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Context: The study investigated the regulation of matrix metalloproteinases (MMP)-9 in obesity-associated insulin resistance in humans.

Objectives: The objectives of the investigation were to study MMP-9 regulation by insulin resistance and pioglitazone treatment in impaired glucose tolerant subjects using adipose tissue biopsies and study the mechanism of MMP-9 regulation by pioglitazone in adipocyte cultures.

Research Design: 86 nondiabetic, weight-stable subjects between 21 and 66 yr of age were recruited in a university hospital research center setting. All subjects underwent a sc adipose tissue incisional biopsy from the lower abdominal wall and insulin sensitivity testing using a frequently sampled iv glucose tolerance test. Impaired glucose-tolerant subjects were randomized to receive metformin or pioglitazone for 10 wk. To study the mechanism of MMP-9 regulation in adipocytes, cells were treated with pioglitazone or protein kinase Cα antisense oligomers, and MMP-9 levels were examined.

Results: There was a positive correlation between MMP-9 and body mass index ($r = 0.40$, $P < 0.01$) and negative correlation between MMP-9 and insulin sensitivity ($r = -0.46$, $P < 0.001$). The improvement in insulin sensitivity from pioglitazone resulted in a $52 \pm 0.2\%$ reduction in MMP-9 mRNA. Fractionation of adipose tissue indicated that MMP-9 was mostly in the stromal vascular fraction. Pioglitazone also decreased MMP-9 in 3T3-F442A adipocytes and THP1 macrophages. Coculture of adipocytes with macrophages augmented MMP-9 expression in adipocytes and pioglitazone decreased MMP-9 in both adipocytes and macrophages.

Conclusion: These data indicate that MMP-9 is elevated in insulin resistance and is reduced by pioglitazone. **(*J Clin Endocrinol Metab* 95: 2993–3001, 2010)**

The loss of the balance between food intake and energy expenditure results in a massive expansion of adipose tissue, which is defined as obesity. Obesity is a growing epidemic and is associated with metabolic diseases such as insulin resistance, hypertension, and cardiovascular disease (1). Chronic inflammation is one of the major consequences, which results in dysfunction of adipose tissue in obese patients (2).

With the expansion of adipose tissue and the enlargement of fat cells, there is a considerable need for constant remodeling of the stromal matrix (3). Matrix metalloproteinases (MMPs) are proteolytic enzymes that are responsible for remodeling extracellular matrix by affecting the degradation and turnover of connective tissue and basement membrane proteins such as collagen, proteoglycans, and elastin. MMPs are a family of more than 23 members

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Abbreviations: ADHASC, Adult-derived human adipocyte stem cell; BMI, body mass index; IGT, impaired glucose tolerant; LPL, lipoprotein lipase; MMP, matrix metalloproteinase; NGT, normal glucose tolerant; PKC, protein kinase C; PMA, phorbol myristyl acetate; PPAR, peroxisomal proliferator-activated receptor; S_I, insulin sensitivity; SVF, stromal vascular fraction.

and each MMP is a product of a different gene, yet there are shared structural features, and all MMPs are multiple domains containing proteins with a catalytic zinc-binding domain (4, 5). There are several studies suggesting that MMPs play an important role in obesity-mediated adipose tissue remodeling (6). Studies on the Pima Indian population indicated that MMP-9 levels are increased in the stromal vascular fraction of obese subjects (7), suggesting the role of MMP-9 in the expansion/remodeling of the obese adipose tissue.

A number of previous studies linked MMP9 to protein kinase C (PKC)-α, which in turn has been implicated in the development of insulin resistance. Obesity and insulin resistance are associated with elevated nonesterified fatty acids, and the formation of cellular diacylglycerol after adipocyte lipolysis activates many PKC isoforms, including PKCα (8, 9). Up-Regulation of MMP-9 has been demonstrated in bronchial epithelial cells in response to phorbol myristyl acetate (PMA) treatment, and the increase in MMP-9 was inhibited by isoform-specific PKC inhibitors (10). The transcriptional activation of MMP-9 was identified as PKCα-nuclear factor-κB mediated and *in vitro* studies with hepatocarcinoma cells demonstrated that PKC-dependent nuclear factor-κB activation is essential for MMP-9 induction by PMA (11).

This study examined the expression of MMP-9 in the adipose tissue of subjects with a wide range of body mass index (BMI) and insulin sensitivity (S_I), and the regulation of MMP-9 by the insulin sensitizer pioglitazone both *in vivo* and *in vitro*. MMP-9 was increased in insulin-resistant subjects and was decreased by pioglitazone for 10 wk along with an improvement in insulin sensitivity and the inhibition of MMP-9 expression by pioglitazone was mediated by PKCα-dependent mechanisms.

Subjects and Methods

Human subject recruitment

We recruited generally healthy nondiabetic subjects by local advertisement. All subjects provided written, informed consent under protocols approved by the local institutional review board, and studies were conducted at the University of Arkansas Medical Center/Central Arkansas Veterans Healthcare System-General Clinical Research Center. Subjects were included if fasting glucose was less than 126 mg/dl and 2-h postchallenge glucose was less than 200 mg/dl determined by an initial 75-g oral glucose tolerance test. Subjects classified as normal glucose tolerant (NGT) had fasting glucose of less than 110 mg/dl and 2-h glucose of less than 140 mg/dl, and impaired glucose-tolerant (IGT) subjects had 2-h glucose of 140–199 mg/dl. Eighty-six nondiabetic subjects were recruited for this study (70 women and 16 men), of whom 41 had IGT. The subjects were weight stable and between 21 and 66 yr of age. Subjects with a history of coronary artery disease were excluded. All subjects underwent a sc adipose tissue incisional biopsy from the lower abdominal wall and insulin sensitivity testing using a frequently sampled iv glucose tolerance test. IGT subjects were randomized to receive metformin or pioglitazone, with a 2-wk dose escalation followed by 8 wk at 1000 mg twice per day of metformin or 45 mg daily of pioglitazone. After 10 wk of treatment, the oral and iv glucose tolerance tests and biopsy were repeated. Many of the subjects described in this study are the same subjects who have been included in previous studies (12, 13).

Insulin sensitivity measurement

S_I was measured by insulin-modified frequently sampled iv glucose tolerance test using a glucose infusion of 11.4 g/m^2 and an insulin injection of 0.04 U/kg, as described previously (14). Plasma insulin was measured using a chemiluminescent assay (Molecular Light Technology Research, Ltd., Cardiff, Wales, UK), and plasma glucose was determined by duplicate determinations using the glucose oxidase method. S_I was calculated from the insulin and glucose data using the MINMOD program (15).

RNA isolation and real-time RT-PCR

Total RNA from adipose tissue was extracted using an RNeasy lipid tissue minikit from QIAGEN (Valencia, CA). The quantity and quality of the isolated RNA was determined using Agilent 2100 Bioanalyzer (Palo Alto, CA). One microgram of total RNA was reverse transcribed using random hexamer primers with TaqMan RT reagents (Applied Biosystems, Foster City, CA). Reverse-transcribed RNA was amplified with SYBR Green PCR master mix (Applied Biosystems) plus 0.3 mM of gene-specific upstream and downstream primers during 35 cycles on a Rotor-Gene 3000 using real-time thermal cycler (Corbett Research, Sydney, Australia). Each cycle consisted of denaturation at 94 C for 20 sec, annealing at 58 C for 20 sec, and extension at 72 C for 20 sec. Amplified 18S expression was used as a standard control to normalize the differences in individual samples. There was no correlation between 18S RNA and BMI or S_I, and similar results were obtained when mRNA data were expressed in terms of total RNA. The primer sequences for human MMP-9 and 18S RNA, as well as mouse MMP-9 and PKCα, are described below. All data were expressed in relation to 18S RNA and standard curves were generated using pooled cDNA from the samples being assayed. Therefore, the data represent arbitrary units, which accurately compare samples within each assay but do not necessarily accurately compare samples between different assays. All samples were analyzed twice; to avoid genomic DNA contamination, primers were also designed spanning an intron. The primers used for amplification are as follows: human MMP-9, forward, 5′-CCTTCTACGGCCACTACT-3′, reverse, 5′-ATGGCGTCGAAGATGTTCA-3′; mouse MMP-9, forward, 5′-CCAACTATGACCAGGATAAAC-3′, reverse, 5′-TTCTTGTCAGTGTCGAAGTTC-3′; mouse PKCα, forward, 5′-CCCATTCCAGAAGGAGATGA-3′, reverse, 5′-CGTTGACGTATTCCATGACG-3′; 18S, forward, 5′-TTCGAACGTCTGCCCTATCAA-3′, reverse, 5′-ATGGTAGGCACGGCGACTA-3′.

Fractionation of adipocytes and stromal vascular fraction (SVF) from whole adipose tissue

Adipocytes and SVF are separated from adipose tissue specimens obtained by biopsy using procedures described earlier (13). Adipose tissue was digested with 5 volumes of collagenase type 1 (Worthington, Freehold, NJ) containing 3% BSA and 5

mM dextrose in 20 mM HEPES (pH 7.4). After digestion the collagenase was inactivated by the addition of 5% serum, and adipocytes and stromal vascular cells were separated by centrifugation at 1000 × *g*. To ensure that the preparation of adipocytes was relatively free of other cell types, the floating layer of adipocytes was washed in PBS and recovered after a second low-speed centrifugation. In previous studies, 10 times more lipoprotein lipase (LPL) was found in the adipocyte fraction, compared with the SVF, and 14 times more CD68 was expressed by the SVF, compared with the adipocyte fraction (13, 16). RNA was isolated from the adipocyte and stromal vascular fractions using lipid RNAeasy kit (QIAGEN).

Cell culture and differentiation

3T3-F442A adipocytes were obtained from Dr. Howard Green (Harvard Medical School, Boston, MA). Cells were maintained in DMEM (Life Technologies, Inc., Grand Island, NY), supplemented with 10% calf serum. For experiments, cells were grown to confluence and stimulated to differentiate in DMEM containing 10% fetal bovine serum and 100 nM insulin for 14 d. For pioglitazone treatment, adipocytes differentiated for 14 d were cultured in plain DMEM with the specified concentration of pioglitazone (0, 1.0, 3.0, or 10.0 μM) added for 24 h, and medium was assayed for MMP-9 using Western blot or gelatinase B zymography to measure activity.

Culture and treatment of THP1 cells

Human monocytes THP1 cells (American Type Culture Collection, Manassas, VA) are maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin. To obtain macrophages, cells were cultured in serum-free culture medium with 250 nM phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate) for 3 d to differentiate the THP1 monocyte to macrophages, followed by culture in the absence of phorbol ester. Cells were treated with pioglitazone as specified for 24 h in serum-free medium to measure MMP-9 expression and secretion.

Adipocyte-macrophage coculture experiments

Adipocytes were derived by differentiation of adult-derived human adipocyte stem cells (ADHASCs) obtained from normal women undergoing adipose liposuction, based on previously described procedures (13). The preadipocyte fraction was cultured in DMEM/Ham's F10 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 50 U penicillin and 50 μg streptomycin per milliliter. For experiments, preadipocytes were grown to confluence on polystyrene membrane inserts with 0.4-μm pore size and pore density of $4 \times 10^5/cm^2$ in six-well culture dishes (Corning, Corning, NY) and differentiated as described previously (17). Adipocytes were differentiated for 14 d or until 80% differentiation before coculturing with THP1 macrophages. THP-1 cells were differentiated into macrophages using tetradecanoyl phorbol acetate as described above. THP1 cells were scraped and counted for replating into companion six-well plates. In this culture system, ADHASC adipocytes and THP1 macrophages are cultured together in the same well on two different surfaces, which are 0.9 mm apart, exchanging the same medium. Adipocytes and macrophages are cocultured for 48 h. In experiments examining the effect of pioglitazone, the cultures or cocultures were treated with 1.5 μM pioglitazone or DMSO (vehicle for pioglitazone) for 24 h. At the completion of the treatment, adipocytes and macrophages were washed in PBS and lysed independently in RNA lysis buffer. All coculture experiments were performed in triplicates and repeated for confirmation of results.

Transfection of adipocytes with oligonucleotides

Oligonucleotides were synthesized with phosphoorothioate containing 2-methoxyethyl modification at positions 1–5 and 15–20 (Isis Pharmaceuticals, Carlsbad, CA). The sequences were as follows: Isis 14012 antisense PKCα, 5′-CAGCCATGGTTCCCCCAAC-3′; and Isis 17250 scrambled oligomer, 5′-CCAGTCACTCGCACCATCGC-3′. All oligomers were synthesized using an Applied Biosystems 380B automated DNA synthesizer as described previously. Differentiated 3T3-F442A adipocytes were transfected using serum-free DMEM containing 15 μg/ml Lipofectin (Invitrogen) and 1.0 μM PKCα antisense oligomer for 16 h and incubated in differentiation medium for 72 h. In some experiments, pioglitazone 1 μM was added during the final 24 h. Control cultures were treated similarly but with Lipofectin and scrambled oligomer or Lipofectin alone in the presence of DMEM (18, 19).

Gelatin zymography

Gelatinolytic MMP activity was determined on substrate impregnated gels. Briefly, samples were separated on sodium dodecyl sulfate-10% polyacrylamide gels containing 1 mg/ml gelatin B (Difco, Detroit, MI) under nonreducing conditions. These gels were washed twice with 2.5% Triton X-100, incubated for 24 h at 37 C in 0.25 M Tris-HCl, 1 M NaCl, 25 mM CaCl2 (pH 7.4), and stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/water (30:10:60). The clear lysed areas (white bands) on the stained gels were measured with a densitometer (Bio-Rad) (20).

Western blot analysis of MMP-9

Medium from THP1 cells or 3T3-F442A adipocytes treated as specified in *Results* and figure legends were assayed for MMP-9 by Western blot analysis on 10% SDS-PAGE. MMP-9 antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA).

Statistical analysis

Data were presented as mean ± SE. Student's *t* tests were performed to determine the statistical significance of MMP9 expression among different experimental groups. $P < 0.05$ was considered significant.

Results

Adipose tissue MMP-9 mRNA levels are correlated with obesity and insulin resistance

To determine whether MMP-9 in adipose tissue was associated with obesity or insulin resistance, we examined MMP-9 mRNA expression (expressed in relation to 18s RNA) in adipose tissue biopsy samples of 86 nondiabetic subjects with varying BMI (19–40 kg/m^2) and S_I (1–13.64 × 10^{-5} × min/pmol·liter). As shown in Fig. 1A, MMP-9 mRNA was higher in subjects with higher BMI ($r = 0.40$, $P < 0.0001$, $n = 86$), and MMP-9 was inversely

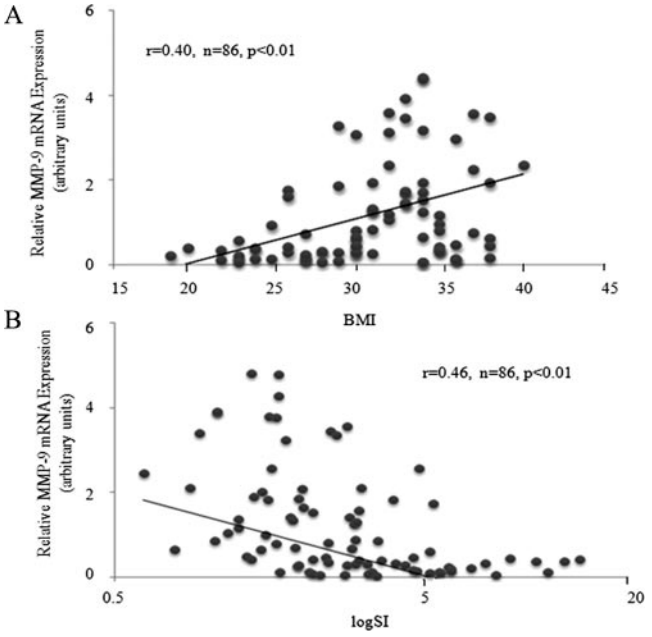

FIG. 1. Adipose expression of MMP-9: correlation with BMI and S_I. MMP-9 mRNA was quantitated in the adipose tissue of 86 subjects, as described in *Subjects and Methods*, by real-time RT-PCR and was expressed relative to endogenous 18S RNA. A, MMP-9 expression in relation to BMI. B, MMP-9 expression relative to SI (log scale). S_I was measured by frequently sampled iv glucose tolerance test.

correlated with S_I ($r = -0.46$, $P < 0.0001$, $n = 86$) (Fig. 1B). Consistent with this stronger relationship between S_I and MMP-9, IGT subjects, who were more insulin resistant than NGT subjects (IGT: S_I 1.9 ± 0.16; NGT: S_I 3.75 ± 0.38, $P < 0.001$) demonstrated higher expression of MMP-9 (IGT: 1.7 ± 0.27; NGT: 1.1 ± 0.17, $P = 0.05$). As expected, BMI and S_I were correlated with each other ($r = -0.62$, $P < 0.0001$, $n = 86$).

Pioglitazone treatment decreased MMP-9 expression

A number of studies have demonstrated macrophage infiltration into the adipose tissue of insulin-resistant subjects along with a decrease in inflammation after thiazolidinedione treatment (13). To determine whether improvement in S_I after pioglitazone treatment altered adipose expression of MMP-9, 41 subjects with IGT were randomized to receive either pioglitazone or metformin for 10 wk. Only subjects for whom pre- and posttreatment adipose samples was available were included in the analysis. As described in previous studies, treatment with pioglitazone, but not metformin, resulted in an improvement in S_I (12, 13). Adipose tissue MMP-9 expression was measured in these subjects before and after pioglitazone or metformin treatment. Pioglitazone treatment caused a significant decrease in MMP-9 mRNA (Fig. 2). MMP-9 mRNA levels were not changed significantly after metformin treatment.

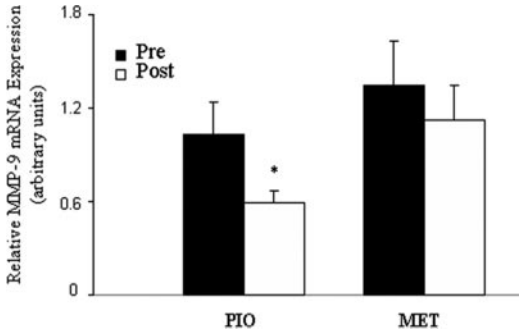

FIG. 2. Effect of pioglitazone or metformin on MMP-9 expression. IGT subjects were treated with either pioglitazone (PIO; n = 20) or metformin (MET; n = 21) for 10 wk. MMP-9 mRNA was measured in adipose tissue by real-time PCR as described in *Subjects and Methods* and normalized to endogenous 18S RNA. The values expressed as arbitrary units represent changes from baseline after drug treatment (means ± SEM). *, $P < 0.05$ *vs.* baseline.

MMP-9 in adipocytes, macrophages, and cellular fractions of adipose tissue

The decrease in adipose MMP-9 from pioglitazone treatment could have resulted from the change in insulin sensitivity or other pleiotropic effects or could have resulted from a direct effect of pioglitazone on cells in adipose tissue. To better understand the cellular origins and regulation of MMP-9 in adipose tissue, we examined MMP-9 expression in adipocytes and in the stromal vascular fraction of adipose tissue using a collagenase digestion to fractionate the cells (21). Although MMP-9 was expressed in both fractions, approximately 9-fold more MMP-9 mRNA was found in the stromal vascular fraction of subjects (adipocytes: 0.17 ± 0.03 U; SVF: 1.55 ± 0.35 U, $P < 0.002$). This finding raised the question of whether adipocyte MMP-9 contributed significantly to total adipose MMP-9 expression and whether adipocyte MMP-9 was subject to any regulation.

To assess the expression and regulation of MMP-9 in adipocytes, experiments were performed with both the mouse 3T3-F442A cell line differentiated for 14 d (Fig. 3) and human ADHASCs (Table 1). To assess the direct effects of pioglitazone on 14 d differentiated 3T3-F442A adipocytes, MMP-9 activity was studied in culture medium using gelatin zymography in parallel with the measurement of MMP-9 mRNA levels in the adipocytes after treatment of the adipocytes with 1.0, 3.0, and 10 μM pioglitazone for 24 h. MMP-9 secretion decreased with increasing concentration of pioglitazone (Fig. 3A), and both 3 and 10 μM pioglitazone decreased MMP-9 secretion by more than 50% ($P < 0.05$). To examine whether pioglitazone altered MMP-9 mRNA expression, 3T3-F442A adipocytes were treated for 24 h with 3 μM pioglitazone. MMP-9 mRNA expression also decreased by 50% after pioglitazone treatment ($P < 0.05$) (Fig. 3B). Because the SVF contains adipocyte precursors, we examined MMP-9

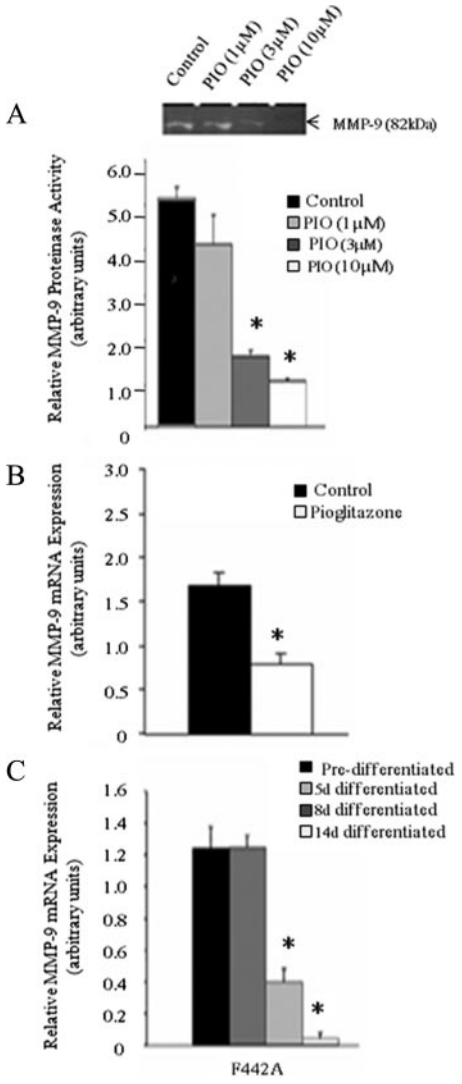

FIG. 3. Effect of pioglitazone on MMP-9 activity and mRNA in 3T3-F442A adipocytes. A, 3T3-F442A adipocytes were treated with increasing concentrations pioglitazone (PIO; 1, 3, and 10 μM) in serum-free medium for 24 h, and MMP-9 activity was measured using gelatin zymography assay. Clear areas on the Coomasie stained gels are quantitated by densitometry and represented as arbitrary units. B, Effect of 3 μM pioglitazone treatment for 24 h on MMP-9 mRNA in 3T3-F442A cells fully differentiated for 14 d. MMP-9 mRNA was quantitated using real-time PCR, as described in *Subjects and Methods*. The results represent three separate experiments performed in duplicates as means ± SEM. C, Change in MMP-9 mRNA expression during differentiation was quantitated on d 0, 5, 8, and 14 after initiation of differentiation of 3T3-F442Aadipocytes. *, $P < 0.05$.

mRNA expression on d 0, 5, 8, and 14 after induction of differentiation of 3T3-F442A adipocytes. MMP-9 expression was relatively high in undifferentiated preadipocytes and decreased by 75% by d 8 after differentiation and decreased with further differentiation on d 14 (Fig. 3C). Thus, both preadipocytes and adipocytes express MMP-9, and adipocyte MMP-9 is subject to regulation by pioglitazone.

Because macrophages are present in adipose tissue, we examined the expression and regulation of MMP-9 by

TABLE 1. MMP-9 and LPL expression in adipocytes

Cell culture	MMP-9	LPL
Adipocytes alone	0.0035 ± 0.0002	0.35 ± 0.02
Adipocytes + pioglitazone	0.0040 ± 0.0002	1.12 ± 0.2[a]
Adipocytes cocultured with macs	0.47 ± 0.07[b]	0.37 ± 0.09
Adipocytes cocultured with macs + pioglitazone	0.28 ± 0.04[b]	1.26 ± 0.25[a]

Data are mean ± SD; MMP-9 and LPL are expressed relative to 18S RNA in arbitary units. macs, Macrophages.

[a] $P < 0.05$ *vs.* cells not treated with pioglitazone.

[b] $P < 0.05$ *vs.* ADHASC adipocytes cultured alone.

pioglitazone on THP1 macrophages in culture. THP1 macrophages were treated with pioglitazone (1.5 or 3 μM) for 24 h. MMP-9 mRNA expression was studied in control and pioglitazone-treated THP1 macrophages. As shown in Fig. 4A, pioglitazone had no effect on MMP-9 mRNA expression. However, when MMP-9 secretion into the medium was measured by Western blot, THP-1 macrophages demonstrated a 45 ± 10% ($P < 0.05$) reduction in MMP-9 after pioglitazone treatment (Fig. 4B).

To determine whether there are interactions between adipocytes and macrophages that may explain the changes that occur in whole adipose tissue, coculture experiments were performed using human ADHASC adipocytes and the human THP1 macrophage cell line. As described in *Subjects and Methods*, human ADHASC adipocytes were grown and differentiated on inserts and then cocultured for 48 h with THP-1 macrophages, which were grown and differentiated in six-well plates to determine the effects of

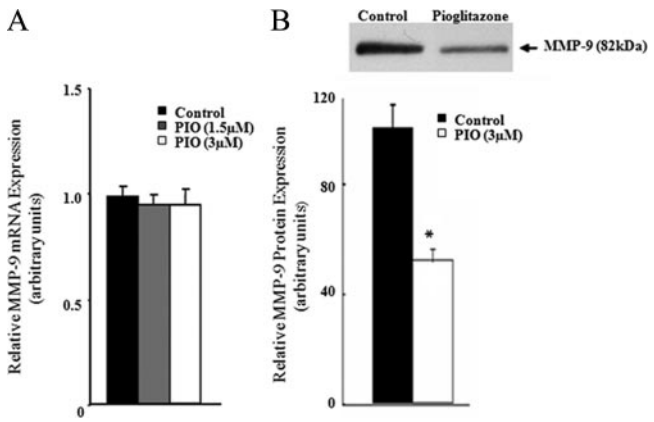

FIG. 4. Effect of pioglitazone on MMP-9 protein and mRNA expression in THP1 macrophage cells. THP1 macrophages were differentiated as described in *Subjects and Methods* and treated with pioglitazone (PIO; 1.5 or 3 μM) for 24 h in serum-free medium. A, MMP9 mRNA expression was measured by real-time PCR and expressed relative to 18S RNA. B, MMP-9 protein secretion was measured in medium by Western blots using specific antibodies as described in *Subjects and Methods*. Relative expression on MMP-9 secretion is expressed as arbitrary units. *, $P < 0.05$ *vs.* control.

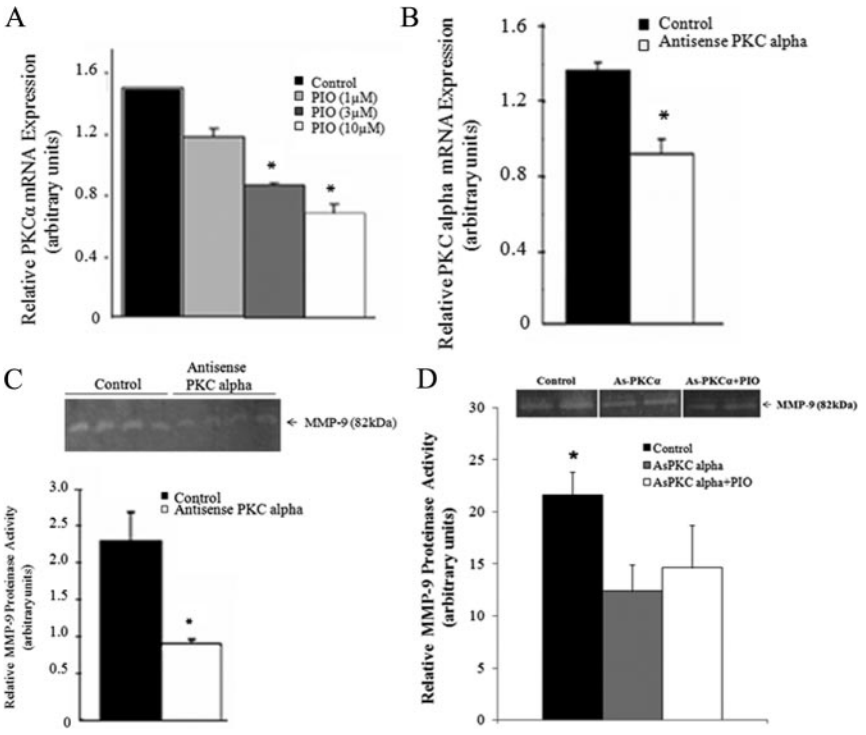

FIG. 5. Dose-dependent effect of pioglitazone treatment on PKCα mRNA and effect of PKCα depletion on MMP-9 activity in 3T3-F442A adipocytes. A, Adipocytes were treated with increasing concentration of pioglitazone (PIO; 1, 3, and 10 μM) for 24 h, and PKCα mRNA was measured using real-time PCR. B, The decrease in PKCα after treatment of cells with antisense oligos to PKCα. C, Effect of PKCα depletion on MMP-9 activity is measured using gelatin zymography assay, as described in *Subjects and Methods*, in fully differentiated 3T3-F442A cells. D, Fully differentiated 3T3-F442A adipocytes were treated with PKCα antisense oligos with and without treatment with pioglitazone 1 μM for 24 h. The results represent three separate experiments performed in duplicates as means ± SEM. *, $P < 0.05$.

the shared medium on adipocyte MMP-9 expression. As shown in Table 1, relative MMP-9 gene expression was very low in cultured human adipocytes. After coculture with macrophages, however, there was greater than 100-fold increase in adipocyte MMP-9 expression. Although the addition of pioglitazone to the coculture had no effect on the low levels of MMP-9 in adipocytes alone, the addition of pioglitazone decreased the adipocyte expression of MMP-9 in the adipocyte/macrophage coculture. As expected, LPL expression increased in the adipocytes in response to pioglitazone, both when adipocytes were cultured alone and in the presence of macrophages, but coculture with macrophages did not affect adipocyte LPL expression.

Pioglitazone-induced MMP-9 regulation is PKCα mediated

Previous studies showed that PMA induced PKCα activation and up-regulated MMP-9 expression in bronchial epithelial cells and hepatocarcinoma cells (10, 11). To determine whether pioglitazone treatment affected MMP-9 expression through PKCα-dependent mechanisms, 3T3-F442A adipocytes were treated with increasing concentrations of pioglitazone, and PKCα expression was measured. As shown in Fig. 5A, pioglitazone treatment resulted in a dose-dependent decrease in PKCα mRNA expression, with a 50% decrease in PKCα mRNA expression at 3 and 10 μM pioglitazone ($P < 0.05$). To examine the effects of PKCα on MMP-9 more directly, 3T3-F442A adipocytes were treated with antisense oligos to PKCα. In previous studies, these antisense oligos were demonstrated to be specific to the PKCα isoform (18). Treatment of adipocytes with 1 μM antisense oligomers to PKCα caused a modest decrease (45 ± 10%) in PKCα mRNA expression (Fig. 5B) and also resulted in a 60 ± 5% ($P < 0.05$) decrease in MMP-9 secretion into the medium, using zymography to measure MMP-9 activity (Fig. 5C). Because pioglitazone decreased both MMP-9 and PKCα and PKCα knockdown inhibited MMP-9, these data suggested that the effects of pioglitazone occurred through PKCα. To obtain further evidence for this, adipocytes were treated with the antisense RNA to PKCα and also with pioglitazone. As shown in Fig. 5D, PKCα antisense RNA treatment resulted in a decrease in MMP-9 activity, using zymography. The addition of pioglitazone to PKCα antisense RNA-treated cells did not have an additive effect and did not decrease MMP-9 activity further.

Discussion

Although there is a significant correlation between obesity and insulin resistance, the mechanisms underlying this relationship are not completely understood (2, 22). Recent studies have been focused on the potential role of the adipose tissue extracellular matrix. Adipose tissue is an organ composed of cells that can undergo considerable volumetric expansion during the progression from the lean to obese state. Such an expansion of fat cell size would require a pliant extracellular matrix, and recent studies suggested that the absence of such a pliant matrix could lead to adipose tissue inflammation, which characterizes the adipose tissue of subjects with insulin resistance and other features of the metabolic syndrome (23).

Tissue remodeling is a normal physiological process regulated by a balance between synthesis and degradation

of the extracellular matrix. Although there is significant correlation between obesity and insulin resistance, the pathophysiology of this relationship is not completely understood (24). MMPs are proteolytic enzymes that play an essential role in extracellular matrix remodeling. MMPs are involved in two important events of this process and control proteolysis and adipogenesis during obesity-mediated fat mass development (25). Several previous studies suggested a role for MMPs in adipose tissue remodeling. Obese mice expressed higher levels of numerous MMPs (6), and studies in Pima Indians indicated that MMP-9 levels are increased in the SVF of adipose tissue in obese subjects (7). No other study has carefully examined the regulation of MMP-9 in humans, and in this study we examined the relationship between MMP-9 and insulin resistance and the response to insulin sensitizers, and we performed studies to examine the cellular regulation of MMP-9 in adipose tissue.

To understand the role of MMP-9 in obesity and insulin resistance, we measured MMP-9 in humans over a wide range of obesity and insulin resistance. MMP-9 expression correlated positively with BMI and negatively with insulin sensitivity. Some of the subjects in this study had been treated with the insulin sensitizers pioglitazone or metformin, and previous studies have described the clinical findings in these subjects more fully (12). Although pioglitazone and metformin both improve insulin sensitivity, they have different mechanisms of action. Pioglitazone is a peroxisomal proliferator-activated receptor (PPAR)-γ agonist that targets adipose tissue and reduces inflammation and results in a decrease in lipotoxicity (12, 26). Metformin reduces blood glucose levels by decreasing hepatic glucose output; the precise target for metformin is not clear, but increases in AMP kinase have been described *in vitro* (27, 28). Pioglitazone treatment resulted in a decrease in MMP-9 expression in adipose tissue.

To assess the direct effects of pioglitazone on adipocytes, we treated fully differentiated 3T3-F442A adipocytes in culture with this PPARγ agonist. Because 3T3-F442A cells do not require a PPARγ agonist for differentiation, they are responsive to pioglitazone. The addition of pioglitazone to adipocytes resulted in a decrease in MMP-9 expression and activity. In adipose tissue, however, most of the MMP-9 expression was from the stromal vascular fraction (20), which is composed of preadipocytes, macrophages, and other cells. Preadipocytes expressed higher levels of MMP-9 than adipocytes after induction of differentiation. Although these data indicate that MMP-9 is decreased with induction of adipocyte differentiation, MMP-9 is also decreased with pioglitazone treatment of mature adipocytes. Although mature adipocytes are fully differentiated, it is possible that pioglitazone further induces adipocyte differentiation and thereby reduces MMP-9.

To assess the direct effects of pioglitazone in macrophages, we treated THP-1 macrophages with pioglitazone and observed a decrease in MMP-9 protein, although no change in THP-1 MMP-9 mRNA, suggesting posttranscriptional regulation of macrophage MMP-9. It is of interest that pioglitazone decreases MMP-9 in both adipocytes and macrophages, but in adipocytes the regulation occurs at the level of mRNA, whereas in THP1 macrophages, the regulation is posttranscriptional. It would be of interest to better define the pathways of macrophage MMP-9 regulation, which may be different from in adipocytes.

With obesity and insulin resistance, adipose tissue contains a higher proportion of macrophages, and the interaction between these cell types becomes more important. To better understand the interactions between adipocytes and macrophages and the origin of the elevated MMP-9 expression, we cocultured human ADHASC adipocytes, which were induced to differentiate from stem cells, with THP-1 macrophages. Although the ADHASC adipocytes expressed only low levels of MMP-9, coculture with macrophages resulted in a greater than 100-fold increase in adipocyte MMP-9 expression, and this up-regulation of MMP-9 in the ADHASCs was attenuated by the addition of pioglitazone. Pioglitazone did not have a significant effect on ADHASC adipocyte MMP-9. Adipose stem cells require a thiazolidinedione for differentiation and therefore may be less responsive to the subsequent addition of pioglitazone. These data indicate that macrophage secretory products have a specific role to increase adipocyte MMP-9 expression, even in the absence of a tissue that is undergoing remodeling. It cannot be determined from these data whether the pioglitazone was acting on the adipocytes or macrophages. Although pioglitazone has direct effects on adipocytes, macrophages also express PPARγ, and the thiazolidinediones have well-described antiinflammatory effects on macrophages (26, 29). On the other hand, other studies found direct effects of PPARγ agonists on MMP-9 activity. Rosiglitazone treatment inhibited MMP-9 activity in murine mammary tumor cells, and this inhibition of MMP-9 activity occurred through PPARγ activation (30).

Several previous studies suggested a connection between the activation of PKC and MMP-9 regulation. The treatment of hepatocarcinoma cells with PMA, which are activators of diacylglycerol-dependent conventional PKC activation, increased the secretion of MMP-9, and this increase was inhibited by PKC inhibitors (11). In a similar manner, PMA treatment of endothelial cells activated PKCα and caused an increase in MMP-9 activity and ex-

pression (10). In addition, other studies found decreases in PKCα activity after treatment with a thiazolidinedione (31, 32). Our data are consistent with these findings and indicate that MMP-9 is regulated by pioglitazone through PKCα-mediated mechanisms. Pioglitazone treatment of 3T3-F442A adipocytes caused a dose-dependent decrease in PKCα mRNA expression and MMP-9 activity. We also demonstrate that PKCα depletion using antisense oligomers inhibited MMP-9 secretion in 3T3-F442A adipocytes, and the treatment of cells with both PKCα antisense oligos and pioglitazone yielded no further decrease in MMP-9 activity. We therefore propose that the pioglitazone-mediated decrease in MMP-9 could be caused by the inhibition of PKCα in adipocytes, and this may counteract the increased activation of PKC that occurs in response to the elevation in free fatty acids that occurs with obesity and insulin resistance. Pioglitazone is known to promote differentiation in adipocytes, and both PKCα and MMP-9 levels could also be regulated by adipocyte lipid accumulation.

In summary, these data examine the expression and activity of MMP-9 in adipose tissue and adipose cells. MMP-9 expression in adipose tissue is increased with obesity and insulin resistance and is increased in adipocytes in response to coculture with macrophages. This increase in MMP-9 is ameliorated by treatment with the thiazolidinedione pioglitazone, both in humans and *in vitro*, and this effect appears to occur through the PPARγ-mediated inhibition of PKCα. These data are consistent with the emerging image of adipose tissue in obese subjects that is in a state of chronic inflammation and remodeling.

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References

1. **Hossain P, Kawar B, El Nahas M** 2007 Obesity and diabetes in the developing world—a growing challenge. N Engl J Med 356:213–215
2. **Rasouli N, Kern PA** 2008 Adipocytokines and the metabolic complications of obesity. J Clin Endocrinol Metab 93:S64–S73
3. **Halberg N, Wernstedt-Asterholm I, Scherer PE** 2008 The adipocyte as an endocrine cell. Endocrinol Metab Clin North Am 37:753–768, x-xi
4. **Krane SM, Inada M** 2008 Matrix metalloproteinases and bone. Bone 43:7–18
5. **Visse R, Nagase H** 2003 Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92:827–839
6. **Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E, Tartare-Deckert S** 2003 Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. J Biol Chem 278:11888–11896
7. **Nair S, Lee YH, Rousseau E, Cam M, Tataranni PA, Baier LJ, Bogardus C, Permana PA** 2005 Increased expression of inflammation-related genes in cultured preadipocytes/stromal vascular cells from obese compared with non-obese Pima Indians. Diabetologia 48:1784–1788
8. **Farese RV, Standaert ML, Arnold TP, Yamada K, Musunuru K, Hernandez H, Mischak H, Cooper DR** 1994 Preferential activation of microsomal diacylglycerol/protein kinase C signaling during glucose treatment (*de novo* phospholipid synthesis) of rat adipocytes. J Clin Invest 93:1894–1899
9. **Itani SI, Zhou Q, Pories WJ, MacDonald KG, Dohm GL** 2000 Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. Diabetes 49:1353–1358
10. **Shin Y, Yoon SH, Choe EY, Cho SH, Woo CH, Rho JY, Kim JH** 2007 PMA-induced up-regulation of MMP-9 is regulated by a PKCα-NF-κB cascade in human lung epithelial cells. Exp Mol Med 39:97–105
11. **Hah N, Lee ST** 2003 An absolute role of the PKC-dependent NF-κB activation for induction of MMP-9 in hepatocellular carcinoma cells. Biochem Biophys Res Commun 305:428–433
12. **Rasouli N, Raue U, Miles LM, Lu T, Di Gregorio GB, Elbein SC, Kern PA** 2005 Pioglitazone improves insulin sensitivity through reduction in muscle lipid and redistribution of lipid into adipose tissue. Am J Physiol Endocrinol Metab 288:E930–E934
13. **Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G, Peterson CA, McGehee RE, Kern PA** 2005 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. Diabetes 54:2305–2313
14. **Bergman RN, Finegood DT, Ader M** 1985 Assessment of insulin sensitivity *in vivo*. Endocr Rev 6:45–86
15. **Boston RC, Stefanovski D, Moate PJ, Sumner AE, Watanabe RM, Bergman RN** 2003 MINMOD Millennium: a computer program to calculate glucose effectiveness and insulin sensitivity from the frequently sampled intravenous glucose tolerance test. Diabetes Technol Ther 5:1003–1015
16. **Yao-Borengasser A, Rassouli N, Varma V, Bodles AM, Rasouli N, Unal R, Phanavanh B, Ranganathan G, McGehee Jr RE, Kern PA** 2008 Stearoyl-CoA desaturase 1 (SCD1) gene expression increases following pioglitazone treatment and is associated with PPARγ responsiveness. J Clin Endocrinol Metab 93:4431–4439
17. **Varma V, Yao-Borengasser A, Bodles AM, Rasouli N, Phanavanh B, Nolen GT, Kern EM, Nagarajan R, Spencer 3rd HJ, Lee MJ, Fried SK, McGehee Jr RE, Peterson CA, Kern PA** 2008 Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. Diabetes 57:432–439
18. **Ranganathan G, Song W, Dean N, Monia B, Barger SW, Kern PA** 2002 Regulation of lipoprotein lipase by protein kinase Cα in 3T3–F442A adipocytes. J Biol Chem 277:38669–38675
19. **Unal R, Pokrovskaya I, Tripathi P, Monia BP, Kern PA, Ranganathan**

G 2008 Translational regulation of lipoprotein lipase in adipocytes: depletion of cellular protein kinase Cα activates protein kinase A C subunit binding to the 3′ untranslated region of the LPL mRNA. Biochem J 413:315–322
20. **Croissandeau G, Chretien M, Mbikay M** 2002 Involvement of matrix metalloproteinases in the adipose conversion of 3T3–L1 preadipocytes. Biochem J 364:739–746
21. **Rodbell M** 1964 Metabolism of isolated fat cells. I. Effects of hormone on glucose metabolism and lipolysis. J Biol Chem 239:375–380
22. **Olefsky JM** 2008 Fat talks, liver and muscle listen. Cell 134:914–916
23. **Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR** 2009 Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. Diabetes 58:718–725
24. **de Luca C, Olefsky JM** 2008 Inflammation and insulin resistance. FEBS Lett 582:97–105
25. **Boden G** 2008 Obesity and free fatty acids. Endocrinol Metab Clin North Am 37:635–646, viii–ix
26. **Bodles AM, Varma V, Yao-Borengasser A, Phanavanh B, Peterson CA, McGehee Jr RE, Rasouli N, Wabitsch M, Kern PA** 2006 Pioglitazone induces apoptosis of macrophages in human adipose tissue. J Lipid Res 47:2080–2088
27. **Musi N, Goodyear LJ** 2006 Insulin resistance and improvements in signal transduction. Endocrine 29:73–80
28. **Ciaraldi TP, Kong AP, Chu NV, Kim DD, Baxi S, Loviscach M, Plodkowski R, Reitz R, Caulfield M, Mudaliar S, Henry RR** 2002 Regulation of glucose transport and insulin signaling by troglitazone or metformin in adipose tissue of type 2 diabetic subjects. Diabetes 51:30–36
29. **Consoli A, Devangelio E** 2005 Thiazolidinediones and inflammation. Lupus 14:794–797
30. **Magenta G, Borenstein X, Rolando R, Jasnis MA** 2008 Rosiglitazone inhibits metastasis development of a murine mammary tumor cell line LMM3. BMC Cancer 8:47
31. **Yamagishi S, Ogasawara S, Mizukami H, Yajima N, Wada R, Sugawara A, Yagihashi S** 2008 Correction of protein kinase C activity and macrophage migration in peripheral nerve by pioglitazone, peroxisome proliferator activated-γ-ligand, in insulin-deficient diabetic rats. J Neurochem 104:491–499
32. **Haneda M, Koya D, Kikkawa R** 2001 Cellular mechanisms in the development and progression of diabetic nephropathy: activation of the DAG-PKC-ERK pathway. Am J Kidney Dis 38:S178–S181