Original Article

Variation in the Adiponutrin Gene Influences Its Expression and Associates With Obesity

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Adiponutrin is one of three recently identified adipocyte lipases. Surprisingly, these proteins also retain transacylase activity, a hitherto unknown pathway of triacylglycerol synthesis in the adipocytes. This may enable them to participate in both anabolic and catabolic processes. The adiponutrin gene (*ADPN*) is downregulated by fasting and upregulated by refeeding, suggesting a role in lipogenesis. Experiments in human adipocytes confirmed that the gene is upregulated in response to insulin in a glucose-dependent fashion. Obese subjects had increased levels of subcutaneous and visceral abdominal adipose tissue *ADPN* mRNA. Visceral *ADPN* mRNA expression was correlated to measures of insulin sensitivity (fasting insulin and homeostasis model assessment). We also studied genetic variation in *ADPN* and its relation to obesity, lipolysis, and mRNA expression. Two *ADPN* polymorphisms showed association with obesity. Carriers of the obesity-associated variants showed a lesser increase in the levels of adipose tissue *ADPN* mRNA and an increased basal lipolysis. Our results suggest that obese subjects that are insulin resistant and/or carriers of the obesity-associated *ADPN* alleles fail to upregulate the gene and that upregulation of adiponutrin may be an appropriate response to orchestrate energy excess. *Diabetes* 55:826–833, 2006

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

HOMA, homeostasis model assessment; HSL, hormone-sensitive lipase; iPLA2, identical to calcium-independent phospholipase A2; SNP, single nucleotide polymorphism.

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Obesity is a growing health problem associated with development of type 2 diabetes and coronary heart disease. Obesity results from interaction between genetic, environmental, and psychosocial factors. Collectively, these factors alter the balance between energy intake and expenditure (1–3). Energy is stored as triacylglycerol in adipose tissue found at subcutaneous or visceral abdominal sites. The release of energy is governed by several circulating factors, including insulin, cortisol, growth hormone, and cathecholamines, ultimately by regulating the rate of lipolysis (4). Hormone-sensitive lipase (HSL), an adipocyte triacyl- and diacylglycerol lipase, is believed to be the rate-limiting enzyme in the lipolytic process. However, recent findings have identified three different proteins that seem to complement HSL, particularly concerning triacylglycerol hydrolysis under nonstimulated conditions (4–8). Surprisingly, these proteins also exhibit transacylase activity (6). This may enable them to participate in both lipolysis and lipogenesis, in the latter case defining a previously unknown pathway of triglyceride synthesis in adipocytes.

The expression of one of these proteins, a membrane-associated protein called adiponutrin, is increased during adipogenesis and strongly influenced by nutritional status (5). In both rodents and humans in vivo and in mice preadipocyte cell lines in vitro, fasting results in very low or undetectable adiponutrin mRNA levels, whereas refeeding, particularly with a carbohydrate-rich diet, increases expression (5,9–13). This indicates that adiponutrin may promote adipocyte energy storage or recycling and plays an anabolic lipogenic rather than catabolic lipolytic role in adipocyte metabolism (6). Adiponutrin mRNA, encoded by *ADPN*, is highly expressed in adipocytes but also in different regions of the brain (5,11). *Adpn* mRNA levels are increased in *fa/fa* obese Zucker rats, a rodent model of obesity (5). Furthermore, genome-wide scans searching for susceptibility loci for type 2 diabetes and related phenotypes have shown association to a marker on human chromosome 22q13 in the proximity of *ADPN* (14,15). The locus harboring the *Adpn* gene in rats (7q34) has been associated with increased body weight (16,17).

Taken together, these observations identify *ADPN* as a candidate gene for obesity and related traits. Here, we investigated *ADPN* mRNA expression in human obesity, its regulation in human adipocytes, and whether polymorphisms in the gene are associated with *ADPN* mRNA expression, adipocyte lipolysis, and obesity.

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RESEARCH DESIGN AND METHODS

Obese, nondiabetic Scandinavian Caucasian subjects (234 in total; 63 men and 171 women, median age 42 years [33–52], and BMI 40.3 kg/m^2 [35.5–45.3]) from the obesity outpatient clinic at the University Hospital MAS in south of Sweden were randomly and individually matched for age (±5 years) and sex with nondiabetic, normal-weight subjects participating in the Botnia study (63 men and 171 women; median age 43 years [34–53], and BMI 22.6 kg/m^2 [21.3–23.8]) (18–20). Diabetes status was based on a previous diagnosis, on-going hypoglycemic medication, and fasting plasma glucose according to World Health Organization criteria. Biopsies of subcutaneous and visceral abdominal fat for mRNA expression studies were obtained from 36 nondiabetic, obese patients undergoing bariatric surgery (4 men and 32 women, median age 36 years [29–48], BMI 41.6 kg/m^2 [37.7–45.1], and waist circumference 118 cm [106–129]) and from 5 nondiabetic, nonobese patients undergoing abdominal elective surgery (2 men and 3 women, mean age 72 years [51–77], and BMI 26 kg/m^2 [24–29]); all of these subjects were fasted overnight but were in a stable metabolic state (19). DNA was available from 32 of these obese subjects, and data for homeostasis model assessment (HOMA) calculation was available for 23 subjects. Needle biopsies of abdominal subcutaneous adipose tissue for lipolysis studies were obtained from 130 obese patients (33 men and 97 women, median age 38 years [29–48], and BMI 39.4 kg/m^2 [35.3–43.5]) and 100 lean subjects (38 men and 62 women, median age 36 years [30–47], and BMI 23.4 kg/m^2 [21.5–24.2]). They were consequently recruited either by local advertisement or from the obesity clinic at the hospital to study the influence of genetic variance on adipocyte lipolysis regulation. They were all healthy and, except for obesity, free of metabolic disease. All were living in the Stockholm area and at least second generation Scandinavian. None was completely sedentary or involved in athletic performances. None had undergone a slimming effort or experienced a change (>1 kg) in body weight during the last 6 months before study, according to self-report. DNA was available for all patients included in the lipolysis study. All patients gave their written consent, and the local ethics committees approved the study.

Genotyping. DNA was extracted from whole blood by conventional methods as previously described or by using QiaGen MiniPrep (QiaGen, Hilden, Germany) at the DNA/RNA Genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö University Hospital (Malmö, Sweden). Seven single nucleotide polymorphisms (SNPs) were chosen from the National Center for Biotechnology Information and HapMap databases (http://www.ncbi.nlm.nih.gov and http://www.hapmap.org, respectively) positioned ~3–5 kb apart starting in the 5′ promoter region upstream of the transcription start in the 23.8-kb-long gene coding for *ADPN* to the untranslated region 3′ of the gene (Fig. 3). Two additional SNPs were chosen for genotyping after analyzing HapMap data using the Tagger program (predicting tagSNPs, SNPs that could explain most part of the variation in a gene) (Paul de Bakker; http://www.broad.mit.edu/mpg/tagger/). Sequences for primers and probes will be given on request. The chosen SNPs were genotyped using Assay by Design from Applied Biosystems (Foster City, CA) for TaqMan allelic discrimination performed with ABI 7900HT in 5-μl reaction volumes with 5 ng DNA and Universal TaqMan 2× PCR MasterMix according to manufacturer's recommendations (Applied Biosystems). Success rate for all genotyping was >99%, and there was no deviation from Hardy-Weinberg equilibrium (Supplement 1, which is detailed in the online appendix [available at http://diabetes.diabetesjournals.org]).

Expression analysis. Total RNA from biopsies was extracted using TRI REAGENT according to manufacturer's recommendations (Sigma, St. Louis, MO). Preadipocytes of human origin were differentiated and treated as previously described (19,21). The adipocytes were treated for 4 h with glucose (1, 5, and 25 mmol/l), palmitate (C16:0) (0.2, 0.5, and 1.6 mmol/l), and oleate (C18:1) (0.2, 0.5, and 1.6 mmol/l) in presence or absence of 1 nmol/l insulin, duplicates for each condition. RNA was extracted using RNeasy mini kit (QiaGen). cDNA was synthesized using Superscript II RNase H$^-$ Reverse Transcriptase and random hexamer primers (Life Technologies, Frederick, MD). Samples were analyzed with real-time PCR using the ABI 7900HT sequence detection system in 10-μl reaction volumes with 50 ng cDNA and Universal TaqMan 2× PCR MasterMix according to manufacturer's recommendations (Applied Biosystems). All samples were run in triplicate, and data were calculated using the standard curve method and expressed as a ratio to the endogenous control cyclophilin A (arbitrary units). Cyclophilin A was chosen because this housekeeping gene transcript has been shown to be stable in microarray studies comparing expression in adipose tissue between obese and nonobese subjects (22). Primers and probe for *ADPN* (NM_025225) were designed using Primer Express software (Applied Biosystems): forward primer, 5′-ATTTGCAACTTGCTACCCATTAGG-3′; reverse primer, 5′-CATGTCACCAGTCTCTGGACAATC-3′; and probe, 5′-(FAM)TAATGTCTTATGTAATGCTGCCCTGTACCCTGC(TAMRA)-3′ (MWG-Biotech, Edsberg, Germany).

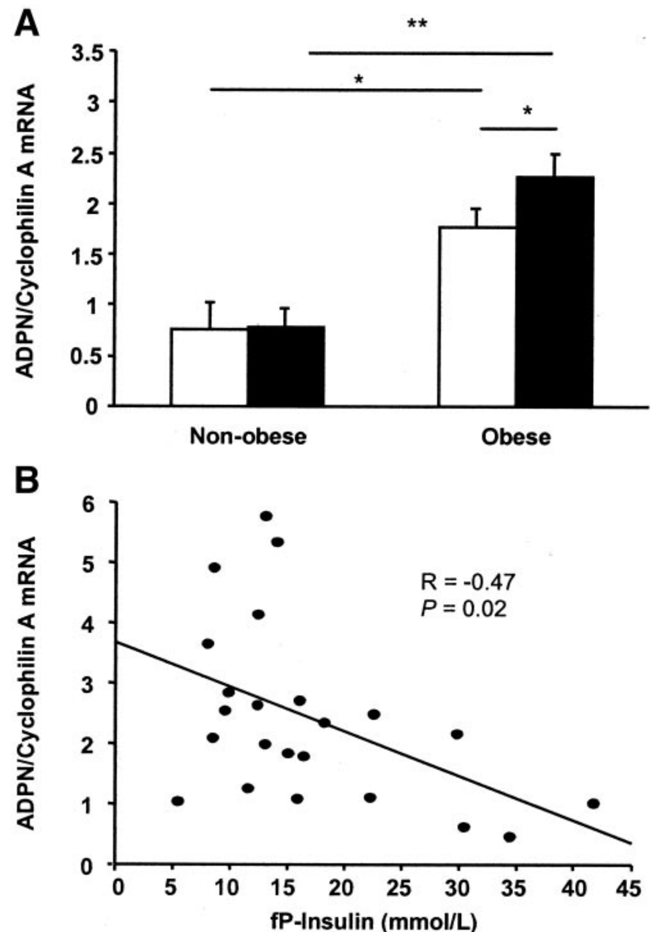

FIG. 1. *A*: Expression of *ADPN* mRNA in subcutaneous (□) and visceral (■) fat depots in 5 nonobese and 36 obese subjects. *B*: Inverse correlation between visceral *ADPN* mRNA expression and fasting plasma (fP)-insulin levels in 23 obese subjects. RNA was extracted, and *ADPN* mRNA levels were quantified by real-time RT-PCR and normalized to the level of cyclophilin A mRNA. Samples were analyzed in triplicate, and data are presented as means ± SE. *$P < 0.05$, **$P < 0.01$.

Lipolysis study. Adipose samples (1–2 g) were obtained by needle biopsy from the abdominal subcutaneous area under local anesthesia. The adipose tissue was collagenase treated, and isolated fat cells were collected and subject to lipolysis experiments as previously described (23,24).

Statistical analysis. Allele frequencies were compared using the McNemar test and genotype frequencies using χ^2 statistics. We used permutation testing (10.000 permutations) to correct for multiple testing. Wilcoxon's signed rank test was used for paired comparisons, Mann-Whitney *U* test was used for unpaired comparisons, and Spearman correlation was used for estimating relationships between variables. Kruskal-Wallis was used for testing the difference in expression between genotype groups. ANOVA was used to test for difference in expression in the adipocyte cultures when comparing more than two treatments. A P value <0.05 was considered statistically significant. Data are presented as median with interquartile range (25th–75th percentile) in parentheses or as means ± SE. All statistical calculations were performed using Number Cruncher Statistical Systems 2000 software (NCSS, Kaysville, UT).

RESULTS

We examined the *ADPN* mRNA expression in adipose tissue biopsies obtained from fasted nonobese or obese subjects undergoing cholecystectomy or bariatric surgery (Fig. 1*A*). *ADPN* mRNA expression levels were significantly higher in obese compared with nonobese humans in both subcutaneous (2.3- ± 0.2-fold, $P = 0.026$) and visceral adipose tissue (2.9- ± 0.3-fold, $P = 0.006$). The expression levels in visceral fat were significantly higher than in

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subcutaneous fat in the obese subjects (P = 0.027). Visceral and subcutaneous *ADPN* mRNA levels were correlated to each other (R = 0.46, P = 0.003; n = 41). Insulin resistance characterized the obese participants in the biopsy study (HOMA 2.91 [2.27–4.52], median age 35 years [29–49], and BMI 41.4 [37.6–45.2], n = 23). These 23 subjects were not different from the other participants in the biopsy study concerning age, sex, and BMI (data not shown). Visceral but not subcutaneous *ADPN* mRNA expression was inversely correlated to fasting plasma insulin (R = −0.47, P = 0.02, n = 24; Fig. 1*B*) and HOMA (R = −0.43, P = 0.04, n = 23). Accordingly, visceral *ADPN* mRNA was significantly lower (1.81 [0.97–2.37] vs. 2.73 [2.00–4.71], P = 0.03) in subjects with HOMA values above median (>2.91) compared with subjects with HOMA below median (<2.91), respectively. There was no correlation between BMI and either subcutaneous (R = 0.02, P = 0.9) or visceral (R = −0.08, P = 0.6) *ADPN* mRNA expression. The nonobese subjects were significantly older than the obese subjects, but we found no correlation between *ADPN* mRNA expression and age (data not shown).

We next examined the regulation of *ADPN* mRNA expression in human adipocytes in vitro. The circulating levels of glucose, free fatty acids, and insulin are all elevated in the postprandial state, which resembles an insulin-resistant state. To differentiate the effect of carbohydrates and fatty acids from that of insulin, we treated human cultured adipocytes with various concentrations of glucose, palmitate, and oleate in the presence or absence of insulin for 4 h (Fig. 2). Increasing the glucose concentration alone did not affect *ADPN* mRNA expression significantly. Adding insulin together with glucose resulted in increased expression (P < 0.05) with a significant trend toward increased expression with increasing glucose concentrations (ANOVA, P = 0.025; Fig. 2*A*). By contrast, inclusion of free fatty acids had no effect on *ADPN* mRNA expression (ANOVA, P = 0.78 and 0.84 for the effect of insulin in the presence of palmitate and oleate, respectively; Fig. 2*B* and *C*). Time-course experiments performed in three independent cell cultures at 5 mmol/l glucose suggested that the effect of insulin on *ADPN* mRNA is rapid, seen within 1 h, and transient, returning to basal levels within 6 h (data not shown).

Because both disease manifestation and gene expression may be influenced by genetic variation, we performed a case-control association study to examine whether variants in the adiponutrin gene was associated with obesity. Nine SNPs in the adiponutrin gene were genotyped in an obesity case-control cohort matched for age and sex (Fig. 3). All SNPs were in Hardy-Weinberg equilibrium, and they were equally distributed among males and females (data not shown). Two SNPs showed significant association with obesity: the tagSNP rs2072907, located in intron 5 (P_{Allele} = 0.0013, $P_{Genotype}$ = 0.0068), and rs1010022, located in intron 7 (P_{Allele} = 0.0023, $P_{Genotype}$ = 0.0056). The common alleles of both SNPs were overrepresented in obese compared with lean subjects (Tables 1 and 2). The difference in allele frequency between obese and lean subjects was significant after correcting for multiple testing (by permutation) for both rs2072907 (P = 0.015) and rs1010022 (P = 0.021). A third SNP, rs2076211, located in intron 5, also showed allelic and genotype association with obesity, the common allele being overrepresented in obese subjects (P_{Allele} = 0.016, $P_{Genotype}$ = 0.046). However, the difference in allele frequency was not significant after

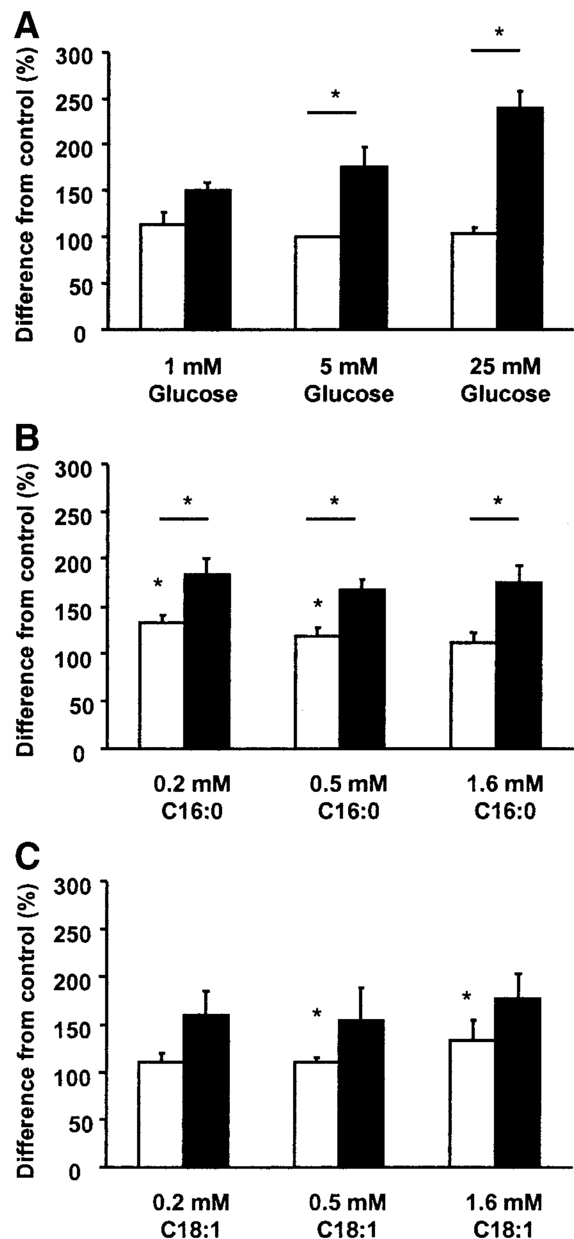

FIG. 2. The *ADPN* mRNA expression in cultured human adipocytes treated with (■) or without (□) 1 nmol/l insulin and 1, 5, and 25 mmol/l glucose (*A*); 0.2, 0.5, or 1.6 mmol/l palmitate (C16:0) (*B*); or 0.2, 0.5, or 1.6 mmol/l oleate (C18:1) (*C*). Three separate cultures were performed, duplicate for each condition. Expression is related to the endogenous control cyclophilin A, and data are presented as percentage of control set as treatment with 5 mmol/l glucose without insulin. **P **< 0.05 compared with control unless indicated otherwise.

correcting for multiple testing (P = 0.13). SNP rs738409 (Ile148Met), located in exon 3, showed significant difference in allele but not genotype frequency between obese and lean subjects, the common allele being overrepresented in obese subjects (P_{Allele} = 0.023, $P_{Genotype}$ = 0.12). But again, the difference in allele frequency was not sustained after correcting for multiple testing (P = 0.19). The remaining five SNPs (rs9626055, located in the 5′ region upstream of the putative *ADPN* mRNA transcription initiation site; rs2076212, a second coding SNP located in exon 2 leading to an amino acid substitution (Gly115Cys); rs5764034, located in intron 3; the tagSNP rs3810622, located in intron 8; and the 3′-untranslated region SNP,

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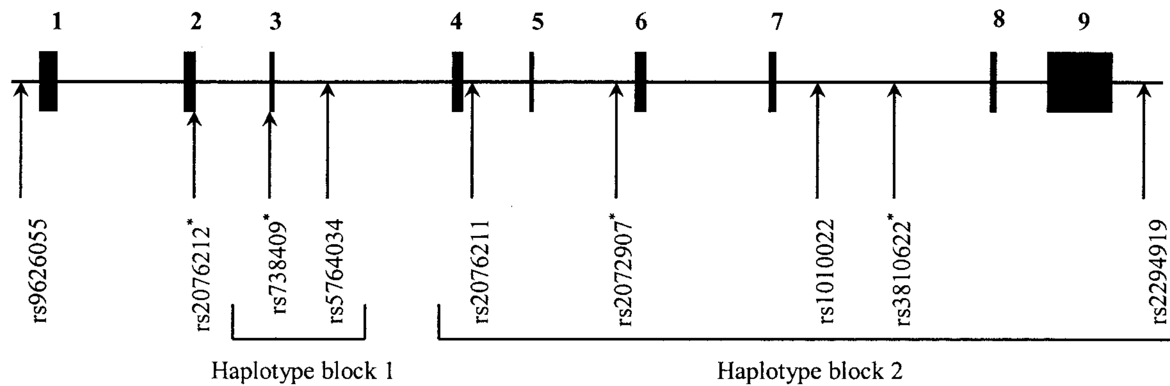

FIG. 3. Schematic presentation of the adiponutrin (*ADPN*) gene and the SNPs genotyped in this study. ■, the nine exons. Two blocks were constructed in the haplotype analysis, indicated below the rs numbers (NCBI SNP indentification numbers) . *Predicted tagSNPs using the Tagger program (Paul de Bakker; http://www.broad.mit.edu/mpg/tagger/).

rs2294919) showed no significant association with obesity (Tables 1 and 2).

Haplotype analysis was performed using the Haploview software (25). Haplotype blocks were determined by the method based on D′ values (26). Two haplotype blocks, the first consisting of rs738409 and rs5764034, and the second consisting of rs2076211, rs2072907, rs1010022, rs3810622, and rs2294919, showed strong linkage disequilibrium, with high pairwise magnitude of linkage disequilibrium (D′ > 0.8) and a logarithms of odds score >2.0. The linkage disequilibrium between the two obesity-associated SNPs rs2072907 and rs1010022 is very high (r^2 = 0.96 and D′ = 1.00 [0.98–1.00]). Two of the haplotypes, one in the first block (rs738409G/rs5764034C) and the other in the second block (rs2076211T/rs2072907C/rs1010022G/rs3810622T/rs2294919C), were significantly more common among the control subjects compared with the obese cases (P = 0.023 and 0.012, respectively). The significance of these findings disappeared when adjusting for multiple testing (Supplement 2, which is detailed in the online appendix).

The obesity-associated *ADPN* SNPs are located in introns that may contain regulatory elements for gene expression (27). We therefore genotyped the participants of the biopsy study and re-examined *ADPN* mRNA according to *ADNP* genotype (Fig. 4). None of the biopsy study subjects were homozygous for the less common variant alleles. Homozygous carriers of the obesity-associated alleles for SNPs rs2072907 and rs1010022 had significantly lower *ADPN* mRNA expression in subcutaneous tissue (P = 0.043 and 0.018, respectively; Fig. 4*A* and *B*). There was no significant difference in visceral expression between genotype carriers. The decrease in subcutaneous compared with visceral *ADPN* mRNA levels noted in all obese subjects (Fig. 1*A*) was restricted to homozygous carriers of the risk alleles for SNPs rs2072907 (P = 0.020) and rs1010022 (P = 0.023; Fig. 4*A* and *B*).

Because *ADPN* mRNA expression differed between carriers of the obesity-associated SNPs and because increased gene expression may indicate increased protein levels and function, we next explored whether adipocytes from different genotype carriers behave differently with respect to in vitro lipolysis. As shown in Table 3, nonstimulated lipolysis was significantly higher in obese carriers of the obesity-associated SNPs (P = 0.025 for both rs1010022 and rs2072907). No such differences were observed between nonobese genotype carriers. There were no differences in maximal lipolysis in response to isoprenaline or norepinephrine (Table 3) nor any differences concerning half-maximal doses for these lipolytic agents or lipid content per cell between different genotype carriers (data not shown).

DISCUSSION

In this study, we found that two common variants in the gene encoding adiponutrin, a novel adipocyte lipase/transacylase, show association to obesity. *ADPN* mRNA expression levels were increased in adipose tissue of obese subjects, and the degree of upregulation was influ-

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TABLE 1
Allelic frequencies for SNPs in an obesity case-control material

SNP	Major/minor allele	Lean		Obese		McNeamar P value	χ^2	Permutation P value
		1	2	1	2			
rs9626055	C/T	0.979	0.021	0.979	0.021	1.00	0.0	1.00
rs2076212*	Gly/Cys(G/T)	0.891	0.109	0.882	0.118	0.65	0.31	1.00
rs738409*	Ile/Met(C/G)	0.733	0.267	0.788	0.212	0.023	5.14	0.19
rs5764034	C/A	0.622	0.378	0.597	0.403	0.34	0.67	0.99
rs2076211	C/T	0.792	0.208	0.848	0.152	0.016	5.87	0.13
rs2072907*	G/C	0.759	0.241	0.835	0.165	0.0013	9.84	0.015
rs1010022	A/G	0.767	0.233	0.840	0.160	0.0023	9.09	0.021
rs3810622*	T/C	0.601	0.399	0.552	0.448	0.082	3.01	0.52
rs2294919	C/T	0.780	0.220	0.748	0.252	0.22	1.87	0.79

Data are presented as frequency. P values calculated using McNemar test. P values <0.05 are considered significant. 1, major allele; 2, minor allele. *Predicted tagSNPs using the Tagger program (Paul de Bakker; http://www.broad.mit.edu/mpg/tagger/).

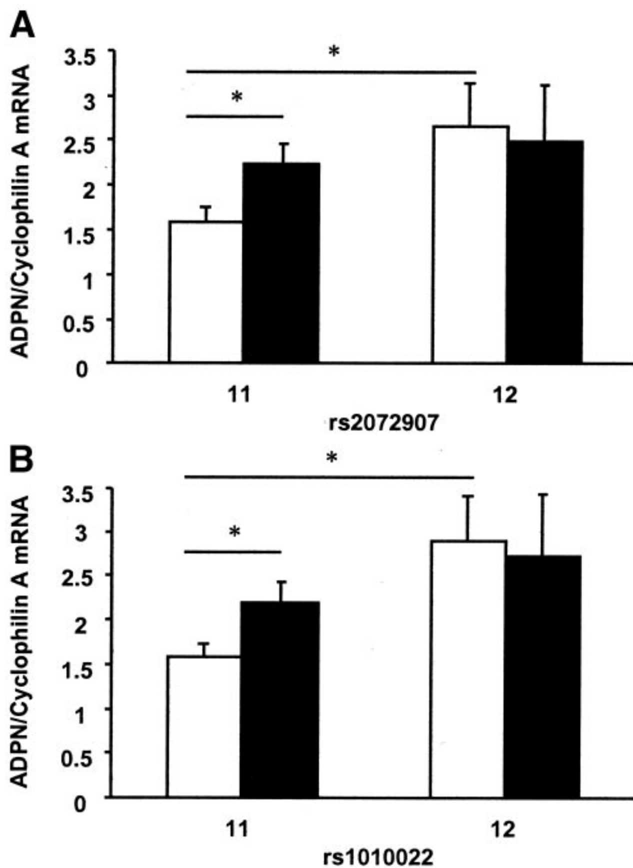

FIG. 4. Expression of *ADPN* mRNA in subcutaneous (□) and visceral (■) fat depots from obese subjects divided on genotype carriers of tagSNP rs2072907 (2/23 [men/women] carriers of 11 genotype, median age 36 [29–48] years, and BMI 42.2 [37.7–44.6] kg/m^2; and 1/6 [men/women] carriers of 12 genotype, median age 40 [34–50] years, and BMI 39.8 [35.1–43.7] kg/m^2) (*A*) and SNP rs1010022 (2/24 [men/women] carriers of 11 genotype, median age 36 [29–50] years, and BMI 41.8 [37.8–44.3] kg/m^2; and 1/5 [men/women] carriers of 12 genotype, median age 38 [34–49] years, and BMI 40.7 [34.4–44.4] kg/m^2) (*B*). RNA was extracted, and *ADPN* mRNA levels were quantified by real-time RT-PCR and normalized to the level of cyclophilin A mRNA. Samples were analyzed in triplicate, and data are presented as means ± SE. *P < 0.05. 1, major allele; 2, minor allele.

enced by ADPN genotype and, in the visceral adipose tissue, by insulin sensitivity. In vitro experiments using human adipocytes confirmed the nutritional influence on *ADPN* mRNA regulation and suggest that the upregulation of *ADPN* expression seen in the presence of carbohydrates requires insulin. The obesity-associated *ADPN* mRNA increase may in other words be secondary to the hyperinsulinaemia that often characterize obesity. These findings suggest a possible protective effect of adiponutrin during obesity. Obesity-associated *ADPN* SNPs were associated with lower *ADPN* mRNA expression and increased basal adipocyte lipolysis, suggesting that the predominant role of adiponutrin in obese subjects may be anabolic lipogenic, i.e., reflecting the transacylase activity, rather than catabolic lipolytic.

Until recently, HSL was the only known triacylglycerol lipase in the adipocyte (4,28). Surprisingly, HSL knockout mice retained a lean phenotype, residual lipase activity, and accumulation of diacylglycerol rather than triacylglycerol, suggesting the existence of important complimentary lipases (29–32). Adiponutrin (identical to calcium-independent phospholipase A2 ε [iPLA2ε]), along with two other recently discovered lipases, desnutrin (identical to adipose triglyceride lipase or iPLA2ζ) and GS-2 (identical to iPLA2η), are prime candidates for this lipase activity (5–8). An updated view of adipocyte lipolysis is emerging in which one or more of these proteins are responsible for the first step of lipolysis, the triacylglycerol hydrolysis step; HSL is responsible for the second diacylglycerol hydrolysis step; and monoglyceride lipase is responsible for the final step (4). Although the three complimentary lipases show similarities, such as their predominant or exclusive expression in adipose tissue and induction of expression during the early stages of adipocyte differentiation, their respective genes seem to be differentially regulated, and it is possible that they serve different functions. For example, the mRNA expression of the gene encoding iPLA2ζ (*PNPLA2*) is transiently induced during fasting, as would be expected for an enzyme involved in energy release, whereas that of *ADPN* is decreased. Upon refeeding, *ADPN* mRNA expression is then strongly induced by carbohydrates, shown either by a high-carbohydrate diet given to rats or by exposing 3T3-L1 mouse adipocytes to glucose (5,7,9–12). In addition, although *ADPN* mRNA is increased in rodent obesity, the expression of *PNPLA2* seems to be downregulated (5,7). Although *ADPN* mRNA expression increased with increasing concentrations of glucose in our cultured human adipocytes, a marked increase in the expression of *ADPN* was not seen until after adding insulin. This further differentiates the regulation of *ADPN* from that of *PNPLA2*, which is downregulated by insulin in mouse 3T3-L1 cells (33). Meanwhile, the inclusion of the fatty acids palmitate and oleate had no effect on *ADPN* mRNA expression analogous

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TABLE 2
Genotypic frequencies for SNPs in an obesity case-control material

SNP	Lean			Obese			χ^2	P value
	11	12	22	11	12	22		
rs9626055	0.957	0.043	0	0.957	0.043	0	0	1.00
rs2076212*	0.786	0.209	0.004	0.774	0.218	0.009	0.40	0.82
rs738409*	0.521	0.423	0.056	0.611	0.355	0.034	4.26	0.12
rs5764034	0.361	0.524	0.116	0.365	0.464	0.172	3.38	0.18
rs2076211	0.614	0.356	0.030	0.721	0.253	0.026	6.14	0.046
rs2072907*	0.560	0.397	0.043	0.701	0.269	0.030	9.99	0.0068
rs1010022	0.568	0.397	0.034	0.709	0.261	0.030	10.36	0.0056
rs3810622*	0.339	0.524	0.137	0.313	0.476	0.210	4.32	0.12
rs2294919	0.598	0.363	0.038	0.573	0.350	0.077	3.19	0.20

Data are presented as frequency. P values calculated using χ^2 test. P values <0.05 are considered significant. 1, major allele; 2, minor allele.
*Predicted tagSNPs using the Tagger program (Paul de Bakker; http://www.broad.mit.edu/mpg/tagger/).

TABLE 3
Lipolysis in human isolated adipocytes from subcutaneous needle biopsies based on genotypes for SNP rs2072907 in nonobese and obese subjects

Stimulation	11	12	22	11 + 12	*P* value
Nonobese					
n (men/women)	27/38	10/22	1/3	37/60	0.61 (0.87)
Age (years)	36 (30–47)	37 (33–46)	32 (28–48)	36 (31–47)	0.85 (0.61)
BMI (kg/m^2)	23.4 (21.8–24.2)	23.1 (21.2–24.2)	23.5 (17.8–24.3)	23.4 (21.6–24.2)	0.81 (0.91)
Basal lipolysis (μg glycerol · g lipid^{-1} · 2 h^{-1})	0.79 (0.52–1.30) [64]	0.76 (0.53–1.24) [32]	1.44 (0.45–1.55) [3]	0.78 (0.53–1.25) [96]	0.80 (0.51)
Isoprenaline	6.27 (4.52–8.80) [63]	6.56 (4.26–8.63) [29]	7.90 (4.37–8.25) [3]	6.27 (4.41–8.66) [92]	0.98 (0.82)
Norepinephrine	3.19 (2.02–4.65) [62]	3.25 (2.39–5.96) [32]	2.81 (2.74–4.74) [3]	3.25 (2.21–4.92) [94]	0.73 (0.89)
Obese					
n (men/women)	20/69	11/21	2/7	31/90	0.40 (0.82)
Age (years)	39 (28/48)	37 (30–46)	47 (25–52)	38 (29–48)	0.95 (0.77)
BMI (kg/m^2)	40.4 (35.6–44.8)	38.3 (33.4–41.1)	35.9 (31.4–40.4)	39.5 (35.4–43.7)	0.063 (0.13)
Basal lipolysis (μg glycerol · g lipid^{-1} · 2 h^{-1})	1.28 (0.79–1.83) [89]	1.32 (0.76–1.92) [32]	0.68 (0.43–0.96) [9]	1.30 (0.79–1.83) [121]	0.025 (0.007)
Isoprenaline	4.57 (3.13–5.75) [88]	4.19 (3.15–4.88) [31]	3.56 (1.80–4.33) [9]	4.41 (3.13–5.57) [119]	0.13 (0.055)
Norepinephrine	3.08 (2.10–4.06) [88]	2.77 (2.27–3.86) [32]	2.57 (1.31–3.09) [9]	2.90 (2.14–4.00) [120]	0.30 (0.12)

Data are presented as median with interquartile range within parenthesis (25th–75th percentile) and number of observations within brackets [*n*]. The *P* value within parentheses represents the comparison between the genotypes 11 + 12 and 22. *P* values are calculated using Kruskal-Wallis or Wilcoxon's test. 1, major allele; 2, minor allele.

to the lack of effect of a high-fat diet on *Adpn* expression in rats (9). Adipose tissue *Adpn* mRNA levels in mice follow the serum levels of insulin, but in contrast to our findings, insulin itself only had a minor effect on *Adpn* mRNA expression in 3T3-L1 cells (5). It is thus possible that adiponutrin gene expression is subject to species-specific regulation. Importantly, however, 3T3-L1 mouse adipocytes differ in many ways from the primary human adipocytes used here (21).

We conclude from these experiments that the effect of carbohydrates on *ADPN* mRNA expression is dependent on insulin. To date, the promoter of the adiponutrin gene has not been analyzed, but it is likely that insulin responsive transcription factor(s) plays an important role. For example, Baulande et al. (5) have shown that the expression of adiponutrin in 3T3-L1 cells and rat adipose tissue follows the same pattern as the expression of adipocyte determination and differentiation factor–1/sterol regulatory element-binding protein–1c, a transcription factor that mediates important insulin effects (34) both during 3T3-L1 adipocyte differentiation and in the white adipose tissue of rats during feeding, fasting, and refeeding conditions (5).

In vivo, obese subjects with decreased insulin sensitivity had decreased *ADPN* expression, at least in the visceral adipose tissue. The limited number of participants in this study makes the interpretation of correlations suggestive, but our results are supported by findings in the first human report on adiponutrin. Liu et al. (13) found significantly higher insulin sensitivity in subjects with increased expression of *ADPN* mRNA in subcutaneous adipose tissue and an inverse correlation between *ADPN* mRNA and the level of glycemia and/or indirect measures of insulin resistance. We also found increased *ADPN* mRNA levels in both subcutaneous and visceral adipose tissue from obese subjects. Although a similar trend observed by Liu et al. (13) in subcutaneous adipose tissue failed to reach statistical significance (visceral adipose tissue was not available), they did find a 36 and 58% decrease in *ADPN* mRNA expression after caloric restriction after 2 and 21 days, respectively.

Genome-wide scans from both humans and rats have shown association and linkage to the region harboring the gene encoding adiponutrin with type 2 diabetes, plasma insulin levels, and body weight (3,14–17). Because no previous study of the role of genetic variation in this gene on obesity-related variables has been performed, we selected nine SNPs covering the entire gene. We found two intronic SNPs showing association to obesity. SNPs are not independent of each other, and historical recombination results in patterns of SNPs inherited together in the form of haplotypes (35). Haplotypes constructed from data available from the public database HapMap showed high recombination in the 5′ end of *ADPN*, whereas SNPs in the 3′ end are inherited together in a large haplotype block (http://www.hapmap.org). Our present haplotype analysis shows the same structure. Haplotypes containing the nonobese alleles (rs738409G, rs2072907C, and rs1010022G) were more common among the lean control subjects, but the significance of these findings disappeared when adjusting for multiple testing. By necessity, constructing haplotypes will create subgroups and decrease the statistical power of the analysis. Until replicated in other populations, these results should be viewed with caution. TagSNPs were predicted to capture all or most of the variation in the large 3′ haplotype (Paul de Bakker; http://www.broad.mit.edu/mpg/tagger/). The haplotype contained two intronic tagSNPs (rs2072907 and rs3810622) of which only rs2072907 was associated with obesity. It was also the only obesity-associated tagSNP that showed

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significant differences in adipose tissue *ADPN* mRNA levels and adipocyte lipolysis, supporting the contribution of genetic variation in *ADNP* to obesity. Although our study populations are genetically homogenous and carefully matched and phenotyped, our findings need to be confirmed in larger populations. One must also consider the possibility that the SNPs described here are nonfunctional and only in linkage disequilibrium with the causal variant(s).

Our findings concerning insulin regulation of *ADPN* mRNA expression are analogous to the strong enhancing effect of insulin and glucose on lipogenic enzymes such as fatty acid synthase and acetyl-CoA reductase (36). In fact, every aspect of *ADPN/Adpn* expression is reminiscent of a lipogenic enzyme (37). It was therefore interesting to note that basal lipolysis was increased in adipocytes from obese carriers of *ADPN* SNPs associated with obesity and low mRNA expression, possibly reflecting a lack of adiponutrin transacylase activity. A transacylase uses mono- and diacylglycerols as acyl donors/acceptors instead of using glycerol phosphate, dihydroxyacetone phosphate, or monoacylglycerol as initial acyl acceptors in the acyl-CoA–dependent enzymatic synthesis of triglycerides (6,38). It should be pointed out that such an acyl-CoA–independent process has been described in intestinal mucosa cells but that its existence and relative contribution in human adipocytes is largely unknown (4,6,38). These data provide a feasible mechanism by which the adiponutrin gene is associated with obesity. It is possible that the upregulation of *ADPN*, and hence the ability to increase triacylglycerol synthesis through transacylation, is an appropriate response aimed at compensating for the imbalance in triglyceride homeostasis. Such a protective mechanism would reduce the risk of systemic increases in free fatty acids and their consequences. Depending on the concomitant lipase activity of adiponutrin, other non-HSL lipases, and HSL itself, they may form a futile cycle to dissipate an increased caloric load (28). In obese, insulin-resistant subjects or in carriers of the associated *ADPN* risk alleles in whom the expression is insufficiently increased, the net result would be an increased basal rate of lipolysis. The finding that basal lipolysis was increased in obese but not in nonobese carriers of the risk allele further strengthens the functionality of this association. Importantly, there were no apparent differences in adipocyte cell size or lipid content per cell between genotype carriers. Such differences may otherwise have biased the results, because it is known that obese subjects have larger adipocytes that show increased basal lipolysis (24). The fact that the *ADPN* alleles that were associated with obesity were also associated with decreased adipose tissue *ADPN* expression and increased nonstimulated adipocyte lipolysis suggests that it may be worthwhile to examine involvement in phenotypes associated with obesity rather than obesity per se. Certainly, the possible role of adiponutrin in the pathogenesis of the metabolic syndrome and type 2 diabetes should be subject to future investigation.

In conclusion, genetic variation in *ADPN* is associated with obesity, *ADPN* mRNA expression levels, and basal adipocyte lipolysis. The gene is upregulated in obese subjects, but the upregulation is dependent on both genotype and insulin. The regulation of *ADPN* is reminiscent of a protein involved in lipogenesis rather than lipolysis. We therefore hypothesize that this is the main action of adiponutrin under conditions of energy excess such as in obesity. The relative contribution of the lipase and transacylase activities of adiponutrin requires further investigation. This is particularly urgent concerning the transacylation pathway of triacylglycerol synthesis in adipocytes. This is a completely new concept that may turn out to be important both in the understanding and treatment of obesity-related conditions such as type 2 diabetes and cardiovascular disease.

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REFERENCES

1. Kahn BB, Flier J: Obesity and insulin resistance. *J Clin Invest* 106:473–481, 2000
2. Flier JS: Obesity wars: molecular progress confronts an expanding epidemic. *Cell* 116:337–350, 2004
3. Bell CG, Walley AJ, Froguel P: The genetics of human obesity. *Nat Rev Genet* 6:221–234, 2005
4. Zechner R, Strauss JG, Haemmerle G, Lass A, Zimmermann R: Lipolysis: pathway under construction. *Curr Opin Lipidol* 16:333–340, 2005
5. Baulande S, Lasnier F, Lucas M, Pairault J: Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. *J Biol Chem* 276:33336–33344, 2001
6. Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, Gross RW: Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* 279:48968–48975, 2004
7. Villena JA, Roy S, Sarkadi-Nagy E, Kim KH, Sul HS: Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem* 279:47066–47075, 2004
8. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R: Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306:1383–1386, 2004
9. Polson DA, Thompson MP: Adiponutrin mRNA expression in white adipose tissue is rapidly induced by meal-feeding a high-sucrose diet. *Biochem Biophys Res Commun* 301:261–266, 2003
10. Polson DA, Thompson MP: Macronutrient composition of the diet differentially affects leptin and adiponutrin mRNA expression in response to meal feeding. *J Nutr Biochem* 15:242–246, 2004
11. Bertile F, Raclot T: Differences in mRNA expression of adipocyte-derived factors in response to fasting, refeeding and leptin. *Biochim Biophys Acta* 1683:101–109, 2004
12. Wiesner G, Morash BA, Ur E, Wilkinson M: Food restriction regulates adipose-specific cytokines in pituitary gland but not in hypothalamus. *J Endocrinol* 180:R1–R6, 2004
13. Liu YM, Moldes M, Bastard JP, Bruckert E, Viguerie N, Hainque B, Basdevant A, Langin D, Pairault J, Clement K: Adiponutrin: a new gene regulated by energy balance in human adipose tissue. *J Clin Endocrinol Metab* 89:2684–2689, 2004
14. Ghosh S, Watanabe RM, Valle TT, Hauser ER, Magnuson VL, Langefeld CD, Ally DS, Mohlke KL, Silander K, Kohtamaki K, Chines P, Balow J Jr, Birznieks G, Chang J, Eldridge W, Erdos MR, Karanjawala ZE, Knapp JI, Kudelko K, Martin C, Morales-Mena A, Musick A, Musick T, Pfahl C, Porter R, Rayman JB: The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study: I. An autosomal genome scan for genes that predispose to type 2 diabetes. *Am J Hum Genet* 67:1174–1185, 2000
15. Watanabe RM, Ghosh S, Langefeld CD, Valle TT, Hauser ER, Magnuson VL,

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Mohlke KL, Silander K, Ally DS, Chines P, Blaschak-Harvan J, Douglas JA, Duren WL, Epstein MP, Fingerlin TE, Kaleta HS, Lange EM, Li C, McEachin RC, Stringham HM, Trager E, White PP, Balow J Jr, Birznieks G, Chang J, Eldridge W: The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. II. An autosomal genome scan for diabetes-related quantitative-trait loci. *Am J Hum Genet* 67:1186–1200, 2000

16. Galli J, Li LS, Glaser A, Ostenson CG, Jiao H, Fakhrai-Rad H, Jacob HJ, Lander ES, Luthman H: Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nat Genet* 12:31–37, 1996
17. Gauguier D, Froguel P, Parent V, Bernard C, Bihoreau MT, Portha B, James MR, Penicaud L, Lathrop M, Ktorza A: Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. *Nat Genet* 12:38–43, 1996
18. Groop L, Forsblom C, Lehtovirta M, Tuomi T, Karanko S, Nissen M, Ehrnstrom BO, Forsen B, Isomaa B, Snickars B, Taskinen MR: Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* 45:1585–1593, 1996
19. Ridderstråle M, Carlsson E, Klannemark M, Cederberg A, Kosters C, Tornqvist H, Storgaard H, Vaag A, Enerback S, Groop L: *FOXC2* mRNA expression and a 5′ untranslated region polymorphism of the gene are associated with insulin resistance. *Diabetes* 51:3554–3560, 2002
20. Suviolahti E, Oksanen LJ, Ohman M, Cantor RM, Ridderstråle M, Tuomi T, Kaprio J, Rissanen A, Mustajoki P, Jousilahti P, Vartiainen E, Silander K, Kilpikari R, Salomaa V, Groop L, Kontula K, Peltonen L, Pajukanta P: The SLC6A14 gene shows evidence of association with obesity. *J Clin Invest* 112:1762–1772, 2003
21. Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E, Debatin KM, Hauner H: Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* 25:8–15, 2001
22. Nair S, Lee YH, Rousseau E, Cam M, Tataranni PA, Baier LJ, Bogardus C, Permana PA: Increased expression of inflammation-related genes in cultured preadipocytes/stromal vascular cells from obese compared with non-obese Pima Indians. *Diabetologia* 48:1784–1788, 2005
23. Reynisdottir S, Eriksson M, Angelin B, Arner P: Impaired activation of adipocyte lipolysis in familial combined hyperlipidemia. *J Clin Invest* 95:2161–2169, 1995
24. Large V, Reynisdottir S, Langin D, Fredby K, Klannemark M, Holm C, Arner P: Decreased expression and function of adipocyte hormone-sensitive lipase in subcutaneous fat cells of obese subjects. *J Lipid Res* 40:2059–2065, 1999
25. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265, 2005
26. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D: The structure of haplotype blocks in the human genome. *Science* 296:2225–2229, 2002
27. Fedorova L, Fedorov A: Introns in gene evolution. *Genetica* 118:123–131, 2003
28. Raben DM, Baldassare JJ: A new lipase in regulating lipid mobilization: hormone-sensitive lipase is not alone. *Trends Endocrinol Metab* 16:35–36, 2005
29. Osuga J, Ishibashi S, Oka T, Yagyu H, Tozawa R, Fujimoto A, Shionoiri F, Yahagi N, Kraemer FB, Tsutsumi O, Yamada N: Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc Natl Acad Sci U S A* 97:787–792, 2000
30. Wang SP, Laurin N, Himms-Hagen J, Rudnicki MA, Levy E, Robert MF, Pan L, Oligny L, Mitchell GA: The adipose tissue phenotype of hormone-sensitive lipase deficiency in mice. *Obes Res* 9:119–128, 2001
31. Okazaki H, Osuga J, Tamura Y, Yahagi N, Tomita S, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Kimura S, Gotoda T, Shimano H, Yamada N, Ishibashi S: Lipolysis in the absence of hormone-sensitive lipase: evidence for a common mechanism regulating distinct lipases. *Diabetes* 51:3368–3375, 2002
32. Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, Zechner R: Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *J Biol Chem* 277:4806–4815, 2002
33. Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M, Fasshauer M: Isoproterenol, TNFα, and insulin downregulate adipose triglyceride lipase in 3T3–L1 adipocytes. *Mol Cell Endocrinol* 240:43–49, 2005
34. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM: Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101:1–9, 1998
35. Clark AG: The role of haplotypes in candidate gene studies. *Genet Epidemiol* 27:321–333, 2004
36. Foufelle F, Gouhot B, Péorier JP, Perdereau D, Girard J, Ferré P: Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. *J Biol Chem* 267:20543–20546, 1992
37. Foufelle F, Girard J, Ferré P: Regulation of lipogenic enzyme expression by glucose in liver and adipose tissue: a review of the potential cellular and molecular mechanisms. *Advan Enzyme Regul* 36:199–226, 1996
38. Coleman RA, Lee DP: Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* 43:134–176, 2004

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