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## ***In Vivo* and *ex Vivo* Regulation of Visfatin Production by Leptin in Human and Murine Adipose Tissue: Role of Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase Signaling Pathways**

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Visfatin is an adipogenic adipokine with increased levels in obesity, properties common to leptin. Thus, leptin may modulate visfatin production in adipose tissue (AT). Therefore, we investigated the effects of leptin on visfatin levels in 3T3-L1 adipocytes and human/murine AT, with or without a leptin antagonist. The potential signaling pathways and mechanisms regulating visfatin production in AT was also studied. Real-time RT-PCR and Western blotting were used to assess the relative mRNA and protein expression of visfatin. ELISA was performed to measure visfatin levels in conditioned media of AT explants, and small interfering RNA technology was used to reduce leptin receptor expression. Leptin significantly ( $P < 0.01$ ) increased visfatin levels in human and murine AT with a maximal response at leptin  $10^{-9}$  M, returning to baseline at leptin  $10^{-7}$  M. Importantly, ip leptin administration to C57BL/6 *ob/ob* mice further supported leptin-induced visfatin protein production in omental AT ( $P < 0.05$ ). Additionally, soluble leptin receptor levels rose with concentration dependency to a maximal response at leptin  $10^{-7}$  M ( $P < 0.01$ ). The use of a leptin antagonist negated the induction of visfatin and soluble leptin receptor by leptin. Furthermore, leptin-induced visfatin production was significantly decreased in the presence of MAPK and phosphatidylinositol 3-kinase inhibitors. Also, when the leptin receptor gene was knocked down using small interfering RNA, leptin-induced visfatin expression was significantly decreased. Thus, leptin increases visfatin production in AT *in vivo* and *ex vivo* via pathways involving MAPK and phosphatidylinositol 3-kinase signaling. The pleiotropic effects of leptin may be partially mediated by visfatin. (***Endocrinology* 150: 3530–3539, 2009**)

**O**besity, in particular visceral adiposity, has reached epidemic proportions and is associated with serious cardio-metabolic sequelae including insulin resistance, diabetes, dyslipidemia, hypertension, and cardiovascular disease, all features of the metabolic syndrome (1). Greater understanding of the underlying molecular mechanisms is required to explain the physiological implications of obesity.

Apart from its role in energy storage, adipose tissue (AT) produces several hormones and cytokines that have wide-

-ranging effects on carbohydrate and lipid metabolism. These effects play an important role in the pathogenesis of the metabolic syndrome (2). The expression and circulating levels of leptin, for example, positively correlate with adiposity (3). Besides its major roles in food intake and energy homeostasis (4), leptin has pleiotropic effects, for example, on immunity, angiogenesis, and reproduction (5). Also, leptin has been shown to enhance insulin action in mouse models of obesity and diabetes (6).

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Abbreviations: AT, Adipose tissue; FBS, fetal bovine serum; IRS, insulin receptor substrate; LTM, leptin triple mutant; MEK, MAPK kinase; OB-R, leptin receptor; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride; si, small interfering; SLR, soluble leptin receptor.

Visfatin (pre-B cell colony-enhancing factor) is a novel adipokine reported to be preferentially produced by visceral AT in humans and mice (7). Visfatin appears to have insulin-mimetic actions; it apparently binds to and activates the insulin receptor at a site distinct from that of insulin (7). Visfatin has also been shown to induce the phosphorylation of insulin receptor substrate (IRS)-1, and IRS-2; binding of phosphatidylinositol 3-kinase (PI3K) to IRS-1 and IRS-2; and phosphorylation of Akt and MAPK (7). Interestingly, leptin also phosphorylates IRS-2 and leads to PI3K/Akt phosphorylation (8). In addition, plasma levels of visfatin correlate positively with visceral adiposity (7). Furthermore, plasma concentrations of visfatin are increased in insulin resistance states, for example, in the polycystic ovary syndrome (9) and type 2 diabetes mellitus (10). More recently, in addition to its adipogenic potential (7), visfatin has also been shown to promote angiogenesis (11), properties common to that of leptin.

With the aforementioned in mind, we studied the effect of leptin on visfatin production in human and murine AT *in vivo* and *ex vivo* and investigated the potential signaling pathways and mechanisms regulating leptin-induced visfatin production.

## Materials and Methods

### Animals

Adult male C57BL/6J wild-type mice ( $n = 48$ ), C57BL/6 *ob/ob* ( $n = 20$ ), and C57BL/Ks *db/db* ( $n = 12$ ) mice were obtained from Harlan Ltd. (Loughborough, UK). All mice were housed individually and maintained under pathogen-free conditions with controlled temperature and humidity and a 12-h light (0700–1900 h), 12-h dark (1900–0700 h) cycle as well as free access to standard mice diet and water. The standard diet consisted of 70% carbohydrate, 10% fat, and 20% protein, with an energy density of 3.85 kcal/g.

For *in vivo* experiments, adult male C57BL/6 *ob/ob* mice ( $n = 8$ ; four treated, four untreated) were used. In the treatment group, recombinant murine leptin (PeproTech, Rocky Hill, NJ), dissolved in PBS at a concentration of 13 mg/ml, was administered by two daily (0900–1100 and 2100–2300 h) ip injections of 5 mg/kg per mouse over 24 h. In the control group, twice-daily equivalent volume injections of PBS without leptin were administered instead.

All animals were culled by cervical dislocation at 0900 h and sc and omental AT were dissected and placed into sterile DMEM (Invitrogen, Paisley, UK) for primary AT culture or immediately snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  (*in vivo* experiments). The overall weights for all mice used were C57BL/6J:  $30 \pm 5$  g; C57BL/6 *ob/ob*:  $55 \pm 4$  g; C57BL/Ks *db/db*:  $53 \pm 5$  g. All procedures were performed in accordance with the U.K. Guidance on the Operation of Animals (Scientific Procedures) Act (1986).

### Subjects

After an overnight fast, omental AT samples were obtained (0800–1000 h) from healthy subjects ( $n = 6$ ). Omental AT from subjects was placed into sterile medium 199 (Sigma-Aldrich, Gillingham, UK) for primary AT culture. The local research ethics committee approved the study and all patients gave their informed consent, in accordance with the guidelines in The Declaration of Helsinki 2000. Exclusion criteria for the study included known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes mellitus, hypertension (blood pressure greater than 140/90 mm Hg), and renal impairment (serum creatinine,  $>120$   $\mu\text{mol/liter}$ ). None of the subjects had been prescribed any medications known to influence nutrient metabolism for at least 6 months before the study.

### Primary explant culture

AT organ explants were cultured using methods described by Fried and Moustaid-Moussa (12). Briefly, AT was minced into 5- to 10-mg [ $\sim 1$  mm (3)] fragments, washed with a 230- $\mu\text{m}$  mesh (filter no. 60; Sigma-Aldrich), and rinsed with sterile PBS warmed to 37 C. Samples were then transferred to six-well plates containing 3 ml DMEM (Invitrogen; murine) or media 199 (Invitrogen; human) supplemented with 50  $\mu\text{g/ml}$  gentamicin and 1% fetal bovine serum (FBS; Sigma-Aldrich) and cultured for 15 min, 20 min, or 24 h with or without the addition of human/mouse leptin (Sigma-Aldrich), leptin antagonist, *i.e.* recombinant leptin triple mutant (LTM; Protein Laboratories Rehovot, Rehovot, Israel), MAPK kinase (MEK)inhibitor (U0126; Calbiochem, San Diego, CA), and/or PI3K inhibitor (LY294002; Calbiochem) in a 37 C incubator under an atmosphere of 5%  $\text{CO}_2$ -95% air.

### Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen) containing 25 mM glucose and 10% FBS at 37 C with 5%  $\text{CO}_2$ -95% air. Two days after confluence (d 0), adipocyte differentiation was induced by incubation with DMEM, 25 mM glucose, and 10% FBS supplemented with insulin (10  $\mu\text{g/ml}$  insulin), dexamethasone (0.25  $\mu\text{M}$ ), rosiglitazone (10  $\mu\text{M}$ ), and isobutylmethylxanthine (0.5 mM) until d 2. Then cells were fed with DMEM, 25 mM glucose, and 10% FBS until d 10. Before each experiment, 3T3-L1 adipocytes were serum starved overnight in DMEM containing 0.5% BSA.

### Total RNA extraction and cDNA synthesis

Total RNA was isolated using RNeasy minikit from 3T3-L1 adipocytes (QIAGEN, Crawley, UK) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (Fermentas, York, UK) and random hexamers (Promega, Southampton, UK) as primers.

### RT-PCR

Quantitative PCR of mouse visfatin and mouse leptin receptor were performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). PCRs were carried out in a reaction mixture consisting of 5.0  $\mu\text{l}$  reaction buffer and 2.0 mM  $\text{MgCl}_2$  (Biogene, Kimbolton, UK), 1.0  $\mu\text{l}$  of each primer (10 ng/ $\mu\text{l}$ ), 2.5  $\mu\text{l}$  cDNA, and 0.5  $\mu\text{l}$  of Light Cycler DNA master SYBR Green I (Roche, Mannheim, Germany). Protocol conditions consisted of denaturation of 95 C for 15 sec, followed by 40 cycles of 94 C for 1 sec, 58 C for 10 sec, and 72 C for 12 sec, followed by melting curve analysis. For analysis, quantitative amounts of visfatin were standardized against the housekeeping gene  $\beta$ -actin. The RNA levels were expressed as a ratio, using  $\delta$ - $\delta$  method for comparing relative expression results between treatments in real-time PCR (13). The sequences of the sense and antisense primers used were: mouse visfatin (142 bp) 5'-CTTGTTTCAGTCTGGTATCC-3' and 5'-GCGAAGAGACTCCTCTGTAA-3'; mouse leptin receptor (182 bp) 5'-AGGCGCAGCCTGTATTGTCC-3' and 5'-CACGTTGGTGGCGAGTCAAG-3'; and  $\beta$ -actin (198 bp) 5'-AAGAGAGGTATCCTGACCCT-3' and 5'-TACATGGCTGGGTGTTGAA-3'. Ten microliters of the reaction mixture(s) were subsequently electrophoresed on a 1% agarose gel and visualized by ethidium bromide, using a 1-kb DNA ladder (Invitrogen) to estimate the band sizes. As a negative control for all the reactions, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. RNAs was assayed from three independent biological replicates.

### Small interfering (si) RNA transfection

Chemically synthesized Stealth interference RNA siRNA directed toward mouse leptin receptor (OB-R) was obtained from Invitrogen. The target sequence for siOB-R is 5'-GCCUGAAGUCAUGAUGAU-3' corresponding to the position of 495–513 relative to the start codon on OB-R. 3T3-L1 cells were cultured and transfected with siRNA (1  $\mu\text{M}$ ) using Lipofectamine 2000 (Invitrogen) transfection reagent according to

the manufacturer's instructions. Two days later, siRNA mediated down-regulation of OB-R mRNA and protein levels were analyzed by quantitative PCR and Western blotting. A nonsilencing RNA duplex (RNA interference explorer control siRNA duplex) was used as a negative control, according to manufacturer's protocol (Gene Link, Hawthorne, CA).

### Sequence analysis

The PCR products from samples were purified from the 1% agarose gel using the QIAquick gel extraction kit (QIAGEN). PCR products were then sequenced in an automated DNA sequencer, and the sequence data were analyzed using Blast nucleic acid database searches (National Centre for Biotechnology Information, Bethesda, MD), confirming the identity of our products.

### Western blotting

Western blotting was used to assess: 1) leptin receptor levels in 3T3-L1 adipocytes; 2) visfatin levels in AT; 3) soluble leptin receptor (SLR) levels in conditioned media; and 4) phospho-AKT, phospho-Erk1/2, total AKT, and total Erk1/2 in AT. Protein lysates were prepared by homogenizing 3T3-L1 adipocytes or AT in radioimmunoprecipitation lysis buffer (Upstate, Lake Placid, NY) according to the manufacturer's instructions. Also, conditioned media of primary omental AT culture from C57BL/6J mice were subjected to Western blotting for the SLR. Equal amounts of Laemmli buffer [5 M urea, 0.17 M sodium dodecyl sulfate, 0.4 M dithiothreitol, and 50 mM Tris-HCl (pH 8.0)] were added, mixed, and placed in a boiling water bath for 5 min. All samples were then allowed to cool at room temperature. The proteins in protein lysates (35  $\mu$ g/lane) and conditioned media (30  $\mu$ l/well) were separated by SDS-PAGE (8% resolving gel) and transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. PVDF membranes were then blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% BSA for 2 h.

The PVDF membranes were incubated with primary rabbit-antihuman/antimouse antibody for visfatin (Abcam, Cambridge, UK; 1:10,000 dilution); primary rabbit-antihuman/antimouse antibody for leptin receptor (Abcam; 1:1500 dilution); primary rabbit-antihuman/antimouse antibody for  $\beta$ -actin (Cell Signaling Technology, Beverly, MA; 1:1000 dilution); primary rabbit-antihuman/antimouse antibody for phospho-Erk1/2 (Cell Signaling Technology; 1:1000 dilution); primary rabbit-antihuman/antimouse antibody for total Erk1/2 (Cell Signaling Technology; 1:1000 dilution); primary rabbit-antihuman/antimouse antibody for phospho-AKT (Cell Signaling Technology; 1:1000 dilution); or primary rabbit-antihuman/antimouse antibody for total AKT (Cell Signaling Technology; 1:1000 dilution) overnight at 4 C. The membranes were washed thoroughly for 60 min with TBS-0.1% Tween 20 before incubation with the secondary antirabbit horseradish peroxidase-conjugated Ig (Dako, Ely, UK; 1:2000) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; Amersham, Little Chalfont, UK). Human and mouse visfatin peptide (Axxora, Nottingham, UK) were used as positive controls and water as the negative control (data not shown).

### ELISA analysis

Visfatin levels were determined in conditioned media from human and mouse primary omental AT cultures, which were measured using commercially available EIA (EIA kit; Phoenix Pharmaceuticals, Belmont, CA; human) and ELISA (ELISA kit; MBL International, Woburn, MA; mouse) kits, with intraassay coefficient of variations of 5.0 and 5.8%, respectively.

### Statistics

Differences between two groups were assessed using the unpaired *t* test. Data involving more than two groups were assessed by ANOVA with Bonferroni's test for *post hoc* analysis. For Western immunoblotting experiments, the densities were measured using a scanning den-

sitometer coupled to scanning software (ImageQuant; Molecular Dynamics, Amersham, Little Chalfont, UK).  $P < 0.05$  was considered significant.

## Results

### Concentration-dependent effects of leptin on visfatin mRNA expression in 3T3-L1 adipocytes

Real-time RT-PCR analysis corrected over  $\beta$ -actin showed a significant increase of visfatin mRNA expression by leptin in 3T3-L1 adipocytes. This was concentration dependent with a maximal response at  $10^{-9}$  M (Fig. 1A;  $P < 0.05$ ,  $P < 0.01$ , respectively), returning to baseline values at  $10^{-7}$  M (Fig. 1A;  $P > 0.05$ ). Subsequent sequencing of the PCR products confirmed gene identity.

### mRNA expression and protein levels of mouse leptin receptor (OB-R) in siOB-R transfected 3T3-L1 adipocytes vs. control siRNA 3T3-L1 adipocytes

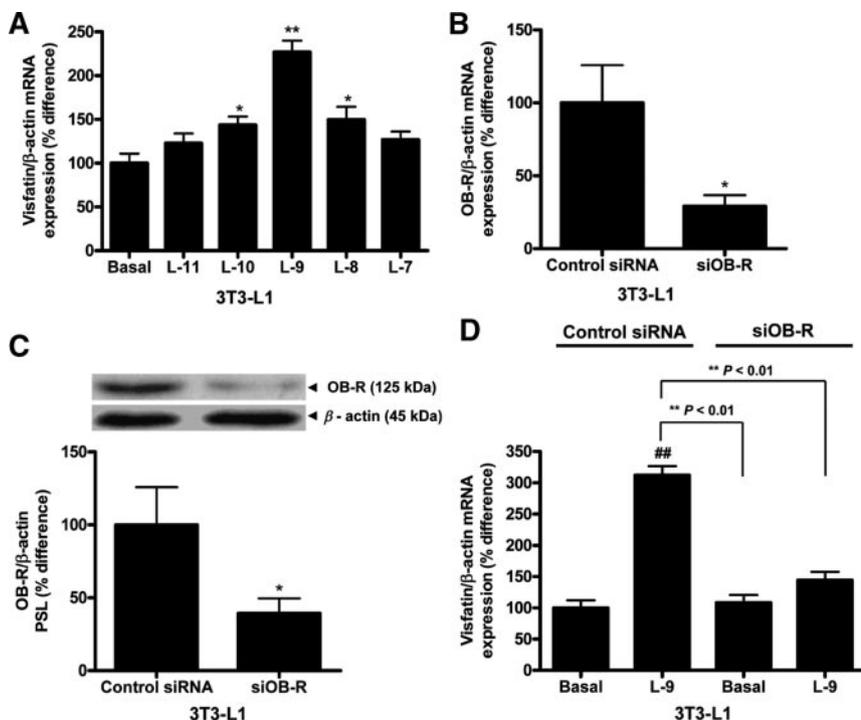
Real-time RT-PCR corrected over  $\beta$ -actin and Western blotting analyses showed a significant decrease of OB-R in siOB-R transfected 3T3-L1 adipocytes compared with control 3T3-L1 adipocytes (Fig. 1, B and C; \*,  $P < 0.05$ ,  $P < 0.05$ , respectively).

### Effects of leptin on visfatin mRNA expression in siOB-R transfected 3T3-L1 adipocytes and control siRNA 3T3-L1 adipocytes

Real-time RT-PCR analysis corrected over  $\beta$ -actin showed that visfatin mRNA expression was significantly increased by leptin ( $10^{-9}$  M)-treated control siRNA 3T3-L1 adipocytes when compared with basal (control siRNA 3T3-L1 adipocytes) (Fig. 1D; ##,  $P < 0.01$ ). There were no significant differences between basal (siOB-R transfected 3T3-L1 adipocytes) and leptin ( $10^{-9}$  M)-treated siOB-R transfected 3T3-L1 adipocytes (Fig. 1D;  $P > 0.05$ ). There were also no significant differences between basal (control siRNA 3T3-L1 adipocytes) and basal (siOB-R transfected 3T3-L1 adipocytes) (Fig. 1D;  $P > 0.05$ ). Visfatin mRNA expression was significantly higher in leptin ( $10^{-9}$  M)-treated control siRNA 3T3-L1 adipocytes when compared with basal (siOB-R transfected 3T3-L1 adipocytes) as well as leptin ( $10^{-9}$  M)-treated siOB-R transfected 3T3-L1 adipocytes (Fig. 1D; \*\*,  $P < 0.01$ ,  $P < 0.01$ , respectively).

### Concentration-dependent effects of leptin on visfatin protein production in C57BL/6 mouse sc and omental AT explants

Once again, visfatin protein production was significantly increased by leptin in C57BL/6 mouse omental AT explants, dose dependently, and as before the maximal response was at  $10^{-9}$  M (Fig. 2A;  $P < 0.05$ ,  $P < 0.01$ , respectively), returning to baseline values at  $10^{-7}$  M (Fig. 2A;  $P > 0.05$ ). Similar findings were noted in visfatin protein production by leptin in mouse subcutaneous AT explants; however, these were not statistically significant (data not shown). The detected protein for visfatin and  $\beta$ -actin had apparent molecular masses of 55 and 45 kDa, respectively (Fig. 2A, insets).



**FIG. 1.** A, Concentration-dependent effects of leptin  $10^{-11}$  M (L-11), leptin  $10^{-10}$  M (L-10), leptin  $10^{-9}$  M (L-9), leptin  $10^{-8}$  M (L-8), and leptin  $10^{-7}$  M (L-7) on visfatin mRNA expression in 3T3-L1 adipocytes at 4 h were assessed by real-time RT-PCR, compared with basal (no supplement). Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , respectively. B, OB-R mRNA expression relative to  $\beta$ -actin is significantly decreased in 3T3-L1 adipocytes transfected with siOB-R compared with control siRNA, using real-time RT-PCR. Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by unpaired *t* test. \*,  $P < 0.05$ . C, Leptin receptor (OB-R) protein levels relative to  $\beta$ -actin is significantly decreased in 3T3-L1 adipocytes transfected with siOB-R compared with control siRNA, using Western blotting. Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by unpaired *t* test. \*,  $P < 0.05$ . PSL, Phospho-stimulated light units. D, Effects of leptin on visfatin mRNA expression in siOB-R transfected 3T3-L1 adipocytes and control siRNA 3T3-L1 adipocytes at 4 h were assessed by real-time RT-PCR, compared with basal (no supplement). Visfatin mRNA expression was significantly increased by leptin  $10^{-9}$  M (L-9) in control siRNA 3T3-L1 adipocytes when compared with basal (no supplement; control siRNA 3T3-L1 adipocytes). ##,  $P < 0.01$ . Visfatin mRNA expression was significantly higher in leptin  $10^{-9}$  M-treated control siRNA 3T3-L1 adipocytes when compared with basal (siOB-R transfected 3T3-L1 adipocytes) as well as leptin  $10^{-9}$  M-treated siOB-R transfected 3T3-L1 adipocytes. \*\*,  $P < 0.01$ . Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test.

### Concentration-dependent effects of leptin on visfatin protein production in human omental AT explants

Like 3T3-L1 and murine AT, visfatin protein production was significantly increased by leptin ( $10^{-11}$  M,  $10^{-9}$  M) in human omental AT explants (Fig. 2B;  $P < 0.05$ ,  $P < 0.01$ , respectively). Similarly, we noted that at a dose of leptin  $10^{-7}$  M, visfatin net protein production was significantly less than that of leptin  $10^{-9}$  M ( $P < 0.05$ ) (Fig. 2B, *insets*).

### Effects of leptin and leptin triple mutant on leptin-induced visfatin protein production in C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice omental AT explants

Given the above findings, we sought to investigate these effects of leptin in the leptin-deficient C57BL/6 *ob/ob* mouse and the hyperleptinemic C57BL/Ks *db/db* mouse with or without the use of the leptin triple-mutant antagonist. Visfatin protein production was significantly increased by leptin ( $10^{-11}$  M,  $10^{-9}$  M)

in C57BL/6 *ob/ob* mouse omental AT explants (Fig. 2C;  $P < 0.01$ ,  $P < 0.001$ , respectively) and by leptin  $10^{-9}$  M in C57BL/Ks *db/db* mouse omental AT explants (Fig. 2D;  $P < 0.05$ ). Also, leptin-induced visfatin protein production was significantly decreased by LTM ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M) in C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice omental AT explants (Fig. 2, E and F;  $P < 0.05$ ,  $P < 0.01$ , respectively) (Fig. 2, E and F, *insets*). It was interesting to note that in these mutant mice, once again at a higher dose of leptin ( $10^{-7}$  M), we observed that visfatin production returned to baseline levels (Fig. 2, C and D;  $P > 0.05$ ) (Fig. 2, C and D, *insets*).

### Concentration-dependent effects of leptin on visfatin levels in conditioned media

#### C57BL/6 mouse omental AT explants

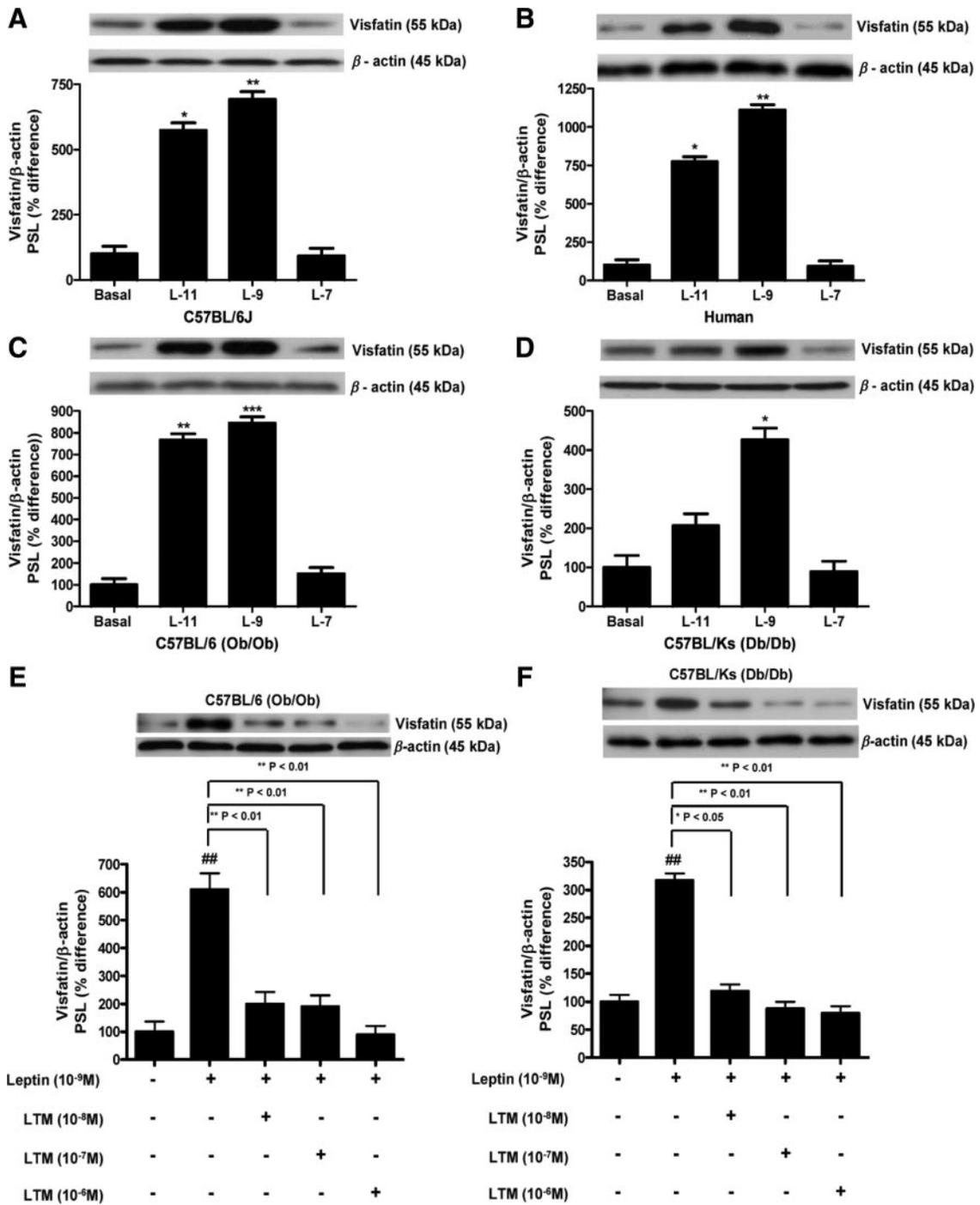
Visfatin levels, measured by ELISA, were significantly increased by leptin ( $10^{-11}$ ,  $10^{-9}$  M) in conditioned media of C57BL/6 mouse omental AT explants (Fig. 3A;  $P < 0.05$ ,  $P < 0.01$ , respectively). Leptin at a dose of  $10^{-7}$  M had significantly less visfatin compared with the lower concentrations of leptin ( $P < 0.05$ ).

#### Human omental AT explants

Visfatin levels were significantly increased by leptin ( $10^{-11}$ ,  $10^{-9}$  M) in conditioned media of human omental AT explants (Fig. 3B;  $P < 0.05$ ,  $P < 0.01$ , respectively). There was no significant difference in visfatin levels in conditioned media with leptin  $10^{-7}$  M treatment compared with baseline levels (Fig. 3B;  $P > 0.05$ ), and levels were significantly less compared with lower concentrations of leptin ( $P < 0.05$ ).

### Effects of leptin and leptin triple mutant on leptin-induced visfatin levels in conditioned media of C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice omental AT explants

Visfatin levels were significantly increased by leptin ( $10^{-11}$ ,  $10^{-9}$  M) in conditioned media of C57BL/6 *ob/ob* mouse omental AT explants and by leptin  $10^{-9}$  M in conditioned media of C57BL/Ks *db/db* mouse omental AT explants (Fig. 3, C and D;  $P < 0.01$ ,  $P < 0.05$ , respectively). Importantly, leptin-induced visfatin levels in conditioned media were significantly decreased by LTM ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M) in C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice omental AT explants (Fig. 3, E and F;  $P < 0.05$ ,  $P < 0.01$ , respectively). Again, at a leptin concentration of  $10^{-7}$  M, visfatin levels were significantly lower than leptin at a concentration of  $10^{-9}$  M ( $P < 0.05$ ), approaching baseline values (Fig. 3, C and D;  $P > 0.05$ ).

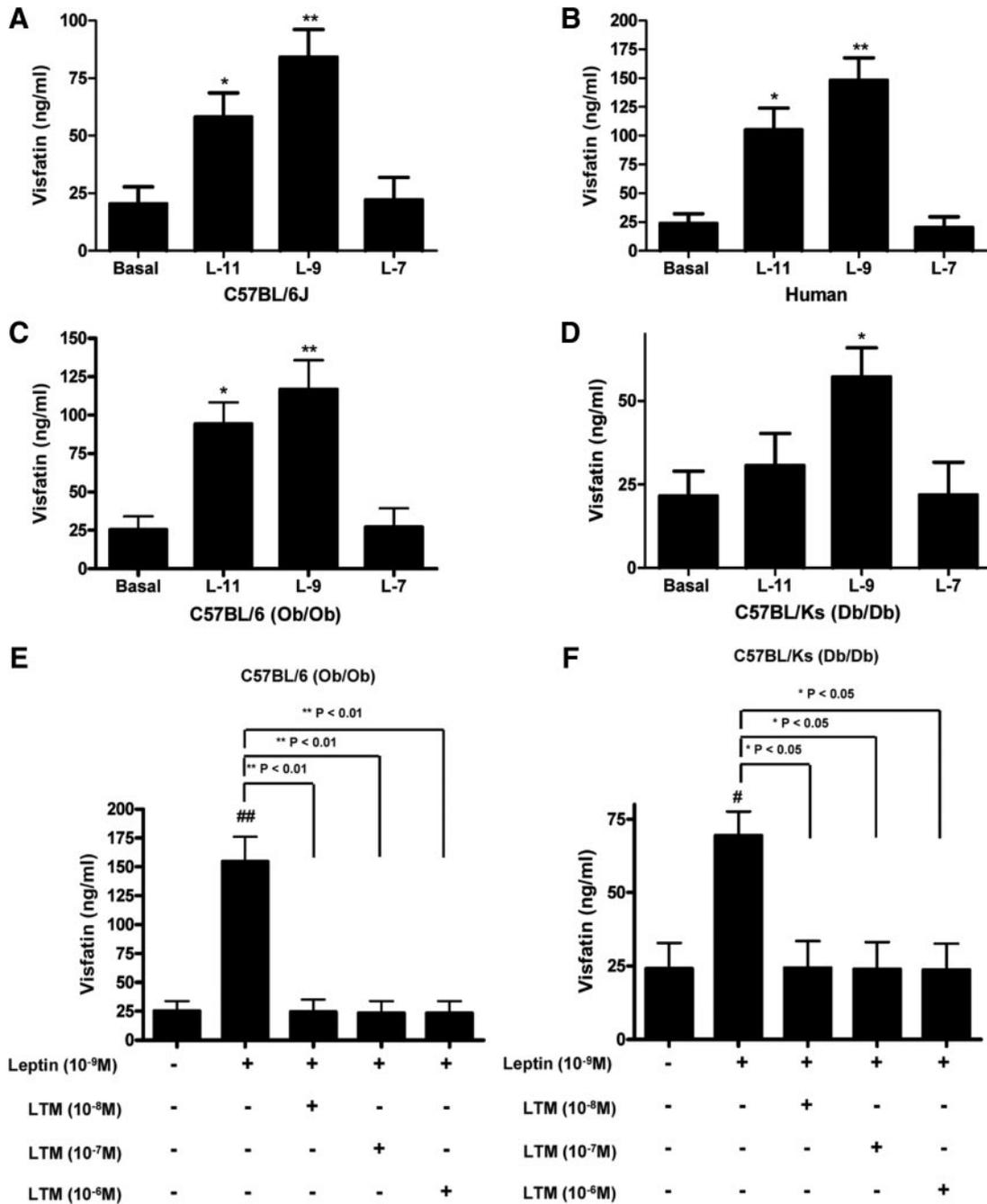


**FIG. 2.** A and B, Concentration-dependent effects of leptin  $10^{-11}$  M (L-11), leptin  $10^{-9}$  M (L-9), leptin  $10^{-7}$  M (L-7) on visfatin protein production in C57BL/6J mouse and human omental AT explants at 24 h were assessed by Western blotting, compared with basal (no supplement). Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . PSL, Phospho-stimulated light units. C and D, Concentration-dependent effects of leptin  $10^{-11}$  M (L-11), leptin  $10^{-9}$  M (L-9), and leptin  $10^{-7}$  M (L-7) on visfatin protein production in C57BL/6 *ob/ob* and C57BL/Ks *db/db* mouse omental AT explants at 24 h were assessed by Western blotting, compared with basal (no supplement). Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . PSL, Phospho-stimulated light units. E and F, Concentration-dependent effects of a mouse leptin antagonist *i.e.* LTM on leptin-induced visfatin protein production in C57BL/6 *ob/ob* and C57BL/Ks *db/db* mouse omental AT explants at 24 h were assessed by Western blotting, compared with leptin ( $10^{-9}$  M), without LTM. Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . PSL, Phospho-stimulated light units.

**Effect of leptin on SLR levels in conditioned media of C57BL/6 mouse omental AT explants**

Leptin (24 h of stimulation) concentration-dependently increased SLR levels in conditioned media of C57BL/6 mouse

omental AT explants. However, it was only at a concentration of  $10^{-7}$  M that this increase was significant (Fig. 4A;  $P < 0.01$ ). Similar findings were noted after 36 and 48 h of stimulation with leptin, respectively (see supplemental Figs. 1 and 2, published as



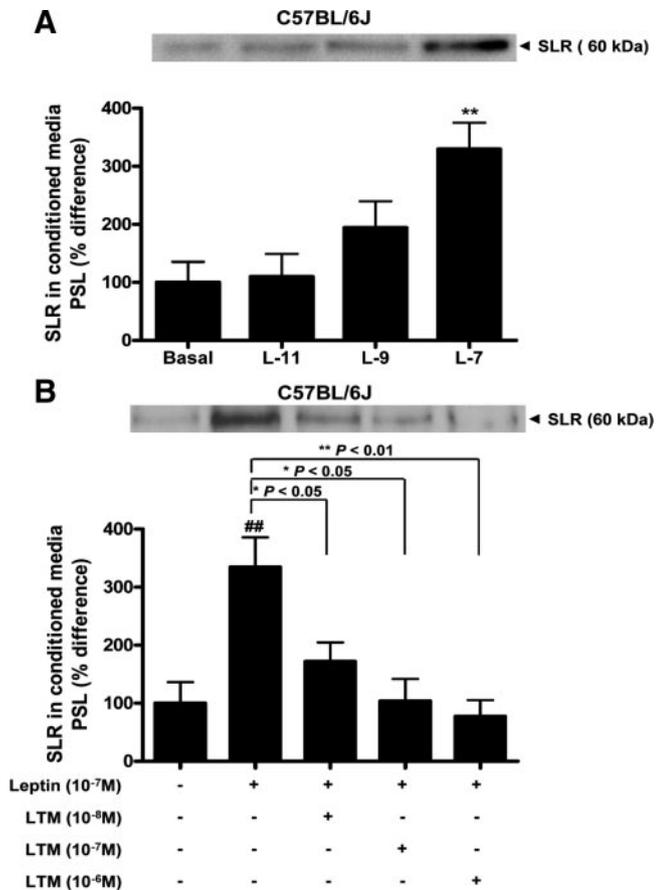
**FIG. 3.** A–D, Concentration-dependent effects of leptin  $10^{-11}$  M (L-11), leptin  $10^{-9}$  M (L-9), and leptin  $10^{-7}$  M (L-7) on visfatin levels in conditioned media of C57BL/6J mouse, human, C57BL/6 *ob/ob* mouse, and C57BL/Ks *db/db* mouse omental AT explants at 24 h were assessed by ELISA, compared with basal (no supplement). Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni’s test. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ . E and F, Concentration-dependent effects of a mouse leptin antagonist *i.e.* LTM on leptin-induced visfatin levels in conditioned media of C57BL/6 *ob/ob* and C57BL/Ks *db/db* mouse omental AT explants at 24 h were assessed by ELISA, compared with leptin ( $10^{-9}$  M), without LTM. Six independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; #,  $P < 0.05$ ; ##,  $P < 0.01$ .

supplemental data on The Endocrine Society’s Journals Online web site at <http://endo.endojournals.org>). The detected protein for SLR had an apparent molecular mass of 60 kDa (Fig. 4A, *inset*). Also, we found that both short and long leptin receptor levels were concentration-dependently increased by leptin ( $10^{-11}$ ,  $10^{-9}$ , and  $10^{-7}$  M) treatments, respectively, in C57BL/6 mouse omental AT explants. However, these were not statistically significant (data not shown). Finally, we found that the maximal effect of leptin on SLR levels at a dose of  $10^{-7}$  M was significantly

decreased by LTM in C57BL/6 mouse omental AT explants (Fig. 4B;  $P < 0.05$ ,  $P < 0.05$ , and  $P < 0.01$ , respectively) (Fig. 4B, *inset*).

**Concentration-dependent effects of leptin on PI3K and MAPK signaling pathways in C57BL/6 mouse omental AT explants**

PI3K and MAPK signaling pathways were significantly activated dose dependently by leptin in C57BL/6 mouse omental AT explants. AKT phosphorylation and Erk1/2 phosphorylation



**FIG. 4.** A, Concentration-dependent effects of leptin  $10^{-11}$  M (L-11), leptin  $10^{-9}$  M (L-9), and leptin  $10^{-7}$  M (L-7) on SLR levels in conditioned media of C57BL/6 mouse omental AT explants at 24 h were assessed by Western blotting, compared with basal (no supplement). Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test. \*\*,  $P < 0.01$ . PSL, Phospho-stimulated light units. B, Concentration-dependent effects of a mouse leptin antagonist *i.e.* LTM on leptin-induced SLR levels in conditioned media of C57BL/6J mouse omental AT explants at 24 h were assessed by Western blotting, compared with leptin ( $10^{-7}$  M), without LTM. Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . PSL, Phospho-stimulated light units.

were significantly increased concentration dependently by leptin in C57BL/6 mouse omental AT explants (Fig. 5, A and B: \*,  $P < 0.05$ , \*\*\*,  $P < 0.001$ , respectively). The detected protein for phosphorylated AKT, total AKT, phosphorylated Erk1/2 and total Erk1/2 had apparent molecular masses of 44/42, 44/42, 60, and 60 kDa, respectively (Fig. 5, A and B, insets).

### Role of PI3K and MAPK signaling pathways on leptin-induced visfatin levels

#### C57BL/6 mouse omental AT explants

Western blotting revealed that leptin-induced visfatin protein production was significantly decreased by the PI3K inhibitor (LY294002;  $50 \mu\text{M}$ ) and the MEK inhibitor (U0126;  $10 \mu\text{M}$ ) in C57BL/6 mouse omental AT explants ( $P < 0.05$  and  $P < 0.05$ , respectively) (Fig. 5C, inset).

#### Conditioned media of C57BL/6 mouse omental AT explants

ELISA analysis showed that leptin-induced visfatin levels were significantly decreased by LY294002 ( $50 \mu\text{M}$ ) and U0126 ( $10 \mu\text{M}$ ) in conditioned media of C57BL/6J mouse omental AT explants (Fig. 5D;  $P < 0.05$  and  $P < 0.01$ , respectively).

#### Effect of ip leptin injection on visfatin protein production in omental AT of C57BL/6 *ob/ob* mouse

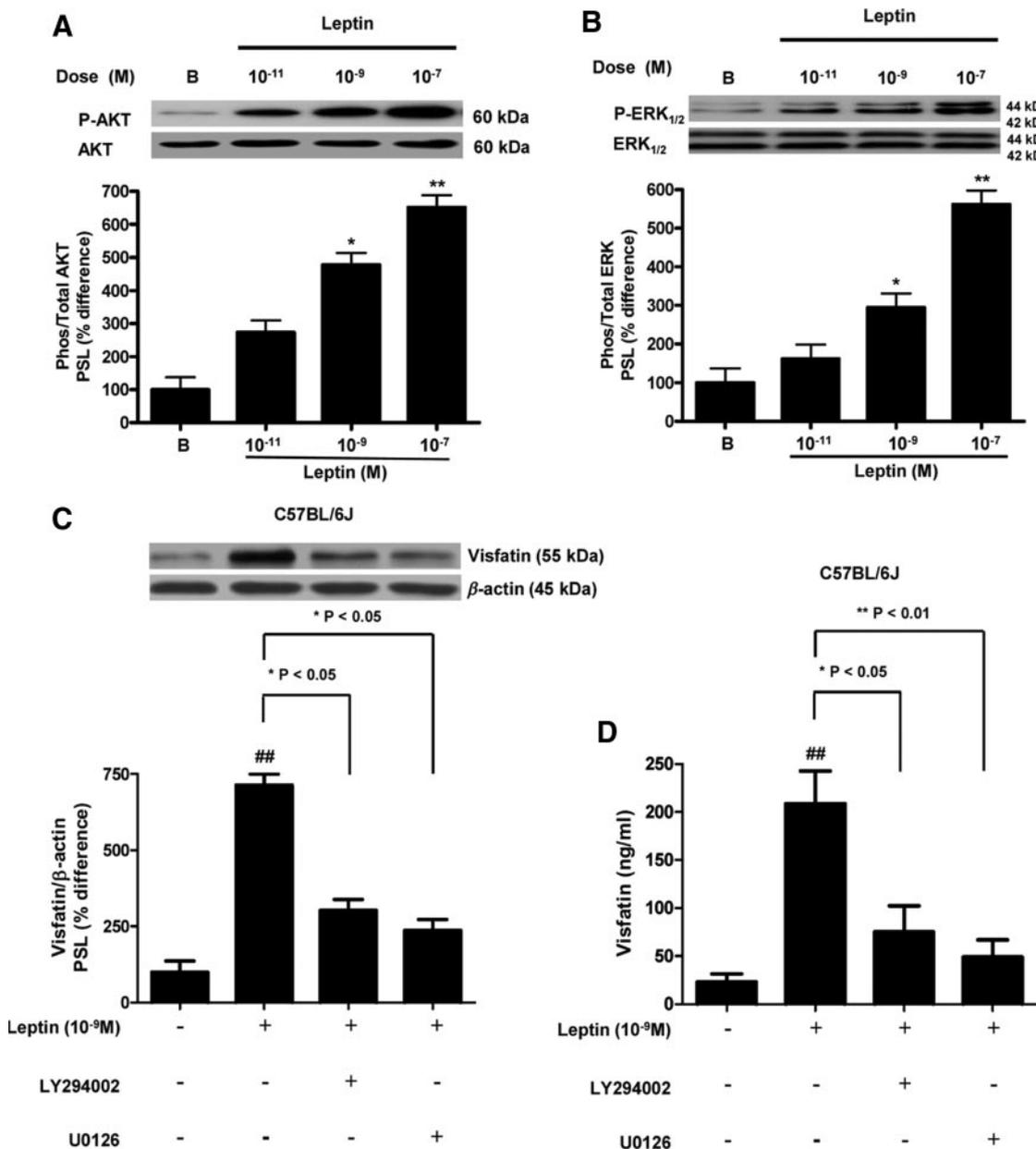
Because leptin increased visfatin protein production in omental AT *ex vivo*, in particular with respect to C57BL/6 *ob/ob* mouse, we administered ip leptin injections ( $5 \text{ mg/kg}$  per mouse) twice daily, about 12 h apart over 24 h in C57BL/6 *ob/ob* mice. The leptin injections significantly increased visfatin protein production in omental AT in these mice compared with controls (without leptin treatments) (Fig. 6;  $P < 0.05$ ).

### Discussion

We report here that leptin is a regulator of visfatin in human and murine AT as well as 3T3-L1 adipocytes. Initial experiments on 3T3-L1 adipocytes revealed that leptin increased visfatin mRNA expression concentration dependently with a maximum response at leptin  $10^{-9}$  M. This concentration is physiologically relevant in both mice and humans (14, 15). Interestingly, this effect was attenuated with a very high concentration of leptin. In view of our subsequent evidence that leptin increased visfatin protein production in omental AT *ex vivo*, in particular using AT from C57BL/6 *ob/ob* mice, we administered leptin ip ( $5 \text{ mg/kg}$  per mouse) to these mice. Leptin significantly increased visfatin protein production in omental AT in these mice compared with controls (without leptin treatments) (Fig. 6;  $P < 0.05$ ). When the leptin receptor (OB-R) gene was knocked down using siRNA, visfatin expression significantly decreased, confirming the regulation of visfatin by leptin via OB-R. Also, in both subcutaneous and omental AT of C57BL/6 mice, there was a similar pattern of leptin-induced visfatin protein production.

Given that visfatin production is greater in visceral AT (7), we subjected omental AT from human, C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice to leptin treatments and once again found the same pattern of leptin-induced visfatin protein production. The somewhat unforeseen result in C57BL/Ks *db/db* mice highlights a possible role of the membrane bound short leptin receptor (OB-Ra) in leptin-induced visfatin protein production, given that the other membrane bound short leptin receptor isoforms (OB-Rc, OB-Rd, OB-Rf) are not known to mediate leptin signaling (5). Additionally, we measured visfatin levels in conditioned media of all of the omental AT explants and found similar changes in leptin-induced visfatin protein production.

The observed regulation of visfatin by leptin was further supported by studies in which omental AT of C57BL/6 *ob/ob* and C57BL/Ks *db/db* mice was treated with leptin in the presence of a mouse leptin antagonist (LTM). In these studies there was a significant concentration-dependent decrease in leptin-induced visfatin protein production and a significant concentration-dependent decrease in leptin-induced visfatin levels in conditioned

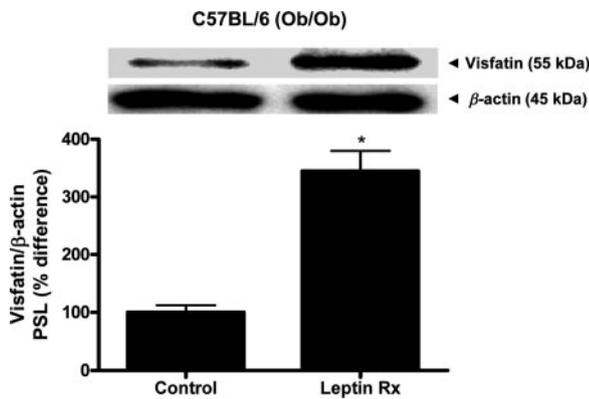


**FIG. 5.** A and B, Concentration-dependent effects of leptin on PI3K and MAPK signaling pathways in C57BL/6J mouse omental AT explants at 15 and 20 min, respectively, compared with basal (B) (no supplement). Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Group comparison by ANOVA and *post hoc* Bonferroni's test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . PSL, Phospho-stimulated light units; P-AKT, phospho-Akt; P-ERK, phospho-ERK; Phos/Total, phosphorylation of the total. C, Effects of PI3K (LY294002) and MEK (U0126) inhibitors on leptin-induced visfatin protein production in C57BL/6 mice omental AT explants at 24 h were assessed by Western blotting, compared with leptin ( $10^{-9}$  M) without inhibitors. Data are expressed as percent difference of median of basal. Six independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*,  $P < 0.05$ . D, Effects of PI3K (LY294002) and MEK (U0126) inhibitors on leptin-induced visfatin levels in conditioned media of C57BL/6 mice omental AT explants at 24 h were assessed by ELISA, compared with leptin ( $10^{-9}$  M) without inhibitors. Six independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; ##,  $P < 0.01$ .

media, respectively. The precise mechanism of leptin-induced visfatin production and regulation remains unclear.

The apparently diminished response to a very high concentration of leptin with respect to visfatin production may be partly explained by the novel finding of significantly increased SLR levels by leptin  $10^{-7}$  M in conditioned media of C57BL/6J mice omental AT explants. Furthermore, we showed that this phenomenon, similar to the up-regulation of visfatin by leptin, was significantly decreased by the mouse leptin antagonist (LTM). Binding of leptin

with SLR decreases the bioavailability of leptin to membrane bound leptin receptors, and this attenuates the biological actions of leptin (16). We also found that in omental AT explants of C57BL/6 mice, levels for both the short and long leptin receptors were higher during exposure to leptin ( $10^{-11}$  to  $10^{-7}$  M), although this did not achieve statistical significance. These data are in keeping with those of Cohen *et al.* (17), who reported induction of plasma SLR levels as well as an increase of liver leptin receptor (both short and long) expression by leptin administration in C57BL/6 mice.



**FIG. 6.** Effect of ip leptin injection (5 mg/kg per mouse) (Leptin Rx) administered twice daily, about 12 h apart on visfatin protein production in C57BL/6 *ob/ob* mice omental AT at 24 h were assessed by Western blotting, compared with control C57BL/6 *ob/ob* mice (without leptin treatments). Data are expressed as percent difference of median of C57BL/6 *ob/ob* mice omental AT of controls. Four independent experiments were performed and each experiment was carried out in three replicates. Differences between groups were assessed using the unpaired *t* test. \*, *P* < 0.05. PSL, Phospho-stimulated light units.

In obesity, circulating leptin (18) and visfatin (19, 20) levels appear to be increased, although one study reported reduced circulating visfatin levels with obesity (21). Interestingly, Krzyzanowska *et al.* (22) showed that massive weight loss in morbidly obese patients after gastroplastic surgery was accompanied by an increase in circulating visfatin levels. We tentatively propose that the present data may serve to explain the apparent paradox with visfatin levels observed in obese states, notably an increase in soluble leptin receptors or leptin resistance in morbidly obese states. The present observations reinforce the notion that leptin, specifically free bioavailable leptin, up-regulates visfatin production in AT. It is important to bear in mind that the regulation of visfatin in AT is probably multifactorial. In relation to this, TNF- $\alpha$  has recently been shown to increase levels of visfatin in human visceral AT (23). Further studies are needed to elucidate the role of other factors that regulate visfatin in AT.

To tease out the intracellular signaling pathways involved in the regulation of visfatin production by leptin, we subjected omental AT of C57BL/6 mice to leptin in the presence of inhibitors of MAPK and PI3K. This significantly decreased leptin-induced visfatin protein production and decreased leptin-induced visfatin levels in conditioned media. The rationale for studying these signaling pathways was based on our observations in C57BL/Ks *db/db* mice [short leptin receptor (OB-Ra) present; long leptin receptor (OB-Rb) absent], which exhibited a dose-dependent increase in leptin-induced visfatin protein production, albeit not as prominently as observed in C57BL/6 and C57BL/6 *ob/ob* mice. The MAPK and PI3K signaling pathways are known to functionally signal through both the short (OB-Ra) and long (OB-Rb) leptin receptors (8, 24). Our findings suggest an important role of both MAPK and PI3K signaling pathways in the regulation of visfatin production by leptin in AT. These findings may have important implications in other tissues, *i.e.* in the kidney, in which leptin signaling is maintained through the function of the short leptin receptor, OB-Ra (25). Also, it has recently been suggested that OB-Ra plays an important role in the pathogenesis of nonalcoholic steatohepatitis (26), an obser-

vation that is of interest, given that the liver is another important site of visfatin production (7).

Finally, *in vivo* experiments using ip leptin injections to C57BL/6 *ob/ob* mice further supported leptin-induced visfatin protein production in omental AT.

Collectively these observations suggest that the diverse actions of leptin in AT may be mediated partially by visfatin. Equally, the various functions of visfatin may be driven, in part, by leptin, illustrating the need for further studies. It would be of interest to know whether the effects of leptin on visfatin production are also applicable to other tissues. Additionally, the present data raise the possibility of cross talk between leptin and visfatin, possibly involving the MAPK and PI3K signaling pathways.

In conclusion, leptin and visfatin may therefore play a coordinated role in various bodily functions, including adipogenesis. Unraveling of the varied signaling mechanisms that link leptin with visfatin may further elucidate the pathogenesis of obesity and associated disorders.

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