The mobilization of fat stored in adipose tissue is mediated by hormone-sensitive lipase (HSL) and the recently characterized adipose triglyceride lipase (ATGL), yet their relative importance in lipolysis is unknown. We show that a novel potent inhibitor of HSL does not inhibit other lipases. The compound counteracted catecholamine-stimulated lipolysis in mouse adipocytes and had no effect on residual triglyceride hydrolysis and lipolysis in HSL-null mice. In human adipocytes, catecholamine- and natriuretic peptide–induced lipolysis were completely blunted by the HSL inhibitor. When fat cells were not stimulated, glycerol but not fatty acid release was inhibited. HSL and ATGL mRNA levels increased concomitantly during adipocyte differentiation. Abundance of the two transcripts in human adipose tissue was highly correlated in habitual dietary conditions and during a hypocaloric diet, suggesting common regulatory mechanisms for the two genes. Comparison of obese and nonobese subjects showed that obesity was associated with a decrease in catecholamine-induced lipolysis and HSL expression in mature fat cells and in differentiated preadipocytes. In conclusion, HSL is the major lipase for catecholamine- and natriuretic peptide–stimulated lipolysis, whereas ATGL mediates the hydrolysis of triglycerides during basal lipolysis. Decreased catecholamine–induced lipolysis and low HSL expression constitue a possibly primary defect in obesity. Diabetes 54:3190–3197, 2005

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Studies on human preadipocytes. Differentiation of preadipocytes was carried out as described previously (30). In brief, cells from the monovascular fraction of collagenase-treated adipose tissue were isolated and differentiated in 22-mm wells using serum-free culture medium supplemented with 10 μmol/l rosiglitazone and 0.2 mmol/l 3-isobutyl-1-methylxanthine for 3 days and maintained in culture medium without rosiglitazone and the methylxanthine until the end of differentiation. On average, the cells of obese or nonobese subjects were investigated at day 15. Apparent fat-cell size of the differentiated preadipocytes was determined in the last 5 days of the culture (12 nonobese and 38 obese subjects). The lipids formed microdroplets, which accumulated into one ellipsoidal cluster. The area of each “lipid ellipse” was measured with a calibrated microscope, and mean area in each subject was determined from 10 to 26 measures and represented fat cell size. The average number of measurements in each obese and nonobese subject was similar (15 ± 25 and 60 ± 30, respectively). Lipolysis was performed as described previously (30). The glycerol concentration was determined in the medium. The average protein content was similar in obese (19 ± 6 μg/well) and nonobese (19 ± 8 μg/well) subjects. Leptin in the medium was measured using Quantikine Human Leptin Immunoassay (R&D Systems). We used cell protein content determined using BCA Protein Assays Reagent kit (Fierce) as a denominator for lipolysis and leptin secretion. The protein content reflected the number of adipocytes because the same level of differentiation was obtained in the two groups. On 7 nonobese and 14 obese subjects, it was possible to isolate 100 μg of proteins from three pooled incubation wells. These proteins were separated by SDS-PAGE and transferred to polyvinyldine fluoride membranes by Western blot. Two blots were performed. The blots were probed with antibodies against human HSL (9) and β3-adrenergic receptor (Santa Cruz). The proteins were detected with chemiluminescence, and the band intensity was assessed by the National Institutes of Health Image program.

Results
Effect of an inhibitor of HSL on lipases and lipolysis. The role of HSL and non-HSL lipases in acylglycerol hydrolysis and adipose tissue lipolysis was investigated using BAY, an isoxazolone inhibitor of HSL (20). BAY specificity was determined using purified preparations of lipases (Fig. 1A). Human and rat HSL were potently inhibited in a concentration-dependent fashion, whereas no effect was observed on pancreatic lipase, lipoprotein lipase, and monocYTE Glycerol lipase. Importantly, BAY had no effect on extracts from Cos7 cells expressing ATGL, a recently identified adipocyte tissue triglyceride hydrolase that may participate in fat mobilization (Fig. 1B) (16). The effect of BAY was tested on the enzyme activity of adipose tissue extracts from wild-type and HSL-null mice. In wild-type mice, BAY caused a concentration-dependent decrease of triolein hydrolysis (Fig. 1C). At 1 μmol/l, BAY induced a 90% inhibition in extracts from wild-type mice, whereas no inhibition was observed in HSL-null mice (Fig. 1D). The residual enzyme activity observed in HSL-null animals was similar to the triglyceride hydrolase activity seen after BAY inhibition in wild-type mice. Thus, BAY does not inhibit non-HSL triglyceride lipases. Lipolysis assays were performed on isolated adipocytes from wild-type and HSL-null mice (Fig. 2). Inactivation of the HSL gene revealed that there was only a partial ablation of

anonyl-[2H]oxazol-5-one), thereafter named BAY, was evaluated on enzymatic activities of purified lipase preparations using 1(3)-monooeyl-2(3)-monooeyl glycerol (MOME) for rat and human HSL, monooe for mouse monoglyceride lipase, and triolein for bovine lipoprotein lipase and porcine pancreatic lipase as substrates (21,22). MOME is a diacylglycerol analog. It binds and is not a substrate for

Mononacylglycerol Lipase. Cos7 cells were transfected with the pcDNA3, pcDNA3-HSL, and pcDNA3-ATGL vectors (17,23). Triolein hydrolysis was measured on cellular extracts.

Generation and analysis of HSL-null mice. HSL-null mice were generated by targeted disruption of the HSL gene in 129Sv-derived embryonic stem cells (14,24). The animals were killed after an overnight fast according to the Institut National de la Santé et de la Recherche Médicale animal care ethical guidelines. Adipose tissue samples from wild-type and HSL-null mice were homogenized in 4 vol homogenization buffer (0.25 mol/l sucrose, 1 mmol/l EDTA, pH 7.0, 1 mmol/l diithioerythritol, 20 μg/ml leupeptin, and 20 μg/ml antipain) and centrifuged at 15,000g for 4°C for 30 min, to obtain fat-free infranatants on which in vitro enzymatic activities were performed (21).

Protein concentrations were determined using the Bio-Rad Protein Assay. Adipose tissue lipolysis was investigated by

Studies on freshly isolated human fat cells and adipose tissue pieces. One portion (−300 mg) of adipose tissue was used to study leptin release (27). The remaining adipose tissue was used for lipolysis experiments after isolation of fat cells by collagenase digestion (10). Glycerol in the medium was determined as described previously (28). FFAs were quantified using a sensitive chemiluminescence method (29). Glycerol and FFA release were expressed in relation to the lipid weight of the incubated cells, the amount of adipocyte proteins or per cell number. Fat-cell size and number were determined as described previously (10). The concentration of proteins did not differ between nonobese and obese subjects (108 ± 11 and 112 ± 14 ng/100 μl fat cells, n = 8 and 14, respectively). In the methodological study of basal lipolysis on 251 subjects, subcutaneous adipocyte tissue was cut into small pieces (10–20 mg) and incubated under basal conditions in the same type of

aluminum buffer as used for isolated fat cells (300 mg tissue/300 ml aluminum buffer). Glycerol release was determined after 2 h of incubation and related to the amount of lipids.

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Statistical methods. The limiting factor in the first cohort was the amount of protein available for Western blot from the differentiated preadipocytes. Based on previous studies (30), we estimated that sufficient amounts of protein (100 μg) could be obtained from ~20% of the subjects and that it would be easier to recruit obese rather than nonobese subjects, because biopsies of very large amounts of adipose tissue need to be performed. Using published data on HSL expression level in adipose tissue because biopsies of large amounts of adipose tissue need to be obtained, we estimated that sufficient amounts of adipose tissue would be twice as easy to recruit obese rather than nonobese subjects. Based on previous studies (30), we estimated that sufficient amounts of protein (100 μg) could be obtained from ~20% of the subjects and that it would be easier to recruit obese rather than nonobese subjects, because biopsies of very large amounts of adipose tissue need to be performed. Using published data on HSL expression level in adipose tissue because biopsies of large amounts of adipose tissue need to be obtained, we estimated that sufficient amounts of adipose tissue would be twice as easy to recruit obese rather than nonobese subjects. Based on previous studies (30), we estimated that sufficient amounts of protein (100 μg) could be obtained from ~20% of the subjects and that it would be easier to recruit obese rather than nonobese subjects, because biopsies of very large amounts of adipose tissue need to be performed. Using published data on HSL expression level in adipose tissue because biopsies of large amounts of adipose tissue need to be obtained, we estimated that sufficient amounts of adipose tissue would be twice as easy to recruit obese rather than nonobese subjects.

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lipolysis in fat cells. BAY at 10 nmol/l partially inhibited isoprenaline-induced glycerol release, whereas 1 μmol/l BAY had an almost full inhibitory effect (Fig. 2A). BAY caused a concentration-dependent inhibition of isoprenaline-induced glycerol release in fat cells from wild-type mice, whereas basal lipolysis was not influenced (Fig. 2B). In HSL-null mice, isoprenaline did not induce lipolysis, and BAY had no inhibitory effect. Similar results were obtained for glycerol and FFA release (Fig. 2B and C). These inhibition data show that β-adrenoceptor–mediated lipolysis is entirely mediated by HSL in mouse fat cells, whereas a non-HSL lipase contributes to basal lipolysis.

Lipolysis studies on freshly isolated human fat cells are presented in Fig. 3. Isoprenaline concentration-response curves were shifted to the right using maximally effective antilipolytic concentrations of insulin and prostaglandin E2 with a preserved maximal lipolytic effect of the β-adrenoceptor agonist (Fig. 3A). This represents the classical decrease of agonist potency induced by antilipolytic molecules acting at the receptor level. In contrast, blockade of HSL activity by BAY caused an inhibition of the maximal glycerol release induced by isoprenaline. These data are consistent with the notion that the enzyme catalyzes the rate-limiting step of adipocyte lipolysis. At 1 μmol/l BAY, basal glycerol release was markedly reduced in contrast to the findings in mice. We also compared the effect of increasing concentrations of BAY on the inhibition of basal, atrial natriuretic peptide–induced (10⁻⁷ mol/l), and isoprenaline-induced (10⁻⁸ mol/l) glycerol release (Fig. 3B). Lipolysis was reduced in a concentration-dependent manner by BAY. Half-maximal effective concentration of BAY was ~10⁻⁸ mol/l. We also investigated, in parallel, inhibition of glycerol and fatty acid release (Fig. 3C). BAY inhibited isoprenaline- and atrial natriuretic peptide–induced glycerol and fatty acid release. However, basal glycerol release but not basal fatty acid release was blunted by the inhibitor. Experiments investigating the effect of BAY on human fat cell lipolysis were independently performed in two laboratories and gave similar results (data not shown). To determine whether the procedure to isolate fat cells modified basal lipolysis, basal glycerol release from subcutaneous adipose tissue pieces and isolated adipocytes were compared (supplementary Fig. 1, online appendix [available at http://diabetes.diabetesjournals.org]). A strong positive correlation was observed between the two preparations (r = 0.6; P < 0.001).
However, the rate of glycerol release was ~50% more rapid from isolated cells than tissue pieces. A similar relationship was obtained when nonobese and obese were analyzed separately (\( r = 0.54 \) and \( r = 0.57 \), respectively). Although the true basal lipolysis rate may be overestimated because collagenase isolation may relieve antilipolytic effects induced by inhibitory factors produced by cells of the stromavascular fraction, mechanistic studies of basal lipolysis on isolated fat cells are valid. BAY inhibited a large fraction of triglyceride hydrolysis and the entirety of diglyceride hydrolysis in human adipocyte extracts (Fig. 3D). These results suggest that HSL mediates the hydrolysis of triglycerides under stimulated conditions and the hydrolysis of diglycerides under both stimulated and basal conditions.

**HSL and ATGL gene expression in human adipose tissue.** We studied the relationship between ATGL and HSL gene expression in human adipose tissue. ATGL and HSL mRNA were found to be highly expressed in mature adipocytes but not in the stromavascular fraction that contains preadipocytes, endothelial cells, resident macrophages, and lymphocytes (Fig. 4A). ATGL and HSL mRNA expression increased concomitantly during the course of conversion of human preadipocytes to adipocytes (Fig. 4B). The expression of the two lipase transcripts was investigated in subcutaneous adipose tissue from a cohort of 80 obese women. HSL and ATGL mRNA levels were strongly correlated (\( r = 0.8, P < 0.0001 \)) (Fig. 4C). On an independent group of 24 women, the positive correlation was confirmed in habitual dietary conditions (\( r = 0.6, P < 0.005 \)) but also observed after a 10-week hypocaloric diet (\( r = 0.9, P < 0.0001 \)). The individual variations induced by the hypocaloric diet were highly correlated (\( r = 0.8, P < 0.0001 \)) (Fig. 4D). The relationship in the expression of the two genes is specific because no correlation was observed between ATGL or HSL mRNA and the transcript for adiponutrin, a protein with high homology to ATGL also expressed in adipose tissue (data not shown) (18). It appears therefore that HSL and ATGL are tightly coregulated in human adipose tissue.

**Comparison of nonobese and obese subjects.** Having established the essential role of HSL in catecholamine- and

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**FIG. 3. Effect of BAY on human fat cell lipolysis.**

(A) Antilipolytic effects of BAY (\( \bullet \), \( 10^{-5} \) mol/l; \( \square \), \( 10^{-7} \) mol/l), insulin (\( \circ \), \( 10^{-8} \) mol/l), and prostaglandin E2 (\( \Delta \), \( 10^{-7} \) mol/l) on isoprenaline-induced glycerol release. (B) Dose-response curve of isoprenaline without antilipolytic agents (\( n = 4 \)). (C) Effect of BAY on basal (\( \square \)) and isoprenaline-induced (\( \bullet \)) glycerol release (\( n = 4 \)). (D) Inhibition by BAY (\( 10^{-5} \) mol/l) of basal, isoprenaline-induced (\( \bullet \)), and atrial natriuretic peptide–induced (ANP, \( 10^{-7} \) mol/l) glycerol and FFA release (\( n = 6 \)). (E) BAY (\( 10^{-5} \) mol/l) inhibition of triglyceride (triolein) and diglyceride (MOME) hydrolyase activity in human adipose tissue extracts (\( n = 4 \)).

**FIG. 4. HSL and ATGL gene expression in human adipose tissue.**

HSL and ATGL mRNA levels were quantified by reverse transcription-quantitative PCR and normalized with 18S rRNA levels. (A) HSL and ATGL mRNA levels in isolated adipocytes and stromavascular fraction (SVF) from human adipose tissue (\( n = 6 \)). (B) Expression of HSL, ATGL, and peroxisome proliferator–activated receptor \( \gamma \) (PPAR\( \gamma \)) mRNA during human preadipocyte differentiation (\( n = 5 \)). (C) Relationship between HSL and ATGL mRNA levels in subcutaneous adipose tissue from 80 obese women. (D) Relationship between HSL and ATGL mRNA variation during a hypocaloric diet in subcutaneous adipose tissue from 24 obese women. HSL and ATGL mRNA levels were measured before and after a 10-week hypocaloric diet. The variations are calculated as follows for each individual: (mRNA level after the diet − mRNA level before the diet)/mRNA level before the diet.

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natriuretic peptide–stimulated human fat cell lipolysis, we investigated whether defects of adipocyte lipolysis and HSL expression were found in human obesity. A population of 81 obese and 42 nonobese subjects was studied. Circulating leptin concentrations were 37 ± 33 ng/ml in the obese subjects and 12 ± 12 ng/ml in the nonobese subjects (P < 0.001). Corresponding values for plasma insulin were 12.5 ± 6.9 and 6.6 ± 3.6 mU/l (P < 0.001) and for plasma glucose, 5.4 ± 1.9 and 4.9 ± 0.5 mmol/l, respectively (P < 0.001).

Lipolysis data from mature fat cells and preadipocytes differentiated in primary culture were compared in Fig. 5. We also measured leptin secretion as a control adipocyte function. In freshly isolated mature fat cells, catecholamine-induced (norepinephrine) and \( \beta \)-adrenoceptor–induced (isoprenaline) lipolysis were decreased by 40% (P = 0.002 and P < 0.001, respectively) in obesity (Fig. 5A). Leptin secretion was increased by 80% (P < 0.001). We expressed lipolysis data per protein content because, first, the protein concentration is similar in large mature adipocytes and increased leptin secretion in fat cells from subcutaneous adipose tissue of obese subjects (7,27).

To find out whether the lipolysis defect is primary or secondary to obesity, we used preadipocytes prepared from the same biopsies as the mature fat cells. The fraction of preadipocytes that differentiated into adipocytes was similar in the nonobese and in the obese subjects: 60 ± 20% and 57 ± 18%, respectively. The size of the cells was also similar in the obese and in the nonobese subjects: 1.8 ± 0.4 \( \times 10^{-9} \) m\(^2\) and 1.5 ± 0.3 \( \times 10^{-9} \) m\(^2\), respectively. Differentiated preadipocytes displayed similar leptin secretion in obese and nonobese subjects, whereas norepinephrine- and isoprenaline-stimulated lipolysis was reduced by 60–70% in the obese subjects (P < 0.001) (Fig. 5B). As a ratio of stimulated to basal lipolysis, a similar pattern was found (supplementary Table 1, online appendix). Thus, a blunted lipolysis without a change in leptin secretion in preadipocytes from obese subjects suggests that altered lipolysis is not secondary to the obese state.

We have previously shown a decreased HSL expression in mature subcutaneous fat cells of obese subjects (9). The very limited amount of stromavascular cells precluded protein analysis on preadipocytes of all subjects. However, power calculation indicated that we could detect differences between obese and nonobese subjects with 14 obese and 7 nonobese subjects (see statistical methods). HSL expression was decreased by 60% in cells from obese subjects (Fig. 5C). There was no difference between groups in the protein expression of the \( \beta \)-\( \beta \)-adrenoceptor.

**DISCUSSION**

The hydrolysis of the triglycerides stored in the fat cell is a complex phenomenon involving lipases and proteins associated with the lipid droplet (4,33). Based on the present data and earlier reports, the following model for lipase activation can be proposed in human fat cells (Fig. 6). ATGL and HSL both possess the capacity to hydrolyze triglycerides in vitro (16,34). However, only HSL shows a significant diglyceride lipase activity (35). Although HSL has the capacity to hydrolyze monoglycerides in vitro, monoglyceride lipase, which is not under hormonal control, is required to obtain complete hydrolysis of monoglycerides in vivo (36). Triglycerides are hydrolyzed at a lower rate than diglycerides, indicating that the first step of lipolysis is rate limiting (37). In human fat cells, catecholamines and natriuretic peptides stimulate lipolysis through \( \beta \)-adrenoceptors and the natriuretic peptide receptor A, respectively. Stimulation of the two pathways leads to an increase in cAMP and cGMP levels, respectively (4). Both protein kinases A and G phosphorylate and activate HSL at least in part through translocation of the enzyme from the cytosol to the lipid droplet (38–40). In line with the role of HSL in triglyceride hydrolysis under...
stimulated conditions, ATGL is not phosphorylated by protein kinase A and does not appear to be acutely regulated by isoprenaline (16). Our data clearly demonstrate that the two major and independent activation pathways converge on HSL. However, our data show that non-HSL lipases contribute to the hydrolysis of triglycerides into diglycerides under basal conditions. Although ATGL seems to play a predominant role in basal lipolysis, it cannot be excluded that other adipose tissue enzymes with the capacity to hydrolyze triglycerides play a role (16-17,19). The coordinated variation in ATGL and HSL gene expression during adipocyte differentiation and in various dietary conditions suggests that the two genes belong to a common regulatory network with tight transcriptional control.

Primary alterations in the ability to mobilize lipids stored in adipose tissue could occur in obesity. Moreover, a primary defect in leptin secretion could be present and induce a leptin-resistant state with inadequate feedback signals between caloric intake and energy demand. In freshly isolated fat cell preparations, we could confirm earlier studies demonstrating increased leptin secretion and decreased catecholamine-induced lipolysis in obesity (7,27). In differentiated preadipocytes, catecholamine-induced lipolysis was markedly reduced although leptin secretion was normal. These findings were obtained in a very large cohort (81 obese and 42 nonobese subjects). Because the preadipocytes were differentiated in vitro and kept in serum-free medium for 2 weeks, it is very likely that any confounding environmental influence could be excluded (41). This notion is further supported by the finding that basal lipolysis was increased in freshly isolated fat cells but decreased in differentiated preadipocytes of the obese subjects. It has recently been demonstrated that obesity is characterized by a low-grade inflammatory state of adipose tissue with an increase in macrophage number (42,43). It may be argued that macrophage-derived cytokines could influence lipolysis in adipose tissue of the obese subjects and explain the differences between lean and obese subjects. However, data from catecholamine-induced lipolysis were similar in freshly isolated fat cells and differentiated preadipocytes.

From the different cell types of the stromavascular fraction, only preadipocytes survive in the serum-free conditions of culture (41), excluding the confounding contribution of factors produced by the other cells on the differentiation process. One cannot exclude stable epigenetic effects resulting from paracrine or endocrine effects on preadipocytes during the early development of adipose tissue. This appears unlikely because lipolysis was selectively altered in differentiated preadipocytes from obese subjects, whereas the level of differentiation, cell size, leptin secretion, and expression of β2-adrenoceptors were not affected. Our data therefore favor an adipocyte-autonomous defect of lipolysis in obesity.

The impaired HSL expression most likely contributes to the lipolytic defect observed in obesity. As demonstrated here, HSL activity is rate-limiting for catecholamine-induced lipolysis. We showed earlier that the level of HSL expression is related to the lipolytic capacity in mature fat cells (11). The protein expression of this enzyme was markedly reduced, whereas the protein expression of the β2-adrenoceptor, another potential candidate (7), was not altered in differentiated preadipocytes from obese subjects. The coregulation between HSL and ATGL gene expression observed in different dietary conditions suggests that the expression of ATGL may be altered in obesity. A lower expression of ATGL has been reported in white adipose tissue of genetically obese mice (18). It remains to be determined whether other lipases are important in adipocyte lipolysis and whether coregulation with HSL is observed. The link between HSL and obesity is also supported by genetic studies that show association with obesity and impaired lipolytic activity of subcutaneous fat cells (44-46). The physiological significance of lipolysis and HSL defects in obesity may be seen in two ways. A lipolytic defect could contribute to the development of obesity through impairment in the mobilization of fat stores. However, data from HSL-null mice do not support this hypothesis. The animals are lean and resistant to genetic and diet-induced obesity (12,15,47). Alternatively, the defect may constitute an early, possibly primary, event in obesity that protects against excessive FFA release. Accordingly, HSL deficiency in mice causes a reduction in plasma FFA levels (12,13). The lipid profile of HSL-null mice somewhat resembles that of patients treated with nicotinic acid, which acts, at least in part, through inhibition of adipose tissue lipolysis (3,48). In that context, a strong rationale can be seen for the development of adipose tissue HSL inhibitors such as BAY in the treatment of the metabolic syndrome (20,49,50).

By summary, this study demonstrates that HSL is the major lipase catalyzing the rate-limiting step in stimulated lipolysis in humans, whereas ATGL participates in basal lipolysis. Decreased catecholamine-induced lipolysis but not leptin production in subcutaneous adipose tissue of obese subjects is an early, possibly primary, defect that is linked to decreased protein expression of HSL.

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