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Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis

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Abstract

Aims/hypothesis. We investigated the role of the adipocyte-specific protein perilipin for lipolysis in humans.
Methods. Perilipin protein content and lipolysis rates were measured in human subcutaneous fat cells of non-obese (n=10) and obese (n=117) women. Single nucleotide polymorphisms in the perilipin gene were examined in obese subjects.
Results. Basal and noradrenaline-induced rates of lipolysis were two to fourfold increased (p<0.01) and perilipin protein content decreased 50% (p=0.005) in adipocytes of the obese women. In subjects matched for body mass index and fat-cell volume, a high rate of lipolysis was associated with a low adipocyte content of perilipin (p=0.01). Adipocyte content of perilipin was inversely correlated with the circulating concentrations of glycerol (r=0.62) and non-esterified fatty acids (n=0.49). A gene polymorphism (rs891460 A/G) in intron 6 was common. In AA subjects basal and noradrenaline induced lipolysis were 50 to 100% times more rapid (p≤0.01) and the adipocyte perilipin content was about 80% reduced (p=0.005) as compared to GG carriers. Intermediate values were found in AG carriers.
Conclusions/interpretation. Perilipin seems important for the regulation of lipolysis in human fat cells. Obesity and a polymorphism in the perilipin gene associate with decreased protein content of perilipin and increased basal (unstrained) and noradrenaline-induced lipolysis. Low perilipin content also associate with high in vivo lipolytic activity. Perilipin could be a factor behind impaired lipolysis in insulin-resistant conditions. [Diabetologia (2003) 46:789–797]

Keywords Catecholamines, fat cells, obesity, single nucleotide polymorphism, women.

Increased circulating non-esterified fatty acids derived from adipocyte lipolysis is frequently observed in obesity and Type 2 diabetes mellitus [1]. These fatty acids could cause insulin resistance in skeletal muscle and liver and also impair insulin secretion from pancreatic beta cells [2, 3, 4, 5]. Although it is well established that circulating NEFA concentrations are increased in human obesity, the mechanisms behind this phenomenon are less clear and cannot be readily explained by alterations in the action of the major lipolysis-regulating hormones, which are insulin, and catecholamines [6, 7, 8, 9]. However, it is unanimously shown that the unstrained (i.e. basal) lipolysis rate in fat cells is increased in the obese state irrespective of the mode of expression (per cell or per weight unit of adipose tissue) [10]. It is possible that the increase in fat-cell size, which is a hallmark of most forms of obesity, plays a role for enhanced basal lipolysis rate in adipose tissue of obese subjects because there is a strong correlation between the basal rate of lipolysis and fat-cell volume [10].

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Abbreviations: ADA, Adenosine deaminase; ANCOVA, analysis of covariance; dcAMP, dibuturyl cyclic AMP; DASH, dynamic allele specific hybridization; HSL, hormone sensitive lipase; NCBI, National Center for Biotechnology Information; OD, optical density; SNP, single nucleotide polymorphism.

The regulation of basal lipolysis is not known but the adipocyte specific protein perilipin is an attractive candidate according to several animal studies [11]. The protein is located at the surface of intracellular lipid droplets in adipocytes and is believed to protect the lipid droplet from being hydrolyzed by lipase activity [11]. The murine perilipin gene generates a number of differently spliced mRNAs resulting in at least four isoforms. The A form is the most abundant in fat cells, although small amounts of perilipin B are also expressed [11]. The C and D isoforms are almost exclusively expressed in stereoidgenic cells [11]. Overexpression of perilipin in fat-cell lines inhibits tumour necrosis factor-α mediated stimulation of lipolysis [12, 13]. Perilipin ablation in mice leads to an increased lipolysis rate in fat cells [14, 15]. Perilipin overexpression or ablation has effects on basal as well as catecholamine-stimulated lipolysis [12, 13, 14, 15, 16]. Perilipin-null mice are also lean and protected from obesity [14, 15]. It is possible that perilipin is involved in the regulation of the size of adipocyte. Acylglycerols constitute more than 95% of the fat-cell volume and it has been shown in cell lines that stored neutral lipids in adipocytes regulating perilipin production by post-translational effects [16]. A cDNA for perilipin A has been cloned; the corresponding mRNA is expressed only in adipose tissue and the mammary gland [17].

So far there is no information available on the functional role of perilipin in humans. We investigated the perilipin protein content and lipolysis in subcutaneous fat cells of 117 obese and 10 lean women. In the obese women we also examined whether polymorphism in the perilipin gene could be of importance for lipolysis regulation.

Methods

Subjects. This study comprised 10 non-obese control women and 117 obese women who participated in an ongoing investigation of lipolysis regulation in subcutaneous adipose tissue. The study was approved by the hospital's committee on ethics. It was explained in detail to each participant and their informed consent was obtained. All women were healthy except for being obese; none were on regular medication. None were completely sedentary or involved in athletic activity. Obesity was defined according to the criteria of the World Health Organization with the cut-off point for BMI being 30 kg/m^2. The subjects came to the laboratory in the morning after an overnight fast. Height and weight were measured and venous plasma and serum samples were obtained for the measurement of cholesterol, triglycerides, glucose, glycerol, NEFA and insulin concentrations, as described previously [18] and the buffy coat was kept at −70°C for genotyping. Thereafter a subcutaneous adipose tissue biopsy (1–3 g) was obtained from the abdominal subcutaneous area [18]. About 300 mg of tissue was immediately frozen in liquid nitrogen and stored at −70°C for subsequent protein determination. The remaining tissue was used for lipolysis experiments.

Lipolysis in fat cells. The adipose tissue was collagenase-treated and isolated fat cells were prepared and used for lipolysis experiments as described previously [18]. Fat-cell size and number were measured and diluted cell suspensions (2%, vol/vol) were incubated in an albumin-containing buffer (pH 7.4) containing glucose (1 g/l) and ascorbic acid (0.1 g/l) for 2 h at 37°C with air as gas phase. Glycerol release into the medium was subsequently determined and used as an index of lipolysis and expressed asmol $10^{-6}{\cdot}2\ h^{-1} \cdot$ cells 10^{-7} of glycerol. The adipocytes were incubated in the absence or presence of either noradrenaline (a natural catecholamine), terbutaline (a selective β_2-adrenoceptor agonist), dobutamine (a selective β_1-adrenoceptor agonist), clonidine (a selective α_2-adrenoceptor agonist) or insulin. In the insulin experiments 10^{-3} mol/l of 8-bromo cyclic AMP (a phosphodiesterase sensitive cyclic AMP analogue) was added to the buffer. In the clonidine and insulin experiments 1 U/ml of adenosine deaminase (ADA) was added to remove any traces of adenosine in the incubation medium which might otherwise interfere with basal lipolysis and the antilipolytic effect of the used agents [20]. Each agent was added in increasing concentrations (10^{-15}–10^{-3} mol/l), depending on the drug. In the non-obese women we only investigated basal, noradrenaline- and ADA-induced lipolysis. The concentration-response curves were analysed in two ways. For receptor-selective agents (terbutaline, dobutamine, clonidine and insulin) half- maximum effective concentration was calculated (EC_{50}). This was converted to pD_2 (negative logarithm of molar EC_{50}-value) which is a measure of receptor sensitivity and function [21]. For noradrenaline, glycerol release at the maximum effective concentration was determined and responsiveness was calculated as the maximum glycerol release minus the value with no agent present (i.e. basal). For each agent and in each subject a plateau of response was obtained, so it was always possible to accurately measure responsiveness and pD_2.

Determination of perilipin protein The protein studies, which required about 300 mg of adipose tissue, were done by using Western blot experiments exactly as described [22]. Because the lipolysis protocol in the lean was limited and required only small amounts of fat cells, a tissue sample from each of the non-obese women could be removed for protein analysis. In the obese women priority was given to the extensive lipolysis protocol precluding us from obtaining tissue for protein determination from all of these women. Proteins were extracted and concentrations were determined as described previously [22]. A total of 300 mg of frozen adipose tissue was homogenized in 0.6 ml of protein lysis buffer (1% Triton X-100, Tris-HCl, pH 7.6 supplemented with protease inhibitor). After centrifugation at 4°C the infranatant was isolated and protein concentration was measured. To study if significant amounts of perilipin protein were retained in the fat cake, adipose tissue of three lean and eight obese subjects was subjected to protein extraction as described above. After centrifugation the fat cake was removed and subjected to methanol-$CHCl_3$ extraction as described [15]. Similar concentrations of protein were detected in all adipose tissue extracts (5–6 μg/ml). Proteins that were extracted from the fat cake were dissolved in 0.6 ml of the lysis buffer described above. No protein was detected in any sample from the fat cakes. Since the detection limit for protein in the assay is 0.01 μg/μl the methodological data suggest that insignificant amounts of proteins (<0.2%) are retained in the fat cake. Thus, essentially all protein was extracted in the infrantant. Perilipin protein expression was investigated using a commercially available antibody directed towards the N-terminal portion of the protein (Progen Biotechnik, Heidelberg, Germany). Hormone sensitive lipase (HSL) expression was

also studied using a polyclonal antibody (generously donated by Prof. C. Holm, Lund University). A total of 100 µl of protein was loaded on polyacylamide gels and separated by SDS-PAGE. Proteins were transferred to polyvinylidine fluoride membranes (Amersham, Little Chafford, UK). Proteins were detected by chemiluminescence using Super Signal (Pierce Chemical, Rockford, Ill., USA). The optical density (OD) of the protein bands was measured by scanning and quantification by the NIH image software provided by National Institute of Health (Bethesda, Md., USA). With regard to perilipin, OD determinations were only made on the A band corresponding to perilipin A. In methodological experiments different amounts of protein from the same adipose tissue extract was transferred to the same SDS-PAGE and perilipin A was quantified as described above. There was a linear relation between the amount of protein added and the OD reading of perilipin A (r=0.986, intercept not different from zero; t value=–0.9).

Genotyping. Because only 10 non-obese women were included all genotyping was carried out on the 117 obese women. DNA was extracted from blood as described [23]. The gene for human perilipin A (National Institute of Health access number XM-007694) has nine exons and is located on chromosome 15q26 [17]. The genomic sequence and allele information for SNPs in perilipin were obtained from the SNP database at the US National Center for Biotechnology Information (NCBI). The SNPs were referred to by their Reference Cluster ID (rs#) as given at the NCBI web-site (http://www.ncbi.nlm.nih.gov/SNP/). For Genotyping we used the dynamic allele-specific hybridization (DASH) method [24, 25], using protocols available from Dynametrix (Hillsboro, Ore., USA) at the web site (http://www.dynametrix-ltd.com). Assays were run on a dedicated melting curve analysis instrument equipped with suitable built in software (Thermo Hybaid, Ashford, Middlesex, UK). Genotypes were scored from fluorescence curves as described previously [24]. The primer sequences used for PCR amplification were modified so as to amplify a DNA product with least stable secondary structures (Table 1), using Michael Zuker's program *mFold* [26] (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi). Each of the two alleles were probed independently using specific oligo-primers (allelic variations are underlined in Table 1). All PCR reactions were done on a MJ Research PTC-225 (Wellesley, Mass., USA). The DNA polymerase (AmpliTAQGold, Applied Biosystems, Foster City, Calif., USA) was activated by heating the PCR mixture at 98°C for 10 min. The amplification was carried out by 40 cycles of 98°C (10 s) and 55°C (30 s). Representative genotyping experiment is depicted in Fig. 1. The Y-axis indicates the negative derivate (d) of fluorescence against temperature (i.e., rate of negative change) for the temperature range in the X-axis. Melting curves illustrate differences in melting temperature profiles for the homozygous and heterozygous samples assayed for SNP rs894160, in intron 6 of the perilipin gene. The melting temperature was raised between 40°C to 65°C, with an interval of 0.03°C. The line having a single peak in the darker (i.e. pink) zone indicates the melting of a probe with a single mismatch (A/A alleles) and the line with a single peak in the lighter zone (i.e. light blue) indicates melting of probes with no mismatch (perfect match, G/G allele). The line with double peaks (one peak in each zone) represents a heterozygote (A/G alleles). Non-polymorphic SNPs (like rs894161 and rs 894162, showed peaks in either one of the zones (match or mismatch), but not in both.

In silico analysis. Information of the SNPs on their possible role in a functional intron-control element was provided from a search of the TRANSFAC database [27].

Table 1. Primer and probe sequences for amplification of genomic sequences with possible polymorphisms in perlipin gene

dbSNP: rs ID number	5′ primer—biotinylated	allele	3′ primer	probes	polymorphic
rs894160	GTCTCTGTTTGTGTGGCTCCC	A/G	GGTATCTACTGAGGCACATTCT	ATTCTAAACCTCTAGGG ATTCTAAACTTCTAGGG	yes
rs894161	ACTCTGGTTCATTGAATTTTTC	G/T	ATACTCCTCCCAGATCTTTTAA	ATCTTTTAAGAGTGAAA ATCTTTTAAGCGTGAAA	no
rs894162	CTTCAGCATGGGCAGTTGAGTCAG	A/T	TGCTCTGAGGGAGGATGGTAGCAG	TGGTAGCAGATAAGCTGA TGGTAGCAGATTTGCTGA	no
rs1561724	CCTGAGAACATTCTCTCTTCCA	C/G	CTCTTAGAACTGAAGCCCCAGC	CCCCAGCCCACGTTGGA CCCCAGCCCAGGTTGGA	no
rs1561726	AGCAGGTGTATCTCTGATGCATAC	A/T	AGGGAAGGGAAAAGAGTTGCCACCAG	CACCAGAGAGTGGGTAT CACCAGAGAGCGGGTAT	no
rs1052700	CTGATTGTTCCCTTCAAAGTAG	A/T	CTCTGATGAATATCCTCTGATG	TGATGATCTAGGCTCCC TGATGATCAAGGCTCCC	yes
rs1036750	TAAGCCCAGAAAAGAAAATAAA	C/T	CTTGTTTGCTGGCCTTTATGCT	TATGCTTTAGTATTGTT	no

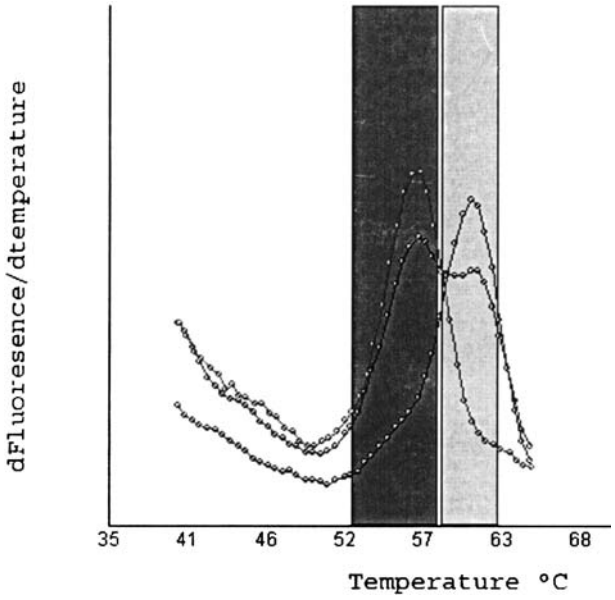

Fig. 1. Graph representing typical results from DASH genotyping

Statistics. Values are means ± standard error of the mean (SE). They were analysed using a Student's paired or unpaired *t* test, single or multiple regression analysis and ANOVA with a correction for age or BMI (ANCOVA), when indicated. Some results with Western blot might not be normally distributed so non-parametric tests were also used. A two-sided *p* value of 0.05 or less was considered to be statistically significant.

Results

Clinical findings. As expected the obese women had higher insulin and glucose concentrations than the non-obese women. Plasma triglycerides tended also to be increased in the obese group. In addition, fat-cell volume was increased almost twofold in the obese subjects and the difference in mean BMI between groups was 17 kg/m^2 (Table 2).

Comparison of perilipin in non-obese and obese women. In a first set of experiments we investigated the relation between perilipin protein content, obesity and the rate of lipolysis. Perilipin was assessed on western blot. One blot could handle up to 19 samples. Therefore all 10 non-obese and 27 randomly chosen obese women were studied using two separate blots. One obese sample was divided into two equal parts and was put on each blot. This sample was used to normalize values between each of the blots. The same amount of protein (100 µg) was used from each subject.

Results with lipolysis and perilipin in the fat cells are also shown in Table 2. The basal rate was about a fourfold increased in obesity (p=0.002). The maximum noradrenaline-induced lipolysis rate was almost twice as rapid in the obese as compared to the lean women (p=0.01). However, when the basal lipolysis rate was substracted to obtain a value of noradrenaline responsiveness there was no difference between the two groups. ADA-induced rate of lipolysis (i.e. basal rate in the absence of adenosine) was more than twofold increasedin the obese state (Table 2, p<0.001). The adipocyte protein amounts of perilipin and HSL were 100% and 50%, respectively higher in the lean as compared to the obese women (p=0.005 for perilipin and p=0.04 for HSL, Table 2).

The two western blot experiments are shown in Fig. 2. The perilipin antibody detected a major band of Mr 57 000 in all subjects and a less intense one of Mr 46 000 in some of the subjects. The HSL antibody detected one major band at Mr 88 000. Perilipin content A showed much greater interindividual variability than HSL content.

Construction of discordant pairs. BMI, basal rate of lipolysis and fat-cell size were significantly interrelated (r=0.6–0.7, p<0.002;Table 3). Perilipin correlated also with BMI, basal lipolysis and fat-cell volume

Table 2. Comparison of non-obese and obese women

Measure	Non-obese	Obese	*p* value
n	10	27	
Age, years	37±3	37±2	0.96
BMI, kg/m^2	23±1	40±1	<0.0001
pl-insulin, mU/l	6±1	19±3	0.0005
pl-glucose, mmol/l	4.8±0.2	5.6±0.2	0.03
pl-triglycerides, mmol/l	1.1±0.3	1.6±0.1	0.35
Fat cell volume, picolitres	412±55	837±34	<0.0001
Lipolysis, µmol/2 h/10^{7}cells			
Basal	3±1	12±2	0.002
ADA	8±1	18±2	0.0003
NA	17±3	28±2	0.01
NA minus basal	14±3	15±1	0.70
Adipocyte perilipin, OD/mm^2	2946±362	1646±239	0.005
Adipocyte HSL, OD/mm^2	1806±311	1299±90	0.04

Values are means ± SE and compared by Student's unpaired *t* test. Pl, fasting plasma; ADA (1 mU/l); NA, value at maximum effective concentration of noradrenaline

Table 3. Correlation matrix for the relationship between basal lipolysis, fat-cell volume, BMI and perilipin content

	Cell volume	BMI	Perilipin
Lipolysis	0.64	0.66	0.55
Cell volume		0.71	0.43
BMI			0.59

The individual values are from the results presented in Table 2. Lean and obese were put together in the analysis. The values were compared with each other by Spearman's rank correlation because we could not ascertain a normal distribution for perilipin values. Results are correlation coefficients. In all correlations the *p* value was 0.009 or less

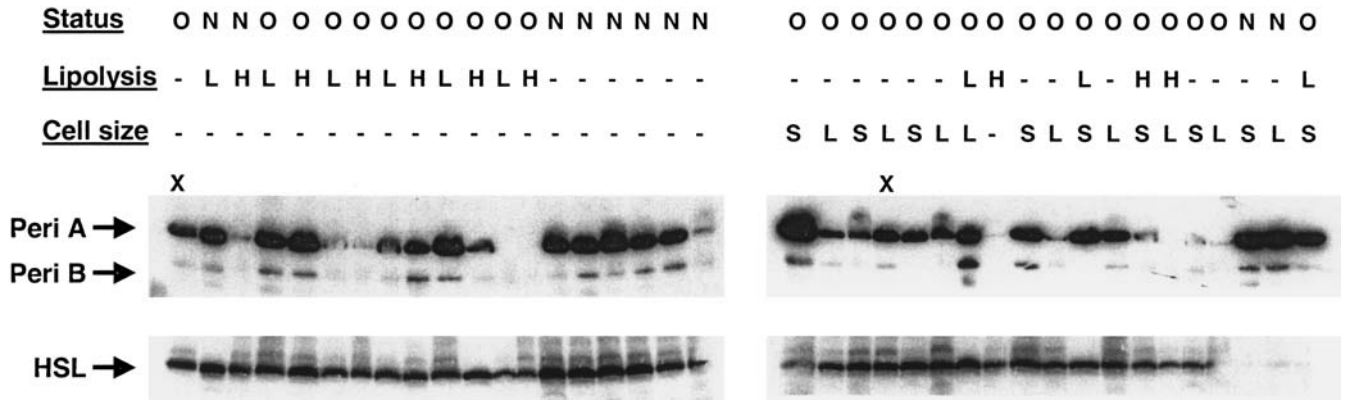

Fig. 2. Two western blots showing protein amounts in adipose tissue of perilipin (peri, upper part) and hormone sensitive lipase (HSL, lower part in 27 obese (O) and 10 non-obese (N) women. X = protein extract from one obese women was used in each of the blotting experiments and served as a tool to normalize optical density reading between blots. Some of the subjects were included in analysis of discordant pairs for either basal lipolysis (L = low lipolytic rate; H = high lipolytic rate) or fat cell size (S = small cells; L = large cells) as described in detail in Results. The western blots showed the occurrence of two perilipin bands at 57 and 46 kDa and one HSL band at 88 kDa

(*r*=0.5–0.6, $p \leq 0.009$; Table 3). Because all factors are strongly interrelated it is impossible, from a correlation analysis, to fully evaluate the individual contributions of cell volume, BMI and lipolysis to perilipin content. Furthermore, perilipin values appeared not to be normally distributed. Nevertheless, perilipin content was an independent regressor for lipolysis (partial *r*=0.37; *p*=0.025) when analysed together with fat-cell volume in a multivariate analysis. Fat-cell volume had a border-line contribution in this model (partial *r*=0.31; *p*=0.056). To make a more thorough evaluation we compared adipocyte perilipin between subjects who were matched for two of the three factors, lipolysis, BMI and fat-cell volume but discordant for the third factor.

To investigate if perilipin was related to lipolysis or fat-cell volume we investigated subsets of women described in Table 2 who were matched for interaction factors. In one set of experiments we matched for BMI as well as basal lipolysis and investigated the role of fat-cell volume. It was possible to select nine non-obese or obese pairs who had almost identical BMI, basal rate of lipolysis and noradrenaline-induced lipolysis within the pair but they differed by about 400 picoliters in fat-cell volume within the pair (Table 4). The adipocyte amounts of perilipin or HSL were almost the same within the pair although the women were discordant for fat-cell volume.

In another set of experiments we matched for BMI as well as fat-cell volume and investigated the role of lipolysis. It was possible to select nine non-obese or obese pairs who had almost identical fat-cell volume and BMI within the pair but who had a marked difference in basal and noradrenaline-induced rates of lipolysis within the pair (Table 5). In these pairs, adipocyte perilipin content was 50% decreased in the women with a high as compared to a low rate of basal lipoly-

Table 4. Comparison of nine pairs discordant for fat-cell volume but matched for BMI and basal lipolysis

Measure	Large cells	Small cells	*p* value
Age, years	40±4	37±4	0.57
BMI, kg/m^2	37±2	36±2	0.59
Fat cell volume, picolitres	990±48	599±53	<0.0001
Lipolysis, μmol/glycerol/2 h/10^{7}cells			
Basal	10±2	9±2	0.44
NA	23±4	23±2	0.96
Adipocyte perilipin, OD/mm^2	1513±3779	1996±409	0.22
Adipocyte HSL, OD/mm^2	1006±165	939±204	0.80

Values are means ± SE. They were compared by Student's paired *t* test. The pairs were selected from the women described in Table 2. See this legend to this table fur further details
NA, noradrenaline at maximum effective concentration

Table 5. Comparison of nine pairs discordant basal rate of lipolysis but matched for BMI and fat-cell volume

Measure	High lipolytic rate	Low lipolytic rate	*p* value
Age, years	37±4	35±4	0.68
BMI, kg/m^2	39±2	38±2	0.37
Fat cell volume, picolitres	817±51	810±53	0.57
Lipolysis, μmol/glycerol/2 h/10^{7}cells			
Basal	21±2	5±1	0.02
NA minus basal	38±3	21±2	0.002
Adipocyte perilipin, OD/mm^2	1110±396	2341±355	0.01
Adipocyte HSL, OD/mm^2	1455±161	1471±205	0.95

Values are means ± SE. NA, noradrenaline at maximum effective concentration (see legend to Tables 2, 4)

sis (p=0.01), whereas the protein amount of HSL was the same within the pair.

Relation between adipocyte perilipin content and circulating lipid metabolites. In order to indirectly study in vivo lipolytic activity we examined the correlation between fasting concentrations of plasma glycerol and serum NEFA and adipocyte perilipin content. An inverse linear relation between glycerol and perilipin (r=–0.617; p=0.0001, n=33) and between NEFA and perilipin (r=–0.485; p=0.03, n=19) was found, i.e. those with high perilipin had low NEFA and glycerol and vice versa. Similar results were obtained when Spearman's rank correlation was used as statistical test.

Genotyping. Seven SNPs (Table 1) were reported in the NCBI-dbSNP database at the time of this investigation. DASH assays were designed for all these SNPs and initially tested on DNA from a random set of 16 women from the investigated cohort. Of the seven SNPs, two (rs1052700 and rs894160) were found to be frequent (Table 1) in this study population (i.e. at least one subject had a variation in the gene sequence). These were located in exon 9 (past the coding sequence, in the 3 untranslated region) and intron 6 respectively. The two SNPs were genotyped in all the 117 obese women. It was not considered meaningful to genotype the few non-obese women.

The frequencies of the rs894160 (A/G) and the rs1052700 (A/T) variations in the perilipin gene were in Hardy-Weinberg equilibrium (p>0.5 by chi-square analysis) and both were relatively common. For rs 1052700 the number of AA, AT and TT subjects were 29, 50 and 38, respectively. For rs 894160 the number of AA, AG and GG subjects were 7, 42 and 68, respectively. The rs1052700 variation showed no statistically significant association with clinical data or lipolysis and was not investigated in detail. In contrast the rs894160 variation (located in intron 6) had an association to lipolysis and was therefore subjected to a thorough analysis.

Clinical data in relation to the rs 894160 perilipin polymorphism. Table 6 shows the clinical results with the rs894160 polymorphism. Mean age did not differ between the three genotypes and the same was true for fasting plasma concentrations of glucose and insulin and lipids. There was also no effect of the polymorphism on mean BMI or mean fat-cell volume.

Lipolysis data in relation to the rs 894160 perilipin polymorphism. An examination of lipolysis in the absence of a lipolytic agent was carried out (Fig. 3). A marked genotypic influence on basal and ADA induced lipolysis was observed (p=0.0008 for basal and p=0.01 for ADA). AA women had twofold higher mean rates than GG women (p<0.005), whereas heterozygotes had intermediate rates (p<0.05). These results were not influenced in an important way by including age or BMI in ANCOVA. Table 6 also shows the mean pD_2 values according to the genotype. No genotype effect on pD_2 for insulin, clonidine, terbutaline or dobutamine was observed.

Furthermore, we made an analysis of the influence of the polymorphism on noradrenaline- induced lipolysis. Because of paucity of AA subjects (n=5 for noradrenaline) we studied the allele by combining AA and AG women. Basal rates of lipolysis were about 50% higher in AA/AG women as compared to GG women (15±1 and 10±1 $mol^{-1} \cdot 2h^{-1} \cdot$cells 10^{-7} of glycerol, respectively; p=0.005). Maximum noradrenaline-

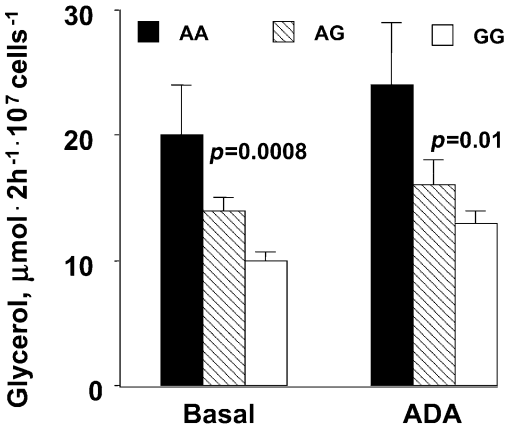

Fig. 3. Effect of the rs 894160 SNP in the perilipin gene on unstrained (basal) lipolysis and lipolysis induced by 1 mU/l of adenosine deaminase (ADA) in isolated fat cells in subjects further described in Table 6. The three genotypes are shown and compared by ANOVA. Values are means ±SE

Table 6. Characteristics of the rs891460 polymorphism in the perilipin gene in obese women as regards clinical findings and adipocyte pD2 values

Measure	GENOTYPE			
	AA (n=7)	*AG* (n=42)	*GG* (n=68)	p-value
Age, years	41±2	40±1	39±1	0.30
BMI, kg/m^2	39.7±0.8	39.1±0.4	38.9±0.3	0.71
Fat cell volume, picoliters	794±55	857±26	826±20	0.50
pl-insulin,mU/l	14.7±1.4	17.1±1.0	16.9±0.8	0.55
pl-glucose, mmol/l	5.3±0.2	5.3±0.1	5.4±0.1	0.93
pl, cholesterol, mmol/l	5.7±0.4	5.5±0.1	5.6±0.1	0.51
pl-triglycerides, mmol/l	1.5±0.2	1.5±0.1	1.7±0.1	0.48
pD_2 values				
Insulin	14.2±0.4	13.5±0.2	13.6±0.2	0.37
Terbutaline	7.5±0.7	7.6±0.2	7.5±0.2	0.93
Dobutamine	7.4±0.4	7.5±0.1	7.6±0.1	0.57
Clonidine	9.3±0.4	9.5±0.2	9.7±0.2	0.55

Values are means ± SE. They were compared by ANOVA; (see legend to Table 2); pl, fasting plasma

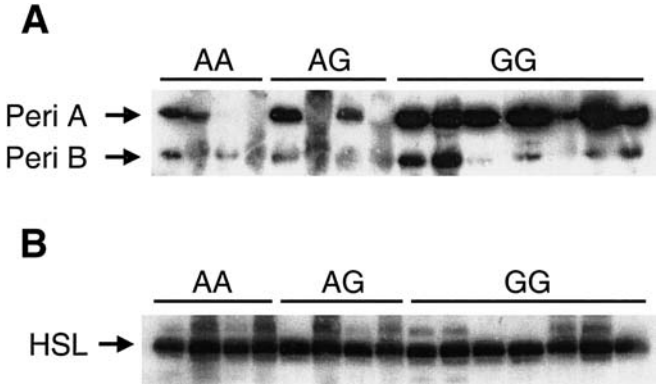

Fig. 4. Effect of the rs894160 SNP in the perilipin gene on adipose tissue protein content. Graph A is perilipin and graph B is hormone sensitive lipase. The individual results with the three genotypes are shown (see legend to Fig. 2)

Table 7. Comparison of obese women with high and low adipocyte perilipin content

Measure	Low	High	*p* value
n	9	18	
Age, years	39±3	36±3	0.51
BMI, kg/m^2	44±1	38±1	0.02
pl-insulin, mU/l	22±4	16±3	0.22
pl-glucose, mmol/l	5.7±0.2	5.6±0.3	0.64
Pl-triglycerides, mmol/l	1.9±0.4	1.5±0.1	0.21
pl-glycerol, µmol/l	162±20	100±8	0.004
s-NEFA, nmol/l	0.89±0.07	0.70±0.03	0.050
Basal lipolysis, µmol/2 h/10^{7}cells	17±3	10±1	0.04

Values are means ± SE and compared by Student's unpaired *t*-test; Pl, fasting plasma; s, fasting serum

induced lipolysis was about 30% higher in AA/AG than in GG women (29±2 and 23±2 mol^{-1}·2 h^{-1}·cells 10^{-7} of glycerol, respectively; p=0.01).

Protein measurements in relation to the rs894160 perilipin polymorphism. We investigated the protein amounts of perilipin and hormone sensitive lipase in fat cells from a subset of the women undergoing genotyping and lipolysis investigations. Because priority was given to lipolysis experiments in the genotyping study the western blot experiments could only be done on a limited number of obese women. We had tissue from 4 AA subjects. All these were used for protein determination together with 4 AG and 7 GG samples, which were chosen at random. The results of the western blot are shown (Fig. 3). The perilipin protein content was markedly decreased in AA subjects in comparison to GG subjects. In contrast, the amount of HSL did not differ between genotypes (Fig. 3) demonstrating that the genotype effect is specific for perilipin. Furthermore, there was very little between subjects' variability in HSL protein amount. The putative perilipin A band was quantified by densitometric scanning. The mean values (OD/mm^2) were 2754±247 for GG, 685±416 for AG and 523±310 for AA (p=0.005 by Kruskal-Wallis test). Thus, perilipin content was about 80% reduced in AA as compared to GG subjects while AG subjects had an intermediate content. We put all available genotyping data plus perilipin western blot data (non-obese plus obese) together in the analysis. The mean values (OD/mm^2) were 232±189 for AA (n=5), 1911±371 for AG (n=12) and 2555±251 for GG (n=20). The genotype effect was significant (p=0.003 by Kruskal-Wallis test).

In silico analysis. Using database search, we examined whether the G/A polymorphism was sited within a consensus DNA binding site for a known transcription factor. No site with complete homology to known transcription factor binding sites could be found.

Subgroup analysis of perilipin protein. We observed a considerable interindividual variation of the perilipin protein content in adipocytes (Figs. 2, 4). In the obese group nine women had values clearly below minus one standard deviation from the mean value. They were categorised as "low perilipin" and compared with the remaining 17 obese women ("high perilipin") (Table 7). The low group had higher BMI and circulating concentrations of NEFA and glycerol than the high group. Furthermore, basal rate of adipocyte lipolysis was much higher among the low group although fat-cell volume did not differ between groups.

Discussion

In this study we investigated the importance of perilipin for lipolysis in human fat cells. We focused on lipolysis, bearing in mind the role of lipid turnover in the pathophysiology of obesity and Type 2 diabetes. Several independent findings (clinical or genetic) point at an important role of perilipin in regulating basal (unstrained) and catecholamine-induced lipolytic activity in human fat cells. Menopausal women were not excluded from the investigation because menopause does not influence lipolysis regulation in human subcutaneous fat cells [28].

Methodological studies showed that the method to detect and quantitate perilipin was valid. We detected a major Mr 57000 and a minor Mr 96000 band. These bands correspond to perilipin A and B according to previous investigations in rodents [29]. It should be noted that the OD values for obese in the polymorphism study are different from those in Table 2. This is due to technical reasons such as in-between blot variation of antibody binding, luminescence intensity and exposure time.

One striking finding was the decreased perilipin content in adipocytes of the obese women. Likewise, obese women had increased basal and maximum nor-

adrenaline-stimulated lipolysis, although noradrenaline minus basal lipolysis was not influenced by obesity, confirming earlier studies [10]. Probably a low perilipin content is directly linked to an increased rate of lipolysis because women with a high rate of adipocyte lipolysis had much lower adipocyte perilipin content than those with a low rate of lipolysis even if the putative influence of BMI status and fat-cell volume was taken into consideration. Furthermore, it was observed that fat-cell volume had no or little influence on adipocyte perilipin content when BMI and basal rate of lipolysis were similar. This suggests that obesity and a high rate of lipolysis are independently associated with low adipocyte perilipin content in humans. The primary and secondary events in these relations have yet to be established. Experimental data are in favour of perilipin as a causative factor. Thus, mice lacking perilipin are lean and protected against development of obesity [14, 15]. These mice have an enhanced rate of lipolysis while over-expression of perilipin in rodent fat-cell lines decreases lipolysis [12, 13, 14, 15]. It is therefore possible that, in humans, obesity might decrease perilipin concentrations in the adipocytes resulting in an increased rate of lipolysis. Clearly this hypothesis must be tested in prospective studies including measurements on reduced body weight of obese subjects. In addition, we only investigated women and there are sex differences in the influence of obesity on subcutaneous adipocyte lipolysis [31].

The comparison of in vivo and in vitro data further suggests a role of perilipin in the regulation of lipolysis. Circulating concentrations of NEFA and glycerol, which indirectly mirror in vivo lipolytic activity, were inversely related to the adipocyte perilipin content. Furthermore, obese women with low perilipin adipocyte content had higher in vivo lipolytic activity (circulating glycerol and NEFA) and higher adipocyte lipolytic activity (in spite of no change in fat-cell volume) in comparison with obese women with high perilipin content.

Another important observation was the association between lipolysis and polymorphism in the perilipin gene. Two common SNPs were detected through a data base search. One (rs894160 A/G), which was located in intron 6, showed a clear relation to adipocyte lipolysis and perilipin protein concentrations. The other SNP (rs1052700), which was located in the 3′ untranslated region, had no apparent phenotypic effect. The rs894160 polymorphism had no effect on clinical variables. This does not exclude such effects of the polymorphism in women with co-morbidity, since we only investigated healthy obese women.

AA women had a twofold increase in the basal lipolytic rate whereas heterozygotes had an intermediate rate in comparison with GG women. This was also observed when adenosine was removed, which could artificially leak out of fat cells and inhibit the basal rate [20]. Furthermore, maximum noradrenaline-induced lipolysis was higher in carriers of the A-allele than those with GG. In contrast, a pD_2 analysis indicated that the polymorphism did not influence the function of adrenergic receptor subtypes or insulin receptors. This suggests that the perilipin polymorphism has effects mainly on post-receptor signalling to lipolysis.

Why is lipolysis increased in carriers of the A-allele? This is probably due to the low adipocyte content of perilipin in these carriers. Perilipin is an abundant protein in adipocytes but the observed marked decrease could have pronounced effects on lipolysis in human fat cells. Reduction of perilipin to very low amounts in mice increased adipocyte lipolysis markedly [14, 15]. Perilipin protein concentrations can be regulated at the transcriptional [12, 13] and post-translational [16] level in adipocyte cell lines. Since we observed a link between the perilipin protein amount and an intronic SNP it is likely that mRNA-related changes could explain the variation in protein amount. Unfortunately, we did not have enough adipose tissue left for quantitative mRNA measurements. There is also a possibility that, since the polymorphism is localized in an intron, it might simply be a marker of some other yet undiscovered functional variation, for example, in the promotor region of perilipin or in a nearby regulatory gene. Such a variation might alter transcription (or translation) of perilipin. In addition, the intron variation in itself might change the tertiary structure of DNA. Intronic regions could play an important role in the regulation of gene expression through a variety of mechanisms and a number of genes have been shown to contain functional intronic control elements [30, 31]. Other functional effects of the polymorphism such as being a binding site for transcription factors seem less likely according to our in silico analysis.

The amount of protein extracted from adipose tissue was very limited. Therefore we could only analyse two different proteins. Because HSL and perilipin are interrelated [11] we picked these two proteins for analysis. The findings with perilipin protein are most likely to be specific for perilipin because the HSL protein, which also is involved in lipolysis regulation, showed quite a different pattern. Although the amount of HSL protein was decreased in obesity, confirming earlier findings in other subjects [32] it was not influenced by the rate of basal lipolysis or by the perilipin polymorphism. If the perilipin changes would just reflect overall variations in fat-cell protein content identical results should be observed with HSL and perilipin in all conditions investigated. When our data and previous [33] data with HSL and adipocyte lipolysis are considered together it seems that HSL protein expression is related to catecholamine-stimulated lipolytic capacity of fat cells whereas perilipin protein expression is related to basal lipolytic capacity.

In summary, this study shows that obesity and a common rs89160 polymorphism in intron 6 of the perilipin gene is associated with low adipocyte perilipin content.

In addition, a low perilipin content is associated with high basal and catecholamine-induced rates of lipolysis in the fat cells and also with a high in vivo lipolytic activity. Taken together the data suggest an important role of perilipin in regulating lipolysis in human fat cells, which could be of pathogenic importance in obesity, Type 2 diabetes and other insulin resistant disorders.

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