

## Decreased *STAMP2* Expression in Association with Visceral Adipose Tissue Dysfunction

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**Context:** Six-transmembrane protein of prostate 2 (*STAMP2*) is a counter-regulator of inflammation and insulin resistance according to findings in mice. However, there have been contradictory reports in humans.

**Objective:** We aimed to explore *STAMP2* in association with inflammatory and metabolic status of human obesity.

**Design, Patients, and Methods:** *STAMP2* gene expression was analyzed in adipose tissue samples (171 visceral and 67 sc depots) and during human preadipocyte differentiation. Human adipocytes were treated with macrophage-conditioned medium, TNF- $\alpha$ , and rosiglitazone.

**Results:** In visceral adipose tissue, *STAMP2* gene expression was significantly decreased in obese subjects, mainly in obese subjects with type 2 diabetes. *STAMP2* gene expression and protein were significantly and inversely associated with obesity phenotype measures (body mass index, waist, hip, and fat mass) and obesity-associated metabolic disturbances (systolic blood pressure and fasting glucose). In addition, *STAMP2* gene expression was positively associated with lipogenic (*FASN*, *ACC1*, *SREBP1*, *THRSP14*, *TR $\alpha$* , and *TR $\alpha$ 1*), *CAV1*, *IRS1*, *GLUT4*, and *CD206* gene expression. In sc adipose tissue, *STAMP2* gene expression was not associated with metabolic parameters. In both fat depots, *STAMP2* gene expression in stromovascular cells was significantly higher than in mature adipocytes. *STAMP2* gene expression was significantly increased during the differentiation process in parallel to adipogenic genes, being increased in preadipocytes derived from lean subjects. Macrophage-conditioned medium (25%) and TNF- $\alpha$  (100 ng/ml) administration increased whereas rosiglitazone (2  $\mu$ M) decreased significantly *STAMP2* gene expression in human differentiated adipocytes.

**Conclusions:** Decreased *STAMP2* expression (mRNA and protein) might reflect visceral adipose dysfunction in subjects with obesity and type 2 diabetes. (*J Clin Endocrinol Metab* 96: 0000–0000, 2011)

Various pathways and factors in adipose tissue such as increased release of free fatty acids and adipokines, and a proinflammatory state, have been proposed to contribute to obesity-associated insulin resistance (1,

2). Six-transmembrane protein of prostate 2 (*STAMP2*; also called TIARP and STEAP4) has been characterized as a counter-regulator of inflammation and insulin resistance in mice (3). *STAMP2* mRNA has been shown to

ISSN Print 0021-972X ISSN Online 1945-7197  
Printed in U.S.A.

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doi: 10.1210/jc.2011-0310 Received February 3, 2011. Accepted July 27, 2011.

Abbreviations: BMI, Body mass index; Ct, threshold cycle; FBS, fetal bovine serum; HRP, horseradish peroxidase; LPS, lipopolysaccharide; MCM, macrophage-conditioned medium; p, phospho-; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; *STAMP2*, six-transmembrane protein of prostate 2; SVC, stromovascular cell; SVF, stromal vascular fraction.

be highly induced by TNF- $\alpha$  exposure (4). STAMP2<sup>-/-</sup> mice displayed an insulin-resistant phenotype with elevated plasma glucose, insulin, and lipids, elevated expression of proinflammatory mediators in white adipose tissue, impairment of insulin-stimulated glucose transport in adipocytes, and reduced adiponectin expression (3). These data suggest that STAMP2 expression may be synthesized as a protective antiinflammatory factor and that enhancing STAMP2 signaling potentially could be a target for human insulin resistance. In this sense, two recent *in vitro* studies in human preadipocytes have shown that STAMP2 contributed to insulin sensitivity in mature human adipocytes, increasing insulin-stimulated glucose uptake (5, 6).

In humans, the *STAMP2* gene is highly expressed in adipose tissue (7). Recently, two studies have reported opposite findings. Arner *et al.* (8) showed that *STAMP2* gene expression was increased in obese subjects in both sc and visceral adipose tissue in five heterogeneous cohorts [sc adipose tissue was studied in three cohorts (in 15 nonobese and 81 obese women, in seven nonobese and seven obese men and women, and in 13 obese men and women), and visceral adipose tissue was studied in two other cohorts (in 24 nonobese and 69 obese men and women and in 20 obese men and women)]. In this last cohort, paired visceral and sc adipose tissue samples was used to compare *STAMP2* mRNA and protein between sc and visceral adipose tissue. On the other hand, decreased *STAMP2* gene expression and protein in visceral adipose tissue has also been described in obese subjects (in a study involving only 12 subjects) (7).

Here, we aimed to investigate *STAMP2* gene expression in both visceral and sc adipose tissue, in adipose tissue fractions and during human adipocyte differentiation according to obesity status. In addition, we also explored *in vitro* the effects of inflammatory agents (TNF- $\alpha$  and macrophage conditioned medium) and a peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonist (rosiglitazone) on *STAMP2* gene expression in human adipocytes.

## Materials and Methods

### STAMP2 expression in adipose tissue stromal vascular fraction (SVF) and in isolated adipocytes

A group of 238 adipose tissue samples (171 visceral and 67 sc depots), from participants, who were recruited at the Endocrinology Department of the Hospital Virgen de la Victoria (Malaga, Spain) and at the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain), were analyzed. 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 biochem-

ical work-up. All subjects gave written informed consent after the purpose of the study was explained to them.

Adipose tissue samples were obtained from sc and visceral depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric by-pass surgery). Both sc and visceral fat were obtained from the abdomen, following standard procedures. To analyze adipose tissue gene expression, tissues were washed, fragmented, and immediately flash-frozen in liquid nitrogen before being stored at  $-80^{\circ}\text{C}$ . To perform the isolation of adipocyte and SVF, tissues were washed three to four times with PBS and suspended in an equal volume of PBS supplemented with 1% penicillin/streptomycin and 0.1% collagenase type I prewarmed to  $37^{\circ}\text{C}$ . The tissue was placed in a shaking water bath at  $37^{\circ}\text{C}$  with continuous agitation for 60 min and centrifuged for 5 min at  $300\text{--}500 \times g$  at room temperature. The supernatant, containing mature adipocytes, was recollected. The pellet was identified as the SVF cell. The adipose tissue fractionation was performed from eight visceral and eight sc depots.

### STAMP2 expression during human preadipocyte differentiation

Isolated preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were plated on T-75 cell culture flasks and cultured at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in DMEM/nutrient mix F-12 medium (1:1, vol/vol) supplemented with 10 U/ml penicillin/streptomycin, 10% fetal bovine serum (FBS), 1% HEPES, and 1% glutamine (all from GIBCO, Invitrogen S.A, Barcelona, Spain). One week later, the isolated and expanded human visceral and sc preadipocytes were cultured ( $\sim 40,000$  cells/ $\text{cm}^2$ ) in 12-well plates with preadipocytes medium (Zen-Bio) composed of DMEM/nutrient mix F-12 medium (1:1, vol/vol), HEPES, FBS, penicillin, and streptomycin in a humidified  $37^{\circ}\text{C}$  incubator with 5%  $\text{CO}_2$ . Twenty-four hours after plating, cells were checked for complete confluence (d 0), and differentiation was induced using differentiation medium (Zen-Bio) composed of preadipocytes medium, human insulin, dexamethasone, isobutylmethylxanthine, and PPAR $\gamma$  agonists (rosiglitazone). After 7 d (d 7), differentiation medium was replaced with fresh adipocyte medium (Zen-Bio) composed of DMEM/nutrient mix F-12 medium (1:1, vol/vol), HEPES, FBS, biotin, panthothenate, human insulin, dexamethasone, penicillin, streptomycin, and amphotericin. Negative control (nondifferentiated cell) was performed with preadipocyte medium during all differentiation processes. TNF- $\alpha$  (100 ng/ml) and macrophage-conditioned medium (MCM, 25%) from THP-1 cells previously treated with 10 ng/ml lipopolysaccharide (LPS) (MCM LPS) was added during both sc and visceral adipocyte differentiation. Fourteen days after the initiation of differentiation, cells appeared rounded with large lipid droplets apparent in the cytoplasm. Cells were then considered mature adipocytes, harvested, and stored at  $-80^{\circ}\text{C}$  for RNA extraction to study *STAMP2* gene expression levels after human adipocyte differentiation. The experiment was performed in triplicate for each sample. The differentiation was monitored with the fatty acid synthase (*FASN*, Hs00188012\_m1; Applied Biosystems Inc., Madrid, Spain) and adiponectin (*Adipoq*, Hs00605917\_m1; Applied Biosystems) expression.

## Effects of TNF- $\alpha$ , MCM, and rosiglitazone on STAMP2 gene expression

After adipocyte differentiation (at d 14), sc adipocytes were incubated with fresh medium (control), fresh medium containing TNF- $\alpha$  (100 ng/ml), or LPS-stimulated MCM (25%) and rosiglitazone (2  $\mu$ M). After 48 h, the supernatants were centrifuged at 400  $\times$  g for 5 min, the cells were harvested, and pellets and supernatants were stored at  $-80$  C for RNA and protein analysis.

To evaluate the insulin action, we measure the phosphorylated (Ser473) [phospho- (p)AKT to total AKT ratio and the phosphorylated (PanTyr) insulin receptor substrate 1 (pIRS1) to IRS1 ratio using PathScan pAkt1 (Ser473) sandwich ELISA kit, PathScan total Akt1 sandwich ELISA kit, PathScan pIRS1 (PanTyr) sandwich ELISA kit, and PathScan total IRS1 sandwich ELISA kit (Cell Signaling Technology, Izasa SA, Barcelona, Spain). The analysis was performed following manufacturer's instructions. After incubation with cell lysates, pAkt (Ser473) protein, total Akt, pIRS1 (PanTyr), or total IRS1 was captured by the coated antibody. After extensive washing, Akt1 and IRS1 mouse antibody was added to detect the captured pAkt1 (Ser473) and pIRS1 (PanTyr) protein, respectively. Horseradish peroxidase (HRP)-linked antimouse antibody was then used to recognize the bound detection antibody. HRP substrate, 3,3',5,5'-tetramethylbenzidine, was added to develop color. The magnitude of absorbance for this developed color (450 nm) was proportional to the quantity of pAkt1 (Ser473) or pIRS1 (PanTyr) protein.

## Study of gene expression

RNA was prepared from these samples using RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA). The integrity of each RNA sample was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was quantified by means of spectrophotometer (GeneQuant; GE Healthcare, Piscataway NJ) reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol.

Gene expression was assessed by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems), using TaqMan and SybrGreen technology suitable for relative genetic expression quantification.

The commercially available and prevalidated TaqMan primer/probe sets used were as follows: endogenous control *PPIA* (4333763, cyclophilin A) and target genes six-transmembrane protein of prostate 2 (*STAMP2* or *STEAP4*, Hs00226415\_m1), fatty acid synthase (*FASN*, Hs00188012\_m1), acetyl-coenzyme A carboxylase  $\alpha$  (*ACC*, Hs00167385\_m1), Spot 14 (*THRSP14*, Hs00930058\_m1), sterol regulatory element-binding protein 1 (*SREBP1*, Hs00231674\_m1), *PPAR $\gamma$*  (Hs00234592\_m1), fatty acid-binding protein 4, adipocyte (*FABP4*, Hs00609791\_m1), adiponectin (*ADIPOQ*, Hs00605917\_m1), IL-6 (*IL6*, Hs00985639\_m1), monocyte chemoattractant protein-1 (*MCP1*, Hs00234140\_m1), IL-8 (*IL8*, Hs00174103\_m1), insulin receptor substrate 1 (*IRS1*, Hs00178563\_m1), solute carrier family 2

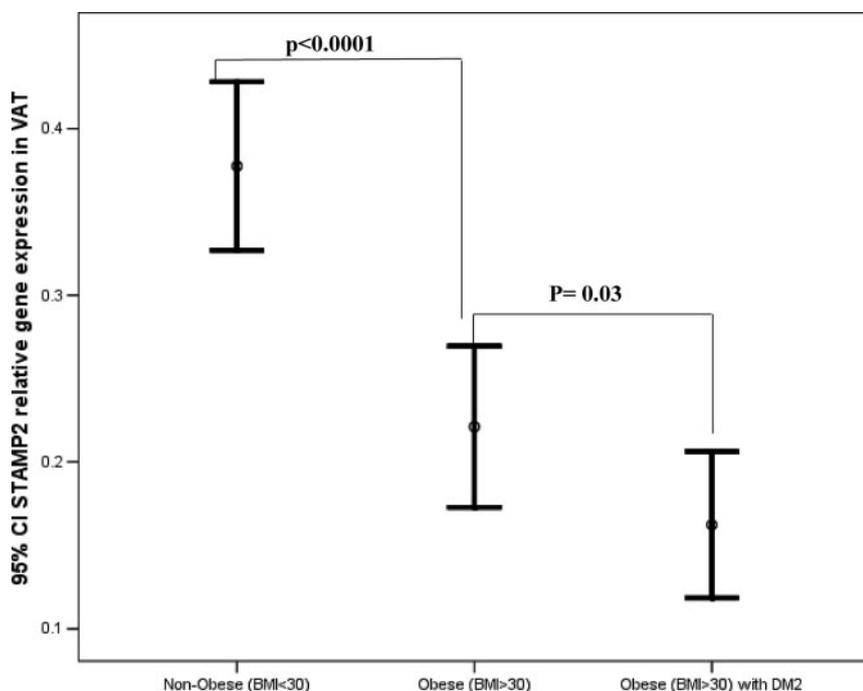
**TABLE 1.** Anthropometric and clinical characteristics of all participants

	Nonobese subjects	Obese subjects	Obese with type 2 diabetes subjects	P
n	74	65	32	
Sex (men/women)	31/43	17/48	7/25	0.6
Age (yr)	48.35 $\pm$ 14.2	46.3 $\pm$ 11.5	48.5 $\pm$ 11.7	0.5
BMI (kg/m <sup>2</sup> )	24.9 $\pm$ 3.1	43.2 $\pm$ 8.4 <sup>a</sup>	42.5 $\pm$ 4.7 <sup>a</sup>	<0.0001
Waist (cm)	85.5 $\pm$ 10.9	118.7 $\pm$ 18.4 <sup>a</sup>	127.5 $\pm$ 15.3 <sup>a</sup>	<0.0001
Hip (cm)	97.9 $\pm$ 8.9	125.4 $\pm$ 19.4 <sup>a</sup>	131.9 $\pm$ 15.1 <sup>a</sup>	<0.0001
SBP (mm Hg)	126.8 $\pm$ 18.2	136.7 $\pm$ 17.9 <sup>a</sup>	146.3 $\pm$ 24.9 <sup>a</sup>	<0.0001
DBP (mm Hg)	76.8 $\pm$ 10.7	80 $\pm$ 10.3	78.5 $\pm$ 13.3	0.2
Fasting glucose (mg/dl)	84.5 $\pm$ 10.2	95 $\pm$ 12.1 <sup>a</sup>	147.9 $\pm$ 64.5 <sup>a,b</sup>	<0.0001
HDL-cholesterol (mg/dl)	56.02 $\pm$ 14.7	59.1 $\pm$ 15.6	51.3 $\pm$ 11.5	0.58
LDL-cholesterol (mg/dl)	121.2 $\pm$ 29.2	119.25 $\pm$ 32.2	105.2 $\pm$ 34.3	0.09
Fasting triglycerides (mg/dl)	103.25 $\pm$ 45.5	129.3 $\pm$ 74.3	161.6 $\pm$ 117.5 <sup>a</sup>	0.002
Gene expression in VAT (RU)				
<i>FASN</i>	0.24 (0.12–0.35)	0.072 (0.04–0.13) <sup>a</sup>	0.04 (0.02–0.07) <sup>a</sup>	<0.0001
<i>ACC1</i>	0.023 (0.01–0.05)	0.017 (0.01–0.03) <sup>a</sup>	0.018 (0.01–0.024) <sup>a</sup>	0.006
<i>THRSP14</i>	0.41 (0.26–0.6)	0.29 (0.16–0.36) <sup>a</sup>	0.19 (0.13–0.2) <sup>a</sup>	<0.0001
<i>SREBP</i> total	0.18 (0.1–0.53)	0.11 (0.06–0.21)	0.08 (0.06–0.12)	0.5
<i>TR<math>\alpha</math></i>	0.08 (0.04–0.13)	0.088 (0.05–0.1)	0.088 (0.04–0.1)	0.9
<i>TR<math>\alpha</math>1</i>	0.054 (0.03–0.09)	0.052 (0.03–0.067)	0.052 (0.03–0.07)	0.7
<i>p53</i>	0.16 (0.14–0.2)	0.17 (0.11–0.24)	0.16 (0.13–0.22)	0.4
Caveolin 1	1.37 (0.99–1.89)	1.09 (0.9–1.2) <sup>a</sup>	1.1 (0.8–1.3) <sup>a</sup>	0.005
<i>GLUT4</i>	0.027 (0.02–0.05)	0.017 (0.01–0.02) <sup>a</sup>	0.015 (0.009–0.019) <sup>a</sup>	0.006
<i>IRS1</i>	0.014 (0.008–0.02)	0.009 (0.006–0.01) <sup>a</sup>	0.008 (0.005–0.01) <sup>a</sup>	<0.0001
<i>CD68</i>	0.105 (0.08–0.15)	0.16 (0.11–0.22) <sup>a</sup>	0.23 (0.19–0.34) <sup>a,b</sup>	<0.0001
<i>CD206</i>	0.019 (0.012–0.03)	0.018 (0.012–0.024)	0.023 (0.017–0.03)	0.5
<i>CD206/CD68</i> ratio	0.17 (0.12–0.23)	0.1 (0.07–0.13) <sup>a</sup>	0.09 (0.07–0.11) <sup>a</sup>	<0.0001
<i>STAMP2</i>	0.35 (0.22–0.47)	0.12 (0.09–0.39) <sup>a</sup>	0.1 (0.07–0.2) <sup>a,b</sup>	<0.0001

DBP, Diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; VAT, visceral adipose tissue.

<sup>a</sup>  $P < 0.05$ , significant differences in obese and type 2 diabetic obese in comparison with lean subjects, by Bonferroni *post hoc* test.

<sup>b</sup>  $P < 0.05$ , significant differences in type 2 diabetic obese in comparison with obese subjects, by Bonferroni *post hoc* test.



**FIG. 1.** *STAMP2* gene expression in visceral adipose tissue according to obesity and type 2 diabetes. CI, Confidence interval.

(facilitated glucose transporter), member 4 (*SLC2A4*, Hs00168966\_m1), p53 (p53, Hs01034249\_m1), CD68 (*CD68*, Hs00154355\_m1), and mannose receptor, C type 1 (*MRC1* or *CD206*, Hs00267207\_m1).

**TABLE 2.** *STAMP2* gene expression in visceral adipose tissue (n = 171) in association with metabolic parameters

	r	P
Age	0.04	0.6
BMI	−0.47	<0.0001
Waist	−0.40	<0.0001
Hip	−0.39	<0.0001
SBP	−0.20	0.015
DBP	−0.10	0.2
Fasting glucose	−0.31	<0.0001
HDL-cholesterol	0.12	0.1
LDL-cholesterol	0.21	0.01
Fasting triglycerides	−0.07	0.3
Gene expression (RU)		
<i>FASN</i>	0.24	0.006
<i>ACC1</i>	0.22	0.02
<i>THRSP14</i>	0.4	<0.0001
<i>SREBP</i> total	0.68	<0.0001
<i>TRα</i>	0.37	<0.0001
<i>TRα1</i>	0.40	<0.0001
<i>p53</i>	−0.34	<0.0001
Caveolin 1	0.25	0.005
<i>GLUT4</i>	0.21	0.02
<i>IRS1</i>	0.45	<0.0001
<i>CD68</i>	−0.15	0.09
<i>CD206</i>	0.36	<0.0001
<i>CD206/CD68</i> ratio	0.53	<0.0001

DBP, Diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RU, relative units; SBP, systolic blood pressure.

The SybrGreen primer sets used were previously validated to give an optimal amplification over serial dilutions of target, and analysis of melting curves demonstrated a specific single product for each gene primer. Primer sequences were as follows: *PPIA* forward and reverse primer sequences 5'-CAAATGCTGGACCCAACACAA-3' and 5'-CCTCCACAATATTCATGCCTTCTT-3', respectively, and *CAV1* forward and reverse primer sequences 5'-AACGATGACGTGGTCAAGATTG-3' and 5'-TC-CAAATGCCGTCAAAACTGT-3', respectively. *TRα* and *TRα1* primer sequences were detailed in a previous study (9).

The RT-PCR TaqMan reaction was performed in a final volume of 25  $\mu$ l. The cycle program consisted of an initial denaturing of 10 min at 95 C and then 40 cycles of a 15-sec denaturing phase at 95 C and a 1-min annealing and extension phase at 60 C. A threshold cycle (*Ct*) value was obtained for each amplification curve and a  $\Delta$ *Ct* value was first calculated by subtracting the *Ct* value for human cyclophilin A (*PPIA*) RNA from the *Ct* value for each sample. Fold changes compared with the endogenous

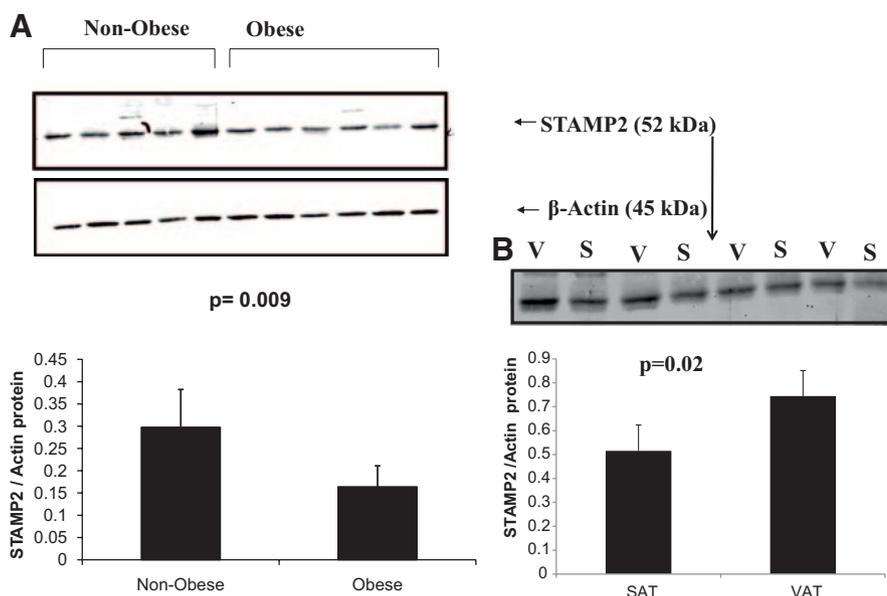
control were then determined by calculating  $2^{-\Delta$ *Ct*, so gene expression results are expressed as expression ratio relative to *PPIA* gene expression according to manufacturers' guidelines.

## Western blot assays

Fat tissue was homogenized in radioimmunoprecipitation assay buffer as we described previously (10). Protein extracts (10  $\mu$ g) were loaded, resolved on 10% SDS-PAGE, and transferred to Hybond ECL nitrocellulose membranes by conventional procedures. Membranes were stained with 0.15% Ponceau red (Sigma-Aldrich, St. Louis, MO) to ensure equal loading after transfer and then blocked with 5% (wt/vol) BSA in Tris-buffered saline with 0.1% Tween 20. The primary antibodies used were 1:4000 rabbit anti-STEAP4 (AV9842) and 1:2000 goat anti- $\beta$ -actin (sc-1616). Blots were incubated with the appropriate IgG-HRP-conjugated secondary antibody. Immunoreactive bands were visualized with ECL-plus reagent kit (GE Healthcare, Uppsala, Sweden). OD of the immunoreactive bands were measured using Image J analysis software.

## Immunofluorescence

Five-micrometer sections of frozen adipose tissue were fixed with 4% paraformaldehyde and PBS. Immunofluorescence staining was performed overnight at 4 C with rabbit anti-STEAP4 antibody at 1:50 dilution and mouse anti-CD68 at 1:25 dilution, washed, and visualized using Alexa Fluor 546 goat antirabbit antibody and Alexa Fluor 488 goat antimouse (1:500; Molecular Probes, Eugene, OR), respectively. As a negative control, the entire immunofluorescence procedure was performed in the absence of primary antibody. The slides were counterstained with 4',6-diamidino-2-phenylindole to reveal nuclei and examined under a Nikon Eclipse 90i microscope.



**FIG. 2.** A, STAMP2 protein levels in visceral adipose tissue comparing nonobese vs. obese subjects; B, comparison of STAMP2 protein levels between sc adipose tissue [SAT (S)] and visceral adipose tissue [VAT (V)].

### Statistical analyses

Statistical analyses were performed using SPSS version 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range. Parameters that did not fulfill normal distribution were mathematically transformed to improve symmetry for subsequent analyses. The relation between variables was analyzed by simple correlation (Spearman's test) and multiple linear regression model analyses. Unpaired *t* test was used to compare clinical variables and *STAMP2* gene expressions and protein levels (densitometry data) according to obesity status and adipose tissue fractions. Unpaired *t* tests were used to evaluate the effects of TNF- $\alpha$ , MCM, and rosiglitazone treatments on adipocytes. The experiments were performed in triplicate. Statistical significance was set at  $P < 0.05$ .

## Results

### *STAMP2* gene expression in visceral and sc adipose tissue

Anthropometric and clinical characteristics of all participants are shown in Table 1. In visceral adipose tissue, *STAMP2* gene expression was significantly decreased in obese subjects [0.11 (0.08–0.33) vs. 0.35 (0.22–0.47) relative expression,  $P < 0.0001$ ] (Table 1 and Fig. 1). Furthermore, within the obese, *STAMP2* gene expression was further decreased in subjects with type 2 diabetes [0.1 (0.07–0.2) vs. 0.12 (0.09–0.39) relative expression,  $P = 0.03$ ] (Table 1 and Fig. 1). In fact, *STAMP2* gene expression was significantly associated with measurements of the obesity phenotype [body mass index (BMI), waist and hip circumference, and fat mass], and obesity-associated metabolic disturbances (systolic blood pressure and fasting

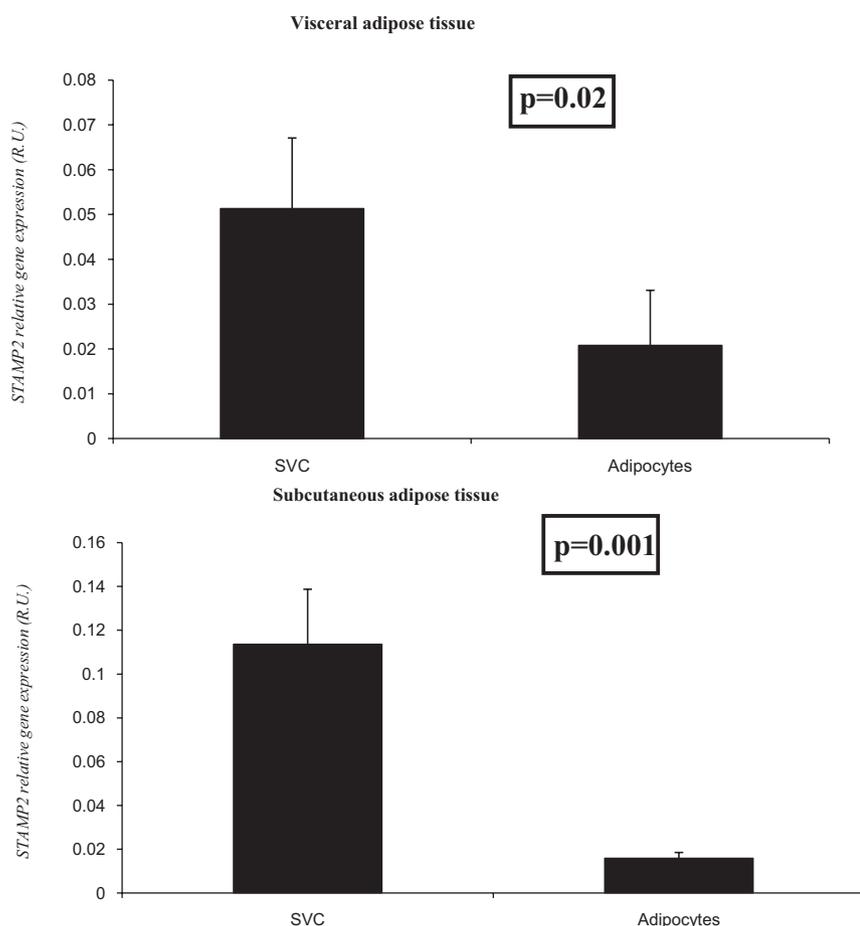
glucose). In addition, *STAMP2* gene expression was positively associated with the expression of lipogenic genes (*FASN*, *ACC1*, *SREBP1*, *THRSP14*, *TR $\alpha$* , and *TR $\alpha$ 1*), caveolin-1 and insulin-related pathway genes (*IRS1* and *GLUT-4*) gene expression (Table 2). After analyzing several multiple linear regression models, BMI ( $P = 0.003$ ) and *FASN* ( $P = 0.01$ ), *ACC1* ( $P = 0.01$ ), *SREBP1* ( $P = 0.001$ ), *THRSP14* ( $P = 0.001$ ), *TR $\alpha$ 1* ( $P = 0.04$ ) and *IRS1* ( $P = 0.001$ ) gene expression contributed independently to *STAMP2* gene expression variance, after controlling for the effects of the age and fasting glucose. *STAMP2* gene expression was not associated with metabolic parameters in sc adipose tissue.

There is evidence indicating that mRNA levels may not necessarily predict the translated protein levels. In this regard, we measured *STAMP2* protein in adipose tissue by Western blot. *STAMP2* protein was significantly decreased in obese subjects (Fig. 2A).

*STAMP2* gene expression tended to be higher in visceral adipose tissue [0.24 (0.1–0.39) vs. 0.12 (0.096–0.15) relative gene expression,  $P = 0.1$ ], and in fact, *STAMP2* protein levels were significantly increased in visceral adipose tissue (Fig. 2B).

We also analyzed *STAMP2* gene expression in stromovascular cells (SVC) and mature adipocytes. In both visceral and sc adipose tissue, *STAMP2* gene expression in SVC was significantly higher than in mature adipocytes (2.5- and 7.2-fold higher,  $P = 0.02$  and  $P = 0.001$ , respectively) (Fig. 3). In addition, *STAMP2* gene expression in visceral adipose tissue-derived SVC tended to be negatively associated with fasting glucose. We also tested CD14 and CD68 (two macrophage markers) gene expression in both fractions and confirmed that CD14 and CD68 are more expressed in SVC. Immunostaining analyses were performed to determine the cellular distribution of *STAMP2* protein in biopsies of adipose tissue. Confirming *STAMP2* mRNA data, immunofluorescence detection showed a bright staining pattern mainly in the SVF cells. This staining is shown in the cytoplasm as well as in the nuclei of a few cells. To determine whether the stained cells were macrophages, costaining analysis was performed using *STAMP2* and CD68 antibodies (Fig. 4).

Finally, to investigate the relationship between *STAMP2* and macrophage markers, we investigated *CD68* and *CD206* [a specific marker of alternative macrophage (M2)] gene expression in adipose tissue. *STAMP2* gene expression



**FIG. 3.** *STAMP2* gene expression in adipose tissue fractions (SVC vs. adipocytes) in both visceral and sc adipose tissue. R.U., Relative units.

was significantly associated with *CD68* ( $r = 0.29$ ;  $P = 0.03$ ) gene expression only in nonobese. However, *STAMP2* gene expression was significantly associated with *CD206* gene expression in all subjects [nonobese ( $r = 0.46$ ;  $P < 0.0001$ ), obese ( $r = 0.54$ ;  $P < 0.0001$ ), and obese with type 2 diabetes ( $r = 0.49$ ;  $P = 0.04$ )] and with the ratio of *CD206* to *CD68* [nonobese ( $r = 0.32$ ;  $P = 0.01$ ), obese ( $r = 0.48$ ;  $P = 0.002$ ), and obese with type 2 diabetes ( $r = 0.48$ ;  $P = 0.045$ )] (Table 2).

### ***STAMP2* gene expression during differentiation of human preadipocytes**

In isolated preadipocytes from lean and obese subjects, *STAMP2* gene expression was significantly increased during the differentiation process in parallel to adipogenic genes (Fig. 5A). *STAMP2* gene expression was positively associated with *FASN* ( $r = 0.87$ ;  $P < 0.0001$ ), *FABP4* ( $r = 0.83$ ;  $P < 0.0001$ ), *PPAR $\gamma$*  ( $r = 0.92$ ;  $P < 0.0001$ ), and adiponectin (*Adipoq*) ( $r = 0.84$ ;  $P < 0.0001$ ) gene expression and negatively with *IL6* ( $r = -0.89$ ;  $P < 0.0001$ ) gene expression.

*STAMP2* (Fig. 5B), *FASN*, *ACC1*, *PPAR $\gamma$* , *Adipoq*, and *FABP4* gene expression in lean subject-derived cells (prea-

dipocytes and adipocytes) were increased in comparison with obese subject-derived cells. On the contrary, in this group, *IL6* gene expression was higher in adipose tissue-derived cells from obese vs. lean subjects.

### **Effects of TNF- $\alpha$ , LPS-induced MCM, and rosiglitazone on *STAMP2* gene expression on human adipocytes and during adipocyte differentiation**

To gain insight in the modulator factors of *STAMP2* gene expression in human adipocytes, we performed the following treatments: TNF- $\alpha$  (100 ng/ml), MCM (25%), and rosiglitazone (2  $\mu$ M). TNF- $\alpha$  administration increased significantly inflammatory gene expression (*IL6*, *IL8*, and *MCP1*), reduced the pIRS to IRS ratio, and did not affect adipogenic genes. MCM administration also increased inflammatory gene expression (*IL6*, *IL8*, and *MCP1*), decreased adipogenic gene expression (*PPAR $\gamma$* , *FASN*, *Adipoq*, and *FABP4*), and reduced pAKT to AKT ratio and total IRS1 levels. Rosiglitazone administration reduced inflammatory gene expression (*IL6*, *IL8*, and

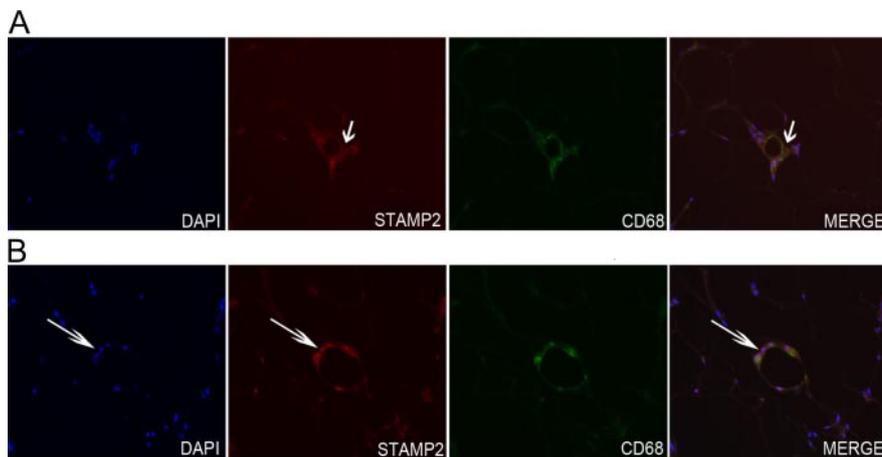
*MCP1*) and increased the gene expression of some adipokines (*Adipoq*, *FABP4*) and pAKT to AKT and pIRS1 to IRS1 ratio (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>).

In parallel with these data, TNF- $\alpha$  (100 ng/ml) and MCM (25%) administration increased significantly *STAMP2* gene expression in human adipocytes. On the contrary, rosiglitazone (2  $\mu$ M) administration decreased significantly *STAMP2* gene expression (Fig. 6A).

Finally, the effects of TNF- $\alpha$  and LPS-induced MCM was evaluated during sc and visceral adipocyte differentiation. TNF- $\alpha$  (100 ng/ml) and MCM (25%) administration potentiated the increase of *STAMP2* gene expression during adipocyte differentiation (Fig. 6B). As expected, these treatments decreased significantly adipogenic gene expression (*FASN*, *PPAR $\gamma$* , and *Adipoq*) and led to increased the expression of the *IL6* gene (Supplemental Fig. 2).

## **Discussion**

*STAMP2* is essential for prevention of excessive inflammation and protection of adipocyte insulin sensitivity and



**FIG. 4.** Immunofluorescence detection of STAMP2 and CD68 in human adipose tissue cryosections. Costaining of adipose tissue macrophages in crown-like structures surrounding an individual adipocyte demonstrates colocalization of both proteins STAMP2 (red) and CD68 (green). The counterstaining of nuclei [4',6-diamidino-2-phenylindole (DAPI)] is shown in blue. A, Using a  $\times 10$  objective; B, using a  $\times 20$  objective. Images are representative of adipose tissue preparations collected from five subjects. The short arrows indicate STAMP2 in macrophages (CD68<sup>+</sup> cells), and long arrows indicate STAMP2 in the nuclei.

systemic glucose homeostasis. Therefore, STAMP2 deficiency is sufficient to spontaneously recapitulate many features of the metabolic syndrome including inflammation, insulin resistance, glucose intolerance, hyperglycemia, dyslipidemia, and fatty infiltration of liver and can markedly exacerbate the metabolic abnormalities of the ob/ob model of severe obesity (3, 5, 6). In agreement with

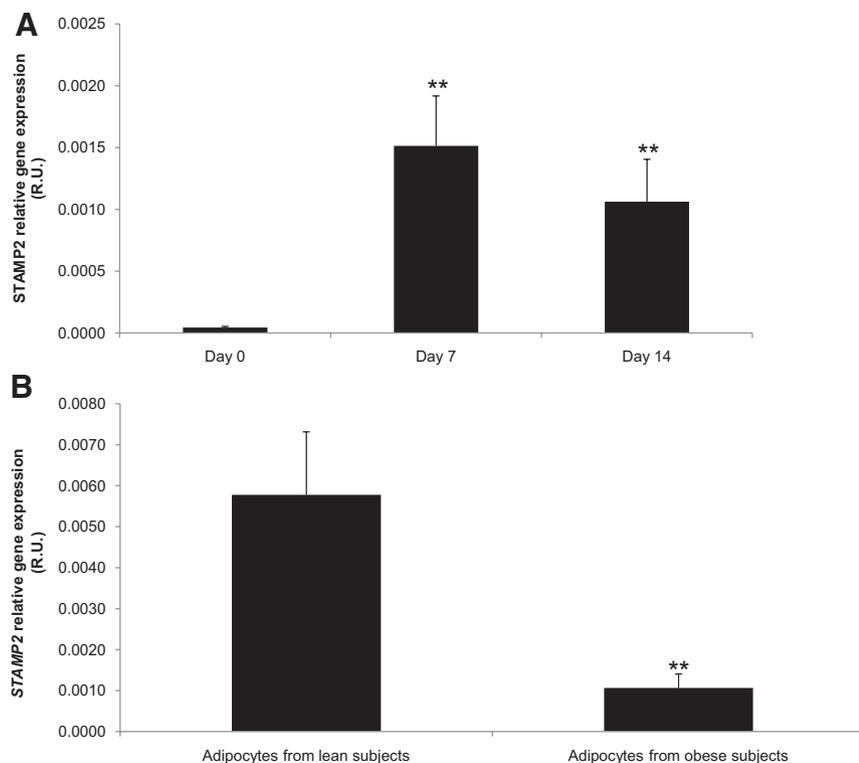
these previous studies, here we found that STAMP2 gene expression in visceral adipose tissue might reflect adipose tissue dysfunction.

The main findings of the present study are as follows.

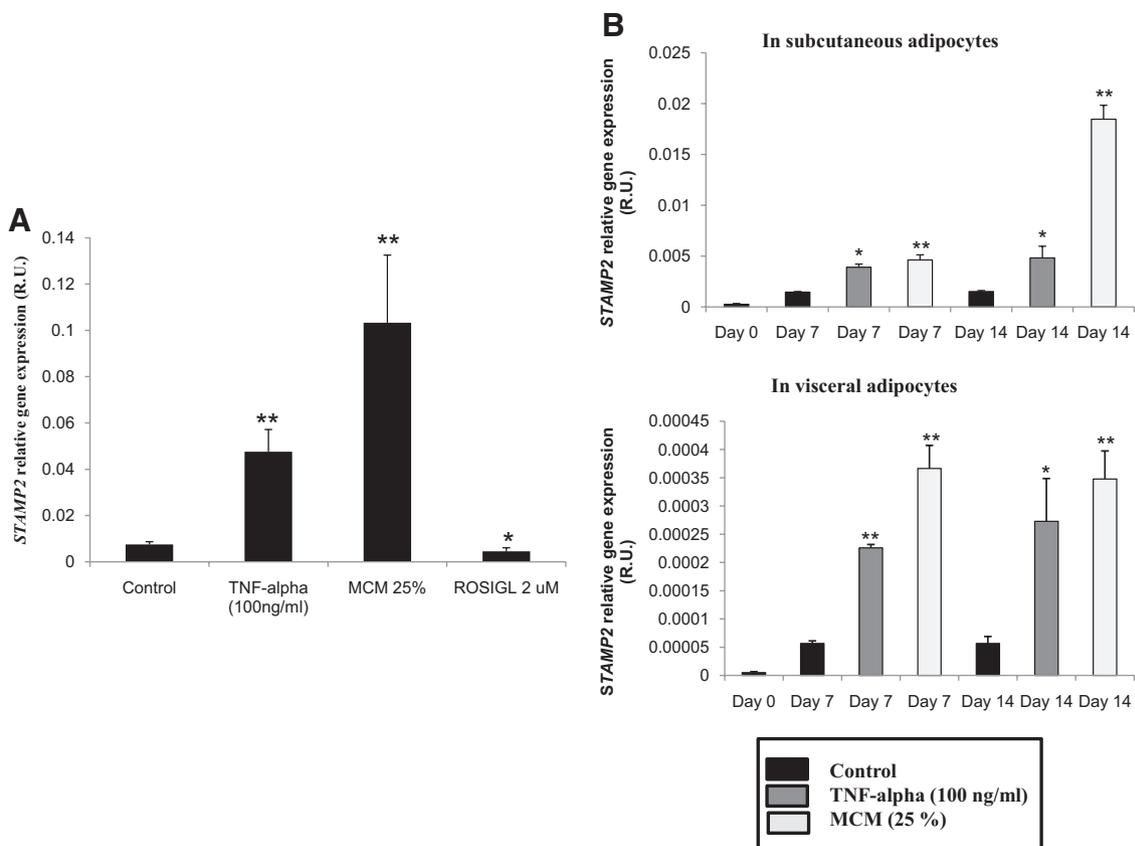
1) This is the first study in humans, to our knowledge, describing a direct association of STAMP2 gene expression with lipogenic genes such as *FASN*, *ACC1*, *THRSP14*, *SREBP1*, thyroid hormone receptors (*TR $\alpha$*  and *TR $\beta$* ), and caveolin-1 (*CAV1*).

The expression of lipogenic genes (such as *FASN* and *ACC1*) in visceral adipose tissue has been consistently found to be decreased in subjects with obesity and insulin resistance (11). STAMP2 gene expression followed the same expression pattern that lipogenic genes in visceral adipose tissue, decreased in subjects with obesity and type 2 diabetes and increasing during adipocyte differentiation. In addition, we found that STAMP2 gene expression in visceral adipose tissue was also associated with *CAV1* gene expression. *CAV1* knockdown adipocytes displayed reductions in insulin-stimulated glucose transport, insulin-triggered glucose transporter 4 (GLUT4) recruitment to the cell surface, and insulin receptor activation (12). Thus, *CAV1* might modulate insulin action in adipocytes, and the direct association between *CAV1* and STAMP2 suggests that these two genes have an important role in insulin action in adipose tissue. In fact, two other insulin signaling pathway-related genes (*IRS1* and *GLUT4*) were significantly associated with STAMP2 gene expression. Another interesting association was the inverse relationship between *p53* and STAMP2 gene expression in visceral adipose tissue. Recently, Minamino *et al.* (13) have reported that *p53* gene expression in adipose tissue promotes inflammation and insulin resistance in mice.

The inverse association between STAMP2 gene expression and obesity-associated metabolic disturbances was mainly found in visceral adipose tissue. Abdominal obesity is a well-known contributor to insulin resistance, attributed in part to the concomitant in-



**FIG. 5.** A, STAMP2 gene expression during human adipocyte differentiation. \*\*,  $P < 0.005$  vs. d 0. B, STAMP2 gene expression in adipocytes according to obesity status. \*\*,  $P < 0.005$  vs. adipocytes from lean subjects. R.U., Relative units.



**FIG. 6.** A, Effects of TNF- $\alpha$ , MCM, and rosiglitazone on *STAMP2* gene expression in human sc adipocytes; B, effects of TNF- $\alpha$  and MCM on *STAMP2* gene expression during human sc and visceral adipocyte differentiation. \*,  $P < 0.05$  vs. control treatment; \*\*,  $P < 0.005$  vs. control treatment. R.U., Relative units.

creases in the circulating concentration of free fatty acids (14, 15). In fact, lipogenic gene expression was associated negatively with metabolic parameters only in visceral adipose tissue (11). In parallel to lipogenic genes, *IRS1* and *GLUT4* gene expression in the same fat depot was significantly decreased in association with obesity.

2) In visceral adipose tissue, *STAMP2* gene expression was significantly decreased in obese subjects, mainly in obese subjects with type 2 diabetes. *STAMP2* gene expression and protein was significantly and inversely associated with obesity phenotype measures and obesity-associated metabolic disturbances (systolic blood pressure and fasting glucose). In addition, *STAMP2* gene expression was significantly decreased not only in whole adipose tissue but also in isolated adipocytes from obese subjects.

These findings are in agreement with other studies in humans and mice (6, 7). However, Arner *et al.* (8) reported in a heterogeneous study involving five cohorts that *STAMP2* gene expression was increased in obese subjects. They also reported that the *STAMP2* gene was also more expressed in sc than in visceral adipose tissue in 20 paired samples. Differences in study design or in the characteristics of the populations evaluated (age and cardiovascular

risk factors) may contribute to explain the differences found.

3) The predominant *STAMP2* gene expression was found in SVC (including monocytes, preadipocytes, and endothelial cells) in comparison with mature adipocytes. Interestingly, monocytes express high levels of *STAMP2* that were inversely associated with obesity-associated cardiovascular alterations (16). There is previous evidence that circulating monocytes in obese subjects are in a pro-inflammatory state, with an increase in nuclear factor- $\kappa$ B transcriptional activity (17) and reduced insulin receptor phosphorylation (18). Previous studies have demonstrated that adipose tissue macrophages derive from circulating monocytes, which migrate into the adipose tissue under the influence of chemokines (19). These infiltrated macrophages in visceral adipose tissue contribute to the overall inflammatory state and interference with insulin signal transduction (19). Thus, the relatively increased expression of *STAMP2* in SVC when compared with adipocytes may be explained by the presence of macrophages in adipose tissue, and this was confirmed with the study of the macrophage markers CD14 and CD68. Here, *STAMP2* gene expression was associated not only with

CD68 but also robustly with CD206 gene expression in visceral adipose tissue. CD206 is a marker of alternative activated macrophages (M2), which are characterized to display antiinflammatory activities. Recently, it has been shown in mice and humans that CD206<sup>+</sup> macrophages in adipose tissue were directly associated with a healthy metabolic profile (20, 21). In fact, the relative levels of CD206<sup>+</sup> macrophages increased after gastric surgery-induced weight loss (22). Considering current findings, we speculate that STAMP2 could be implicated in this anti-inflammatory profile.

4) *STAMP2* gene expression was significantly increased during human adipocyte differentiation in association with adipogenic genes (*FASN*, *ACCI*, *PPAR* $\gamma$ , *Adipoq* and *FABP4*) and inversely associated with *IL6* gene expression. These associations were in agreement with the previous possible antiinflammatory role of STAMP2 reported in 3T3-L1 (3). We have recently investigated the effects of an anti-inflammatory protein on STAMP2 gene expression. Lactoferrin administration led concomitantly to markedly decreased *IL6* and *MCP1* gene expression and to increased STAMP2 gene expression in human adipocytes (23). In sharp contrast, Arner *et al.* reported that *STAMP2* gene expression decreased during adipocyte differentiation. The lack of methodology details and of control parameters of adipocyte differentiation (the expression of adipogenic genes) preclude any comparison between studies (8).

5) Here we confirmed that *STAMP2* gene expression was induced under inflammatory stimuli (TNF- $\alpha$  and MCM) (3, 4, 24). *STAMP2* gene expression increased during early adipocyte differentiation, with the maximum increase at d 7. This increase of *STAMP2* mRNA levels during adipogenesis was potentiated after inflammatory stimuli. Thus, *STAMP2* gene expression is induced after two different stimuli (the early adipogenic program and the inflammatory environment).

The effects of rosiglitazone (2  $\mu$ M) leading to decreased *STAMP2* gene expression were observed in fully differentiated adipocytes. In these particular cells at this stage of differentiation, rosiglitazone did not change other genes that are robustly induced during the early adipogenic program (such as *FASN* and *PPAR* $\gamma$ ) but led to decreased expression of inflammatory genes (*IL6*, *IL8*, and *MCP1*). The antiinflammatory action of rosiglitazone at the cellular and molecular level has been previously and first shown by Mohanty *et al.* (25). However, the paradoxical suppressive effect of rosiglitazone on STAMP2 is still unclear. It is possible that the inhibition of inflammatory signals evoked by rosiglitazone results in decreased requirements of STAMP2 antiinflammatory function, being down-regulated secondarily. It is in this context of de-

creased inflammation in which the compensatory gene expression (*STAMP2*) is also lowered after rosiglitazone in fully differentiated adipocytes.

Otherwise, *IL6* gene expression was induced only by inflammatory mediators and repressed during adipocyte differentiation. The higher the number of differentiated adipocytes in a given sample of adipose tissue, the higher the *STAMP2* gene expression and the lower the *IL6* gene expression. The inverse association of *IL6* with *STAMP2* gene expression should be interpreted in this context.

In conclusion, in agreement with the counterregulatory properties of STAMP2 (3) in the face of nutritional (saturated fatty acids) insults and its association with insulin sensitivity, we found that reduced STAMP2 levels in visceral adipose tissue might be an important contributor to metabolic disturbances (insulin resistance and inflammation-induced adipocyte dysfunction) in this fat depot.

## Acknowledgments

We acknowledge the technical assistance of Gerard Pardo and the administrative help of Roser Rodriguez.

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This work was supported by SAF-2009-10461 and Grant SAF-2008-02073 from the Ministerio de Ciencia e Innovación, Spain, and from the Fundación Mutua Madrileña, Spain.

Disclosure Summary: The authors declare no conflict of interest.

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