Anti-inflammatory effects of short-term pioglitazone therapy in men with advanced diabetic nephropathy

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Agarwal, Rajiv. Anti-inflammatory effects of short-term pioglitazone therapy in men with advanced diabetic nephropathy. Am J Physiol Renal Physiol 290: F600–F605, 2006. First published September 13, 2005; doi:10.1152/ajprenal.00289.2005.—Patients with diabetic nephropathy have a high rate of cardiovascular events and mortality. Nontraditional cardiovascular risk factors such as oxidative stress and inflammation are thought to be particularly important in mediating these events. Studies suggest that thiazolidinediones (TZDs) can reduce the level of nontraditional cardiovascular risk in people with or without diabetes mellitus. Whether this benefit occurs in patients with diabetic nephropathy is unknown. I hypothesized that the TZD pioglitazone will mitigate oxidative stress and inflammation compared with glipizide in patients with overt diabetic nephropathy. Markers of oxidative stress (plasma and urine albumin carbonyl and total protein carbonyls and malondialdehyde), inflammation [white blood cell (WBC) count, C-reactive protein (CRP), plasma IL-6, TNF-α], and plaque stability [matrix metalloproteinase 9 (MMP-9)] were measured in frozen samples obtained from patients with overt diabetic nephropathy participating in a randomized, open-label, blinded end-point, 16-wk trial with glipizide (n = 22) or pioglitazone (n = 22). Pioglitazone therapy in men with advanced diabetic nephropathy reduced WBC count by 1.125/µl (P < 0.001), CRP by 41% (P = 0.042), IL-6 by 38% (P = 0.009), and MMP-9 by 29% (P = 0.016). Specific differential reductions in WBC count of 1.251/µl (P = 0.009) and reduction in IL-6 of 58% with pioglitazone (P = 0.001) were seen compared with glipizide. There were no statistically significant changes observed with plasma TNF-α concentrations or markers of oxidative stress with either hypoglycemic agent. In conclusion, pioglitazone reduces proinflammatory markers in patients with overt diabetic nephropathy, which indicates potentially beneficial effects on overall cardiovascular risk. This surrogate end point needs to be confirmed in trials designed to demonstrate cardiovascular protection.

Chronic kidney disease (CKD) is associated with a high cardiovascular event rate and cardiovascular mortality (11). Data from animals and emerging data from patients with CKD suggest that nontraditional risk factors such as the presence of oxidative stress and inflammation may be especially relevant for the pathogenesis of atherosclerotic and renal disease (9, 14, 21, 32, 35). Type 2 diabetes mellitus is a common cause of CKD and itself is associated with a heightened state of oxidative stress and inflammation (12, 25, 26). Thus patients with CKD due to type 2 diabetes mellitus provide an excellent opportunity to study the role of therapies that ameliorate oxidative stress and inflammation to reduce the progression of cardiovascular disease.

Thiazolidinediones (TZDs) are synthetic peroxisome proliferator-activated receptor (PPAR)-γ agonists, act as insulin sensitizers, and are used in the treatment of type 2 diabetes. Preclinical data demonstrate that TZDs may have beneficial effects on atherosclerosis via regulation of cytokine production through modulation of monocyte-derived macrophages and reduced expression of adhesion molecules on endothelial cells (15, 30). Markers of inflammation such as C-reactive protein (CRP) are powerful, independent predictors of mortality of cardiovascular disease risk in the general population (17, 22) as well as those with CKD (7). Animal studies suggest that TZDs can reduce oxidative stress independently of their ability to reduce hyperglycemia (6, 8, 23, 33, 34).

TZDs reduce inflammatory markers in patients with type 2 diabetes with no overt nephropathy (13) or in patients with coronary artery disease without diabetes mellitus (31). However, it is unknown whether TZDs can reduce oxidative stress and inflammation in patients with overt diabetic nephropathy where these abnormalities are more pronounced. The cardiovascular disease burden in the later stages of CKD is particularly large (19); an improvement in inflammation and oxidative stress would raise the prospects for cardiovascular and renal protection with these agents. I hypothesized that the TZD pioglitazone will mitigate oxidative stress and inflammation compared with glipizide in patients with overt diabetic nephropathy.

Methods

Subjects

Urinary, serum, or plasma biomarkers were analyzed from 44 patients with type 2 diabetes mellitus who completed a 16-wk randomized controlled trial of pioglitazone or glipizide randomly allocated in an equal ratio. The study was blinded to those performing the analyses but open label to the patients and treating physician and has been reported previously (3).

Patients with established nephropathy were recruited from the renal clinic at the Roudebush Veterans Administration Medical Center (Indianapolis, IN). To qualify for inclusion in the study, patients with type 2 diabetes mellitus requiring treatment with oral hypoglycemic drugs or insulin were required to have a urine protein/creatinine ratio of >1.0 g/g on a single voided specimen and a
creatinine clearance of >20 ml/min by the Cockcroft-Gault formula (5). Exclusion criteria included the presence of liver disease, New York Heart Association Class III or IV heart failure, unstable angina, myocardial infarction or stroke in the previous 3 mo, nonsteroidal anti-inflammatory drug use, or body mass index of ≥40 kg/m². The study was approved by the Institutional Review Board of Indiana University and the Research and Development Committee of the Roudebush Veterans Administration Medical Center, and all patients gave their written informed consent.

The primary results, although reported in detail elsewhere, are recapitulated briefly (3). Baseline median 24-h urine protein/g creatinine was 2.6 g (interquartile range (IQR) 1.8–4.2 g) in the pioglitazone group and 2.8 g (IQR, 1.5–5.0 g) in the glipizide group. The glipizide group had an adjusted least mean square difference in proteinuria of 6.1% [95% confidence interval (CI) −11.7 to 23.8%], whereas the pioglitazone group had an adjusted mean reduction of 7.2% (95% CI −24.9 to 10.6%). The adjusted mean reduction with pioglitazone of 13.2% compared with glipizide (95% CI −38.4 to 11.9%) was not statistically significant (P = 0.294). The mean dose of pioglitazone was 33 ± 10 mg, and average exposure time was 3.8 ± 0.7 mo. The mean dose of glipizide was 16 ± 8 mg, and the average exposure time was 3.7 ± 0.8 mo. The mean dose and exposures were calculated by actual amounts and duration of these drugs taken by patients. No relationship between the dose of either hypoglycemic agent and reduction in proteinuria was seen.

Biological samples were obtained on the day of randomization and at week 16 and stored at −84°C until analyzed. Samples were assayed as pairs. Although no significant changes are noted in these analytes on storage under conditions reported here, paired comparisons would presumably mitigate the loss of active molecules, if that were to occur.

Laboratory Assays

Oxidative stress markers. MALONDIALDEHYDE. Malondialdehyde (MDA), a lipid hydroperoxide, is formed by β-scission of peroxidized polyunsaturated fatty acids and was measured by high-performance liquid chromatography following derivatization with thiobarbituric acid (TBA), as reported previously (2). Urinary MDA was analyzed in three spontaneously voided specimens over a period of 3 h. Each specimen was analyzed for MDA and creatinine. The MDA/creatinine ratio was averaged and used further for statistical analysis. Blood was collected in EDTA-containing vacutainers (Beckton-Dickinson), and the total leukocyte [white blood cell (WBC)] count was calculated by our clinical laboratory using the Coulter method (Coulter STK-S, Coulter Electronics, Hialeah, FL).

CRP. CRP was measured with a Cobas Integra 400 analyzer using a particle-enhanced turbidimetric assay (Cobas Integra C-Reactive Protein Latex, Roche Diagnostics, Indianapolis, IN). The intra-assay coefficient of variation was 1.8%, and the interassay coefficient of variation was 2.9% at a mean level of 0.62 mg/dl CRP.

IL-6. IL-6 was assayed in plasma using a sandwich ELISA (Quantikine kit for Human IL-6 Immunoassay; R&D Systems, Minneapolis, MN). A standard curve was generated using a linear curve-fit. The correlation coefficient for standards was greater than 0.99, and the lowest detectable limit was 0.039 pg/ml in undiluted plasma. The intra-assay coefficient of variation was 7.8%, and the interassay coefficient of variation was 7.2%.

TNF-α. TNF-α was assayed in plasma using a sandwich ELISA (Quantikine kit for Human TNF-α Immunoassay, R&D Systems). A standard curve was generated using a linear curve-fit. The correlation coefficient for standards was greater than 0.99 and the lowest detectable limit 0.12 pg/ml in undiluted plasma. The intra-assay coefficient of variation was 5.9% and the interassay coefficient of variation was 12.6%.

Plaque stability marker. MATRIX METALLOPROTEINASE 9. Matrix metalloproteinase 9 (MMP-9) was assayed in plasma using a sandwich ELISA (Quantikine kit for Human MMP-9 Immunoassay; R&D Systems). A standard curve was generated using a four parameter logistic curve-fit. The correlation coefficient for standards was greater than 0.99 and the lowest detectable limit 0.156 ng/ml in 40-fold diluted plasma. The intra-assay coefficient of variation was 1.9% and the interassay coefficient of variation was 7.8%.

Statistical Analysis

Primary analyses involved detecting a difference in markers of oxidative stress, inflammation, and plaque stability between the two study medications. All results, except the WBC count, were highly skewed; therefore, the concentrations of the analytes or the ratios of analyte to urine creatinine concentration were loge transformed. The change in analytes at the final visit was compared with the baseline visit using repeated-measures ANOVA. The antilogarithm of the mean log value was taken as the geometric mean. To assess the relationship between glycemic control and cytokine parameters, partial correlation coefficients between glycemic control (HbA1C) and WBC and log-transformed cytokines were calculated to account for repeated observations in the same patient. A two-sided significance value was set at <0.05. All statistical analyses were conducted using Statistica 7.0 (Statsoft, Tulsa, OK) and SPSS 13.0 for Windows (SPSS, Chicago, IL).

RESULTS

Twenty-one patients in the pioglitazone group and 19 in the glipizide group had paired samples for analyses. In some instances, due to technical reasons, e.g., the presence of hemolysis in plasma samples for MDA or an inadequate amount of protein in the urine for carbonyl estimation, samples could not be analyzed.

Effects on Oxidative Stress

Biomarkers of oxidative stress in plasma and urine are shown in Table 1. Neither plasma MDA, a marker of lipid peroxidation, nor protein or albumin carbonyls, markers of protein oxidation, were reduced with pioglitazone.

Effects on Inflammation Biomarkers

Biomarkers of inflammation in blood and urine are shown in Table 2 and Fig. 1. The WBC count was reduced from 8,530 to 7,405/μl, a reduction of 1,125/μl with pioglitazone (P =
Table 1. Biomarkers of oxidative stress before and after treatment with oral hypoglycemic agent

<table>
<thead>
<tr>
<th></th>
<th>Pioglitazone</th>
<th>Glipizide</th>
<th>Pioglitazone – Glipizide Geometric Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Mean geometric difference (95% CI)</td>
</tr>
<tr>
<td>Plasma oxidative stress markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total carbonyl (DU total carbonyl/DU total protein)</td>
<td>0.99 (1.43)</td>
<td>0.91 (1.48)</td>
<td>0.92 (0.75–1.12)</td>
</tr>
<tr>
<td>Albumin carbonyl (DU albumin carbonyl/DU albumin protein)</td>
<td>0.80 (1.38)</td>
<td>0.81 (1.38)</td>
<td>1.01 (0.92–1.11)</td>
</tr>
<tr>
<td>Urinary oxidative stress markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary MDA/creatinine, μM/g creatinine</td>
<td>1.41 (1.35)</td>
<td>1.43 (1.33)</td>
<td>1.02 (0.94–1.11)</td>
</tr>
<tr>
<td>Urine albumin carbonyl (DU albumin carbonyl/DU albumin protein)</td>
<td>3.96 (1.81)</td>
<td>3.32 (1.82)</td>
<td>0.84 (0.64–1.10)</td>
</tr>
<tr>
<td>Urine albumin (DU albumin albumin protein)</td>
<td>0.39 (2.86)</td>
<td>0.35 (4.87)</td>
<td>0.90 (0.44–1.86)</td>
</tr>
<tr>
<td>Total leukocyte count, ×1000/μl</td>
<td>0.49 (2.88)</td>
<td>0.48 (4.85)</td>
<td>0.99 (0.48–2.06)</td>
</tr>
</tbody>
</table>

Data shown are geometric least-squares mean (SD). CI, confidence interval; MDA, malondialdehyde; DU, densitometric units. Geometric difference is a fold-change. Thus, a mean geometric difference of 0.92 is a 8% reduction. Paired data are on 21 subjects on pioglitazone and 19 subjects on glipizide. MDA levels in plasma and urine were available for 20 subjects treated with pioglitazone and 19 treated with glipizide. Urine total and albumin carbonyls could be estimated in 17 patients who received pioglitazone and 16 patients with glipizide. For the columns indicating pioglitazone minus glipizide difference, >1 indicates a greater relative increase in the pioglitazone group, whereas <1 indicates a greater relative decrease in the pioglitazone group.

Effects on Plaque Stability Marker

Patients treated with pioglitazone experienced a 29% reduction in MMP-9 plasma concentration (P = 0.016) (Table 2). Although patients treated with glipizide had a 24% reduction in MMP-9 plasma concentration, this was not statistically significant (P = 0.064). The comparison of the difference between the differences between the two drugs was not significant. Effect of Glycemic Control on Biomarkers

There was no relationship between HbA1c with either drug and a reduction in WBC (partial correlation coefficient = 0.009, P = 0.009) but increased 1.47-fold with glipizide (P = 0.039). Comparison of the differences between the drugs demonstrated a 58% reduction with pioglitazone (P = 0.001).

There were no statistically significant changes observed with plasma TNF-α concentrations with either drug.

Table 2. Biomarkers of inflammation before and after treatment with oral hypoglycemic agent

<table>
<thead>
<tr>
<th></th>
<th>Pioglitazone</th>
<th>Glipizide</th>
<th>Pioglitazone – Glipizide Geometric Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Difference (95% CI)</td>
</tr>
<tr>
<td>Blood/plasma inflammation markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total leukocyte count, ×1000/μl</td>
<td>8.5 (2.52)</td>
<td>7.4 (2.43)</td>
<td>−1.12 (−1.77–−0.48)</td>
</tr>
<tr>
<td>Plasma CRP, mg/dl</td>
<td>0.94 (3.05)</td>
<td>0.55 (3.74)</td>
<td>0.59 (0.35–0.98)</td>
</tr>
<tr>
<td>Plasma IL-6, pg/ml</td>
<td>2.95 (3.10)</td>
<td>1.82 (2.71)</td>
<td>0.62 (0.43–0.88)</td>
</tr>
<tr>
<td>Plasma MMP-9, pg/ml</td>
<td>4.10 (2.25)</td>
<td>4.09 (1.97)</td>
<td>1.00 (0.83–1.20)</td>
</tr>
<tr>
<td>Atherosclerotic plaque stability marker</td>
<td>Matrix metalloproteinase-9, ng/ml</td>
<td>44.12 (1.99)</td>
<td>31.42 (1.66)</td>
</tr>
</tbody>
</table>

Data shown are geometric least-squares mean (SD) except for total leukocyte count, which is arithmetic least-squares mean. CRP, C-reactive protein. Geometric difference is a fold-change. Thus, a mean geometric difference of 0.52 is a 48% reduction. Paired data are on 21 subjects on pioglitazone and 19 subjects on glipizide. For the columns indicating pioglitazone minus glipizide difference, >1 indicates a greater relative increase in the pioglitazone group, whereas <1 indicates a greater relative decrease in the pioglitazone group.
ANCOVA model, a pioglitazone-attributable reduction in log IL-6 remained significant ($P = 0.008$).

**DISCUSSION**

The primary end point in the study was reduction in proteinuria, which has been reported previously and is not the subject of this report (3). Randomization was balanced with respect to key clinical features. In the pioglitazone group ($n = 22$), the average age was 68 yr, all were men, 86% were white, fasting glucose was 147 mg/dl, HbA1c was 7.7 ± 2.2%, 24-h average ambulatory blood pressure was 148/74.8 mmHg, and iothalamate glomerular filtration rate 36 ml/min. In the glipizide group ($n = 22$), the average age was 64 yr, all were men, 73% were white, fasting glucose was 155 mg/dl, HbA1c was 7.7 ± 2.5%, 24-h average ambulatory blood pressure was 152.8/76.3 mmHg, and iothalamate glomerular filtration rate 52 ml/min. All but five patients in the pioglitazone group and three in the glipizide group were taking an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker.

The major findings of this study are that pioglitazone caused a reduction in markers of inflammation, i.e., total WBC count, CRP, and IL-6, and a reduction in MMP-9 inhibitor that correlate with atherosclerotic plaque stabilization in patients with advanced diabetic nephropathy. Notably, two markers of inflammation, total WBC count and IL-6, were reduced even compared with an active oral hypoglycemic agent, glipizide, thus demonstrating the specific differential action of pioglitazone. The pioglitazone-attributable reduction in WBC count and IL-6 was not attributable to glycemic control alone. TNF-α and markers of oxidative stress were unchanged with either therapy. These findings in patients with advanced diabetic nephropathy, who have the highest cardiovascular risk profile, offer hope that such therapies can improve cardiovascular outcomes.

Our study confirms the data of Haffner et al. (13), who measured serum biomarkers CRP, IL-6, MMP-9, and WBC, in 357 patients with type 2 diabetes mellitus who completed a 26-wk randomized, double-blind, placebo-controlled study, to assess the safety and efficacy of rosiglitazone at two different doses, 2 mg twice daily and 4 mg twice daily (13). No improvement was seen with IL-6 at any of the doses. However, with 4 mg/day rosiglitazone, the CRP level decreased by

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**Fig. 1.** Changes in individual levels of biomarkers are shown. Note the log scale of all biomarkers except total leukocyte count. Box plot reflects the median (horizontal line), interquartile range (the box itself), and 10th and 90th percentiles (the whiskers). Pre reflects the baseline level of the analyte, and post the level at 16 wk. See also Table 2. WBC, white blood cell; CRP, C-reactive protein.
26.8%, WBC count by 10.3%, and MMP-9 by 12.4%. With an 8-mg dose, the CRP level decreased by 21.8%, WBC count by 12.1%, and MMP-9 by 23.4%. Although their study excluded patients who required insulin therapy and those with renal failure (18), the reduction in WBC and MMP-9 was comparable to the two studies. Reduction in both CRP and IL-6 was greater in our study.

In patients with stable coronary artery disease, but without diabetes mellitus, randomized to placebo (n = 44) or rosiglitazone (n = 40) over 12 wk, a 38% reduction in CRP was reported with rosiglitazone, from 0.56 to 0.35 mg/l, but not with placebo (P < 0.005 for change in rosiglitazone vs. placebo group) (31). A 41% CRP reduction that was nearly identical in magnitude to that reported in patients without diabetes mellitus or renal disease confirms the anti-inflammatory efficacy of pioglitazone in a group of patients with much greater inflammation. Notably, in neither of the two studies mentioned above were the glitazones compared with another hypoglycemic agent. Thus our report appears to be the first to compare the differential effects of pioglitazone with another hypoglycemic agent. Therefore, our study appears to be the first to compare the differential effects of pioglitazone with another hypoglycemic agent on inflammation markers.

PPAR-γ is expressed in human monocytes and macrophages, and increased expression of PPAR-γ is seen with activation of these cells (28). PPAR-γ agonists negatively regulate the expression of proinflammatory genes induced during macrophage differentiation and activation (29). Specifically, PPAR-γ activation reduces monocyte secretion of IL-1β, IL-6, and TNF-α. However, PPAR-γ activation does not reduce monocyte TNF-α secretion induced by lipopolysaccharide, suggesting that cytokine production triggered by inflammatory triggers overrides the regulatory control by PPAR-γ (16). These in vitro findings support our results that demonstrate a reduction in IL-6 with pioglitazone but not that of TNF-α. A recent study in obese subjects with diabetes mellitus also failed to show an improvement in TNF-α with rosiglitazone therapy, despite reduction in plasma CRP (24).

In human coronary artery endothelial cells stimulated with oxidized LDL, ANG II, or TNF-α, intracellular superoxide generation is increased but is reduced with pioglitazone in a dose-dependent manner (33). In prediabetic rats treated with pioglitazone, plasma MDA was reduced at 20 wk of treatment and correlated well with arterial wall hypertrophy and stiffness (34). Pioglitazone reduced urinary isoprostanes and lipid peroxides in rats with diet-induced obesity (8). In obese subjects without diabetes, troglitazone improved markers of oxidative stress and endothelial function (10). I observed no changes in plasma or urinary markers of lipid or protein oxidation. It is possible that the salutary effects of TZDs on oxidative stress are mediated largely through glycemic control or are seen at an earlier stage of the disease, which may account for the negative nature of our results.

This study extends our knowledge about glitazones in diabetic nephropathy in several ways. First, the comparison with an active oral hypoglycemic agent in the control group, with equal glycemic control, allowed us to observe the nonglycemic effects of glitazones. Second, the reduction in inflammation markers despite a lack of reduction in proteinuria suggests that it may be valuable to evaluate biomarkers in patients with diabetic nephropathy who have no reduction in proteinuria. Third, the reduction in WBC count empowers the clinician at the bedside to look at this readily available marker of inflammation as evidence of anti-inflammatory response.

Some limitations of our study require consideration. I did not have a group of patients that served as untreated controls. This does not detract from the overall validity of the results because our control group was treated with glipizide, another effective oral hypoglycemic agent. Although the magnitude of anti-inflammatory response with pioglitazone may be underestimated, comparison with an active control group provides more clinically relevant results. For example, paired comparison of MMP-9 before and after pioglitazone was statistically significant, but statistical significance of the difference between drugs was not achieved because glipizide also reduced MMP-9 levels slightly. Another limitation of the study is the small sample size of 44 patients. The long-term significance of the findings of reduced inflammation reported in this study will require studies with “hard” end points.

The findings of this study extend the known anti-inflammatory benefit of TZDs to patients with overt diabetic nephropathy. This benefit was seen despite the use of angiotensin receptor blockers and angiotensin-converting enzyme inhibitors in the majority of the patients. Because there was no reduction in fasting glucose or glycosylated hemoglobin with pioglitazone or an improvement in blood pressure or proteinuria, these improvements in inflammation were independent of glycemia, hemodynamic factors, or protein excretion. Patients with CKD due to diabetes mellitus have an enormous cardiovascular risk that has not been favorably impacted with the use of angiotensin receptor blockers (4, 20). TZDs add an additional drug class to the therapeutic arsenal of angiotensin receptor blockers, statins, and aspirin to favorably influence the cardiovascular risk profile in these patients (27).

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GRANTS

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REFERENCES


