

The L- α -Lysophosphatidylinositol/*GPR55* System and Its Potential Role in Human Obesity

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GPR55 is a putative cannabinoid receptor, and L- α -lysophosphatidylinositol (LPI) is its only known endogenous ligand. We investigated 1) whether *GPR55* is expressed in fat and liver; 2) the correlation of both *GPR55* and LPI with several metabolic parameters; and 3) the actions of LPI on human adipocytes. We analyzed *CB1*, *CB2*, and *GPR55* gene expression and circulating LPI levels in two independent cohorts of obese and lean subjects, with both normal or impaired glucose tolerance and type 2 diabetes. Ex vivo experiments were used to measure intracellular calcium and lipid accumulation. *GPR55* levels were augmented in the adipose tissue of obese subjects and further so in obese patients with type 2 diabetes when compared with nonobese subjects. Visceral adipose tissue *GPR55* correlated positively with weight, BMI, and percent fat mass, particularly in women. Hepatic *GPR55* gene expression was similar in obese and type 2 diabetic subjects. Circulating LPI levels were increased in obese patients and correlated with fat percentage and BMI in women. LPI increased the expression of lipogenic genes in visceral adipose tissue explants and intracellular calcium in differentiated visceral adipocytes. These findings indicate that the LPI/*GPR55* system is positively associated with obesity in humans. *Diabetes* 61:281–291, 2012

In addition to their ability to store triacylglycerol, adipocytes act as endocrine secretory cells (1). A growing number of adipocyte-derived factors have been described, and their contribution to the pathophysiology of metabolic syndrome is being investigated (2). Patients with type 2 diabetes exhibit increased activity of the endocannabinoid system in visceral fat and higher concentrations of endocannabinoids in the blood,

when compared with corresponding controls (3–6). The endocannabinoid system acts through the type 1 and 2 cannabinoid receptors (*CB1* and *CB2*) (4,7,8). *GPR55* is a seven-transmembrane G protein-coupled receptor that shares only 13.5% sequence identity with the *CB1* receptor and 14.4% with the *CB2* receptor (9). The differences between their sequences are in agreement with the distinct relative affinities of ligands established for *CB1* or *CB2* receptors and *GPR55*.

The pharmacology of *GPR55* has provided intricate results (9–13), and a recent article suggests that L- α -lysophosphatidylinositol (LPI) has *GPR55*-independent actions in endothelial cells (14). However, it is generally accepted that LPI is an endogenous ligand of *GPR55* (15–18) because stimulation of this receptor upon LPI treatment evokes an intracellular calcium concentration ($[Ca^{2+}]_i$) rise in several cell types. LPI belongs to the class of lysophospholipids and is generated by phosphatidylinositol hydrolysis via the action of the calcium-dependent phospholipase A2 (19) and calcium-independent phospholipase A1 (20). LPI is involved in numerous physiological actions, including reproduction, angiogenesis, apoptosis, and inflammation, among others (21), which are closely related to adipose tissue biology.

Most studies on the pharmacological properties of *GPR55* have used HEK293 cells transfected with *GPR55*. However, despite its wide distribution (22,23), the physiological function of *GPR55* in vivo remains largely unknown. Recent reports indicate that *GPR55* plays an important role in inflammatory pain (24) and in the regulation of bone physiology by regulating osteoclast number and functions as well as bone turnover in vivo (17), and the LPI/*GPR55* system also has been involved in cancer (12). To our knowledge, there are no available data on the expression of *GPR55* in human tissues or the possible involvement of LPI in energy homeostasis.

Herein, we sought to investigate the potential role of the LPI/*GPR55* system in human adiposity. We demonstrate for the first time that *GPR55* is present in human visceral and subcutaneous adipose tissue (VAT and SAT, respectively), as well as in the liver. It is important that *GPR55* expression in VAT was positively associated with obesity and type 2 diabetes. Consistently, plasma LPI levels were higher in obese patients in comparison with lean subjects. Ex vivo studies using both adipose tissue explants and differentiated primary adipocytes show that LPI increased the expression of genes stimulating fat deposition in VAT explants. Furthermore, in differentiated adipocytes from visceral fat of obese patients, LPI raised $[Ca^{2+}]_i$.

RESEARCH DESIGN AND METHODS

Cohort 1. A total of 95 Caucasian subjects were recruited from healthy volunteers and patients attending the Departments of Endocrinology and Surgery

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at the Clínica Universidad de Navarra. Patients underwent a clinical assessment, including medical history, physical examination, body composition analysis, and comorbidity evaluation. Obesity was classified according to BMI (>30 kg/m²). Body fat was estimated by air-displacement plethysmography (Bod-Pod; Life Measurements, Concord, CA) (25). Obese patients were further subclassified according to three established diagnostic thresholds for diabetes: 1) normoglycemia (NGT), fasting plasma glucose (FPG) <100 mg/dL and plasma glucose (PG) <140 mg/dL 2 h after an oral glucose tolerance test (OGTT); 2) impaired glucose tolerance (IGT), FPG between 100 and 125 mg/dL or PG between 140 and 199 mg/dL 2 h after an OGTT; and 3) type 2 diabetes, FPG >126 mg/dL or PG ≥ 200 mg/dL 2 h after an OGTT (26). Type 2 diabetic subjects were not receiving insulin therapy or medication likely to influence endogenous insulin levels.

The VAT ($n = 68$) and SAT ($n = 59$) samples were collected from patients undergoing either Nissen fundoplication (for hiatus hernia repair in lean volunteers) or Roux-en-Y gastric bypass (for morbid obesity treatment in obese subjects). Tissue samples were immediately frozen and stored at -80°C . Hepatic gene expression levels of *GPR55* were assessed in a subgroup of subjects ($n = 38$), from which 25 of 38 corresponded to the same individuals in whom adipose tissue samples were collected. Although an intraoperative liver biopsy can be performed in obese patients undergoing bariatric surgery, this procedure is not clinically justified in lean subjects. The study was approved, from an ethical and scientific standpoint, by the hospital's ethical committee responsible for research, and written informed consent of participants was obtained.

Cohort 2. VAT samples from 64 consecutive subjects (35 with NGT, 17 with IFG, and 12 with type 2 diabetes) were obtained at the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain). BMI was between 20 and 68 kg/m². Adipose tissue samples were obtained from visceral depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric bypass surgery). All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. Liver and renal diseases were specifically excluded by biochemical workup. All subjects gave written informed consent after the purpose of the study was explained to them. The study was approved, from an ethical and scientific standpoint, by the ethical committee of the Hospital Universitari Dr. Josep Trueta. Adipose tissue samples were washed, fragmented, and immediately flash frozen in liquid nitrogen before storage at -80°C . To perform the isolation of adipocytes and the stromal vascular fraction (SVF), nonfrozen tissues were washed three to four times with phosphate-buffered saline and suspended in an equal volume of phosphate-buffered saline supplemented with 1% penicillin-streptomycin and 0.1% collagenase type I prewarmed to 37°C . The tissue was placed in a shaking water bath at 37°C with continuous agitation for 60 min and centrifuged for 5 min at 300–500g at room temperature. The supernatant, containing mature adipocytes, was recollected. The pellet was identified as the SVF cell.

Blood assays. Plasma samples were obtained by venipuncture, after an overnight fast, from individuals of cohort 1, centrifuged at 1,300g for 10 min, and stored at -80°C . Glucose and insulin, total cholesterol, HDL cholesterol, LDL cholesterol, high-sensitivity C-reactive protein, and leptin were measured as previously described (27–29). Please see Supplementary information for details.

LPI analysis. Plasma samples for the measurement of LPI were obtained from 78 individuals of cohort 1 (Supplementary Table 1). Total LPI was calculated by combining 16:0, 18:0, and 20:4 LPI measurements as previously reported (30). Please see Supplementary information for details.

Ex vivo experiments using SAT and VAT explants. Samples of VAT and SAT were immediately transported to the laboratory (5–10 min), cut into small pieces, and processed as previously described (31). The experiment was performed in five replicates for each adipose tissue sample and treatment. In these experiments, SAT and VAT explants were used. To evaluate cell integrity, lactate dehydrogenase activity released from damaged cells was analyzed by the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions in all treatments. The doses were selected based on previous reports (32,33). Please see Supplementary information for details.

Effect of LPI on $[\text{Ca}^{2+}]_i$ in visceral and subcutaneous adipocytes. Human SVF cells were isolated from VAT and SAT from obese subjects undergoing open abdominal surgery (gastrointestinal bypass) as previously described (34). Please see Supplementary information for details.

RNA extraction and real-time PCR. Adipose tissue and liver RNA isolation was performed as previously described (35). Primers or TaqMan probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript, avoiding genomic DNA amplification (Supplementary Table 2). The cDNA was amplified as previously reported (35). Please see Supplementary information for details.

Western blotting. White adipose tissue (WAT) was homogenized, and protein was extracted as previously reported (36). Please see Supplementary information for details.

Statistical analysis. Statistical analyses were performed using the SPSS 12.0 software statistical package (SPSS, Chicago, IL). Unless otherwise stated,

descriptive results of continuous variables are expressed as mean \pm SE for Gaussian variables. Variables that did not fulfill a normal distribution were logarithmically transformed for subsequent analyses. The relation between variables was analyzed by simple correlation (Pearson χ^2 test) and multiple regression analyses. ANOVA was used to compare NGT, IGT, and type 2 diabetic subjects followed by pairwise post hoc tests. For $[\text{Ca}^{2+}]_i$ measurements, unpaired *t* test was used. Levels of statistical significance were set at $P < 0.05$.

RESULTS

The main anthropometric and biochemical characteristics of the two cohorts are shown in Table 1.

Relative GPR55, CB1, and CB2 mRNA expression in WAT. The gene expression of *GPR55* (Fig. 1A), *CB1* (Fig. 1B), and *CB2* (Fig. 1C) was higher in the VAT of obese patients in comparison with lean subjects from cohort 1. Within obese patients, *GPR55*, *CB1*, and *CB2* mRNA levels were increased in the VAT of subjects with type 2 diabetes when compared with NGT and IGT obese patients (Fig. 1A and C). The expression of the three receptors was also assessed in the SAT, where we failed to detect *CB2* gene expression. The pattern of expression of *GPR55* and *CB1* was quite similar to that observed in VAT. Both *GPR55* (Fig. 1D) and *CB1* (Fig. 1E) were higher in the SAT from obese diabetic patients when compared with obese NGT and IGT patients in cohort 1. Consistent with results obtained in cohort 1, *GPR55* gene expression was increased in the VAT of obese diabetic patients when compared with obese NGT and IGT patients in cohort 2 (Fig. 1F). In accordance with the gene expression data, we found that *GPR55* protein levels were significantly increased in the VAT from obese patients in comparison with lean subjects (Fig. 1G). Similar to that observed for *GPR55* gene expression, the protein levels of this receptor were increased in type 2 diabetic patients when compared with NGT patients (Fig. 1H). In addition, we compared *GPR55* mRNA (Fig. 1I) and protein (Fig. 1J) expression levels between VAT and SAT from the same individuals and found that *GPR55* levels were significantly lower in SAT. Finally, *CB1* mRNA expression was also higher in VAT when compared with SAT (Fig. 1K).

In a multivariate linear regression analysis, BMI emerged as a significant predictor of *GPR55* gene expression, except in men from cohort 2 (Supplementary Table 3). The higher expression of *GPR55* in obese patients in comparison with lean subjects from cohort 1 was in agreement with a positive correlation between VAT *GPR55* levels and body weight (Fig. 2A), BMI (Fig. 2B), circulating LPI levels (Fig. 2C), and percent fat mass (Supplementary Table 4). Given that some groups were not exactly matched for sex, an analysis was performed to verify whether the observed differences were due to sex. A correlation was constructed considering only men or women, and the results showed that VAT *GPR55* expression exhibited significantly higher levels with increasing BMI in both sexes (Supplementary Table 4). On the contrary, we did not find a significant correlation between SAT *GPR55* mRNA levels with any of the variables studied (Supplementary Table 4). Contrary to *GPR55*, VAT *CB1* expression was not correlated to BMI (Supplementary Table 5), although SAT *CB1* expression showed such a correlation, but only in women (Supplementary Table 5).

Similar to cohort 1, we also found that VAT *GPR55* mRNA levels were positively associated with body weight (Fig. 2D), BMI (Fig. 2E), and percent fat mass (Supplementary Table 6) in the subjects of cohort 2. Thus, *GPR55* gene expression levels were increased in the VAT of obese patients in both cohorts, and the correlation of *GPR55* gene expression with weight, BMI, and fat mass was especially

TABLE 1
Anthropometric and analytical characteristics of subjects in cohorts 1 and 2

	Subjects				P
	Lean	Obese NGT	Obese IGT	Obese T2DM	
Cohort 1					
<i>n</i> (men/women)	5 (2/3)	34 (13/21)	27 (14/13)	29 (13/16)	
Age (years)	38 ± 4	38 ± 2	42 ± 2	47 ± 2††	<0.001
BMI (kg/m ²)	21.3 ± 1.1	42.7 ± 1.1**	46.0 ± 1.2**	47.6 ± 1.8**	<0.001
Body fat (%)	26.5 ± 2.8	48.3 ± 1.6**	53.3 ± 1.0**	50.6 ± 1.5**	<0.001
Fasting glucose (mg/dL)	86.8 ± 3.5	89.0 ± 1.7	105.2 ± 2.2	139.9 ± 10.9***,††,##	<0.001
Fasting insulin (μU/mL)	8.2 ± 1.7	17.4 ± 1.6*	18.0 ± 2.3*	20.2 ± 2.6*	0.012
Triglycerides (mg/dL)	68 ± 11	103 ± 8	139 ± 24	191 ± 42	0.066
Cholesterol (mg/dL)	192 ± 10	181 ± 8	208 ± 8	205 ± 8	0.066
LDL cholesterol (mg/dL)	118 ± 10	119 ± 6	130 ± 7	133 ± 8	0.443
HDL cholesterol (mg/dL)	58 ± 2	41 ± 2*	49 ± 2†	42 ± 2*	<0.001
Leptin (ng/mL)	7.8 ± 1.5	48.0 ± 5.4*	60.8 ± 6.4**	61.9 ± 8.5**	0.003
hsCRP (mg/L)	1.8 ± 0.3	6.6 ± 1.3	12.8 ± 2.3*,†	9.0 ± 1.5	0.011
AUC glucose		15.7 ± 0.6	20.3 ± 0.6†††	26.2 ± 1.5†††	0.001
AUC insulin		13.0 ± 1.4	11.8 ± 1.2	7.6 ± 1.3	0.055
2-h glucose (OGTT)		108.5 ± 2.6	168.2 ± 3.7†††	247.7 ± 24.1†††	<0.001
HOMA-IR	1.7 ± 0.3	4.8 ± 0.7	4.7 ± 0.6	7.1 ± 1.3†	<0.05
Cohort 2					
<i>n</i> (men/women)		35 (14/21)	17 (5/12)	12 (4/8)	
Age (years)		48.4 ± 11.6	47 ± 14.2	44 ± 11.1	0.7
BMI (kg/m ²)		39.0 ± 1.6	40.2 ± 1.9	41.2 ± 1.6	0.7
Body fat (%)		40.2 ± 1.02	50.3 ± 1.5	52.1 ± 2.5	0.4
Fasting glucose (mg/dL)		86.6 ± 1.3	110.2 ± 2.1	213.1 ± 19.8	<0.001
Triglycerides (mg/dL)		114.7 ± 15.4	149 ± 17.2	200.7 ± 45.2	0.049
HDL cholesterol (mg/dL)		56.9 ± 3.8	54.2 ± 4.2	47.2 ± 3.5	0.28
LDL cholesterol (mg/dL)		106.1 ± 34.1	114 ± 8.8	124.9 ± 15.1	0.4
Fasting insulin (μU/mL)		12.3 ± 3.1	29.1 ± 4.7	30.2 ± 7.6	0.2§
HOMA-IR		2.6 ± 0.7	8.2 ± 1.2	9.7 ± 1.3	0.09§

Data are mean ± SEM. Differences between groups were analyzed by one-way ANOVA followed by Tukey post hoc tests. T2DM, type 2 diabetes mellitus; hsCRP, high-sensitivity C-reactive protein; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance. **P* < 0.05 vs. lean. ***P* < 0.01 vs. lean. †*P* < 0.05 vs. obese NGT. ††*P* < 0.01 vs. obese NGT. †††*P* < 0.001 vs. obese NGT. ##*P* < 0.01 vs. obese IGT. §These values are representative of only 13 subjects rather than the 64 subjects used for the other parameters.

significant in women in both cohorts (Supplementary Tables 4 and 7). Therefore, we can conclude that *GPR55* gene expression is specifically increased in VAT, but not SAT, of obese subjects.

Relative *GPR55* mRNA expression in adipocytes versus the SVF. We then studied which fraction of the adipose tissue accounted for this increased expression. To address this issue, we used human visceral fat and subcutaneous fat and separated the adipocyte fraction from the SVF. In visceral fat, adipogenic genes (*FASN*, *ACC*, and *SREBP*) were significantly increased in the adipocyte fraction (Supplementary Fig. 1A–C), whereas *CD14* (monocyte marker) was significantly increased in the SVF (Supplementary Fig. 1D). The *GPR55* gene was similarly expressed in both the adipocyte fraction and the SVF (Supplementary Fig. 1E). Similar results were obtained in SAT (data not shown).

Circulating LPI levels in lean and obese subjects. Next, we assessed plasma LPI levels, the only known endogenous ligand of *GPR55*, in lean and obese subjects from cohort 1. We found that total LPI was significantly increased in obese patients in comparison with lean subjects (Fig. 3A). Similar to plasma total LPI levels, we found that all three individual LPI species measured—16:0 LPI (Fig. 3B), 18:0 LPI (Fig. 3C), and 20:4 LPI (Fig. 3D)—also were increased in the three groups of obese patients when compared with lean volunteers, with the exception of 18:0 LPI in type 2 diabetic patients.

Plasma LPI levels were not correlated with body weight or BMI when men and women were analyzed together (data not shown). However, when a correlation was constructed considering only men or women, the results showed that plasma LPI exhibited significantly higher levels with increasing body weight (Fig. 3E), BMI (Fig. 3F), and fat percentage (Fig. 3G) in women but not men (Supplementary Table 7). Finally, we also found that plasma LPI correlates with LDL levels in women but not men (Supplementary Table 7). It is important that circulating levels of total LPI (Fig. 3H), 16:0 LPI (Fig. 3I), 18:0 LPI (Fig. 3J), and 20:4 LPI (Fig. 3K) were not significantly different between men and women.

Effects of LPI on human adipose tissue explants. After demonstrating that VAT *GPR55* and plasma LPI correlate with obesity, we then assessed the direct effects of LPI on explants obtained from SAT and VAT. LPI (1 and 10 μmol/L) increased the expression of genes promoting the synthesis of fatty acids, including fatty acid synthase (Fig. 4A) and acetyl CoA carboxylase (Fig. 4B), in explants from VAT. Both doses of LPI also triggered the expression of peroxisome proliferator-activated receptor γ (PPARγ) (Fig. 4C), which plays an important role in adipocyte differentiation. Other adipokines, such as leptin (Fig. 4D) and adiponectin (Fig. 4E), tended to show a slight increase in their expression, but the differences were not statistically significant. The expression of *GPR55* was significantly upregulated by LPI at both doses (Fig. 4F) in adipocytes from VAT. In contrast to the findings obtained in VAT, LPI did

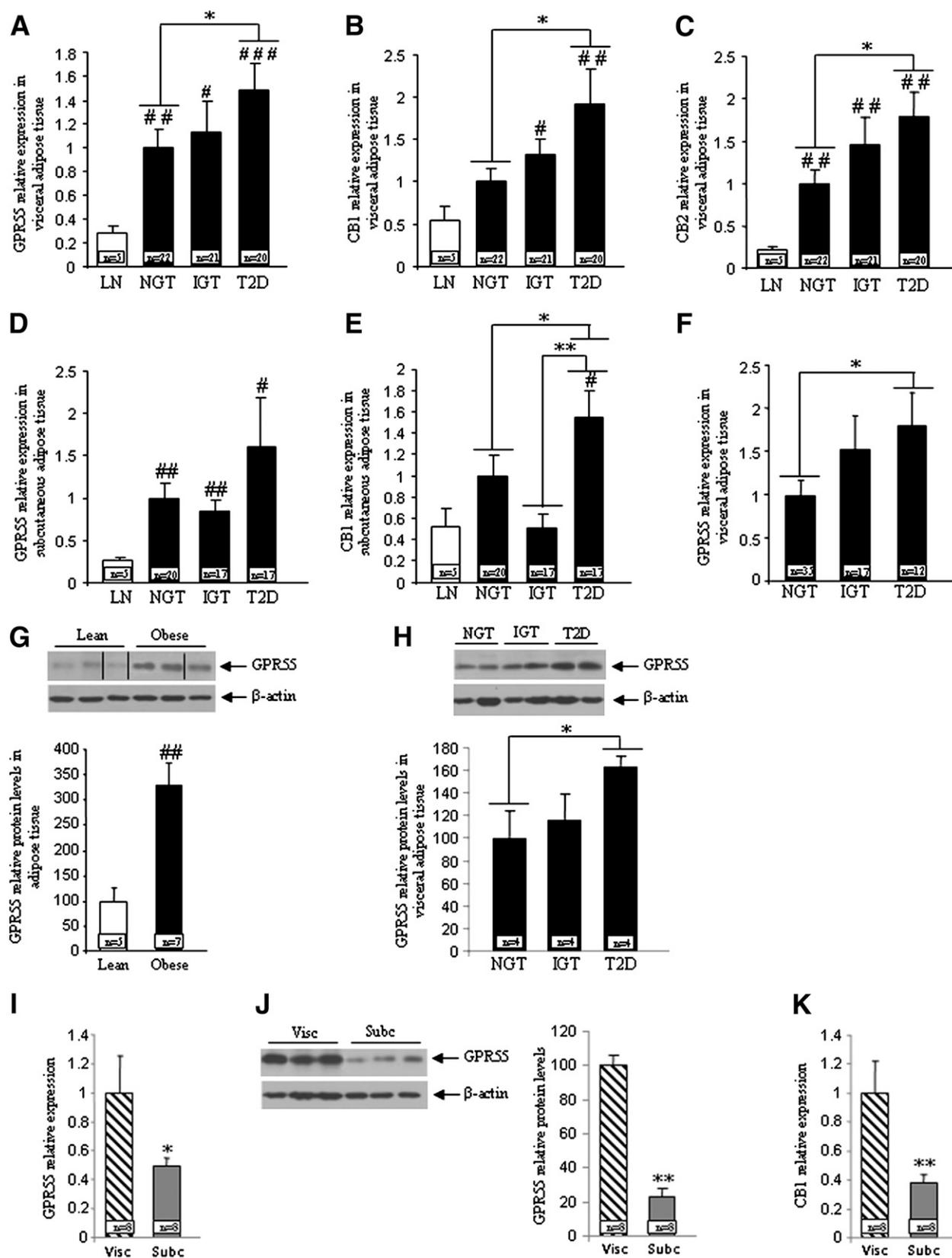


FIG. 1. mRNA expression of GPR55 (A), CB1 (B), and CB2 (C) in VAT obtained from lean and obese subjects in cohort 1. mRNA expression of GPR55 (D) and CB1 (E) in SAT obtained from lean and obese subjects in cohort 1. mRNA expression of GPR55 (F) in VAT obtained from lean and obese subjects in cohort 2. Protein levels of GPR55 (G) in VAT obtained from lean and obese subjects in cohort 2. Dividing lines indicate splittings in the figure. Levels of GPR55 in the three different groups of obese patients (H). mRNA expression of GPR55 in the visceral and subcutaneous fat from the same obese patients (I). Protein levels of GPR55 in the visceral and subcutaneous fat from the same obese patients (J). Obese subjects were subclassified as NGT, IGT, and type 2 diabetic. The relative amounts of mRNA were normalized to the value of NGT. T2D, type 2 diabetes; Visc, visceral; Subc, subcutaneous. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. lean subjects; * $P < 0.05$, ** $P < 0.01$ vs. NGT.

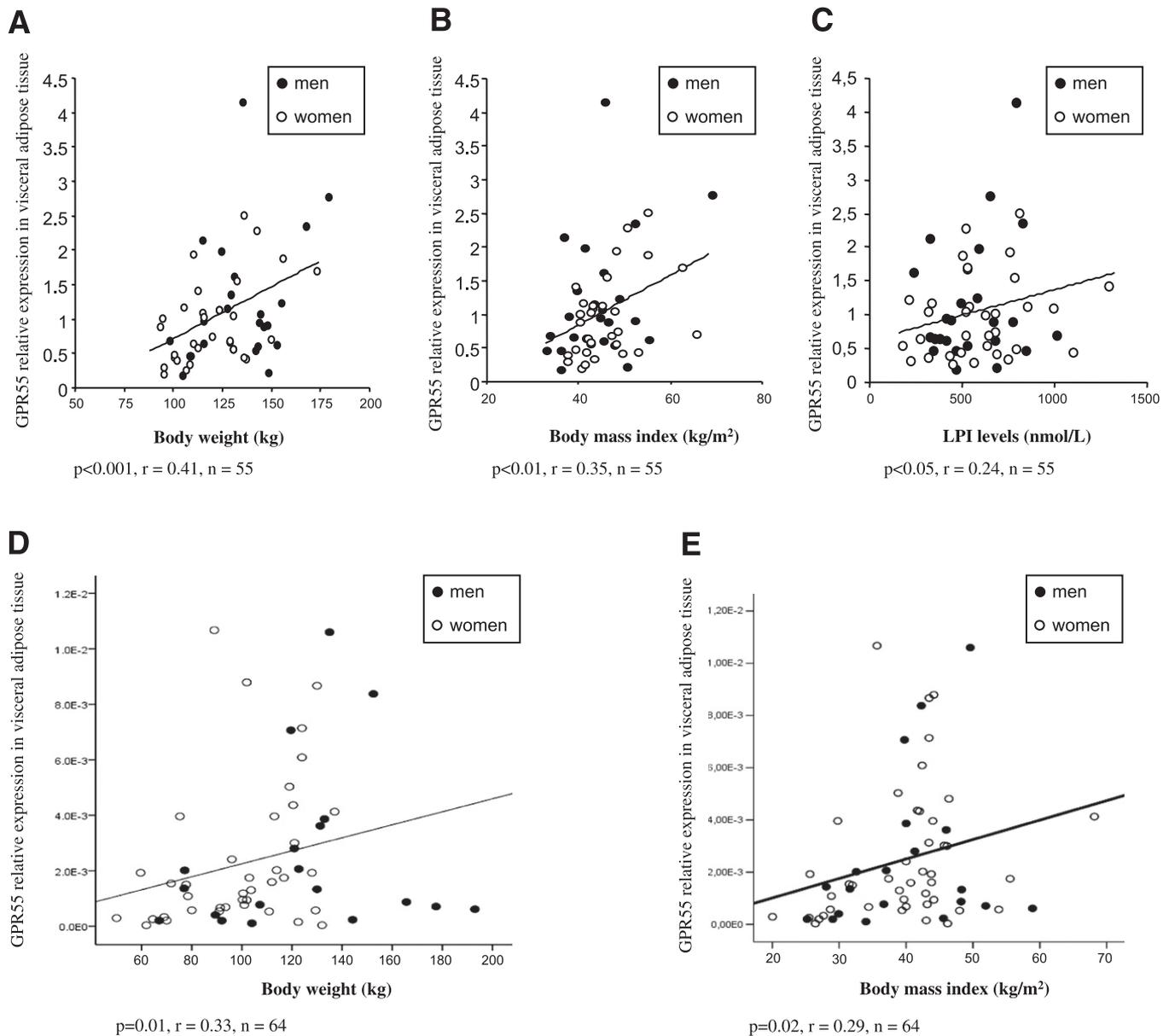


FIG. 2. Correlation between VAT *GPR55* and body weight (A), BMI (B), and circulating LPI (C) in individuals from cohort 1. For cohort 1, we performed the correlations using only obese individuals, and the cutoff value as a diagnosis of obesity was BMI > 30 kg/m². Correlation between VAT *GPR55* and body weight (D) and BMI (E) in individuals from cohort 2.

not modify the expression of any of the studied genes in explants obtained from SAT (Fig. 4G–L).

Effects of LPI on $[Ca^{2+}]_i$ in cultured differentiated human adipocytes. LPI has previously been shown to increase $[Ca^{2+}]_i$ in HEK293 cells expressing GPR55 (15,16) as well as in rat pheochromocytoma PC12 cells (37). Given that increases in $[Ca^{2+}]_i$ have been associated with lipogenesis in adipocytes (38), we next analyzed whether LPI was capable of enhancing $[Ca^{2+}]_i$ in cultured differentiated adipocytes obtained from SVF of VAT and SAT of obese patients. For this purpose, after a 9-day differentiation period, VAT and SAT adipocytes were loaded with the calcium sensitive probe Fura 2-AM (Sigma Aldrich, St. Louis, MO), and $[Ca^{2+}]_i$ was monitored over time (8–10 min) in the absence and presence of LPI. We found that exposure of cells to 5 μ mol/L LPI induced a substantial rise in $[Ca^{2+}]_i$ in 48.4% of differentiated VAT adipocytes (46 of 95 cells; $n = 3$

independent experiments) and in 24.4% of differentiated SAT cells (29 of 119 cells; $n = 3$ independent experiments) (Fig. 5A and C, respectively). In terms of response intensity, differentiated VAT adipocytes treated with 5 μ mol/L LPI exhibited an increase in $[Ca^{2+}]_i$ significantly higher than that observed in differentiated SAT adipocytes ($43.18 \pm 2.77\%$ vs. $19.69 \pm 1.93\%$ above basal levels in VAT and SAT, respectively; $P < 0.001$) (Fig. 5B and D, respectively).

Relative GPR55 mRNA expression in the liver. In addition to its expression in adipose tissue, we also found that *GPR55* is expressed in the liver. No significant differences in hepatic *GPR55* gene expression levels between NGT, IGT, and type 2 diabetic obese patients were observed (Supplementary Fig. 2).

Effect of LPI on adipocyte differentiation of 3T3-L1 cells. When 3T3-L1 cells were treated with LPI (1 and 10 μ mol/L) during 10 days, we found no alteration in the Oil

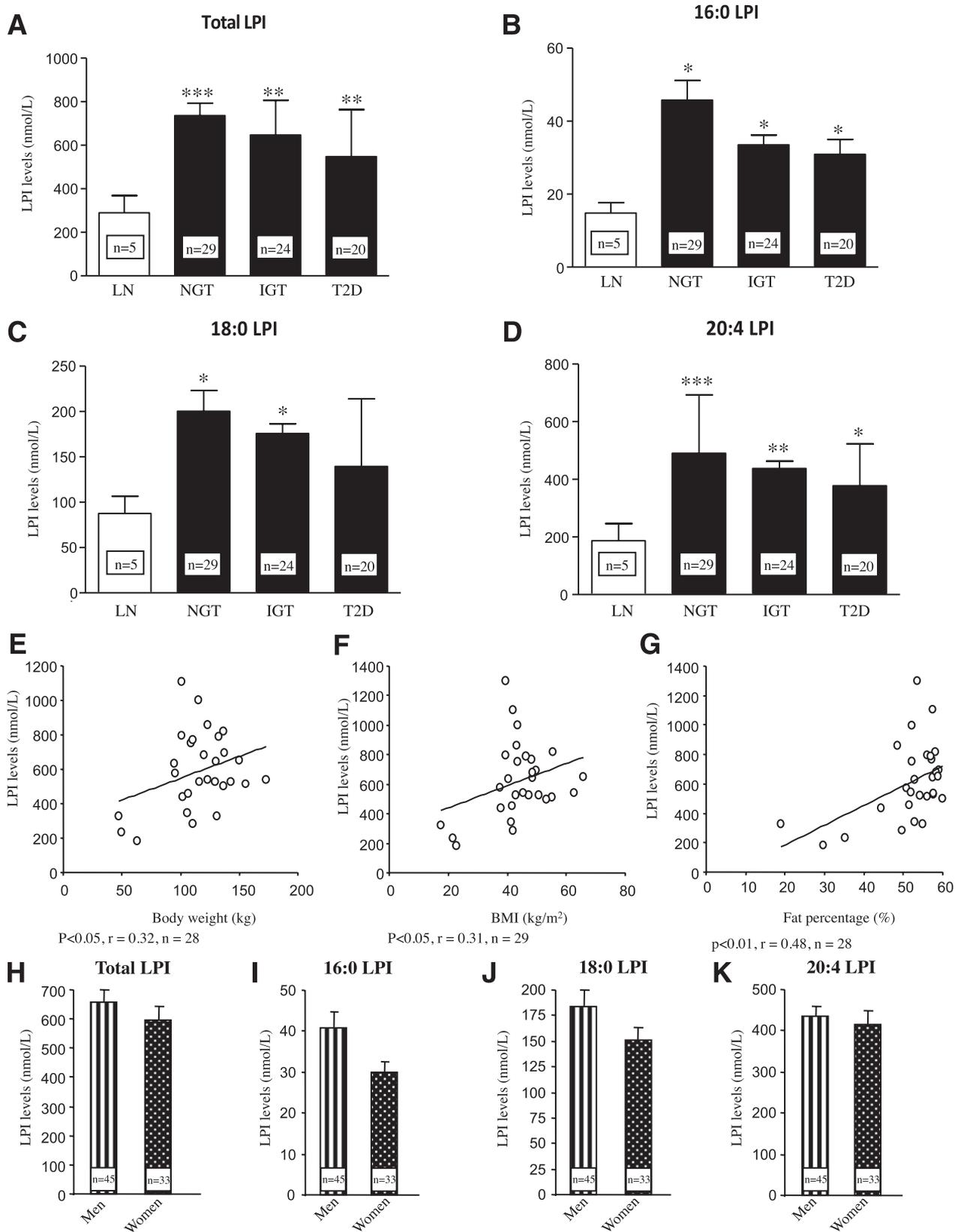


FIG. 3. Circulating levels of total LPI (A), 16:0 LPI (B), 18:0 LPI (C), and 20:4 LPI (D) in the plasma obtained from lean and obese subjects in cohort 1. Correlation between circulating levels (plasma obtained from a subset of individuals from cohort 1) of total LPI and body weight (E), BMI (F), and fat percentage (G) in women. Circulating levels of total LPI (H), 16:0 LPI (I), 18:0 LPI (J), and 20:4 LPI (K) in the plasma obtained from men and women in cohort 1. LN, lean; T2D, type 2 diabetes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

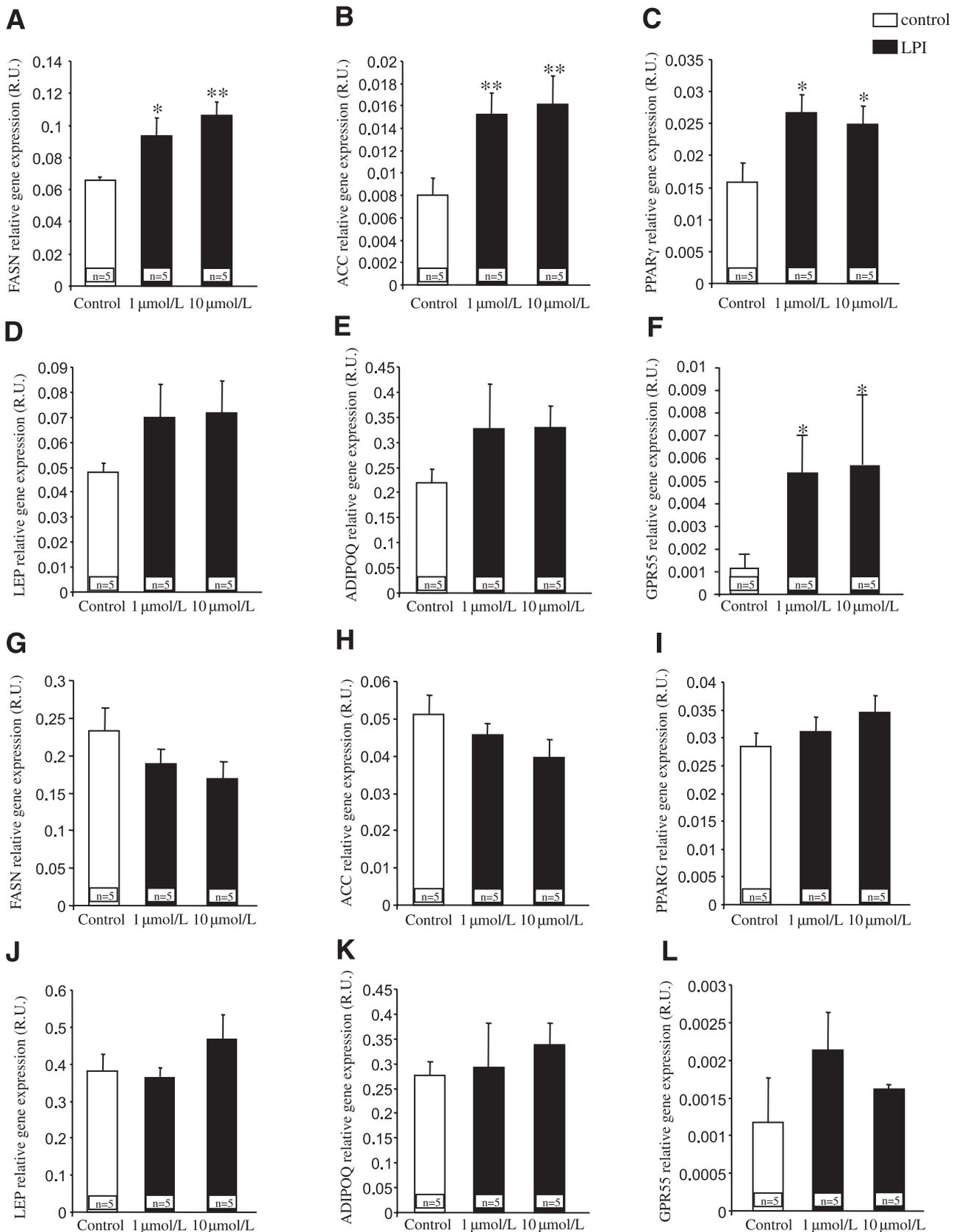


FIG. 4. Ex vivo effects of LPI (1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$) on fatty acid synthase (FASN) (A), acetyl CoA carboxylase (ACC) (B), PPAR γ (C), leptin (LEP) (D), adiponectin (ADIPOQ) (E), and GPR55 (F) in VAT explants. Ex vivo effects of LPI on FASN (G), ACC (H), PPARG (I), LEP (J), ADIPOQ (K), and GPR55 (L) in SAT explants. R.U., relative units. * $P < 0.05$, ** $P < 0.01$.

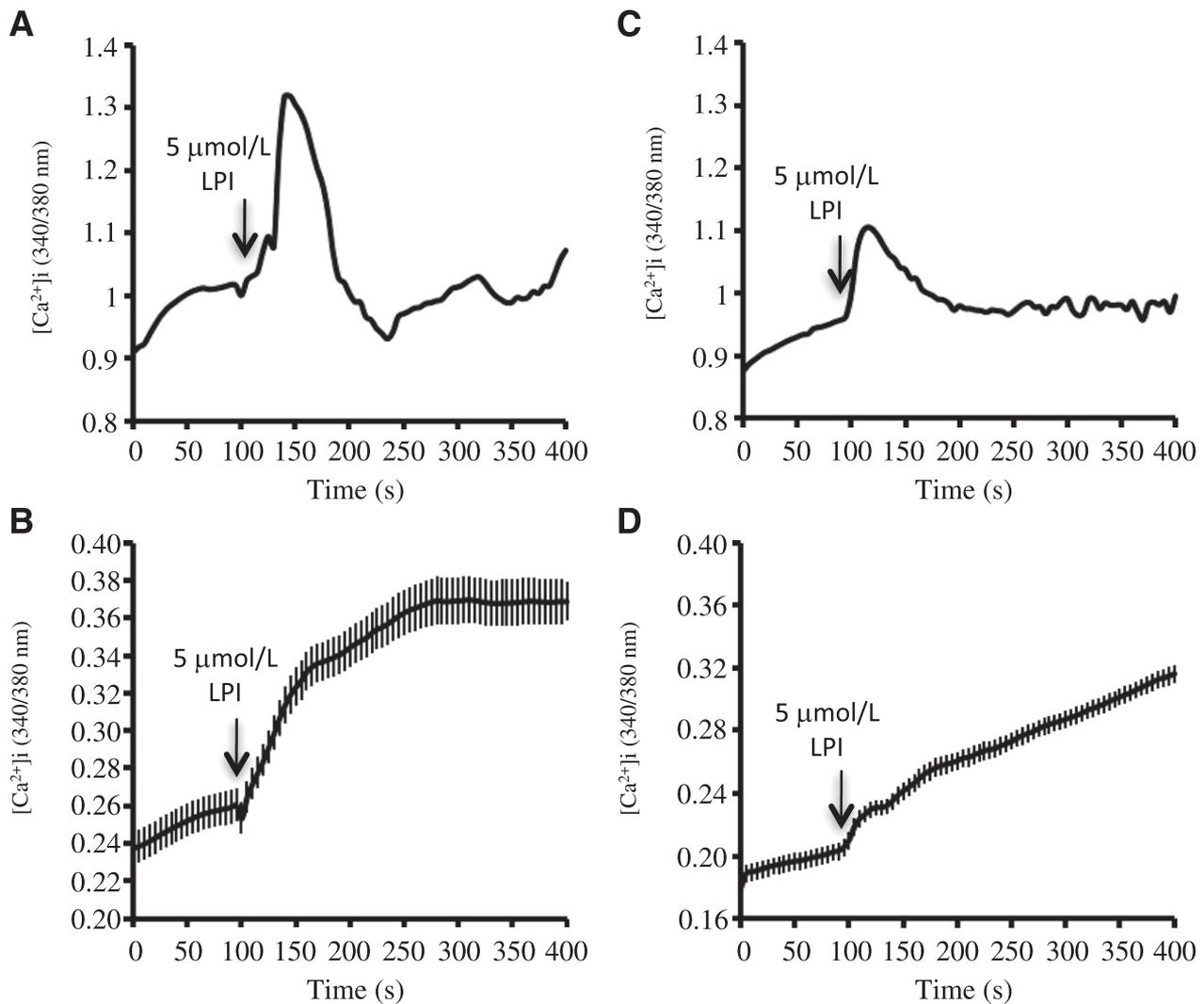


FIG. 5. Representative profiles of the effects of LPI (5 $\mu\text{mol/L}$) on $[\text{Ca}^{2+}]_i$ in cultured differentiated human adipocytes obtained from visceral (A) and subcutaneous (C) fat. Arrows indicate the time of addition of LPI. A Fura-2 dual-wavelength fluorescence imaging system was used to measure $[\text{Ca}^{2+}]_i$, as described in RESEARCH DESIGN AND METHODS. Quantitation of $[\text{Ca}^{2+}]_i$ dynamics in differentiated adipocytes of VAT (B) and SAT (D) responsive to LPI (46 of 95 cells and 29 of 119 cells for VAT and SAT, respectively). Three independent experiments were conducted for both SAT and VAT adipocytes.

Red O staining in comparison with control cells (Supplementary Fig. 3). Thus, these results indicate that LPI may act differently in human and rodent adipocytes.

Relative *GPR55* levels in WAT of obese animals. Mice lacking leptin (Supplementary Fig. 4A) and rats fed a high-fat diet (45% by energy) (Supplementary Fig. 4B) showed significantly decreased *GPR55* mRNA levels in WAT when compared with lean animals. Accordingly, *GPR55* protein levels were also decreased in both obese models (Supplementary Figs. 4C and D). Therefore, these findings suggest that *GPR55* is differentially regulated in humans and rodents.

DISCUSSION

The endocannabinoid system is overactive in WAT of obese patients, thereby contributing to excessive visceral fat accumulation and obesity-associated complications (4,6,39). *GPR55* has been described as a putative receptor for atypical cannabinoids (9,40,41). However, its clinical implications remain largely unknown. Herein, we demonstrate that

GPR55 is expressed in human SAT, VAT, and liver. Our findings, obtained in two independent cohorts, indicate that *GPR55* mRNA levels are increased in VAT and SAT of obese subjects in comparison with lean volunteers. Moreover, VAT but not SAT *GPR55* expression is positively associated with type 2 diabetes. Of interest, correlations between VAT *GPR55* gene levels and weight, BMI, and percent body fat are stronger in women than in men. Consistently, plasma LPI levels are also increased in obese patients and positively correlated with weight, BMI, and percent body fat in women. Finally, we show that in explants obtained from VAT, LPI triggers the mRNA levels of genes promoting lipogenesis, and in cultured differentiated adipocytes obtained from VAT, LPI raises $[\text{Ca}^{2+}]_i$. However, we found that LPI evoked minor responses in adipose tissue explants or in cultured differentiated adipocytes obtained from SAT, suggesting that the LPI/*GPR55* system is particularly important in VAT.

In rodents, *GPR55* mRNA has been detected throughout the central nervous system (22) and in peripheral tissues (23). Our study is the first to show that *GPR55* mRNA is

expressed in human tissues, and its regulation is tissue specific because *GPR55* expression in WAT, but not liver, is particularly upregulated in diabetic patients when compared with NGT obese individuals. Specifically, *GPR55* is found at similar concentrations in both adipocytes and the SVF of fat tissue, indicating that this receptor is also present in other cell types in the SVF, such as mononuclear cells (monocytes, macrophages, and lymphocytes), among others. The current findings are consistent with previous reports demonstrating that *GPR55* is located in human monocytes (17). In obese states, the SVF is largely infiltrated by mononuclear cells in comparison with normal conditions.

Although endocannabinoid levels are increased in obesity (39), data on *CB1* gene expression in WAT is controversial because reduced (39), increased (42), or unchanged (43) expression between obese and lean subjects has been reported. In the current work, we detected higher *CB1* mRNA levels in the adipose tissue of obese patients. Although the discrepancies for those differences are unknown, a plausible explanation might be the different populations used by the studies. Similar to *CB1*, *CB2* and *GPR55* mRNA levels in the VAT of obese subjects were also increased, indicating the lack of a negative feedback loop between circulating endocannabinoids and these three cannabinoid receptors. The similar pattern of expression of the three receptors in obese and diabetic patients also suggests that some of their metabolic functions might be overlapped or compensated. This is an important issue because specific drugs for each receptor also might act through the other two receptors when the drug is administered at high doses or during long-term treatments. As a matter of fact, a recent pharmacological study suggests that low concentrations of rimonabant specifically block *CB1* receptors, but higher doses or long-term treatment also could be targeting *GPR55* (44). Other reports carried out in HEK cells show that the endocannabinoids anandamide and 2-arachidonoylglycerol also have a low affinity by *GPR55* (16,23,45). Thus, *GPR55* activation could be contributing to the biological activity in situations wherein endocannabinoid levels are markedly increased.

Despite activation of *GPR55* by cannabinoids, it is well accepted that LPI is its more potent endogenous ligand known up to date (15–18). Plasma LPI levels previously have been found to be increased in patients with ovarian cancer, thereby considering LPI as a biomarker for this disease (30,46,47). However, to our knowledge, no reports have studied the interaction between LPI levels and metabolism. Similar to *GPR55* expression, we found that plasma LPI is increased in obesity, suggesting that the LPI/*GPR55* system is overactive in obese states. It is well known that visceral fat accumulation represents a key pathophysiologic mechanism for the clustering of metabolic abnormalities, including insulin resistance and type 2 diabetes (48). We found that within obese patients, *GPR55* gene levels in VAT and SAT are significantly increased in diabetic subjects, suggesting that this receptor may play a pivotal role in the development of insulin resistance and the pathogenesis of type 2 diabetes. *GPR55* gene expression in VAT, but not SAT, was positively correlated with several anthropometric parameters, such as body weight and BMI, in the two cohorts examined. It is interesting that when men and women are analyzed separately, VAT *GPR55* shows a stronger correlation with weight, BMI, and fat percentage in women. Marked sex differences have been reported with regard to degrees of insulin resistance, body composition, and energy balance (49). Our results

showing that VAT *GPR55* mRNA expression is correlated with weight, BMI, and percentage body fat in women suggest that estrogens might be relevant to the *GPR55* signaling pathway. Of import, the correlation of plasma LPI levels to weight, BMI, and percentage body fat is almost identical to that of VAT *GPR55* because those correlations were positive in women but not men. This sexual dimorphism in gene expression and circulating levels has been observed previously in other hormones with important metabolic implications, such as leptin gene expression (50) or circulating leptin concentrations (51).

Because both LPI and VAT *GPR55* showed a clear upregulation in obese patients and were correlated with important metabolic parameters, we next sought to investigate the direct actions of this system on adipose tissue explants and isolated adipocytes. Our findings indicate that in explants obtained from VAT, LPI induced lipid storage by stimulating lipogenic genes and promoted adipocyte differentiation by increasing PPAR γ expression. Although *GPR55* was also present in SAT, the treatment of explants from SAT with LPI did not modify the expression of any of the genes studied. Consistent with the gene expression studies and the lipogenic action of $[Ca^{2+}]_i$ in adipocytes (38), LPI elicited a significantly higher effect in $[Ca^{2+}]_i$ in cultured differentiated adipocytes from VAT than in those from SAT, both in terms of percentage of responsive cells and the magnitude of the $[Ca^{2+}]_i$ increase. Indeed, although the doses of LPI tested herein are similar to others used in previous works (32,33), they are higher than plasma concentrations observed in obese patients, but overall, these results indicate that LPI directly favors a condition of lipid deposition within adipocytes from VAT. Given the specific correlations between LPI/*GPR55* and several metabolic parameters in women, further studies will be necessary to address if estrogens can interfere in the biological actions of LPI. In this sense, it is important to point out that body fat is differentially distributed in men and women, with men exhibiting more visceral fat than women, in whom subcutaneous fat predominates (49). The different distribution of fat between both sexes might have clear implications in the metabolic actions of the LPI/*GPR55* system. It is also important to point out that LPI is esterified by the enzyme 1-acylglycerol-3-phosphate-*O*-acyltransferase 3 (AGPAT3), which is ubiquitously expressed in human tissues (52). Thus, it is tempting to speculate that the tissue-specific actions of LPI might be explained by the distinct grade of activity of this enzyme in those fat depots. It is also important to point out that the role of the LPI/*GPR55* system in obesity appears to be species dependent because LPI did not modify adipogenicity in 3T3-L1 cells and *GPR55* mRNA, and protein expression is decreased in obese rodent models compared with their lean controls. Further studies in rodents assessing LPI levels in different pathophysiological models are merited to precisely clarify this issue.

In addition to adipose tissue, we also detected the expression of *GPR55* in the liver. However, we failed to detect significant changes in expression between the various subgroups of obese patients. Although further studies should elucidate the expression of this receptor in other tissues with metabolic functions, including the gastrointestinal tract, skeletal muscle, or pancreas, our current findings suggest that VAT *GPR55* may be the most important one regarding energy homeostasis.

In summary, our findings obtained in two independent cohorts demonstrate that 1) *GPR55* levels are increased in

both VAT and SAT of obese subjects; 2) VAT *GPR55* is positively correlated with weight, BMI, and percentage body fat, particularly in women; 3) hepatic *GPR55* gene expression remains unchanged in obese and diabetic subjects; 4) circulating LPI levels are increased in obesity and correlated with weight, BMI, and fat percentage in women; and 5) LPI increases [Ca^{2+}]_i and the expression of lipogenic enzymes specifically in differentiated adipocytes from VAT. This work suggests that the LPI/*GPR55* system is a new metabolic pathway of clinical relevance. Further studies are required to investigate the potential role of agonists/antagonists for *GPR55* in obesity and insulin sensitivity.

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