The Presence of a Catalytically Inactive Form of Hormone-Sensitive Lipase Is Associated With Decreased Lipolysis in Abdominal Subcutaneous Adipose Tissue of Obese Subjects

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Hormone-sensitive lipase (HSL)-L is a key enzyme in the mobilization of fatty acids from triglyceride stores in adipocytes. A shorter variant of HSL (HSL-S) was detected in humans. This one is generated through in-frame skipping of exon 6 during the processing of HSL mRNA and results in a protein devoid of lipase activity. The role of HSL-S is unknown. The aims of this study were to identify both HSL variants in adipose tissue biopsies and to determine if the presence of HSL-S is correlated to the lipolytic capacity of adipocytes. The study was performed in human abdominal subcutaneous adipocytes from two groups of seven obese subjects. In the group of subjects with both HSL proteins (L+S) group, two immunoreactive bands (80 and 88 kDa) were detected, whereas only the 88-kDa protein was detected in the group with only the wild-type HSL-protein (L group). In the L+S group, the HSL activity was 20% lower ($P < 0.05$) and the (S/S^+) HSL mRNA ratio was twofold higher than in the L group ($P < 0.05$). The maximally lipolytic capacities measured from isolated adipocytes incubated with norepinephrine or other lipolytic agents were 40% lower in the L+S group ($P < 0.05$). These results suggest that the presence of the truncated HSL protein is associated with an impaired adipocyte lipolysis. *Diabetes* 52:1417–1422, 2003

The mobilization of fatty acids from adipose tissue triglycerides provides mammals with the quantitatively most important energy source (i.e., nonesterified fatty acids). Hormone-sensitive lipase (HSL) is a multifunctional lipase that plays a critical role in this process because it is presumed to be the rate-limiting enzyme for hydrolysis of stored triglycerides (1). This intracellular neutral lipase is highly expressed in adipose tissue and catalyzes the hydrolysis of triacylglycerols and diacylglycerols. The activity of lipase is acutely increased by reversible cAMP-dependent phosphorylation, which also leads to its translocation from the cytoplasm to the lipid droplet. Lipolytic hormones such as catecholamines activate the different steps of the lipolytic process leading to the activation of HSL (the so-called lipolytic cascade) (2).

The human HSL gene consists of at least 10 coding exons, of which exon 6 encodes the serine residue of the catalytic triad (3,4). In 1997, Laurell et al. (5) reported the identification of an alternatively spliced form of human HSL that is exclusively expressed in white adipose tissue. In vitro functional analysis showed that skipping exon 6 generated a shorter variant of HSL (HSL-S) (80 kDa compared with 88 kDa for the wild-type HSL protein HSL-L), which, as expected, was devoid of both esterase and lipase activities. In 1998, we reported the presence of two immunoreactive bands (~84 and 88 kDa) in homogenates from obese adipose tissue (6). When a competitive immunoprecipitation experiment was performed, followed by Western blot analysis of the immunoprecipitates, no immunoreactive band at 84 kDa was detected, strongly suggesting that the 84-kDa protein was an HSL variant.

The first goal of this study was to confirm that the 84-kDa immunoreactive protein corresponds to the short form of the HSL variant generated from skipping exon 6. Then we investigated the relationship between the presence of this HSL variant and alterations in the lipolytic capacity of adipose tissue. The study was performed in two groups of obese subjects, with or without the presence of the short form of HSL, as determined by Western blot analysis.

RESEARCH DESIGN AND METHODS

Subjects. A total of 53 obese subjects were screened by HSL Western blot analysis to select 14 obese subjects (2 men and 12 women). They were divided into two groups according to the presence or absence of HSL-S: the group of subjects with both HSL proteins (L+S group) and the group with only the intact 88-kDa HSL protein (L group). The selection of subjects was carried out to get two groups comparable with regard to sex, age, and BMI.

An abdominal subcutaneous fat biopsy was obtained during elective surgery after an overnight fast. The subjects underwent gastric banding because of obesity, which involved a laparotomy. The subjects were drug-free and otherwise healthy. General anesthesia was induced by a short-acting barbiturate and maintained by nitrous oxide and fentanyl. The samples of

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dcAMP, dibutyryl cAMP; HSL, hormone-sensitive lipase; HSL-S, shorter variant of HSL; HSL-L, wild-type HSL protein; L group, group with only the intact 88-kDa HSL protein; L+S group, subjects with the long- and short-form of apo HSL proteins.

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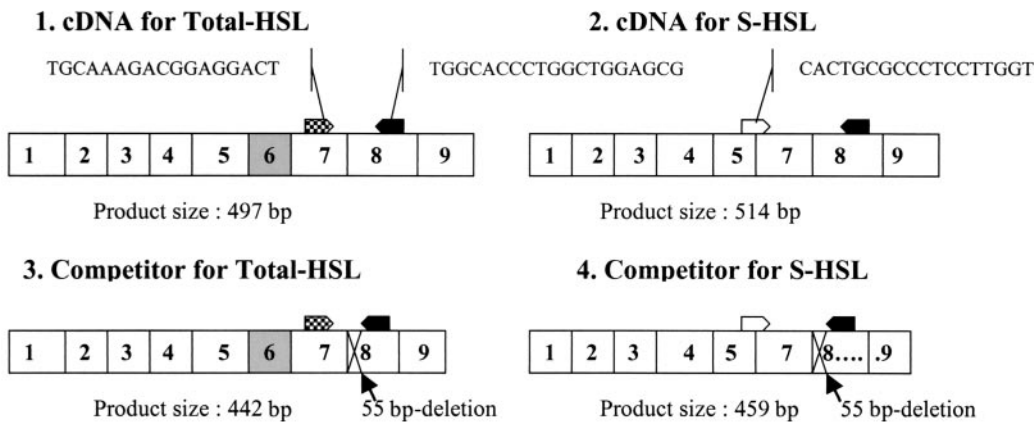

FIG. 1. Strategy of amplification for the assay of mRNA levels of HSL total form, S-HSL, and the corresponding DNA competitors. The respective primers are indicated by the arrows. bp, base pair.

adipose tissue were taken at the beginning of the surgical procedures, within minutes after the skin incision was made, and 20–30 min after the induction of general anesthesia. The study was approved by the Hospital's Committee on Ethics, and all subjects gave their informed consent before entering the study. In the morning of the day before anesthesia was administered, blood pressure was measured and a venous blood sample was obtained after an overnight fast for the determination of glucose, lipids (by the hospital's routine chemistry laboratory), and plasma insulin with a commercial radioimmunoassay kit (Pharmacia-Upjohn, Uppsala, Sweden).

Fat biopsies were immediately transported to the laboratory in saline at 37°C, and ~300 mg of the tissue was frozen in liquid nitrogen for analysis of HSL enzymatic activity, mRNA levels, and protein levels. The rest of the tissue was used immediately for in vitro lipolysis experiments.

Lipolysis experiments. Isolated fat cells were prepared from fresh biopsies through incubation with collagenase according to Rodbell (7). Fat cell size was determined from an aliquot of cells that was suspended in an albumin buffer solution and placed on a glass slide. The diameter of 100 cells was determined by examination with a microscope (Carl Zeiss, Thornwood, NY) equipped with a caliper scale. Mean fat cell volume was then calculated according to the methods developed by Hirsh and Gallian (8). The total lipid content in each incubation was determined gravimetrically after organic extraction with heptane, whereas the number of adipocytes was calculated by dividing the total lipid weight by the mean cell weight.

Isolated fat cells were incubated as reported in detail earlier (9). In brief, adipocytes were incubated in a Krebs-Ringer buffer supplemented with 20 g/l BSA, 0.1 g/l ascorbic acid, and 1 g/l glucose, with or without increasing concentrations of agents acting at different levels of the lipolytic cascade: the nonselective adrenoceptor agonist norepinephrine; the nonselective β-adrenoceptor agonist isoprenaline; forskolin, a direct activator of adenylyl cyclase; and dibutyryl cAMP (dcAMP). The latter molecule is a phosphodiesterase-resistant cAMP analog that activates the cAMP-dependent protein kinase. Glycerol release in the incubation medium was determined after a 2-h incubation at 37°C, using an automated bioluminescence assay (10). Lipolysis rates in the presence or absence of maximal effective agonist concentrations were expressed per gram of triglyceride or per cell number. All agonists caused a dose-dependent increase in glycerol release that reached a plateau at the highest agonist concentrations. The sensitivity to agonist action was defined as the pD2 value, i.e., the negative logarithm of the EC_{50} value (effective agonist concentration causing 50% of maximal effect).

Assay of HSL enzymatic activity. Frozen tissue samples (~300 mg) were homogenized with a knife homogenizer in 0.6 ml of 0.25 mol/l sucrose, 1 mmol/l dithioerythritol, 1 mmol/l EDTA, 20 μg/ml leupeptin, 20 μg/ml antipain, and 1 μg/ml pepstatin A, at pH 7.0 and 4°C. Fat-depleted infranatants were obtained after centrifugation at 12,000*g* and 4°C for 3 h.

The HSL activity assay was performed essentially as described previously (11), using the diolein analog 1(3)-mono[^{3}H]oleoyl-2-oleylglycerol as substrate (12). One unit of enzyme activity is defined as 1 μmol oleic acid released per minute at 37°C. Because the phosphorylated and dephosphorylated forms of the enzyme have the same activity toward diglyceride substrates, only the total amount of activable enzyme in the sample is measured. All samples were analyzed in triplicate, and lipase activity was related to the weight of homogenized adipose tissue.

Western blot analysis of HSL-S and HSL-L. The protein measurement was performed in all subjects using the BCA protein assay (Pierce, Rockford, IL). Equal amounts of total proteins (200 μg) were subjected to SDS-PAGE according to the Laemmli methods under reducing conditions (13) and then transferred to nitrocellulose (as reported in detail earlier [6]). Membranes were incubated with an affinity-purified polyclonal chicken anti-rat HSL antibody (1:1,000) before an anti-chicken IgG alkaline phosphatase conjugate (1:8,000; Sigma, St. Louis, MO) and then briefly incubated with enhanced fluorescence detection reagents (ECF; Amersham, Buckinghamshire, U.K.). Usual exposure times were 15, 30, and 45 s, but they were performed up to 1 h in five of the seven subjects to confirm the absence of the S-protein in the L group. After a stripping treatment (62.5 mmol/l Tris HCl [pH = 6.7], 2% SDS, 100 mmol/l mercaptoethanol for 30 min at 50°C), membranes were first incubated with ECF to control the total removal of the previously detected HSL bands and then incubated with an affinity-purified polyclonal rabbit anti-human exon 6 HSL before an anti-rabbit IgG alkaline phosphatase conjugate (1:8,000) (Sigma). The relative amounts of immunodetectable HSL contained in each lane were determined by scanning with a Fluorimager (Molecular Dynamics, Sunnyvale, CA), followed by analysis using the Image Quant program.

Assay of mRNA levels of HSL total form and HSL-S. RT-competitive PCR was performed to measure the quantity of mRNA from either both forms of HSL (total HSL) or HSL-S alone. Samples were processed simultaneously. Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Specific first-strand cDNA synthesis was performed from 100 ng of total RNA preparations with the thermostable RT (*Tth* DNA polymerase; Promega, Ecully, France) and 15 pmol/l of specific reverse primer (5′-TGG CAC CCT GGC TGG AGC G-3′) as followed 3 min at 60°C, 15 min at 72°C, and 5 min at 99°C in a thermocycler (PCR express; Hybaid, Teddington, U.K.). Thereafter, PCR was carried out with 20 μl of the RT, 10 units of *Taq* polymerase (Life Technologies, Cergy Pontoise, France), 15 pmol/l of primers (as described in Fig. 1), and 5 μl of a defined working solution of the corresponding DNA competitor (Fig. 1). Amplification started with 120 s at 94°C, followed by 40 cycles of PCR amplification (40 s at 94°C, 60 s at 60°C [for total HSL amplification] or 68°C [for HSL-S amplification], and finally 50 s at 72°C). PCR products were resolved on 2% agarose gels, stained with Gel Star (Tebu, Le Perray-en-Yvelines, France), and scanned. The band density was evaluated with the Image Quant program. To correct for differences in nucleotide number, the density ratio of the competitor band to the target was divided by a correction factor, which was 0.89 for the short and total forms of HSL. The logarithm of the corrected ratio was then plotted versus the logarithm of the initial amount of competitor added in the PCR medium. At the competition equivalence point (log ratio = 0), the initial concentration of the target corresponds to the initial concentration of competitor added. When DNA competitor was used, the value obtained at the equivalence point was

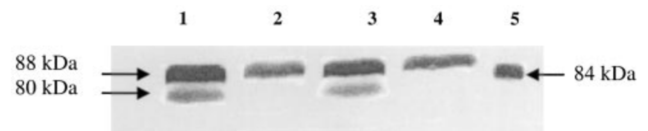

FIG. 2. Western blot analysis of HSL from human adipose tissue. Fat-depleted infranatants (200 μg total protein) were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Immunoreactive proteins were detected with an anti-HSL antibody and enhanced florescence detection (for more details, see RESEARCH DESIGN AND METHODS). The position of the protein detected by the anti-HSL antibody has been calculated from the mobilities of reference proteins. *Lane 5* contained recombinant rat HSL (84 kDa) as standard. The blot shows the results from four representative subjects.

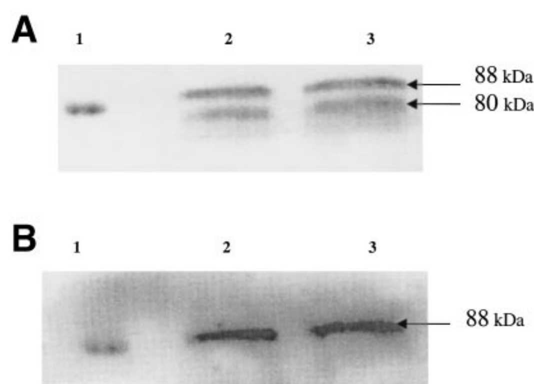

FIG. 3. Western blot analysis of HSL from homogenates of adipose tissue (200 μg of total proteins) of two subjects from the L+S group. Electrophoresis and transfer of proteins onto nitrocellulose membranes were performed as described in RESEARCH DESIGN AND METHODS. Membranes were first incubated with a polyclonal chicken anti-rat HSL before an anti-chicken IgG alkaline phosphatase conjugate (*A*). After an oxidative treatment, membranes were again incubated with a polyclonal rabbit anti-human exon 6 HSL before an anti-rabbit IgG alkaline phosphatase conjugate (*B*). Immunoreactive proteins were detected by enhanced fluorescence. *Lane 1* contained a standard of recombinant rat HSL. *Lanes 2* and *3* contained two homogenates of subjects from the L+S group.

multiplied by factor 2 because the competitor is double-stranded, whereas the target RNA is single-stranded.

Drugs and chemicals. Dibutyryl cAMP, *Clostridium histolyticum* collagenase type I, anti-chicken IgG peroxidase conjugate, antipain, pepstatin A, and leupeptin were obtained from Sigma. Norepinephrine and (−) isoprenaline hydrochloride came from Hässle (Mölndal, Sweden). The 1(3)-mono [^{3}H]oleoyl-2-oleylglycerol was prepared by Dr. L. Krabisch at the Department of Cell and Molecular Biology, Lund University (Lund, Sweden). All other chemicals were of the highest grade of purity commercially available.

Statistical analysis. Values are given as means ± SE. Statistical analysis was performed using the Student's paired t test and correlation using linear regression. All statistical calculations were made using a commercially available computer program (StatView; SAS Institute).

RESULTS

An example of Western blot analysis of adipose tissue from four subjects is shown in Fig. 2. These results allowed us to divide the subjects into two groups, according to the number of HSL immunoreactive bands; subjects with two immunoreactive bands constituted the L+S group, whereas subjects with only the 88-kDa band constituted the L group. The lower molecular band corresponded to the 80-kDa protein, as determined from comparisons with the 88-kDa HSL band and the 84-kDa rat HSL band. To determine the nature of the 80-kDa immunoreactive band, the blot from Fig. 2 was stripped of all antibodies and probed with an anti–exon 6 HSL antibody (directed against the amino acids encoded by exon 6) (Fig. 3). In this analysis, only the 88-kDa protein was detected. After another stripping procedure followed by reprobing with the classic HSL antibodies, both bands were again detected (data not shown).

TABLE 1
Characteristics of obese subjects divided into two groups according to the presence of the HSL-S variant

	L group	L + S group	*P*
n	7	7	—
Age (years)	38.0 ± 5.0	38.0 ± 6.0	NS
BMI (kg/m^2)	43.7 ± 1.7	44.2 ± 1.6	NS
Waist-to-hip ratio	0.99 ± 0.03	0.96 ± 0.04	NS
Glucose (mmol/l)	6.0 ± 0.4	7.6 ± 1.3	NS
Insulin (mU/l)	29.1 ± 6.0	25.7 ± 5.2	NS
Cholesterol (mmol/l)	5.0 ± 0.4	5.3 ± 0.3	NS
Triglycerides (mmol/l)	2.9 ± 0.6	1.8 ± 0.3	NS
Systolic blood pressure (mmHg)	134 ± 8	134 ± 7	NS
Diastolic blood pressure (mmHg)	77 ± 4	84 ± 4	NS
Fat cell volume (pl)	896 ± 89	916 ± 60	NS

Data are means ± SE and are compared using the unpaired Student's t test (NS: $P > 0.05$). (pl, picoliter = 10^{-12} R).

TABLE 2
Pharmacological properties of isoprenaline, β-adrenoceptor agonists, forskolin, and dcAMP in human subcutaneous adipocytes from subjects with or without HSL-S

	Maximal glycerol release*	pD2†
Basal		
L group	1.10 ± 0.19	—
L + S group	0.80 ± 0.13	—
Isoprenaline		
L group	3.21 ± 0.55	—
L + S group	1.95 ± 0.29‡	—
Noradrenaline		
L group	2.46 ± 0.40	6.97 ± 0.34
L + S group	1.36 ± 0.27‡	7.63 ± 0.15
Forskolin		
L group	3.29 ± 0.52	—
L + S group	2.04 ± 0.30‡	—
dcAMP		
L group	3.19 ± 0.46	—
L + S group	2.03 ± 0.32‡	—

Data are means ± SE. n = 7. *Lipolysis rates (μmol · 2 h^{-1} · g^{-1} triglycerides) at the maximal effective agonist concentration with the basal value subtracted. †pD2 = −log EC50 (effective agonist concentration causing 50% of maximal effect); ‡$P < 0.05$: (1 band) vs. (2 bands) group.

The clinical characteristics of the two groups of obese subjects are shown in Table 1. The BMI ranged from 39.2 to 50.9 kg/m^2. As expected from the selection of the subjects, the two groups were comparable in age and BMI. Moreover, there were no statistical differences between the two groups, in any of the other clinical characteristics, such as waist-to-hip ratio, plasma glucose, insulin, cholesterol and triglycerides, blood pressure, and fat cell volume.

Data with basal and maximally stimulated glycerol releases are shown in Table 2. Basal glycerol release values (without any lipolytic agent) were not statistically different between the two groups. However, the rates of isoprenaline, norepinephrine, forskolin, and dcAMP-induced lipolysis at the maximal effective agonist concentrations were significantly lower in the L+S group. The individual values for lipolytic sensitivity of the adrenergic agonists (pD2) were also compared and no difference between the groups was observed. The mean response curves for norepinephrine and forskolin in the two groups are shown in Fig. 4. Norepinephrine caused a dose-dependent stimulation of lipolysis. The L+S group exhibited a reduced response to the addition of norepinephrine, which was evident even when the basal values were not subtracted (data not shown). From 10^{-7} mol/l, mean values were lower in the L+S group and the differences

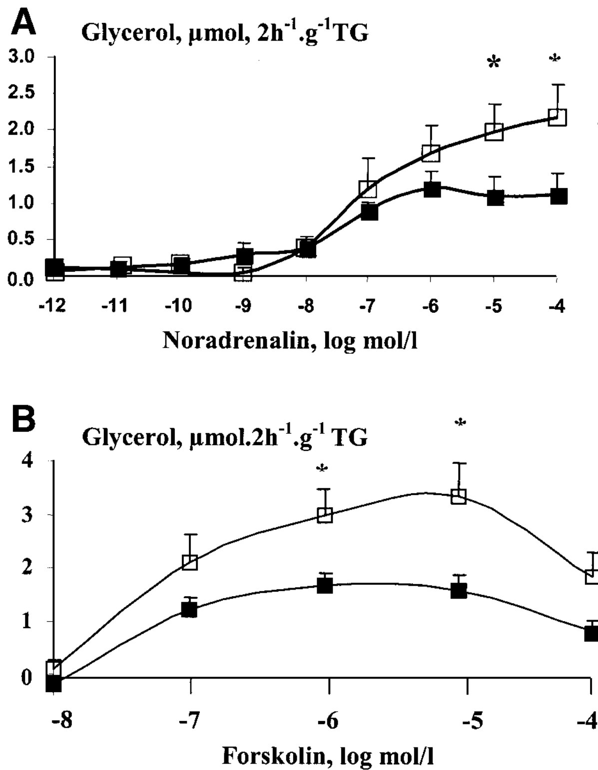

FIG. 4. Mean concentration response curves for norepinephrine (*A*) and forskolin (*B*) in the two groups of subjects. The lipolytic effect was determined in adipocytes from subjects without the short form of HSL (□) and subjects with both forms of HSL (■). Isolated fat cells were incubated with or without the indicated concentrations of the agent, and glycerol release to the medium was measured and used as the lipolysis index, expressed as glycerol release per weight of triglycerides and plotted as absolute rates minus the basal rates. *$P < 0.05$.

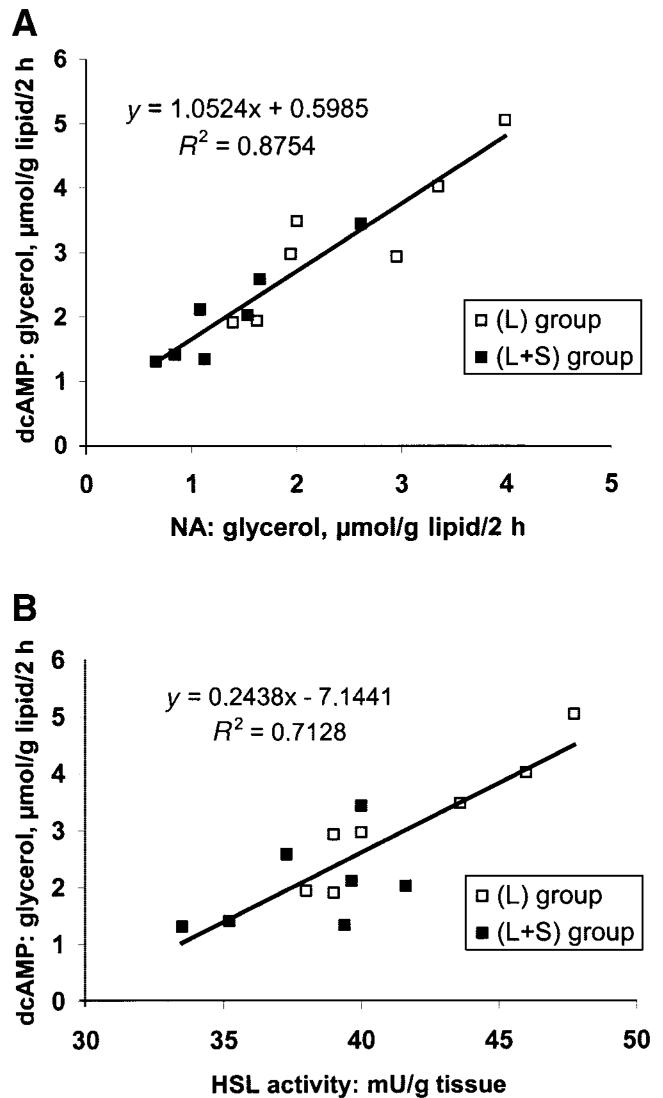

FIG. 5. Correlation between glycerol release induced by a maximal effective concentration of dcAMP and norepinephrine (NA) (*A*) and between HSL enzymatic activity and glycerol release induced by a maximal effective concentration of dcAMP (*B*) in adipocytes from subjects with or without the short form of HSL (L+S and L groups, respectively).

were significant for 10^{-5} and 10^{-4} mol/l ($P < 0.05$). The mean concentration response curve for forskolin again reported lower values for the L+S group, which reached significance for 10^{-6} and 10^{-5} mol/l. Moreover, there was a strong correlation between maximal glycerol release induced by norepinephrine and dcAMP in the whole cohort ($R^2 = 0.88$) (Fig. 5). All significant differences between the two groups persisted for lipolysis when expressed per cell instead of per gram of lipid (data not shown).

The mean enzymatic activity, protein content, and gene expressions of HSL are reported in Table 3. The enzymatic activity was 20% lower in the L+S group ($P < 0.05$), and the density of the 88-kDa HSL protein was not different between the groups. Although the total HSL mRNA levels were not significantly different between the groups, the ratio of HSL-S mRNA to total HSL mRNA was twofold higher in the L+S group. A correlation analysis between lipolysis and HSL enzyme activity was performed using linear regression. As demonstrated in Fig. 5, HSL activity correlated with maximal dcAMP-induced glycerol release ($R^2 = 0.88$). HSL activity also correlated with either maximal isoprenaline or norepinephrine-stimulated glycerol release ($R^2 = 0.79$ and 0.80, respectively) (data not shown). Similar results were obtained when maximal glycerol values were related to fat cell number (data not shown).

DISCUSSION

The immunodetection of HSL using Western blot analysis occasionally revealed a protein band of a lower molecular band in addition to the main 88-kDa protein. Initially described as an 84-kDa protein (2), new Western blot analysis with the Image Quant program (Fig. 3) showed that the lower band has a slightly smaller apparent M_r (molecular ratio) (~80 kDa) than the enzyme from rat adipose tissue (84 kDa). Results from competitive immunoprecipitation experiments, followed by Western blot analysis of the immunoprecipitates, strongly suggested that the 80- and 88-kDa proteins share common epitopes and are structurally related (2). In-frame skipping of exon 6 has been shown to generate a shorter transcript, which is translated into an 80-kDa protein (5). The aims of the study were, first, to define the nature of the 80-kDa protein that we revealed in human subcutaneous adipose tissue and, second, to determine if this protein corresponds to

TABLE 3
Enzymatic activity, protein content, and mRNA level for HSL in both groups of subjects

	L group	L + S group	*P*
HSL activity (mU/mg protein)	44.0 ± 2.2	35.4 ± 2.2	0.039
Intensity of the 88-kDa protein (OD × mm^2)	33,504 ± 10,544	27,599 ± 7,578	0.623
Total HSL mRNA level (attoM mRNA/μg total isolated RNA)	101 ± 38	113 ± 35	0.890
HSL mRNA ratio (short form/total form)	0.07 ± 0.03	0.14 ± 0.07	0.012

Data are means ± SE (n = 7) and were statistically compared by an unpaired Student's t test. OD, optical density. attoM = 10^{-18} mol/l.

the short form of HSL that was described by Laurell et al. (5). Immunodetection experiments with an antibody specifically recognizing the amino acids encoded by exon 6 strongly suggest that the 80-kDa protein corresponds to the polypeptide translated from the transcript generated through the human-specific skipping of exon 6. A final conformation of the identity of the 80-kDa protein will have to await the isolation of sufficient amounts to perform mass spectrometry analysis.

Laurell et al. (5) showed that the truncated enzyme expressed in COS cells did not exhibit any enzyme activity. The second aim of the present study was to determine the relationship between the presence of the truncated HSL protein and the lipolytic function of the adipocyte. For that purpose, HSL enzymatic activity and maximal lipolytic capacity of the adipocyte were determined in the two groups of subjects with or without the truncated HSL protein. Our results clearly show that the maximal lipolytic capacity of adipocytes from the L+S group was 35% lower than in the L group with all lipolytic agents tested in this study. The reduction in the lipolytic capacity was the same regardless of whether lipolysis was stimulated at the level of the adrenoceptors (norepinephrine) or at the level of protein kinase A (with dcAMP). Furthermore, there was a strong linear correlation between glycerol release induced by norepinephrine and dcAMP (R^2 = 0.88). These data strongly suggest that the difference in lipolytic capacity between the two groups is determined at or beyond the level of protein kinase A.

Interestingly, the HSL enzymatic activity was reduced by 20% in the L+S group and there was a good linear correlation between the HSL activity and the lipolytic action of dcAMP (R^2 = 0.71). These results confirm our previous data, showing that HSL enzymatic activity is a major determinant of the lipolytic capacity of human fat cells (2). No difference in the quantity of HSL-L protein was found between the two groups, indicating that there is no compensatory increase in the expression of HSL-L when HSL-S is present.

The mechanism whereby the presence of HSL-S decreases lipolytic capacity is not known. One possible mechanism relates to the finding that HSL appears to be a homodimer (14,15) and that dimerization is essential for function. Thus, one possibility is that oligomerization of HSL-S with HSL-L could render dimers that are devoid of enzymatic activity. Another possibility is that HSL-S, which has been shown to be a substrate for cAMP-dependent protein kinase A, translocates to the lipid droplet upon lipolytic stimulation of the adipocyte, in agreement with what has been established for HSL-L, and thus competes with HSL-L for binding to the lipid droplet (16,17). Detailed molecular and cellular studies will have to be performed to evaluate these possible mechanisms.

HSL gene expression measurements showed that the ratio between the HSL-S RNA level and the total RNA level is twofold higher in the group with both HSL proteins. Interestingly, in the L group, where the HSL-S was not detected by Western blot analysis, the ratio ranged the same between 0.02 and 0.13. With a mean ratio of 0.07, we were unable to quantify any S-protein in the L group. Moreover, there was a good linear correlation between this ratio and the maximal lipolysis rate induced by dcAMP in the 14 subjects (R^2 = 0.66) (data not shown)

In conclusion, this study showed that the HSL-S protein generated as a result of in-frame skipping of exon 6 could influence the maximal lipolytic capacity of the adipocytes from obese subjects, because no compensatory increase in the expression of the normal HSL variant was found with increasing concentrations of the HSL-S form.

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