Pioglitazone Inhibits the Expression of Inflammatory Cytokines From Both Monocytes and Lymphocytes in Patients With Impaired Glucose Tolerance

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Objective—The current study determines whether pioglitazone (PIO) therapy reduces both monocyte and lymphocyte inflammatory activity and their ability to induce inflammation in other tissues.

Methods and Results—Monocyte and lymphocyte cytokine gene and protein expression of interleukin (IL)-6 were first shown to be greater in subjects with impaired glucose tolerance (IGT) than in subjects with normal glucose tolerance. Sixty-six IGT subjects were then randomized to 4.5 months of placebo or PIO therapy. After receiving PIO, subjects had lower triglycerides and higher HDL cholesterol (P<0.05) than did subjects receiving placebo. Monocyte gene and protein expression of IL-1β, IL-6, and IL-8 (and IL-2, IL-6 and IL-8 from lymphocytes) was significantly lower after PIO therapy in the resting state, as well as after lipopolysaccharide (LPS) stimulation (P<0.05 for all). Moreover, IL-6, IL-8, and MCP-1 gene expression were decreased by nearly 50% in human adipocytes exposed to conditioned media from monocytes or lymphocytes from PIO treated subjects.

Conclusion—These results demonstrate that PIO therapy in IGT can reduce proinflammatory gene and protein expression from both monocytes and lymphocytes. This intervention also reduces the inflammatory cross-talk between these immune cells and adipose tissue, which could in turn contribute to the metabolic improvements resulting from PIO therapy. (Arterioscler Thromb Vasc Biol. 2008;28:2312-2318.)

Key Words: thiazolidinediones ■ cytokine ■ monocytes ■ lymphocytes ■ adipose tissue

Several decades of research have demonstrated that atherosclerosis is in part an inflammatory process. Infiltration of the arterial wall by monocytes and lymphocytes and their production of a variety of inflammatory factors contribute to the development and progression of atherosclerosis, as well the formation of vulnerable atherosclerotic plaques. Recent studies have indicated that in a similar fashion, mononuclear infiltration of fat tissue may be an instigating event in the development of insulin resistance and type 2 diabetes. Although the events initiating the recruitment of mononuclear cells into adipose tissue remain unclear, these cells produce a variety of factors that induce adipose tissue inflammation. The interaction between fat cells and infiltrating mononuclear cells may lead to a paracrine loop of inflammation that may contribute to the development of both local and systemic insulin resistance. Although most studies have focused on the interaction of monocytes/macrophages with fat cells, several recent studies have suggested lymphocytes may also be increased in adipose tissue in the setting of obesity or perivascular inflammation and may contribute to both development of diabetes and vascular disease. Thus, infiltration of mononuclear cells into various tissues may play critical roles in the development of several chronic inflammatory disease processes.

Thiazolidinedione (TZD) compounds are peroxisome proliferator-activator receptor gamma (PPARγ) ligands that have shown great potential to reduce insulin resistance and contribute to the delay or prevention of diabetes. They also have been shown to reduce atherosclerosis in animal models and modulate early stages of atherosclerosis by decreasing the intimal-medial thickness of carotid arteries and at least in the case of pioglitazone, reduce major cardiovascular events. Although TZDs are best recognized for their insulin sensitizing activity, they also have potent antiinflammatory activity in tissues and inflammatory cells. It has been proposed that this may occur through both PPARγ receptor-mediated and receptor-independent mechanisms. Numerous studies have shown that TZDs can repress AP1- and NF-κB–dependent expression of inflammatory genes, and this may occur in large part via PPARγ-mediated stabilization of the nuclear receptor corepressor (NCoR) complex. Thiazolidinediones may also drive expression of a variety of antiinflammatory genes, such as adiponectin, heme oxygenase-1, and IL-10.

Given the diverse mechanisms by which TZDs may reduce inflammation, it is not surprising that therapy with TZDs has been shown to reduce multiple plasma markers of inflamma-
that, although it is unclear whether these in vivo effects are attributable to effects on mononuclear cells or other tissues. Previous studies have demonstrated that therapy with TZDs reduce mononuclear NF-kB activity, although the specific effect on protein secretion from these cells was not thoroughly investigated. In addition, these studies were conducted on mixed cell mononuclear preparations. Therefore, the specific in vivo effects of TZDs on individual mononuclear cell types is unknown. Moreover, it has not been evaluated whether mononuclear cells from TZD-treated individuals are less prone to induce inflammation in other tissues and are therefore less capable of supporting the paracrine feed-forward inflammation cycle that has been suggested to contribute to disease development.

Therefore, the current study determines (1) whether monocytes and lymphocytes from individuals with IGT demonstrate greater proinflammatory activity than cells from individuals with normal glucose tolerance (NGT), (2) whether PIO therapy in IGT reduces inflammatory activity in both cell types, and (3) whether monocytes and lymphocytes from PIO treated individuals are less able to induce inflammation in adipocytes.

Materials and Methods

Experimental Design
This study was approved by the Institutional Review Board of the Phoenix VA Health Care System. A 75-g glucose tolerance test was used to classify nondiabetic subjects as having either NGT or IGT according to ADA criteria. Seventy-two subjects between the ages of 21 to 75 had normal or borderline HbA1c, fasting blood glucose between 5.3 to 6.9 mmol/L, and glucose at 120 minutes from 7.8 to 11.1 mmol/L, and were classified as having IGT. Another 33 subjects were identified as having NGT. A subset of age- and gender-matched subjects with NGT or IGT were then evaluated for monocyte and lymphocyte inflammation. Subsequently, 66 subjects with IGT were randomized to receive either placebo or 45 mg pioglitazone per day for an average of 4.5 months (Placebo 143 days versus PIO-treated 141±15 days) in a double-blind study design.

Glucose, Lipid, and Cytokine Assays
Plasma levels of glucose, total cholesterol, triglyceride, LDL cholesterol, HDL cholesterol, and HbA1c were measured in the clinical laboratory of the Phoenix VA Health Care System in subjects at baseline, and for those participating in the intervention phase of the study again at approximately 4.5 months after randomization. EDTA-anticoagulated blood samples were used for rapid isolation of monocyte and lymphocyte inflammation. Subsequently, 66 subjects with IGT were randomized to receive either placebo or 45 mg pioglitazone per day for an average of 4.5 months (Placebo 143 days versus PIO-treated 141±15 days) in a double-blind study design.

RNA Isolation and Real-Time RT-PCR
Total RNA was isolated from peripheral monocytes or lymphocytes using TriZol (Invitrogen) according to the manufacturer’s protocol and treated with DNase I to remove residual DNA contamination (Ambion). First-Strand cDNA synthesis was performed using a

Figure 1. Comparison of expression of IL-6 by monocytes and lymphocytes between NGT and IGT subjects. A, IL-6 was measured in plasma collected on the same day from matched NGT and IGT subjects (n=13 per group). B, Monocyte and lymphocyte IL-6 gene expression. C, IL-6 protein in 24-hour monocyte- or lymphocyte-conditioned media. In all panels, the white bar represents NGT; the black bar represents IGT. *P<0.05 compared to NGT.

Superscript III kit (Invitrogen) according to manufacturer’s protocol. Real-time RT-PCR was performed using iQ SYBR Green Supermix and an iCycler iQ system (BioRad) using the 5ΔΔCt method. IL-1β, IL-2, IL-6, IL-8, and MCP-1 gene expression was normalized to GAPDH or 18s rRNA.

Treatment of Adipocytes With Conditioned Media From Monocytes of Control or Pioglitazone-Treated Subjects
The human Simpson-Golabi-Behmel syndrome (SGBS) adipocyte cell line was a kind gift from Dr Wabitsch (University of Ulm, Germany). Mature SGBS adipocytes were obtained by differentiating preadipocytes as previously described. Mature SGBS adipocytes were incubated for 18 hours with control medium alone (a 1:1 mixture of RPMI supplemented with 10% FBS and DMEM/F12 containing 0.5% BSA), 1 mmol/L tumor necrosis factor (TNF)-α in control medium, or media conditioned by monocytes or lymphocytes from subjects who had received either placebo or pioglitazone treatment diluted 1:1 with DMEM/F12 containing 0.5% BSA. At the end of the treatment, the adipocytes were lysed. Cell lysates served as templates for cDNA synthesis and gene expression was measured as described above. Gene expression in adipocytes exposed to media conditioned by monocytes or lymphocytes was normalized to gene expression in adipocytes exposed only to control medium.

Statistical Analysis
Unless otherwise specified, all table data are mean±SD, and all figure data are mean±SE. ANOVA, t tests were used for comparisons between groups as appropriate. Spearman correlations and multivariable linear regression models were performed to assess relationships between metabolic variables and release of cyto-
kines from mononuclear cells. A value of \( P<0.05 \) was considered significant.

**Results**

To confirm that IGT was associated with increased inflammation, we first compared 13 subjects with NGT with a similar number of subjects with IGT. The groups were, by selection, well-matched for age (NGT: 61.2 ± 6 versus IGT: 60.4 ± 5 years) and BMI (NGT: 31 ± 4 versus IGT: 32 ± 5) and were similar in gender distribution (NGT: 1F/12M versus IGT: 1F/12M) and also had similar fasting glucose (NGT: 5.6 ± 0.5 versus IGT: 5.8 ± 0.3 mmol/L), HbA1c values (NGT: 5.6 ± 0.5% versus IGT: 5.9 ± 0.3%) and lipid values (triglyceride, NGT: 1.7 ± 0.7 versus IGT: 1.7 ± 0.8 mmol/L; total cholesterol, NGT: 4.9 ± 0.6 versus IGT: 4.9 ± 0.9 mmol/L; HDL cholesterol, NGT: 1.1 ± 0.3 versus IGT: 1.0 ± 0.2 mmol/L; LDL cholesterol, NGT: 2.9 ± 0.6 versus IGT: 3.0 ± 0.7 mmol/L). Although BMI trended higher in IGT subjects (32.3 ± 5.2 versus 31.2 ± 4.4 m²/kg), this difference was not significant.

In contrast, 2-hour glucose values were significantly higher in the IGT subjects (8.9 ± 0.8 versus 6.2 ± 0.7 mmol/L, \( P<0.05 \)). Plasma IL-6 concentrations were also significantly elevated in IGT compared to NGT subjects (Figure 1A).

To reduce interassay variation, monocytes and lymphocytes from each IGT subject were isolated on the same day as cells from each matched NGT control. As shown in Figure 1B, the elevated plasma IL-6 was accompanied by significantly higher (more than 3-fold) IL-6 mRNA expression in monocytes and lymphocytes from individuals with IGT. Importantly, this difference in mRNA levels translated to increased secretion of IL-6 protein into conditioned media over 24 hours (Figure 1C), with monocyte/macrophage secretion of IL-6 also nearly 3-fold higher in subjects with IGT. A significant increase in IL-6 secretion from lymphocytes was also present, although secreted levels of IL-6 were substantially lower than those from monocytes/macrophages.

Expression of several other cytokine genes (such as IL-1β and IL-8 in monocytes, and IL-2 and IL-8 in lymphocytes) was also increased 2- to 3-fold (data not shown) in cells from IGT subjects compared to those from NGT subjects. These data confirm that monocytes and lymphocytes from individuals with IGT do demonstrate greater degrees of inflammation and may contribute to increased levels of inflammatory markers in plasma.

Sixty-six individuals with IGT were then randomized to receive 4.5 months of either PIO or placebo therapy. At baseline, both groups had similar age, gender distribution, BMI, blood pressure, fasting and 2-hour glucose, and lipid values (Table). The majority of subjects in both groups at baseline also had impaired fasting glucose (placebo: 68% versus PIO: 74%, \( P=0.6 \)). The percent of individuals that were current smokers, or with a history of hypertension, cardiovascular disease, or ongoing statin use also did not differ between groups (Table). The number of individuals using angiotension inhibitors or angiotension receptor blockers also did not differ between groups (placebo: 22.6% versus PIO: 14.3%, \( P=0.4 \)).

After 4.5 months of therapy there were several differences between groups (Table); HDL cholesterol levels were significantly higher (PIO: 1.2 ± 0.3 versus placebo: 1.0 ± 0.2 mmol/L, \( P<0.05 \)) and triglycerides significantly lower (PIO: 1.5 ± 0.7 versus placebo: 1.9 ± 0.8 mmol/L, \( P<0.05 \)) in those receiving PIO. Of note, plasma levels of IL-6 (placebo: 2.2 ± 1.7 versus PIO: 1.3 ± 0.7 pg/mL) and IL-8...
baseline, there was no significant difference in monocyte mRNA expression of IL-1β, IL-6, and IL-8 between groups randomized to placebo or PIO. After PIO treatment, monocytes demonstrated greatly reduced mRNA levels of all 3 cytokines compared to monocytes of placebo treated subjects. Corresponding reductions were seen for IL-1β, IL-6, and IL-8 protein levels in 24-hour monocyte-conditioned media. White bars = placebo; black bars = PIO; n = 31 per group; *P < 0.05 compared to samples from placebo treated subjects.

As shown in Figure 2A, expression of IL-1β, IL-6, and IL-8 mRNA did not differ in monocytes isolated from placebo and PIO randomized groups at baseline. In contrast, after 4.5 months of PIO therapy, monocytes demonstrated suppressed gene expression of all three cytokines (P < 0.01 in all cases) in comparison with monocytes from subjects receiving placebo (Figure 2B). Consistent with these changes in gene expression, levels of the corresponding proteins secreted into the media during a 24-hour period were also significantly lower in the PIO group (Figure 2C).

As shown in Figure 3A, relative gene expression of IL-2, IL-6, and IL-8 did not differ in lymphocytes isolated from placebo and PIO randomized groups at baseline. In contrast, gene expression of IL-2, IL-6, and IL-8 was significantly suppressed (≈70%) in lymphocytes from PIO-treated subjects (Figure 3B). This also translated to reduced (by ≈50%) cytokine protein expression into conditioned media over 24 hours (Figure 3C), although only differences in IL-6 and IL-8 reached statistical significance.

To determine whether PIO therapy might limit not only basal mononuclear inflammatory activity, but also responses to stress, monocytes from a subset of subjects receiving PIO or placebo treatment (n = 8 per group) were exposed to low concentrations of LPS (1 ng/mL) for 24 hours. As shown in Figure 4, monocytes from subjects receiving PIO demonstrated reduced levels of IL-6 and IL-8 protein secretion into the media in the unstimulated state as expected (left set of bars). When stimulated with LPS, cells from both PIO and placebo individuals responded with increases in cytokine secretion into the media; however, protein secretion was reduced (P < 0.05) by approximately 30% to 40% in cells from PIO-treated subjects but not in placebo-treated subjects.

To assess whether monocyte and lymphocyte cytokine secretion at the end of the 4.5-month treatment period was related to subject characteristics we assessed univariate relationships of concentrations of secreted cytokines with available demographic or metabolic variables. In the entire group, triglycerides at follow-up were correlated with IL-6 (r = 0.33, P < 0.05) and IL-8 (r = 0.39, P < 0.05) secretion by lymphocytes, and with IL-8 secretion by monocytes (r = 0.39, P < 0.05). Even more strikingly, HDL cholesterol at follow-up

(placebo: 5.5 ± 3.1 versus PIO: 3.7 ± 1.2 pg/mL) were significantly lower in individuals who received PIO (P < 0.05).

As shown in Figure 2A, expression of IL-1β, IL-6, and IL-8 mRNA expression of IL-1β, IL-6, and IL-8 between groups randomized to placebo or PIO. A, At baseline, there was no significant difference in monocyte mRNA expression baseline and after 4.5 months of PIO or placebo therapy.
was strongly and inversely related to lymphocyte and monocyte secretion of IL-6 ($r = -0.82$, $r = -0.59$, respectively, $P<0.0001$) and IL-8 ($r = -0.54$, $r = -0.50$, respectively, $P<0.0001$), as illustrated in the supplemental Figure (please see http://atvb.ahajournals.org). In multivariable regression analysis, after adjusting for age, BMI, fasting glucose, lipid levels, and treatment assignment, HDL cholesterol, but not triglycerides, remained significantly associated with concentrations of IL-6 ($P=0.05$) and IL-8 ($P=0.035$) released from monocytes and IL-6 ($P=0.001$) from lymphocytes (but not with IL-8 from lymphocytes, $P=0.08$). Assignment to PIO therapy was also significantly associated with reduced levels of cytokine secretion; however, β estimates for treatment assignment were generally decreased with addition of HDL cholesterol to the multivariable models, indicating that the effect of PIO treatment on inflammation was explained in part by its effects on HDL cholesterol levels.

As monocytes and lymphocytes from PIO treated subjects appeared to have reductions in both basal and stimulated inflammatory responses, we wondered whether cells from these subjects would be less likely to induce activation and inflammation in other cell types that they might interact with that could contribute to the development of insulin resistance. We therefore incubated SGBS human adipocytes with 24-hour conditioned media collected from monocytes or lymphocytes from individuals receiving either PIO or placebo. Media conditioned by monocytes and lymphocytes from placebo treated subjects induced striking increases in adipocyte IL-6, IL-8 and MCP-1 mRNA expression (Figure 5). The increased gene expression induced by conditioned media from inflammatory cells from subjects receiving placebo was in fact greater than that induced by direct addition of TNF-α (1 nmol/L) to the adipocytes (data not shown). This demonstrates the substantial potential of infiltrating monocytes/macrophages and lymphocytes to increase adipose tissue inflammation. Interestingly, this increase in inflammatory gene mRNA was reduced markedly in adipocytes that were treated with conditioned media from either monocytes (panel A) or lymphocytes (panel B) isolated from individuals receiving PIO.

**Discussion**

This study demonstrates several major findings. First, it confirms that subjects with IGT show greater evidence of systemic inflammation than individuals with NGT, even when they have similar ages and measures of obesity. Importantly, this is evident not only from increased plasma markers of inflammation, but also from increased production of cytokines from both monocytes and lymphocytes. Whether the inflammation observed in subjects with IGT is a direct contributor to the development of insulin resistance or simply reflects the many metabolic abnormalities present in these conditions is unknown. Nonetheless, the enhanced mononuclear cell inflammatory activity, once present, is undoubtedly capable of contributing to further disease progression. Although monocytes/macrophages have been implicated in a variety of chronic inflammatory conditions, including the development of diabetes and atherosclerosis, similar evidence is accumulating for the presence and potential contribution of lymphocytes in these same conditions.1,4,7-9,32

This study also helps clarify the potential that PIO treatment has for preventing the development of these chronic inflammatory conditions in individuals with IGT. Four and a half months of PIO treatment not only decreased several markers of systemic inflammation, but demonstrated substan-
tial inhibitory effects on both monocyte and lymphocyte expression of proinflammatory cytokines. We focused on the effects of PIO on IL-1β, IL-2, IL-6, and IL-8 as they are important proinflammatory cytokines that have been associated with, or implicated in, both the recruitment of monocytes and lymphocytes or the activation of both adipose tissue and vascular cells. Moreover, these cytokines are produced by monocytes and lymphocytes in relatively large amounts over relatively short periods of time and thus can be collected and reliably measured during the time frame of the experiments. However, we also found using a more broad-based multiplex bead assay approach that PIO treatment reduced monocyte release of other cytokines such as GM-colony stimulating factor (CSF), TNF-α, and MIP1-β, although concentrations of these factors were lower and less consistently within the assay range. Although numerous in vitro studies have demonstrated that PPARγ receptor agonists may reduce inflammatory responses in diverse cell types,15–18 few studies have convincingly shown the in vivo relevance of these findings to inflammatory cells of TZD-treated individuals.25–27 In the few clinical intervention studies that have examined the effects of TZDs on inflammatory cells, the investigations included small numbers of subjects and did not focus on individual inflammatory cell types or secretion of specific cytokines.25–27

A particularly novel finding of this study was that whereas factors released into the media from freshly isolated monocytes/macrophages and lymphocytes were potent stimulators of adipocyte inflammatory gene expression, this effect was substantially reduced when the cells were from individuals receiving PIO. It has been suggested that after infiltration of these inflammatory cells into various tissues, they may activate neighboring cells in the vasculature, fat tissue, or other organs; these cells in turn may generate their own inflammatory cytokines, leading to a paracrine cascade of inflammation.5,6,33 This type of inflammatory process may therefore contribute to the development of not only atherosclerosis, but also to insulin resistance.2–4,33,34 The current study demonstrates that PIO treatment may reduce the inflammatory activity of both monocytes and lymphocytes, and also provides evidence of the potential of PIO to limit propagation of inflammation in tissues that monocytes and lymphocytes infiltrate.34 This combination of effects may help explain the potent ability of several PPARγ agonists to inhibit or delay diabetes onset as well as reduce progression of atherosclerosis.10–13

An additional unique finding in this study was the strong inverse relationship between HDL cholesterol levels and production of cytokines from both monocytes and lymphocytes. Although the correlation was greatest for HDL cholesterol at the end of the treatment phase (supplemental Figure), baseline and change in HDL cholesterol levels also demonstrated significant (but smaller) inverse associations with IL-6 and IL-8 cytokines released from monocytes and lymphocytes ($r = -0.23$ to 0.62, $P<0.05$ for each cytokine). Additionally, in multivariable regression models, both PIO treatment and HDL cholesterol levels at follow-up significantly contributed to prediction of cytokines released from both cell types. These data raise several interesting points. First, they suggest that HDL particles may have uniquely potent antiinflammatory effects on circulating inflammatory cells. Although it has been increasingly recognized that HDL particles may have antiinflammatory and antioxidative activity in vitro,35 it has been difficult to more directly demonstrate this in humans. The strong inverse relationship between HDL cholesterol levels and secretion of cytokines from freshly isolated monocytes and lymphocytes certainly supports the notion that HDL particles may be modifying inflammatory activity of these cells in vivo. Second, as inclusion of HDL cholesterol levels in multivariable regression models appears to lessen (but not abolish) the association of PIO treatment assignment with mononuclear cytokine secretion, while improving the overall variance in cytokine concentrations explained by these models, it suggests that part of the PIO antiinflammatory effect on these cells in vivo may be explained through its well-recognized ability to increase HDL cholesterol. This is certainly consistent with a recent trial that demonstrated that the ability of PIO therapy to reduce progression of atherosclerosis (as measured by change in carotid intima-media thickness) appears in part explained by its capacity to raise HDL levels.36 These data overall suggest that PIO treatment in individuals with IGT may have both direct and indirect antiinflammatory effects on monocytes and lymphocytes in vivo.

In summary, these studies provide evidence that PIO has potent antiinflammatory effects in vivo on both monocytes and lymphocytes in individuals with IGT. This may directly contribute to reductions in systemic inflammation and may facilitate inhibition of inflammation in other tissues, such as adipose or vascular tissue by limiting inflammatory cross-talk with other cell types (as illustrated with adipocytes in this study). The combination of these effects may have important implications for preventing development of conditions with chronic inflammatory underpinnings, including type 2 diabetes and atherosclerosis.

Acknowledgments
We acknowledge the excellent clinical project assistance provided by Linda McDonald, RN.

Sources of Funding
This work was supported by the office of Research and Development, Medical Research Service, Department of Veterans Affairs and a grant from Takeda Pharmaceutical Inc (to P.D.R).

Disclosures
Peter Reaven is a consultant for Takeda Pharmaceutical Inc. He was also a member of the Merck & Company, Inc. Speakers Bureau in 2007. He was a member of the Merck & Company, Inc. Speakers Bureau in 2007.

References


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Arterioscler Thromb Vasc Biol. 2008;28:2312-2318; originally published online September 25, 2008;
doi: 10.1161/ATVBAHA.108.175687
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Supplemental Figure.** Plasma HDL cholesterol levels at follow-up were strongly and inversely related to monocyte and lymphocyte secretion of IL-6 and IL-8. IL-6 and IL-8 released into conditioned media by monocytes (Panel A and B) or lymphocytes (Panel C and D). Plasma HDL was measured after five months treatment with PIO or placebo. HDL cholesterol at follow-up was strongly and inversely related to lymphocyte and monocyte secretion of IL-6 (r= -0.82, r= -0.59, respectively, p < 0.0001) and IL-8 (r= -0.54, r= -0.50, respectively, p < 0.0001).