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Adipose Triglyceride Lipase and Hormone-Sensitive Lipase Protein Expression Is Decreased in the Obese Insulin-Resistant State

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Aim/Hypothesis: Obesity is associated with increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG is mediated by hormone-sensitive lipase (HSL) and the recently discovered adipose triglyceride lipase (ATGL). The aim of the present study was to examine whether ATGL and HSL mRNA and protein expression are altered in insulin-resistant conditions. In addition, we investigated whether a possible impaired expression could be reversed by a period of weight reduction.

Methods: Adipose tissue biopsies were taken from obese subjects ($n = 44$) with a wide range of insulin resistance, before and just after a 10-wk hypocaloric diet. ATGL and HSL protein and mRNA expression was determined by Western blot and quantitative RT-PCR, respectively.

Results: Fasting insulin levels and the degree of insulin resistance (using the homeostasis model assessment index for insulin resistance)

were negatively correlated with ATGL and HSL protein expression, independent of age, gender, fat cell size, and body composition. Both mRNA and protein levels of ATGL and HSL were reduced in insulin-resistant compared with insulin-sensitive subjects ($P < 0.05$). Weight reduction significantly decreased ATGL and HSL mRNA and protein expression. A positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction was observed. Finally, ATGL and HSL mRNA and protein levels seem to be highly correlated, indicating a tight coregulation and transcriptional control.

Conclusions: In obese subjects, insulin resistance and hyperinsulinemia are strongly associated with ATGL and HSL mRNA and protein expression, independent of fat mass. Data on weight reduction indicated that also other factors (e.g. leptin) relate to ATGL and HSL protein expression. (*J Clin Endocrinol Metab* 92: 2292–2299, 2007)

OBESITY IS CHARACTERIZED by increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG (lipolysis) is mediated by hormone-sensitive lipase (HSL). For more than 30 yr, the paradigm has been that HSL is the rate-limiting enzyme responsible for TAG breakdown. Studies in HSL knockout mice (1–6) raised doubt on the rate-limiting role of HSL in TAG metabolism and suggested that at least one additional lipase in adipose tissue should be active that preferentially hydrolyzes the first ester bond of the TAG molecule. Recently, a new TAG lipase that belongs to a gene family characterized by the presence of a patatin domain was

identified (7–9). Zimmermann *et al.* (9) termed this new non-HSL lipase adipose triglyceride lipase (ATGL), being predominantly expressed in adipose tissue.

An impaired catecholamine-induced lipolysis and a reduced HSL expression in preadipocytes and differentiated adipocytes is observed in obesity (10–12). This blunted catecholamine-induced lipolysis has been proposed to be a cause of excessive accumulation of body fat. Indeed, studies in first-degree relatives of obese subjects demonstrate an impaired catecholamine-mediated lipolysis (13). Furthermore, the impaired catecholamine-induced lipolysis did not improve after weight loss, indicating that it may be an early factor in the development or maintenance of increased fat stores (14–17). A plausible other interpretation is that this reduced lipolytic response is an appropriate down-regulation of lipolysis per unit fat mass (FM) to prevent an excessive fatty acid outflow from the expanded FM and to prevent worsening of the insulin-resistant (IR) state. In line, fasting insulin concentrations have been shown to be inversely related to fatty acid efflux from adipose tissue (18). Moreover, insulin down-regulates ATGL and HSL mRNA expression in 3T3-L1 adipocytes, and HSL mRNA expression is increased

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Abbreviations: Adj.Vol.OD, Adjusted volume OD; ATGL, adipose triglyceride lipase; BMI, body mass index; FCV, fat cell volume; FCW, fat cell weight; FFM, fat-free mass; FM, fat mass; HOMA_{IR}, homeostasis model assessment index for insulin resistance; HSL, hormone-sensitive lipase; IR, insulin resistant; IS, insulin sensitive; PPAR- γ , peroxisome proliferator-activated receptor- γ ; TAG, triacylglycerol.

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in adipocytes from insulin-deficient animals (19–22). In addition, ATGL is down-regulated in animal models for insulin resistance (ob/ob and db/db), and HSL knockout animals show signs of impaired insulin sensitivity in adipose tissue and skeletal muscle (4, 7). Thus, there seems to be a negative relationship between insulin, ATGL, and HSL expression.

The aim of the present study was to investigate whether the degree of insulin resistance and hyperinsulinemia are, independently of FM, related to an impaired ATGL and HSL protein expression in a group of overweight-obese subjects with a wide range of insulin resistance, selected from an existing cohort. In addition, we investigated the impact of weight loss by means of a hypocaloric diet (low-fat *vs.* medium-fat diet) on adipose tissue ATGL and HSL protein levels. To the best of our knowledge, this is the first time that ATGL protein levels are measured in human adipose tissue.

Subjects and Methods

Subjects

This study is part of the European multicenter study NUGENOB (for Nutrient-Gene Interactions in Human Obesity), which has been described in detail previously (23–25). Only the overweight-obese subjects from the Maastricht center participated in this part of the study. The basic selection criteria for overweight-obese subjects were 20–50 yr of age and a body mass index (BMI) of at least 26 kg/m². Exclusion criteria were as follows: weight change of more than 3 kg within the 3 months before the study start; drug-treated hypertension, diabetes, or hyperlipidemia; thyroid disease; surgically treated obesity; pregnancy; alcohol or drug abuse; and participation in other simultaneous ongoing trials. All subjects were recruited by means of an advertisement in a local newspaper and were informed in detail about the investigation, and their consent was obtained before participating in the study. From the 116 participants at the Maastricht center, a selection of 22 insulin-sensitive (IS) and 22 IR subjects was made. Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA_{ir}) calculated from fasting glucose and insulin according to the equation of Matthews *et al.* (26). The median for HOMA_{ir} in the total Maastricht cohort was 2.19 (range, 0.4–9.9). Subjects above the 50th percentile of HOMA_{ir} were assigned as IR, and subjects below the 50th percentile were assigned as IS. Before entering the study, all subjects were in good health as assessed by medical history and physical examination. The Medical Ethical Review Committee of Maastricht University approved the study protocol, and the clinical investigations were performed according to the Declaration of Helsinki.

Study design

A clinical investigation day took place before and just after a 10-wk dietary intervention with either low-fat or medium-fat diets (see *Dietary intervention*). Subjects arrived at the clinical research center at 0800 h after a 12 h overnight fast and a 3-d run-in period, in which they had to avoid excessive physical activity and alcohol consumption, as described previously in detail (23). During this day, the subjects underwent anthropometric measurements (see below), and an adipose tissue biopsy was taken (see *Adipose tissue biopsy*). In addition, a venous basal blood sample was drawn for additional analysis (see *Biochemical analysis*).

Dietary intervention

Subjects followed one of two energy-restricted diets: a medium-fat (n = 23) or a low-fat (n = 21) diet. Data on the different diets and how the diet was controlled have been published previously (23). The target macronutrient composition of the two diets was as follows: for the low-fat diet, 20–25% of total energy was provided by fat, and the corresponding figure for the medium-fat diet was 40–45%. Both diets derived 15% of total energy from protein and the remainder (60–65 and 40–45 for the low-fat and medium-fat diets, respectively) from carbohydrates. Both diets were designed to provide 600 kcal/d less than the

individual estimated energy expenditure based on resting metabolic rate, measured using a ventilated hood system, expressed in kilocalories per day and multiplied by 1.3.

Anthropometric measurements

After subjects voided their bladder, body weight was determined on a calibrated electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the nearest 1 cm were made midway between the lower rib and iliac crest with the participant standing upright. Height was measured using a wall-mounted stadiometer. BMI was calculated as body weight in kilograms divided by squared height in meters. FM and fat-free mass (FFM) were assessed using multifrequency bioimpedance (QuadScan 4000; Bodystat, Douglas, Isle of Man, UK). The percentage body fat was calculated from total FM (in kilograms) and body weight.

Adipose tissue biopsy

A sc adipose tissue biopsy was taken from the abdominal region early in the morning after an overnight fast. The second biopsy was taken in wk 10 of the dietary intervention. Biopsies were performed under local anesthesia (0.5% Xylocaine and 0.5% Lidocaine; AstraZeneca, Zoetermeer, The Netherlands) on the left or right side of the abdomen about 5 cm lateral from the umbilicus using a Hepafix Luer lock syringe (Braun Medical, Bethlehem, PA) and a 146 × 3 1/2 in. (2.10 × 80 mm) Braun Medical Sterican needle. The biopsy was washed in physiological saline and stored in a sterile polypropylene tube at –80 C until additional analysis.

Biochemical analysis

Plasma glucose concentrations (ABX Diagnostics, Montpellier, France) were measured on a COBAS MIRA automated spectrophotometer (Roche Diagnostica, Basel, Switzerland). TAG (Sigma, St. Louis, MO), FFA (NEFA C kit; Wako Chemicals, Neuss, Germany), and glycerol (Roche Molecular Biochemicals, Mannheim, Germany) were measured on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica). Standard samples with known concentrations were included in each run for quality control. Plasma insulin and serum leptin were measured with a double antibody RIA [Insulin RIA 100 (Kabi-Pharmacia, Uppsala, Sweden) or Human Leptin RIA kit (Linco Research, St. Charles, MO)].

Fat cell volume (FCV) and fat cell weight (FCW)

Fat cell characteristics were determined in a subset of the same cohort (n = 39; 19 IS/20 IR; HOMA_{ir}, 1.4 ± 0.1 *vs.* 4.7 ± 0.5; P < 0.01). Weight loss after diet was the same among these subjects as in the whole cohort (data not shown). Also, with respect to other metabolic parameters, this subgroup was comparable with the group in which ATGL and HSL protein and mRNA expression was determined (see *Results*). Adipose tissue was subjected to collagenase treatment, and the mean FCV and FCW were determined as described previously (27).

Sample preparation

Approximately 200 mg adipose tissue was ground to a fine powder under liquid nitrogen and homogenized in 200 μl of ice-cold buffer: 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (catalog no. C9426; Sigma), 65 mM dithiothreitol (catalog no. 161-0611; Bio-Rad, Hercules, CA), protease inhibitor (catalog no. P8340; Sigma) and phosphatase inhibitor cocktail (catalog no. P5726; Sigma). The homogenate was vortexed for 5 min and centrifuged at 20,000 × g for 30 min at 4 C. The supernatant was carefully collected, and aliquots were stored at –80 C. The protein concentration was determined by the Bradford-based protein assay (catalog no. 500-0006; Bio-Rad).

Western blot analysis

Ten micrograms of protein were separated using 10% SDS-PAGE and then transferred to a nitrocellulose membrane. An affinity-purified polyclonal antibody was raised in rabbit against a 15 amino acid peptide

(amino acids 386–400, GRHLPSRLPEQVERL) of human ATGL (Eurogentec, Seraing, Belgium). In Western blot analysis, a single band at 56 kDa was detected that disappeared after preincubation of the antibody with the peptide. This band corresponds to the predicted molecular mass of the human ATGL protein (9). In addition, when COS cells were transfected with cDNA coding for human ATGL, also a single band of 56 kDa was observed after Western blot of extracted cellular protein. HSL was detected using a rabbit polyclonal antibody, raised and purified against recombinant human HSL. The HSL antibody has been validated previously (28). The secondary antibody was a horseradish peroxidase-conjugated antirabbit Ig (DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL+; Amersham Biosciences, Little Chalfont, UK) and a Kodak Image Station (Eastman Kodak, Glostrup, Denmark). Quantification of antigen-antibody complexes was performed using Quantity One 1-D analysis software (Bio-Rad). Optical density units are expressed as adjusted volume [adjusted volume OD (Adj.Vol.OD) = sum of pixels inside the volume boundary \times area of a single pixel (in mm²) – the background volume]. Differences in loading were adjusted to β -actin protein levels, and an isolated mature adipocyte lysate was included as positive control.

Adipose tissue mRNA analysis

ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects (13 IS/13 IR; 14 females and 12 males) before and after the diet. Total RNA was extracted from adipose tissue using the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA concentration and purity were assessed spectrophotometrically. An Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France) was used to confirm the integrity of the RNA. From each sample, 1 μ g of total RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) and random hexamer primers (Invitrogen). HSL and ATGL mRNAs were quantified using pre-made gene expression assays (Applied Biosystems). Ribosomal RNA, 18S, was used as control to normalize gene expression.

Statistics

All variables were checked for normal distribution, and variables with a skewed distribution were ln-transformed to satisfy conditions of normality.

First, univariate regression analysis was performed to identify variables that contribute to ATGL and HSL protein expression and to changes in protein expression induced by weight loss. Subsequently, a multivariate regression analysis was performed with ATGL or HSL

protein levels as dependent variables and age, gender, FM, FFM, waist circumference, circulating insulin, and leptin levels as independent variables (model 1). The same model was repeated with HOMA_{IR} as independent variable instead of insulin (model 2). To study the impact of weight reduction, changes in ATGL or HSL protein level were entered as dependent variable in the multivariate regression model with age, gender, change in FM, FFM, circulating insulin, and leptin as independent variables. ATGL and HSL mRNA and protein levels were compared between IS and IR subjects using Student's unpaired *t* test. Anthropometric and metabolic parameters and HSL and ATGL mRNA and protein levels were compared before and after the diets using Student's paired *t* test. The differential effect of the diets was assessed with analysis of covariance using diet as fixed factor. To avoid multicollinearity in the regression model, independent variables with a correlation greater than 0.8 were not simultaneously included in the model. The impact of the independent variables is described as unstandardized β or regression coefficients. A *P* value of ≤ 0.05 was considered statistically significant. All analyses were performed using SPSS for Mac OS X version 11.0 (SPSS, Chicago, IL).

Results

Characteristics of the study subjects

Anthropometric and metabolic characteristics of the study subjects before and after a 10-wk hypocaloric diet are displayed in Table 1. Extensive data on the effects of the hypocaloric diet in the total NUGENOB cohort were reported previously (23). The diet resulted in significant loss of body weight (before *vs.* after, 98.7 ± 3.2 *vs.* 90.0 ± 3.3 kg; *P* < 0.001), FM (37.4 ± 1.6 *vs.* 30.7 ± 1.5 kg; *P* < 0.001), and a significantly decreased BMI (34.1 ± 0.7 *vs.* 31.3 ± 0.7 kg/m²; *P* < 0.001). In addition, circulating fatty acids (506 ± 24 *vs.* 418 ± 22 μ M/liter; *P* = 0.016), glycerol (105 ± 11 *vs.* 83 ± 8 μ M/liter; *P* = 0.003), and leptin (24.7 ± 2.3 *vs.* 14.5 ± 1.5 ng/ml; *P* < 0.001) decreased. There were no significant differences in fasting glucose, insulin, and HOMA_{IR}. As reported previously, the low-fat and medium-fat diets resulted in similar changes in anthropometric and metabolic parameters (23, 25).

TABLE 1. Anthropometric and metabolic parameters before and after a 10-wk hypocaloric diet

	Before		After	
	IS	IR	IS	IR
n (female/male)	22 (16/6)	22 (12/10)	18 (13/5)	17 (9/8)
Age (yr)	39.7 \pm 1.5	44.4 \pm 5.0*		
Weight (kg)	91.0 \pm 3.6	106.4 \pm 4.8*	84.2 \pm 3.7**	96.2 \pm 5.1**
Height (m)	1.68 \pm 0.02	1.71 \pm 0.02		
BMI (kg/m ²)	32.1 \pm 0.7	36.2 \pm 0.9*	29.7 \pm 0.8**	32.9 \pm 1.0**
Waist (cm)	105 \pm 3	115 \pm 3*	99 \pm 3**	105 \pm 3.8**
Hip (cm)	113 \pm 8	117 \pm 2	108 \pm 2**	112 \pm 2.1**
WHR	0.93 \pm 0.02	0.98 \pm 0.02	0.91 \pm 0.02**	0.94 \pm 0.03**
%BF	38.2 \pm 1.6	38.4 \pm 1.6	33.6 \pm 1.7**	34.6 \pm 1.8**
FFA (μ M/liter)	504 \pm 30	507 \pm 37	418 \pm 29	418 \pm 35
Free glycerol (μ M/liter)	114 \pm 21	95 \pm 8	89 \pm 13**	76 \pm 8**
TG (μ M/liter)	937 \pm 69	1476 \pm 125*	932 \pm 80	1230 \pm 136
Glucose (mmol/liter)	5.2 \pm 0.1	5.7 \pm 0.1*	5.1 \pm 0.1	5.4 \pm 0.1
Insulin (μ U/ml)	7.2 \pm 0.4	15.8 \pm 1.4*	7.4 \pm 0.6	12.4 \pm 1.4
HOMA _{IR}	1.7 \pm 0.1	4.0 \pm 0.4*	1.7 \pm 0.2	3.1 \pm 0.4
Leptin (ng/ml)	23 \pm 3	26 \pm 4	14 \pm 2**	15 \pm 2**
FCV (pl) ^a	775 \pm 36	869 \pm 33*	655 \pm 36**	744 \pm 29**
FCW (ng) ^a	709 \pm 33	795 \pm 24*	600 \pm 33**	681 \pm 27**

Values are mean \pm SEM. %BF, Percentage body fat; FFA, free fatty acids; WHR, waist/hip ratio. *, *P* < 0.05 IS *vs.* IR, unpaired *t* test; **, *P* < 0.05 before *vs.* after, paired *t* test.

^a FCV and FCW are measured in a subgroup of the same cohort (n = 39; 19 IS/20 IR) with similar characteristics.

Relationship between the degree of insulin resistance and adipose tissue ATGL and HSL protein levels

Univariate regression analysis indicated a negative correlation between HOMA_{ir}, fasting insulin, and ATGL or HSL protein levels (all $P < 0.05$), whereas age, gender, body composition (waist, FM, FFM), and levels of circulating leptin were not significantly related to ATGL or HSL protein levels (all $P > 0.10$). Multivariate regression analysis, shown in Table 2, indicated the same negative correlation between HOMA_{ir} (ATGL β -coefficient, -1.33 , $P = 0.045$; HSL β -coefficient, -0.965 , $P = 0.039$) (Table 2), fasting insulin (ATGL β -coefficient, -1.41 , $P = 0.048$; HSL β -coefficient, -1.07 , $P = 0.032$) (Table 2), and ATGL or HSL protein levels. These data indicate that the IR state rather than FM *per se* causes the decrease in adipose tissue ATGL and HSL protein levels.

To illustrate the impact of insulin resistance on ATGL and HSL protein levels, subjects were assigned as IS or IR based on HOMA_{ir} (see *Subjects and Methods, Subjects*). Anthropometric and metabolic characteristics of IS and IR subjects are displayed in Table 1. Adipose tissue ATGL and HSL protein levels were found to be dramatically reduced in IR compared with IS obese subjects. ATGL protein levels were decreased by 72% in IR compared with IS obese subjects (IR vs. IS, 2.6 ± 1.3 vs. 9.3 ± 3.6 Adj.Vol.OD; $P = 0.025$) (Fig. 1B), whereas the corresponding figure for HSL was 57% (6.6 ± 2.3 vs. 15.4 ± 3.0 Adj.Vol.OD; $P = 0.001$) (Fig. 1B). ATGL and HSL protein levels were highly correlated (β -coefficient, 1.05; $r = 0.568$; $P = 0.0001$) (Fig. 2), indicating that ATGL and HSL protein levels might be tightly coregulated in adipose tissue of obese subjects.

As indicated in *Subjects and Methods*, FCV and FCW were determined in a subset of IS ($n = 19$) and IR ($n = 20$) subjects from the same cohort with similar characteristics with respect to HOMA_{ir} (1.5 ± 0.1 vs. 4.7 ± 0.5 ; $P < 0.01$), body fat percentage (42.5 ± 1.1 vs. 42.8 ± 2.0 ; $P = 0.742$), and waist (108 ± 2 vs. 114 ± 2 ; $P = 0.031$) and similar diet-induced changes. FCV and FCW were significantly higher (12%; $P = 0.039$) in IR compared with IS subjects and decreased significantly after weight loss ($P < 0.01$) (Table 1). When ATGL and HSL protein expression was corrected for mean FCV or

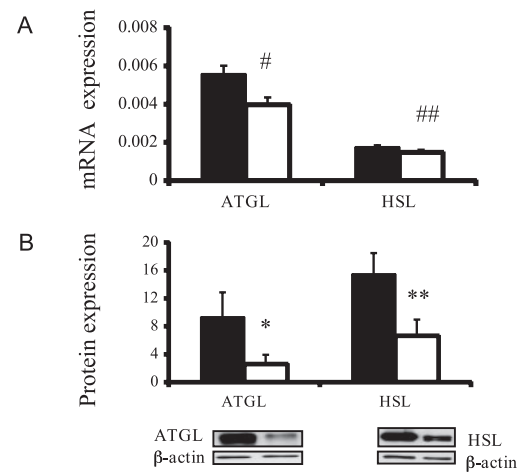


FIG. 1. ATGL and HSL mRNA and protein expression in sc adipose tissue of IS (black bars) vs. IR (white bars) overweight-obese subjects. A, ATGL and HSL mRNA expression was significantly lower in IR ($n = 13$) compared with IS ($n = 13$) subjects (#, $P = 0.006$; and ##, $P = 0.057$, respectively). mRNA levels were quantified by quantitative RT-PCR and normalized with 18S rRNA. B, In line, ATGL and HSL protein expression was significantly lower in IR ($n = 22$) compared with IS ($n = 22$) subjects (*, $P = 0.025$; and **, $P = 0.001$, respectively). ATGL and HSL protein expression was quantified using Western blot, and expression was related to the structural protein β -actin. IS vs. IR using unpaired Student's t test statistics. Values are mean \pm SEM.

FCW, the difference between groups remained significant (for ATGL: IR vs. IS, 2.6 ± 0.6 vs. 3.9 ± 0.5 , $P < 0.01$; for HSL: IR vs. IS, 9.2 ± 1.5 vs. 13.4 ± 1.6 , $P < 0.01$).

ATGL and HSL mRNA expression in IS vs. IR subjects

Additionally, ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects. In agreement with the protein expression data, ATGL and HSL mRNA expression were significantly lower in IR ($n = 13$) compared with IS ($n = 13$) obese subjects ($P = 0.006$ and $P = 0.057$, respectively) (Fig. 1A). Also, a positive correlation was found for ATGL and HSL mRNA expression (β -coefficient, 0.531; $r = 0.253$; $P = 0.005$), suggesting that the two

TABLE 2. Determinants of ATGL and HSL protein levels in multivariate regression analysis

Dependent variable (n = 44)	ATGL expression			HSL expression		
	B ^b	95% CI	P value	B ^b	95% CI	P value
Model 1						
Age (yr)	5.98E-02	-3.37E-02 to 0.15	0.199	5.29E-02	-1.24E-02 to 0.12	0.107
Gender						
Male	0	0		0	0	
Female	-0.606	-4.27 to 3.06	0.736	-0.72	-3.27 to 1.85	0.570
Waist (cm)	-3.77E-02	-0.13 to 5.15E-02	0.392	-1.01E-02	-7.23E-02 to 5.22E-02	0.742
FFM (kg)	-5.79E-03	-0.13 to 0.12	0.926	-2.14E-02	-0.11 to 6.72E-02	0.622
FM (kg)	2.78E-02	-9.57E-02 to 0.15	0.646	3.51E-02	-5.13E-02 to 0.12	0.410
Insulin (μ U/ml) ^a	-1.41	-2.81 to -1.57E-02	0.048	-1.07	-2.05 to -9.95E-02	0.032
Leptin (ng/ml) ^a	7.79E-03	-4.97E-02 to 6.53E-02	0.782	9.43E-03	-3.08E-02 to 4.96E-02	0.633
Model 2						
HOMA _{ir} ^a	-1.33	-2.63 to -3.43E-02	0.045	-0.965	-1.88 to -5.14E-02	0.039

Model 2 includes HOMA_{ir} instead of circulating insulin levels in addition to the variables indicated in model 1. CI, Confidence interval; E, exponent 10.

^a Values are entered in the model after being ln-transformed.

^b Unstandardized β -coefficient.

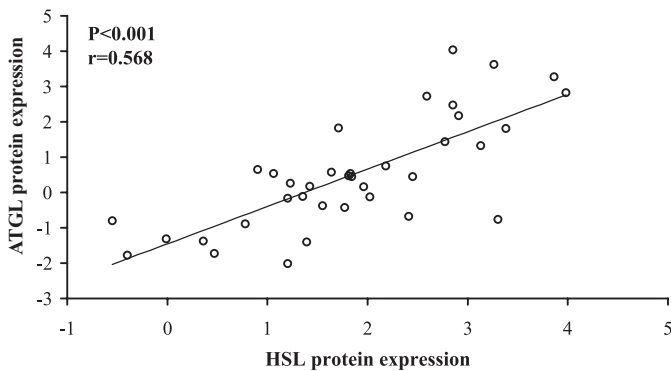


FIG. 2. Correlation between ATGL and HSL protein levels in sc adipose tissue of overweight-obese subjects. ATGL and HSL protein levels were quantified by Western blot analysis (values are Adj.Vol.OD mm²). ATGL and HSL protein levels are entered after being ln-transformed.

enzymes belong to a common regulatory network with tight transcriptional control.

Effect of weight reduction on adipose tissue ATGL and HSL protein levels

A 10-wk hypocaloric diet resulted in a decreased adipose tissue ATGL (before *vs.* after, 5.7 ± 1.8 *vs.* 1.4 ± 0.4 Adj.Vol.OD; $P = 0.04$) (Fig. 3B) and HSL (before *vs.* after, 10.8 ± 1.9 *vs.* 5.9 ± 1.3 Adj.Vol.OD; $P = 0.023$) (Fig. 3B) protein level. When ATGL and HSL protein expression was corrected for mean FCV or FCW, the difference remained significant (for ATGL: before *vs.* after, 3.2 ± 0.5 *vs.* 2.1 ± 0.4 , $P = 0.02$; for HSL: before *vs.* after, 11.1 ± 1.8 *vs.* 7.5 ± 1.4 , $P < 0.01$). Low-fat and medium-fat diets resulted in similar changes in ATGL and HSL protein levels. To find the effect of changes in anthropometric and metabolic parameters on ATGL and

HSL protein levels, univariate and multivariate regression analysis was applied (Table 3). Univariate regression analysis indicated a positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction ($P < 0.05$), whereas age, gender, changes in body composition (FM, FFM), and insulin were not significantly related to changes in ATGL protein level (all $P > 0.10$). Multivariate regression analysis (Table 3) indicated the same positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction (β -coefficient, 6.05×10^{-2} ; $P = 0.023$) (Table 3). In addition, univariate regression analysis indicated a positive correlation between the decrease in FFM and the decrease in HSL protein level after weight reduction ($P < 0.05$), whereas age, gender, changes in FM, circulating levels of insulin, and leptin were not significantly related to changes in HSL protein level (all $P > 0.10$). Multivariate regression analysis (Table 3) indicated the same positive correlation between the decrease in FFM and decrease in HSL protein level after weight reduction (β -coefficient, 0.67; $P = 0.020$) (Table 3).

Finally, the correlation between ATGL and HSL protein levels found during habitual dietary conditions was also observed after a 10-wk hypocaloric diet (β -coefficient, 0.99; $r = 0.484$; $P < 0.0001$). Also, changes in ATGL and HSL induced by weight loss highly correlated to each other (β -coefficient, 1.01; $r = 0.503$; $P = 0.004$) (Fig. 4). This coordinated variation in ATGL and HSL expression during various dietary conditions suggests that the two enzymes share a common regulatory network.

ATGL and HSL mRNA expression after the diet

In line with the protein expression data, a 10-wk hypocaloric diet significantly lowered adipose tissue ATGL ($P = 0.001$) and HSL ($P = 0.007$) mRNA expression (Fig. 3A). Low-fat and medium-fat diets resulted in similar changes in ATGL and HSL mRNA expression. A strong positive correlation was found between ATGL and HSL mRNA expression after the diet (β -coefficient, 0.761, $r = 0.578$; $P < 0.0001$). The changes in ATGL and HSL mRNA expression induced by weight loss tended to correlate positively ($P = 0.135$).

Discussion

Our study, for the first time, examined the relationship between adipose tissue ATGL and HSL mRNA and protein expression and whole-body insulin sensitivity in a group of obese subjects. The major finding of the present study is that a reduced ATGL and HSL mRNA and protein expression is associated with insulin resistance independent of FM. Weight reduction decreased, rather than increased, ATGL and HSL mRNA and protein expression. When ATGL and HSL protein expression was corrected for mean FCV or FCW, the differences between groups remained significant. In addition, ATGL and HSL mRNA and protein expression seem to be tightly coregulated in adipose tissue, suggesting that they belong to a common regulatory network.

Our data indicate that the degree of insulin resistance and hyperinsulinemia in obesity rather than the increase in FM and body fat distribution *per se* is associated with a reduced ATGL and HSL protein and mRNA level. Because we only

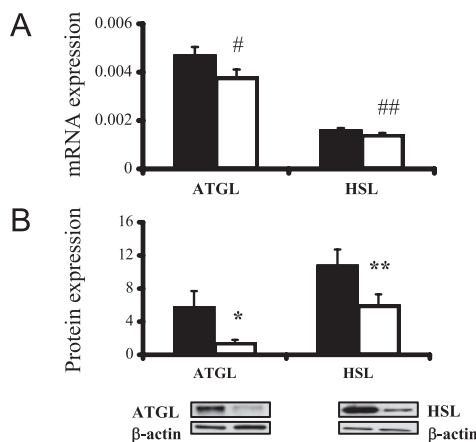


FIG. 3. ATGL and HSL mRNA (A) and protein (B) expression before (black bars) and after (white bars) a 10-wk hypocaloric diet. A, The hypocaloric diet resulted in significantly decreased ATGL and HSL mRNA expression ($n = 26$; #, $P = 0.001$; and ##, $P = 0.007$, respectively). mRNA levels were quantified by quantitative RT-PCR and normalized with 18S rRNA. B, In line, a significantly decreased ATGL and HSL protein expression was observed after the diet (*, $P = 0.04$; and **, $P = 0.023$, respectively). ATGL and HSL protein expression was quantified using Western blot, and expression was related to the structural protein β -actin. Before *vs.* after using paired Student's *t* test statistics. Values are mean \pm SEM.

TABLE 3. Determinants of change in ATGL and HSL protein level after a 10-wk hypocaloric diet in multivariate regression analysis

Dependent variable (n = 35)	Change in ATGL			Change in HSL		
	B ^b	95% CI	P value	B ^b	95% CI	P value
Model						
Age (yr)	4.32E-03	-4.17E-02 to 5.03E-02	0.841	-6.28E-02	-0.18 to 5.17E-02	0.242
Gender						
Male	0	0		0	0	
Female	-0.14	-1.22 to 0.94	0.781	-0.76	-3.88 to 2.35	0.587
ΔFFM (kg)	4.95E-02	-9.39E-02 to 0.19	0.464	0.67	0.14 to 1.21	0.020
ΔFM (kg)	4.38E-02	-0.18 to 0.27	0.676	0.16	-0.16 to 0.49	0.283
ΔInsulin (μU/ml) ^a	-6.12E-02	-0.14 to 1.47E-02	0.105	3.24E-02	-0.14 to 0.20	0.676
ΔLeptin (ng/ml) ^a	6.05E-02	9.70E-03 to 0.11	0.023	1.04E-02	-0.16 to 0.18	0.889

CI, Confidence interval; E, exponent 10.

^a Values are entered in the model after being ln-transformed.

^b Unstandardized β-coefficient.

studied expression in abdominal sc fat, we cannot rule out depot-specific differences in ATGL and HSL expression. For instance, it has been shown that HSL mRNA expression is significantly different in sc and visceral adipose tissue, a finding that could not be confirmed for ATGL mRNA expression (29). There is accumulating evidence from *in vitro* and animals studies that insulin reduces HSL and ATGL expression. It is documented that insulin down-regulates ATGL and HSL mRNA levels in 3T3-L1 adipocytes in a dose-dependent manner (19, 21, 22). More interestingly, ATGL is down-regulated in a mouse model for insulin resistance by 50% (7). In accordance, our data indicate a 72% reduction in adipose tissue ATGL protein levels of IR compared with IS obese subjects. Moreover, HSL mRNA levels are increased in adipocytes from insulin-deficient streptozotocin-treated rats compared with controls, suggesting a negative effect of insulin on HSL expression (20).

Weight reduction decreased, rather than increased, ATGL and HSL protein and mRNA expression levels with no effect of diet composition and independent of changes in FM. This seems consistent with Viguerie *et al.* (25) reporting a similar decrease in HSL mRNA for the low-fat and medium-fat diets. This down-regulation of key enzymes for triglyceride breakdown and the increase in lipoprotein lipase mRNA level after weight loss (30) potentially enhances lipid storage and makes additional weight loss more difficult. In contrast, Mairal *et al.*

(29) showed that adipose tissue ATGL mRNA expression was unchanged and HSL mRNA expression increased after long-term weight reduction in obese subjects. It should be mentioned that, in this study, the second biopsy was taken 2–4 yr after surgery. A factor explaining the inconsistent findings may be that different conditions are compared. In the present study, subjects were investigated while still on the energy-restricted diet (second biopsy taken just at the end of the diet). The negative energy balance produced by the energy-restricted diet is known to modify profoundly adipocyte metabolism, particular the lipolytic pathway, making it impossible to differentiate between the chronic effect of weight reduction *per se* and the acute effect of energy restriction. Interestingly, the decrease in leptin correlated positively with the decrease in ATGL expression after energy restriction, independent of changes in FM, FFM, and circulating insulin levels. Flier (31) advocate that this decrease in leptin concentration serves as an important signal from fat to the brain that the body is starving. In addition, it has been proposed that an important function of leptin is to confine storage of triglycerides to adipocytes (*i.e.* to affect adipose tissue lipolysis) and to prevent triglyceride storage in non-adipocytes (*e.g.* myocytes), protecting them from lipotoxicity (32).

In obese subjects, we observed no strict relationship between FM and ATGL or HSL expression, and fat cell size *per se* was not important for our findings. When the obese state has already developed, insulin resistance and hyperinsulinemia seem to be the major determinants for ATGL and HSL protein expression. This seems in line with the observation of a negative correlation between fasting insulin and *in vivo* fatty acid outflow per unit of adipose tissue in IR conditions (18), suggesting that a reduced expression of ATGL and HSL may be a secondary phenomenon to insulin resistance. It can be speculated that hyperinsulinemia may down-regulate adipose tissue lipolysis and thereby prevent worsening of the IR state (33, 34). In the present study, weight loss had no significant effect on insulin sensitivity. To fully elucidate the effect of insulin resistance on ATGL and HSL expression, an intervention should be performed that significantly improves insulin sensitivity [*e.g.* exercise training or treatment with a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist]. It has been shown that ATGL is subject to transcriptional control by PPAR-γ-mediated signals (22). In addition, Festuccia

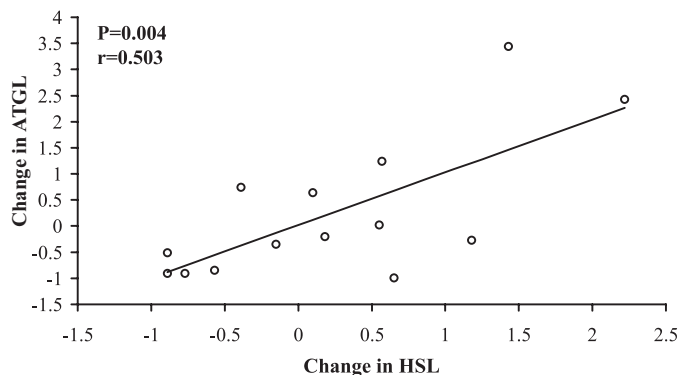


FIG. 4. Correlation between change in ATGL and HSL protein levels after a hypocaloric diet. ATGL and HSL protein levels were measured before and after a 10-wk hypocaloric diet using Western blot analysis (values are Adj.Vol.OD mm²). The changes are calculated as follows for each individual: (protein level after the diet – protein level before the diet)/protein level before the diet.

et al. (35) recently showed that treatment of mice with the PPAR- γ agonist rosiglitazone significantly increased ATGL and HSL mRNA expression, indicating that an improved insulin sensitivity increases adipose tissue ATGL and HSL expression. Finally, we cannot rule out that a decreased ATGL and HSL expression is a primary defect in obesity. Interestingly, ATGL-deficient mice have an increased fat storage in adipose and nonadipose tissues (36). Furthermore, studies in first-degree relatives of obese subjects have demonstrated an impaired lipolytic function of adipocytes, suggesting also that primary adipocyte lipolysis defects are present in obesity (13). Expression of HSL is markedly decreased in sc adipocytes and differentiated preadipocytes from obese subjects, suggesting a decreased HSL expression to be a primary defect in obesity (11, 12, 16, 25). Also, several studies suggest that genetic variation in the *HSL* and *ATGL* genes are associated with obesity and type 2 diabetes mellitus (37, 38). Additional research is needed to elucidate the exact order of events.

The coregulation between ATGL and HSL protein levels or mRNA expression (39) during different dietary conditions suggests that the two enzymes belong to a common regulatory network with tight transcriptional control. A recent study indicated that HSL is the major lipase catalyzing the rate-limiting step in stimulated lipolysis, whereas ATGL participates in basal lipolysis (39). Insufficient time has passed since the discovery of ATGL to understand the nature of its regulation. However, from the limited data available, it appears that, compared with HSL, ATGL is not a direct target for protein kinase A-mediated phosphorylation and is localized on the lipid droplet in the basal and hormone-stimulated state of the cell (9). These observations suggest that ATGL is not activated by phosphorylation and translocation to the lipid droplet as demonstrated for HSL. Instead, an activator protein regulates ATGL activity: CGI-58 (comparative gene identification 58) (40, 41). It will be important to establish whether the decreased HSL and ATGL protein and mRNA expression observed in IR subjects is also accompanied by a decreased activity of both enzymes.

In conclusion, ATGL and HSL expression are decreased in the obese IR state. When the obese state has already developed, insulin resistance or hyperinsulinemia seem to be the major determinant of ATGL and HSL protein expression independent of FM. Conversely, there are also indications that a reduced ATGL and HSL protein expression is a primary defect in obesity. Weight reduction decreased ATGL and HSL expression, independent of circulating insulin and FM, indicating that also other factors (*e.g.* leptin) relate to ATGL and HSL protein expression in obese subjects.

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