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*J Immunol* 2009; 183:2729-2740; Prepublished online 20 July 2009;
doi: 10.4049/jimmunol.0804341
http://www.jimmunol.org/content/183/4/2729

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The Peroxisome Proliferator-Activated Receptor γ Agonist Pioglitazone Improves Cardiometabolic Risk and Renal Inflammation in Murine Lupus

Wenpu Zhao,* Seth G. Thacker,* Jeffrey B. Hodgin,‡ Hongyu Zhang,‡ Jeffrey H. Wang,‡ James L. Park,‡ Ann Randolph,‡ Emily C. Somers,* Subramaniam Pennathur,‡ Matthias Kretzler,† Frank C. Brosius III,† and Mariana J. Kaplan2*

Individuals with systemic lupus erythematosus (SLE) have a striking increase in the risk of premature atherosclerosis, a complication preceded by significant subclinical vascular damage. A proposed mechanism leading to accelerated vascular disease in SLE is an imbalance between vascular damage and repair, as patients with this disease display significant abnormalities in phenotype and function of endothelial progenitor cells. In addition, individuals with SLE have a higher incidence of insulin resistance which may further contribute to the increased cardiovascular risk. This study examined the role of the peroxisome proliferator activated receptor γ agonist pioglitazone in improving endothelial function, endothelial progenitor cell numbers and functional capacity, metabolic parameters, and disease activity in the lupus-prone murine model New Zealand Black/New Zealand White (NZB × NZW)F1. Ten-week-old prenephritic female NZB/NZW F1 mice were exposed to 10 or 25 mg/kg/day of oral pioglitazone or vehicle for 15 or 24 wk. Mice exposed to pioglitazone exhibited pronounced enhancement in endothelial-dependent vasorelaxation of thoracic aortas and in endothelial progenitor cell function, as assessed by the capacity of bone marrow-derived endothelial progenitor cells to differentiate into mature endothelial cells. Pioglitazone-treated mice showed improvement in insulin resistance, adipokine, and lipid profile. Kidneys from pioglitazone-treated mice showed significant decreases in immune complex deposition, renal inflammation, T cell glomerular infiltration, and intrarenal synthesis of TNF-α, IL-1β, and VCAM-1. These results indicate that peroxisome proliferator-activated receptor γ agonists could serve as important tools in the prevention of premature cardiovascular disease and organ damage in SLE.


Importantly, SLE is associated with a striking increase in the risk of premature cardiovascular (CV) complications due to accelerated atherosclerosis (2), which significantly contributes to morbidity and mortality in this patient population (3). Although traditional risk factors may play a role in this increased propensity, they do not seem to fully account for this complication (4). As such, immune dysregulation characteristic of lupus may play a prominent role in the development of premature atherosclerosis. However, the exact etiology of increased CV risk in SLE remains unclear. A significant proportion of individuals with SLE have evidence of subclinical vascular disease which may precede atherosclerosis development. These subclinical abnormalities include endothelial dysfunction (with preservation of vascular smooth muscle function) (5) and coronary perfusion abnormalities (6). As a potential mechanism explaining premature endothelial dysfunction and accelerated atherosclerosis development in SLE, our group and others have recently reported that patients with lupus develop a striking imbalance between vascular damage (apoptosis) and repair (vasculogenesis) (5, 7, 8). Indeed, SLE patients have decreased numbers and abnormal function of circulating endothelial progenitor cells (EPCs) (7). Importantly, similar abnormalities in EPCs have clearly been identified by numerous groups as risk factors for premature CV disease in various diseases including diabetes mellitus, hypertension, and rheumatoid arthritis (9–11). SLE patients also have a higher incidence of insulin resistance and the metabolic syndrome which may further contribute to vascular dysfunction and may be triggered by immune dysregulation (12). Interestingly, lupus-prone mice also show evidence of endothelial dysfunction (13, 14), abnormal EPC phenotype and function (13), and insulin resistance (15).
Although advances in immunosuppressive treatments have decreased organ damage in lupus, no drug to this date has proven to prevent or abrogate atherosclerosis development in patients with SLE. The thiazolidinediones (TZDs) are a class of drugs used in the treatment of type 2 diabetes mellitus. In addition to their role in improving insulin resistance and other metabolic parameters, these drugs are potent peroxisome proliferator-activated receptor γ (PPAR-γ) agonists and induce significant pleiotropic antiatherogenic and anti-inflammatory effects (16–19). PPARs play a crucial role in the regulation of energy metabolism and cell differentiation, control of inflammation, and vascular biology (20). Importantly, these drugs can improve EPC phenotype and function (21) and overall vascular function in various conditions, independent of glucose control (22, 23), and some reports suggest a beneficial effect in the prevention of renal injury in experimental conditions (24, 25). We have hypothesized that TZDs could represent a useful tool in the treatment of SLE by improving endothelial function, EPC phenotype and function, overall CV risk, and, potentially, disease activity. We now report the results of the administration of the PPAR-γ agonist pioglitazone to lupus-prone mice on CV, metabolic, and renal parameters.

Materials and Methods

Animals and drug treatment

Protocol was reviewed and approved by the University of Michigan’s Committee on Use and Care of Animals. Female New Zealand Black/ New Zealand White (NZB × NZW)F1 (referred in the text as NZB/W) mice were purchased from The Jackson Laboratory and housed in individual cages in a specific-pathogen-free barrier facility at the University of Michigan.

Pioglitazone (Takeda Pharmaceuticals) was suspended in a vehicle of 1:1 mixture of Ora-plus (Paddock Laboratories) and raspberry-flavored sugar-free gelatin (Jell-O brand, purchased commercially) as previously described by us (26). NZB/W mice (10 wk old, prenephritic) were fed daily either 10 mg/kg pioglitazone (n = 20), 25 mg/kg pioglitazone (n = 20), or vehicle only (n = 20). The mice routinely ate the entire amount provided. The doses chosen were based on those safely tolerated in other murine models in the literature (27, 28) and were also based on the Pioglitazone Summary Basis of Approval from the Food and Drug Administration (http://www.fda.gov/cder/foi/nda/99/021073A_Acts_ho.html). In addition, these doses approximate the therapeutic serum levels that are effective in humans (29).

One-half of the mice were euthanized at 25 wk (a time point where florid renal immune complex deposition is observed but renal function is overall preserved) and the other half at 34 wk of age (a time point where nephrotic range proteinuria and renal dysfunction are apparent). An additional group of five NZB/W mice was left untreated and euthanized at ~36 wk of age to assess the effect of in vitro addition of pioglitazone to bone marrow EPCs in their capacity to differentiate into mature endothelial cells (see below).

Assessment of vascular function

Studies were performed as previously reported by us (30). After euthanasia with pentobarbital, thoracic aortas were excised, cleaned, and cut into 2-mm length rings. Endothelium was left intact and aortic rings were mounted in a myograph system (Danish Myo Technology). Vessels were bathed with warmed and aerated (95% O2/5% CO2) with a physiological salt solution. Aortic rings were set at 700 mg of passive tension, equilibrated for 1 h, and washed every 20 min. Before performing concentration response curves, the vessels were contracted with physiological salt solution containing 100 mM potassium chloride. Vessels were washed and contracted again with physiological salt solution containing 100 mM potassium chloride. Cumulative concentrations of phenylephrine (PE) (10−9 to 10−6 mol/L) were added to the bath to establish a concentration-response curve. Acetylcholine (Ach) (10−9 to 10−6 mol/L) was added cumulatively to the bath to examine endothelium-dependent relaxation. PE and Ach were washed out of the vascular preparation at the end of the concentration response, and the aortic rings were again recontracted with PE and allowed to reach a stable plateau in the contraction. Endothelium-independent relaxation (vascular smooth muscle response) was assessed by the cumulative addition of sodium nitroprusside (SNP; 10−9 to 10−8 mol/L) to the bath. Ach and SNP relaxation were expressed as a percentage of the PE contraction as previously described (30).

EPC quantification

After euthanasia, femurs and tibias were washed and their ends were excised and flushed with ice-cold MACS buffer (Miltenyi Biotec). Cells were filtered through a 40-μm cell strainer (BD Biosciences) to obtain a single-cell suspension. Spleens were also forced through a 40-μm cell strainer, followed by passage over the same cell strainer to obtain a single-cell suspension. Bone marrow cells (30–60 × 10^6) were depleted of lineage-positive cells using a mouse lineage depletion kit (Miltenyi Biotec) according to the manufacturer’s recommendation. Spleen cells were depleted of B and T cells using anti-CD3 (eBioscience) and anti-CD19 (BioLegend) mAbs, respectively, following a similar protocol used for the bone marrow depletion. Blood was obtained via cardiac puncture after mice where fully anesthetized with pentobarbital. Blood was collected in a heparinized syringe and PBMCs were obtained through Histopaque 1083 gradient (Sigma-Aldrich). Bone marrow and spleen lineage-depleted cells and PBMCs were stained with fluorochrome-conjugated Abs to murine CD34 and VEGF-R2 (eBioscience) to determine EPC numbers, as previously described (31, 32). EPC apoptosis was assessed by annexin V staining (BD Bioscience) per the manufacturer’s recommendations. FACs was performed using a FACSCalibur (BD Biosciences) followed by analysis with FlowJo (Tree Star).

Assessment of EPC differentiation into mature endothelial cells

Bone marrow-derived EPCs were obtained as above and plated onto fibronectin-coated plates (BD Biosciences) at a density of 1 × 10^6 million cells/cm^2 in EGM-2 Bulletkit medium (Lonza) supplemented with 5% heat-inactivated FBS. Pioglitazone potassium salt (Cayman Chemical) was dissolved in distilled water and added to culture in a volume of 5 μL to obtain final concentrations of 2 or 10 μmol/L. Medium was changed after 72 h in culture, then every 48 h. At different time points, cells were incubated with Bandeiraea (Griffonia) Simplicifolia Lectin 1 (Isolectin B4) (BS-1; Vector Laboratories) and 1’-diododecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI)-acetylated low density lipoprotein (ac-LDL; Biomedical Technologies) for 4 h to assess the capacity of EPCs to differentiate into mature endothelial cells. Cells were then analyzed by fluorescent microscopy using a Leica DMIRB fluorescent inverted microscope. Images were acquired with an objective magnification of ×20 (×200 total magnification) using an Olympus DP50BW camera and the acquisition software Olympus-BSW. Final processing was done with Adobe Photoshop CS2. Five representative images from each well were counted to estimate total number of mature endothelial cells as assessed by cells that costain with BS-1 and ac-LDL.

Quantification of metabolic parameters

Body weight was recorded at 10, 25, and 34 wk of age using a scale. An ELISA was performed for quantification of serum leptin (Linco Research), serum adiponectin (R&D Systems), and serum insulin (Linco Research). Serum glucose levels were determined by glucose oxidase (Pointe Scientific). Insulin resistance was calculated with the Homeostasis Model Assessment using the HOMA-2 calculation. The HOMA calculator was downloaded from the web site of the Oxford Center for Diabetes, Endocrinology and Metabolism (www.dtu.ox.ac.uk/homa). Total plasma cholesterol, high density lipoprotein (HDL), and triglyceride (Roche Diagnostics) concentrations were measured using enzymatic kits on an automated analyzer (Roche Diagnostics) according to the manufacturer’s instructions. LDL was calculated as: total cholesterol – HDL – (triglycerides/5). All of these measurements were performed at 25 and 34 wk of age.

Determination of serum autoantibodies

Mice were bled via the tail vein approach; serum was isolated and frozen at −80°C. Anti-dsDNA Abs were quantified at 25, 30, and 34 wk of age by ELISA (Alpha Diagnostic).

Determination of renal function

Proteinuria was determined by collecting spot urine at 21, 25, 30, and 34 wk of age using Bio-Rad assay reagent per the manufacturer’s instructions. Urine albumin and creatinine were quantified with a mouse Albwell ELISA kit and a creatinine companion kit (Exocell). Urine protein/creatinine and albumin/creatinine ratios were calculated.

Serum blood urea nitrogen (BUN) was quantified using VETTEST 8008 IDEXX Laboratories. Serum creatinine was quantified using an Agilent Technologies 6410 Triple Quadrupole Mass Spectrometer System equipped with an Agilent 1200 series HPLC system and an electro spray

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source. A known amount of [3H]creatinine was spiked into each plasma sample. Full-scan mass spectrum revealed a molecular ion of m/z 114 and m/z 117 for authentic creatinine and [3H]creatinine, respectively. The transitions of m/z 114 to m/z 44 and m/z 117 to m/z 47 were monitored in multiple reaction monitoring mode for authentic and [3H]creatinine for the above transitions as described previously (33, 34). Serum electrolytes (sodium, chloride, and potassium) from 34-wk-old mice were quantified on the VetLyte Electrolyte Analyzer, model VETTEST 8008 (IDEXX Laboratories).

**Kidney harvesting and renal histopathology**

Mice were anesthetized with pentobarbital and kidneys were perfused with PBS (Invitrogen) at a rate of ~12 ml/min for 5 min and removed. A section of the cortex was snap frozen in liquid nitrogen and stored at ~80°C for immunofluorescence staining, while the other cortical section was fixed with 4% buffered formalin, embedded in paraffin, and sectioned at 4-μm thickness. Masson trichrome-stained sections were examined and graded by one of the authors (J.B.H.) in a blinded manner following procedures previously described by us (26). In brief, a semi-quantitative scoring system (0, no involvement; 0.5, minimal involvement of <10%; 1, mild involvement (10–30% section); 2, moderate involvement (31–60% of section); and 3, severe (>60% of section)) was used to assess 13 different parameters of activity and chronicity (mesangial hypercellularity, mesangial deposits, mesangial sclerosis, endocapillary cellular infiltrate, subepithelial and subendothelial deposits, capillary thrombi, capillary sclerosis, cellular or organized crescents, synechiae, tubular atrophy, and interstitial fibrosis). An activity and chronicity index was generated by compiling scores from groups of related parameters (for activity: mesangial hypercellularity, mesangial deposits, and endocapillary cellular infiltrate; for chronicity: interstitial fibrosis, tubular atrophy, synechiae, organized crescents, and capillary sclerosis).

Immunofluorescence staining was performed as described previously (35). IgG deposition was evaluated on frozen kidney sections stained with Alexa Fluor 594 F(ab')2 of goat anti-mouse IgG (H + L) at a concentration of 20 μg/ml (Invitrogen). C3 deposition was examined by staining kidney sections with FITC-goat anti-mouse C3 10 μg/ml (Immunology Consultant Lab). Glomerular staining was graded according to intensity on a 0–4+ scale (0, no staining; 4+, maximum intensity staining) as described by us (26).

Quantification of T cell, B cell, and macrophage infiltration in kidney tissue was performed by immunohistochemistry, as previously described by our group and others (26, 35, 36). The following Abs were used to assess infiltration in kidney sections: rabbit anti-mouse CD3 (DakoCytomation), CD4 (Abcam), and CD8 (Abcam); rabbit anti-mouse CD20 (Millipore) for B cells, and rat anti-mouse F4/80 (Abcam) for macrophages. Cell infiltration was analyzed and graded by one of the authors (J.B.H.) in a blinded fashion. Positive cells were counted in 30 random glomeruli/mouse and a score derived.

**Quantification of renal cytokines and adhesion molecules by real-time PCR**

Total RNA was isolated from kidney sections with Tri-pure (Roche Diagnostics). Reverse transcription and real-time TaqMan PCR analyses for IL-1β, TNF-α, and VCAM-1 were performed as described previously (37, 38). In brief, total RNA was reverse-transcribed and PCR was conducted using TaqMan Universal PCR Master Mix (Applied Biosystems), Gene Expression Assay Mix, and cDNA. Real-time PCR analyses were performed with the Applied Biosystems PRISM 7900HT Detection System according to the manufacturer’s instructions. The expression level of the 18S subunit of ribosomal RNA was used as a housekeeping gene. For IL-1β, TNF-α, VCAM-1, and 18S, assays-on-demand gene expression products were used as described in the manufacturer’s protocol (Applied Biosystems). All PCR were performed in duplicate. Controls consisting of distilled water were negative in all runs.

**Statistical analysis**

The underlying distributions of the outcome variables were first examined and nonparametric analyses were indicated for the majority of the variables. To compare variables associated with PPAR doses of either 10 or 25 mg/kg vs vehicle, the Wilcoxon rank sum test was used. For comparison of variables across all three PPAR dose categories, the Kruskal-Wallis equality-of-populations rank test was used. When appropriate, parametric analyses were also conducted (two-sample t tests or one-way ANOVA). Separate analyses were conducted for mice sacrificed at 25 or 34 wk. Analyses were performed with StataCorp 10.1. A value of p < 0.05 was considered statistically significant. For measurements of endothelial function, the median effective concentration (EC50) for agent-induced relaxation in aortic rings was calculated by nonlinear regression analysis (GraphPad Prism). EC50 between two groups was analyzed by Student’s t test. Unless otherwise specified, results represent mean ± SEM.

**Results**

**Pioglitazone improves endothelial function in lupus-prone mice**

At 34 wk of age, endothelial-dependent vasorelaxation of precontracted thoracic aortas was markedly enhanced by pioglitazone, particularly in the animals that received doses of 25 mg/kg/day (p = 0.02 when comparing vehicle-treated mice with 25-mg/kg/day pioglitazone-treated mice; p = 0.07 when comparing vehicle-treated mice with mice treated with 10 mg/kg pioglitazone; Fig. 1B). In contrast, endothelium-independent vasorelaxation at 34 wk of age, assessed by the response of precontracted thoracic aortas to SNP, was not affected by pioglitazone (Fig. 1D). Both endothelium-dependent and endothelium-independent responses showed no difference in responses to pioglitazone in younger (25-wk-old) mice (Fig. 1, A and C). Overall, these results suggest that pioglitazone improves endothelial function in lupus-prone mice with established disease.

**Pioglitazone improves EPC function in lupus-prone mice**

We have previously reported that humans with lupus as well as NZB/W F1 mice have decreased numbers of circulating EPCs, increased EPC apoptosis, and decreased capacity of EPCs to differentiate into a mature endothelium under proangiogenic conditions (7, 13). Since TZDs have been reported to improve EPC numbers and function in other conditions (21, 39), we assessed the role of pioglitazone in total numbers of EPCs and numbers of apoptotic EPCs in different compartments (blood, bone marrow, and spleen), as well as the effect of this drug on in vitro differentiation of bone marrow EPCs into mature endothelial cells.

At 25 and 34 wk, pioglitazone administration did not result in significant differences in EPC numbers or EPC apoptosis in bone marrow, blood, or spleen (data not shown). However, when graded doses of pioglitazone were added in vitro to bone marrow EPCs from NZB/W mice, a very significant improvement in the capacity of these cells to differentiate into mature endothelial cells was observed. As shown in Fig. 2, while EPCs from NZB/W mice had poor capacity to form an endothelial cell monolayer under in vitro proangiogenic stimulation, similar to what we have reported in human SLE (7), cells exposed to 10 μM pioglitazone showed increased numbers of cells that expressed mature endothelial cell markers at 7 days in culture. These results suggest that pioglitazone improves EPC function in lupus-prone mice.

**Metabolic parameters in lupus-prone mice improve with administration of pioglitazone**

Previous reports suggest that NZB/W mice are overweight, with increased body fat, insulin resistance, and overall deleterious metabolic parameters (15). Pioglitazone administration resulted in decreases in insulin levels (Fig. 3B), as glucose levels remained stable after pioglitazone administration (Fig. 3B). These results suggest that pioglitazone improves insulin handling in lupus-prone mice with established disease.

Analysis of plasma adipokines (leptin and adiponectin), molecules important in the development of metabolic abnormalities triggered by inflammation, revealed that pioglitazone treatment
had positive effects in NZB/W mice. Plasma leptin has been reported to be increased in murine SLE, possibly secondary to “leptin resistance” due to increased body fat (15). Increased leptin levels in these mice have been associated with macrophage infiltration of renal and adipose tissue (15). Pioglitazone treatment at 25 mg/kg/day resulted in significant decreases of serum leptin levels at 25 and 34 wk of age, when compared with vehicle-treated mice (Fig. 3). Lower doses of pioglitazone (10 mg/kg/day) resulted in significant decreases of serum leptin at 34 wk of age, with a trend to lower levels at earlier time points (Fig. 3). Adiponectin is an adipose-secreted hormone with anti-inflammatory properties. In contrast to leptin, low levels of adiponectin have been associated with obesity and insulin resistance (40) and administration of recombinant adiponectin to rodents results in increased glucose uptake and fat oxidation in muscle, reduced hepatic glucose production, and improved whole-body insulin sensitivity (41, 42). Previous reports indicate that TZDs, including pioglitazone, stimulate adiponectin secretion from mice and human adipocytes in other diseases (43, 44). In our study, adiponectin levels significantly decreased as mice aged and developed active lupus. Importantly, adiponectin levels increased in the pioglitazone-treated mice (Fig. 3). The kinetics of adiponectin elevations differed between the groups receiving the two doses of pioglitazone used. Adiponectin levels significantly increased in the 10 mg/kg/day group by 34 wk but not at the earlier time point (Fig. 3). Adiponectin is an adipose-secreted hormone with anti-inflammatory properties. In contrast to leptin, low levels of adiponectin have been associated with obesity and insulin resistance (40) and administration of recombinant adiponectin to rodents results in increased glucose uptake and fat oxidation in muscle, reduced hepatic glucose production, and improved whole-body insulin sensitivity (41, 42). Previous reports indicate that TZDs, including pioglitazone, stimulate adiponectin secretion from mice and human adipocytes in other diseases (43, 44). In our study, adiponectin levels significantly decreased as mice aged and developed active lupus. Importantly, adiponectin levels increased in the pioglitazone-treated mice (Fig. 3). The kinetics of adiponectin elevations differed between the groups receiving the two doses of pioglitazone used. Adiponectin levels significantly increased in the 10 mg/kg/day group by 34 wk but not at the earlier time point (Fig. 3).
in other conditions (47). At 25 wk of age, there were no statistically significant differences in lipid profiles between the three treatment groups (total cholesterol: vehicle, 179 ± 8 mg/dl; 10 mg/kg/day pioglitazone, 184 ± 11.4 mg/dl; and 25 mg/kg/day pioglitazone, 179 ± 8.6 mg/dl; triglycerides: vehicle, 86 ± 12.6 mg/dl; 10 mg/kg/day pioglitazone, 87 ± 11.6 mg/dl; and 25 mg/kg/day pioglitazone, 75 ± 14.3 mg/dl; HDL cholesterol: vehicle, 104 ± 7 mg/dl; 10 mg/kg/day pioglitazone, 105 ± 7 mg/dl; and 25 mg/kg/day pioglitazone, 102.5 ± 5.4 mg/dl; LDL cholesterol: vehicle, 58 ± 5.3 mg/dl; 10 mg/kg/day pioglitazone, 62.1 ± 7.5 mg/dl; and 25 mg/kg/day pioglitazone, 61.2 ± 3.9 mg/dl; results represent mean ± SEM of nine independent experiments). However, at 34 wk of age, pioglitazone treatment (25 mg/kg/day) resulted in statistically significant decreases in total cholesterol (Fig. 4A) with a trend toward a decrease in the 10-mg/kg/day group. Furthermore, there were decreases in LDL cholesterol (observed with both doses of pioglitazone) and increases in HDL cholesterol (observed with 10 mg/kg/day of pioglitazone) at 34 wk of age (Fig. 4, C and D).

There were not statistically significant differences in triglyceride levels at 34 wk of age (Fig. 4B). Overall, at this time point, there was a 40% increase in HDL with 10-mg/kg/day pioglitazone treatment (25 mg/kg/day pioglitazone, 184 ± 11.4 mg/dl; and 25 mg/kg/day pioglitazone, 179 ± 8.6 mg/dl; triglycerides: vehicle, 86 ± 12.6 mg/dl; 10 mg/kg/day pioglitazone, 87 ± 11.6 mg/dl; and 25 mg/kg/day pioglitazone, 75 ± 14.3 mg/dl; HDL cholesterol: vehicle, 104 ± 7 mg/dl; 10 mg/kg/day pioglitazone, 105 ± 7 mg/dl; and 25 mg/kg/day pioglitazone, 102.5 ± 5.4 mg/dl; LDL cholesterol: vehicle, 58 ± 5.3 mg/dl; 10 mg/kg/day pioglitazone, 62.1 ± 7.5 mg/dl; and 25 mg/kg/day pioglitazone, 61.2 ± 3.9 mg/dl; results represent mean ± SEM of nine independent experiments). However, at 34 wk of age, pioglitazone treatment (25 mg/kg/day) resulted in statistically significant decreases in total cholesterol (Fig. 4A) with a trend toward a decrease in the 10-mg/kg/day group. Furthermore, there were decreases in LDL cholesterol (observed with both doses of pioglitazone) and increases in HDL cholesterol (observed with 10 mg/kg/day of pioglitazone) at 34 wk of age (Fig. 4, C and D). There were not statistically significant differences in triglyceride levels at 34 wk of age (Fig. 4B). Overall, at this time point, there was a 40% increase in HDL with 10-mg/kg/day pioglitazone treatment (25 mg/kg/day pioglitazone, 184 ± 11.4 mg/dl; and 25 mg/kg/day pioglitazone, 179 ± 8.6 mg/dl; triglycerides: vehicle, 86 ± 12.6 mg/dl; 10 mg/kg/day pioglitazone, 87 ± 11.6 mg/dl; and 25 mg/kg/day pioglitazone, 75 ± 14.3 mg/dl; HDL cholesterol: vehicle, 104 ± 7 mg/dl; 10 mg/kg/day pioglitazone, 105 ± 7 mg/dl; and 25 mg/kg/day pioglitazone, 102.5 ± 5.4 mg/dl; LDL cholesterol: vehicle, 58 ± 5.3 mg/dl; 10 mg/kg/day pioglitazone, 62.1 ± 7.5 mg/dl; and 25 mg/kg/day pioglitazone, 61.2 ± 3.9 mg/dl; results represent mean ± SEM of nine independent experiments). However, at 34 wk of age, pioglitazone treatment (25 mg/kg/day) resulted in statistically significant decreases in total cholesterol (Fig. 4A) with a trend toward a decrease in the 10-mg/kg/day group. Furthermore, there were decreases in LDL cholesterol (observed with both doses of pioglitazone) and increases in HDL cholesterol (observed with 10 mg/kg/day of pioglitazone) at 34 wk of age (Fig. 4, C and D).

Effect of pioglitazone on lupus disease activity
There were no significant differences between treatment groups in serum levels of anti-dsDNA at different time points (Fig. 5A). Similarly, there were no statistically significant differences in renal function between treatment groups as assessed by protein:creatinine and albumin:creatinine ratios at various time points and serum BUN and creatinine at 30 and 34 wk of age (Fig. 5 and data not shown). Similarly, no hyperkalemia was observed in the mice by 34 wk of age. In general, serum electrolyte levels (sodium, potassium, and chloride) did not significantly differ between groups (potassium: vehicle, 2.25 ± 0.34 milliequivalents (mEq)/L; 10 mg/kg/day pioglitazone, 3.6 ± 0.4 mEq/L; 25 mg/kg/day pioglitazone, 2.58 ± 0.4 mEq/L; sodium: vehicle, 154.2 ± 12 mEq/L; 10 mg/kg/day pioglitazone, 146.3 ± 1.7 mEq/L; and 25 mg/kg/day pioglitazone, 151.5 ± 5.9 mEq/L; chloride: vehicle, 99.7 ± 7 mEq/L; 10 mg/kg/day pioglitazone, 101.7 ± 3.59 mEq/L; and 25 mg/kg/day pioglitazone, 104.5 ± 4.6 mEq/L).

On histological analysis, however, kidneys from mice treated with the two different doses of pioglitazone showed a significant decrease in immune complex deposition at 25 wk of age (Fig. 6). Furthermore, in the vehicle-treated group, mesangial expansion and prominent endocapillary hypercellularity was evident in nearly all glomeruli. In contrast, glomeruli from mice treated with the low dose of pioglitazone (10 mg/kg/day) demonstrated an expanded mesangium with only rare evidence of an endocapillary component (Fig. 6). Indeed, capillary inflammation in 10-mg/kg/day pioglitazone-treated mice was significantly decreased (score of
7.8 ± 5 in vehicle-treated mice vs 0.7 ± 0.8 in pioglitazone-treated mice, 10 mg/kg/day; \( p < 0.05 \)). Although there were no statistically significant differences in activity and chronicity indices between vehicle-treated and pioglitazone-treated mice at this time point, there was a trend for mice treated with 10 mg/kg/day of pioglitazone to have a lower activity index and decreased inflammation (Fig. 6). By 34 wk of age, there were no differences between groups in immune complex deposition or renal activity/chronicity (Fig. 6 and data not shown). When assessing the cell subsets present in renal inflammatory infiltrates, both groups of pioglitazone-treated mice displayed significant reductions in T cell infiltration at both 25 and 34 wk of age (Fig. 7, \( A \) and \( B \)), while there were no significant differences in macrophage or B cell infiltration scores (data not shown).

Since PPAR-\( \gamma \) agonists down-regulate proinflammatory cytokines and adhesion molecules (48), which may play a pathogenic role in lupus nephritis, we assessed renal mRNA expression of TNF-\( \alpha \), IL-1\( \beta \), and VCAM-1 in kidneys from the three treatment groups. Kidney tissue from pioglitazone-treated mice displayed significant down-regulation of IL-1\( \beta \) and TNF-\( \alpha \). At 25 wk of age,
IL-1β mRNA was decreased in kidneys from both 10- and 25-mg/kg/day pioglitazone-treated mice. This effect was sustained by 34 wk of age in the group treated with 25 mg/kg/day of pioglitazone (Fig. 7C). TNF-α mRNA expression in renal tissue was overall decreased in the pioglitazone-treated groups at both time points and this was significantly marked at 34 wk of age in the mice treated with the 25-mg/kg/day dose (Fig. 7D). With regard to VCAM-1, pioglitazone-treated groups showed overall a decrease in its renal expression, which was close to statistically significant ($p < 0.07$; Fig. 7E). Overall, these results demonstrate that pioglitazone induces down-regulation of proinflammatory cytokines and adhesion molecules in kidneys from lupus-prone mice.

**Discussion**

Inflammatory processes play a fundamental role in the initiation, propagation, and complications of atherosclerosis (49). This is well illustrated by the observations that autoimmune and chronic inflammatory disorders are associated with the development of accelerated CV disease, as exemplified by the striking increase in atherosclerotic risk in individuals with SLE (50). Although the use of various immunosuppressive treatments has resulted in better control of organ damage in SLE, premature CV complications develop in this patient population at an accelerated rate and represent one of the most significant causes of morbidity and mortality. Therefore, finding strategies that prevent or improve endothelial dysfunction (a strong predictor of atherosclerosis development (51, 52)) and decrease metabolic abnormalities associated with premature vascular damage are considered an important priority in the health care of this patient population.

PPAR-γ agonists are members of the nuclear receptor superfamily of ligand-dependent transcription factors which are expressed in a variety of cells including adipocytes, endothelial cells, macrophages, vascular smooth muscle cells, lymphocytes, and dendritic cells (47). PPAR-γ can modulate crucial cellular events such as growth factor release, cytokine production, cell proliferation and migration, extracellular matrix remodeling, and cell cycle progression (53). PPAR-γ agonists also have potent antioxidant effects (54). Several lines of evidence suggest that PPAR-γ exerts anti-inflammatory effects by negatively regulating the expression of proinflammatory genes induced during macrophage differentiation and activation (16). Once tissue injury and inflammatory responses ensue, there is PPAR-γ...
up-regulation which inhibits the activation of the transcription factors NF-κB, AP-1, NFAT, and STAT (55). This attenuates synthesis of important proinflammatory cytokines (IL-1β, IL-6, TNF-α, and IFN-γ), adhesion molecules (ICAM-1 and VCAM-1), and other inflammatory and prothrombotic mediators (inducible NO synthase, cyclooxygenase 2, CD40L, plasminogen activator inhibitor 1, and matrix metalloproteinases 9 and 1) (15, 48, 56–59), all crucial in target organ damage including the endothelium and the kidney. Indeed, in our study, pioglitazone-treated mice displayed down-regulation of renal mRNA expression of IL-1β, TNF-α, and VCAM-1.

Given the role of PPAR-γ pathways on immune modulation, adipogenesis, lipid metabolism, and glucose homeostasis, they represent an attractive target for CV risk reduction. Synthetic PPAR-γ agonists have been found not only to increase insulin sensitivity, modify lipid profiles, and decrease blood pressure, but also to reduce biomarkers of inflammation that are crucial in the development of atherosclerosis (60). These clinical effects, in addition to extensive in vitro and in vivo studies assessing anti-inflammatory and antiatherosclerotic effects of these agonists, indicate that these drugs may be powerful agents in CV complication prevention, beyond pure glucose control.

In this study, treatment with the PPAR-γ agonist pioglitazone resulted in pronounced enhancement in endothelium-dependent vasorelaxation, functional capacity of EPCs, and various metabolic parameters associated with increased CV risk, including body weight, insulin resistance, and lipid and adipokine profiles that characterize the metabolic syndrome. These results in a murine model of lupus point at a favorable effect of TZDs on CV risk that may translate to beneficial effects in the human disease.

In addition to their anti-inflammatory properties, TZDs can restore vasodilatory responses, improve endothelial function, and suppress atherosclerosis progression in various animal models of vascular injury (61, 62). We have now found pronounced enhancement in endothelial-dependent vasorelaxation in lupus-prone mice. The observation that this improvement was primarily seen at later time points (when mice have developed clinical manifestations of lupus) and not at earlier time points (when mice have not developed clinical disease) is likely due to the fact that significant endothelial dysfunction only becomes apparent in murine lupus once overt clinical disease has developed (15). The exact mechanisms for the improvement of endothelial function by pioglitazone in lupus are not known but are likely to be multifactorial, given the pleiotropic effect of these drugs on inflammatory parameters, vasculogenesis and proatherogenic metabolic abnormalities mentioned above. Indeed, TZDs can inhibit carotid plaque (63) and ameliorate endothelial dysfunction (64) in patients with diabetes independent of glucose control (65). TZDs also ameliorate markers of endothelial cell activation and improve endothelial function in nondiabetics with CV disease or risk factors for atherosclerosis (22, 66–69).
Numerous reports have indicated that PPAR signaling pathways play critical roles in the regulation of a variety of biological processes within the CV system and PPAR-γ agonists inhibit the expression of genes that contribute to atherogenesis (53). PPAR-γ isoforms have been characterized in multiple vascular cell types and prevent in vitro vascular smooth muscle cell growth (70–72). Furthermore, various cell types potentially involved in the formation of the atherosclerotic plaque (including T cells, monocytes, macrophages, and dendritic cells) have been reported to undergo phenotypic and functional changes in response to TZDs (17–19, 72). In addition, as now demonstrated in murine lupus, TZDs can improve atherosclerosis risk by improving lipid profiles, promoting improvements in adipokine levels, and overall decreasing levels of biomarkers that are crucial in plaque development (73). Indeed, pioglitazone has beneficial effects on low HDL cholesterol levels that characterize atherogenic dyslipidemia (74), which was corroborated in this study by observing significant decreases in total cholesterol and a substantial increase in HDL cholesterol in pioglitazone-treated NZB/W mice with established disease.

TZDs have also been reported to improve phenotype and function of EPCs (75), with enhancement of vascular repair after endothelial cell damage. Although in our study we did not observe an increase in EPC numbers or decreases in EPC apoptosis in various compartments, pioglitazone treatment did lead to an increased capacity of bone marrow-derived EPCs in differentiating into mature endothelial cells. These improvements may account, at least in part, for the amelioration of vascular function observed on the treated mice. Indeed, abnormal EPC phenotype and/or function have been associated with increases in overall CV risk and vascular dysfunction in various human and murine systems (10, 76). The reasons for the lack of effect of pioglitazone on EPC numbers in vivo are unclear. It is possible that EPC increases occur at an earlier time point than the ones measured in this study and are not sustained but, nevertheless, provide beneficial effects in vascular function as lupus progresses. It is also possible that the beneficial effect of pioglitazone on vascular repair is primarily by improving EPC function, even if EPC numbers are not affected. Finally, various doses of pioglitazone may have differential effects on EPCs and other parameters and this could be explored in the future.

Adipose tissue is a massive source of bioactive substances known as adipokines, including leptin and adiponectin. Hypertrophied adipocytes release chemokines that induce macrophage accumulation in adipose tissue. Accumulated macrophages in obese adipose tissue produce proinflammatory cytokines and NO, and these inflammatory changes induce adipocytokine dysregulation (77). The latter is characterized by a decrease in insulin-sensitizing and anti-inflammatory adipocytokines and an increase in proinflammatory adipocytokines. Extensive work in this area has shown that adipocytokine dysregulation induces obesity-related metabolic disorders, the so-called metabolic syndrome. Adiponectin expression is detectable during an intermediate stage of adipogenesis and represents the most abundant protein secreted by adipose tissue. Unlike most other adipokines, plasma adiponectin levels are reduced in animal models of obesity and insulin resistance (40). In contrast, increased leptin levels in individuals with cardiovascular disease have been associated with poor prognosis (78).

In our study, pioglitazone resulted in an overall beneficial adipokine profile, with increases in adiponectin and decreases in leptin serum levels in lupus-prone mice. Furthermore, serum insulin levels decreased in mice with established disease without associated changes in glucose. This was reflected as improvement in insulin sensitivity. Overall, these results indicate a beneficial effect of this drug on proatherogenic metabolic abnormalities described in murine and human lupus.

Although no significant differences were observed in serum autoantibody levels and renal function with pioglitazone treatment, there were significant decreases in kidney immune complex deposition and evidence of decreased renal inflammation in younger mice. Kidneys from pioglitazone-treated mice display decreased infiltration of T lymphocytes and decreased expression of proinflammatory cytokines and adhesion molecules considered relevant to the development of lupus nephritis. The high frequency of T cells in interstitial infiltrates of patients with lupus nephritis suggests a contribution of these cells to local pathogenesis (79). Indeed, 60% of inflammatory cells in patients with lupus nephritis have been found to be T cells, while high levels of T cells in urine correlate with disease severity (80, 81). Although renal macrophage infiltration did not differ between treatment groups, it appears that those infiltrating macrophages and potentially other APCs present in the kidney of pioglitazone-treated mice are less activated and therefore synthesize decreased amounts of proinflammatory cytokines. Although the precise roles of TNF-α in SLE remain a matter of discussion, various reports indicate that this molecule can be deleterious in murine lupus (82, 83) and that TNF-α blockade decreases disease severity and improves renal disease in various animal models of SLE (84, 85). TNF-α is also overexpressed in human lupus nephritis (86–88) and refractory cutaneous disease (89) and this increase is associated with worsening kidney histological activity (90). With regard to IL-1β, this cytokine is also up-regulated in murine lupus nephritis (82), induced at least in part by anti-dsDNA Abs. Renal increases in IL-1β expression have been associated with mesangial matrix increases (91). Both TNF-α and IL-1β have been shown to sequentially enhance VCAM-1 expression in murine lupus nephritis, a phenomenon that may further enhance the capacity of the endothelium to interact with inflammatory cells and the development of renal damage (92). Indeed, VCAM-1 expression in murine lupus nephritis parallels disease severity (92). Recent studies suggest that onset of proliferative glomerulonephritis and proteinuria in NZB/W mice is associated with activation of the renal endothelium (36). It is possible that pioglitazone could decrease renal inflammation, at least in part, by modulating endothelial activation in lupus glomerulonephritis, as assessed by detecting down-regulation of adhesion molecules characteristic of an activated endothelium. Given the pleiotropic effects of pioglitazone on various cellular and humoral immune mediators (17–19, 72), it is possible that various other mechanisms play a role in down-regulating inflammation in lupus kidneys by this drug, which should be explored in future studies.

These results indicate that further exploring the role of pioglitazone as adjuvant therapy when combined with other treatments targeting SLE activity and renal involvement is warranted, given the potential positive effects on organ damage prevention in this disease. This is particularly important given the positive and attractive effects on endothelial health observed in these experiments. Additional beneficial effects of TZDs on renal disease have been previously reported. TZDs inhibit podocyte injury and loss (25, 34), ameliorate renal injury in experimental diabetes (93), limit cyclosporine nephrotoxicity (94), and attenuate fibrosis-related genes in kidneys (24).

Very recently, Praharjum et al. (95) reported the effects of another TZD, rosiglitazone, on disease activity and plaque development in a mouse model of premature atherosclerosis and SLE that was developed by breeding lupus-prone Fas ligand-deficient (gld) mice with atherosclerosis-prone apolipoprotein E-deficient (apoE−/−) mice to create mice deficient in both molecules (gld.
apoE<sup>−/−</sup>). Administration of rosiglitazone to these mice reduced antinuclear Ab titers, renal disease, and atheroma size through modulation of adiponectin (95). The group did not report on the effects of rosiglitazone on lipids and other metabolic parameters and EPC phenotype/function in the gld.apoE<sup>−/−</sup> model (95). Nevertheless, taken together with our findings, it appears that TZDs are overall beneficial in murine lupus models with regard to cardiometabolic risk and disease activity. Given evidence in humans that rosiglitazone has deleterious effects on HDL cholesterol (96) whereas pioglitazone causes improvement in lipid profiles (97), it is possible that despite an overall beneficial class effect of TZDs, there could be differential effects on cardiovascular risk development among various PPAR-γ agonists. Given the potential beneficial role of both rosiglitazone and pioglitazone in now two models of murine lupus, TZDs should be further explored as adjuvant therapy for lupus disease activity in both murine and human systems.

In conclusion, the PPAR-γ agonist pioglitazone was well tolerated in a murine model of SLE and resulted in improvement of several parameters of CV risk and renal inflammation when given early in the course of the disease. PPAR-γ agonists may represent attractive candidate drugs for improving endothelial function and reducing end-organ damage in SLE.

Acknowledgments

We thank Dr. Charles Burant for helpful discussions and the Michigan Metabolomics and Obesity Center (University of Michigan) for technical support. Takeda Pharmaceuticals provided pioglitazone powder.

Disclosures

Dr. M. J. Kaplan has received investigator-initiated grant funding from Takeda Pharmaceuticals.

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