Antioxidative, Antinitrative, and Vasculoprotective Effects of a Peroxisome Proliferator–Activated Receptor-γ Agonist in Hypercholesterolemia

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Background—Peroxisome proliferator–activated receptor (PPAR) signaling pathways have been reported to exert anti-inflammatory effects and attenuate atherosclerosis formation. However, the mechanisms responsible for their anti-inflammatory and antiatherosclerotic effects remain largely unknown. The present study tested the hypothesis that a PPARγ agonist may exert significant endothelial protection by antioxidative and antinitrative effects.

Methods and Results—Male New Zealand White rabbits were randomized to receive a normal (control) or a high-cholesterol diet and treated with vehicle or rosiglitazone (a PPARγ agonist) 3 mg · kg\(^{-1}\) · d\(^{-1}\) for 5 weeks beginning 3 weeks after the high-cholesterol diet. At the end of 8 weeks of a high-cholesterol diet, the rabbits were killed, and the carotid arteries were isolated. Bioactive nitric oxide was determined functionally (endothelium-dependent vasodilatation) and biochemically (the phosphorylation of vasodilator-stimulated phosphoprotein, or P-VASP). Vascular superoxide production, PPARγ, gp91\(^{phox}\), and inducible nitric oxide synthase (iNOS) expression, and vascular ONOO\(^{-}\) formation were determined. Hypercholesterolemia caused severe endothelial dysfunction and reduced P-VASP, despite a marked increase in iNOS expression and total NO\(_x\) production. Treatment with rosiglitazone enhanced PPARγ expression, improved endothelium-dependent vasodilatation, preserved P-VASP, suppressed gp91\(^{phox}\) and iNOS expression, reduced superoxide and total NO\(_x\) production, and inhibited nitrotyrosine formation.

Conclusions—The PPARγ agonist rosiglitazone exerted a significant vascular protective effect in hypercholesterolemic rabbits, most likely by attenuation of oxidative and nitrative stresses. The endothelial protective effects of PPARγ agonists may reduce leukocyte accumulation in vascular walls and contribute to their antiatherosclerotic effect.

**Key Words:** hypercholesterolemia ● endothelium ● inflammation ● atherosclerosis

Hypercholesterolemia and the subsequent formation of atherosclerosis is one of the most important risk factors for ischemic heart disease.\(^1\) Considerable evidence exists that hypercholesterolemia causes endothelial dysfunction, a prerequisite of atherosclerosis, in conduit vessels and small arteries. Numerous mechanisms have been proposed to explain this pathological alteration, including deficiencies of arginine supply, alteration of signaling mechanisms, alterations of nitric oxide synthase (NOS) expression or one of the cofactors involved in NOS activation, and increased destruction of nitric oxide (NO).\(^1\) Among these proposed mechanisms, superoxide inactivation of NO has been thought to be the most important mechanism of endothelial dysfunction in hypercholesterolemia. However, the enzymatic sources responsible for this increased superoxide production remain largely unknown.

Vasodilator-stimulated phosphoprotein (VASP), a family member of proline-rich proteins, was first characterized as a major phosphorylated protein in platelets and endothelial cells after stimulation with vasodilators such as prostaglandins and NO donors. Recent studies have demonstrated that VASP is the downstream target of both cAMP- and cGMP-dependent protein kinases (cAK and cGK), and reduced VASP phosphorylation (P-VASP) is a sensitive monitor of defective NO/cGMP signaling.\(^2\) However, the diagnostic and pathological significances of this newly identified phosphoprotein have not been determined in real pathological settings, such as hypercholesterolemia.

Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily. Emerging evidence indicates that the PPAR signaling pathways play critical roles in the regulation of a variety of...
biological processes within the cardiovascular system.\(^3\) Considerable evidence indicates that treatment with PPAR\(_\gamma\) agonists improves endothelial function in diabetic animal models and diabetic patients.\(^4\) However, whether this endothelial protective effect is secondary to improved glucose metabolism by the drug or PPAR\(_\gamma\) agonists exert direct endothelial protection remains unknown.

Several recent studies have demonstrated that PPAR agonists improve atherosclerosis by ameliorating systemic metabolic risk factors for atherogenesis and inflammatory events that occur within the artery wall.\(^5,6\) However, although substantial evidence exists that endothelial dysfunction is one of the earliest events in hypercholesterolemia and plays a critical role in the development of atherosclerosis,\(^1\) whether or not PPAR agonists may preserve vascular endothelial function and thus retard atherosclerosis has not been investigated previously.

Therefore, the purposes of the present study were to (1) provide direct evidence that the NO/cGMP/cGK signaling pathway is impaired in hypercholesterolemia, (2) identify possible enzymatic sources responsible for increased superoxide production in hypercholesterolemic vessels, (3) determine whether the PPAR\(_\gamma\) agonist rosiglitazone (RSG) may attenuate endothelial dysfunction in hypercholesterolemia, and (4) investigate the potential mechanisms by which PPAR agonists may exert their vasoprotective effects.

### Methods

#### Animals

Adult male New Zealand White rabbits (Covance, Denver, Pa) were randomly assigned to one of the following groups: control (C, normal diet, \(n=8\)), hypercholesterolemia (HC, 1% cholesterol diet for 8 weeks, \(n=10\)), and HC treated with RSG, a PPAR\(_\gamma\) agonist (oral gavage, 3 mg kg\(^{-1}\) d\(^{-1}\) for 5 weeks beginning 3 weeks after HC, \(n=10\)). At the end of 8 weeks of a high-cholesterol diet, rabbits were anesthetized, and tissue samples were collected immediately. The experiments were performed in adherence to National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

#### Determination of Endothelium-Dependent, NO-Mediated Vasorelaxation

Freshly harvested carotid arteries were placed into ice-cold Krebs-Henseleit buffer and cut into rings 3 to 4 mm long. Endothelial function was examined as described in our previous studies by comparing the vasorelaxation responses to acetylcholine (ACh), an endothelium-dependent vasodilator, with those to acidified NaNO\(_2\), an endothelium-independent vasodilator.\(^7\) Endothelial dysfunction was described as reduced vasorelaxation to ACh with a normal response to acidified NaNO\(_2\).

### Vascular Superoxide Production

Superoxide production was measured by lucigenin-enhanced chemiluminescence as described previously.\(^8\) Superoxide production was expressed as relative light units (RLU) per second per mg vessel dry weight (RLU \(\cdot\) mg\(^{-1}\) \(\cdot\) s\(^{-1}\)).

#### Immunoblotting

Proteins from tissue homogenate were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted with monoclonal antibodies against gp91\(^{phox}\), PPAR\(_\gamma\), endothelial NOS (eNOS) (Santa Cruz), VASP, and P-VASP (A.G. Scientific). The blot was developed with Super Signal Femto Reagent (Pierce) and visualized with a Kodak Image Station 400. The blot densities were analyzed with Kodak 1D software.

#### Determination of Total NO\(_x\) Content in Carotid Artery Tissue

Vascular NO and its in vivo metabolic products (NO\(_2\) and NO\(_3\)), collectively known as NO\(_x\), were determined by use of a chemiluminescent NO detector (Siever 280i) as described in our previous study.\(^9\)

#### Quantification of Tissue Nitrotyrosine Content

Nitrotyrosine content, a footprint of in vivo ONOO\(^-\) formation, was determined by an ELISA method described in our recent publication.\(^10\) The results were presented as pmol nitrotyrosine/mg protein.

#### Immunohistological Detection of PPAR\(_\gamma\), Inducible NOS, gp91\(^{phox}\), and Nitrotyrosine

Carotid arteries were removed and stored in 10% formalin for \(<48\) hours. Fixed artery segments were dehydrated and embedded in paraffin, and sections were cut at 5 \(\mu\)m and mounted onto glass slides. Immunohistochemical detections of PPAR\(_\gamma\), gp91\(^{phox}\), inducible NOS (iNOS), and nitrotyrosine were performed.

#### Statistical Analysis

All values in the text, table, and figures are presented as mean \(\pm\) SEM of \(n\) independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc \(t\) test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn’s post hoc test. Probabilities of \(P\leq0.05\) were considered to be statistically significant.

### Results

#### RSG Had No Effects on Plasma Lipid Profiles After a High-Cholesterol Diet

There were no significant differences in plasma lipid profiles before the onset of the study (time 0), after 3 weeks of high-cholesterol diet, or at the end of 5 weeks of RSG treatment (at 8 weeks, cholesterol, \(P=0.4953\); LDL, \(P=0.3541\) between vehicle and RSG-treated group) (Table). These results indicate that RSG failed to improve plasma lipid profiles in this diet-induced hypercholesterolemic
model; therefore, any vascular protective effects observed cannot be attributed to this mechanism.

**RSG Attenuated Hypercholesterolemia-Induced Endothelial Dysfunction**

In carotid artery rings isolated from control rabbits, ACh induced a concentration-dependent vasorelaxation. In contrast, carotid artery rings isolated from hypercholesterolemic rabbits treated with vehicle showed a severe right-shifting of their dose-response curve to ACh. Treatment with RSG markedly preserved ACh-induced vasorelaxation, indicating that the PPARγ agonist preserved endothelial function in hypercholesterolemic animals (Figure 1A). Addition of acidified NaNO₂, an exogenous NO donor, resulted in comparable and full relaxations in rings from all 3 groups, indicating that smooth muscle response to exogenous NO was normal in these vascular segments (Figure 1B).

Previous studies have demonstrated that many antioxidants preserve endothelial function in animals subjected to hypercholesterolemia. To explore the possibility that RSG may act as an antioxidant and thus improve endothelial function, RSG was added directly into the tissue bath containing carotid artery rings from vehicle-treated hypercholesterolemic rabbits. Preincubation of these rings with RSG (5 μmol/L for 30 minutes) failed to improve ACh-induced vasorelaxation (maximal relaxation, 60±5.9%). However, preincubation with MnTE-2-PyP³⁺ (50 μmol/L), a cell-permeable superoxide dismutase mimetic, markedly improved ACh-induced vasorelaxation (79±6.9%, P<0.01). Taken together, these results demonstrated that RSG attenuated hypercholesterolemia-induced endothelial dysfunction when administered in vivo via mechanisms other than direct superoxide scavenging.

**RSG Preserved Hypercholesterolemia-Induced Impairment of the NO/cGMP/cGK Signaling Pathway**

To determine the signaling pathway involved in hypercholesterolemia-induced endothelial dysfunction and to further document the vasoprotective effect of RSG, we examined P-VASP, a novel marker for NO/cGMP/cGK signaling, in vascular tissues. As illustrated in Figure 2A and summarized in Figure 2B, P-VASP was significantly diminished in vascular tissues from vehicle-treated rabbits, suggesting that hypercholesterolemia impaired NO/cGMP/cGK signaling. Treatment with RSG significantly enhanced P-VASP compared with vehicle-treated animals, indicating that RSG treatment maintained the integrity of a critical signaling system by which NO exerts most of its physiological effects.

**Antioxidant Effect of RSG as a Mechanism Responsible for Its Vascular Protection**

Considerable evidence suggests that increased superoxide generation and subsequent NO degradation is the major cause of endothelial dysfunction associated with hypercholesterolemia. To further identify the enzymatic source(s) responsible for superoxide generation under this particular pathological condition and, more importantly, to determine whether a PPARγ agonist may attenuate superoxide generation in hypercholesterolemic vessels, thus improving endothelial function, superoxide generation was assayed directly. Consistent with previously reported results, a 2.2-fold increase in superoxide generation was observed in vessels from HC rabbits receiving vehicle (98.5±7.2 versus 44.3±10.1 RLU·mg⁻¹·s⁻¹ in control, P<0.01). In vivo treatment with RSG markedly reduced superoxide production (56.5±7.1 RLU·mg⁻¹·s⁻¹, P<0.01 versus vehicle). Moreover, in vitro preincubation
(30 minutes, 37°C) of the vascular segments from HC rabbits with MnTE-2-PyP \(5 \times 10^{-6}\) mol/L, 28.2 ± 3.6 RLU · mg\(^{-1}\) · s\(^{-1}\), \(P<0.01\) versus vehicle) or DPI (an NADPH oxidase inhibitor, 100 μmol/L, 37.9 ± 5.8, \(P<0.01\) versus vehicle) but not with oxypurinol (100 μmol/L, 86.6 ± 4.4 RLU · mg\(^{-1}\) · s\(^{-1}\), \(P>0.05\) versus vehicle) or \(N^\circ\)-monomethyl-L-arginine (94.6 ± 7.1 RLU · mg\(^{-1}\) · s\(^{-1}\), \(P>0.05\) versus vehicle) markedly inhibited superoxide generation from HC rabbits, suggesting that NADPH oxidase is the primary source for increased \(O_2^\cdot\) in hypercholesterolemic vessels. Short-term incubation of carotid artery segments from hypercholesterolemic animals with RSG (5 μmol/L, 30 minutes) did not inhibit \(O_2^\cdot\) production, further indicating that RSG has no intrinsic antioxidant properties.

To further ensure that NADPH oxidase plays a major role in hypercholesterolemia-induced oxidative stress and that a PPARγ agonist may exert its antioxidant effect by suppressing NADPH oxidase expression, 2 additional experiments were performed. As illustrated in Figure 3A, the degree of gp91\(^{\text{phox}}\) staining was markedly increased in the vascular endothelium and in the adventitia of hypercholesterolemic rabbits receiving vehicle. This staining was significantly attenuated by RSG treatment. Western blotting demonstrated that hypercholesterolemia resulted in a 3.6-fold increase in gp91\(^{\text{phox}}\) expression and treatment with RSG significantly reduced its expression (Figure 3, B and C). Taken together, these results provide direct evidence that NADPH oxidase expression is upregulated by hypercholesterolemia and that PPARγ agonists may inhibit NADPH oxidase expression in hypercholesterolemic vessels, thus reducing superoxide production, preventing NO degradation, and improving endothelial function.

**Antioxidative Effect of RSG as a Mechanism Responsible for Its Vasculoprotection**

Having demonstrated that RSG exerted significant antioxidant effects and preserved endothelial function in hypercholesterolemia, we further investigated the effect of RSG on tissue nitrative stress associated with hypercholesterolemia. Despite a marked reduction in endothelium-dependent vasorelaxation and an impaired NO/cGMP/cGK signaling pathway, the total NO production, as determined by NO\(_x\) content, in vascular tissue was markedly increased (Figure 4). Treatment with RSG reduced total NO\(_x\) production compared with HC rabbits treated with vehicle. Hypercholesterolemia did not alter eNOS levels in the carotid artery, and treatment with RSG had no effect on its expression (representative Western blot presented in Figure 4; samples from 5 animals per group were studied). However, endothelial and smooth muscle expression of iNOS was significantly increased in vascular samples from hypercholesterolemic rabbits, and this was markedly attenuated by RSG treatment (Figure 4). To provide direct evidence that increased NO production (probably from iNOS) was inactivated by increased superoxide (probably from NADPH oxidase) before it could reach its normal physiological target (guanylate cyclase) and that RSG could block this reaction, we further determined the tissue nitroty-
ROS content by ELISA and its distribution by immunohistochemistry. As illustrated in Figure 5A, strong nitrotyrosine staining was detected in endothelial cells and smooth muscle cells from hypercholesterolemic rabbits, and treatment with RSG reduced nitrotyrosine staining. Quantitative ELISA results indicated that a 6.8-fold increase in nitrotyrosine content was observed in hypercholesterolemic rabbit tissue, and this increased nitrotyrosine content was markedly reduced by in vivo administration of RSG (Figure 5B).

RSG Upregulated PPARγ Expression in the Carotid Artery
Recent studies have demonstrated that PPARγ expression is reduced under certain pathological conditions, such as pulmonary hypertension, and that in vitro treatment with PPARγ agonists upregulates PPARγ expression. To determine whether hypercholesterolemia may alter PPARγ expression and in vivo treatment with RSG may upregulate PPARγ expression, 2 additional experiments were performed. As illustrated in Figure 6, in carotid arteries isolated from control rabbits, PPARγ is expressed in both endothelial cells and adventitial tissue. Hypercholesterolemia had no significant effect on PPARγ expression in adventitial tissue. However, PPARγ expression in endothelial cells was virtually abolished, and the total PPARγ protein content as measured by Western blot was significantly reduced (57% of control, n=5). Most interestingly, in vivo treatment with RSG in hypercholesterolemic rabbits upregulated PPARγ expression to a level even higher than that seen in control rabbits (1.7-fold increase over control, n=5).

Discussion
The present study confirmed previous reports that hypercholesterolemia induces endothelial dysfunction by increased O2− generation rather than decreased NO production. Moreover, using a novel molecular marker of the NO/cGMP/cGK signaling pathway, we have demonstrated that P-VASP at Ser239 is markedly reduced in diet-induced hypercholesterolemic vessels. In addition, our results provide the first direct evidence that NADPH oxidase but not xanthine oxidase or dysfunctional NOS is the primary source for O2− in vascular tissues from diet-induced hypercholesterolemic animals.

Impaired NO/cGMP/cGK in Hypercholesterolemic Vessels
The VASP was originally discovered as a substrate for both cAMP- and cGMP-dependent protein kinases in human platelets, but subsequent studies have demonstrated that VASP is expressed ubiquitously in many types of cells. Although its downstream signaling molecules and physiological roles remain unknown, P-VASP represents a novel biochemical marker for monitoring the NO-stimulated soluble guanylate cyclase (sGC)/cGK pathway and endothelial integrity in vascular tissue. Decreased P-VASP has been reported in vascular tissues from nitroglycerin-induced nitrate tolerance, chronic angiotensin II exposure, and spontaneous hyperlipidemic Watanabe rabbits. Our present study demonstrated for the first time that diet-induced hypercholesterolemia had no effect on VASP expression (total VASP); however, P-VASP was markedly reduced.

NADPH Oxidase as a Major O2− Source in Hypercholesterolemic Vessels
Increasing evidence indicates that NADPH oxidase is the predominant O2− source in both endothelial and smooth
muscle cells, and increased NADPH oxidase activity has been found in human atherosclerotic vessels and diabetic vessels. In a recent study, Warnholtz et al reported that NADPH-oxidase activity in aortic homogenates from hyperlipidemic Watanabe rabbits and cholesterol-fed rabbits was increased significantly. In accordance with this study, we have demonstrated that O$_2^-$ production is markedly inhibited by in vitro treatment with DPI but not N$^\omega$-monomethyl-L-arginine in carotid arteries from diet-induced hypercholesterolemic rabbits. In addition, we have provided, for the first time, direct evidence that expression of gp91$^\text{phox}$, a membrane-bound subunit of NADPH oxidase, is markedly increased in endothelial and adventitial tissue from hypercholesterolemic rabbits. These results provide firm evidence that NADPH oxidase is a significant source of O$_2^-$ in hypercholesterolemic vessels.

**Vascular Protective Effects of a PPARγ Agonist and Its Potential Mechanisms**

The most important finding of the present study is that treatment with RSG markedly improved NO bioavailability in a nondiabetic animal model. There are several potential mechanisms by which a PPAR agonist may exert its vascular protection in hypercholesterolemia. PPARγ activators have been found to decrease triglyceride concentrations and increase HDL cholesterol. The improvement in lipid profile may thus attenuate hypercholesterolemia-induced endothelial dysfunction. However, this mechanism is unlikely to contribute to the vascular protection exerted by RSG (a PPARγ agonist), because this treatment failed to improve the lipid profile in our experiment. PPARγ agonists improve insulin sensitivity and glucose metabolism in the diabetic patient. However, it is unlikely that insulin resistance plays a significant role in endothelial dysfunction associated with diet-induced hypercholesterolemia.

The present experiment demonstrated that RSG most likely achieves its endothelial protection via inhibition of O$_2^-$ production and subsequent NO degradation. A variety of antioxidants, such as ascorbate, α-tocopherol, and cardedol, have been reported to improve endothelial dysfunction induced by hypercholesterolemia. However, unlike these antioxidants, RSG failed to reduce superoxide production or improve endothelium-dependent vasodilatation after in vitro incubation with hypercholesterolemic vessels. Therefore, our results suggest that PPARγ agonists may inhibit superoxide-producing enzyme(s) expression and/or increase antioxidant enzyme(s) expression. In a recent study, Inoue et al demonstrated that in vitro treatment of endothelial cells with pioglitazone increased CuZn-superoxide dismutase gene expression and inhibited p22$^\text{phox}$ and p47$^\text{phox}$ expression. In the present study, we have provided direct evidence that in vivo RSG treatment markedly attenuated the expression of gp91$^\text{phox}$, a membrane-bound subunit of NADPH oxidase, in hypercholesterolemic vessels.

Emerging evidence suggests that in addition to oxidative stress, nitrosative stress also plays a significant role in tissue injury. Recent in vitro studies have demonstrated that PPARγ agonists exert significant anti-inflammatory effects, as evidenced by reduced adhesion molecule expression and decreased iNOS expression. In addition, Linscheid et al recently reported that in addition to its inhibitory effect on iNOS gene expression, RSG may inhibit NO production by reducing tetrahydrobiopterin production in adipocytes. Our present study demonstrated that treatment with a PPARγ agonist in vivo exerted significant antiinflammatory effects, as evidenced by reduced iNOS expression, total NO production, and nitrotyrosine formation. This unique effect has not been reported with other treatments such as angiotensin II type 1 receptor antagonists (Bay 10-6734) and ascorbate, although these treatments also reduce oxidative stress. Taken together, our present study demonstrated for the first time that the PPARγ agonist RSG exerted significant endothelial protective effects by reducing both oxidative stress (reduced superoxide generation) and nitrosative stress (prevented excessive NO production and ONOO$^-$ formation).

The endothelial protective effects of PPARγ agonists are of clinical significance. The endothelium plays a crucial role in the process of atherosclerotic disease. Endothelial dysfunction causes upregulation of adhesion molecules, enhances migration of monocytes into the vessel wall, and increases proliferation of smooth muscle cells. Therefore, therapeutic interventions that are capable of preserving the NO/cGMP signaling pathway and improving endothelial function, such as PPARγ agonists, may have tremendous application in the treatment of cardiovascular diseases.

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**References**


