interexperimental comparison.

RNAi. RNAi in human preadipocytes was essentially performed as described previously using short-interfering RNA (siRNA) oligonucleotides (25). For each oligonucleotide, optimal transfection conditions were determined in separate titration experiments using different amounts of siRNA oligonucleotides (Qiagen, Hilden, Germany) and transfection reagent RNAiFect (Qiagen). Cells at day 12 of differentiation (a time point where the cells are almost fully differentiated) were transfected with or without ATGL or HSL siRNAs (Qiagen). The siRNA sequences were as follows (sense strand): ATGL 5'-CGG CGA GAA UGU CAU UAU, HSL 5'-GCC UGC UUC AAA CCA AAG A. To control for unspecific RNAi effects, control cells were transfected with nonsilencing siRNA oligos without known similarities to human sequences (Qiagen). Cells were incubated for 24 h, a time point where a significant gene silencing effect was observed. Conditioned cell media aliquots were then analyzed for glycerol content while cells were lysed for RNA or protein isolation as indicated above to confirm gene silencing.

RNA analysis. Total RNA from preadipocyte cultures was extracted using the RNAeasy mini kit (Qiagen). RNA concentration and purity were assessed spectrophotometrically. A bioanalyzer (Agilent 2100; Agilent Technologies, Kista, Sweden) was used to confirm RNA integrity. Total RNA (1 μg) was reverse transcribed using a kit (OmniScript) and random hexamers (Invitrogen, Tästrup, Denmark). To minimize methodological errors resulting from variation in cDNA synthesis, cDNA synthesis was performed simultaneously for all subjects included in the analysis. PCR conditions and primers for HSL, ATGL, and 18S have been described previously (22). The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by agarose gel electrophoresis. Quantitative real-time PCR was performed in an iCycler IQ (Bio-Rad Laboratories, Hercules, CA). In RNAi experiments, ATGL and HSL mRNA levels were reduced significantly to <20% of that in control cells, and each condition was repeated at least three times with cells
from different donors. Experiments where gene silencing was not efficient were discarded and not used for further analysis.

Statistical analysis. Values are given as means ± SE. They were compared with Student’s unpaired t-test, ANOVA, post hoc tests, and linear regression analysis using standard software packages. A P value of 0.05 or less was considered to be statistically significant. It is difficult to find young, lean, and otherwise healthy women with PCOS. Before recruitment, we made a power calculation for the comparison of lean, young women with or without PCOS based on previous findings with HSL protein expression and lipolysis (8). We can detect a 20% difference between groups in either result with P < 0.05 and with a power of 80% by investigating, as performed in this study, 12 control and eight PCOS subjects.

Drugs and chemicals. BSA (fraction V, lot no. A-9418), glucose, glycerol kinase, and norepinephrine were obtained from Sigma Chemical. All chemicals used were of the highest grade of purity that was commercially available.

RESULTS

Clinical data. The clinical findings in cohorts 1–3 are shown in Table 1. The obese subjects were insulin resistant and somewhat older than the other groups. Healthy lean and PCOS women had almost identical BMI, age, and insulin sensitivity, but the PCOS subjects had a fourfold higher testosterone-to-SHBG ratio.

Methodological experiments. Similar concentrations of protein (3–6 µg/µl) were obtained in the tissue extracts. However, no protein was detected in the extracts from the fat cake (detection limit is 0.01 µg/µl). From these findings, we calculated that <0.2% of all protein in adipose tissue following extraction for Western blot remained in the fat cake. In Western blot analysis of tissue extracts, a single band at 56 kDa was detected, which disappeared after preincubation of the antibody with the immunizing peptide. This band corresponds to the predicted molecular mass of human ATGL protein. Specificity of the antibody was further confirmed by Western blot of protein lysates from COS cells transfected with cDNA coding for human ATGL. We used these cells to ensure the specificity of the antibody since COS cells lack ATGL. Here, a single 56-kDa band was observed (Fig. 1A).

Glycerol release following downregulation of HSL and ATGL by RNAi. We used RNAi to determine the effects of selective lipase downregulation on basal and catecholamine (i.e., isoprenaline)-stimulated lipolysis in differentiated human preadipocytes. This system was chosen since we have recently developed efficient RNAi protocols in these cells (25) and since freshly isolated mature fat cells display a limited survival in vitro (hours). Lipolysis was determined as glycerol release. We also attempted to measure FFA release using a sensitive chemiluminescence method as well as gas chromatography, but the concentrations were below threshold sensitivity for the assays (data not shown). To control for nonspecific effects of siRNA, scrambled nonsilencing oligonucleotides were used to transfect control cells under otherwise identical conditions. Using either siRNA directed against HSL or ATGL, we were able to downregulate HSL and ATGL mRNA in the same order of magnitude (15 ± 2.5 and 12 ± 4%, respectively, n = 4, Fig. 1B). There was no effect of ATGL oligonucleotides on HSL mRNA expression or vice versa (Fig. 1B) nor of control (scrambled) oligonucleotides (data not shown). Moreover, downregulation of mRNA resulted in a clearly observable reduction of the cognate protein down to ∼30% (35 ± 3.5 and 32 ± 2.5%, for HSL and ATGL vs. control, respectively, P < 0.01, n = 4, Fig. 1C). Following downregulation of HSL, a 35% decrease of both basal (P = 0.028, Fig. 1D) and isoprenaline-stimulated lipolysis (P = 0.017, Fig. 1E) was observed. In contrast, RNAi knock down with siRNA oligonucleotides specific for ATGL decreased basal lipolysis by 40% (P = 0.018, Fig. 1D) but had no effect on isoprenaline-induced lipolysis (P = 0.73, Fig. 1E).

Lipolysis in hMSCs. The role of HSL for lipolysis in adipocyte precursor cells was examined using the selective HSL inhibitor BAY at the maximum effective concentration (Fig. 2). This inhibitor does not influence any other lipase apart from HSL, including ATGL. We chose to use a recently established cell system based on hMSC isolated from human adipose tissue. These cells can be efficiently differentiated in vitro into adipocytes and display all the morphological and functional characteristics of human adipocytes (3). Furthermore, these are the only human cells that can be used to block HSL from the earliest stage of differentiation since preadipocytes are committed to the adipogenic lineage. hMSC were allowed to proliferate and then differentiate into fat cells. Thereafter, a 3-h lipolysis experiment was performed. Inhibition of HSL, with BAY after adipose differentiation (con-bay) decreased basal lipolysis by one-half and almost completely counteracted lipolysis stimulated by isoprenaline, norepinephrine, the α2-adrenergic receptor inhibitor yohimbine, a cAMP analog (DbcAMP), and ANP (which stimulates lipolysis via the cGMP pathway) compared with control cells not exposed to BAY (con-con, Fig. 2, P < 0.05, n = 5). Almost identical results as with con-bay-treated cells were obtained with hMSC-derived adipocytes continuously exposed to BAY during the entire experiment, i.e., during the differentiation process and the lipolytic experiment (bay-bay). In contrast, cells exposed to BAY during differentiation but not during the lipolytic assessment (bay-con) displayed a lipolytic response that was very similar to control cells (con-con). BAY treatment did not influence adipocyte differentiation of hMSCs according to measurements of GAPDH activity (data not shown).

Lipolysis and lipase protein expression in lean controls and obese and lean PCOS subjects. To compare lipolysis and HSL or ATGL levels in subjects with different lipolytic capacity, we investigated lean, obese, and PCOS subjects. Catecholamine-induced lipolysis (in mature adipocytes) and protein expression (in adipose tissue) of ATGL and HSL were determined. The maximal lipolytic capacity, expressed as norepinephrine-stimulated lipolysis over basal, was significantly and markedly blunted in obese and PCOS compared with adipocytes from lean subjects (9.2 ± 1.7, 2.7 ± 0.2, and 3.0 ± 0.7 for lean, obese, and PCOS, respectively, mean ± SD, P < 0.0001, Fig. 3A). Basal rate of glycerol release was similar in lean controls and PCOS vs. control, respectively, P = 0.056, data not shown). HSL protein levels were 50–65% decreased in PCOS and obese subjects (Fig. 3B, P = 0.0022). Post hoc analysis of HSL data revealed a significant difference between lean vs. obese (P = 0.0005) and PCOS vs. lean (P = 0.046), but there was no significant difference between obese and PCOS. In contrast, there was no difference in ATGL protein levels between the three groups (P = 0.96, Fig. 3C). The mean value for obese and PCOS was identical (1.75 RU). The value for lean controls was 10% lower than that for obese.
Actin expression was similar in the three groups and was used to correct values to enable comparison between blots.

Because obese women were somewhat older than lean and PCOS women, values for lipolysis and protein expression were also corrected for age in the ANOVA analysis (i.e., ANCOVA). This did not alter the outcome of the results. We also excluded the obese undergoing general surgery. The results with the remaining eight obese investigated as the lean and PCOS women were the same as for the whole group except that this obese subgroup had almost the same mean age as the two other groups (30.5 yr).

The expression of HSL and ATGL protein from the obese, lean, and PCOS subjects was plotted against maximal lipolytic capacity (expressed as norepinephrine/basal lipolysis). There was a positive correlation between HSL levels and lipolysis (Fig. 3D, $P < 0.0001$, $r = 0.72$). In contrast, we observed no significant correlation between ATGL protein expression and lipolysis although a slight negative trend was observed (data not shown, $P = 0.15$, $r = -0.23$). We also expressed norepinephrine-induced glycerol release in absolute values corrected for either per gram lipids or per $10^7$ cells and correlated this with lipase expression. When expressing lipolysis per gram lipids, results did not differ from those obtained using norepinephrine over basal lipolysis (data not shown). However, no correlation between lipolysis and lipase expression was obtained using glycerol release per number of fat cells (data not shown). Basal glycerol release did not correlate with ATGL or HSL expression. Furthermore, insulin or HOMA levels did not correlate with either ATGL or HSL expression.

These data were obtained by combining the results from two separate Western blots corrected for protein expression by β-actin to allow comparison between blots. However, the same results were obtained if the gels were analyzed separately (data not shown).
DISCUSSION

The role of ATGL for human fat cell lipolysis is not clear. Previous comparisons of mRNA and enzyme activity for HSL and ATGL have demonstrated clear differences in their regulation and hydrolase activity. In this work, we have assessed the relative importance of ATGL and HSL protein for human fat cell lipolysis. We compared the protein levels and lipolysis in two independent conditions with altered HSL function and lipolysis, i.e., subjects with obesity and PCOS. We also determined the effect of gene knock down of the two enzymes and inhibition of HSL during and/or after adipogenesis. With regard to ATGL expression, we developed a human-specific ATGL antibody that was very selective since it recognized only one protein band with the expected molecular size on extracts from adipose tissue or cells transfected with cDNA coding for human ATGL. The selectivity of the HSL antibody
is well documented. Some protein extraction methods do not sufficiently remove lipid droplet-associated proteins (including lipases) from the fat cake of adipose tissue protein extracts. Methodological experiments revealed that no or insignificant amounts of protein remained in the fat cake using our protein extraction protocol. In addition, the HSL inhibitor has no effect on other lipases at the concentration used in this study.

Because of the difficulties in recruiting lean PCOS subjects, the number of these women and their matched controls was limited. However, our power calculation before recruitment showed that the number of subjects was large enough to significantly detect the observed differences. Although there was a small difference in age between obese and the other two study groups, age correction of results and a subgroup analysis showed that there was no significant effect of age on these results. We found a positive correlation between HSL, but not ATGL, protein levels and lipolytic capacity in response to natriuretic peptides (30). Overall, this indicates that HSL is more important in regulating catecholamine-stimulated lipolysis, at least when considering complete hydrolysis of TG in glycerol and FFA.

There may also be incomplete lipolysis resulting in the formation of DG from TG (2), and the DG pool in human adipose tissue, although small (~1% of all lipids), is subject to rapid turnover (5). We can therefore not exclude the possibility that ATGL, with its high affinity to TG, is more important than HSL for the regulation of incomplete lipolysis (TG to DG), resulting in release of FFA but not glycerol that occurs in human fat cells. To answer this question, it is necessary to measure DG and FFA. We have developed among the most sensitive assays for the measurements of glycerol (end product of DG analysis) and FFAs in human fat cells. Unfortunately, the intracellular levels of DG and the amounts of FFAs released from our primary cultures and the intracellular levels of DG were below the detection limit of our methods. Therefore, at present, such experiments cannot be performed. However, our results are valid for complete hydrolysis of TG or DG, which always results in the release of glycerol.

Little is known regarding the regulation of ATGL expression in humans although adipose tissue mRNA expression was not altered by obesity. This was in contrast to HSL mRNA, which was significantly downregulated in this condition (22). We presently compared activation of basal lipolysis with lipase protein expression in subcutaneous adipose tissue of lean, obese, and lean PCOS women. Norepinephrine-induced lipolysis and HSL expression is decreased in the latter two states, as reviewed in Ref. 1. Obesity is associated with insulin resistance, and some PCOS women are also insulin resistant. The PCOS subjects included in this study displayed no insulin resistance, presumably because they were young and lean. This confirms findings in a similar cohort of PCOS women investigated previously (8). We could also confirm previous findings from our laboratory, namely that lean, non-insulin-resistant PCOS, and obese insulin-resistant women have a blunted catecholamine-induced lipolysis and reduced HSL expression (6, 8). More important is the concomitant observation that ATGL protein expression is not altered at all in obesity or PCOS. These results give further support to the notion that ATGL plays a less important role in regulating catecholamine-stimulated lipolysis in human fat cells. It is possible, though, that there is not a simple one-to-one relationship between HSL expression and lipolysis activation. First, there was a considerable dispersion in the relationship between HSL and lipolysis in Fig. 3D. Second, although lipolysis activation was almost identical in obese and PCOS (Fig. 3A), HSL expression was slightly but not significantly lower in obese than in PCOS women (Fig. 3B).

Although the data discussed so far favor HSL over ATGL in lipolytic regulation by catecholamines, they only provide indirect evidence for this hypothesis. However, direct proof is obtained from the RNAi experiments. We have recently optimized conditions for RNAi in primary cultures of human adipocytes (25), and we are currently perfecting a similar approach in hMSC-derived adipocytes. These studies demonstrate that gene knock down of HSL results in decreased basal and catecholamine-stimulated lipolysis, whereas siRNAs directed against ATGL only inhibit basal glycerol release. It may appear strange why an 85% reduction in mRNA for either ATGL or HSL only reduces basal lipolysis by 35–40% and stimulates lipolysis by 35% (the latter for HSL). It should be noted, though, that siRNA only inhibit protein expression transiently and have no effect on protein degradation. The endogenous half-lives of ATGL and HSL proteins are not known. Moreover, although protein expression for both lipases was clearly reduced using their cognate siRNA oligonucleotide, protein levels in these primary cultures were still easily detectable. As discussed above, there is probably a nonlinear relationship between the amounts of lipase (HSL and ATGL) present in the adipocyte and the lipolytic activity. Thus considerable lipolytic activity is present in cells where lipase expression has been reduced by ~70%. It would of course be of interest to assess the effects of double knock down by RNAi. At present, however, this is not feasible because of off-target effects that are yet be resolved in our cellular system. The ideal experiments would be to induce long-term stable reductions of these lipases. Unfortunately, such experiments are presently impossible to perform because of the lack of established human fat cell lines. The use of plasmid vectors is also not an option because the transfection efficiency is too low in human adipocytes. Nevertheless, basal and isoprenaline-induced lipolysis was simultaneously measured. It is evident that, for ATGL, the obtained reduction of this lipase only affects basal lipolysis. Even if data with double knock down could be generated, the outcome would in no way change the present interpretation of our single knock down experiments.

The RNAi results obtained in these primary human cells contrast findings in a murine preadipocyte cell line (16). We used the same protocols (decreasing gene expression of ATGL or HSL with siRNA and measuring basal and isoprenaline-induced glycerol release). The earlier study (16) also reported data on fatty acids. As mentioned above, we were not able to measure these lipids although we used an ultrasensitive bioluminescence assay. Nevertheless, the murine study showed that ATGL is important for basal and stimulated lipolysis, whereas HSL is mainly required for stimulated lipolysis in adipocytes. This is not the only regulatory aspect of lipolysis that is subject to major species differences when results of human and murine fat cells are compared (for reviews, see Ref. 1). For example, rodent adipocytes use the β3-adrenoceptor for catecholamine-induced lipolysis, whereas human fat cells respond mainly to the prolipolytic β- and β2-adrenoceptors and the antihipolytic α2A-adrenoceptor. Moreover, human fat cells display a unique prolipolytic response to natriuretic peptides (30). Overall, this
warrants caution in interpreting data obtained from murine fat cell models and extrapolating them to the human level.

The strongest evidence for a superior role of HSL among lipases in regulating human fat cell lipolysis can be obtained from our studies in hMSCs. When these cells were subjected to complete HSL inhibition during proliferation, differentiation, and lipolysis experiments, basal lipolysis was inhibited by 50%, whereas stimulated lipolysis (using a range of different agonists) was almost completely blunted. Identical results were obtained if HSL was temporarily inhibited only during the lipolysis experiment. Conversely, hMSC-derived adipocytes exposed to the HSL inhibitor during the entire differentiation process, but where the HSL inhibitor was withdrawn immediately before the lipolytic experiment, displayed essentially the same lipolytic activity as control cells. These data suggest that ATGL cannot replace HSL during chronic inhibition of the latter lipase and that HSL can immediately reestablish its lipolytic role when it is disinhibited. This is independent of the route of stimulation by either catecholamines, by direct activation of protein kinase A or through activation of cGMP.

Detailed clinical characteristics were available for cohorts 1–3 (partly summarized in Table 1) but not for cohort 4. It could be argued that the results obtained in cells from cohort 4 could be influenced by specific clinical conditions of the donors. However, we find this less likely since all donors were requested to be otherwise healthy and free of medication. Moreover, these immature cells from the stroma-vascular portion of adipose tissue were isolated and differentiated in vitro for 2–3 wk, thereby avoiding confounding environmental factors normally present when freshly isolated fat cells are used. In fact, we have previously shown that this system is efficient in establishing primary and secondary causes to alterations in fat cell function [17, 31].

On the basis of the present and previous (17, 22) comparisons of ATGL and HSL in humans, we propose the following model on their respective roles in lipolytic regulation in human fat cells. HSL is of greater importance than ATGL in regulating complete hydrolysis of TG (to glycerol) following catecholamine or natriuretic peptide stimulation in normal conditions and obesity or PCOS. However, both lipases are important for the regulation of basal complete hydrolysis of TG to glycerol. We admit that we cannot preclude the possibility that ATGL may have roles in lipolysis that are not revealed until more is known regarding the details of this lipase (e.g., phosphorylation, intracellular localization, and protein–protein interactions). In fact, recent data suggest that the regulation of enzyme activity of HSL and ATGL is more complex than previously recognized (10). However, such more advanced studies of lipase function/activity can, for the moment, only be performed in fat cell lines and not in primary cells used in the present work. Furthermore, specific ATGL inhibitors that are essential for a detailed enzymatic analysis are not yet available. In addition, we cannot excluded the possibility that our use of different human fat cell models, which was mandatory to answer all questions raised in the present work, could obscure some differences regarding ATGL vs. HSL function. Finally, this study has only examined HSL and ATGL. It is possible that additional lipases are active in human fat cells that may have yet undefined roles in the regulation of lipolysis. However, the importance of such lipases is doubtful, since HSL and ATGL are responsible for >95% of TG hydrolytic activity in mouse fat cells (28).

In summary, this study suggests that, in contrast to findings in rodents, HSL is more important than ATGL in regulating catecholamine-induced lipolysis in human fat cells under normal and insulin-resistant conditions. However, both ATGL and HSL regulate basal lipolytic activity of human fat cells. The role of these lipases in partial hydrolysis of TG to DG remains to be defined.

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