

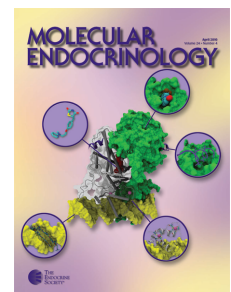
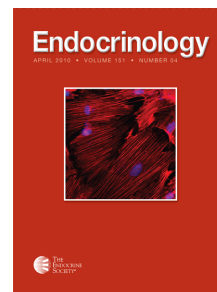
# Endocrinology

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## Identification of Macrophage Inhibitory Cytokine-1 in Adipose Tissue and Its Secretion as an Adipokine by Human Adipocytes

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Macrophage inhibitory cytokine-1 (MIC-1), a divergent member of the TGF- $\beta$  superfamily, is involved in the control of multiple cellular processes and mediates cachexia through the inhibition of appetite. Adipose tissue as an endocrine organ secretes proteins (adipokines) that regulate energy homeostasis and other cellular functions. This study investigated whether MIC-1 is expressed in adipose tissue and whether MIC-1 is a secretory product of adipocytes. Mouse and human adipose tissues were collected from different depots. 3T3-L1 preadipocytes and human preadipocytes were induced to differentiate into adipocytes in cell culture. MIC-1 mRNA was detected in the major mouse adipose depots (epididymal, perirenal, sc). In these depots, MIC-1 gene expression was evident in both isolated mature adipocytes and stromal-vascular cells. In 3T3-L1 adipocytes, MIC-1 mRNA was detected before and after differentiation. MIC-1 mRNA and protein secretion were evident in human preadipocytes as well as differentiated adipocytes. MIC-1 production by human adipocytes was stimulated by H<sub>2</sub>O<sub>2</sub> and 15d-prostaglandin J<sub>2</sub>. In addition, recombinant MIC-1 increased adiponectin secretion by differentiated human adipocytes. MIC-1 mRNA and protein were also observed in human sc and visceral fat. MIC-1 mRNA levels were positively correlated with adiponectin mRNA. Moreover, MIC-1 mRNA was negatively associated with body mass index and body fat mass in human subjects. We conclude that MIC-1 is expressed in adipose tissue and secreted from adipocytes and is therefore a new adipokine. MIC-1 may have a paracrine role in the modulation of adipose tissue function and body fat mass. (*Endocrinology* 150: 1688–1696, 2009)

**M**acrophage inhibitory cytokine (MIC)-1 is a divergent member of the TGF- $\beta$  superfamily (1). The cDNA sequence of MIC-1 is identical with several other sequences, including growth differentiation factor-15 (2, 3), placental bone morphogenetic protein (4), placental transforming growth factor- $\beta$  (5, 6), prostate-derived factor (7), and nonsteroidal anti-inflammatory drug-activated protein-1 (8). MIC-1 mRNA encodes a secreted protein, resulting from cleavage of a propeptide to give rise to the mature form as a 25-kDa dimer, which contains seven conserved cysteine residues in the carboxyl terminal (1, 2, 9). MIC-1 is distributed in various tissues, being highly expressed in macrophages and epithelial cells of the choroid plexus, prostate, lung, kidney proximal tubules, and intestinal mucosa (7, 10, 11).

MIC-1 was initially considered to function as a macrophage

inhibitor (1), but further studies suggested that it has other roles, involving the regulation of cellular processes including the cell cycle (12), proliferation (13), differentiation (14), and apoptosis (15). MIC-1 expression can be induced by stress conditions such as tissue injury (16, 17) and malignancy (18). MIC-1 is overexpressed by a variety of cancers, which probably relates to its antitumorigenic and proapoptotic properties (19, 20). MIC-1 has also been implicated as a cachexia mediator inducing weight loss because MIC-1 tumor-bearing mice and MIC-1-transgenic mice exhibit a marked decrease in body weight and total fat mass (21, 22). Furthermore, serum MIC-1 levels are higher in prostate cancer patients with cachexia than those without (21). The cachectic effects of MIC-1 have been attributed to its central actions on appetite control in the hypothalamus, inhibiting the

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Abbreviations: BAT, Brown adipose tissue; BMI, body mass index; HOMA-IR, homeostasis model of insulin resistance; MIC-1, macrophage inhibitory cytokine-1; PG, prostaglandin; PPAR, peroxisomal proliferator-activated receptor; SV, stromal vascular; TBS, Tris-buffered saline; WAT, white adipose tissue; ZAG, zinc- $\alpha$ 2-glycoprotein.

orexigenic mediator neuropeptide Y and stimulating the anorexic proopiomelanocortin (21). However, to date, little is known of the presence and potential actions of MIC-1 in adipose tissue.

White adipose tissue (WAT) is recognized as an important endocrine organ that not only stores triglyceride as energy but also regulates energy homeostasis and other metabolic processes (23). Adipose tissue has cross talk with the brain, liver, and skeletal muscle through its secreted proteins (adipokines). Over the last decade, a growing number of adipokines have been identified such as leptin, adiponectin, visfatin, chemerin, and zinc- $\alpha$ -2-glycoprotein (ZAG), which modulate appetite, nutrient metabolism, insulin sensitivity, stress responses, and inflammation in an autocrine/paracrine and/or endocrine manner (23, 24). Given the diverse roles of MIC-1, adipose tissue could be important in the physiological function of this factor.

The aim of this study was therefore to examine whether MIC-1 is expressed in adipose tissue, both murine and human, and whether MIC-1 is a secretory product of adipocytes. The study also investigated factors that modulate MIC-1 production and the potential role of MIC-1 in adipocytes.

## Materials and Methods

### Tissue collection

Fifteen-week-old male mice (C57BL/6J) from Harlan (Bicester, Oxon, UK) were housed at an ambient temperature of  $22 \pm 1$  C under a 12-h light, 12-h dark cycle (lights on at 0700 h) and fed *ad libitum* (CRM diet; Labsure, Poole, UK). The mice were killed by cervical dislocation and the following tissues rapidly dissected and frozen in liquid nitrogen: WAT (epididymal, perirenal, and sc depots), interscapular brown adipose tissue (BAT), intestine, spleen, liver, heart, kidneys, lungs, and brain. Mouse colonic adenocarcinoma was kindly provided by Professor M. J. Tisdale (Aston University, Birmingham, UK). Nine-week-old male obese (*ob/ob*) and lean (*ob/+*) mice were purchased from Harlan. Epididymal adipose tissue was collected after mice were killed. The tissues were stored at  $-80$  C until analysis. All animal studies were carried out in accordance with the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986, in the fully licensed premises of the University of Liverpool.

Human adipose tissue samples were obtained during elective abdominal surgery (cholecystectomy or bariatric surgery) from patients [12 males and 14 females, aged  $44 \pm 12$  yr (mean  $\pm$  SD) (range 19–76 yr), body mass index (BMI)  $44.8 \pm 17.7$  kg/m<sup>2</sup> (mean  $\pm$  SD, range 19–80 kg/m<sup>2</sup>)]. Body composition was assessed by whole-body electrical bioimpedance analysis (Bodystat 1500; Bodystat, Isle of Man, UK), allowing the measurement of total fat mass. Blood samples were taken, and plasma obtained after centrifugation and stored at  $-80$  C. All subjects were free from major illnesses (e.g. infection, diabetes, cancer) at the time of surgery. Fully informed and written consent was obtained in all cases and the study protocol was approved by the Sefton Research Ethics Committee. After removal of adipose tissue from omental (visceral) and abdominal sc sites, the samples were immediately frozen in liquid nitrogen and stored at  $-80$  C until use.

### Cell culture

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were cultured as described previously (25). Differentiation of the cells was initiated 2 d after confluence by incubation for 2 d in DMEM containing 10% fetal calf serum, 1  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine and 2  $\mu$ M insulin. After induction, cells were maintained in feeding medium (DMEM with 10% fetal calf serum and 2  $\mu$ M insulin), which was changed every other day. Differentiation into adi-

pocytes was confirmed by accumulation of lipid droplets observed under the microscope. The cells were either maintained for up to 16 d after differentiation and sampled every 2–4 d for the study of MIC-1 expression during differentiation or maintained for 10 d and incubated with specific agents for 24 h. The agents used were as follows: mouse leptin (0.1 or 2  $\mu$ M; Sigma, Poole, Dorset, UK), mouse IL-1 $\beta$  (5 ng/ml or 20 ng/ml; Sigma), mouse TNF- $\alpha$  (10 or 30 nM; Sigma), and 15d-prostaglandin (PG) J<sub>2</sub> (3 or 10  $\mu$ M; Alexis Biochemicals, Nottingham, UK). The addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 0.1, 0.25, 0.5, and 2 mM; Sigma) was for 4 h. The control cells had no agent added to the media. After the completion of treatment, cells were collected in Trizol reagent (Invitrogen, Carlsbad, CA) and stored at  $-80$  C until analysis.

Human primary adipocytes isolated from sc adipose tissue and culture media were obtained from Zen-Bio (Research Triangle Park, NC). The cells were from a female donor (24 yr old) with a BMI of 22 kg/m<sup>2</sup>. Cells were maintained and induced to differentiation according to the manufacturer's protocol. Briefly, cells were maintained in preadipocyte medium containing DMEM/Ham's F12 (1:1, vol/vol), 10% fetal calf serum, 15 mM HEPES, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B. At confluence, cells were induced in differentiation medium supplemented with 0.25 mM 3-isobutyl-1-methyl-xanthine, 1  $\mu$ M dexamethasone, and 10  $\mu$ M of a peroxisomal proliferator-activated receptor (PPAR)- $\gamma$  agonist for 7 d. Cells were then cultured with adipose medium containing DMEM/Ham's F12 (1:1, vol/vol), 3% fetal calf serum, 1  $\mu$ M dexamethasone, 15 mM HEPES, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B, and the medium changed every 3 d. The differentiated cells at d 14 after differentiation were treated with human TNF- $\alpha$  (30 ng/ml), H<sub>2</sub>O<sub>2</sub> (0.5 mM), 15d-PGJ<sub>2</sub> (5  $\mu$ M), or recombinant human MIC-1 (0.05, 0.5, or 5 ng/ml; R&D Systems, Abingdon, UK) for 24 h. The control cells had no agent added to the media. Cells were harvested in Trizol reagent (Invitrogen) or lysis buffer and stored at  $-80$  C until analysis.

### RNA extraction and cDNA synthesis

Total RNA was extracted from tissues or cells using Trizol and the RNA concentration determined from the absorbance at 260 nm. First-strand DNA was reverse transcribed from 0.5  $\mu$ g of total RNA using a Reverse-iT first-strand synthesis kit (ABgene, Epsom, UK) in a final volume of 10  $\mu$ l.

### RT-PCR

MIC-1 gene expression in tissues and adipocytes was detected by RT-PCR. One microliter of reverse transcription reaction was amplified with 20  $\mu$ M of each primer and 1.1 $\times$  Reddy Mix PCR master mix (ABgene) in a volume of 25  $\mu$ l. The following primer pairs were used: mouse MIC-1, 5'-CGC CCT GGC AAT GCC TGA ACA ACG AC-3' (forward), 5'-GCA CGC GGT AGG CTT CGG GGA GAC C-3' (reverse); mouse  $\beta$ -actin, 5'-TGC TGT CCC TGT ATG CCT CT-3' (forward), 5'-AGG TCT TTA CGG ATG TCA ACG-3' (reverse); human MIC-1, 5'-ACT GCT GGC AGA ATC TTC GT-3' (forward), 5'-AAT GAG CAC CAT GGG ATT GT-3' (reverse); human MAC-1, 5'-CAT AGC CAG CGG ATA GCA-3' (forward), 5'-GCA ACT GTA GTT TCA GGG TC-3' (reverse). Primers for human adiponectin, ZAG, and  $\beta$ -actin were as reported previously (26, 27). PCR was performed on a thermal cycler (Hybaid, Middlesex, UK), with an initial denaturation at 94 C for 2 min followed by 32 cycles for MIC-1, 25 cycles for adiponectin, 33 cycles for ZAG, and 23 cycles for  $\beta$ -actin, consisting of denaturation at 94 C for 20 sec, annealing at optimized temperature for 1 min, and extension at 72 C for 30 sec. Negative controls (without template) were also carried out. To confirm the identity of PCR products, the products were sequenced commercially (MWG, Ebersberg, Germany). PCR products were separated on a 1% agarose gel and images recorded with a Kodak 1D Image Analysis System (Kodak Digital Science, Rochester, NY).

### Real-time PCR

Real-time PCR amplification was performed in a final volume of 12.5  $\mu$ l, containing cDNA (equivalent to 12.5 ng of RNA), optimized concentrations of primers, TaqMan probe FAM-TAMRA, and a master mix

made from quantitative PCR core kit (Eurogentec, Southampton, UK) using a Mx3005P detector (Stratagene, La Jolla, CA). Except for human MIC-1, the sequences of primers and probes were as described previously (26). The sequences for human MIC-1 (accession no. NM\_004864) were: 5'-CCA TGG TGC TCA TTC AAA AGA C-3' (forward), 5'-GGA AGG ACC AGG ACT GCT CAT-3' (reverse), 5'-FAM-TGA CTT GTT AGC CAA AGA CTG CCA CTG CA-TAMRA-3' (probe). PCR amplification was performed in duplicate using a 96-well plate and the PCR cycling conditions were as follows: 50 C for 2 min and 95 C for 10 min followed by 40 cycles (95 C for 15 sec, 60 C for 1 min). Blank controls without cDNA were run in parallel.  $\beta$ -Actin was used as a reference gene. All samples were normalized to the  $\beta$ -actin values and the results expressed as fold changes of cycle threshold (Ct) value relative to controls using the  $2^{-\Delta\Delta Ct}$  formula.

### Western blotting

Proteins were isolated from mouse WAT, BAT, and cultured adipocytes (ZenBio). Ten percent of homogenates from human WAT samples were prepared in a buffer of 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4), and protein concentration was determined by the BCA method. Tricine-sodium dodecyl sulfate electrophoresis (28) of the samples was performed on 10% polyacrylamide slab gels (Mini Protean Tetra; Bio-Rad, Hercules, CA) using the same aliquots of sodium dodecyl sulfate-solubilized proteins (20–30  $\mu$ g/slot). Proteins from the gel were blotted onto a nitrocellulose membrane (Hybond C EXTRA; Amersham Bioscience, Amersham, Buckinghamshire, UK) by wet transfer (Trans Blot; Bio-Rad) at 100 V for 1 h.

The membrane was blocked in 1 $\times$  Tris-buffered saline (TBS) containing 0.1% Tween 20 and 2% BSA overnight at +4 C with constant agitation. Immunoblotting was performed using a polyclonal antibody against mouse MIC-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution or a polyclonal antibody against human MIC-1 (R&D Systems) at 0.1  $\mu$ g/ml. In both cases, antibodies were diluted in TBS containing 0.1% Tween 20 and 2% BSA and incubation was overnight at +4 C. Blots were then incubated with an antioat horseradish peroxidase secondary antibody (R&D Systems) at a dilution of 1:2000. Signal was detected on film by chemiluminescence (West Pico kit, Pierce, Rockford, IL). The size of the proteins detected was estimated with PageRuler protein markers (Fermentas, Hanover, MD). For the control of protein loading, blots were stripped and incubated with a mouse monoclonal anti- $\alpha$ -tubulin antibody (Sigma) diluted at 1:5000 in TBS containing 0.1% Tween 20 and 2% BSA and subsequently incubated with horseradish peroxidase conjugated rabbit antimouse IgG (Bio-Rad) at 1:2000.

### Measurement of MIC-1, adiponectin, and ZAG protein by ELISA

Zen-Bio cells were differentiated as described above. For determining the secretion of MIC-1, culture medium was collected at the indicated time point before and after differentiation. For examining the effects of 15d-PG<sub>2</sub>, TNF- $\alpha$ , and H<sub>2</sub>O<sub>2</sub> on MIC-1 or adiponectin secretion, cells at d 14 after differentiation were incubated with the indicated concentration of 15d-PG<sub>2</sub> or TNF- $\alpha$  for 24 h or H<sub>2</sub>O<sub>2</sub> for 4 h. The medium from each sample was collected and centrifuged at 1000 rpm for 10 min to remove cell debris with the supernatant being stored at -80 C until analysis. The concentration of MIC-1 or adiponectin in each sample was determined using ELISA kits (both from R&D Systems, Abingdon, UK) according to the manufacturer's protocol. To determine the effects of MIC-1 on ZAG and adiponectin release from adipocytes, cells at d 14 after differentiation were incubated with the indicated concentrations of recombinant human MIC-1 (R&D Systems) for 24 h. The concentration of ZAG in the medium was measured using an ELISA kit (BioVendor, Modrice Czech Republic) and adiponectin concentration determined as above.

### Measurement of plasma insulin and glucose

Fasting plasma insulin levels were determined by ELISA (BioSource Europe, Nivelles, Belgium). Plasma glucose concentrations were mea-

sured using the glucose hexokinase method with the Advia 1650 system (Bayer UK, Newbury, UK). Insulin resistance was estimated using the homeostasis model of insulin resistance (HOMA-IR) with the formula: HOMA-IR = fasting insulin (international units per milliliter)  $\times$  fasting glucose (millimoles per liter)/22.5.

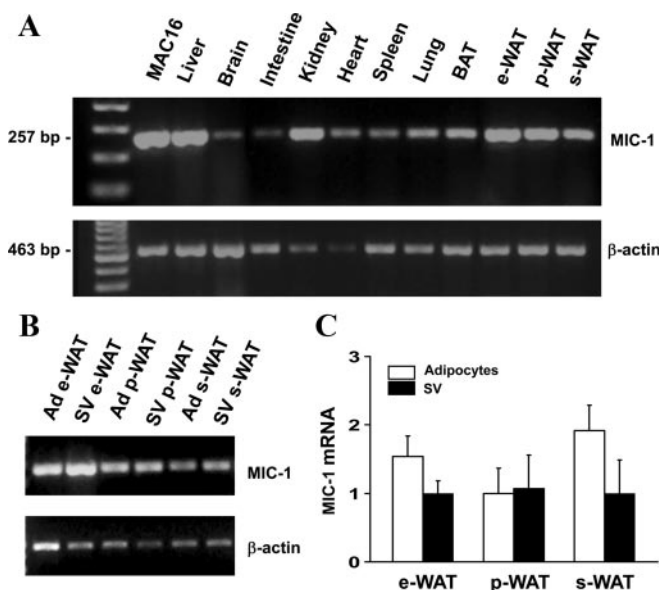
### Statistical analysis

Data are expressed as means  $\pm$  SEM. Differences between two groups were analyzed by Student's unpaired *t* test, and a paired *t* test was used for comparing depot expression. Differences between multigroups were analyzed by ANOVA coupled with Bonferroni's *t* tests. The association between MIC-1 and adiponectin mRNA was assessed with Pearson's correlation test. Differences were considered as statistically significant when *P* < 0.05.

## Results

### MIC-1 gene expression in mouse adipose tissue

In the initial studies, the gene expression pattern of MIC-1 was examined in mouse tissues by RT-PCR. A strong signal was detected in the mouse colonic adenocarcinoma tumor and the liver, and a moderate signal was present in interscapular BAT and the WAT depots (Fig. 1A). Three different white fat depots were examined (epididymal, perirenal, and sc) and each showed a marked signal for MIC-1 mRNA. The 257-bp PCR product was sequenced and found to be 100% identical with the corresponding region of murine MIC-1 cDNA. Whether the *MIC-1* gene was expressed in mature adipocytes and/or in stromal vascular (SV) fraction of white fat was then examined. As shown in Fig. 1B, the MIC-1 transcript was detected in both mature adipocytes and in the cells of the SV fraction. Next, the relative MIC-1 mRNA levels in the two fractions was determined by real-time PCR in each of the three WAT depots (epididymal,



**FIG. 1.** *MIC-1* gene expression in various mouse tissues. A, RT-PCR analysis of RNA isolated from tissues of adult male C57B6/J mice. B, *MIC-1* gene expression by RT-PCR in isolated mature adipocytes (Ad) and the SV fraction, obtained by collagenase digestion of adipose tissue. C, *MIC-1* mRNA levels in isolated mature adipocytes and the SV fraction by real-time PCR; data are means  $\pm$  SEM for groups of five to six. e-WAT, Epididymal adipose tissue; p, perirenal; s, sc; BAT, interscapular BAT.

perirenal, and sc), and no significant differences between adipocytes and the SV cells were apparent (Fig. 1C). We further assessed MIC-1 expression in epididymal adipose tissue harvested from *ob/ob* mice and their lean counterparts. mRNA levels of MIC-1 were increased by 2.7-fold in *ob/ob* mice relative to lean controls [ $1.00 \pm 0.25$  (lean) *vs.*  $2.70 \pm 0.34$  (obese),  $P < 0.01$ ].

### Expression and regulation of MIC-1 in 3T3-L1 adipocytes

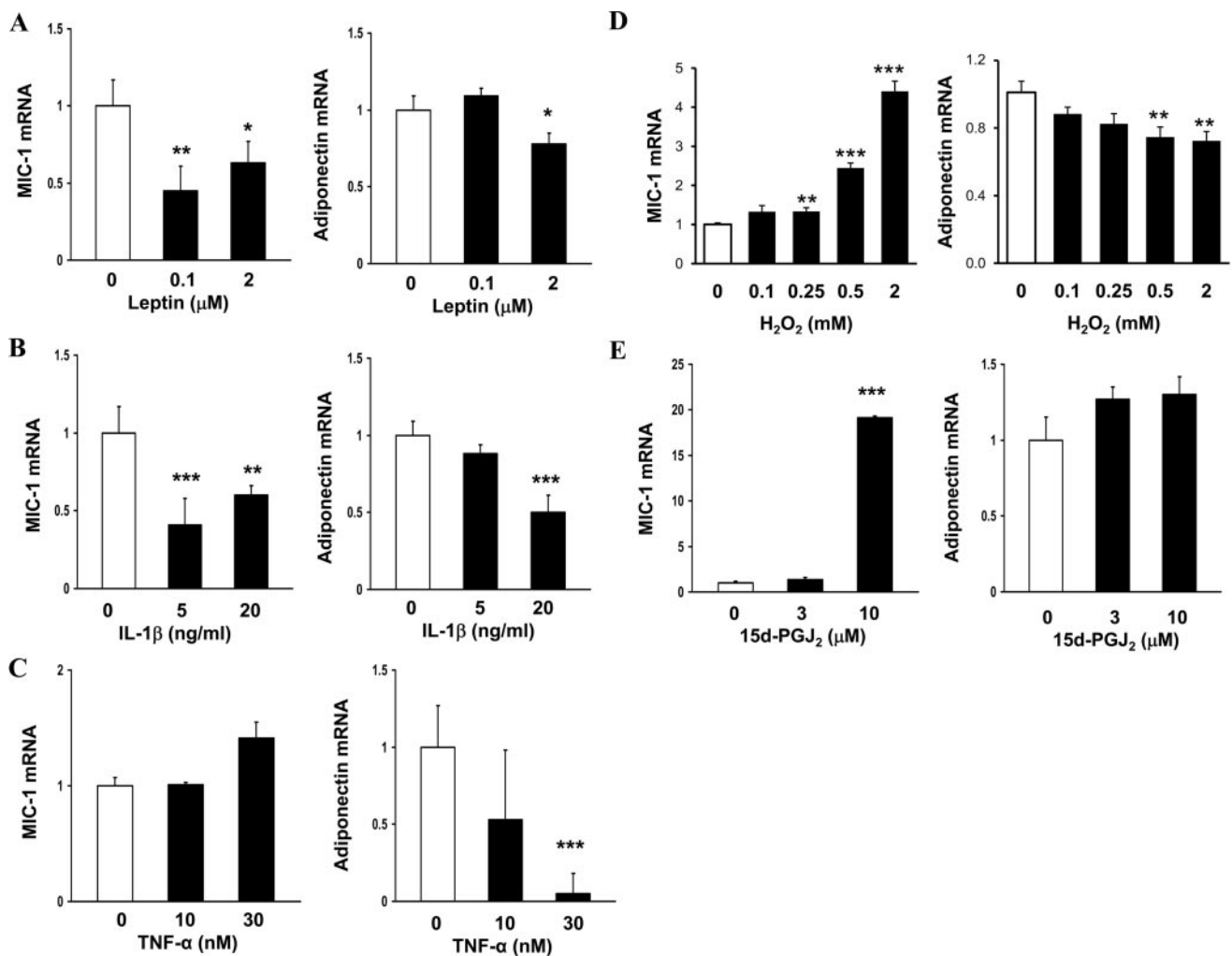
Because the *MIC-1* gene was expressed in freshly isolated mouse adipocytes, we further investigated its expression in 3T3-L1 adipocytes in culture. Examination by RT-PCR indicated that the MIC-1 signal was evident both before and after the induction of differentiation of the cells to adipocytes; consistently, MIC-1 mRNA was detectable in 3T3-L1 preadipocytes as well as differentiated adipocytes using real-time PCR (data not shown).

To investigate the possible regulation of MIC-1 expression, the effects of leptin, proinflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ), H<sub>2</sub>O<sub>2</sub> (an oxidative stress inducer), and a PPAR $\gamma$  agonist 15d-PGJ<sub>2</sub> were examined on MIC-1 gene expression in differ-

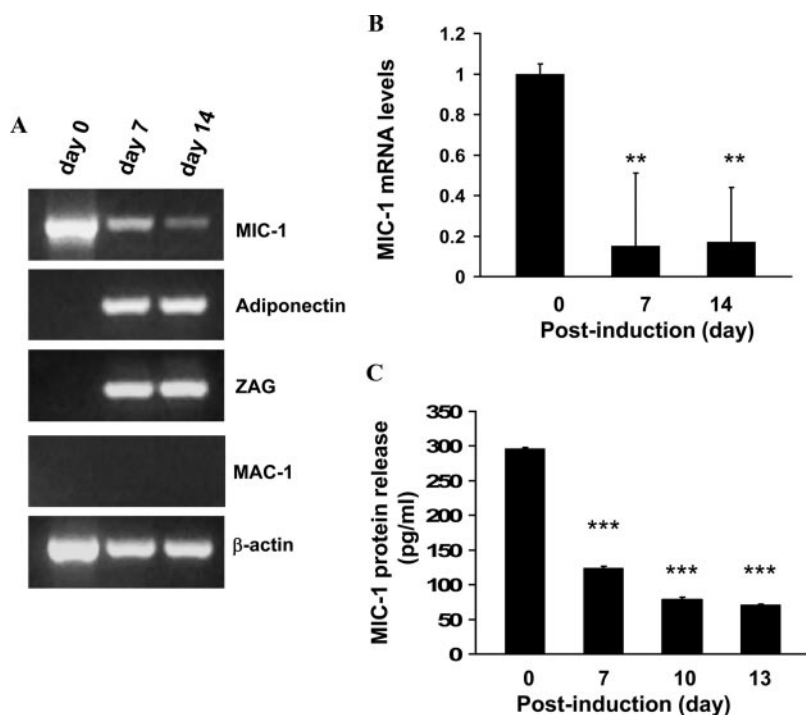
entiated 3T3-L1 adipocytes (d 10). To assess the specificity of the effect of these agents on MIC-1 expression, mRNA levels of adiponectin were also determined.

Leptin led to a reduction in MIC-1 mRNA levels, which decreased by 55% at the lower dose ( $P < 0.01$ ) and 37% at the higher dose ( $P < 0.05$ ) (Fig. 2A). IL-1 $\beta$  treatment resulted in a 59% fall in MIC-1 mRNA level at the lower dose ( $P < 0.001$ ) and a 40% decrease at the higher dose ( $P < 0.01$ ) (Fig. 2B). However, addition of TNF- $\alpha$  had no effect on MIC-1 mRNA (Fig. 2C). Treatment with H<sub>2</sub>O<sub>2</sub> for 4 h induced a dose-dependent increase in MIC-1 mRNA levels. The rise began at the 0.1 mM dose (by 33%) and was 4.4-fold higher compared with the controls at the highest dose (2 mM,  $P < 0.001$ ) (Fig. 2D). 15d-PGJ<sub>2</sub> led to a substantial (19-fold) increase in MIC-1 mRNA levels at the higher dose ( $P < 0.001$ ) (Fig. 2E).

Leptin and IL-1 $\beta$  led to a decrease in adiponectin mRNA at the higher dose ( $P < 0.05$  and  $P < 0.001$ , respectively) (Fig. 2, A and B). A 20-fold reduction of adiponectin mRNA was induced by TNF- $\alpha$  at the higher dose ( $P < 0.01$ ) (Fig. 2C). H<sub>2</sub>O<sub>2</sub> treatment caused a dose-dependent fall in adiponectin mRNA (Fig.



**FIG. 2.** Regulation of *MIC-1* gene expression in 3T3-L1 adipocytes. Effects of leptin (A), IL-1 $\beta$  (B), TNF- $\alpha$  (C), H<sub>2</sub>O<sub>2</sub> (D), and 15d-PGJ<sub>2</sub> (E) on MIC-1 and adiponectin mRNA levels in 3T3-L1 adipocytes. Cells were harvested at d 11 after the induction of differentiation and incubated for 24 h in media to which each agent was added. Leptin (0.1 or 2  $\mu$ M), IL-1 $\beta$  (5 or 20 ng/ml), TNF- $\alpha$  (10 or 30 nM), and 15d-PGJ<sub>2</sub> (3 or 10  $\mu$ M). The addition of H<sub>2</sub>O<sub>2</sub> at the indicated concentrations (0.1, 0.25, 0.5, and 2 mM) was for 4 h. Control cells received no addition. MIC-1 and adiponectin mRNA levels were determined by real-time PCR. Data are means  $\pm$  SEM for groups of four to five, presented as changes relative to controls. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with controls.



**FIG. 3.** MIC-1 gene expression and protein secretion in human adipocytes. A, RT-PCR of MIC-1 gene expression in comparison with adiponectin, ZAG, MAC-1, and  $\beta$ -actin (reference gene) in ZenBio preadipocytes (d 0, before induction of differentiation) and differentiated adipocytes at d 7 and 14 after induction. B, MIC-1 mRNA levels in ZenBio cells before and after differentiation by real-time PCR. C, Secretion of MIC-1 protein by human adipocytes, measured as MIC-1 protein concentrations in the medium of ZenBio cells before and after differentiation. Data are means  $\pm$  SEM for groups of three, presented as changes relative to the mean value of d 0. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with d 0.

2D). In contrast, adiponectin mRNA levels were unaffected by 15d-PGJ<sub>2</sub> (Fig. 2E).

### Expression and secretion of MIC-1 by human adipocytes

Because MIC-1 mRNA was present in murine 3T3-L1 adipocytes, we further explored whether the MIC-1 gene was also expressed by human adipocytes. As shown in Fig. 3A, a clear signal for MIC-1 mRNA was detected by RT-PCR in human preadipocytes (d 0), and adipocytes at d 7 and 14 after differentiation. Sequence analysis of the PCR product demonstrated 100% identity with the corresponding region of human MIC-1 cDNA. Expression of adiponectin and ZAG mRNA was similarly examined for comparison with MIC-1, and the mRNA for both adipokines was detected in differentiated adipocytes only at d 7 and 14, consistent with our previous report (27).

We also assessed whether gene expression of MIC-1 in preadipocytes was due to the contamination of macrophages; this was not the case because MAC-1, a macrophage marker, was not expressed in preadipocytes or mature adipocytes (Fig. 3A). Further quantitation of MIC-1 mRNA by real-time PCR showed that the expression levels were 6-fold higher in preadipocytes (d 0) than in differentiated adipocytes (d 7 and 14) (both  $P < 0.01$ ; Fig. 3B). We next examined whether MIC-1 is secreted by human adipocytes and is therefore an adipokine. Consistent with the gene expression results, MIC-1 protein was detected by ELISA in the medium of both preadipocytes (d 0) and adipocytes at d 7, 10, and 13 after differentiation. The MIC-1 levels were higher in

preadipocytes than in differentiated adipocytes (all  $P < 0.001$ ; Fig. 3C).

### Regulation of MIC-1 expression and secretion in human adipocytes

The effects of TNF- $\alpha$ , H<sub>2</sub>O<sub>2</sub>, and 15D-PGJ<sub>2</sub> on the expression and secretion of MIC-1 were then studied in human adipocytes. In agreement with the results with 3T3-L1 cells, addition of TNF- $\alpha$  had no effect on MIC-1 mRNA, and MIC-1 protein levels in the medium were unaffected by the treatment (Fig. 4A). In contrast, H<sub>2</sub>O<sub>2</sub> induced a large increase in MIC-1 mRNA (by 8-fold,  $P < 0.01$ ) in human adipocytes, consistent with the finding in 3T3-L1 cells (Fig. 4B). In parallel, the concentration of MIC-1 in the medium with H<sub>2</sub>O<sub>2</sub> treatment increased (by 50%) compared with the controls ( $P < 0.01$ ; Fig. 4B). Similar to the stimulatory effect observed in 3T3-L1 cells, 15d-PGJ<sub>2</sub> treatment led to a major increase in MIC-1 mRNA level (by 18-fold) in human adipocytes ( $P < 0.001$ ; Fig. 4C). Concurrently, the concentration of MIC-1 protein in the medium of cultured adipocytes was elevated (by 43%) with 15d-PGJ<sub>2</sub> ( $P < 0.01$ ; Fig. 4C).

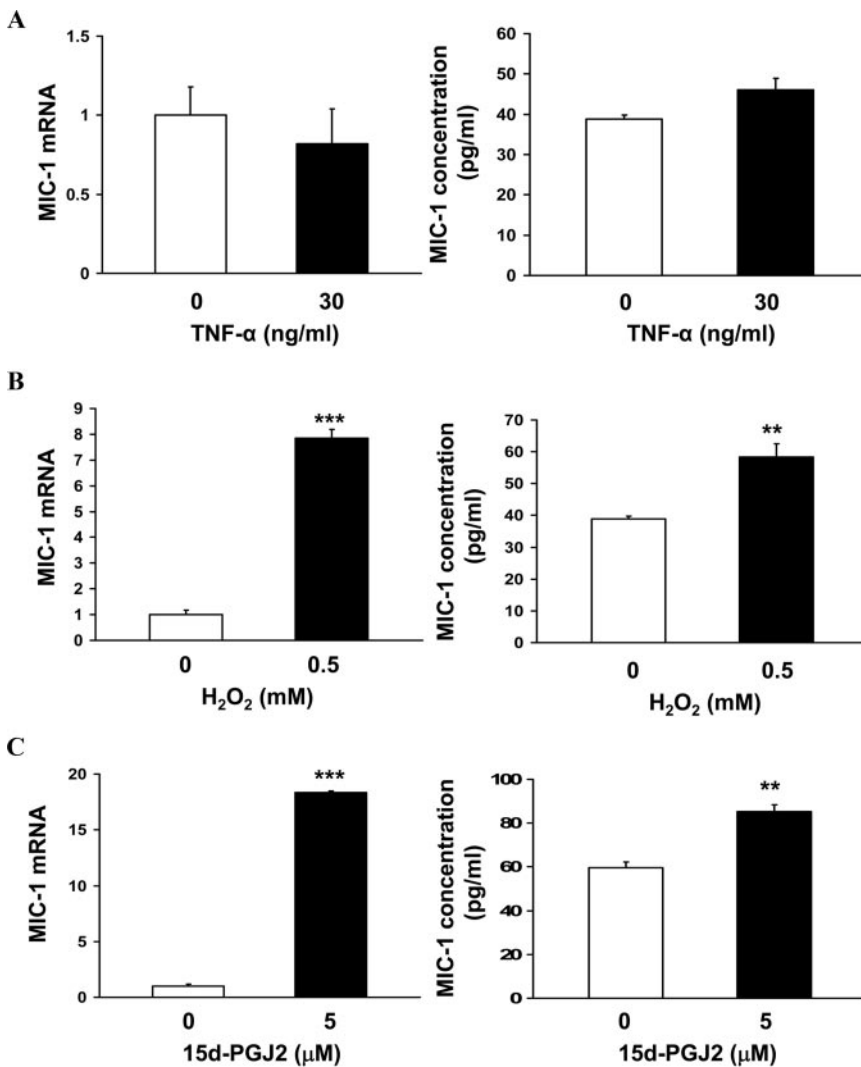
### The effect of recombinant human MIC-1 on adipokine release

To explore whether MIC-1 may have an autocrine/paracrine role in the modulation of adipokine production by adipocytes, ZAG and adiponectin protein release from human adipocytes that were exposed to recombinant human MIC-1 for 24 h was examined. There was no significant ( $P > 0.05$ ) effect of MIC-1 on ZAG release (Fig. 5A). However, an increase in adiponectin secretion into the medium was evident with MIC-1 treatment; this was statistically significant at a dose of 0.5 ng/ml (by 69%;  $P < 0.01$ ) and 5 ng/ml (by 53%;  $P < 0.05$ ) (Fig. 5B).

### MIC-1 expression in human white fat

In the final studies, visceral and sc adipose tissue from a group of obese subjects were probed for MIC-1 mRNA by RT-PCR, and a signal was evident from both fat depots (Fig. 6A). In a comparison using paired samples, real-time PCR measurements indicated that MIC-1 mRNA levels were higher (>2-fold,  $P < 0.05$ ) in sc than in visceral fat (Fig. 6B). The presence of both preprotein and mature MIC-1 was also detected by Western blotting in both visceral and sc fat (Fig. 6C). Both forms of MIC-1 were higher in sc than in visceral fat samples (1.4-fold for preprotein, 4.5-fold for mature protein), but neither difference was found to be statistically significant, probably due to a limited number of available samples (Fig. 6D).

Furthermore, MIC-1 mRNA levels were positively correlated with adiponectin mRNA in both the visceral ( $r = 0.43$ ,  $P < 0.05$ ) and sc depot ( $r = 0.65$ ,  $P < 0.01$ ) (Fig. 6E). To explore whether MIC-1 gene expression was associated with body fat or insulin sensitivity, we examined the correlation between MIC-1 expres-



**FIG. 4.** Effects of TNF- $\alpha$  (A), H<sub>2</sub>O<sub>2</sub> (B), and 15d-PGJ<sub>2</sub> (C) on MIC-1 mRNA level and protein secretion in human adipocytes. ZenBio cells were harvested at d 14 after the induction of differentiation and incubated for 24 h in media containing TNF- $\alpha$  (30 ng/ml), H<sub>2</sub>O<sub>2</sub> (0.5 mM), or 15d-PGJ<sub>2</sub> (5  $\mu$ M). Control cells received no addition. MIC-1 mRNA levels by real-time RT-PCR were expressed relative to the controls. MIC-1 protein concentrations in the medium were measured using ELISA. Data are means  $\pm$  SEM for groups of four to six. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with controls.

sion and indexes of adiposity and insulin sensitivity. As shown in Fig. 6, F and G, MIC-1 mRNA was negatively correlated with BMI (visceral:  $r = -0.53$ ,  $P < 0.01$ ; sc:  $r = -0.56$ ,  $P < 0.05$ ) and total fat mass (visceral:  $r = -0.47$ ,  $P < 0.05$ ; sc:  $r = -0.49$ ,  $P < 0.05$ ). However, there was no significant association between MIC-1 mRNA and plasma insulin (visceral:  $r = -0.21$ ,  $P > 0.05$ ; sc:  $r = -0.31$ ,  $P > 0.05$ ) or between MIC-1 mRNA and HOMA-IR (visceral:  $r = -0.18$ ,  $P > 0.05$ ; sc:  $r = -0.29$ ,  $P > 0.05$ ).

## Discussion

In the present study, we demonstrate for the first time that the *MIC-1* gene, which encodes macrophage inhibitory cytokine-1, a divergent member of the TGF- $\beta$  superfamily, is expressed by adipose tissues. MIC-1 mRNA was detected in the major white adipose tissue depots (epididymal, perirenal, and sc) and in brown fat of mice. In white fat, MIC-1 mRNA was evident

in both freshly isolated adipocytes and the stromal vascular fraction, and the expression levels were similar in the two fractions of adipose tissue. The stromal vascular fraction of white fat is recognized to contain several cell types in addition to preadipocytes, including fibroblasts, vascular endothelial cells, mast cells, and macrophages (29). Our cell culture studies clearly indicate that MIC-1 is synthesized by preadipocytes as well as mature adipocytes, but whether the nonfat cells in adipose tissue also synthesize the factor is unclear. However, expression of MIC-1 has been demonstrated in a number of cell types including macrophages (15).

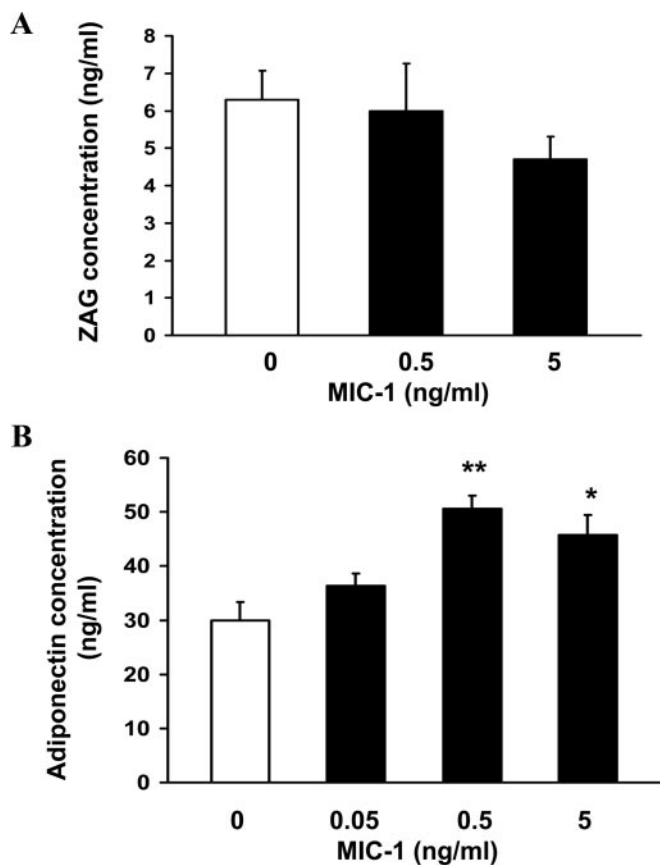
We further assessed MIC-1 expression in adipose tissue of genetically obese (*ob/ob*) mice, and there was an increase in MIC-1 mRNA levels in *ob/ob* mice compared with their lean controls. Because *ob/ob* mice are leptin deficient, this result may suggest that leptin could be one of the factors modulating MIC-1 expression.

In both 3T3-L1 cells and human primary adipocytes, the *MIC-1* gene is expressed before and after the induction of differentiation into adipocytes. This is in contrast to the expression pattern of the other two adipokines examined, ZAG and adiponectin, both of which are expressed only in differentiated adipocytes, as observed in this study and previously (27). Thus, MIC-1 may play a role in the function of both preadipocytes and differentiated adipocytes.

A further important finding of the present study is the demonstration that MIC-1 is a secretory product of adipocytes.

MIC-1 has been shown to be a secreted protein in the case of several other cell types, including cardiomyocytes (30), primary erythroblasts (31), and keratinocytes (14). Here MIC-1 protein was found to be secreted by preadipocytes as well as differentiated human adipocytes. Consistent with the gene expression data, MIC-1 protein release was higher in preadipocytes than mature adipocytes. Several adipokines, including IL-6 (32) and nerve growth factor (33), are like MIC-1 secreted at higher levels by preadipocytes than mature adipocytes, which suggests that preadipocytes could be a significant source of these proteins. Notwithstanding this difference, our data indicate that MIC-1 is a novel adipokine. As such, it is possible that, like other adipokines, MIC-1 could influence adipose tissue function in an autocrine/paracrine fashion, both directly and indirectly.

Several factors were shown to influence MIC-1 expression and secretion by adipocytes. The effects of potential mediators on MIC-1 gene expression were initially studied in 3T3-L1 cells. Leptin led to a small reduction in MIC-1 mRNA levels, suggest-



**FIG. 5.** Effects of MIC-1 on ZAG (A) and adiponectin (B) secretion by human adipocytes. Cells were harvested at d 14 after the induction of differentiation and incubated for 24 h in media containing recombinant human MIC-1. Control cells received no addition. Protein concentrations in the medium were measured by ELISA. Data are means  $\pm$  SEM for groups of four to five. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with controls.

ing the potential involvement of leptin. Similar to the result of leptin, a small inhibitory effect on MIC-1 gene expression was observed with IL-1 $\beta$ . Because both leptin and IL-1 $\beta$  are known to have proinflammatory effects, MIC-1 is probably not part of the proinflammatory response in adipocytes. This view is reinforced by the lack of effect of the major pleiotropic, proinflammatory cytokine TNF- $\alpha$  on MIC-1 gene expression, whereas TNF- $\alpha$  markedly reduced adiponectin mRNA level. However, the present study demonstrated that treatment with H<sub>2</sub>O<sub>2</sub> induces a dose-dependent increase in MIC-1 mRNA levels; this may suggest that MIC-1 is involved in the stress response of adipocytes. The effect of 15d-PGJ<sub>2</sub>, a natural ligand that activates PPAR $\gamma$ , on MIC-1 gene expression was also assessed, and it led to a marked increase in MIC-1 mRNA level.

We further investigated whether MIC-1 production in human adipocytes was also affected by some of these agents. Consistent with the observation in 3T3-L1 cells, TNF- $\alpha$  had no effect on MIC-1 mRNA levels in human adipocytes. MIC-1 protein release was also unaffected by TNF- $\alpha$ , although the induction of MIC-1 mRNA and protein by TNF- $\alpha$  has been reported in macrophages (1, 15). The cell-specific responses of MIC-1 expression to TNF- $\alpha$  presumably reflect the different immunological nature of adipocytes compared with macrophages.

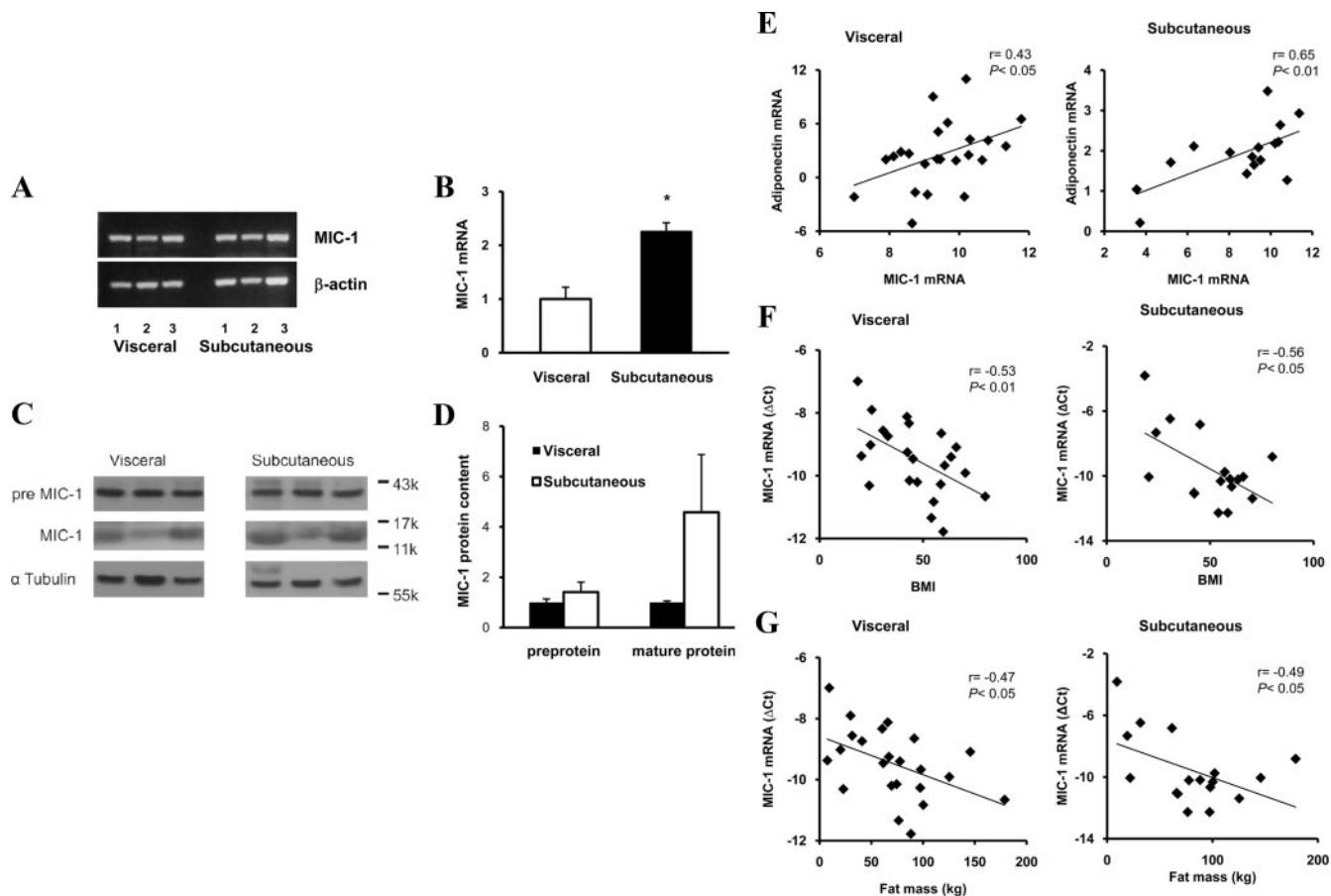
H<sub>2</sub>O<sub>2</sub> induced a marked increase in MIC-1 gene expression in human adipocytes, in line with the observations in 3T3-L1 adipocytes, and MIC-1 protein secretion was also increased with the treatment. As an inducer of oxidative stress, H<sub>2</sub>O<sub>2</sub> has been shown to strongly increase MIC-1 gene transcripts in human lung epithelial cells (34) and protein expression in human macrophages (15). MIC-1 expression in humans is also up-regulated by other stress conditions such as tissue injury, cancer, and anoxia (16, 18, 35). The induction of MIC-1 production in adipocytes by H<sub>2</sub>O<sub>2</sub> may suggest a role for the cytokine in defense against stress in adipose tissue. Although the exact molecular pathways in adipocytes await future experiments, MIC-1 is considered as a prominent downstream mediator of p53 function in cellular stress signaling (36).

MIC-1 production in human adipocytes is stimulated by 15d-PGJ<sub>2</sub>, and the effect is comparable in scale with that observed with 3T3-L1 cells and is accompanied by a rise in MIC-1 protein release. Our findings thus indicate the involvement of 15d-PGJ<sub>2</sub> in modulating MIC-1 synthesis and secretion in adipocytes. 15d-PGJ<sub>2</sub> has been reported to induce MIC-1 protein expression, promoting apoptosis in cancer cells (37). In addition, 15d-PGJ<sub>2</sub> has been linked to the suppression of monocyte activation (38), inhibition of angiogenesis (39), and antiinflammatory responses (40). Interestingly, a potential antiinflammatory function of MIC-1 has been reported in macrophages because MIC-1 attenuated LPS-induced TNF- $\alpha$  secretion (1). Thus, the major stimulatory effect of 15d-PGJ<sub>2</sub> on MIC-1 production reported here may imply potential roles for the cytokine in adipocytes.

In the present study, the putative role of MIC-1 in adipose tissue was explored by examining its effects on the secretion of other adipokines that are involved in adipocyte metabolism. ZAG is a recently identified adipokine that has been implicated in adipose atrophy in cancer cachexia (26, 27, 41). Because MIC-1 mediates cachexia, we assessed whether MIC-1 affects ZAG production in adipocytes, thereby influencing fat mass. As observed in this study, MIC-1 had no significant effect on ZAG protein release by adipocytes so that MIC-1 is unlikely to play a key role in the regulation of ZAG production. Adiponectin, abundantly expressed in adipocytes, is an insulin-sensitizing adipokine (42). In contrast to other adipokines whose levels increase with fat mass, circulating adiponectin is inversely associated with adiposity (43). It is evident that adiponectin acts through activation of AMP kinase to stimulate glucose uptake and fatty acid oxidation and decreases vascular inflammation (44). We found that MIC-1, at physiologically relevant concentrations, increased adiponectin release from human adipocytes. This suggests that MIC-1 could act as a paracrine signal to stimulate adiponectin production, and therefore, MIC-1 may well be important in modulating adiponectin action, including its insulin-sensitizing effect.

Finally, the present study shows expression of the MIC-1 gene and protein in human adipose tissue, both visceral and sc. Interestingly, MIC-1 mRNA levels were higher in the sc than the visceral depot, as has been reported previously for leptin, for example (45). Moreover, there is a positive association between MIC-1 mRNA and adiponectin mRNA levels in the two fat depots. Given the stimulatory effect of MIC-1 on adiponectin secretion by human adipocytes observed in this study, a potential





**FIG. 6.** *MIC-1* gene and protein expression in human adipose tissue. **A**, RT-PCR analysis of RNA isolated from human visceral and sc fat. **B**, *MIC-1* mRNA levels in paired human visceral and sc fat by real-time PCR. Data are means  $\pm$  SEM (n = 11). \*, *P* < 0.05 compared with the mean value of visceral fat. **C**, Western blot of *MIC-1* preprotein and mature protein in visceral and sc fat. **D**, *MIC-1* protein levels in paired human visceral and sc fat samples. Data are means  $\pm$  SEM (n = 5). **E**, Comparison of mRNA levels of *MIC-1* and adiponectin in visceral (Pearson correlation, *r* = 0.43, *P* < 0.05, n = 23) and sc depot (Pearson correlation, *r* = 0.65, *P* < 0.01, n = 16). **F**, Comparison of *MIC-1* mRNA levels and BMI in visceral (Pearson correlation, *r* = -0.53, *P* < 0.01, n = 23) and sc depot (Pearson correlation, *r* = -0.56, *P* < 0.05, n = 17). **G**, Comparison of *MIC-1* mRNA levels and body fat mass in visceral (Pearson correlation, *r* = -0.47, *P* < 0.05, n = 23) and sc depot (Pearson correlation, *r* = -0.49, *P* < 0.05, n = 17).

functional link between the two adipokines in adipose tissue is a possibility, but further studies are needed to substantiate this. In addition, our study shows that *MIC-1* mRNA levels are decreased with increased body adiposity in humans with a wide range of BMI and body fat mass. This suggests that *MIC-1* may have a role, directly or indirectly such as through up-regulation of adiponectin, in modulating body fat accumulation. However, there is a lack of significant association between *MIC-1* expression and plasma insulin or HOMA-IR. Whether adipose tissue-derived *MIC-1* is strongly influenced by insulin requires further studies.

In conclusion, our study clearly demonstrates that the *MIC-1* gene is expressed in the major adipose depots in mice and humans and in mature adipocytes. We also show that *MIC-1* is a secretory product of both preadipocytes and differentiated adipocytes. Recombinant *MIC-1* enhances adiponectin release from adipocytes, suggesting that *MIC-1* is a positive regulator of adiponectin. In addition, *MIC-1* expression in human adipose tissue is negatively associated with body fat mass and may influence adiposity. Finally, *MIC-1* is a novel adipokine that may well have a paracrine role in the modulation of adipose tissue function and could even be a potential target for obesity treatment.

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