Treatment of spontaneously hypertensive rats with rosiglitazone ameliorates cardiovascular pathophysiology via antioxidant mechanisms in the vasculature

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Submitted 6 May 2009; accepted in final form 14 June 2009

Potenza MA, Gagliardi S, De Benedictis L, Zigrino A, Tiravanti E, Colantuono G, Federici A, Lorusso L, Benagiano V, Quon MJ, Montagnani M. Treatment of spontaneously hypertensive rats with rosiglitazone ameliorates cardiovascular pathophysiology via antioxidant mechanisms in the vasculature. Am J Physiol Endocrinol Metab 297: E685–E694, 2009. First published June 16, 2009; doi:10.1152/ajpendo.00291.2009.—Oxidative stress contributes to cardiovascular complications of diabetes, in part, by reducing the bioavailability of nitric oxide (NO). We investigated the mechanisms whereby the insulin sensitizer rosiglitazone may ameliorate oxidative stress in the vasculature of spontaneously hypertensive rats (SHR). Nine-week-old SHR were treated by gavage for 7 wk with rosiglitazone (5 mg·kg⁻¹·day⁻¹) or vehicle control. Treatment of SHR with rosiglitazone lowered systolic blood pressure, reduced fasting plasma insulin and asymmetrical dimethylarginine, and increased insulin sensitivity (when compared with vehicle treatment). In vessel homogenates and serum from rosiglitazone-treated SHR, SOD activity was enhanced, while 8-iso-PGF₂α (lipid peroxidation product) was reduced (when compared with samples from vehicle-treated SHR). Moreover, expression of p22phox (catalytic subunit of NADPH oxidase) as well as nitrotyrosine and superoxide content were all reduced in the aortas of rosiglitazone-treated SHR. In mesenteric vascular beds (MVB) isolated ex vivo from rosiglitazone-treated SHR, NO-dependent vasodilator actions of insulin were improved when compared with MVB from vehicle-treated SHR. Acute pretreatment of MVB from vehicle-treated SHR with apocynin (NADPH oxidase inhibitor) enhanced vasodilator actions of insulin (results comparable to those in MVB from rosiglitazone-treated SHR). In Langendorff heart preparations from rosiglitazone-treated SHR, ischemia/reperfusion injury caused infarcts 40% smaller than in hearts from vehicle-treated SHR. Acute pretreatment of hearts from vehicle-treated SHR with apocynin produced similar results. Finally, rosiglitazone treatment of endothelial cells in primary culture reduced superoxide induced by insulin-resistant conditions. We conclude that rosiglitazone therapy in SHR increases SOD activity and decreases p22phox expression in the vasculature to reduce oxidant stress leading to an improved cardiovascular phenotype.

by reduced bioavailability of nitric oxide (NO), contributes to cardiovascular complications of diabetes (59). NO bioavailability is reduced when NO reacts with superoxide anion (O₂⁻) to generate peroxynitrites (ONOO⁻), strong oxidants that compromise cellular function by irreversibly altering nucleic acids, lipids, and proteins (5). Under physiological conditions, O₂⁻ produced by NADPH oxidases is scavenged by antioxidant enzymes including SOD (23, 43). Imbalance in cell redox status resulting from excessive production of reactive oxygen species (ROS) and/or insufficient antioxidant capacity promotes both endothelial dysfunction and insulin resistance (4, 12, 13, 20, 31). Therefore, therapeutic strategies that diminish oxidative stress by restoring physiological redox balance are attractive treatment approaches for diseases characterized by both endothelial dysfunction and insulin resistance (see Ref. 36 for review).

Thiazolidinediones (TZD) are synthetic ligands for peroxisome proliferator-activated receptor-γ (PPARγ) that act as insulin sensitizers in metabolic targets including skeletal muscle, liver, and adipose tissue (74). In addition, TZDs have anti-inflammatory actions in macrophages and vascular endothelium that may oppose atherosclerosis (35, 48, 76). This is reflected in the ability of TZD treatment to reduce serum C-reactive protein (63) and carotid intima-media thickness (45). Moreover, TZDs have direct actions in vascular endothelium to increase expression of endothelial NO synthase (eNOS) and production of NO (8, 22). In spontaneously hypertensive rats (SHR), a genetic model of hypertension with features of overweight and insulin resistance resembling human metabolic syndrome (60), treatment with rosiglitazone restores the balance between phosphatidylinositol 3-kinase and MAPK-dependent branches of insulin-signaling pathways in the vascular endothelium resulting in improved endothelial function, decreased circulating levels of endothelin-1 (ET-1), and reduced systemic blood pressure (61). Thus the ability of rosiglitazone to improve insulin action in vascular endothelium may contribute to its antihypertensive effects in diabetic patients (65).

Additional mechanisms by which TZDs exert beneficial vascular effects may involve decreased oxidative stress (7). In primary endothelial cells, TZDs directly stimulate both NO-dependent branches of insulin-signaling pathways in the vascular endothelium resulting in improved endothelial function, decreased circulating levels of endothelin-1 (ET-1), and reduced systemic blood pressure (61). Thus the ability of rosiglitazone to improve insulin action in vascular endothelium may contribute to its antihypertensive effects in diabetic patients (65).

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oxidase-derived \( \text{O}_2^- \) (3). Thus rosiglitazone therapy may improve cardiovascular functions through direct actions in the vascular endothelium that decrease oxidative stress and increase NO bioavailability. In the present study, we investigated the mechanisms whereby rosiglitazone therapy reduces oxidative stress in the vasculature to ameliorate endothelial dysfunction and improve cardiovascular function in SHR rats.

**METHODS**

**Animal experiments.** All procedures were performed in accordance with Guidelines and Authorization for the Use of Laboratory Animals (Italian Government, Ministry of Health). Male spontaneously hypertensive rats (SHR) and age-matched normotensive Wistar-Kyoto (WKY) control rats were from Charles River (Calco, Italy). Nine-week-old SHR were randomized into two groups and treated daily for 7 wk by gavage with vehicle alone or rosiglitazone (5 mg·kg\(^{-1}\)·day\(^{-1}\)). This dose of rosiglitazone was chosen according to previous studies where it proved effective in lowering blood pressure, improving endothelial function, and decreasing insulin resistance in SHR (61). Nine-week-old WKY controls were given vehicle daily for 7 wk. Daily food intake was measured by weighing the amount provided the previous day. Daily water intake was measured by weighing the amount of standard chow left after removal of vascular tissue. Plasma insulin and asymmetrical dimethylarginine (ADMA) were measured using ELISA kits (Linco Research and Alexis Biochemicals, respectively). Blood glucose concentrations were determined using a diagnostic glucometer (Accu-Check Active; Roche Diagnostics, Germany). Insulin sensitivity was estimated using the quantitative insulin-sensitivity check index [\( \text{QUICKI} = 1/[(\log(\text{insulin}) + \log(\text{glucose}))]/32 \)] (32).

**Reagents.** Noradrenaline hydrochloride, ACH, apocynin, SOD, and \( N^\text{a} \)-nitro-l-arginine methyl ester (l-NNAME) were from Sigma-Aldrich. Insulin was from Novo Nordisk, and rosiglitazone was from Alexis Biochemicals. Final dilutions of all drugs were prepared in modified Krebs-Henseleit solution immediately before use. Stock solutions of rosiglitazone were in ethanol (1%). Final dilutions were in drinking water (~4× dilution). Vehicle-treated WKY and SHR received the same amount of ethanol as drug-treated animals.

**Evaluation of vascular function ex vivo.** Mesenteric vascular beds (MVB) were isolated and removed from rats after treatment with vehicle or rosiglitazone for 7 wk as described previously (60).

**Evaluation of myocardial function in isolated hearts.** Hearts from SHR treated for 7 wk with vehicle or rosiglitazone were isolated and perfused according to the Langendorff technique as described previously (60).

**Measurement of SOD activity in serum and tissue homogenates.** Total SOD activity in serum and tissue homogenates was measured using an immunoenzymatic SOD activity assay kit (Cayman Chemicals). Tissues were processed according to instructions provided by the manufacturer.

**Oxidative fluorescence microtopography.** Dihydroethidium (DHE; Molecular Probes) was used to evaluate \( \text{O}_2^- \) production in cells and in vessels in situ as described previously (6, 52). Bovine aortic endothelial cells (BAECs) in primary culture from Lonzà (Basel, Switzerland) were maintained in EGM-MV medium with glucose at either normal (4.5 mM) or high concentration (HG; 25 mM) for 24 h. Some groups of cells under HG conditions were also treated with rosiglitazone (20 \( \mu \text{M} \)) for an additional 24 h before incubation with DHE (3 \( \mu \text{M} \), 30 min, 37°C). Unfixed, frozen aortas cut into 12-\( \mu \text{m} \)-thick sections were prehydrated with PBS (10 min) and then incubated with DHE. Cells or sections were washed, stained with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA), and mounted on a coverslip. In each experiment, parallel samples were pretreated with SOD (180 U/ml, 30 min). Results visualized using a Zeiss Axiosvert TS100 epifluorescence microscope with appropriate filters were captured with a CCD camera using identical settings for each image acquisition. Densitometric analysis for merged DHE and DAPI fluorescence was performed using AxioVision Rel 4.6.3 software. Fluorescence was quantified by counting the number of pixels in identical fields for each group, and data were normalized to results from control samples (6).

**Measurements of lipid peroxidation in tissue homogenates and serum.** Levels of 8-iso-PGF\(_2\alpha\) were measured in homogenates of MVB or aorta or in serum using a commercially available ELISA kit (Assay Designs, Ann Arbor, MI) according to instructions provided by the manufacturer.

**Immunohistochemistry.** Thoracic aortas embedded in paraffin were cut into 5-\( \mu \text{m} \) sections. Deparaffinized sections heated to unmask antigens were subsequently blocked (PBS, 1% BSA, 5% FCS, and 10% donkey normal serum, 60 min) and then incubated overnight at 4°C with primary antibodies against p22phox (Santa Cruz Biotechnology) or nitrotyrosine (Upstate Biotechnology, Lake Placid, NY). Sections subsequently incubated with donkey anti-rabbit IgG (60 min) and horseradish streptavidin-peroxidase (Vector Labs; 30 min) were counterstained with Mayer’s hematoxylin (Microstain).

**Measurement of p22phox mRNA.** Total RNA was prepared from BAECs by homogenization in the Trizol reagent (Life Technologies) and mRNA quantified using RT-PCR according to standard methods. The following oligonucleotides were used as forward and reverse primers for bovine p22phox mRNA, respectively: 5′-TAT TGG TCT GCC TGG TGG AA-3′ and 5′-AGG ACC TTC AAA GCT TCA-3′. Total RNA was isolated from SHR treated for 7 wk with vehicle or rosiglitazone (20 \( \mu \text{M} \), 30 min) and stored at -80°C.

**Statistical analysis.** Results were expressed as means ± SE of \( n \) experiments (\( n \) = number of rats). Two-way ANOVAs for repeated measures followed by Bonferroni’s correction or Student’s \( t \)-tests (paired or unpaired) were used where appropriate. Values of \( P < 0.05 \) were considered to indicate statistical significance.

**RESULTS**

**Treatment of SHR with rosiglitazone improves metabolic and cardiovascular phenotypes.** Consistent with our previous findings (60, 61), 16-wk-old SHR that had undergone treatment with vehicle alone for 7 wk were overweight and hypertensive with substantial fasting hyperinsulinemia (but normoglycemia) when compared with age-matched WKY control rats (Table 1). Insulin sensitivity as assessed by the surrogate index QUICKI (42, 49) was significantly reduced in vehicle-treated SHR. Circulating levels of ADMA (endogenous inhibitor of NO synthase and circulating marker of oxidative stress; Refs. 70, 72) tended to be higher in vehicle-treated SHR when compared with WKY rats (although this did not quite reach statistical significance, \( P = 0.08 \)). When compared with vehicle-treated SHR, SHR treated with rosiglitazone (5 mg·kg\(^{-1}\)·day\(^{-1}\)) for 7 wk had comparable body weights and fasting glucose levels. Although a slight tendency to increased food and water intake was observed in rosiglitazone-treated SHR, no significant difference in these parameters was found between this group and vehicle-treated SHR for the period of our study (Table 1).

In accordance with its known insulin-sensitizing actions, treatment of SHR with rosiglitazone reduced fasting plasma...
Rosiglitazone promotes antioxidant mechanisms in the vasculature.

Table 1. Physiological and biochemical parameters obtained from 16-wk-old WKY and SHR rats treated with vehicle or rosiglitazone (5 mg·kg⁻¹·day⁻¹) for 7 wk

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WKY Vehicle Tx</th>
<th>SHR Vehicle Tx</th>
<th>SHR Rosiglitazone Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>330±10</td>
<td>370±4*</td>
<td>385±3*</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>33±2</td>
<td>35±6</td>
<td>39±8</td>
</tr>
<tr>
<td>Daily water intake, ml</td>
<td>30±5</td>
<td>29±4</td>
<td>34±9</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>138±3</td>
<td>220±7*</td>
<td>189±9†</td>
</tr>
<tr>
<td>ADMA, µM</td>
<td>1.60±0.10</td>
<td>1.95±0.18</td>
<td>0.92±0.07*</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>114±3</td>
<td>108±2</td>
<td>104±3</td>
</tr>
<tr>
<td>Fasting plasma insulin, ng/ml</td>
<td>1.1±0.2</td>
<td>13.9±2.0†</td>
<td>0.8±0.1§</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.283±0.007</td>
<td>0.218±0.003†</td>
<td>0.331±0.004§</td>
</tr>
</tbody>
</table>

Data shown are means ± SE for each group of rats (n = 24 for each group). Statistical comparisons were made using two-tailed unpaired Student’s t-test. Parameters are as described in MATERIALS AND METHODS. ADMA, asymmetrical dimethylarginine; Tx, treatment. *P < 0.001 vs. WKY; †P < 0.001 vs. SHR treated with vehicle; ‡P < 0.001 vs. spontaneously hypertensive rats (SHR; vehicle); §P < 0.0001 vs. SHR (vehicle).

We next evaluated vascular function in MVB isolated from 16-wk-old WKY and SHR rats treated daily with vehicle or rosiglitazone for 7 wk (Fig. 1). As we reported previously (60), insulin-stimulated vasodilation was significantly impaired in MVB isolated from vehicle-treated SHR (when compared with MVB from WKY rats; Fig. 1). By contrast, in MVB isolated from rosiglitazone-treated SHR, the ability of insulin to stimulate vasorelaxation was greater than that observed in MVB isolated from vehicle-treated SHR (Fig. 1). Indeed, at lower doses of insulin (1–10 nM), insulin-stimulated vasorelaxation in MVB from rosiglitazone-treated SHR was even greater than that observed in MVB from WKY rats. Taken together, the physiological and biochemical changes observed in SHR treated with rosiglitazone suggest that therapy with this insulin sensitizer ameliorates both metabolic and hemodynamic derangements in a genetic model of hypertension with features of human metabolic syndrome.

Rosiglitazone treatment reduces oxidative products, increases SOD activity, and decreases p22phox expression in the circulation and vasculature of SHR. Oxidative stress may play a key role in cardiovascular pathophysiology associated with insulin resistance (36). Since rosiglitazone treatment of SHR substantially reduced circulating ADMA levels (Table 1), we hypothesized that rosiglitazone treatment of SHR improves cardiovascular function, in part, by reducing oxidative stress in the circulation and vasculature. When compared with WKY rats, the levels of 8-iso-PGF₂α (lipid peroxidation product) were elevated in both serum and MVB homogenates from vehicle-treated SHR (Table 2). Moreover, activity of SOD (an antioxidant enzyme that scavenges O₂⁻) was reduced in both serum and in homogenates of vascular tissues from vehicle-treated SHR when compared with samples from WKY rats. Treatment of SHR with rosiglitazone for 7 wk reduced 8-iso-PGF₂α levels in both serum and MVB from SHR when compared with vehicle-treated SHR (Table 2). We also measured the reduction in systolic blood pressure when compared with vehicle-treated SHR. Moreover, plasma ADMA levels were markedly reduced to levels below those observed in WKY rats (Table 1), suggesting that rosiglitazone treatment caused a substantial decrease in oxidative stress in SHR. In normotensive control WKY rats, in vivo treatment with rosiglitazone did not significantly affect any of the physiological parameters evaluated (data not shown).

Table 2. 8-iso-PGF₂α concentration and SOD activity in serum and vasculature of 16-wk-old WKY and SHR rats treated with vehicle or rosiglitazone (5 mg·kg⁻¹·day⁻¹) for 7 wk

<table>
<thead>
<tr>
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<th>SHR Vehicle Tx</th>
<th>SHR Rosiglitazone Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 8-iso-PGF₂α, ng/ml</td>
<td>5.2±1.0</td>
<td>40.8±7.6*</td>
<td>22.7±4.0†</td>
</tr>
<tr>
<td>Serum SOD activity, U/ml</td>
<td>17.4±1.9</td>
<td>7.3±1.2*</td>
<td>9.6±0.5‡</td>
</tr>
<tr>
<td>8-iso-PGF₂α in MVB, ng/mg wet tissue</td>
<td>11.5±1.8</td>
<td>27.6±3.7*</td>
<td>21.8±0.3†</td>
</tr>
<tr>
<td>SOD activity in MVB, U/g wet tissue</td>
<td>23.2±1.5</td>
<td>10.8±3.7*</td>
<td>39.5±11.6†</td>
</tr>
<tr>
<td>SOD activity in aorta, U/g wet tissue</td>
<td>89.4±11.1</td>
<td>32.4±5.9*</td>
<td>82.8±7.7‡</td>
</tr>
</tbody>
</table>

Data shown are means ± SE for each group (n = 6 for each group). Statistical comparisons were made using two-tailed unpaired Student’s t-test. Parameters are as described in MATERIALS AND METHODS. MVB, mesenteric vascular beds. *P < 0.001 vs. WKY; †P < 0.05 vs. SHR (vehicle); ‡P < 0.001 vs. SHR (vehicle).
pared with samples from vehicle-treated SHR. SOD activity in serum as well as in aorta and MVB homogenates was increased by treatment of SHR with rosiglitazone (when compared with vehicle-treated SHR). Along these same lines, increased production of $O_2^-$ evident in aortic rings from vehicle-treated SHR was absent in aortic rings from rosiglitazone-treated SHR (Fig. 2). That is, markedly increased nuclear staining in DHE-treated samples (indicating enhanced $O_2^-$ production) was observed in aortic rings from vehicle-treated SHR when compared with those from WKY rats. DHE-treated aortic rings from rosiglitazone-treated SHR had an appearance similar to that of samples from WKY rats and also to that of aortic rings from vehicle-treated SHR acutely preincubated in vitro with SOD. Furthermore, as assessed by immunohistochemistry, expression of p22phox (catalytic subunit of NADPH oxidase that produces $O_2^-$) and levels of nitrotyrosine (increased by peroxinitrite) were both substantially increased in vessels from vehicle-treated SHR when compared with samples from WKY rats (Fig. 3). By contrast, immunostaining of vessels from rosiglitazone-treated SHR for p22phox and nitrotyrosine yielded results that were similar to those observed in aortic sections from WKY. Taken together, these findings suggest that reduced oxidative stress in the vasculature may underlie improvements in the cardiovascular phenotype of SHR resulting from rosiglitazone therapy.

**Effects of chronic rosiglitazone therapy to improve cardiovascular function in SHR are mimicked by acute inhibition of NADPH oxidase.** Impaired insulin-mediated vasodilation in MVB from SHR is related to decreased endothelial NO bioavailability (60). This may be secondary to increased oxidant stress resulting from production of ROS. To help evaluate this possibility, insulin-stimulated vasorelaxation in MVB from vehicle-treated SHR was assessed in the absence and presence of acute pretreatment with apocynin (an inhibitor of NADPH oxidase; Fig. 4A). Importantly, acute pretreatment of MVB from vehicle-treated SHR with apocynin significantly augmented insulin-dependent vasodilation (Fig. 4A). This effect of apocynin was completely inhibited in the presence of l-NAME, a competitive inhibitor for NO synthase (Fig. 4A). These results suggest that increased $O_2^-$ production from vascular NADPH oxidase may underlie endothelial dysfunction in SHR. When these experiments were repeated using MVB from rosiglitazone-treated SHR, the maximal insulin-stimulated vasorelaxation (in the absence of apocynin pretreatment) was comparable to that seen in MVB from vehicle-treated SHR acutely pretreated with apocynin (Fig. 4B). Apocynin pretreatment of MVB from rosiglitazone-treated SHR did not further improve endothelial function (Fig. 4B). However, in the presence of l-NAME, insulin-stimulated vasorelaxation was substantially impaired in MVB from rosiglitazone-treated SHR (Fig. 4B), resembling the results in MVB from vehicle-treated SHR in the absence of apocynin pretreatment (Fig. 4A). Thus acute pretreatment of MVB from vehicle-treated SHR with apocynin mimics the improved NO-dependent vasorelaxation observed in MVB from rosiglitazone-treated SHR.

We next investigated whether a 7-wk therapy with rosiglitazone may also protect against myocardial ischemia/reperfusion injury. Hearts isolated from SHR treated for 7 wk with

![Fig. 2. Treatment of SHR with rosiglitazone for 7 wk reduces superoxide production in aorta. In situ detection of $O_2^-$ in aortic rings was performed by visualizing red fluorescence of tissue sections loaded with dihydroethidium (DHE) as described in MATERIALS AND METHODS. Representative fluorescent photomicrographs from 3 independent sets of experiments are shown. Red fluorescence in sections loaded with DHE is indicative of $O_2^-$ production (top); blue fluorescence in sections stained with DAPI indicates cell nuclei (middle). Merged images of DHE and DAPI staining are shown at bottom.](http://ajpendo.physiology.org/ by 10.220.33.1 on March 30, 2017)
either vehicle or rosiglitazone were perfused according to the method of Langendorff (Fig. 5). Heart rate and heart weight were comparable among all groups of animals (data not shown). Isolated hearts from vehicle-treated and rosiglitazone-treated SHR had comparable function during aerobic perfusion as assessed by a variety of hemodynamic parameters (Fig. 5, B, C, and D at baseline time point B). In hearts from vehicle-treated SHR, 30 min of ischemia followed by 120 min reperfusion resulted in infarction of >50% of left ventricular volume with severely impaired cardiac function (Fig. 5). By contrast, myocardial infarct size was ~40% smaller in hearts from rosiglitazone-treated SHR (Fig. 5A). Consistent with reduction in infarct size, postischemic recovery of ventricular function was also improved in hearts from rosiglitazone-treated SHR, as assessed by coronary flow, left-ventricular end-diastolic pressure, and left ventricular developed pressure (Fig. 5, B, C, and D). Acute preincubation of hearts from vehicle-treated SHR with apocynin resulted in reduction in infarct size and improvement in left-ventricular end-diastolic pressure and left ventricular developed pressure that were comparable to that seen in hearts from rosiglitazone-treated SHR (Fig. 5). Thus as with endothelial function (Fig. 4), the effects of rosiglitazone therapy to protect against myocardial ischemia/reperfusion injury were mimicked by acute treatment of hearts in vitro with apocynin. This provides additional support for the hypothesis that rosiglitazone therapy in SHR improves cardiovascular pathophysiology by reducing oxidant stress.

**Fig. 3.** Expression of p22phox and nitrotyrosine containing proteins in aortas from WKY and SHR rats treated with vehicle or rosiglitazone (5 mg·kg\(^{-1}\)·day\(^{-1}\)) for 7 wk. Aortas from WKY and SHR rats were prepared as described in MATERIALS AND METHODS. Representatives photomicrographs of aortas immunostained with primary antibodies against p22phox or nitrotyrosine are shown at ×400 magnification (scale bar = 200 μm).

**DISCUSSION**

Reciprocal relationships between insulin resistance and endothelial dysfunction underlie the clustering of hypertension, overweight, endothelial dysfunction, and insulin resistance observed in both SHR rats and humans with the metabolic syndrome (34, 50). This helps to explain why SHR or humans evaluated \(\text{O}_2^-\) production in primary endothelial cells incubated with normal (4.5 mM) or high (25 mM) concentrations of glucose in the absence or presence of rosiglitazone treatment (Fig. 6). Incubation of BAECs with high glucose concentrations increased production of nuclear \(\text{O}_2^-\) levels (as assessed by the DHE-to-DAPI fluorescence ratio; Ref. 6). Importantly, treatment of BAECs with rosiglitazone (20 μM) for 24 h reduced nuclear DHE staining to levels similar to those obtained in BAEC cultured with high glucose and treated acutely in vitro with exogenous SOD (180 U, 30 min). We observed similar results in cells where production of \(\text{O}_2^-\) was stimulated by exposure to high insulin concentrations plus wortmannin (conditions mimicking insulin resistance with compensatory hyperinsulinemia; Ref. 47; Fig. 6, D and E). In addition to superoxide levels, the mRNA expression of p22phox was increased in BAEC cells exposed to insulin-resistant conditions. Interestingly, in vitro treatment with rosiglitazone substantially reduced mRNA levels of p22phox under these conditions (Fig. 6C). Thus rosiglitazone has direct antioxidant actions in the vascular endothelium that may contribute to improved cardiovascular phenotypes observed in rosiglitazone-treated SHR.
Rosiglitazone therapy reduces oxidative stress in vasculature of SHR. Correlations between increased ROS levels, impaired metabolic control, and higher risk of cardiovascular events suggest that oxidative stress is a critical mechanism underlying the pathogenesis of both insulin resistance and endothelial dysfunction (19, 24, 36). Indeed, a number of studies suggest that oxidative stress contributes independently to both insulin resistance in metabolic and vascular tissues (2, 58, 64), as well as to endothelial dysfunction and atherosclerosis in the vasculature (14, 33, 75). Consistent with our previous report (61), in this study, SHR exhibited systolic hypertension, metabolic insulin resistance, and endothelial dysfunction that were all reduced by rosiglitazone treatment. Important new findings of the present study include the observations that circulating levels of ADMA and elevated levels of 8-iso-PGF$_2$α, O$_2$-, and nitrotyrosine in serum or vascular tissues in SHR were all reduced substantially by rosiglitazone therapy. High circulating levels of ADMA, increased amounts of lipid peroxides, and nitrosylated proteins in vascular and smooth muscle tissues are known markers of oxidant stress characteristically seen with endothelial dysfunction, atherosclerosis, and diabetes (11, 53, 69, 72). The effects of rosiglitazone therapy to reduce markers of oxidative stress in SHR that we observed were comparable to results obtained by others using specific antioxidant treatments in SHR to reduce blood pressure or prevent progression of hypertension (56, 67). It is unlikely that these changes in markers of oxidative stress were secondary to the metabolic actions of rosiglitazone, since SHR in our study were not hyperglycemic and fasting glucose levels were similar among SHR treated with vehicle or with rosiglitazone. In fact, in primary endothelial cells, we demonstrated that the effects of rosiglitazone treatment to reduce O$_2$- production in cells incubated with insulin plus Wortmannin (to mimic conditions of metabolic insulin resistance in SHR) were comparable to effects seen in cells treated with exogenous SOD. Similar results were obtained when ROS was induced with high glucose concentrations. However, this is less relevant to SHR that do not have overt hyperglycemia. Thus the ability of systemic rosiglitazone therapy to reduce oxidative stress in the vasculature may be due to direct or indirect antioxidant actions of rosiglitazone in vascular endothelium. In normotensive control WKY rats, in vivo treatment with rosiglitazone did not significantly affect any of the physiological parameters evaluated or other markers of oxidative stress (data not shown). This may be explained by low levels of systemic oxidative stress in WKY. Our results in SHR are in line with previous studies demonstrating actions of TZDs to oppose oxidative stress in the vasculature of animal models with atherosclerosis or other cardiovascular diseases (46, 51, 76).

Rosiglitazone treatment increases SOD activity and decreases p22phox expression in the circulation and the vasculature of SHR. Increased production of ROS, and of O$_2$- in particular, may result from both reduced activity of scavenging enzymes such as SOD and catalase as well as increased expression and activation of prooxidant enzymes including NADPH oxidases (1, 36, 77). Therefore, one potential mechanism for rosiglitazone therapy to reduce oxidative stress in the vasculature may be to increase expression or activity of scavenging enzymes and decrease expression of enzymes responsible for O$_2$- production. Of note, in vessels from rosiglitazone-treated SHR, SOD activity was substantially increased, while treated with ACE inhibitors, ARBs (antihypertensive agents that improve endothelial dysfunction), or TZDs (antidiabetic agents that improve insulin sensitivity) show simultaneous improvement in both cardiovascular and metabolic phenotypes (37–39, 55, 61). We previously demonstrated that rosiglitazone therapy in SHR restores the balance between phosphatidylinositol 3-kinase-dependent and MAPK-dependent branches of insulin-signaling pathways in vascular endothelium to reduce endothelial dysfunction (61). In the present study, we focused on the ability of rosiglitazone to reduce oxidative stress in the vasculature as an additional mechanism contributing to beneficial cardiovascular actions of rosiglitazone therapy.
p22phox expression was markedly decreased (when compared with vessels from vehicle-treated SHR). In addition to p22phox, several other components of the NOX complex may be involved in oxidative stress under insulin-resistant conditions (36). However, among the numerous elements of the NADPH oxidase enzyme, p22phox is considered the catalytic subunit (23). Therefore, p22phox levels are more directly related to NADPH oxidase activity. Nevertheless, it remains possible that other NOX subunits may also be altered by rosiglitazone therapy. Changes in enzyme activity and expression may help to explain the reductions in markers of oxidative stress in the vasculature we observed after rosiglitazone therapy. Our findings are consistent with previous reports demonstrating effects of PPARγ ligands to reduce vascular oxidative stress by altering expression and/or activation of key redox enzymes. For example, increased expression of p22phox observed in endothelial cells incubated with phorbol esters is opposed by troglitazone treatment (30). Along these same lines, rosiglitazone treatment of DOCA-salt rats decreases production of O2 in the vasculature (29), while treatment of db/db mice with rosiglitazone reduces NADPH oxidase-derived production of O2 and enhances catalase activity in coronary arterioles (3). TZD treatment also reduces expression of several subunits of NADPH oxidase (including p47phox and Nox-2) in the vasculature of obese or diabetic rodents (16, 27) and hypercholesterolemic rabbits (71).

Effects of chronic rosiglitazone therapy to improve cardiovascular function in SHR are mimicked by acute inhibition of NADPH oxidase. In this study, we found that the salutary cardiovascular effects of rosiglitazone therapy included improved vasodilator actions of insulin similar to results we reported previously (61). Although MVB is considered as a prototype of resistance vessels (60), distinct vascular districts possess vessel specificity that may account for important differences in organ and tissue functions. Thus changes in the vascular reactivity of MVB do not necessarily imply that similar changes may occur in other vascular districts. Nevertheless, the fact that parameters of vascular oxidative stress are ameliorated by rosiglitazone in a vascular district not directly involved in glucose uptake (such as skeletal muscle arteries) provides additional evidence that the vascular effects of rosiglitazone do not completely depend on metabolic amelioration. Endothelial dysfunction and hypertension contribute importantly to impaired cardiac function and increase susceptibility to myocardial ischemia/reperfusion injury (18). Oxidative stress may also play a role in the pathogenesis of contractile dysfunction and structural damage in the myocardium (28, 44). Since endothelial dysfunction, systolic blood pressure, and markers of oxidative stress in the vasculature were all improved by rosiglitazone treatment, we reasoned that rosiglitazone therapy may also afford cardiac protection in SHR. Indeed, we found that rosiglitazone treatment conferred substantial protection against myocardial ischemia/reperfusion injury in SHR that has not previously been reported. To help support the idea that the antioxidant actions of rosiglitazone may be responsible for its ability to improve cardiovascular phenotypes, we performed ex vivo experiments where MVB and hearts from vehicle-treated SHR were preincubated with apocynin. Apocynin is an inhibitor of NADPH oxidase that prevents the assembly of the NADPH oxidase subunits p47phox and gp91phox resulting in decreased NADPH-oxidase-dependent O2 formation (73). In addition, apocynin may have direct antioxidant properties in the endothelium (25, 66). Interestingly, the results from experiments with MVB and hearts from vehicle-treated SHR that were preincubated with apocynin mimicked the results from similar experiments performed with MVB and hearts isolated from rosiglitazone-treated SHR. That is, vessels and hearts from vehicle-treated SHR subjected to acute antioxidant treatment ex vivo had improved function similar to that resulting from chronic ros-
Rosiglitazone therapy in vivo. Apocynin pretreatment did not result in further improvement of endothelial function in MVB from rosiglitazone-treated SHR. This may be due to the fact that rosiglitazone therapy had already substantially decreased expression of p22phox.

Despite cell and animal studies suggesting that rosiglitazone may have beneficial cardiovascular actions (74), results from several recent large clinical trials have raised the alarming possibility that TZD therapy in patients with diabetes may paradoxically increase, rather than decrease, cardiovascular morbidity and mortality (17, 21, 40, 41, 54, 57, 68). Given the preclinical data available on the potential cardiovascular benefits of TZDs, it is not clear why some large studies (9, 15) show an increased risk for adverse cardiovascular outcomes after TZD therapy. One possibility is that the cardiovascular risk/benefit profile of TZD therapy may change depending on whether patients are early in the course of the disease with reversible functional alterations or late in the course of disease with irreversible structural damage. However, it should also be pointed out that differences in the rosiglitazone-dependent effect on vascular function between our investigation and large clinical trials may result from the relatively short period of rosiglitazone administration in this study. It remains possible that a subset of patients with reversible cardiovascular disease may derive both metabolic and cardiovascular benefits from rosiglitazone therapy.

In conclusion, rosiglitazone therapy in SHR reduces markers of oxidative stress in the circulation and vasculature that may be explained by increased SOD activity and decreased expression of p22phox. Thus in addition to improving insulin signaling in metabolic tissues and the vasculature, rosiglitazone may have important antioxidant mechanisms of action. These properties may contribute to the beneficial effects of rosiglitazone therapy on endothelial and cardiac function in SHR.
REFERENCES


ROSIGLITAZONE PROMOTES ANTIOXIDANT MECHANISMS IN THE VASCULARITY


