Original Article

Expression of CD68 and Macrophage Chemoattractant Protein-1 Genes in Human Adipose and Muscle Tissues
Association With Cytokine Expression, Insulin Resistance, and Reduction by Pioglitazone

Gina B. Di Gregorio,[1,2] Aiwei Yao-Borengasser,[1,2] Neda Rasouli,[1,2] Vijayalakshmi Varma,[1,2] Tong Lu,[1,2] Leslie M. Miles,[1,2] Gouri Ranganathan,[1,2] Charlotte A. Peterson,[1,3] Robert E. McGehee,[4] and Philip A. Kern[1,2]

To examine the role of adipose-resident macrophages in insulin resistance, we examined the gene expression of CD68, a macrophage marker, along with macrophage chemoattractant protein-1 (MCP-1) in human subcutaneous adipose tissue using real-time RT-PCR. Both CD68 and MCP-1 mRNAs were expressed in human adipose tissue, primarily in the stromal vascular fraction. When measured in the adipose tissue from subjects with normal glucose tolerance, covering a wide range of BMI (21–51 kg/m^2) and insulin sensitivity (S_I) (0.6– 8.0 × 10^{-4} min^{-1} · µU^{-1} · ml^{-1}), CD68 mRNA abundance, which correlated with the number of CD68-positive cells by immunohistochemistry, tended to increase with BMI but was not statistically significant. However, there was a significant inverse relation between CD68 mRNA and S_I ($r = -0.55, P = 0.02$). In addition, there was a strong positive relationship among adipose tissue CD68 mRNA, tumor necrosis factor-α (TNF-α) secretion in vitro ($r = 0.79, P < 0.005$), and plasma interleukin-6 ($r = 0.67, P < 0.005$). To determine whether improving S_I in subjects with impaired glucose tolerance (IGT) was associated with decreased CD68 expression, IGT subjects were treated for 10 weeks with pioglitazone or metformin. Pioglitazone increased S_I by 60% and in the same subjects reduced both CD68 and MCP-1 mRNAs by >50%. Furthermore, pioglitazone resulted in a reduction in the number of CD68-positive cells in adipose tissue and reduced plasma TNF-α. Metformin had no effect on any of these measures. Thus, treatment with pioglitazone reduces expression of CD68 and MCP-1 in adipose tissue, apparently by reducing macrophage numbers, resulting in reduced inflammatory cytokine production and improvement in S_I. *Diabetes* 54:2305–2313, 2005

The increasing prevalence of obesity over the last few decades has been associated with a parallel increase in diabetes and metabolic syndrome (1–3). Although there is a significant correlation between obesity, insulin resistance, and metabolic syndrome, the pathophysiology of these relationships are not well understood.

Knowledge about the metabolic syndrome has moved forward with the description of numerous adipose secretory products, including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, plasminogen activator inhibitor 1, resistin, IL-1, leptin, and adiponectin (4–7). There is growing evidence that adipose secretory products are important determinants of insulin resistance, either through a traditional (circulating) hormonal effect or through local effects in adipose tissue. The expression and secretion into plasma of these and other cytokines has likened metabolic syndrome to a chronic state of inflammation, and this complex elaboration of inflammatory cytokines forms the basis for insulin resistance in liver and muscle and for tissue lipid accumulation (8).

Two recent studies (9,10) demonstrated that obesity is associated with significant infiltration of adipose tissue by macrophages. The expression of macrophage markers was highly correlated with obesity in mice, and the resident macrophages were responsible for the expression of most of the tissue TNF-α and IL-6, although previous studies demonstrated TNF-α expression by human adipocytes (11). Limited human studies were performed and demonstrated a correlation between adipose tissue macrophage markers and BMI (10). Important questions that remain unanswered include the relationship between adipose tissue macrophage infiltration and insulin resistance in

TABLE 1
Primer sequences

18S forward	5′TTCGAACGTCTGCCCTATCAA3′
18S reverse	5′ATGGTAGGCACGGCGACTA3′
MCP-1 forward	5′CCCCAGTCACCTGCTGTTAT3′
MCP-1 reverse	5′AGGTGACTGGGGCATTGATT3′
CD68 forward	5′GCTACATGGCGGTGGAGTACAA3′
CD68 reverse	5′ATGATGAGAGGCAGCAAGATGG3′

humans, along with the relationship to cytokine expression and the response to insulin sensitizer drugs.

The thiazolidinedione class of drugs, which include pioglitazone and rosiglitazone, are agonists for peroxisome proliferator–activated receptor-γ (PPAR-γ), which is a transcription factor present at high levels in adipocytes (12). The treatment of diabetic patients with these drugs results in an improvement in peripheral insulin sensitivity, which involves improved glucose transport, mostly into skeletal muscle (13). However, PPAR-γ is also expressed by macrophages (14,15), and recent studies have suggested that PPAR-γ agonists, such as the thiazolidinediones, may have broad effects on suppressing macrophage-mediated inflammation (16).

To better understand the relationship between adipose tissue macrophage infiltration and insulin resistance, we measured the expression of transcripts encoding CD68, a macrophage transmembrane protein (17), as well as macrophage chemoattractant protein-1 (MCP-1) in well-characterized subjects with obesity and insulin resistance. In addition, we treated subjects with impaired glucose tolerance (IGT) with either pioglitazone or metformin and examined these markers of adipose tissue inflammation.

RESEARCH DESIGN AND METHODS

We recruited generally healthy subjects without diabetes by local advertisement. All subjects provided written, informed consent under protocols approved by the local institutional review board, and studies were conducted on the University of Arkansas for Medical Sciences/Central Arkansas Veterans Health Care System General Clinical Research Center. Subjects were included if their fasting glucose was <110 mg/dl and 2-h postchallenge glucose was <199 mg/dl. Based on an initial 75-g oral glucose tolerance test, subjects were classified as having either normal glucose tolerance (NGT) (fasting blood glucose <6.1 mmol/l and 2-h glucose <11.1 mmol/l) or IGT (2-h glucose 7.8–11.1 mmol/l). A total of 42 subjects were recruited, of which 18 had NGT and 24 had IGT. All 42 subjects were weight stable, 35–65 years of age, and 36 were women. Subjects with a history of coronary artery disease or who were being treated with fibrates, ACE inhibitors, and angiotensin II receptor blockers were excluded. Insulin sensitivity was measured using a frequently sampled intravenous glucose tolerance test (FSIGTT), and all subjects underwent an incisional subcutaneous adipose tissue biopsy from the lower abdominal wall and a muscle biopsy from the vastus lateralis. Subjects with IGT were then randomized to receive either metformin or pioglitazone for a 2-week dose escalation followed by 8 weeks at a maximum dose (1,000 mg metformin twice daily or 45 mg pioglitazone daily). After 10 weeks of

treatment, the oral and intravenous glucose tolerance tests and biopsies were repeated.

Adipose tissue and cells. To isolate different cell fractions from adipose tissue, subcutaneous fat from a biopsy was digested with collagenase according to the method of Rodbell (18) and the adipocytes separated from the stromal vascular fraction by centrifugation. Additional human adipocyte preparations were prepared by the differentiation of preadipocytes obtained from discarded adipose tissue from normal women undergoing liposuction. The stromal vascular fraction was isolated, placed into culture, and induced to differentiate into adipocytes as described previously. In brief, the lipoaspirate is washed in Krebs-Ringer bicarbonate solution (KRB) and digested with 1 vol collagenase type I (1 g/l KRB with 1% BSA) for 1 h at 37°C with intermittent shaking. The stromal vascular fraction was separated from the floating adipocytes by centrifugation, resuspended in growth media (Dulbecco's modified Eagle's medium/Hams-F12, 10% fetal bovine serum, and 15 mmol/l HEPES) and plated in tissue culture flasks at a density of 3,500 cells/cm^2. Following two passages, cells were plated in either 12- or 24-well culture dishes at a density of ~35,000 cells/cm^2, allowed to reach confluence, treated with adipocyte-inducing media (growth media supplemented with 100 nmol/l/l insulin, 1.0 μmol/l dexamethasone, 0.25 mmol/l isobutylmethylxanthine, 0.033 mmol/l biotin, 0.017 mmol/l pantothenate, and 1.0 μmol/l rosiglitazone) for 3 days, and followed for an additional 3 days of the same supplementation without isobutylmethylxanthine and rosiglitazone (19). Differentiation is assessed by staining cells for lipid with Oil Red O and the detection of adipocyte-specific mRNA and/or protein expression. In the experiments described below, ~50–60% of the differentiated cells stained positively with Oil Red O.

Insulin sensitivity. In all patients, insulin sensitivity was measured by FSIGTT, although different tests were used with different subjects. For the NGT subjects, the classic tolbutamide-modified FSIGTT was performed using 11.4 g/m^2 glucose at time 0 and an injection of tolbutamide (125 mg/m^2) at 20 min, followed by MinMod analysis (20). Since tolbutamide for parenteral administration is no longer available, the IGT subjects were studied using an insulin-modified intravenous glucose tolerance test using 0.04 units/kg insulin instead of tolbutamide, as described elsewhere (21). Although the measures of insulin sensitivity (S_I) derived from these different procedures correlate strongly with each other and with the euglycemic clamp (22), the values are different, hence the data from NGT and IGT subjects were analyzed separately. Insulin was measured using an immunochemiluminescent assay (MLT assay; Molecular Light Technology, Cardiff, Wales, U.K.), and glucose was measured in duplicate by a glucose oxidase assay. Insulin sensitivity was calculated from the insulin and glucose data using the MinMod program (23).

Hormone measurements. TNF-α and IL-6 proteins were quantified using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN), as described previously (24). TNF-α was measured in the medium of adipose tissue following a 2-h culture after biopsy, as described previously (24). IL-6 was measured in plasma. These measures of adipose tissue TNF-α secretion and plasma IL-6 have previously been demonstrated to correlate with insulin resistance (24).

Total RNA isolation and real-time RT-PCR. Total RNA from adipose tissue was isolated using an RNeasy Lipid Tissue Mini kit from Qiagen (Valencia, CA), following the manufacturer's instruction. Total RNA from muscle biopsies was isolated using an Ultraspec RNA Isolation System kit from Biotex (Houston, TX), according to the manufacturer's instruction. The quantity and quality of the isolated RNA was determined by ultraviolet spectrophotometry and formaldehyde-agarose gel electrophoresis, respectively. One microgram of total RNA was reverse transcribed using random hexamer primers with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Reverse-transcribed RNA was amplified with 1× SYBR Green PCR Master Mix (Applied Biosystems) plus 0.3 μmol/l gene-specific upstream and downstream primers during 55 cycles on a Rotor-Gene 3000 Real-Time Thermal Cycler (Corbett Research, Sydney, Australia). Each cycle consisted of denaturation

TABLE 2
CD68 and MCP-1 mRNA in cells and tissue

Cell/tissue	CD68*	MCP-1*	Adiponectin*
Whole adipose tissue	1.15 ± 0.32	1.14 ± 0.23	2.3 ± 0.12
Adipocytes from adipose tissue	0.38 ± 0.03	0.25 ± 0.004	2.3 ± 0.01
Stromal fraction from adipose tissue	5.61 ± 0.22	1.27 ± 0.09	0.25 ± 0.01
Adipocytes from cultured preadipocytes	0.36 ± 0.06	0.83 ± 0.02	1.1 ± 0.004
Muscle tissue	0.05 ± 0.01	0.03 ± 0.003	0.01 ± 0.001

Data are means ± SE. *The pooled RNA from all the samples was used for a standard curve. Hence, the data are expressed relative to each other.

TABLE 3
Characteristics of NGT subjects

	Age (years)	BMI (kg/m^2)	S_I	TG (mmol/l)	LDL (mmol/l)	HDL (mmol/l)	FBG (mmol/l)
Means ± SE	37 ± 3.3	37 ± 2.4	3.3 ± 0.6	1.3 ± 0.3	3.1 ± 0.2	1.2 ± 0.1	5.1 ± 0.1
Range	23–61	21–51	0.59–8.1	0.5–3.4	1.7–4.0	0.9–1.8	4.6–5.5

To convert glucose from mmol/l to mg/dl, multiply by 18; cholesterol mmol/l to mg/dl, multiply by 38.67; and triglyceride mmol/l to mg/dl, multiply by 88.57. S_I is presented as 10^{-4} min$^{-1} \cdot \mu U^{-1} \cdot ml^{-1}$. FBG, fasting blood glucose; TG, triglycerides.

at 94°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s. Amplified 18S expression was used as standard control to normalize the differences in individual samples. The primer sequences for CD68, MCP-1, and 18S are listed in Table 1. All data were expressed in relation to 18S RNA, where the standard curves were generated using pooled RNA from the samples assayed. Therefore, the data represent arbitrary units, which accurately compare each set of samples to each other but do not necessarily accurately compare samples between different assays. The Ct values of the PCRs were generally between 20 and 30 for all assays. All samples were analyzed twice with and without reverse transcriptase, and no amplification was seen in the samples in the absence of reverse transcriptase.

Immunohistochemistry. Immunohistochemical detection of macrophages was performed on frozen 8-μm–thick human adipose tissue cryostat sections and fixed in 4% paraformaldehyde for 5 min, followed by methanol for 5 min. The sections were blocked for endogenous peroxidase activity by incubation in 0.3% H_2O_2 in PBS overnight at 4°C. For CD68 immunostaining, mouse anti-human CD68 monoclonal antibody (clone EBM-11; Dako, Milan, Italy) was used at a dilution of 1:150 in blocking solution (ImmPRESS Reagent Anti mouse Ig; Vector Laboratories, Burlingame, CA) overnight at 4°C, followed by peroxidase-coupled anti-mouse IgG (ImmPRESS Reagent; Vector labs, Burlingame, CA) for 1 h at room temperature. Another marker for monocyte/macrophages is CD115 (also known as CSF1R) (25). For CD115 immunostaining, rat anti-human CD115 antibody (Research Diagnostic, Flanders, NJ) was used at a dilution of 1:60 in PBS overnight at 4°C, followed by horseradish peroxidase–goat anti-rat IgG (Zymed Laboratories, San Francisco, CA) in PBS for 1 h at room temperature. The fluorescein-based tyramide signal amplification fluorescence system (PerkinElmer Life Sciences, Boston, MA) was used for detection of both antigens according to the protocol provided. The slides were rinsed with water and washed in methanol for 10 min to remove the lipid droplets. Sections were visualized with a Nikon Eclipse E600 microscope using Nikon Plan Fluor 20×/0.50 objective and photographed with a Photometrics CoolSnapES camera at room temperature. For macrophage quantitation, adipocytes and macrophages were counted from 8–10 fields and the macrophages expressed as a percentage of the total adipocytes counted.

Statistical analysis. Correlation analysis was performed with real-time PCR data collected on NGT subjects with significance determined to be $P = 0.05$. Baseline and post-treatment variables within groups were compared by paired t test. Analysis of trends was performed using linear regression. All data are expressed as means ± SE.

RESULTS

Detection of CD68 and MCP-1 mRNA in cell fractions. To better identify the cells that express CD68 and MCP-1, we measured mRNA levels using real-time RT-PCR in human whole adipose tissue and fractionated tissue samples, as well as in muscle tissue (Table 2). The expression of adiponectin, an adipocyte-specific gene, was also measured as an indicator of adipocyte gene expression. All data were expressed as arbitrary units in relation to 18S RNA, and all of the samples were from NGT subjects. Human subcutaneous adipose tissue from biopsies were digested with collagenase and centrifuged to separate the adipocytes from the cell pellet, commonly referred to as the stromal vascular fraction. As shown in Table 2, CD68 gene expression in whole adipose tissue was higher than the level in adipocytes derived from adipose tissue. On the other hand, the stromal vascular fraction of adipose tissue expressed over 10-fold more CD68 mRNA than the adipocyte fraction and ~5-fold more than the whole adipose tissue. As expected, adiponectin mRNA accumulated to 10-fold higher levels in adipose tissue and adipocytes than in the stromal vascular fraction.

Because the adipocytes from an adipose tissue digestion could contain adherent stromal cells or macrophages, we induced human preadipocytes to differentiate into adipocytes, as described in RESEARCH DESIGN AND METHODS. Macrophages are unlikely to survive long-term culture and multiple passages; therefore, these cultures probably contained no macrophages. As shown in Table 2, the expression of CD68 gene expression was similar to that found in the adipocytes derived from adipose tissue. CD68 mRNA accumulation was lower in skeletal muscle compared with adipose tissue but was detectable. The expression of MCP-1 mRNA in adipose tissue was low in adipocytes from adipose tissue but was similar in whole adipose tissue, stromal fraction, and cultured adipocytes. MCP-1 was also expressed at a low level in muscle. Thus, these data suggest that the stromal vascular fraction contains the majority of the CD68-expressing cells, with low-level CD68 expression in the adipocyte fraction and very low levels in muscle. The expression of MCP-1 mRNA was
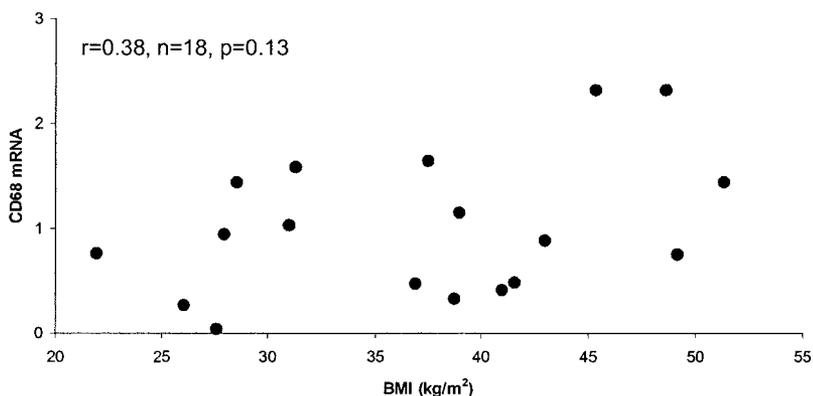

FIG. 1. Relationship between CD68 gene expression and BMI. CD68 was expressed in relation to 18S RNA in NGT subjects covering a range of BMI.
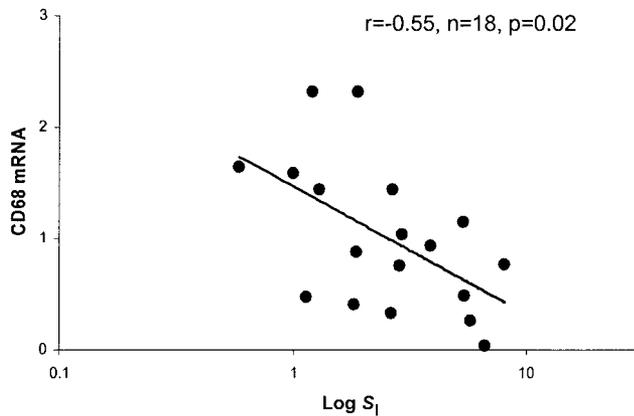
FIG. 2. Relationship between CD68 gene expression and S_I. CD68 was expressed in relation to 18S RNA in NGT subjects. In each subject, insulin sensitivity was determined using the FSIGTT and expressed as S_I (10^{-4}min^{-1} · μU^{-1} · ml^{-1}). S_I was log transformed.

more uniform throughout adipose tissue and was even expressed in cultured adipocytes, suggesting that MCP-1 is expressed by a number of cell types.

Relationship among CD68 mRNA, obesity, and insulin resistance in NGT subjects. CD68 gene expression was analyzed in the adipose tissue samples from 18 subjects with NGT. As shown in Table 3, these subjects were mostly women spanning a wide range of BMI and S_I, which permitted a detailed characterization of gene expression in relation to these parameters. Although there was a tendency for higher CD68 mRNA levels with obesity, there was much variability among subjects, and this relationship was not statistically significant ($r = 0.38, P = 0.13$) (Fig. 1). This relationship between CD68 mRNA levels and obesity was similar using percentage of body fat instead of BMI. However, the relationship between CD68 expression and insulin resistance was stronger. Among these NGT subjects, those with the highest levels of S_I, who were the most insulin sensitive, had the lowest levels of CD68 mRNA levels ($r = -0.55, P = 0.02$) (Fig. 2).

Relationship between CD68 gene expression and cytokines. Adipose tissue expresses many proteins, including inflammatory cytokines, and some of these cytokines are expressed by adipose tissue resident macrophages. In previous studies, TNF-α secretion from adipose tissue was independently associated with insulin resistance, as was plasma IL-6 (24). Therefore, we examined the relationship
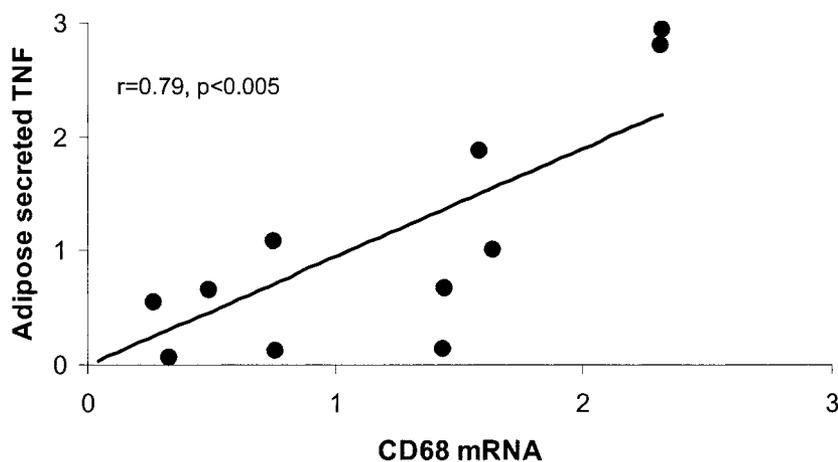
between adipose tissue cytokine expression and CD68 by measuring TNF-α secretion and plasma levels of IL-6 in subjects with NGT. As shown in Fig. 3, there was a significant positive association between CD68 expression and TNF-α secretion from adipose tissue. In addition, there was a significant relationship between CD68 expression and plasma IL-6 (Fig. 4). However, there was no relationship between CD68 expression and leptin ($r = 0.29, P = 0.25$) or adiponectin ($r = -0.35, P = 0.20$) (data not shown). Thus, adipose tissue CD68 gene expression was strongly linked to the cytokines TNF-α and IL-6, which are primarily expressed by monocyte/macrophages, and not related to the expression of leptin and adiponectin, which are adipocyte proteins.

Effects of insulin sensitizers on CD68 and MCP-1 gene expression and serum cytokine levels. As described above, we recruited 24 subjects with IGT and randomized these subjects to treatment for 10 weeks with either pioglitazone or metformin. The characteristics of these IGT subjects are shown in Table 4. Total RNA was extracted from the adipose tissue of these subjects, and CD68 and MCP-1 gene expression was quantitated. As shown in Fig. 5, a 10-week treatment with pioglitazone resulted in a reduction in adipose tissue CD68 expression ($P < 0.002$), whereas there was no suggestion of a decrease in CD68 by metformin treatment. Along with the reduction in CD68 gene expression, subjects treated with pioglitazone also demonstrated a significant increase in S_I, whereas subjects treated with metformin demonstrated no improvement in insulin sensitivity (Table 4). In addition, pioglitazone, but not metformin, treatment resulted in a significant decrease in plasma TNF-α. Although there was a trend toward lower IL-6, this did not reach significance (Table 5).

To determine whether the differences in mRNA levels of CD68 with insulin resistance and changes in response to pioglitazone treatment were related to changes in tissue macrophages, immunohistochemistry was performed on whole adipose tissue samples. Staining of adipose tissue sections was performed with an anti-CD68 antibody to determine whether the number of CD68-positive cells was affected or whether the intensity of the staining in similar numbers of positive cells changed. The number of CD68-positive cells in adipose tissue was decreased following pioglitazone treatment (compare Fig. 6*A* and *B* and the quantitation of CD68 staining in *G*). In addition, we per-

FIG. 3. Relationship between CD68 gene expression and TNF-α secretion. The adipose tissue sample from each subject was minced and placed into serum-free medium, and TNF-α secretion was measured at 2 h as pg/μg DNA.

TABLE 4
Characteristics of IGT subjects

	Age	BMI (kg/m^2)	S_I before	S_I after	TG (mmol/l)	LDL (mmol/l)	HDL (mmol/l)	FBG (mmol/l)
Pioglitazone	48 ± 2	34 ± 1.1	1.15 ± 0.11	1.84 ± 0.2*	2.5 ± 0.5	2.8 ± 0.3	1.2 ± 0.1	5.1 ± 0.2
Metformin	50 ± 2	33 ± 1.0	1.44 ± 0.24	1.47 ± 0.16	1.9 ± 0.3	2.5 ± 0.2	1.3 ± 0.1	4.9 ± 0.2

Data are means ± SE. To convert glucose from mmol/l to mg/dl, multiply by 18; cholesterol mmol/l to mg/dl, multiply by 38.67; and triglyceride mmol/l to mg/dl, multiply by 88.57. S_I is presented as $10^{-4}\ \text{min}^{-1} \cdot \mu U^{-1} \cdot ml^{-1}$. *$P < 0.001$ vs. "before." FBG, fasting blood glucose. TG, triglyceride.

formed CD68 staining in adipose tissue from NGT subjects. When comparing adipose tissue samples from a relatively insulin-resistant subject (S_I $2.5 \times 10^{-4}\ \text{min}^{-1} \cdot \mu U^{-1} \cdot ml^{-1}$) with an NGT subject who was relatively insulin sensitive (S_I $4.5 \times 10^{-4}\text{min}^{-1} \cdot \mu U^{-1} \cdot ml^{-1}$), there was high CD68 mRNA and frequent CD68-positive cells in the insulin-resistant subject and lower CD68 mRNA and fewer positive cells in the insulin-sensitive subject (compare Fig. 6C and D). The images shown are representative of the fields examined, and weakly positive cells, which might be expected if there were a generalized inhibition of CD68 expression without a change in macrophage number, were not seen. To further demonstrate that the changes in CD68 expression and staining was due to changes in macrophage number, immunostaining was performed with CD115, another macrophage marker (25). As shown in Fig. 6D and E, pioglitazone treatment resulted in a decrease in the number of cells staining for CD115. Thus, these data suggest that the changes in CD68 mRNA levels primarily reflect changes in the number of resident macrophages in the adipose tissue.

Whereas CD68 is a surface marker expressed predominantly by macrophages, MCP-1 is a macrophage chemoattractant protein that is expressed by many tissues, including adipocytes (26,27). To determine whether the treatment of subjects with insulin sensitizers led to a change in MCP-1 gene expression, MCP-1 mRNA levels were measured in adipose tissue of the IGT subjects before and after treatment with metformin and pioglitazone. As shown in Fig. 7, pioglitazone, but not metformin, led to a significant decrease in the expression of MCP-1 mRNA in adipose tissue. Thus, treatment with pioglitazone reduces the expression of the mRNA encoding the macrophage marker CD68, as well as the expression of MCP-1.

The improvement in S_I following treatment with pioglitazone results predominantly from improved peripheral glucose transport in muscle, and low levels of both CD68 and MCP-1 mRNAs were expressed by muscle. To determine whether the insulin sensitizers affected macrophage infiltration in muscle, CD68 and MCP-1 mRNAs were measured in skeletal muscle biopsies from the same subjects before and after treatment. As shown in Fig. 8, low levels of both transcripts were detectable in muscle, and there was no change following treatment with the insulin sensitizers.

DISCUSSION

Many studies have characterized obesity, metabolic syndrome, and diabetes as a state of chronic inflammation (8,28), including vascular inflammation (29). The precise origin and etiology of this inflammation has remained unclear, although there is much evidence that the expanding adipose tissue mass in obesity is central to this inflammatory condition. Since the original description of TNF-α expression by adipose tissue of rodents and humans (11,30,31), many studies have demonstrated a strong association between obesity and insulin resistance and the expression of many cytokines. In addition to numerous studies in humans that demonstrated an association between cytokines and insulin resistance (24), knock-out mouse experiments generally confirmed the importance of many cytokines in the development of features of metabolic syndrome (32).

Recent studies have suggested that an important source of many cytokines in adipose tissue is macrophages (9,10). According to these studies, which were performed predominantly in rodents, circulating monocytes of bone
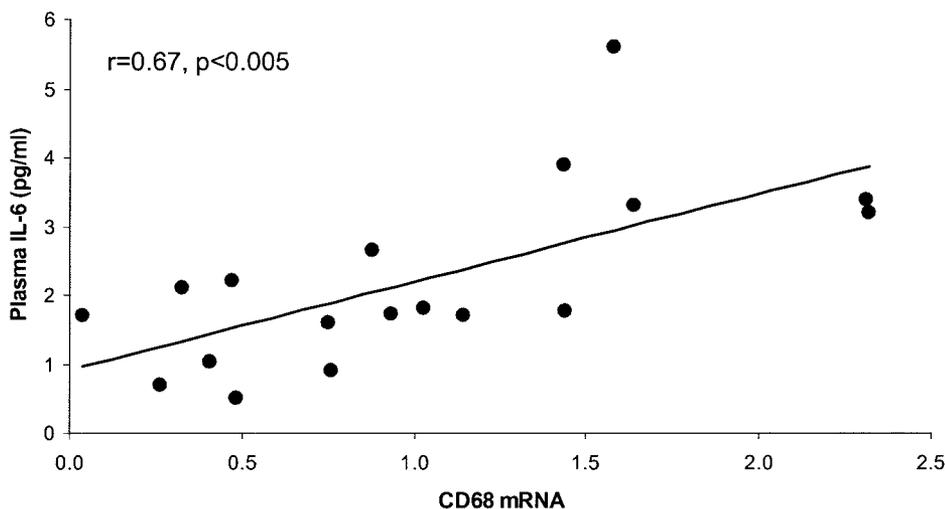
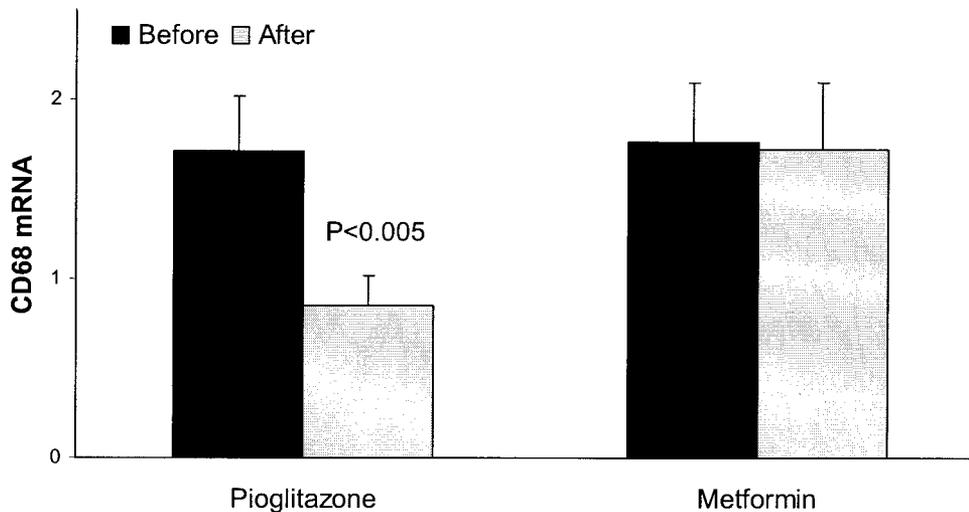FIG. 4. Relationship between CD68 gene expression and plasma IL-6.

FIG. 5. Effect of insulin sensitizers on CD68 gene expression from adipose tissue. Adipose tissue biopsies were performed before and after treatment of IGT subjects with pioglitazone or metformin, as described in RESEARCH DESIGN AND METHODS, for 10 weeks. Data represent arbitrary units standardized to 18S RNA.

marrow origin become resident tissue macrophages. Adipose tissue from obese rodents contains more macrophages, and these adipose tissue macrophages account for most of adipose tissue TNF-α expression and a substantial amount of IL-6 expression. In human adipose tissue, a correlation was observed between BMI and CD68 expression (10).

In the present study, we examined CD68 and cytokine expression in human adipose tissue samples. To demonstrate the cell types associated with CD68 gene expression, we separated the adipocyte fraction from the stromal vascular fraction (containing macrophages, preadipocytes, and other cells). Very low levels of CD68 mRNA were found in the adipocyte fraction compared with the stromal-vascular-macrophage fraction, where CD68 mRNA levels were >10-fold higher than in the adipocyte fraction. In addition to macrophages, the human stromal vascular fraction contains preadipocytes that can be cultured long term and induced to differentiate into adipocytes. After several passages, these cultures are essentially pure differentiated adipocytes, based on the ability of cells to accumulate lipid. When we examined CD68 mRNA levels in these preadipocyte-derived human adipocytes, we again found very low expression of CD68, although it is important to note that CD68 was detectable in adipocytes. Thus, CD68 is a useful macrophage marker; however, it is not entirely specific for macrophages, since it is found in the adipose tissue stroma and also at a very low level in adipocytes, whether the adipocytes are derived from the floating fraction following a collagenase digestion or from preadipocyte differentiation in culture.

The expression of CD68 mRNA was examined in relation to a number of clinical features of the subjects. In

TABLE 5
Effect of pioglitazone and metformin on plasma cytokines

	Pioglitazone		Metformin	
	Before	After	Before	After
Plasma TNF-α (pg/ml)	3.3 ± 0.17	2.7 ± 0.12*	3.6 ± 0.57	3.3 ± 0.5
Plasma IL-6 (pg/ml)	2.5 ± 0.64	1.89 ± 0.29	2.4 ± 0.44	1.9 ± 0.23

Data are means ± SE. *$P < 0.05$ vs. "before."

NGT subjects, there was a trend toward an increase in CD68 gene expression with BMI; however, this relationship was not significant. In addition, there was a significant inverse relationship between CD68 mRNA and S_I, demonstrating that CD68 gene expression, an indicator of macrophage number, is more associated with insulin resistance than with obesity per se. This relationship between CD68 mRNA and S_I was further supported by the relationship between CD68 mRNA and cytokine expression. In subjects with higher CD68 gene expression in adipose tissue, there was also a higher level of TNF-α secretion from adipose tissue into the culture medium and a higher level of plasma IL-6. In previous studies, we demonstrated that adipose tissue–secreted TNF-α and plasma IL-6 were strongly associated with insulin resistance (24). Since TNF-α and IL-6 are both produced either exclusively or predominantly by adipose tissue macrophages, the relationship among adipose tissue CD68 gene expression, TNF-α, and IL-6 suggests that macrophages are important in the expression of these cytokines and therefore important in the development of insulin resistance.

In a previous study, Weisberg et al. (10) demonstrated a statistically significant relationship between CD68 gene expression and BMI in humans. In our subjects, there was a positive relationship between CD68 gene expression and BMI, but this was not statistically significant. This analysis was performed on NGT subjects, whereas in the previous study no indication of glycemic status was given, and there may have been additional differences in the study subjects. Our studies were performed primarily in women, and the sample size would not have permitted a detection of a subtle relationship between CD68 and obesity. Overall, our data would suggest that macrophage accumulation in adipose tissue is a feature more of insulin resistance and metabolic syndrome than of obesity, although obesity is a part of metabolic syndrome.

MCP-1 is a protein produced by many different cell types, including endothelial cells, monocytes, and adipocytes, in response to inflammatory stimuli (27,33–36). MCP-1 is one of a large family of chemokines that attract macrophages and T-cells to sites of inflammation, although other possible actions of MCP-1 have been suggested, since the receptor for MCP-1 is found in many cells, including adipocytes (27). Obese subjects demon-
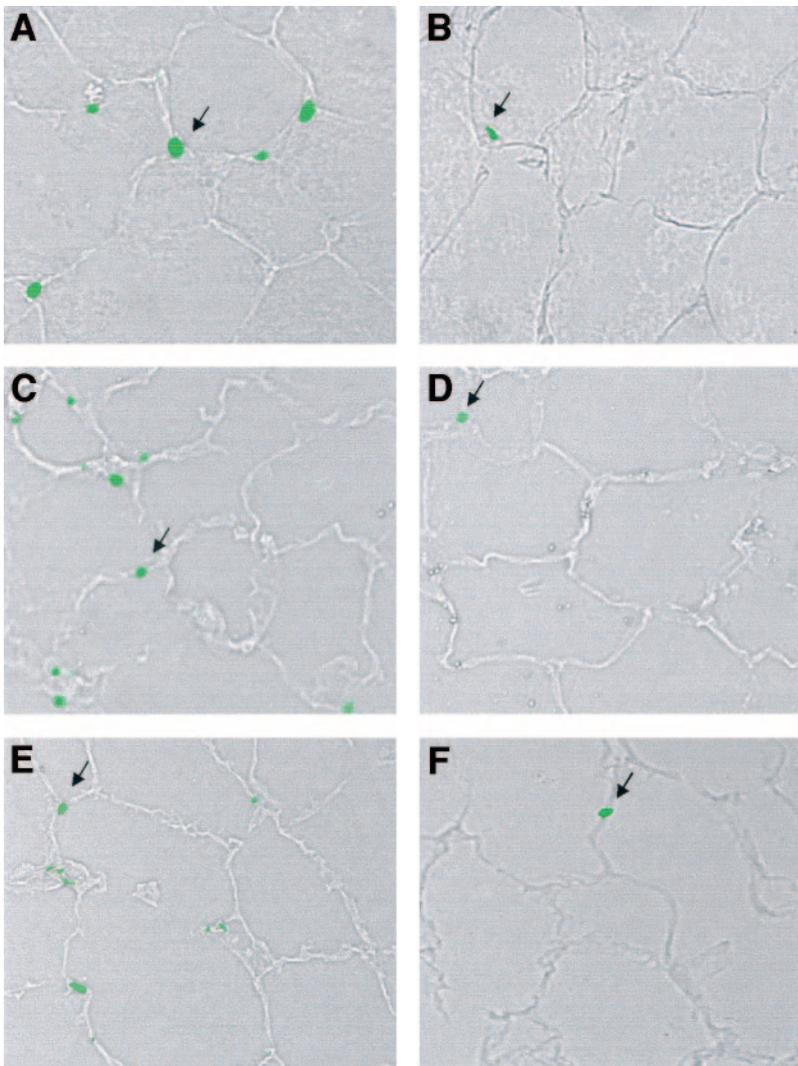
FIG. 6. Immunohistochemistry of adipose tissue for CD68 and CD115. Human adipose tissue samples were sectioned and stained with antibodies for macrophage markers, as described in RESEARCH DESIGN AND METHODS. Positive cells are stained green, and representative positive cells are marked with an arrow. *A*: CD68 staining of adipose tissue from a representative IGT subject before pioglitazone treatment (CD68 mRNA 3.69 units). *B*: Same subject after pioglitazone (CD68 mRNA 1.33 units). *C*: NGT subject with low S_I (CD68 mRNA 2.71 units). *D*: NGT subject with high S_I (CD68 mRNA 0.32 units). *E*: CD115-positive cells in human adipose tissue of an IGT patient before pioglitazone treatment. *F*: Same subject after pioglitazone treatment. *G*: Quantitation of macrophage CD68 staining in five subjects before and after pioglitazone. CD68-positive cells were normalized to adipocyte number and expressed as a percentage. $*P < 0.005$ vs. baseline.

strated higher levels of MCP-1 gene expression (34), and MCP-1 expression was increased in adipocytes by insulin (26). A polymorphism in the MCP-1 gene resulted in decreased plasma MCP-1 levels and less insulin resistance (37). Because MCP-1 is expressed by adipocytes and plays an important role in monocyte recruitment, it is possible that obesity-related adipocyte expression of MCP-1 results in the recruitment of resident monocytes, which then elaborate inflammatory cytokines, resulting in insulin resistance and many of the features of metabolic syndrome.

In our studies, treatment of IGT subjects with pioglita-

zone, but not metformin, resulted in decreased expression of both CD68 and MCP-1 genes and a decrease in CD68-positive cells in adipose tissue, along with an improvement in insulin resistance. Based on studies in rodent adipose tissue (9,10) and our studies localizing CD68 to the stromal vascular fraction, the reduction in CD68 suggests that macrophage abundance in adipose tissue was reduced by pioglitazone treatment. This conclusion was supported by the observation that an additional macrophage marker, CD115, showed a decrease in macrophage number in response to pioglitazone. On the other hand, the reduction
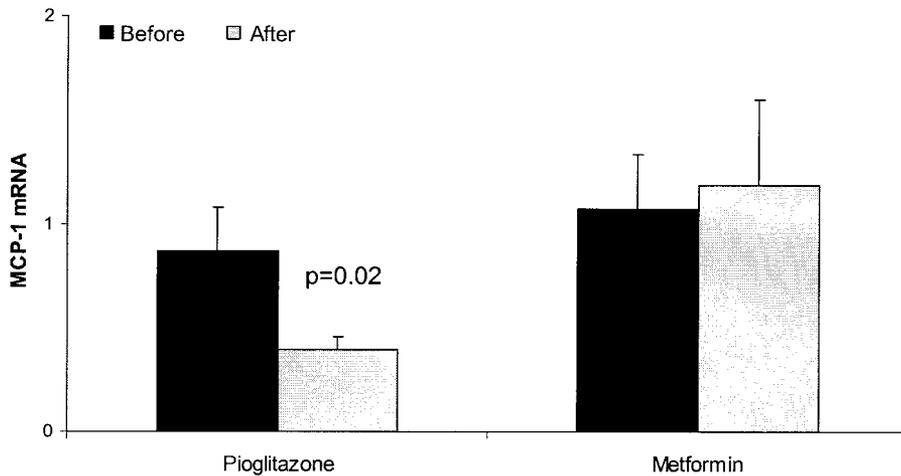
FIG. 7. Effect of insulin sensitizers on MCP-1 gene expression from adipose tissue. See methods described in legend to Fig. 5.

in MCP-1 by pioglitazone provides less information on the cell type involved, since MCP-1 is expressed by adipocytes and by cells in the stromal vascular fraction. Similarly, it is not clear by what mechanism pioglitazone affected the expression of CD68 and MCP-1. Macrophages express PPAR-γ (14), and therefore it is possible that thiazolidinediones directly affect macrophage expression of these proteins or induce apoptosis of macrophages (15). Indeed, numerous studies have suggested a role for mac-

rophage PPAR-γ agonists in the reduction of foam cell formation in the atherosclerotic plaque (38). Alternatively, it is possible that the primary target of thiazolidinediones is the adipocyte. If thiazolidinediones were to inhibit adipocyte expression of MCP-1, this may result in decreased recruitment of macrophages into adipose tissue and hence decreased CD68 expression, along with decreased expression of other inflammatory cytokines, such as TNF-α and IL-6. Another possible mediator of thiazolidinedione-mediated decreases in MCP-1 and CD68 is adiponectin, which is increased in response to thiazolidinedione treatment and which is associated with many aspects of inflammation (39,40). Thus, the effects of pioglitazone on CD68 and MCP-1 are entirely consistent with other anti-inflammatory properties of these insulin sensitizers, although the precise mechanism for this effect is unknown.

Although insulin resistance primarily reflects resistance to muscle glucose transport and muscle expresses low levels of CD68 and MCP-1, neither pioglitazone nor metformin had any effect on muscle expression. These data would suggest that the effects of pioglitazone on peripheral glucose disposal are not due to anti-inflammatory effects in muscle per se and more likely due to changes in adipose tissue and then to secondary effects on muscle.
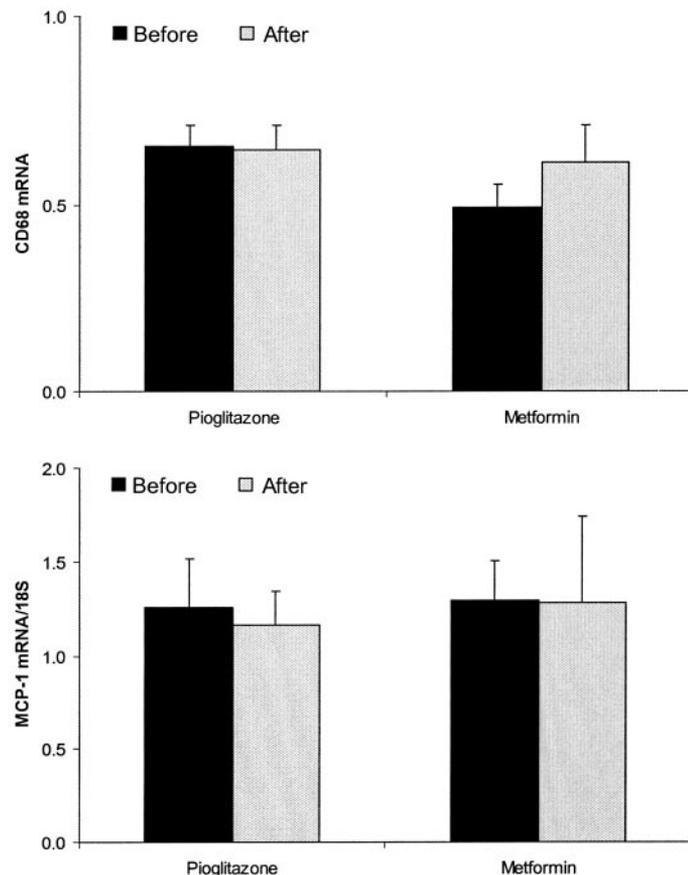
FIG. 8. Effects of insulin sensitizers on muscle gene expression. Subjects with IGT were treated for 10 weeks with either metformin or pioglitazone, and CD68 and MCP-1 mRNA levels were measured in skeletal muscle. The values represent relative values, standardized to 18S RNA. The pooled RNA from all the samples was used for a standard curve. Hence, the data are expressed relative to each other.

REFERENCES

1. Flegal KM, Carroll MD, Ogden CL, Johnson CL: Prevalence and trends in obesity among US adults, 1999–2000. *JAMA* 288:1723–1727, 2002
2. Mokdad A, Serdula MK, Dietz WH, Bowman B, Marks J, Koplan J: The spread of the obesity epidemic in the United States, 1991–1998. *JAMA* 282:1519–1522, 1999
3. Harris MI: Diabetes in America: epidemiology and scope of the problem. *Diabetes Care* 21 (Suppl. 3):C11–C14, 1998
4. Flier JS: The adipocyte: storage depot or node on the energy information superhighway? *Cell* 80:15–18, 1995
5. Flier JS: The missing link with obesity? *Nature* 409:292–293, 2001
6. Kern PA: Potential role of TNF-alpha and lipoprotein lipase as candidate genes for obesity. *J Nutr* 127:1917S–1922S, 1997
7. Hotamisligil GS, Spiegelman BM: Tumor necrosis factor α: a key component of the obesity-diabetes link. *Diabetes* 43:1271–1278, 1994

8. Hotamisligil GS: Inflammatory pathways and insulin action. *Int J Obes Relat Metab Disord* 27 (Suppl. 3):S53–S55, 2003

9. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003

10. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003

11. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB: The expression of tumor necrosis factor in human adipose tissue: regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 95:2111–2119, 1995

12. Vidalpuig AJ, Considine RV, Jimenezlinan M, Werman A, Pories WJ, Caro JF, Flier JS: Peroxisome proliferator-activated receptor gene expression in human tissues: effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest* 99:2416–2422, 1997

13. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP: The effect of insulin on the disposal of intravenous glucose: results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 30:1000–1007, 1981

14. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK: The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391:79–82, 1998

15. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B: Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 273:25573–25580, 1998

16. Castrillo A, Tontonoz P: Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. *Annu Rev Cell Dev Biol* 20:455–480, 2004

17. Holness CL, Da Silva RP, Fawcett J, Gordon S, Simmons DL: Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem* 268:9661–9666, 1993

18. Rodbell M: Metabolism of isolated fat cells. I. Effects of hormone on glucose metabolism and lipolysis. *J Biol Chem* 239:375–380, 1964

19. Van Harmelen V, Skurk T, Rohrig K, Lee YM, Halbleib M, Aprath-Husmann I, Hauner H: Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. *Int J ObesRelat Metab Disord* 27:889–895, 2003

20. Bergman RN, Finegood DT, Ader M: Assessment of insulin sensitivity in vivo. *Endocr Rev* 6:45–86, 1985

21. Bergman RN, Prager R, Volund A, Olefsky JM: Equivalence of the insulin sensitivity index in man derived by the minimal model method and the euglycemic glucose clamp. *J Clin Invest* 79:790–800, 1987

22. Saad MF, Steil GM, Kades WW, Ayad MF, Elsewafy WA, Boyadjian R, Jinagouda SD, Bergman RN: Differences between the tolbutamide-boosted and the insulin-modified minimal model protocols. *Diabetes* 46:1167–1171, 1997

23. Bergman RN, Ider YZ, Bowden CR, Cobelli C: Quantitative estimation of insulin sensitivity. *Am J Physiol* 236:E667–E677, 1979

24. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity-associated insulin resistance. *Am J Physiol Endocrinol Metab* 280:E745–E751, 2001

25. Olweus J, Thompson PA, Lund-Johansen F: Granulocytic and monocytic differentiation of CD34hi cells is associated with distinct changes in the expression of the PU 1-regulated molecules, CD64 and macrophage colony-stimulating factor receptor. *Blood* 88:3741–3754, 1996

26. Sartipy P, Loskutoff DJ: Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 100:7265–7270, 2003

27. Gerhardt CC, Romero IA, Cancello R, Camoin L, Strosberg AD: Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Mol Cell Endocrinol* 175:81–92, 2001

28. Rajala MW, Scherer PE: Minireview: The adipocyte–at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 144:3765–3773, 2003

29. Hsueh WA, Law R: The central role of fat and effect of peroxisome proliferator-activated receptor-gamma on progression of insulin resistance and cardiovascular disease. *Am J Cardiol* 92:3J–9J, 2003

30. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993

31. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995

32. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610–614, 1997

33. Linton MF, Fazio S: Macrophages, inflammation, and atherosclerosis. *Int J ObesRelat Metab Disord* 27 (Suppl. 3):S35–S40, 2003

34. Christiansen T, Richelsen B, Bruun JM: Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J ObesRelat Metab Disord* 29:146–150, 2005

35. Fasshauer M, Klein J, Kralisch S, Klier M, Lossner U, Bluher M, Paschke R: Monocyte chemoattractant protein 1 expression is stimulated by growth hormone and interleukin-6 in 3T3–L1 adipocytes. *Biochem Biophys Res Commun* 317:598–604, 2004

36. Wang B, Jenkins JR, Trayhurn P: Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-{alpha}. *Am J Physiol Endocrinol Metab* 288:E731–E740, 2005

37. Simeoni E, Hoffmann MM, Winkelmann BR, Ruiz J, Fleury S, Boehm BO, Marz W, Vassalli G: Association between the A-2518G polymorphism in the monocyte chemoattractant protein-1 gene and insulin resistance and type 2 diabetes mellitus. *Diabetologia* 47:1574–1580, 2004

38. Castrillo A, Tontonoz P: PPAR-s in atherosclerosis: the clot thickens. *J Clin Invest* 114:1538–1540, 2004

39. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPAR-gamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094–2099, 2001

40. Trayhurn P, Wood IS: Adipokines: inflammation and the pleiotropic role of white adipose tissue. *BrJ Nutr* 92:347–355, 2004