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# Monocyte Chemoattractant Protein-1 Release Is Higher in Visceral than Subcutaneous Human Adipose Tissue (AT): Implication of Macrophages Resident in the AT

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**Human adipose tissue (AT) produces several adipokines including monocyte chemoattractant protein (MCP)-1, involved in the pathogenesis of atherosclerosis.**

**Objective: Human AT cultures, isolated adipocytes, and stromal-vascular cells were used to investigate the relationship among AT-resident macrophages, MCP-1, and adiposity and the regulation of MCP-1.**

**Results: mRNA levels of specific macrophage markers (CD68 and CD14) are correlated with adiposity in sc AT and visceral AT ($P < 0.05$). MCP-1 production is higher in stromal-vascular cells *vs.* adipocytes ($P < 0.01$) and correlates with macrophage markers in both AT compartments ($P < 0.05$). MCP-1 release is higher in obese subjects ($P < 0.05$) and in VAT ($P < 0.01$), but after adjusting for AT-resident macrophages, the differences disappear. MCP-1 is stimulated by IL-1$\beta$, TNF-$\alpha$, IL-8, IL-4, and IL-6 + IL-6-soluble receptor and is decreased by dexamethasone, IL-10, metformin, and thiazolidinediones.**

**Discussion: MCP-1 is correlated with specific macrophage markers, adiposity, and AT localization, but the relationship seems to be related to the number of AT-resident macrophages. Despite this, MCP-1 may be involved in obesity-related health complications, and the decrease of MCP-1 by metformin and thiazolidinediones suggests that these antidiabetic compounds have antiinflammatory properties improving the low-grade inflammatory state observed in obesity. (*J Clin Endocrinol Metab* 90: 2282–2289, 2005)**

OBESITY, ESPECIALLY VISCERAL obesity, is associated with a low-grade inflammatory state (1) insulin resistance, development of type 2 diabetes, and premature atherosclerosis (2). Although no clear link has been established between obesity and the associated deleterious health complications, accumulating evidence suggests the involvement of adipose tissue (AT)-derived proteins, collectively known as adipokines [*e.g.* adiponectin (3), IL-6 (4), IL-8 (5, 6), and TNF-$\alpha$ (7)]. The production and release of IL-6 (8) and IL-8 (9) have previously been reported to be increased in the visceral AT (VAT) depot and in relation to obesity; recently, the antiinflammatory adipokine, adiponectin, was found to be lower in VAT compared with sc AT (SAT) (10), suggesting that the VAT depot is more proinflammatory. Except for leptin and adiponectin, adipokines are not exclusively produced by the adipocytes, but inflammatory cells such as monocytes and macrophages within the AT may contribute to the production (11, 12).

The chemokine monocyte chemoattractant protein (MCP)-1 has recently been added to the growing list of adipokines (13). MCP-1 has been shown to be produced by macrophages and endothelial cells via an activation of the nuclear transcription factor-$\kappa$B pathway (14). MCP-1 recruits monocytes, leukocytes, and other inflammatory cells in response to an inflammatory challenge (15). Circulating MCP-1 has been found to be increased in animal models of obesity (*ob/ob* mice and diet-induced obesity mice) compared with lean littermates (16, 17) and reduced after weight loss (17). In humans, circulating MCP-1 has been found to be associated with cardiovascular disease (18) and is elevated in type 2 diabetic patients compared with nondiabetics (19). Recently, we found that MCP-1 mRNA levels in human AT samples correlated with measures of adiposity and that circulating MCP-1 was reduced after weight loss in severely obese subjects (20).

A growing body of evidence suggests that inflammatory processes are involved in the pathogenesis of atherosclerosis and cardiovascular disease (21). Blockade of MCP-1 through transfection of an N-terminal deletion mutant in the *MCP-1* gene decreased progression of atherosclerosis in apolipoprotein E-knockout mice prone to develop severe cardiovascular disease (22). In mice with diet-induced obesity, elevated MCP-1 levels were found to be associated with an increase in activated (CD11b-positive) monocytes (17) and uptake of oxidized LDL in monocytes through activation of scavenger receptors (23), supporting the hypothesis that MCP-1 may play an important role in the atherosclerotic process within the vessel wall (24). *In vitro*, high glucose (up to 35 mM) increased MCP-1 production in human vascular endothelial cells (25), and MCP-1 blunted the insulin-stimulated glucose uptake in 3T3-L1 adipocytes (16), suggesting an involvement of MCP-1 in obesity-related insulin resistance.

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Abbreviations: AT, Adipose tissue; BMI, body mass index; $C_T$, threshold cycle; ETYA, 5,8,11,14-eicosatetraynoic acid; IL-6sR, IL-6 soluble receptor; MCP, monocyte chemoattractant protein; MIF, migration inhibitory factor; PPAR, peroxisome proliferator-activated receptor; $r_P$, Pearson correlation coefficient; SAT, sc AT; SV, stromal-vascular; TZD, thiazolidinedione; VAT, visceral AT.

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Because AT-derived MCP-1 is known to originate from adipocytes and stromal-vascular (SV) cells, the relationship among MCP-1, adiposity, and macrophages resident in whole-human AT cultures was investigated in two AT depots using two known macrophage-specific markers: CD68 (26) and CD14 (27). Secondly, mRNA levels of MCP-1 and other adipokines [*e.g.* TNF-α, IL-6, and IL-8] were compared with mRNA levels of CD68, CD14, and the adipocyte-specific protein leptin in isolated human adipocytes and the adjacent SV fraction. Finally, the regulation of MCP-1 release in relation to adiposity, AT localization, other adipokines, and antidiabetic compounds [*e.g.* thiazolidinediones (TZDs) and metformin] were investigated in whole human AT cultures.

## Subjects and Methods

### *Subjects*

SAT was obtained from 15 healthy normal to overweight women [mean body mass index (BMI), 24.6 ± 1.3 kg/m$^2$] and from five obese subjects (four males and one female; mean BMI, 39.9 ± 1.4 kg/m$^2$). Paired VAT and SAT samples were obtained from five normal to overweight women (mean BMI, 25.1 ± 1.3 kg/m$^2$) undergoing surgery due to gynecological diseases and from five obese women (mean BMI, 43.0 ± 3.3 kg/m$^2$) undergoing laparoscopic adjustable silicone gastric band surgery for the treatment of morbid obesity. None of the subjects received any medication known to influence AT metabolism.

### *Isolated adipocytes and whole AT cultures*

As described (5), the AT was transported to the laboratory and washed several times in isotonic NaCl. Adipocytes were isolated by collagenase digestion (0.15 mg/g AT) of AT fragments in 10 mmol/liter HEPES buffer for 45–60 min at 37 C. The isolated adipocytes were washed three times in buffer containing 5% albumin. The isolated adipocytes were resuspended in medium 199 containing 1% BSA and 25 mM HEPES. Finally, the 200-μl cell suspension containing 10% adipose cells, corresponding to approximately 100,000 adipocytes in each tube, was snap-frozen in liquid nitrogen and kept at −80 C for later RNA extraction. After the initial collagenase digestion, the SV fraction remaining was centrifuged for 15 min at 6300 × *g*, resuspended in 9 ml buffer, and filtered through a nylon mesh. This procedure was repeated three times, after which the supernatant was removed, and the SV fraction was snap-frozen in liquid nitrogen and kept at −80 C for later RNA extraction. For the whole AT incubations, a total amount of 500 mg AT was minced into fragments less than 10 mg. AT fragments were preincubated overnight (for ~15 h) and incubated for up to 72 h because a maximal effect of IL-1β-stimulated MCP-1 release was observed at that time point ($P < 0.001$, data not shown).

AT was incubated with IL-1β (2 μg/liter), TNF-α (10 μg/liter), dexamethasone (50 nM), IL-6 (50 μg/liter), IL-6 (50 μg/liter), plus the IL-6 soluble receptor (IL-6sR) (100 μg/liter), IL-8 (1 mg/liter), IL-10 (10 μg/liter), or IL-4 (10 μg/liter). Separate incubations using IL-6 alone or IL-6 + IL-6sR were chosen because studies have shown that incubation with IL-6 + IL-6sR but not IL-6 elicits biological effects in human AT (28–30). In addition, AT was incubated with metformin (0.1, 1, or 10 mM), TZDs, and peroxisome proliferator-activated receptor (PPAR)-γ agonists: ciglitazone (50 μM), troglitazone (10 μM), pioglitazone (10 μM), or the PPARα agonist 5,8,11,14-eicosatetraynoic acid (ETYA) (15 μM). All incubations were performed as duplicates. Culture medium from the different incubations was kept at −20 C until protein analysis, and the AT fragments were snap-frozen in liquid nitrogen and kept at −80 C for later RNA extraction.

### *MCP-1 protein*

MCP-1 protein levels were assessed using a specific human ELISA method (MCP-1 DuoSet; R&D Systems Europe Ltd., Abingdon, UK) with an intraassay coefficient of variation of 8.1% (n = 12). Samples were diluted 1:100 to be within the assay range (31.2–1000 ng/liter). The amount of protein in the medium from paired SAT and VAT incubations was normalized for cellular DNA content as previously described (31).

### *Determination of mRNA levels*

For mRNA determination, the following oligonucleotide primer pairs were used: MCP-1, 5′ CGACATCCTGGAACTGCCCTACC 3′ and 5′ CACTGTGCCGCTCTCGTTCAC 3′; TNF-α, 5′ CGAGTGACAAGCCTGTAGC 3′ and 5′ GGTGTGGGTGAGGAGCACAT 3′; IL-8, 5′ TTGGCAGCCTTCCTGATTTC 3′ and 5′ AACTTCTCCACAACCCTCTG 3′; IL-6, 5′ AAATGCCAGCCTGCTGACGAAG 3′ and 5′ AACAACAATCTGAGGTGCCCATGCTAC 3′; leptin, 5′ GATGACACCAAAACCCTCATC 3′ and 5′ GCCACCACCTCTGTGGAGTAG 3′; CD68, 5′ GCTACATGGCGGTGGAGTACAA 3′ and 5′ ATGATGAGAGGCAGCAAGATGG 3′; and CD14, 5′ TAAAGGACTGCCAGCCAAGC 3′ and 5′ AGCCAAGGCAGTTTGAGTCC 3′. β-Actin was used as housekeeping gene: 5′ACGGGGTCACCCACACTGTGC 3′ and 5′CTAGAAGCATTTGCGGTGGACGATG 3′.

RNA was isolated from 250 mg AT using Trizol reagents (Life Technologies, Inc., Roskilde, Denmark). cDNA was made with random hexamer primers using the GeneAmp PCR kit (Applied Biosystems, Foster City, CA). When using the number of threshold cycles ($C_T$) as indications of the amount of mRNA in the adipocyte and SV fractions, no difference was found (24.8 ± 0.7 $C_T$ *vs.* 24.0 ± 0.3 $C_T$, $P = 0.32$). Quantification of target gene (MCP-1, TNF-α, IL-6, IL-8, leptin, CD68, or CD14) mRNA was expressed relative to the housekeeping gene (β-actin) mRNA, except in Fig. 3D, where MCP-1 was the target gene and CD68 was used as a housekeeping gene to evaluate the impact of resident macrophages on MCP-1 mRNA levels in the different AT depots. Quantification was performed with a SYBR-Green real-time PCR assay using an iCycler PCR machine from Bio-Rad (Hercules, CA) as previously described (32). In brief, PCR amplification was performed with PCR master mix-containing target primers, Hot Star Taq DNA polymerase, and SYBR-Green and PCR buffer. All samples were determined as duplicates. Samples were incubated for an initial denaturation at 95 C for 10 min, followed by 40 PCR cycles each consisting of 95 C for 30 sec, 57 C for 30 sec, and 74 C for 60 sec. Relative gene expression of MCP-1, TNF-α, IL-6, IL-8, leptin, CD68, or CD14 to β-actin was calculated as described in User Bulletin no. 2 (1997) from PerkinElmer Cetus (Norwalk, CT).

### *Statistical analysis*

The SPSS statistical packet (SPSS, Chicago, IL) was used for the calculations. Normality was tested using the Kolmogorov-Smirnov test. The association between AT-resident macrophages and adiposity was investigated using the Pearson correlation coefficient ($r_p$). When comparing AT incubations from different subjects as well as AT incubations from subjects with different degrees of adiposity, an unpaired Student's *t* test was applied. When comparing AT depots from the same individual, a paired *t* test was applied. Because comparison between adipocyte fractions *vs.* SV fraction from the same individual did not pass the normality test, a nonparametric Mann-Whitney test was applied. Threshold for significance was set at $P < 0.05$.

### *Ethics*

Informed, written consent was obtained from all subjects. The Ethical Committee of Aarhus approved the study.

## Results

### *AT-resident macrophages, MCP-1, and adiposity*

The macrophage-specific markers CD68 and CD14 were significantly associated with adiposity ($r_p = 0.63$, $P < 0.05$, Fig. 1A) and ($r_p = 0.56$, $P < 0.05$, Fig. 1A) in SAT. Similar results were obtained in VAT (CD68, $r_p = 0.78$, $P < 0.01$; CD14, $r_p = 0.81$, $P < 0.01$; data not shown). In the SAT depot, MCP-1 mRNA levels correlated with mRNA levels of CD68 ($r_p = 0.66$, $P < 0.01$, Fig. 1B) and CD14 ($r_p = 0.55$,

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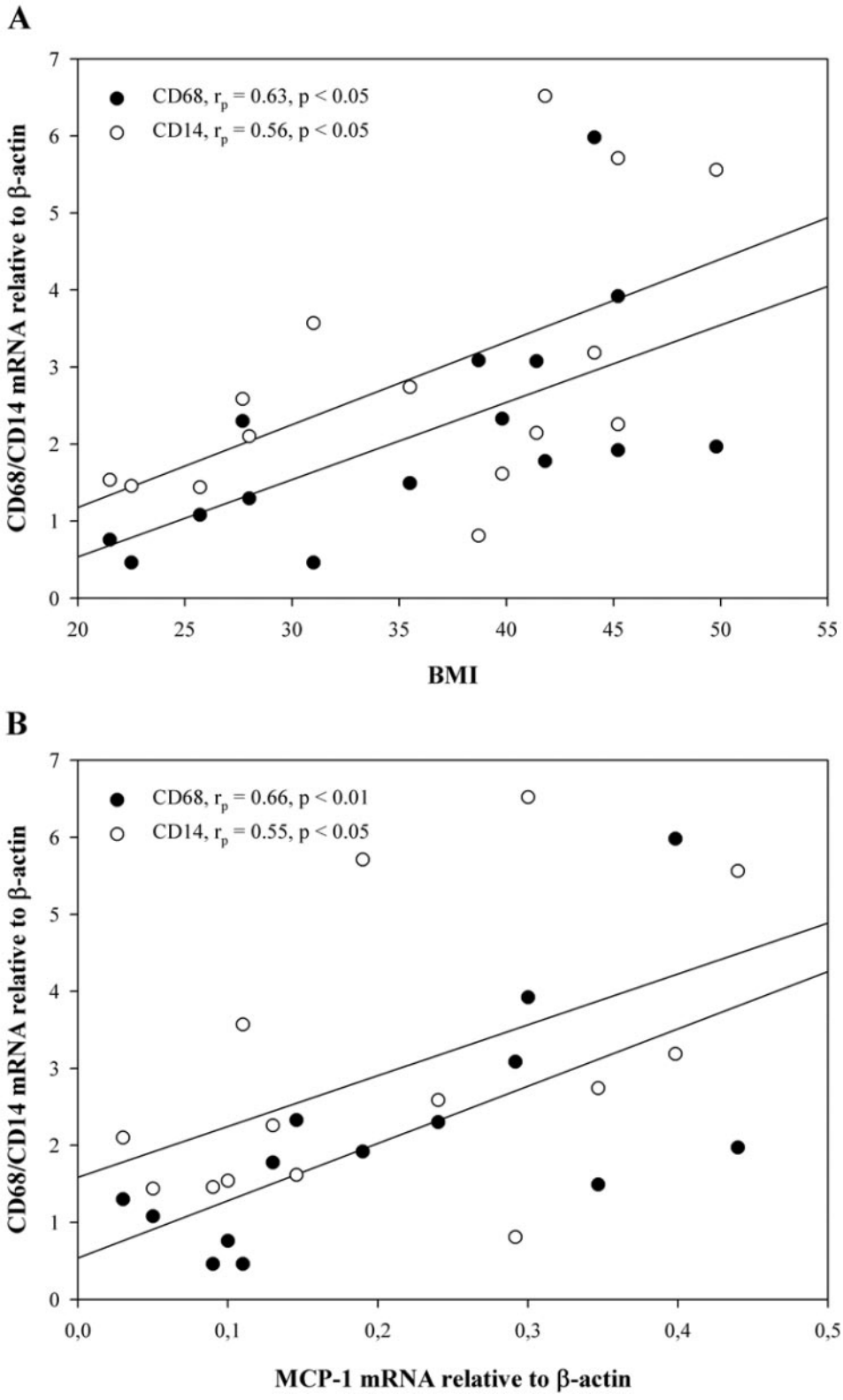


FIG. 1. Correlation between macrophage-specific markers, adiposity, and MCP-1. Correlation between mRNA levels of the macrophage-specific markers, CD68 and CD14, and BMI (kilograms per meter squared) (A) or MCP-1 mRNA levels (B) in SAT biopsies. n = 15, with a BMI range of 21.5–49.8 kg/m$^2$. Bivariate correlation with $r_p$.

$P < 0.05$, Fig. 1B). In VAT MCP-1 correlated with CD68 ($P < 0.05$, data not shown), but the association with CD14 did not reach statistical significance in that depot ($P = 0.07$, data not shown).

*Isolated adipocytes vs. SV fraction*

Leptin mRNA levels in the SV fraction were approximately 1% of that in the isolated adipocyte fraction ($P < 0.01$, Fig. 2). Opposite to leptin, mRNA levels of the macrophage-

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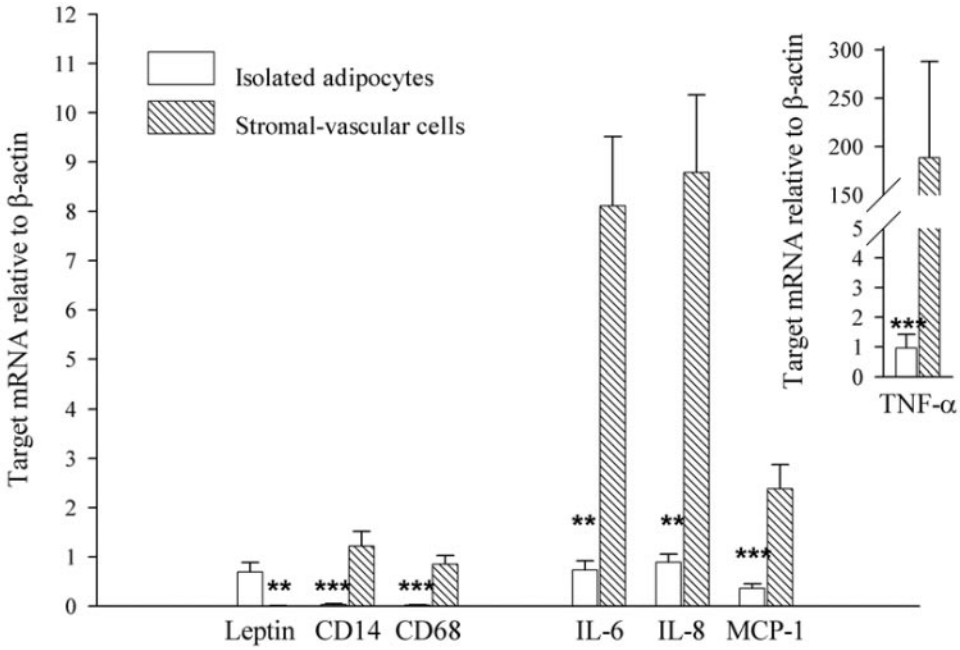


FIG. 2. Adipokine production in adipocytes *vs.* SV cells. Adipokine (MCP-1, IL-6, and IL-8) mRNA levels in the adipocyte fraction *vs.* the SV fraction of human AT was compared with that of the adipocyte-specific marker leptin and macrophage-specific markers CD68 and CD14. Due to the high expression of TNF-$\alpha$ in the SV fraction, this adipokine is presented as an insert. Data represent mean values ± SEM (n = 9). **, $P < 0.01$; ***, $P < 0.001$; adipocyte fraction *vs.* SV fraction.

specific markers CD68 and CD14 in the adipocyte fraction were approximately 2% of that in the SV fraction ($P < 0.001$, Fig. 2). MCP-1, IL-6, and IL-8 mRNA levels in isolated adipocytes were between 10 and 15% of that in the SV fraction ($P < 0.001$, Fig. 2). TNF-$\alpha$ mRNA levels in isolated adipocytes were approximately 5% of that in the SV fraction ($P < 0.001$, Fig. 2). The observation that CD68 and CD14 mRNA were detectable in the adipocyte fraction and leptin mRNA in the SV fraction might be due to minor contamination with mature adipocytes and macrophages, respectively.

*Effects of adiposity and AT localization*

MCP-1 release from AT obtained from obese subjects was about 7-fold higher compared with AT from lean subjects (1.0 ± 0.3 $\mu$g protein/$\mu$g DNA *vs.* 0.2 ± 0.03 $\mu$g protein/$\mu$g DNA, $P < 0.01$, Fig. 3A). VAT displayed a 2-fold higher MCP-1 release compared with SAT in both lean ($P < 0.01$, Fig. 3A) and obese ($P < 0.05$, Fig. 3A) subjects. Similar results were obtained for MCP-1 and CD68 mRNA levels in the two compartments (Fig. 3, B and C). When looking at the relationship between CD68 and MCP-1 mRNA levels, the impact of obesity and AT localization disappeared (Fig. 3D).

*Effects of cytokines on MCP-1 release*

The proinflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ increased MCP-1 release by more than 2-fold ($P < 0.001$, Fig. 4) in the AT. IL-6 + IL-6sR, IL-8, and IL-4 increased MCP-1 release by 20–30% ($P < 0.05$, Fig. 4). Dexamethasone and IL-10 exerted antiinflammatory effects on MCP-1, reducing the release by approximately 25% ($P < 0.001$, Fig. 4).

*Effects of antidiabetic compounds*

In SAT incubations, metformin decreased MCP-1 release in a dose-dependent manner by up to 40% ($P < 0.01$, Fig. 5A). The TZDs and PPAR$\gamma$ agonists ciglitazone, pioglitazone, and troglitazone decreased MCP-1 release by up to 20% ($P < 0.01$, Fig 5B), but the PPAR$\alpha$ agonist ETYA had no effect on MCP-1 release (Fig. 5B). In the VAT depot, MCP-1 release was decreased by metformin ($P < 0.05$, data not shown) and troglitazone ($P < 0.05$, data not shown) to the same extent as found in the SAT samples.

## Discussion

In the present paper, the regulation of AT-derived MCP-1 and the involvement of AT-resident macrophages were studied in human AT *in vitro*. It is demonstrated for the first time that MCP-1 release is higher in obese compared with lean subjects and in VAT compared with SAT. Interestingly, the relationship between MCP-1, adiposity, and AT localization disappears after adjusting for AT-resident macrophages using the macrophage-specific marker CD68. The finding that MCP-1 release is higher in the VAT depot is in accordance with reports on other adipokines, IL-6 (8), and IL-8 (9). However, we have recently reported that the association between IL-8 and adiposity in SAT samples was attributable to SV cells rather than adipocytes (9). Taken together with the finding that approximately 10–15% of MCP-1, IL-6, and IL-8 is derived from the adipocyte fraction compared with the SV fraction, it suggests that the higher production and release of IL-6 and IL-8 in relation to obesity and AT localization may be related to an increased number of macrophages resident in the human AT under these conditions.

MCP-1 release from whole AT cultures was highly regulated and increased by proinflammatory cytokines and chemokines such as IL-1$\beta$, TNF-$\alpha$, IL-4, and IL-8, and decreased by the antiinflammatory cytokine IL-10 and the corticosteroid, dexamethasone. The effects of IL-1$\beta$ and TNF-$\alpha$ are similar to the effects reported in other cell-types (33, 34). Interestingly, when IL-6 was incubated with its soluble receptor (IL-6sR), it displayed proinflammatory properties in-

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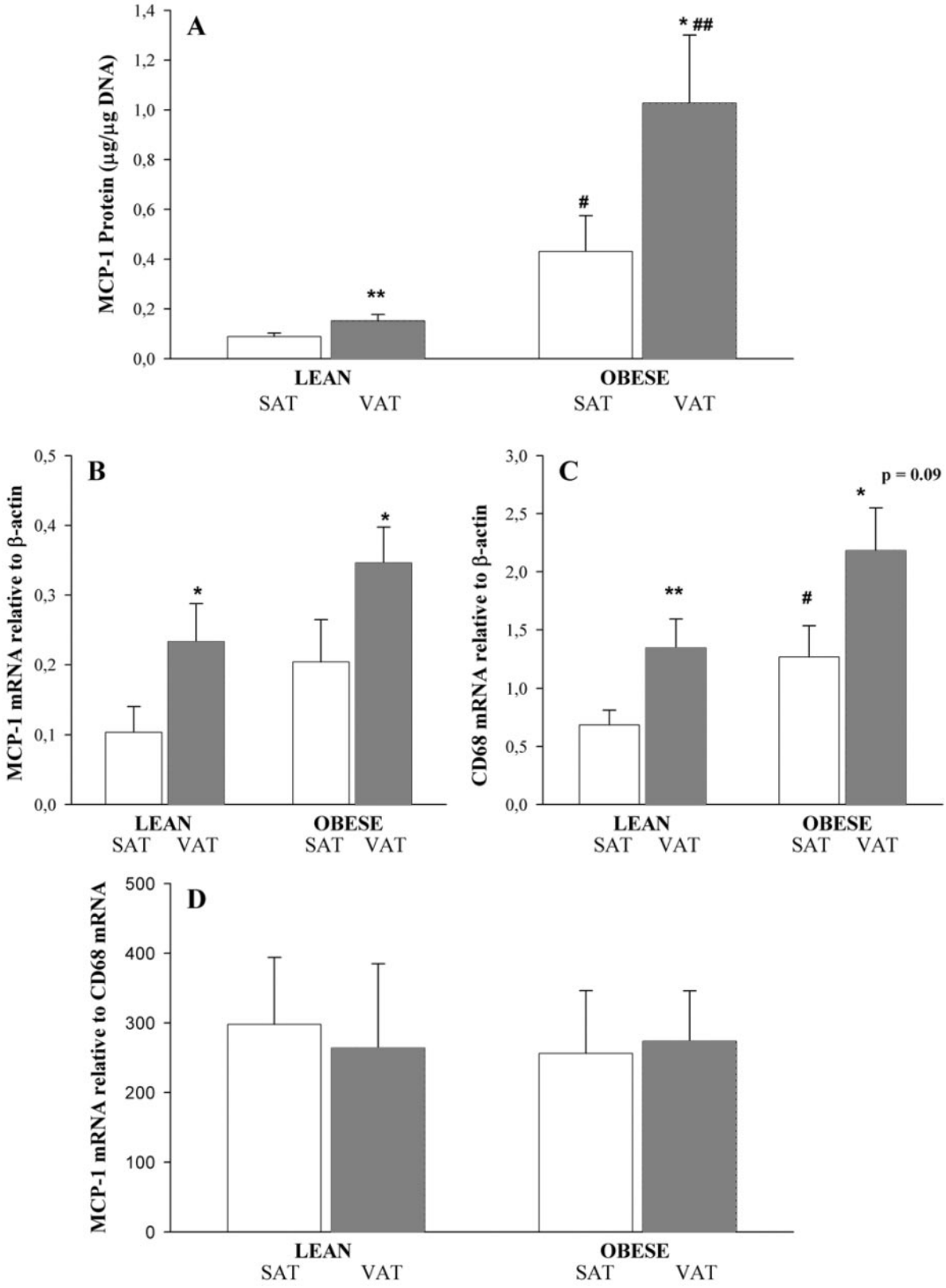


FIG. 3. MCP-1 release and relationship between MCP-1 and CD68 production in SAT and VAT. MCP-1 protein levels (A), MCP-1 mRNA levels (B), and CD68 mRNA levels (C). As described in *Subjects and Methods*, MCP-1 mRNA quantification was done relative to CD68 mRNA to evaluate the impact of resident macrophages on MCP-1 mRNA levels in the two AT depots (D). All investigations were performed in whole AT cultures obtained from the SAT or VAT depot from lean (mean BMI, 24 kg/m$^2$, n = 5) and obese (mean BMI, 47 kg/m$^2$, n = 5) subjects and incubated for 72 h. Data represent mean values ± SEM. *, $P < 0.05$; **, $P < 0.01$ (SAT *vs.* VAT); #, $P < 0.05$; ##, $P < 0.01$ (lean *vs.* obese).

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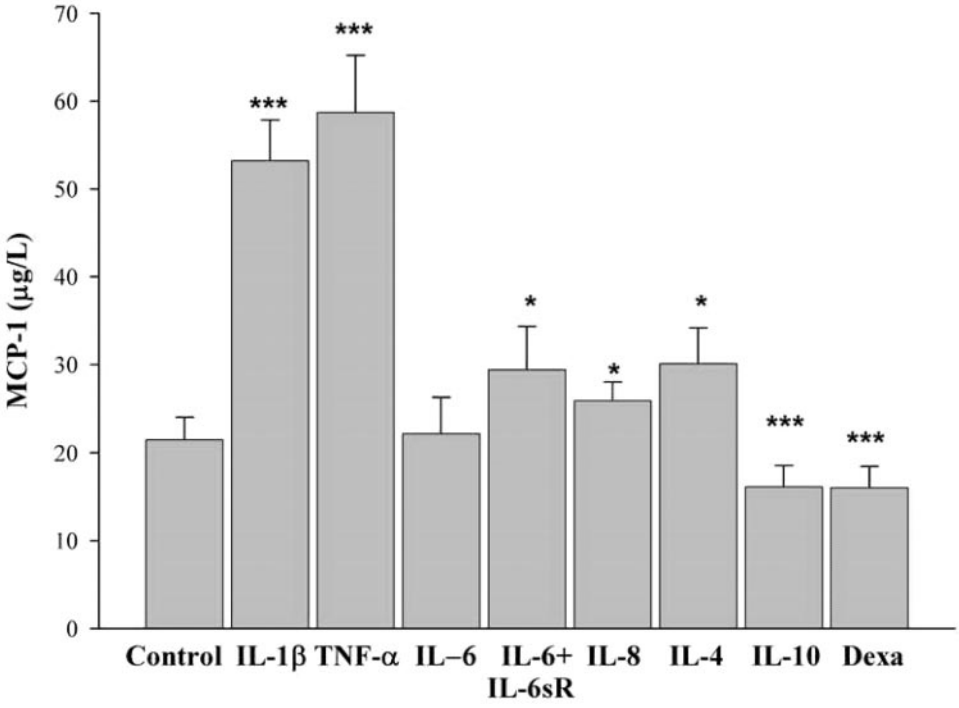


FIG. 4. Regulation of MCP-1 release in whole AT cultures. Human AT cultures were incubated with IL-1$\beta$ (2 $\mu$g/liter), TNF-$\alpha$ (10 $\mu$g/liter), dexamethasone (Dexa; 50 nM), IL-6 (50 $\mu$g/liter), IL-6 (50 $\mu$g/liter) plus IL-6sR (100 $\mu$g/liter), IL-8 (1 mg/liter), IL-10 (10 $\mu$g/liter), or IL-4 (10 $\mu$g/liter) for 48 h. Data represent mean values ± SEM (n = 6). *, $P < 0.05$; ***, $P < 0.001$ compared with control incubations.

creasing MCP-1 release, whereas incubation with IL-6 alone was without any effect on MCP-1 release. This finding is somewhat in contrast to a recent report where incubation with IL-6 alone increased MCP-1 production in 3T3-L1 adipocytes (34). However, the discrepancy may be related to differences in model system (*e.g.* murine 3T3-L1 adipocytes *vs.* whole human AT cultures) (29), and we have previously demonstrated that coincubation with IL-6sR seems to be necessary in the latter *in vitro* model using human AT (30). The findings presented suggest that the higher MCP-1 release from obese subjects may be the result of paracrine activation within the AT by other adipokines also known to be elevated in the obese state (9, 35, 36).

Antidiabetic compounds such as TZDs are known to decrease MCP-1 production in human vascular endothelial cells (33), epithelial cells (37), and plasma from obese subjects (38), probably by inhibition or decrease in the generation of reactive oxygen species acting through intracellular pathways such as the nuclear transcription factor-$\kappa$B (38) or the p38 MAPK (25). In the present paper, it is demonstrated for the first time that oral antidiabetic agents such as TZDs (*e.g.* ciglitazone, troglitazone, and pioglitazone) and the biguanide, metformin, reduce MCP-1 release in SAT and VAT. The *in vitro* findings are in line with two recent papers in which obese type 2 diabetic subjects treated with rosiglitazone or obese nondiabetic subjects treated with metformin displayed a significant reduction in the inflammatory markers MCP-1 and migration inhibitory factor (MIF)-1 (39, 40). TZDs and metformin are used in the treatment of obesity-related type 2 diabetes, and the agreement between the *in vitro* results presented in the present paper and the *in vivo* results (39, 40) further substantiate an antiinflammatory effect of these compounds. Both MCP-1 and MIF-1 are suggested to be involved in the development of atherosclerosis (41), suggesting that treatment with these antidiabetic agents may be of therapeutic value beyond glycemic control.

The finding that MCP-1, IL-6, and IL-8 production is approximately 7- to 8-fold higher in the SV fraction compared with the adipocyte fraction is essentially in line with a recent report on IL-6 and IL-8 release in these two AT fractions (11). By the use of macrophage-specific markers (*e.g.* CD68 and CD14), a novel association was found between AT-resident macrophages and adiposity in VAT samples. In addition, a correlation was found between macrophage markers and adiposity in SAT samples, which essentially is in accordance with two recent papers (26, 42). The background for the increased macrophage infiltration of the human AT in obesity is still not elucidated. However, circulating mononuclear cells from obese subjects are in a proinflammatory state (higher production of TNF-$\alpha$, IL-6, and MIF-1) (43), implicating that these cells when trapped in the AT may be part of the inflammatory process within the AT. The adipokines MCP-1 and IL-8 have the ability to attract monocytes/macrophages (44), and their receptors are expressed in both the adipocyte fraction and the SV fraction of human AT (13), suggesting that the two chemokines may be of importance in the recruitment/attraction of monocytes to the AT as indicated by Wiesberg *et al.* (26). However, the relationship and interaction between MCP-1 and IL-8 from the adipocyte fraction and the SV fraction including the impact of adipocytes from obese *vs.* lean subjects requires further investigations.

In conclusion, the present paper demonstrates that MCP-1 is produced in both the adipocyte fraction and the SV fraction of human AT. MCP-1 release is higher in obese subjects and in VAT although the MCP-1 production seems to

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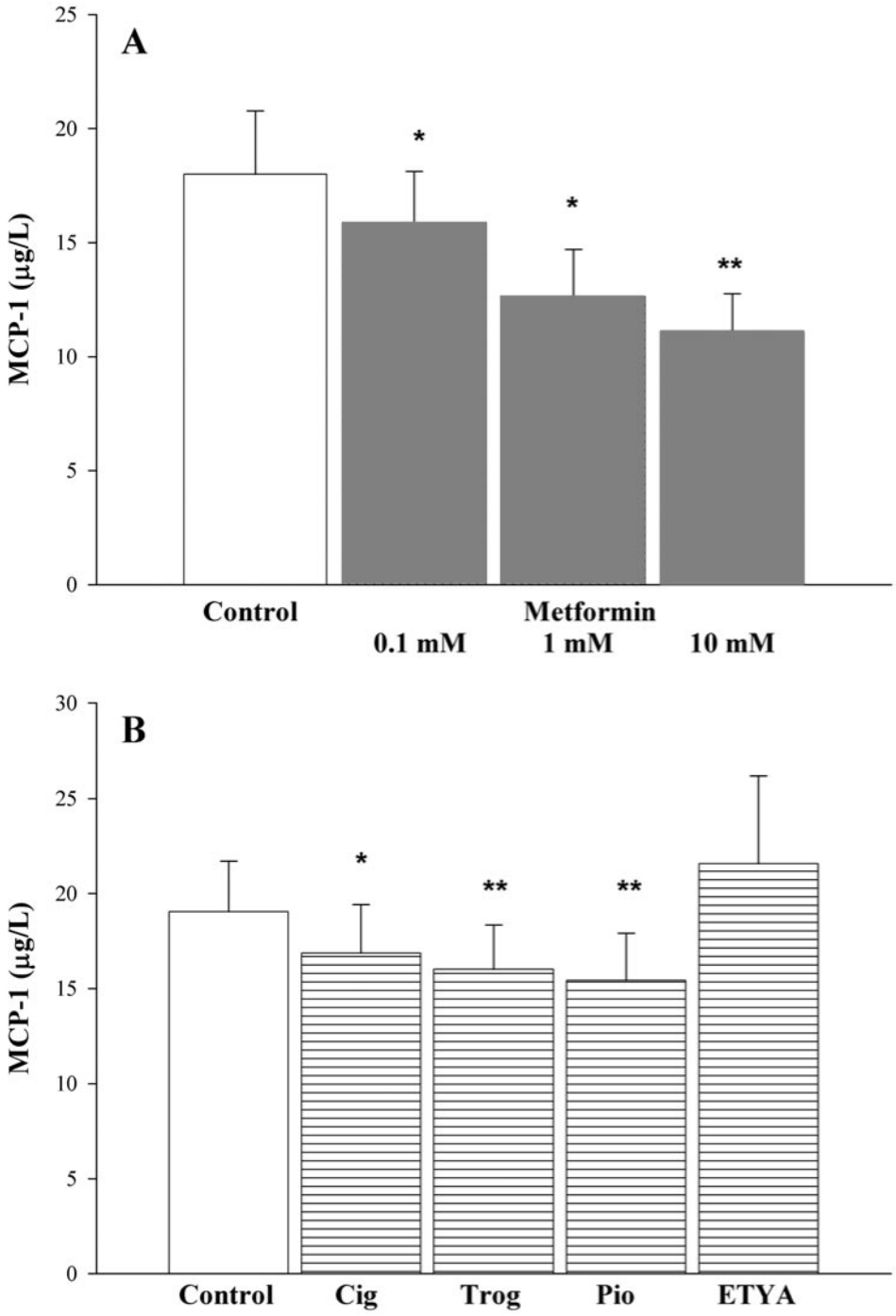


FIG. 5. Regulation of MCP-1 release by antidiabetic compounds. Human SAT was incubated with: A, metformin (0.1, 1, or 10 mM); and B, TZDs [ciglitazone (Cig; 50 μM), troglitazone (Trog; 10 μM), pioglitazone (Pio; 10 μM), or ETYA (15 μM)] for 48 h. Data represent mean values ± SEM (n = 6). *, $P < 0.05$; **, $P < 0.01$ compared with control incubations.

be attributable to the number of macrophages resident in the AT. However, regardless from which cell types within the AT theses adipokines are derived, visceral obesity is associated with a general low-grade inflammatory process and an increased risk of developing type 2 diabetes and cardiovascular disease (1, 2, 44), and the attenuation of MCP-1 release by the antidiabetic compounds, metformin, and TZDs supports the hypothesis that these compounds have antiinflammatory properties improving the low-grade inflammatory state observed in obesity.

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