Rosiglitazone attenuates NF-κB-mediated Nox4 upregulation in hyperglycemia-activated endothelial cells

Clintoria R. Williams, Xianghuai Lu, Roy L. Sutliff, and C. Michael Hart

Address of reprint requests and other correspondence: C. M. Hart, Division of Pulmonary and Critical Care Medicine, Atlanta VAMC (151-P), 1670 Clairmont Rd., Decatur, GA 30033 (e-mail: Michael.Hart3@va.gov).

Submitted 12 July 2011; accepted in final form 8 May 2012

Rosiglitazone attenuates NF-κB-mediated Nox4 upregulation in hyperglycemia-activated endothelial cells. Am J Physiol Cell Physiol 303: C213–C223, 2012. First published May 9, 2012; doi:10.1152/ajpcell.00227.2011—Vascular complications, a major cause of morbidity and mortality in diabetic patients, are related to hyperglycemia-induced oxidative stress. Previously, we reported that rosiglitazone (RSG) attenuated vascular expression and activity of NADPH oxidases in diabetic mice. The mechanisms underlying these effects remain to be elucidated. We hypothesized that RSG acts directly on endothelial cells to modulate vascular responses in diabetes. To test this hypothesis, human aortic endothelial cells (HAECs) were exposed to normal glucose (NG; 5.6 mmol/l) or high glucose (HG; 30 mmol/l) concentrations. Select HAEC monolayers were treated with RSG, caffeic acid phenethyl ester (CAPE), diphenyleneiodonium (DPI), small interfering (si)RNA (to NF-κB/p65 or Nox4), or Tempol. HG increased the expression and activity of the NADPH oxidase catalytic subunit Nox4 but not Nox1 or Nox2. RSG attenuated HG-induced NF-κB/p65 phosphorylation, nuclear translocation, and binding to the Nox4 promoter. Inhibiting NF-κB with CAPE or siNF-κB/p65 also reduced HG-induced Nox4 expression and activity. HG-induced H2O2 production was attenuated by siRNA-mediated knockdown of Nox4, and HG-induced HAEC monocyte adhesion was attenuated by treatment with RSG, DPI, CAPE, or Tempol. These results indicate that HG exposure stimulates HAEC NF-κB activation, Nox4 expression, and H2O2 production and that RSG attenuates HG-induced oxidative stress and subsequent monocyte-endothelial interactions by attenuating NF-κB/p65 activation and Nox4 expression. This study provides novel insights into mechanisms by which the thiazolidinedione rosiglitazone favorably modulates endothelial responses in the diabetic vasculature.

p22phox, NoxA1, NoxO1, rac-1, and Poldip2 (26). Diverse stimuli including hyperglycemia, oxidatively modified lipoproteins, and advanced glycation end products induce NADPH oxidase activation (45) by stimulating the assembly of a functional NADPH oxidase complex. Furthermore, accumulating evidence indicates that nuclear transcription factors, such as activator protein-1 (31) and nuclear factor-κB (NF-κB; Refs. 29, 32) are involved in the transcriptional regulation of NADPH oxidase subunit expression. Thiazolidinediones (TZDs), such as rosiglitazone (RSG) and pioglitazone, belong to a class of insulin-sensitizing medications that mediate their metabolic effects through the ligand-activated nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ). In addition to improving metabolic derangements in diabetes, TZDs also exert beneficial effects on the vasculature of both diabetic and nondiabetic individuals by reducing atherogenic events (10). We (18) previously demonstrated that the expression of Nox1, Nox2, and Nox4 as well as O2·− generation was increased in the aortas of leptin receptor-deficient mice and that daily treatment with RSG for 1 wk reversed the increased expression and activity of vascular NADPH oxidases in diabetic mice. RSG-mediated reversal of these diabetic vascular derangements was observed in the absence of significant improvements in systemic metabolic derangements (18), suggesting that RSG exerted direct antioxidative effects on the vasculature. However, the molecular mechanisms responsible for the effects of RSG on vascular oxidative stress in diabetes were not elucidated.

NF-κB activation plays a key role in the pathogenesis of diabetic complications (4, 41). NF-κB proteins constitute a family of redox-sensitive, nuclear transcription factors that control the expression of genes linked to endothelial activation. Importantly, the transcriptional function of NF-κB is tightly controlled by a family of regulatory proteins, IκB, whose primary role involves inhibiting NF-κB activation. Previous reports demonstrated that hyperglycemia activates NF-κB (17, 38) and that PPARγ ligands attenuate NF-κB signaling (11). Collectively, these reports led us to postulate
that PPARγ activation with TZD ligands would suppress hyperglycemia-induced NF-κB activation in human aortic endothelial cells (HAECs) and thereby attenuate the upregulation of NADPH oxidase and endothelial oxidative stress. Because mechanisms involved in the regulation of NADPH oxidase expression can be stimulus and cell specific (5), the current study examined hyperglycemia-induced NADPH oxidase expression in HAECs in vitro. The current findings extend our previous report that RSG attenuates hypoxia-induced increases in Nox4 expression in human pulmonary artery smooth muscle cells in vitro (29) and in the mouse lung in vivo (37). The current results demonstrate that TZDs as well as molecular or pharmacological approaches to inhibit NF-κB reduce oxidative stress in human systemic macrovascular endothelial cells by attenuating hyperglycemia-induced, NF-κB-mediated Nox4 expression. Our findings emphasize that Nox4 plays a significant role in hyperglycemia-induced oxidative stress in HAECs and that TZDs, through direct effects on endothelial Nox4 expression, can reduce vascular oxidative stress and dysfunction.

**METHODS**

**Cell culture.** HAECs (Clonetics, San Diego, CA) were grown at 37°C in 5% CO₂ in endothelial cell growth medium (EGM; Lonza, Walkersville, MD) supplemented with 10% FBS, 10 ng/ml human epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 12 μg/ml bovine brain extract. At 75% confluence, the culture medium was changed to 2% FBS-containing EGM. Because hyperglycemia constitutes a defining metabolic derangement common to both type 1 and type 2 diabetes that stimulates endothelial ROS generation, HAECs were then exposed to media containing either normal glucose concentration (5.5 mmol/l), clinically relevant in vitro high glucose concentration (30 mmol/l), or an osmotic control (mannitol; 24.5 mmol/l in normal glucose) for 24 or 72 h. Experimental groups received fresh media every 24 h. During the final 24-h of exposure to normal glucose or high glucose media, select HAEC monolayers were treated with 10 μmol/l RSG, 10 μmol/l caffeic acid phenethyl ester (CAPE), 10 μmol/l diphenylethenoidammonium (DPL), 10 μmol/l Tempol, or 0.01% vehicle (DMSO). In separate experiments, select HAEC monolayers were transfected (GeneSilencer, Genlantis, San Diego, CA) with small interfering (si)RNA oligonucleotides against NF-κB/p65 (100 nmol/l), Nox4 (35 nmol/l), or scrambled oligonucleotides before glucose exposure.

**Analysis of Nox4 expression.** Previously, we (18) demonstrated that RSG treatment attenuated the enhanced expression of the catalytic subunits of NADPH oxidases-Nox1, Nox2, and Nox4 in the aortas of leptin receptor-deficient diabetic mice. Initially, quantitative real-time PCR was employed to measure mRNA levels of Nox4 in glucose-stimulated HAECs in vitro. Total RNA was isolated from HAECs with Trizol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Nox4 cDNA was amplified using SYBR Green (Bio-Rad, Hercules, CA). All data were normalized to the 9s content of the same sample. Additionally, Western blot analysis was employed to detect protein levels of Nox4 in vitro. Cell lysates were resolved in 10% Bis-Tris gels (Invitrogen), transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), and probed with primary antibodies specific for Nox4 (provided by Dr. David Lambeth, Emory University) or actin (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunodetection was performed using a chemiluminescence method (SuperSignal; Pierce Biotechnology, Rockford, IL).

**Assessment of endothelial activation.** Endothelial activation was estimated with a monocyte adhesion assay, as previously reported (16). Human monocytes (THP1; 10 × 10⁶/ml) labeled with the fluorescent probe, calcein (AnaSpec, Fremont, CA) were incubated with confluent HAEC monolayers for 1 h at 37°C. Monolayers were gently washed with KRP buffer to remove nonadhered
THP1 monocytes. To assess the adhesion of THP1 monocytes to HAEC monolayers, attached THP1 were quantified by measuring fluorescence intensity on a Victor plate reader (PerkinElmer) at excitation and emission wavelengths of 488 and 520 nm, respectively.

**Statistical analysis.** For all experiments, statistical analysis was performed by one way ANOVA followed by post hoc analysis with the Student-Newman-Keuls test to detect differences between experimental groups. A value of \( P < 0.05 \) was considered statistically significant.

**Fig. 1.** Rosiglitazone (RSG) attenuated high glucose (HG)-induced Nox4 expression and activity in human aortic endothelial cells (HAECs). Nox4 expression and activity were examined in HAECs exposed to normal glucose (NG) or HG concentrations for 24 or 72 h ± RSG or diphenyleneiodonium (DPI) treatment during the final 24 h. A: Nox4 mRNA levels were measured by quantitative real-time PCR and normalized to the 9s content of the same sample. B: Nox4 and actin protein levels were detected by Western blot analysis. Representative Western blots of 3 independent experiments are shown. C: intracellular reactive oxygen species (ROS) were measured using the ROS-sensitive fluorescent probe 2',7'-dihydrodichlorofluorescein (DCF)-AM. D: \( \text{H}_2\text{O}_2 \) release was measured using an Amplex red assay. Data are expressed as percentage of control and represent the means ± SE of 6–9 independent experiments. *\( P < 0.05 \) vs. NG; #\( P < 0.05 \) vs. HG.
RESULTS

RSG attenuated hyperglycemia-induced endothelial NADPH oxidase expression and activity. Since (18) previously observed that RSG attenuated Nox1, Nox2, and Nox4 expression in the vascular wall of diabetic mice, the effects of RSG on high glucose-induced HAEC Nox expression was examined in vitro. While increases in Nox4 mRNA and protein levels were observed following either 24- or 72-h of high glucose exposure (Fig. 1), Nox1 mRNA and protein levels were not increased in HAECS exposed to high glucose concentrations (data not shown), whereas Nox2 protein levels were increased following high glucose exposure at 24 but not 72 h (data not shown). Treatment with RSG for the duration of exposure to high glucose for 24 h or during the final 24 h of the 72-h high glucose exposure was sufficient to produce comparable and significant reductions in Nox4 mRNA and protein levels (Fig. 1) and reduced Nox2 protein levels at 24 h (data not shown). Consistent with these results, treatment with the TZD PPARγ ligand pioglitazone also reduced high glucose-induced increases in Nox4 mRNA levels in HAECS exposed to high glucose concentrations for 72 h (data not shown). Taken together, these results indicate that sustained hyperglycemia caused persistent increases in Nox4 but not Nox1 or Nox2 expression in HAECS and that treatment with RSG or pioglitazone attenuated increases in Nox4.

To further determine if high glucose-induced Nox4 expression was associated with enhanced NADPH oxidase activity, ROS production stimulated by hyperglycemia was measured following treatment with RSG, DPI (nonspecific NADPH oxidase inhibitor), or siNox4. Figure 1C illustrates that compared with HAECS exposed to normal glucose concentrations, high glucose exposure for 24 or 72 h increased ROS production, as measured by DCF fluorescence (detects H2O2 and other reactive species). Treatment with RSG or DPI significantly reduced high glucose-induced ROS levels (Fig. 1C). Additionally, DPI treatment caused a small but statistically significant decrease in basal ROS levels in normal glucose-exposed HAECS at 72 h (Fig. 1C). Exposure to high glucose concentrations for 24 or 72 h also increased H2O2 production in HAECS (measured with Amplex red assay), and these increases were attenuated by RSG or DPI (Fig. 1D). RSG also reduced H2O2 production in HAECS exposed to normal glucose concentrations at 24 or 72 h, whereas DPI reduced H2O2 production in HAECS exposed to normal glucose concentrations at 72 h only. Because Nox4 is unique among the Nox1–5 isoforms in that it generates H2O2 rather than superoxide (1, 48), we employed siRNA to Nox4 and measured hyperglycemia-induced H2O2 production. In HAECS transfected with siRNA against Nox4, basal Nox4 mRNA (Fig. 2A) and protein (Fig. 2B) levels were reduced compared with scrambled-RNA transfected cells. Additionally, the reduction in Nox4 expression was accompanied by significant decreases in basal and high glucose-induced H2O2 generation (Fig. 2C). These findings indicate that Nox4 is a major source of H2O2 generation in HAECS exposed to hyperglycemia and suggest that RSG-mediated reductions in Nox4 are a critical feature of its ability to attenuate hyperglycemia-induced oxidative stress.

RSG attenuated hyperglycemia-induced endothelial NF-κB/p65 activation. NF-κB activation is implicated in the pathogenesis of diabetic complications (41). To assess the effects of RSG on NF-κB activation, nuclear and cytoplasmic NF-κB/p65 and cytoplasmic IκB-α protein levels were examined by Western blot. Figure 3A illustrates that exposure to high glucose for 24 h stimulated NF-κB/p65 activation as indicated by increased nuclear NF-κB/p65, decreased cytoplasmic NF-κB/p65, and reduced cytoplasmic IκB-α protein levels compared with HAECS exposed to normal glucose. In contrast, exposure to equivalent mannitol concentrations (an osmotic control for elevated glucose concentrations) failed to activate
NF-κB (Fig. 3A). Treatment with RSG for the 24-h duration of glucose exposure attenuated high glucose-induced NF-κB/p65 nuclear translocation (Fig. 3, A and B) and prevented reductions in cytoplasmic IkB-α protein levels (Fig. 3A). Similarly, exposure to high glucose for 72 h stimulated sustained nuclear translocation of NF-κB/p65 (Fig. 3, A and B) and decreased cytoplasmic IkB-α protein levels (Fig. 3A). Furthermore, Fig. 3 illustrates that treatment with RSG during the final 24 h of the 72-h high glucose stimulation was sufficient to attenuate these indices of NF-κB activation. Consistent with these results, RSG treatment also decreased high glucose-induced NF-κB/p65 phosphorylation (Fig. 3C). Collectively, these results indicate that RSG can either prevent or reverse NF-κB/p65 activation following hyperglycemia for 24–72 h in HAECs.

The Nox4 promoter contains NF-κB/p65 binding elements (29). To further confirm the role of reduced NF-κB activation in the ability of RSG to inhibit hyperglycemia-induced Nox4 expression, NF-κB/p65 binding to the Nox4 promoter was examined by chromatin immunoprecipitation assay. Compared with normal glucose, high glucose concentrations stimulated binding of NF-κB/p65 to the Nox4 promoter in HAECs and RSG attenuated NF-κB/p65 binding to the Nox4 promoter (Fig. 3D). Taken together, these findings demonstrate that RSG reduces glucose-induced oxidative stress by preventing NF-κB activation and p65 binding to the Nox4 promoter thereby preventing increased Nox4 expression and H2O2 production in glucose-treated HAECs.

CAPE attenuated hyperglycemia-induced endothelial NADPH oxidase expression and activity. The role of glucose-induced NF-κB activation on Nox4 expression was further confirmed using a pharmacological approach. Glucose-induced HAEC NF-κB/p65 activation and endothelial Nox4 expression and activity...
were examined following treatment with the NF-κB inhibitor, CAPE. As illustrated in Fig. 4, CAPE attenuated increases in Nox4 mRNA (Fig. 4A) and protein (Fig. 4B) levels caused by 24 or 72 h of high glucose exposure. CAPE treatment also significantly reduced high glucose-induced ROS production (Fig. 4C). Additionally, CAPE reduced ROS levels in HAECs exposed to normal glucose (Fig. 4C). Similarly, CAPE also attenuated H₂O₂ production in HAECs caused by exposure to high glucose concentrations for 24 or 72 h (Fig. 4D). Furthermore, CAPE caused modest but significant reductions in H₂O₂ production by HAECs exposed to normal glucose concentrations (Fig. 4D). Taken together, these results confirm that NF-κB/p65 activation participates in glucose-mediated up-regulation of HAEC Nox4 expression and activity.

Fig. 4. CAPE attenuated HG-induced Nox4 expression and activity in HAECs. Nox4 expression and activity were examined in HAECs exposed to NG or HG concentrations for 24 or 72 h ± caffeic acid phenethyl ester (CAPE) treatment during the final 24 h. A: Nox4 mRNA levels were measured by quantitative real-time PCR and normalized to the 9s content of the same sample. B: Nox4 and actin protein levels were detected by Western blot analysis. Representative Western blots from 3 independent experiments are shown along with mean densitometric intensity ± SE. C: intracellular ROS were measured using the ROS-sensitive fluorescent probe DCF-AM. D: H₂O₂ release was measured using an Amplex red assay and normalized to total protein. Data are expressed as percentage of control and represent the means ± SE of 6–9 independent experiments. *P < 0.05 vs. NG; #P < 0.05 vs. HG.
siNF-κB/p65 attenuated hyperglycemia-induced endothelial NADPH oxidase expression and activity. To more specifically confirm the role of NF-κB/p65 in high glucose-induced Nox4 expression, HAECs were treated with scrambled oligonucleotides or siRNA against NF-κB/p65. As illustrated in Fig. 5A, transfection with si-NF-κB/p65 significantly reduced basal NF-κB/p65 mRNA and protein levels in HAECs exposed to normal glucose concentrations. Furthermore, siRNA-mediated knockdown of NF-κB/p65 also reduced both basal and high glucose-induced Nox4 mRNA levels (Fig. 5B) and H₂O₂ production (Fig. 5C). These findings demonstrate that NF-κB/p65-mediated Nox4 expression plays a critical role in HAEC oxidative stress in response to hyperglycemia.

RSG, CAPE, DPI, or Tempol attenuated hyperglycemia-induced endothelial activation. In addition to stimulating NF-κB activation and Nox4 expression and activity, hyperglycemia increases endothelial activation and subsequent monocyte binding. As shown in Fig. 6A, high glucose exposure for 24 h, but not mannitol (osmotic control), increased THP1 monocyte adhesion to HAEC. Enhanced monocyte adhesion was reduced when HAECs were treated with RSG, CAPE, DPI, or Tempol (Fig. 6A). Similarly, Fig. 6B illustrates that monocyte adhesion to HAECs was significantly increased after 72 h of high glucose exposure and significantly reduced in HAECs treated with RSG, CAPE, DPI, or Tempol. Collectively, these findings demonstrate that RSG attenuates hyperglycemia-induced endothelial activation to a degree comparable to pharmacological inhibitors of NF-κB (CAPE), NADPH oxidases (DPI), or an antioxidant (Tempol).

**DISCUSSION**

Oxidative stress derived from NADPH oxidases is recognized as a critical mediator in the development and progression of vascular disease (27). Previously, our laboratory (18) demonstrated that the catalytic subunits of NADPH oxidase, Nox1, Nox2, and Nox4, were upregulated in the vasculature of leptin receptor-deficient mice and that treating these diabetic mice with RSG for 1 wk attenuated increases in vascular NADPH oxidase expression and activity. The vascular effects of short-term RSG treatment were independent of significant improvements in metabolic abnormalities in the diabetic mice (18). These results suggested that reductions in vascular NADPH oxidase expression and activity following RSG treatment were more likely attributable to direct actions of RSG on vascular wall cells rather than to overall improvements in diabetes-associated metabolic derangements. However, the antioxidant mechanisms of RSG on the diabetic vasculature were not...
elucidated. The goal of the current study was to define the mechanisms by which RSG modulates the responses of vascular wall cells to hyperglycemia using an in vitro model employing HAECs.

Since NF-κB is a critical mediator in the pathogenesis of diabetic complications (41), the role of NF-κB in the effects of RSG on diabetic vascular wall cells was investigated. Consistent with other reports (40, 51), our findings confirmed that hyperglycemia stimulated IkB degradation and NF-κB/p65 activation, phosphorylation, and nuclear translocation. The present study established that RSG treatment attenuated hyperglycemia-induced NF-κB/p65 nuclear translocation by inhibiting IkB degradation in endothelial cells. Although the mechanism for this effect was not directly addressed in the current study, Marfella et al. (33) demonstrated that RSG intervention reversed the enhanced activity of the ubiquitin-proteasome degradation system and associated increases in IkB degradation and NF-κB activity in atherosclerotic plaques from individuals with diabetic vascular disease. Taken together, these studies indicate that RSG modulates NF-κB activation in vascular wall cells in diabetes.

Previous studies (30, 32) identified NF-κB response elements within the promoter regions of NADPH oxidase subunits in smooth muscle cells. In this study, we investigated whether NF-κB/p65 signaling regulates Nox4 expression in endothelial cells. By employing the NF-κB inhibitor, CAPE, and siRNA against NF-κB/p65, our results demonstrated that NF-κB mediates Nox4 upregulation in endothelial cells exposed to hyperglycemia. While diabetic vasculature has been characterized by increased expression of Nox1, Nox2, and Nox4 (18), high glucose concentrations in HAECs in the current study persistently increased only Nox4. These findings suggest that upregulation of Nox1 and Nox2 in diabetic vasculature may be either spatially or temporally separated from Nox4 upregulation or that factors in the diabetic circulation above and beyond hyperglycemia are required to persistently increase Nox1 and Nox2 expression in the vascular wall. We (29, 32) recently reported that RSG attenuated NF-κB/p65 binding to the Nox4 promoter in pulmonary artery smooth muscle cells exposed to hypoxia. The current results extend those findings by demonstrating that RSG attenuated hyperglycemia-induced NF-κB/p65 phosphorylation, nuclear translocation, and p65 binding to the Nox4 promoter. These findings provide additional insights into the mechanisms by which RSG treatment reduces enhanced NADPH oxidase expression in the aortas of diabetic mice (18).

With the use of pharmacological and molecular approaches, the current report demonstrates that Nox4 plays a central role in hyperglycemia-induced oxidative stress in human vascular endothelial cells. This conclusion is consistent with the findings of previous reports that DPI, but not specific inhibitors for several other ROS-generating flavoproteins, such as xanthine oxidase, nitric oxide synthase and mitochondrial electron transport chain, attenuates hyperglycemia-induced oxidative stress in bovine aortic endothelial cells (19). Furthermore, RSG attenuated hyperglycemia-induced NADPH oxidase-derived

Fig. 6. RSG, CAPE, DPI, or Tempol attenuated THP1 monocyte binding to HG-activated HAECs. Monocyte adhesion to endothelial cells was examined in HAECs exposed to NG, HG, or high mannitol concentrations for 24 h (A) or 72 h (B) ± RSG, CAPE, DPI, or Tempol treatment, during the final 24 h. Endothelial activation was assessed by measuring fluorescence of calcine-labeled monocytes bound to endothelial cells. Data are expressed as percentage of control and represent the means ± SE of 6–9 independent experiments. *P < 0.05 vs. NG; #P < 0.05 vs. HG.
oxidative stress by preventing AMPK-mediated assembly of functional NADPH oxidase complexes in human umbilical vein endothelial cells (7). Our results extend these findings to demonstrate that RSG regulates Nox4 upregulation in hyperglycemia-exposed endothelial cells. Our findings illustrate that pharmacological or siRNA-mediated inhibition of NF-κB prevented hyperglycemia-induced upregulation of Nox4 and subsequent ROS production.

It is well recognized that NF-κB is linked to many genes associated with endothelial dysfunction (43). Endothelial dysfunction constitutes a critical derangement in the pathogenesis of diabetic vascular disease (6, 20, 53). Current evidence indicates that enhanced vascular oxidative stress plays an important role in endothelial dysfunction (25, 36). Endothelial activation is a hallmark of endothelial dysfunction and is characterized by enhanced proinflammatory cytokine and chemokine release as well as increased cellular adhesion molecule expression. These endothelial alterations promote monocyte adhesion to initiate the pathogenesis of atherosclerosis. Our results demonstrate that RSG attenuates hyperglycemia-induced NF-κB activation and increased Nox4 expression to reduce endothelial activation. These results are consistent with previous evidence that NF-κB inhibitors reduced glucose-mediated monocyte adhesion to human umbilical vein endothelial cells (34), that RSG prevented TNFα-stimulated HAEC-monocyte adhesion (21), and that RSG reduced circulating markers of endothelial activation in patients with vascular disease (46). In conjunction with the current findings, these reports indicate that RSG attenuates hyperglycemia-induced endothelial activation by suppressing NF-κB and the expression and activity of Nox4 as well as other potential NF-κB-regulated targets.

While RSG therapy improved endothelial dysfunction in diabetic patients (23, 35, 42), suggesting beneficial effects of RSG on the vascular endothelium, two meta-analyses indicated that RSG increased the incidence of myocardial infarction in diabetic patients (39, 47). In contrast, treatment with pioglitazone did not increase cardiovascular endpoints in diabetic patients (12, 13, 28, 52). The mechanisms underlying increased cardiovascular morbidity in diabetic patients treated with RSG have not been defined. These reports suggest that these adverse effects of RSG may be more related to the drug rather than to a thiazolidinedione class effect. Our results confirm that both RSG and pioglitazone attenuated glucose-induced Nox4 expression in HAECs, suggesting that PPARγ ligands may represent a novel therapeutic strategy for reducing the stimulated expression of selected NADPH oxidase subunits in vascular wall cells. Coupled with evidence that new classes of PPARγ-targeted drugs can preserve therapeutic benefits without causing adverse side effects (8), this indicates that therapeutic strategies targeting PPARγ merit additional investigation.

In summary, we investigated mechanisms by which the PPARγ ligand RSG attenuates vascular endothelial oxidative stress. Our findings indicate the NF-κB/p65-Nox4 axis as an important target of RSG in vascular endothelial cells following hyperglycemia. As illustrated in Fig. 7, our results support a signaling cascade in which high glucose concentrations stimulate NF-κB activation to enhance Nox4 expression and oxidative stress. These events contribute to endothelial cell activation and enhanced endothelial cell-monocyte interactions. Treatment with RSG attenuated this cascade by preventing NF-κB activation, Nox4 upregulation, oxidative stress, and increased endothelial-monocyte interactions. Because ROS can activate NF-κB, Nox4-generated ROS could lead to a self-perpetuating cycle of NADPH oxidase-derived ROS activating NF-κB thereby promoting persistent Nox4 expression (Fig. 7). PPARγ ligands can provide a novel therapeutic strategy to attenuate diabetes-associated alterations in endothelial gene expression and oxidative stress. These findings illustrate that in addition to regulating traditional gene targets, ligands for the PPARγ receptor can exert potent and potentially disease modifying effects on cells and tissues in a variety of pathophysiological scenarios. A more thorough understanding of the mechanistic underpinnings for PPARγ ligand effects will hopefully facilitate the optimal application of these compounds to disease prevention and therapy.

GRANTS
This work was supported by R01DK074518 from the National Institute of Diabetes and Digestive and Kidney Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: C.R.W., R.L.S., and C.M.H. conception and design of research; C.R.W. and X.L. performed experiments; C.R.W. and X.L. analyzed...
data: C.R.W., X.L., R.L.S., and C.M.H. interpreted results of experiments; C.R.W. and X.L. prepared figures; C.R.W. drafted manuscript; C.R.W., X.L., R.L.S., and C.M.H. edited and revised manuscript; C.R.W. and C.M.H. approved final version of manuscript.

REFERENCES


