Pioglitazone reduces inflammatory responses of human adipocytes to factors secreted by monocytes/macrophages

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1Research Service and 3Department of Medicine, Section of Metabolism, Endocrinology, and Nutrition, Phoenix Veterans Affairs Health Care System, Phoenix, Arizona; and 2Division of Pediatric Endocrinology and Diabetes, University of Ulm, Ulm, Germany

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Permana PA, Zhang W, Wabitsch M, Fischer-Posovszky P, Duckworth WC, Reaven PD. Pioglitazone reduces inflammatory responses of human adipocytes to factors secreted by monocytes/macrophages. Am J Physiol Endocrinol Metab 296: E1076–E1084, 2009—First published February 24, 2009; doi:10.1152/ajpendo.91013.2008.—Infiltration of monocyte-derived macrophages into adipose tissue may contribute to tissue and systemic inflammation and insulin resistance. We hypothesized that pioglitazone (Pio) could specifically reduce the inflammatory response of adipocytes to factors released by monocytes/macrophages. We show that macrophage factors (Mq-factors) greatly increase expression levels of proinflammatory adipokines, chemokines, and adhesion molecules in human subcutaneous and visceral adipose tissue (SAT and VAT) as well as in adipocytes (up to several hundredfold of control). Compared with SAT, VAT showed enhanced basal and Mq-factor-induced inflammatory responses. Mq-factors also induced greater lipolysis in adipocytes, as assessed by concentrations of glycerol released from the cells (196 ± 13 vs. 56 ± 7 μM in control, P < 0.05). Pretreatment of adipose tissue or adipocytes with Pio reduced these responses to Mq-factors (by 13–86%, P < 0.05) and prevented Mq-factor suppression of adiponectin expression. Furthermore, Pio pretreatment of adipocytes and macrophages tended to further reduce inflammatory responses of adipocytes to Mq-factors and monocyte adhesion to Mq-factor-activated adipocytes. In support of these in vitro data, media conditioned by monocytes isolated from impaired glucose-tolerant subjects treated with Pio (compared with placebo) induced release of lower concentrations of proinflammatory adipokines and glycerol (100 ± 7 vs. 150 ± 15 μM, P < 0.05) from adipocytes. In summary, Pio decreases inflammatory responses in adipose tissue/cells induced by monocytes/macrophages by acting on either or both cell types. These beneficial effects of Pio may attenuate proinflammatory responses resulting from monocyte/macrophage infiltration into adipose tissue and suppress tissue inflammation resulting from the interaction between both cell types.

INFLAMMATION, characterized by increased concentrations of circulating proinflammatory factors, has been postulated to contribute to the association among obesity, insulin resistance, and diabetes (15). Adipose tissue is increasingly recognized as an important contributor to this condition of chronic systemic inflammation (1). This metabolically active tissue secretes many proinflammatory adipokines, such as IL-6 (61), IL-8 (50), and monocyte chemoattractant protein-1 (MCP-1) (10). Concentrations of these adipokines correlate with measures of obesity (50) and insulin resistance (7, 24), as well as increased risk for type 2 diabetes (22, 41) and cardiovascular disease (12). High concentrations of these proinflammatory adipokines may directly contribute to the development of insulin resistance, diabetes, and cardiovascular disease by affecting key cellular functions. For example, IL-6 impairs in vivo insulin signaling in liver (28) and skeletal muscle (25), MCP-1 decreases insulin-stimulated glucose uptake in adipocytes (45) and recruits monocytes to atherosclerotic lesions (59), and IL-8 mediates angiogenesis promoting atherogenesis (46).

The production of many adipokines appears to be modulated by the interaction between adipocytes and macrophages in adipose tissue (27). The percentage of monocyte-derived macrophages in adipose tissue correlates positively with measures of obesity (57, 60) and insulin resistance (11). Recent studies further indicate that adipose tissue expansion correlates with the accumulation of a subset of adipose tissue macrophages (ATMs) with a more proinflammatory phenotype (5, 36). These macrophages express major proinflammatory cytokines, such as TNF-α (5) and IL-1β (36). In turn, these macrophage-secreted cytokines can induce the transcription of many proinflammatory adipokines in human adipocytes (16). TNF-α also induces adipose tissue production of chemokines (18, 45) and adhesion molecules (43), thereby potentially facilitating further monocyte recruitment into adipose tissue. Importantly, the inflammatory response in adipocytes is induced to a greater extent by a physiological mixture of factors secreted by macrophages (Mq-factors), including, but not limited to, TNF-α and IL-1β, than the individual macrophage cytokines (40, 51).

Adipocytes activated by Mq-factors not only secrete higher concentrations of proinflammatory adipokines, but they also have increased rates of lipolysis (40). A product of lipolysis, free fatty acids (FFAs), may facilitate further proinflammatory cross talk between adipocytes and monocytes/macrophages by activating a proinflammatory subpopulation of ATMs, the number of which is increased in obese ob/ob mice or wild-type mice fed a high-fat diet (37). FFAs induce these inflammatory responses in macrophages (31, 32, 37) via activation of c-Jun NH2-terminal kinase (37) and NF-kB pathways (31, 32, 37, 52). Thus the proinflammatory cross talk between adipocytes and monocytes/macrophages goes in both directions and may be self-propagating. The roles of Mq-factors and FFAs in the cross talk between murine 3T3-L1 adipocytes and RAW264 macrophages have been investigated (40, 51). However, it is not clear whether human adipose tissue and adipocytes respond similarly to Mq-factors.

 Regulation of inflammation in adipose tissue resulting from the interaction between monocytes/macrophages and adipocytes may alleviate systemic inflammation associated with obesity. Potential antagonists of adipose tissue inflammation...
include thiazolidinedione (TZD) insulin-sensitizing agents. In vivo TZD treatment appears to polarize circulating human monocytes to become anti-inflammatory (M2) macrophages (4). Nevertheless, there were conflicting data on whether in vivo TZD treatment reduced the number of ATMs in murine models (49, 60) and human subjects (11, 29). In vitro TZD treatment results in reduced basal secretion of proinflammatory adipokines, such as IL-6 from mouse 3T3-L1 adipocytes (30) and IL-8 from human adipose tissue (6). In vitro exposure of monocytes to TZDs attenuates chemotaxis of human THP-1 monocytes (26) and adhesion of human U937 monocytes to endothelial cells (54). TZDs also attenuate production of inflammatory cytokines, such as TNF-α and IL-1β, from human peripheral monocytes (23, 63) and murine macrophages (8, 58).

Despite the above-mentioned data on the anti-inflammatory effects of TZDs on adipocytes and monocytes/macrophages, little is known about their potential ability to directly reduce the proinflammatory interaction between the different cell types. To investigate this potential anti-inflammatory effect of TZDs, we have used pioglitazone (Pio) as an investigative agent with the most clinically favorable benefits in improving insulin sensitivity while reducing major cardiovascular events (13). We tested the hypothesis that Pio treatment of human adipose tissue from subcutaneous and visceral depots (SAT and VAT), as well as human adipocytes, can decrease their inflammatory responses to factors secreted by monocytes/macrophages. We also investigated the effects of Pio treatment of monocytes/macrophages, separately or combined with Pio treatment of adipocytes, on inflammatory responses of adipocytes. Furthermore, we determined the ability of media conditioned by monocytes isolated from impaired glucose-tolerant (IGT) subjects treated with Pio vs. placebo to induce inflammatory responses in adipocytes.

MATERIALS AND METHODS

**Adipose tissue biopsy.** Abdominal SAT and VAT samples were obtained from 10 male patients who were undergoing elective abdominal surgery (e.g., ventral hernia repair, colostomy takedown) at Phoenix Veterans Affairs Health Care System as approved by the Institutional Review Board. The subjects were generally in good health. As determined by medical history, physical examination, and laboratory tests, including white blood count, none had acute inflammatory conditions before surgery. One subject had diet-controlled type 2 diabetes and four subjects were taking statins, but none were taking TZD or steroids or nonsteroidal anti-inflammatory agents. Anthropometric and metabolic variables of the subjects are described in Table 1.

**Culture and treatments of tissue and cells.** Immediately after the adipose tissue biopsy, the tissue samples were cleaned of any connective tissue and minced into ~10- to 20-mg fragments. Before experiments, the tissue fragments were incubated in basal medium (DMEM-Ham’s F-12 (DMEM/F12) medium (Invitrogen, Carlsbad, CA) supplemented with 33 mM L-glutamine, 17 μM pantothenate, and 1% penicillin-streptomycin-ampicillin B) containing 0.01 mg/ml human transferrin, 0.7 nM insulin (Novo Nordisk, Princeton, NJ), 2.5 nM dexamethasone, and 15 nM HEPES for 4–7 days, as previously described (17). The supplements to culture media were obtained from Sigma-Aldrich (St. Louis, MO).

For experiments, adipose tissue samples were pretreated with 10 μM [a kind gift from Takeda Pharmaceuticals North America (Deerfield, IL); with concentration and treatment duration based on a previous study (47)] or DMSO (vehicle, at 0.02% final concentration) for 3 days. The samples were subsequently incubated for 18 h with control medium (basal medium containing 0.5% BSA) or medium conditioned by human U937 macrophages [containing macrophage-secreted (Mγ) factors; prepared as described below] in the presence of Pio or vehicle. The SAT and VAT samples were then washed and subjected to RNA or protein extraction or further incubated in basal medium containing 0.5% BSA for 24 h to obtain tissue-conditioned media. (The scheme of this experimental procedure is shown in supplemental Fig. 1A in the online version of this article.) Because of the small amounts (~0.5–1 g) of tissue obtained from each subject, not all experiments could be performed on each sample. However, each individual experiment type was performed on SAT and VAT samples from the same patient.

U937 monocytes were cultured according to the manufacturer’s protocol (American Type Culture Collection, Manassas, VA) and used in monocyte adhesion assay (see below). To obtain medium conditioned by U937 macrophages, we differentiated U937 monocytes in the growth medium containing 125 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 1–2 days. When indicated, the differentiation was carried out in the presence of 10 μM Pio or vehicle. The resulting U937 macrophage monolayers were washed and incubated for 24 h in basal medium containing 0.5% BSA to obtain macrophage-conditioned medium (Mγ-factors). Immediately before use in treatment of adipose tissue or adipocytes, the medium was diluted with an equal volume of fresh basal medium containing 0.5% BSA. All cultures were maintained at 37°C and 5% CO₂.

We also cultured the human Simpson-Golabi-Behmel syndrome (SGBS) adipocyte cell line as described previously (55). The SGBS adipocytes have morphology, biochemistry, differentiation, and functional activities similar to isolated human adipocytes prepared from healthy subjects (55). Mature SGBS adipocytes were pretreated with 10 μM Pio or vehicle for 24 h and then incubated for 18 h with control medium or Mγ-factors (in the presence of Pio or vehicle; see supplemental Fig. 1B). In a different set of experiments, the Mγ-factors were obtained as media conditioned by macrophages that had been differentiated in the presence of 10 μM Pio (see experimental design in supplemental Fig. 1B). At the end of the incubation with Mγ-factors or control medium, the treatment media of these adipocytes were flash-frozen in liquid nitrogen for lipolysis assay. For lipolysis assay, concentrations of glycerol in the medium were measured using a commercially available kit (Sigma-Aldrich). In some experiments, the treated adipocytes were subsequently subjected to RNA or protein extraction or further incubated in basal medium containing 0.5% BSA for 24 h to obtain tissue-conditioned media. In other experiments, control or Mγ-factor-activated adipocytes were subjected to adhesion

Table 1. Characteristics of subjects from whom abdominal SAT and VAT were obtained during elective surgery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>59 ± 13</td>
<td>60</td>
<td>30–76</td>
</tr>
<tr>
<td>Height, cm</td>
<td>176 ± 5</td>
<td>178</td>
<td>165–180</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>94 ± 14</td>
<td>91</td>
<td>81–120</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30 ± 4</td>
<td>29</td>
<td>25–38</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>141 ± 12</td>
<td>139</td>
<td>126–162</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>81 ± 9</td>
<td>81</td>
<td>67–96</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>5.6 ± 0.7</td>
<td>5.4</td>
<td>4.8–6.2</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>2.1 ± 0.8</td>
<td>2.1</td>
<td>0.5–3.1</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>4.8 ± 0.9</td>
<td>4.9</td>
<td>3.0–5.8</td>
</tr>
<tr>
<td>HDL</td>
<td>1.1 ± 0.3</td>
<td>1.1</td>
<td>0.7–1.7</td>
</tr>
<tr>
<td>LDL</td>
<td>2.7 ± 0.7</td>
<td>2.8</td>
<td>1.6–3.7</td>
</tr>
</tbody>
</table>

Laboratory data were obtained from a preelective surgery examination of 10 men (unless otherwise noted). SAT and VAT, subcutaneous and visceral adipose tissue; BMI, body mass index; SBP and DBP, systolic and diastolic blood pressure. *Data from 8 subjects.
assays (see below) using U937 monocytes that had been pretreated with 10 μM Pio or vehicle (see experimental design in supplemental Fig. 1C).

In separate experiments, nonpretreated adipocytes were incubated with media conditioned by human primary monocytes (designated Mono-factors). These media were obtained from 24-h culture of monocytes freshly isolated from a subset (n = 19) of IGT individuals who had been randomized to receive placebo (n = 9) or 45 mg/day Pio (n = 10) for an average of 4.5 mo; this group was a subset of a larger group in a recently published study (63). The characteristics of this study cohort have been described elsewhere (63). In the previous study (63), we showed that media conditioned by monocytes isolated from IGT subjects treated with Pio vs. placebo induced the mRNA expression levels of several proinflammatory adipokines in adipocytes. In the present study, we further investigated the effects of the conditioned media on adipokine release and lipidolysis as measures of inflammatory responses in adipocytes. The treatment media of adipocytes that had been incubated with Mono-factors were flash-frozen in liquid nitrogen for lipidolysis (glycerol) assays. The treated adipocytes were washed extensively and incubated with control medium for 24 h to obtain medium conditioned by the adipocytes for subsequent ELISAs (see experimental design in supplemental Fig. 1D).

Monocyte adhesion assay. Adhesion of monocytes to SGBS adipocytes was assessed using labeled U937 monocytes, as previously described (40). Briefly, U937 monocytes were labeled with 5 μM carboxyfluorescein diacetate-succinimidyl ester (Invitrogen), washed with DMEM/F12 medium supplemented with 0.1% BSA, and then added to experimental SGBS adipocytes (seeded at 10⁴ cells per well of a standard 24-well tissue culture plate and differentiated at ~100% confluency). Rotation of the plate at 64 rpm for 30 min at 37°C allowed the labeled monocytes to adhere to the adipocyte monolayers. The cell monolayers were then washed extensively with PBS and dissolved in 0.5 ml of 0.2 N NaOH. Fluorescence was measured at the bottom of the well by a Wallac Victor² plate reader at 485-nm excitation and 535-nm emission.

RNA isolation and quantitative real-time PCR. Total RNA from adipose tissue, SGBS adipocytes, U937 monocytes, or macrophages was isolated using TRIzol (Invitrogen) and the Aurum Total RNA Mini kit (Bio-Rad, Hercules, CA), respectively, according to the manufacturer’s recommendations. cDNA synthesis was carried out as previously described (40). Subsequently, quantitative real-time PCR was done using primers for IL-6, IL-8, adiponectin, macrophage inflammatory protein 1α (MIP-1α), MCP-1, ICAM-1, VCAM-1, TNF-α, IL-1β, and integrin molecule CD11b (see supplemental Table 1 for primer sequences and quantitative real-time PCR conditions). The mRNA levels of the above-mentioned genes were normalized to those of β-actin by the cycle threshold (∆∆CT) method (34). Fold changes of gene expression were calculated by the 2-∆∆CT method (34).

Protein isolation and protein assay. Cytoplasmic protein from SGBS adipocytes was isolated according to a published protocol (2) with slight modifications. Briefly, the cells were washed extensively with PBS and lysed in chilled extraction buffer containing 0.5 mM MgCl₂, 10 mM HEPES, 10 mM KCl, and 1 mM phenylmethylsulfonyl fluoride. After the cell lysates were kept on ice for 15 min, Igepal CA-630 solution was added to a final concentration of 0.5%. The mixtures were vortexed vigorously for 10 s and then centrifuged for 30 s at 16,100 g. The supernatants containing cytoplasmic proteins were collected and stored at −80°C for subsequent ELISA. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

ELISA. Commercially available ELISA kits (R & D Systems) were used to measure the protein concentrations of IL-6, IL-8, MCP-1, and adiponectin in media conditioned by adipose tissue or SGBS adipocytes and soluble ICAM-1 in cytoplasmic protein extracts of adipocytes.

**Statistical analysis.** Values are means ± SE from three or more experiments for each data point. The mRNA expression levels were not normally distributed and, thus, were transformed logarithmically to approximate normal distribution for statistical analyses. Paired Student’s t-test was used for comparisons between Pio-treated and control samples for all assay results, except the ELISA results of SGBS adipocytes treated with media conditioned by primary monocytes (for which Wilcoxon’s signed-rank test was used because of the non-Gaussian distribution of the values). One-way repeated-measures ANOVA with Dunnett’s post test was used for comparisons of multiple experimental conditions. P < 0.05 was considered significant.

**RESULTS**

Factors secreted by macrophages induce inflammation in adipose tissue and adipocytes. We determined the proinflammatory effects of factors secreted by macrophages on human adipose tissue by incubation of SAT and VAT with medium conditioned by U937 macrophages (Mq-factors) for 18 h (see supplemental Fig. 1A). Mq-factors greatly stimulated the mRNA expression levels of several inflammation response genes that encode proteins with diverse functions, including circulating adipokines (IL-6 and IL-8), chemokines (MIP-1α and MCP-1), and adhesion molecules (ICAM-1 and VCAM-1) in SAT and VAT (Fig. 1A). Notably, in unstimulated conditions, the mRNA levels of these genes, except MCP-1, were generally higher in VAT than SAT. Similarly, all tested gene expression levels, except those of MCP-1, in VAT activated by Mq-factors tended to be higher than the corresponding levels in the similarly treated SAT from the same subject. These increased gene expression levels in VAT were accompanied by increased protein concentrations, as illustrated by enhanced secretion of the representative adipokines IL-6 and IL-8 from the tissue (Fig. 1B).

Mq-factors induced inflammatory responses not only in adipose tissue, but also specifically in adipocytes. SGBS adipocytes incubated with Mq-factors (see supplemental Fig. 1B) showed increased mRNA levels and protein concentrations of the above-mentioned inflammation response genes compared with nontreated adipocytes (Fig. 2, A and B). In addition, incubation of adipocytes with Mq-factors resulted in increased lipolysis, as indicated by an almost fourfold higher concentration of glycerol released by the adipocytes into the medium (196 ± 13 vs. 56 ± 7 μM in control, P < 0.05; Fig. 2C).

**Pio alleviates inflammation in adipose tissue and adipocytes induced by Mq-factors.** To investigate the potential anti-inflammatory effects of Pio on adipose tissue, we pretreated SAT and VAT samples ex vivo with Pio or vehicle and then incubated them with Mq-factors (see supplemental Fig. 1A). Pio attenuated the expression levels of several representative inflammation response genes (secreted adipokine IL-6, chemokine MCP-1, and adhesion molecule VCAM-1) in SAT and VAT activated by Mq-factors (Table 2). Pio suppressed IL-6 expression levels to a greater extent in SAT (to 39 ± 8% of SAT treated with Mq-factors alone, P < 0.05) than in VAT (to 77 ± 13% of VAT treated with Mq-factors alone, not significant) obtained from the same donors, whereas it reduced MCP-1 and VCAM-1 expression levels to a similar extent in the different tissue types (to ~54% of tissue treated with Mq-factors alone, P < 0.05).
Similar to its effects on adipose tissue, Pio also attenuated M<sub>q</sub>-factor-induced inflammation in SGBS adipocytes (Table 3; see supplemental Fig. 1B). Specifically, Pio reduced the induction of mRNA levels and protein concentrations of proinflammatory adipokines. Furthermore, Pio pretreatment of adipocytes modestly reduced lipolysis induced by M<sub>q</sub>-factors (from 196 ± 13 to 167 ± 7 µM, P = 0.1).

Pio not only suppressed the inflammation response of adipocytes induced by M<sub>q</sub>-factors, but it also attenuated M<sub>q</sub>-factor suppression of adiponectin expression (Fig. 3). Thus, in addition to directly increasing adiponectin mRNA and protein expression (19), Pio prevents its downregulation in response to M<sub>q</sub>-factors.

**Pretreatment of adipocytes or monocytes/macrophages with Pio reduces inflammatory responses of adipocytes to factors secreted by monocytes/macrophages.** Having demonstrated that Pio has direct anti-inflammatory effects on adipose tissue and adipocytes activated by M<sub>q</sub>-factors, we assessed whether pretreatment of monocytes/macrophages with Pio would reduce their proinflammatory effects on adipocytes. We further determined whether combined pretreatment of adipocytes as well as monocytes/macrophages with Pio would have greater anti-inflammatory effects than individual pretreatment of each cell type with Pio.

We pretreated SGBS adipocytes with Pio or vehicle and then incubated the cells with control medium or M<sub>q</sub>-factors obtained from U937 macrophages that had been differentiated in the presence of Pio or vehicle (see supplemental Fig. 1B). We...
Table 2. Pio reduces mRNA expression levels of inflammation response genes in adipose tissue samples activated by Mcf-factors

<table>
<thead>
<tr>
<th>Gene Transcript/Protein</th>
<th>mRNA Levels in Adipocytes</th>
<th>Protein Concentration in Media Conditioned by Adipocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>68±9*</td>
<td>69±8*</td>
</tr>
<tr>
<td>IL-8</td>
<td>53±13*</td>
<td>84±7*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>61±14*</td>
<td>73±3*</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>55±12*</td>
<td>70±8*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>14±6*</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Values are means ± SE. mRNA levels and protein concentrations were determined in adipocytes (n = 5–7 experiments) activated by Mcf-factors in the presence of 10 μM pioglitazone (Pio) and were presented as percentage of levels obtained in concomitant experiments without Pio. *P < 0.05 vs. tissue samples treated with Mcf-factors alone. †P < 0.05 vs. VAT obtained from the same donors.

Fig. 2. Effects of pioglitazone (Pio) on expression levels of adiponectin in SGBS adipocytes activated by Mcf-factors. Adipocytes were pretreated with Pio or vehicle for 24 h and then incubated for 18 h with basal medium (control) or with Mcf-factors. A: mRNA levels of adiponectin in adipocytes. Values (means ± SE of 4 experiments) are presented as fold change from control samples treated with vehicle (set at 1). B: ELISA of protein concentrations of adiponectin secreted by adipocytes at the end of the experiment. Values are means ± 4–8 experiments. *P < 0.05 vs. mRNA or protein concentrations of adiponectin in adipocytes activated by Mcf-factors alone.

Fig. 3. Effects of pioglitazone (Pio) on expression levels of adiponectin in SGBS adipocytes activated by Mcf-factors. Adipocytes were pretreated with Pio or vehicle for 24 h and then incubated for 18 h with basalmedium (control) or with Mcf-factors. A: mRNA levels of adiponectin in adipocytes. Values (means ± SE of 4 experiments) are presented as fold change from control samples treated with vehicle (set at 1). B: ELISA of protein concentrations of adiponectin secreted by adipocytes at the end of the experiment. Values are means ± 4–8 experiments. *P < 0.05 vs. mRNA or protein concentrations of adiponectin in adipocytes activated by Mcf-factors alone.

Table 3. Pio suppresses inflammation responses in adipocytes activated by Mcf-factors

<table>
<thead>
<tr>
<th>Gene Transcript/Protein</th>
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<th>Protein Concentration in Media Conditioned by Adipocytes</th>
</tr>
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<tbody>
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<td>IL-6</td>
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<td>ICAM-1</td>
<td>55±12*</td>
<td>70±8*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>14±6*</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Values are means ± SE. mRNA levels and protein concentrations were determined in adipocytes (n = 5–7 experiments) activated by Mcf-factors in the presence of 10 μM Pio and were presented as percentage of levels obtained in concomitant experiments without Pio. N/D, not determined. *P < 0.05.

subsequently determined the concentrations of IL-6 and MCP-1 as well as glycerol released from these treated adipocytes. The data shown above are confirmed in Fig. 4, A and B, which shows that Pio treatment of adipocytes alone reduced their inflammatory responses to Mcf-factors compared with control adipocytes. Mcf-factors from Pio-treated macrophages also elicited decreased inflammatory responses in control (vehicle-treated) adipocytes (Fig. 4, A and B). This latter effect might be explained, at least in part, by data showing reduced gene expression levels of TNF-α and IL-1β in Pio-treated macrophages (52 ± 6% and 57 ± 7% of control untreated cells, respectively, P < 0.05). Finally, combined Pio pretreatment of adipocytes and macrophages more consistently reduced the adipocyte production of IL-6 and MCP-1 as well as glycerol (Fig. 4, A and B) compared with Pio treatment of each individual cell type.

In a separate set of experiments, we also investigated the effects of Pio on the adhesion of monocytes to SGBS adipocytes (see supplemental Fig. 1C). The number of monocytes that adhered to Mcf-factor-activated adipocytes (Fig. 4C) was nearly double the number that adhered to untreated adipocytes. This increased level of monocyte adhesion was reduced to almost background adherence level by pretreatment of the adipocytes with Pio before incubation with Mcf-factors (Fig. 4C). In turn, pretreatment of the monocytes with Pio led to a similar reduction of monocyte adhesion to adipocytes activated by Mcf-factors (Fig. 4C). This latter effect might be due, at least in part, to the monocytes’ lower expression levels of CD11b (an integrin molecule necessary for monocyte adhesion), which were reduced to 39 ± 11% of vehicle-treated monocytes (P < 0.05). Not surprisingly, Pio pretreatment of adipocytes and monocytes also normalized the level of monocyte adhesion to the Mcf-factor-activated adipocytes (Fig. 4C).

We then incubated SGBS adipocytes for 18 h with media conditioned by monocytes (Mono-factors) isolated from subjects treated with Pio or placebo (see supplemental Fig. 1D). The protein concentrations of IL-6, IL-8, and MCP-1 produced by the adipocytes incubated with Mono-factors of the Pio-treated group were generally lower than those produced by adipocytes incubated with Mono-factors of the placebo group (Fig. 4D). Furthermore, adipocytes incubated with Mono-factors of the Pio-treated group released lower concentrations of glycerol (by ~34%), indicating a lower rate of lipolysis, than those incubated with Mono-factors of the placebo group (Fig. 4E). Taken together, these results show that monocytes isolated from subjects treated with Pio induced inflammatory responses in adipocytes to a lesser extent than monocytes isolated from subjects treated with placebo.
Fig. 4. Pretreatment of adipocytes or monocytes/macrophages or both cell types with Pio reduces inflammatory response of adipocytes to factors secreted by monocytes/macrophages. A: M₆-factors were obtained as conditioned media from U937 macrophages that had been differentiated in medium containing 10 μM Pio or vehicle. SGBS adipocytes that had been pretreated with 10 μM Pio or vehicle were incubated with these conditioned media for 18 h. Then the adipocyte monolayer was washed and incubated in basal medium containing 0.5% BSA for 24 h. Concentrations of IL-6 and MCP-1 in the latter medium were determined using ELISA. Average IL-6 and MCP-1 concentrations produced by adipocytes differed among treatments (P < 0.05, by ANOVA). *P < 0.05 (by Dunnett’s multiple comparison test) vs. control condition, in which both cell types were pretreated with vehicle.

B: concentrations of glycerol released by the adipocytes in A into the medium during incubation with M₆-factors. Average glycerol concentrations produced by adipocytes differed among treatments (P < 0.05 by ANOVA). *P < 0.05 (by Dunnett’s multiple comparison test) vs. control condition, in which both cell types were pretreated with vehicle.

C: adipocytes were pretreated with 10 μM Pio or vehicle for 24 h and then incubated for 18 h with basal medium (control) or with M₆-factors from untreated U937 macrophages. U937 monocytes that had been pretreated for 24 h with 10 μM Pio or vehicle were used for assay of monocyte adhesion to the adipocytes. Values (means ± SE) are presented as fold induction compared with control condition, in which both cell types were pretreated with vehicle. *P < 0.05 (by Dunnett’s multiple comparison test) vs. control condition, in which both cell types were pretreated with vehicle.

D: ELISA of protein concentrations of IL-6, IL-8, and MCP-1 in medium conditioned by adipocytes that had been incubated for 18 h with conditioned media from monocytes (Mono-factors) of subjects treated with placebo or Pio for 4.5 mo. Values are means ± SE of 8–9 per group. *P < 0.05; #P < 0.1 vs. adipocytes exposed to conditioned media from monocytes of subjects in placebo group.

E: lipolysis in treated adipocytes in D assessed as concentration of glycerol released into the medium during treatment. Values are means ± SE (n = 7–8). *P < 0.05 vs. adipocytes exposed to conditioned media from monocytes of subjects in placebo group.
DISCUSSION

This study demonstrates several major points. First, M\(\alpha\)-factors induce inflammation in SAT and VAT. M\(\alpha\)-factors increased the mRNA expression levels and protein production not only of adipokines, which circulate as endocrine factors (e.g., IL-6, IL-8), but also chemokines (e.g., MCP-1) and adhesion molecules (e.g., ICAM-1, VCAM-1), which may act locally to facilitate macrophage infiltration into adipose tissue. VAT showed not only greater basal inflammatory levels, but also a greater tendency for enhanced M\(\alpha\)-factor-stimulated proinflammatory responses, than SAT. These greater proinflammatory responses of VAT, coupled with a higher percentage of macrophage infiltration in VAT than SAT (9), may contribute to the higher production of proinflammatory adipokines from this depot (14). The overall augmented proinflammatory state of VAT may help explain the frequently reported association between VAT (or its clinical estimates, such as waist circumference) and obesity-related comorbidities (21).

As a major cell type in VAT and SAT, adipocytes may be a key target of M\(\alpha\)-factors. Indeed, M\(\alpha\)-factors significantly increased adipocyte production of proinflammatory adipokines, perhaps in part via TNF-\(\alpha\)-mediated activation of NF-\(\kappa\)B (43). M\(\alpha\)-factors also increased adipocyte lipolysis, releasing FFAs. M\(\alpha\)-factors may exert this latter effect, in part via TNF-\(\alpha\)-mediated suppression of lipin-1, a key enzyme in triglyceride synthesis (35), and of perilipin, a protein associated with lipid droplets (44). The adipokines and FFAs produced by adipocytes activated with M\(\alpha\)-factors can, in turn, exert proinflammatory effects on adipocytes (38) and monocytes/macrophages (51, 52, 62), creating a self-propagating cycle of inflammation in adipose tissue.

A second major and novel finding of this study is that Pio directly suppressed M\(\alpha\)-factor-induced inflammation in adipose tissue and adipocytes. Despite the differential predisposition to inflammation in VAT and SAT, Pio suppressed M\(\alpha\)-factor-induced inflammation to a relatively similar extent in both tissue types. In adipocytes activated by M\(\alpha\)-factors, Pio inhibited the production of a wide variety of inflammation response genes at the mRNA and protein levels. In addition, Pio suppressed M\(\alpha\)-factor-induced lipolysis, presumably in part by alleviating TNF-\(\alpha\) suppression of perilipin (48) and by inducing glicersonogenesis (53). Inasmuch as FFAs, especially saturated FFAs, appear to exert proinflammatory effects on macrophages and adipocytes (52), Pio’s ability to reduce local FFAs released by M\(\alpha\)-factor-activated adipocytes may further reduce the paracrine proinflammatory interaction between adipocytes and monocytes/macrophages. Pio also alleviated M\(\alpha\)-factor suppression of adiponectin mRNA and protein expression levels in adipocytes. By preserving concentrations of adiponectin, Pio may also indirectly mitigate the inflammatory effects of M\(\alpha\)-factors in adipose tissue. This notion is supported by data (not shown) demonstrating that physiological concentrations of adiponectin reduced the expression levels of various inflammation-related genes in adipocytes activated by M\(\alpha\)-factors. However, adiponectin production induced by Pio does not explain all of Pio’s anti-inflammatory effects: we observed that knockdown of adiponectin gene expression with small interfering RNA did not eliminate Pio’s beneficial effects on adipocytes (data not shown). Taken together, Pio likely suppresses inflammation in adipose tissue and adipocytes via adiponectin-dependent and -independent pathways; the latter effect may involve nuclear receptor corepressor (NCoR)/sumoylation-depen-
sion levels of several tested M2 markers [mannose receptor/CD206, AMAC-1/CLL18, and IL-10 (5)] than monocytes isolated from the placebo-treated group (data not shown). This notion is supported by a recent study showing that this phenotype conversion is facilitated by activation of peroxisome proliferator-activated receptor-γ in circulating monocytes (4). In contrast to the classically activated M1 monocytes/macrophages, the alternatively activated M2 phenotype does not appear to inhibit adipogenesis of preadipocytes or insulin-stimulated glucose uptake in adipocytes (36). Furthermore, the M2 monocytes/macrophages may play a key role in beneficial adipose tissue remodeling (5).

The direct anti-inflammatory effects of Pio on adipocytes and monocytes/macrophages demonstrated in this study may help explain how long-term treatment with Pio (and other TZDs) in humans (11) and rodents (60) reduces proinflammatory macrophage infiltration into adipose tissue. Attenuation of adipose tissue inflammation associated with tissue resident macrophages may comprise a mechanism of action for Pio to improve insulin sensitivity (11, 60).

In summary, this study showed that 1) Mγ-factors induced inflammatory responses in human adipose tissue, to a greater extent in VAT than SAT, and also in adipocytes, and 2) incubation of adipocytes and/or monocytes/macrophages with Pio decreased inflammatory responses in adipocytes activated by factors secreted from the monocytes/macrophages. These results may explain, at least in part, how in vivo anti-inflammatory effects of Pio in adipose tissue help reduce local and systemic inflammation and, thus, contribute to improvement of whole body insulin sensitivity.

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