PPARγ activation, by reducing oxidative stress, increases NO bioavailability in coronary arterioles of mice with Type 2 diabetes

Zsolt Bagi, Akos Koller, and Gabor Kaley

Department of Physiology, New York Medical College, Valhalla, New York 10595

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Bagi, Zsolt, Akos Koller, and Gabor Kaley. PPARγ activation, by reducing oxidative stress, increases NO bioavailability in coronary arterioles of mice with Type 2 diabetes. Am J Physiol Heart Circ Physiol 286: H742–H748, 2004. First published October 9, 2003; 10.1152/ajpheart.00718.2003.—We tested the hypothesis that short-term treatment of mice with Type 2 diabetes mellitus (DM) with rosiglitazone (ROSI), an agonist of peroxisome proliferator-activated receptor-γ, ameliorates the impaired coronary arteriolar dilation by reducing oxidative stress via a mechanism unrelated to its effect on hyperglycemia and hyperinsulinemia. Control and Type 2 DM (db/db) mice were treated with ROSI (3 mg·kg⁻¹·day⁻¹) for 7 days, which did not significantly affect their serum concentration of glucose and insulin. Compared with controls, in db/db mice serum levels of 8-isoprostane and dihydroethydine-detectable superoxide production in carotid arteries were significantly elevated and were reduced by ROSI treatment. In coronary arterioles (diameter, ~80 μm) isolated from db/db mice, the reduced dilations to ACh, the nitric oxide (NO) donor NONOate, and increases in flow were significantly augmented either by in vitro administration of apocynin, an inhibitor of NAD(P)H-oxidase, or by in vivo ROSI treatment, responses that were then significantly reduced by the NO synthase inhibitor L-arginine methyl ester. In aortas of db/db mice, activity of SOD and catalase were reduced, whereas NAD(P)H oxidase activity was enhanced. ROSI treatment enhanced catalase and reduced NAD(P)H oxidase activity but did not affect the activity of SOD. These findings suggest that ROSI treatment enhances NO mediation of coronary arteriolar dilations due to the reduction of vascular NAD(P)H oxidase-derived superoxide production and enhancement of catalase activity. Thus, in addition to the previously revealed beneficial metabolic effects, the antioxidant action of rosiglitazone may protect coronary arteriolar function in Type 2 DM.

superoxide; catalase; NAD(P)H oxidase; rosiglitazone, endothelium

Type 2 diabetes mellitus (DM) is associated with a markedly increased prevalence of ischemic heart disease accounting for the high incidence of death in the diabetic population (26, 29). It has been proposed that in Type 2 DM, prior to and/or in addition to epicardial coronary atherosclerosis, functional changes of the coronary microcirculation can lead to a mismatch of myocardial supply and demand, thereby provoking ischemic episodes in the diabetic heart (7).

Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated transcription factor belonging to the nuclear receptor superfamily (24). PPARγ is a regulator of lipid and glucose metabolism (24) and therefore is the target of insulin sensitizing drugs, such as thiazolidinediones, which are frequently used to treat metabolic complications associated with Type 2 DM (20, 23). It has been shown that in Type 2 diabetic subjects, long-term activation of PPARγ by thiazolidinediones reduces plasma levels of insulin and glucose with the subsequent attenuation of vascular dysfunction (5, 30).

PPARγ is also expressed in vascular tissues, specifically in vascular smooth muscle cells (18) and endothelium (17). Recent studies (20) suggest that activators of PPARγ, via a mechanism that is unrelated to lipid and carbohydrate metabolism, may also protect vascular function in DM. Acute administration of troglitazone, which is unlikely to have metabolic effects, increased forearm occlusion-induced vasodilation in humans (10), whereas a high dose of troglitazone increased skin blood flow in both normal and streptozotocin-induced diabetic rats (11). Also, in porcine pulmonary artery and human umbilical vein endothelial cells in culture, PPARγ ligands increased the release of nitric oxide (NO) (6). Thus these findings lead to the hypothesis that PPARγ ligands have vascular actions, which may be unrelated to their activity to modify metabolic disturbances.

It is well documented that oxidative stress contributes importantly to the development of vascular dysfunction in diabetes (3, 13, 28). In diabetic subjects, NO mediation of vascular responses is impaired (8) primarily by the increased production of reactive oxygen species. In this context, we (2) previously found that in vitro administration of SOD by scavenging superoxide anions restores NO-mediated dilations of isolated coronary arterioles of Type 2 diabetic mice.

At present, there are no functional studies extant, which would support the idea that activation of PPARγ in vivo would, in addition to its metabolic effects, significantly improve NO-mediated oxidative stress-sensitive dilations of microvessels in Type 2 DM. There are many serious complications associated with Type 2 DM, including ischemic heart disease, which is related to coronary microvascular dysfunction (4, 19). Thus it seemed to be important to test the hypothesis that short-term treatment of mice with Type 2 DM with an activator of PPARγ (rosiglitazone) enhances NO mediation of flow-dependent dilation of coronary arterioles by reducing oxidative stress.

Materials and Methods

Experimental protocols. Twelve-week-old male wild-type (control, C57BL/KsJ-db⁺/db⁻, n = 35) and Type 2 diabetic db/db (C57BL/KsJ-db⁻/db⁻, n = 35) mice (Jackson Laboratories) were used (15). Animals were fed standard chow and given tap water freely. Mice were housed in the animal care facility. The Animal Care and Use Committee of New York Medical College approved all protocols. Control (n = 14) and db/db (n = 14) mice were treated with the PPARγ synthetic agonist rosiglitazone (3 mg·kg⁻¹·day⁻¹; Cayman Chemicals) or vehicle for 7 days by oral gavage (0.1 ml/mouse). In

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Address for reprint requests and other correspondence: G. Kaley, Dept. of Physiology, New York Medical College, Valhalla, New York 10595 (E-mail: gabor_kaley@nymc.edu).
separate experiments, control and db/db mice (n = 7–12) were treated with rosiglitazone plus the PPARγ antagonist GW-9662 (3 mg·kg⁻¹·day⁻¹; Cayman Chemicals) or vehicle for 7 days. Mice were then anesthetized with an intraperitoneal injection of xylazine and ketamine (50 and 12 mg/kg, respectively). Blood collected from the femoral artery was centrifuged immediately, and the serum was stored at −80°C. Serum glucose concentrations were measured with commercial glucose assay kits (Sigma). Serum insulin (ALPCO Diagnostics) and 8-isoprostane (Cayman Chemicals) levels were determined with commercially available ELISA kits (9). Aortas were removed from control, db/db, and rosiglitazone-treated db/db mice and cleared of connective tissue. Tissues were homogenized, protein was extracted, and concentrations were determined with the Bradford protein assay (Sigma). Aliquots were then homogenized, protein was extracted, and concentrations were determined with the Bradford protein assay (Sigma). Aliquots were separated by electrophoresis on a 4–15% polyacrylamide gradient gel (Bio-Rad) at 125 V for 1 h and transferred onto a polyvinylidene difluoride membrane. Membranes were incubated with specific polyclonal antibody to PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were revealed with chemiluminescence and visualized autoradiographically. Optical density of bands was quantified by Scion Image software.

Detection of superoxide production. Dihydroethylenediamine (DHE) was used to localize superoxide production (9). Briefly, carotid arteries were fixed in 4% formalin, embedded in optimal cutting temperature (OCT) compound, five db/db, and five rosiglitazone-treated mice. Before being cut, single arteries were incubated with oxygenated physiological salt solution (PSS) for 50 min at 37°C and then stained with DHE (5 × 10⁻⁶ M; Molecular Probes) for 10 min. After being washed, single arteries were embedded and cut with a cryostat, and frozen sections of single arteries were then visualized by fluorescence microscopy (Olympus) and stained with hematoxylin and eosin. The separately obtained ethidium bromide (EB) fluorescent and hematoxylin and eosin images were overlaid using Photoshop 6.0 image software, and the number of EB stained fluorescent nuclei was then counted in five sections obtained from control, db/db, and rosiglitazone-treated db/db mice (2).

Isolation of coronary arterioles. With the use of microsurgery instruments and an operating microscope, a branch of the septal coronary artery (~0.5 mm in length) running intramuscularly was isolated, cannulated, and pressurized as described previously (2) using a pressure servocontrol system (Living Systems Instrumentation). The internal arterial diameter at the midpoint of the arterial segment was measured by videomicroscopy with a microangiometer (Texas Instruments), and changes in arteriole diameter and intraluminal pressure were continuously recorded with the Biopac-MP100 system (Biopac Systems).

Agonist and flow-induced coronary arteriolar responses. Cumulative doses of ACh (10⁻⁷–10⁻⁶ M), the NO donor NONOate (10⁻⁷–10⁻⁶ M), and adenosine (10⁻⁶–10⁻⁴ M) were used to test the function of endothelium and smooth muscle of arterioles. Coronary arteriolar responses were also obtained to step increases in intraluminal flow (0–20 μL/min). Each flow rate was maintained for 5 min to allow the vessel to reach a steady-state diameter. Isolated arterioles were then incubated with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (10⁻⁴ M for 20 min), and agonist- and flow-induced responses were obtained again. In separate experiments flow-induced dilations of coronary arterioles were tested after incubation with apocynin (10⁻⁴ M for 30 min), an inhibitor of NAD(P)H oxidase.

SOD, catalase, and NAD(P)H oxidase activity assays. In enzyme-activity assays, aortas were homogenized and centrifuged according to the protocols provided by the manufacturer. Total SOD and catalase activity were measured with commercial enzyme assay kits (Cayman Chemicals). NAD(P)H oxidase activity was estimated by a modified enzyme activity assay previously described (22, 25). Briefly, aortas were homogenized and centrifuged in HEPES buffer (pH 7.4), and protein concentrations were measured with Bradford reagent. Tetrazolium salt radical detector (Cayman Chemicals) dissolved in HEPES buffer (200 μL) was added to 96-microwell plates. Equal volumes (20 μL) of protein samples were then added to separate wells, and the reaction was initiated by 100 μM NADH and 100 μM NADPH. Absorbs at 450 nm were measured with a plate reader (Bio-Tek) every 10 min for a 1-h period at room temperature. Parallel experiments were carried out in the presence of additional apocynin (10⁻⁴ M), an inhibitor of NAD(P)H oxidase. Changes in absorbances were then normalized to the protein concentrations used.

Data are expressed as means ± SE. Agonist- and flow-induced arteriolar responses were expressed as changes in arteriolar diameter as a percentage of the maximal dilation defined as the passive diameter of the vessel at 80-mmHg intraluminal pressure in a Ca²⁺-free medium. Statistical analyses were performed by two-way repeated-measures ANOVA followed by Tukey’s post hoc test or Student’s t-test as appropriate. P < 0.05 was considered statistically significant.

RESULTS

At 12 wk of age, the body weight, serum glucose, and serum insulin of db/db mice were significantly elevated compared with age-matched lean control mice (Table 1). Seven days of rosiglitazone treatment did not significantly affect body weight, serum glucose, and insulin levels of control and db/db mice (Table 1). These findings indicate that this short period of rosiglitazone treatment had no significant effect on the hyperglycemia and hyperinsulinemia in db/db mice. Serum levels of 8-isoprostane were significantly enhanced in db/db and were significantly reduced by rosiglitazone treatment (Fig. 1A). Expression of PPARγ was not significantly different in aortic vascular tissue of control, db/db, and db/db mice receiving one-wk of rosiglitazone treatment (Fig. 1B).

In situ detection of vascular superoxide production. DHE, an oxidative fluorescent dye, was used to detect superoxide production in situ in the wall of carotid arteries. Compared with controls, fluorescent photomicrographs of EB staining showed an increased number of fluorescence-labeled nuclei in carotid arterial sections of db/db mice, which was significantly reduced by rosiglitazone treatment (Fig. 1C).

Agonist-induced coronary arteriolar dilation. There were no significant differences between active and passive (in Ca²⁺-free PSS) diameters of coronary arterioles from the different aortic vascular tissue of control, db/db, and db/db mice receiving one-wk of rosiglitazone treatment (Fig. 1B).

Table 1. Characteristics of rosiglitazone-treated control and db/db mice including basal diameters and agonist-induced dilations of isolated coronary arterioles

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + ROSI</th>
<th>db/db</th>
<th>db/db + ROSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>34±2</td>
<td>36±3</td>
<td>47±5*</td>
<td>50±2*</td>
</tr>
<tr>
<td>Serum glucose, mM</td>
<td>5.3±0.4</td>
<td>5.1±0.4</td>
<td>19.1±2.1*</td>
<td>18.6±2.3*</td>
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<td>Serum insulin, pM</td>
<td>180±21</td>
<td>188±57</td>
<td>2,542±125*</td>
<td>2,620±334*</td>
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<tr>
<td>Passive diameter, μm</td>
<td>111±9</td>
<td>109±9</td>
<td>113±4</td>
<td>105±6</td>
</tr>
<tr>
<td>Active diameter, μm</td>
<td>79±7</td>
<td>80±7</td>
<td>85±5</td>
<td>82±6</td>
</tr>
<tr>
<td>Dilation to ACh</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>(10⁻⁷ M), %</td>
<td>54±8</td>
<td>49±1</td>
<td>28±8*</td>
<td>45±6</td>
</tr>
<tr>
<td>+ L-NAME</td>
<td>41±3*</td>
<td>39±3*</td>
<td>26±4*</td>
<td>31±2*</td>
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<tr>
<td>Dilation to NONO-ate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10⁻⁷ M), %</td>
<td>46±7</td>
<td>40±4</td>
<td>20±3*</td>
<td>32±5*†</td>
</tr>
<tr>
<td>+ L-NAME</td>
<td>46±2</td>
<td>42±6</td>
<td>17±7*</td>
<td>34±2*</td>
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<tr>
<td>Dilation to adenosine</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>(10⁻⁷ M), %</td>
<td>62±8</td>
<td>65±5</td>
<td>57±4</td>
<td>62±1</td>
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<tr>
<td>+ L-NAME</td>
<td>60±4</td>
<td>61±6</td>
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</tbody>
</table>

Values are means ± SE; n = 7–10 control and db/db mice treated with rosiglitazone (ROSI) or vehicle. L-NAME, Nω-nitro-L-arginine methyl ester.

*P < 0.05 vs. control. †P < 0.05 vs. db/db.
groups of mice (Table 1). In coronary arterioles of db/db mice, dilations to ACh and the NO donor NONOate were significantly reduced and were significantly enhanced by rosiglitazone treatment (Table 1). l-NAME significantly reduced the ACh-induced arteriolar dilations of control, rosiglitazone-treated control, and db/db mice, but it did not affect responses of arterioles from db/db mice (Table 1). Adenosine-induced dilations were not different in vessels of the different groups of animals (Table 1).

Flow-induced coronary arteriolar dilation. Increases in intraluminal flow resulted in substantial dilations of coronary arterioles isolated from control mice, responses that were significantly reduced in arterioles of db/db mice (Fig. 2A). Incubation with apocynin, a NAD(P)H oxidase inhibitor, restored flow-induced dilation to control magnitude (Fig. 2B). Rosiglitazone treatment significantly enhanced flow-induced dilation (Fig. 2B), which was reduced by in vitro administration of l-NAME or by in vivo cotreatment with the PPARγ antagonist GW-9662 (Fig. 2D). In control mice, flow-induced coronary dilations were not affected by rosiglitazone or rosiglitazone and GW-9662 but were reduced by l-NAME (Fig. 2C).
SOD, catalase, and NAD(P)H oxidase activity assays. Compared with control, SOD activity was significantly reduced in aortas of db/db mice, which was unaffected by rosiglitazone treatment (Fig. 3A). Catalase activity was also significantly reduced in aortas of db/db mice but, in contrast to SOD activity, was restored by rosiglitazone treatment (Fig. 3B). NAD(P)H oxidase activity of aortic tissue of db/db mice was significantly enhanced, which was reduced by rosiglitazone treatment (Fig. 3C). In the presence of apocynin, NAD(P)H oxidase activity was significantly reduced in aortas of control and db/db-mice but not in those of rosiglitazone-treated mice (Δabsorbance at 60 min: 0.94 ± 0.04, 1.12 ± 0.11, 1.29 ± 0.13, respectively).

DISCUSSION

The main findings of the present study are that, despite the presence of hyperglycemia and hyperinsulinemia, short-term treatment of Type 2 DM mice with the PPARγ activator rosiglitazone augments NO-mediated, flow-dependent dilations of coronary arterioles; decreases the serum levels of 8-isoprostane; and decreases superoxide levels in carotid arteries. These changes are associated with a reduction in vascular NAD(P)H oxidase activity and enhancement of vascular catalase activity.

PPARγ-targeted thiazolidinediones (23), such as rosiglitazone, are widely used in the treatment of insulin-resistant states, in particular, Type 2 DM, but their complex mechanism of action and effect on blood vessels are not yet understood (20). Emerging evidence indicates that activation of PPARγ, in addition to effects on lipid and carbohydrate metabolism, may have an additional role in protecting vascular function (20). In this context, it has been found previously that troglitazone administration is associated with improvement of forearm occlusion-induced vasodilation in patients with insulin resistance (27) or Type 2 DM (5), although in these studies the exact mechanisms of action of PPARγ activation remain obscure.

The beneficial vascular effects of the PPARγ activator rosiglitazone may be surmised by considering the nature of the dysfunction that develops in Type 2 DM. In DM, elevated levels of reactive oxygen species have been documented, (8, 13), and it has been proposed that either decreased activity of tissue antioxidants, such as SOD and catalase (1, 21), or enhanced activity of reactive oxygen species-producing enzymes, such as NAD(P)H oxidase (14), might contribute to this process. In the present study, we have found that in aortas of db/db mice, the activity of SOD and catalase was significantly reduced, whereas the NAD(P)H oxidase activity was significantly enhanced (Fig. 3), which may result in an enhanced level of vascular reactive oxygen species.

The important functional consequence of increased vascular oxidative stress is indicated by our previous study (2) showing that in coronary arterioles isolated from db/db mice, flow-, ACh-, and NONOate-induced dilations were reduced, which
could well contribute to the impaired regulation of coronary microvascular resistance in Type 2 DM. The reduced dilations to flow, ACh, and NONOate could be reversed by administration of SOD to the organ chamber, suggesting that in coronary arterioles of \(db/db\) mice, an enhanced level of oxidative stress is present in both the endothelial and smooth muscle layers of microvessels, which can interact with either endothelium-derived NO or that which is released from the NO donor \((2)\). Furthermore, our finding that in vitro administration of the NAD(P)H oxidase inhibitor apocynin also restored flow-induced coronary arteriolar dilation indicates that vascular NAD(P)H oxidase is the main source of the enhanced superoxide production in coronary microvessels (Fig. 2A). In line with these findings, Guzik et al. \((14)\) recently have also found enhanced NAD(P)H oxidase-derived superoxide production in the internal mammary artery of Type 2 DM patients.

On the basis of previous findings, we hypothesized that even short-term rosiglitazone treatment of mice with Type 2 DM would, by reducing oxidative stress, prevent the development of vascular dysfunction. In the present study, we have found that compared with controls, in \(db/db\) mice, the serum level of 8-isoprostane (a marker of in vivo lipid peroxidation/oxidative stress) \((21)\) was significantly elevated, which was reduced by rosiglitazone treatment (Fig. 1A), supporting the hypothesis that rosiglitazone has an in vivo antioxidant effect in Type 2 DM.

Next, we showed that vascular tissues express PPAR\(\gamma\) but that the protein levels were not significantly different in control and \(db/db\) mice and that they were unaffected by rosiglitazone treatment (Fig. 2B). However, even short-term rosiglitazone treatment of \(db/db\) mice significantly reduced the enhanced vascular superoxide production in isolated carotid arteries, as detected by DHE staining (Fig. 1B), suggesting that rosiglitazone has an in vivo antioxidant effect in vascular tissues in Type 2 DM.

The important functional consequences of in vivo antioxidant effect of PPAR\(\gamma\) activation on coronary arteriolar function were also addressed. Results of these experiments show that in coronary arterioles isolated from \(db/db\) mice, rosiglitazone treatment significantly enhanced the impaired ACh- and NONOate-induced dilations (Table 1). The specific action of rosiglitazone is indicated by the finding that rosiglitazone did not affect dilation to adenosine (Table 1), a response that is not sensitive to the presence of reactive oxygen species. Rosiglitazone treatment significantly enhanced flow-induced dilations of \(db/db\) mice (Fig. 2B) but did not affect flow-induced responses of control vessels (Fig. 2C). The enhanced flow-induced dilations of rosiglitazone-treated \(db/db\) mice were inhibited by the NO synthase inhibitor L-NAME (Fig. 2D). Also, cotreatment of animals with the PPAR\(\gamma\) activator rosiglitazone and the PPAR\(\gamma\) antagonist GW-9662 inhibited the rosiglitazone-induced enhancement of flow-induced dilations in coronary arterioles of \(db/db\) mice (Fig. 2D), but did not affect control responses (Fig. 2C), suggesting that the beneficial effects of rosiglitazone treatment was likely due to the activation of PPAR\(\gamma\). Collectively, these findings suggest that rosiglitazone, by activating vascular PPAR\(\gamma\), prevents the impairment of NO mediation of coronary arteriolar...
dilations, most likely by enhancing NO bioavailability via a reduction of the level of vascular reactive oxygen species.

Several mechanisms can be proposed to explain the antioxidant effect of rosiglitazone shown in the present study. Previously, it has been found that in endothelial cells in culture, activation of PPARγ by troglitazone or pioglitazone (other thiazolidinediones) reduced the protein expression of the NAD(P)H oxidase subunit p22phox and enhanced the expression of CuZn-SOD (16). Also, the functional PPARγ response element has recently been identified in the rat catalase promoter of brain microvascular endothelial cells (12). These findings suggest that activation of PPARγ may exert an antioxidant activity by favorably altering the expression of specific enzymes participating in the production and/or elimination of reactive oxygen species. As discussed before, in the present study, we found that in aortic vascular tissue of db/db mice, SOD and catalase enzyme activities were significantly decreased, whereas the apocynin-sensitive NAD(P)H oxidase activity was enhanced (Fig. 3). Rosiglitazone treatment significantly augmented the vascular catalase activity of db/db mice, but it did not affect the activity of SOD (Fig. 3). Furthermore, rosiglitazone treatment was accompanied by significant reduction of NAD(P)H oxidase activity, which was not affected by additional apocynin (Fig. 3). These findings together provide in vivo functional evidence supporting the idea that PPARγ activation can induce favorable changes in oxidant/antioxidant enzyme expression, an activity independent of its actions on cellular metabolism.

On the basis of our results, we have delineated the hypothesized role of PPARγ activation affecting coronary arteriolar dilation and lipid peroxidation in Type 2 DM (Fig. 3D). In Type 2 DM, due to the enhanced activity of vascular NAD(P)H oxidase, together with a decreased activity of SOD, the vascular level of superoxide anion is enhanced, which can reduce the NO mediation of agonist- and flow-induced dilations of coronary arteries. In addition, the reduced activity of catalase may result in enhanced hydroxyl radical production leading to enhanced lipid peroxidation in Type 2 DM. Even short-term activation of PPARγ by rosiglitazone reduces NAD(P)H oxidase and enhances catalase activity causing a reduction of superoxide and hydroxyl radical production, thereby enhancing NO mediation of coronary vasodilation and reducing lipid peroxidation in Type 2 DM.

In summary, in the present study, we demonstrated that despite the presence of hyperglycemia and hyperinsulinemia, short-term treatment of Type 2 diabetic mice with the PPARγ activator rosiglitazone augments NO-mediated flow-dependent dilations of coronary arteries by reducing vascular superoxide production via a favorable alteration of oxidant/antioxidant enzyme activities. The functionally important antioxidant activity of the PPARγ ligand revealed in the present study may add to the understanding of the mechanisms by which these agents improve the function of coronary vessels and thus slow or prevent the development of miocardial ischemia in Type 2 DM.

REFERENCES


