Pioglitazone protects the myocardium against ischemia-reperfusion injury in eNOS and iNOS knockout mice

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Ye Y, Lin Y, Manickavasagam S, Perez-Polo JR, Tieu BC, Birnbaum Y. Pioglitazone protects the myocardium against ischemia-reperfusion injury in eNOS and iNOS knockout mice. Am J Physiol Heart Circ Physiol 295: H2436–H2446, 2008. First published October 17, 2008; doi:10.1152/ajpheart.00690.2008.—Endothelial nitric oxide synthase (eNOS) activation with subsequent inducible NOS (iNOS), cytosolic phospholipase A2 (cPLA2), and cyclooxygenase-2 (COX2) activation is essential to statin inhibition of myocardial infarct size (IS). In the rat, the peroxisome proliferator-activated receptor-γ agonist pioglitazone (Pio) limits IS, upregulates and activates cPLA2 and COX2, and increases myocardial 6-keto-PGF₁α levels without activating eNOS and iNOS. We asked whether Pio also limits IS in eNOS−/− and iNOS−/− mice. Male C57BL/6 wild-type (WT), eNOS−/−, and iNOS−/− mice received 10 mg·kg−1·day−1 Pio (Pio+) or water alone (Pio−) for 3 days. Mice underwent 30 min coronary artery occlusion and 4 h reperfusion, or hearts were harvested and subjected to ELISA and immunoblotting. As a result, Pio reduced IS in the WT (15.4 ± 1.4% vs. 39.0 ± 1.1%; P < 0.001), as well as in the eNOS−/− (32.0 ± 1.6% vs. 44.2 ± 1.9%; P < 0.001) and iNOS−/− (18.0 ± 1.2% vs. 45.5 ± 2.3%; P < 0.001) mice. The protective effect of Pio in eNOS−/− mice was smaller than in the WT (P < 0.001) and iNOS−/− (P < 0.001) mice. Pio increased myocardial Ser633 and Ser1177 phosphorylated eNOS levels in the WT, and Ser1177 phosphorylated eNOS levels in the iNOS−/− mice. iNOS was undetectable in all six groups. Pio increased cPLA2, COX2, and PGI2 synthase levels in the WT, as well as in the eNOS−/− and iNOS−/− mice. Pio increased the myocardial 6-keto-PGF₁α levels and cPLA2 and COX2 activity in the WT, eNOS−/−, and iNOS−/− mice. In conclusion, the myocardial protective effect of Pio is iNOS independent and may be only partially dependent on eNOS. Because eNOS activity decreases with age, diabetes, and advanced atherosclerosis, this effect may be relevant in a clinical setting and should be further characterized.

PROTECTION AGAINST ischemia-reperfusion injury may have a potential benefit in three distinct clinical situations: 1) limiting reperfusion injury in patients presenting with ST-elevation acute myocardial infarction before undergoing reperfusion therapy; 2) short- to intermediate-term treatment before elective procedures that may pose a risk for myocardial ischemia, such as cardiac or noncardiac surgery or percutaneous coronary interventions; and 3) long-term treatment in patients with established coronary artery disease to minimize the damage from an effort-induced ischemia or a potential future myocardial infarction. In the present study we studied whether the myocardial protection effects of intermediate-term pretreatment with pioglitazone (Pio) is dependent on nitric oxide (NO) synthases (NOSs), using a model that may be relevant to the second option mentioned above.

Periprocedural myocardial infarction following surgery or percutaneous coronary interventions are associated with mortality and morbidity (39). Of the 11 million adults aged over 50 yr undergoing noncardiac surgery in the United States annually (www.CDC.gov), 3.9% will suffer a major perioperative cardiac event and 1.3% (or 143,000 patients) will have a fatal cardiac event (39). The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been shown to decrease cardiovascular morbidity and mortality when administered before elective surgery or percutaneous coronary interventions (39), and recently, the American College of Cardiology/American Heart Association guidelines gave class IIa recommendation for the initiation of statin therapy before vascular surgery and class IIb recommendation for the initiation of statin therapy in patients with risk factors scheduled for intermediate risk surgery (21). Animal studies have shown that statins protect against ischemia-reperfusion injury and when administered before ischemia (2, 5, 7, 31, 32, 46, 48, 49, 54, 55, 59, 62, 63, 68, 69), or immediately upon reperfusion (4, 18, 62), limit myocardial infarct size (IS). The activation of eNOS is essential for the IS-limiting effects of late ischemic preconditioning (8, 9, 53, 66). Similarly, several investigators have shown that the activation of Akt and endothelial NOS (eNOS) is essential for the myocardial protective effect of statins, since nonspecific NOS inhibitors blunt the IS-limiting effect of statins (5, 63) and statins do not reduce IS in eNOS−/− mice (1, 4, 19, 31, 68, 71). Inducible NOS (iNOS) activation is also essential for mediating the IS-limiting effects of statins. Scalia et al. (49) has shown that simvastatin does not limit IS in iNOS−/− mice. Our laboratory showed that 1400W, a specific iNOS inhibitor, abrogated the IS-limiting effects of atorvastatin in the rat without affecting eNOS (2) and, more recently, that atorvastatin failed to decrease IS in iNOS−/− mice (71). The cardioprotection by preconditioning diminishes with age (17, 20, 30), hypertension (17, 20), diabetes (20), and atherosclerosis (20). The dependence on NOS activation may be a limitation, since it has been shown that both vascular (50, 52) and myocardial (44, 52) eNOS activity decreases with age, diabetes, and advanced atherosclerosis. It has been sug-
gested that the dissociation of cavelon from cavoloce occurs with aging and heart failure, leading to a decrease NOS activity (44).

Thiazolidinediones, a class of drugs with peroxisome proliferator-activated receptor-γ (PPARγ) agonist activity, have been shown to reduce myocardial IS in the rat (29, 58, 60, 69, 73, 74). In a study that compared the IS-limiting effects of atorvastatin and Pio in the rat, our group has found that a 3-day pretreatment with oral Pio at 10 mg·kg⁻¹·day⁻¹ limited IS and caused an insignificant increase in myocardial levels of phosphorylated (p)-Akt, without a detectable change in Ser1177 p-eNOS, and -iNOS levels (69). In contrast, atorvastatin (10 mg·kg⁻¹·day⁻¹) caused a significant increase in myocardial p-Akt, Ser1177 p-eNOS and -iNOS levels (69). Both atorvastatin and Pio upregulated cytosolic phospholipase A₂ (cPLA₂) and cyclooxygenase-2 (COX2) expression and activity, and the IS-limiting effects of both agents were abrogated by COX2 inhibitors (69). Thus it seems that statins and Pio activate cPLA₂ and COX2 by different mechanisms. Moreover, S-nitrosylation of COX2 (2, 6, 34, 71) and cPLA₂ (65) by iNOS is needed for the activation of these enzymes. Thus it is unclear whether Pio can activate COX2 and/or cPLA₂ in the iNOS⁻/⁻ mice. In the present study, we asked whether Pio limits myocardial IS and upregulates cPLA₂ and COX2 expression and activity in the eNOS⁻/⁻ and iNOS⁻/⁻ mice.

METHODS

Male C57BL/6 wild-type (WT), University of North Carolina eNOS⁻/⁻, and iNOS⁻/⁻ mice were purchased from Jackson Laboratory and received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The protocol was approved by Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

Treatment

Protocol 1. Mice received a 3-day pretreatment with Pio (10 mg·kg⁻¹·day⁻¹) suspended in water or water alone, administered by oral gavage once daily. On the fourth day, mice underwent coronary artery ligation for 30 min followed by 4-hr reperfusion (IS protocol) (n = 10–12 in each group) or the mice were euthanized under anesthesia. At 30 min of ischemia, the snare was released and myocardial reperfusion was verified by a change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed, and the mice were recovered from anesthesia. Four hours after reperfusion, the coronary artery was reoccluded, Evans blue dye (3%) was injected into the right ventricle, and the mice were euthanized under deep anesthesia (2, 7, 54, 69).

The prespecified exclusion criteria were the lack of signs of ischemia during coronary artery ligation, the lack of signs of reperfusion after the release of the snare, a prolonged ventricular arrhythmia with hypotension, and an area at risk (AR) ≤ 10% of the left ventricular weight.

Determination of AR and IS

Hearts were excised and the left ventricle was sliced transversely into six sections. Slices were incubated for 10 min at 37°C in 1% buffered (pH = 7.4) 2,3,5-triphenyltetrazolium chloride, fixed in a 10% formaldehyde, and photographed to identify the AR (uncolored by the blue dye), the IS (unstained by 2,3,5-triphenyltetrazolium chloride), and the nonischemic zones (colored by blue dye). The AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice, and the results were summed to obtain the weight of the myocardial AR and IS (2, 7, 54, 69).

Immunoblotting

Myocardial samples from the left ventricular wall were homogenized in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected and the total protein concentration was determined. The protein samples (50 μg) in loading buffer were run in 4–20% Tris·HCl Ready Gel at a 100 V for 2 h until the desired molecular weight bands were separated. After electrophoresis, the gel was equilibrated in transfer buffer (containing 25 mM Tris, 193 mM glycine, 0.1% SDS, and 10% methanol) and the proteins transferred to nitrocellulose membranes. The protein signals were quantified by an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin stain signal. Data are expressed as ratios between the protein and the corresponding β-actin signal density.

6-Keto-PGF₁α and PLA₂ Activity

Myocardial samples of the anterior wall of the left ventricle were washed with a PBS solution (pH 7.4) to remove red blood cells and clots, homogenized in cold PBS (pH 7.4), and centrifuged. The supernatants were collected and stored on ice. Measurements of 6-keto-PGF₁α, the stable metabolite of prostacyclin, and PLA₂ activity were made using immunoassay kits.

COX Activity

Myocardial samples of the anterior wall of the left ventricle were rinsed with 0.05 M Tris buffer (pH 7.4) to remove any red blood cells and clots. The samples were homogenized in 5–10 ml of cold buffer (containing 0.1 M Tris·HCl, pH 7.8, with 1 mM EDTA) per gram tissue and centrifuged at 10,000 g for 15 min at 4°C, and the supernatant was collected and stored on ice. The COX activity assay kit measures the peroxidase activity of COX, assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine at 590 nm. Each myocardial sample was tested in triplicate: the first without an inhibitor; the second with DuP-697, a specific COX2 inhibitor; and the third with Sc560, a specific COX1 inhibitor. COX1 activity was calculated as the difference between the total COX activity in the sample without an inhibitor and the sample with Sc560, and COX2 activity as the difference between total COX activity and COX1 activity.
activity in the sample without an inhibitor and the sample with DuP-697.

Real-Time PCR

PCR primers and probes. We used Applied Biosystems inventoried 20× assay mixes of primers and TaqMan MGB probe (FAM dye labeled) for the target genes (see below) and predeveloped 18S rRNA (VIC dye-labeled probe) TaqMan assay reagent (P/N 4319413E) for endogenous control.

Assays-on-demand (P/N 4331182). The specification of probes used for real-time PCR were as follows: AP2, Mn00495574_m1; CD36, Mn00432403_m1; cPLA2, Mn00447040_m1; COX2, Mn88242-m1; eNOS, Mn00435204_m1; and iNOS, Mn00440485_m1.

Relative quantification of gene expression. Separate tubes (Single-plex) real-time PCR was performed with 40 ng cDNA for both target genes and endogenous control. The reagent that we used was the universal PCR master mix reagent kit (P/N 4304437). The cycling parameters for real-time PCR were UNG activation, 50°C for 10 min; denaturation, 95°C for 15 s, and annealing/extension, 60°C for 1 min (repeat 40 times) on ABI7000. Duplicate threshold cycle (CT) values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems). The amount of target (2-ΔΔCT) was obtained by normalization to endogenous reference (18s) and relative to a calibrator (average of the control samples).

Materials

Pio was provided by Takeda Pharmaceuticals North America (Lincolnshire, IL). ELISA kits for 6-keto-PGF1α, cPLA2 activity, and COX activity were purchased from Cayman Chemicals (Ann Arbor, MI). Polyclonal anti-iNOS antibodies and t-NAME were purchased from Cayman Chemicals (Ann Arbor, MI). ELISA kits for 6-keto-PGF1α activity in the sample without an inhibitor and the sample with L-NAME. Polyclonal anti-iNOS antibodies and Pio was provided by Takeda Pharmaceuticals North America (Lincolnshire, IL). ELISA kits for 6-keto-PGF1α and anti-PGI2 synthase antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-

Statistical Analysis

Data are presented as means ± SE. The significance level α is 0.05. Body weight, left ventricular weight, and the size of the AR and IS (as a percentage of the AR) in protocol 1 were compared using analysis of variance with Sidak corrections for multiple comparisons and in protocol 2 with Student’s t-test. The data on enzyme expression and activity were compared between the Pio-treated and nontreated groups using t-test or Mann-Whitney rank sum test when appropriate. Values of P < 0.05 were considered statistically significant.

The authors had full access to the data and take responsibility for its integrity.

<table>
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<tr>
<th>Table 1. Body weight, LV weight, and AR</th>
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<td>Wild-Type</td>
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<tr>
<td>n</td>
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<tr>
<td>Body weight, g</td>
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<td>LV weight, mg</td>
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<td>AR, mg</td>
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Values are means ± SE; n, number of mice; LV, left ventricular; AR, ischemic area at risk; eNOS-/- and iNOS-/-, endothelial and inducible nitric oxide synthase knockout mice, respectively; Pio+, 10 mg·kg-1·day-1 pioglitazone; Pio-, water alone.

RESULTS

Infarct Size

Protocol 1. A total of 62 mice were included. None of the mice died or was excluded. Body weight, left ventricular weight, and AR size were comparable between the Pio+ and Pio- mice (Table 1). Without Pio pretreatment, IS in the eNOS-/- (44.2 ± 1.9%; P = 0.35) and iNOS-/- mice (45.5 ± 2.3%; P = 0.11) tended to be bigger than in the WT mice (39.0 ± 1.1%). Oral Pio decreased IS in all groups (Fig. 1). However, the myocardial protective effect of Pio was significantly smaller in the eNOS-/- mice (32.0 ± 1.6%) than in the WT (15.4 ± 1.4%; P < 0.001) or iNOS-/- (18.0 ± 1.2%; P < 0.001) mice.

Protocol 2. A total of 19 mice were included. Three mice (2 in the Pio+ t-NAME and 1 in the t-NAME-alone group) died during surgery. Body weight, left ventricular weight, and AR size were comparable between the Pio+ t-NAME and t-NAME-alone groups (Table 2). IS was significantly smaller in the Pio+ t-NAME group than in the t-NAME-alone group (Fig. 2).

Immunoblotting

All immunoblotting analyses were done in hearts that were explanted on the fourth day of the experiment without being subjected to regional ischemia. Pio did not cause a detectable change in total eNOS levels in the WT and iNOS-/- mice. As expected, there was no expression of eNOS in the eNOS-/- mice (Fig. 3, A and B). Pio caused a significant increase in myocardial Ser633 p-eNOS (Fig. 3, A and C) and Ser1177 p-eNOS (Fig. 3, A and D) levels in the WT and iNOS-/- mice. iNOS was undetectable in all six groups (data not shown). There was no difference in neuronal NOS (nNOS) levels among the groups (Fig. 3A).

Pio increased cPLA2 (Fig. 4, A and B), COX2 (Fig. 4, A and C), and PGI2 synthase (Fig. 4, A and D) levels in the WT, as well as in the eNOS-/- and iNOS-/- mice.

Real-Time PCR

We used AP2 and CD36 as markers of PPARγ activation. Pio increased AP2 mRNA levels in the WT and iNOS-/- mice and, to a lesser extent, in the eNOS-/- mice (Fig. 5A). Pio increased CD36 mRNA levels in the WT, as well as in the eNOS-/- and iNOS-/- mice (Fig. 5B). Pio increased eNOS mRNA levels in the WT and iNOS-/- mice (Fig. 5C) and caused a small, yet statistically significant, decrease in iNOS mRNA levels in the WT and eNOS-/- mice (Fig. 5D). Both
cPLA2 (Fig. 5E) and COX2 (Fig. 5F) mRNA levels were increased by Pio in the WT, eNOS−/−, and iNOS−/− mice.

Myocardial 6-Keto-PGF1α Levels and cPLA2 and COX Activity

Pio increased myocardial 6-keto-PGF1α levels in the WT, eNOS−/−, and iNOS−/− mice (Fig. 6A). cPLA2 activity was increased by Pio in the WT and iNOS−/− mice and, to a lesser extent, in the eNOS−/− mice (Fig. 6B). Pio caused a small, yet significant, increase in COX1 activity (Fig. 6C). COX2 activity was increased by Pio, especially in the WT mice, and to a lesser extent in the eNOS−/− and iNOS−/− mice (Fig. 6D).

DISCUSSION

Our main findings are that in contrast to the IS-limiting effect of statins which is eNOS and iNOS dependent (71), Pio limits IS in the WT, as well as in the iNOS−/−, mice. In the eNOS−/− mice, Pio significantly reduced IS; however, the effect was smaller than in the WT and iNOS−/− mice. This may suggest that the protective effect of Pio is partially dependent on eNOS. However, blocking all three NOSs (eNOS, iNOS and nNOS) with L-NAME did not attenuate the IS-limiting effects of Pio. Thus an alternative explanation can be the genetic differences among the various mice strains. Pio increased the expression and activity of cPLA2 and COX2 and myocardial 6-keto-PGF1α levels in all three strains of mice.

Role of eNOS

eNOS is essential for mediating late ischemic preconditioning (8). Most investigators agree that eNOS is essential for the IS-limiting effects of statins, especially when given before ischemia, since nonspecific NOS inhibitors abrogate the IS-limiting effect of statins (5, 63) and statins do not reduce IS in eNOS−/− mice (1, 4, 19, 31, 68, 71). Our group has also shown that in the rat, a 3-day pretreatment with Pio (10 mg·kg−1·day−1) limits IS without causing a detectable change in myocardial Ser1177 p-eNOS levels, although there was a small increase in calcium-dependent NOS activity (69), which could have been attributed to Ser633 phosphorylation by protein kinase A. On the other hand, atorvastatin had a similar magnitude of myocardial protective effect in the rat but induced an increase in Ser1177 p-eNOS levels, although the total eNOS levels did not change (7, 46, 69). In contrast, in the mouse, the same regimen of atorvastatin pretreatment increases myocardial levels of both total eNOS and Ser1177 p-eNOS (71), suggesting that there are some differences between mice and rats in response to drugs. However, the general response is the activation of eNOS. In contrast to the effects of atorvastatin in mice, Pio caused an increase in eNOS mRNA without a detectable change in total eNOS protein levels. In the mouse, in contrast to the rat, Pio caused a small, yet significant, increase in eNOS phosphorylation at both Ser633 and Ser1177, suggesting the activation by protein kinase A (and/or other kinases) (26). Other studies have suggested that PPARγ agonists increase NO production by eNOS without affecting the total eNOS expression (10, 28, 47). Hwang et al. (28) reported that ciglitazone increases the bioavailability of NO by an increased expression of Cu/Zn-superoxide dismutase and a suppression of NADPH oxidase. On the other hand, Cho et al. (12) reported that a prolonged treatment with troglitazone increased calcium-dependent NOS activity in bovine aortic endothelial cells by augmenting eNOS phosphorylation at Ser1179 without a detectable change in the total eNOS levels. Huang et al. (27) showed that Pio increases eNOS phosphorylation at Ser1177 in diabetic mice. Although Pio induced a small increase in eNOS phosphorylation in the WT as well as the iNOS−/− mice, eNOS was not essential for mediating cPLA2, COX2, and PGI2 synthase upregulation and activation. One may conclude that the myocardial protective effect of Pio is partially mediated by eNOS, since IS was significantly larger in the Pio-treated eNOS−/− than in the Pio-treated WT and iNOS−/− mice. Indeed, the nonspecific inhibition of NOS with N6-monomethyl-L-arginine blunted the effect of Pio on insulin resistance without affecting eNOS and iNOS protein levels in rat skeletal muscles (37). It was reported that rosiglitazone

Table 2. Body weight, LV weight, and AR

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<th>L-NAME</th>
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<tr>
<td>n</td>
<td>8</td>
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<td>Body weight, g</td>
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<td>25.1±0.5</td>
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<tr>
<td>LV weight, mg</td>
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<td>122±2</td>
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<tr>
<td>AR, mg</td>
<td>55±1</td>
<td>51±2</td>
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Values are means ± SE; n, number of mice. L-NAME, N⁶-nitro-L-arginine methyl ester.
failed to protect $eNOS^{-/-}$ mice from stunning post ischemia-reperfusion injury (22), since it did so in the WT mice, suggesting that in contrast to rosiglitazone, Pio may have an additional eNOS-independent mechanism of protection. It is plausible that the explanation for the smaller effect of Pio on IS in the $eNOS^{-/-}$ may be related to the genetic background of the strain and not to a partial dependence on eNOS, since L-NAME did not affect the IS-limiting effect of Pio (protocol 2). The dose of L-NAME used in the study (30 mg/kg) is sufficient to block all NOS, as Wolfrum et al. (61) reported that at 15 mg/kg, L-NAME blocked the IS-limiting effects of fasudil.

Role of iNOS

iNOS is essential for mediating late ischemic preconditioning (8). As mentioned in the introduction, iNOS is essential for the IS-limiting effects of statins (2, 49, 71) since iNOS is needed for the activation of COX2 (2, 6, 34, 71) and cPLA2 (65) by S-nitrosylation. Several investigators have suggested that ciglitazone (14), rosiglitazone (15, 16), and Pio (15, 36) reduce iNOS expression and activity in various experimental models. The present study shows that in contrast to statins, Pio did not augment iNOS expression; moreover, Pio limited IS in $iNOS^{-/-}$ mice as it did in the WT mice, whereas statins do not (49, 71), emphasizing the fact that the activating pathways of myocardial protection by statins and Pio are different.

It has been suggested that the other forms of NOS may compensate for the knockout of one of the isoforms. For example, the upregulation of eNOS in $iNOS^{-/-}$ mice can mediate preconditioning by the adenosine A1 receptor agonist, 2-chloro-$N^6$-cyclopentanyladenosine (3). However, the protective effect of 2-chloro-$N^6$-cyclopentanyladenosine was blocked by L-NAME. Wang et al. (57) reported that nNOS mediates late preconditioning in rabbits. In the present experiment, there were no differences in nNOS levels between the WT, $eNOS^{-/-}$, and $iNOS^{-/-}$ mice. Pio did not affect nNOS expression in all three types of mice. Moreover, Pio reduced IS in mice pretreated with L-NAME, a nonselective NOS inhibitor, supporting our conclusion that the protective effect of Pio is NOS independent and that cross coverage among the NOS isoforms cannot explain the protective effect in the knockout mice.

Role of cPLA2 and COX2

Our laboratory has shown that a 3-day pretreatment with atorvastatin upregulates cPLA2 and COX2 expression and activity in rats (2, 7, 69) and mice (71). COX2 inhibition, either with an oral COX2 inhibitor during the 3-day pretreatment...
with atorvastatin (7) or acutely, just before coronary artery occlusion (2), abrogates the IS-limiting effect of atorvastatin in the rat. A 3-day pretreatment with Pio (10 mg·kg⁻¹·day⁻¹) also increases cPLA₂ and COX2 expression and activity in the rat (69). The IS-limiting effect of Pio is abrogated with COX2 inhibition, but not with COX1 inhibition (69).

A previous study suggested that PLA₂ is involved in ischemic preconditioning (51). Atorvastatin increases cPLA₂ expression in the WT, as well as eNOS⁻/⁻ and iNOS⁻/⁻ mice, yet cPLA₂ activity increases by atorvastatin only in the WT mice, suggesting that eNOS and iNOS are essential for cPLA₂ activation (70). Indeed, it has recently been shown that iNOS activates cPLA₂ by S-nitrosylation (65). In contrast, in the present study, Pio increased cPLA₂ expression to a similar extent in the WT, eNOS⁻/⁻, and iNOS⁻/⁻ mice (Fig. 4); however, cPLA₂ activity was augmented to a larger extent by Pio in the WT than in both the eNOS⁻/⁻ and iNOS⁻/⁻ mice (Fig. 6). Thus cPLA₂ activation by Pio may be partially eNOS and iNOS independent.

It has been suggested that COX2 is activated to produce PGH₂ by iNOS-induced S-nitrosylation (2, 6, 34, 71). Pio upregulated COX2 expression in the WT, as well as in the eNOS⁻/⁻ and iNOS⁻/⁻ mice (Fig. 4). Although the augmentation of COX2 activity by Pio was smaller in the eNOS⁻/⁻ and iNOS⁻/⁻ mice than in the WT mice, myocardial 6-keto-PGF₁α levels were comparable among the Pio-treated WT, eNOS⁻/⁻, and iNOS⁻/⁻ mice (Fig. 6). The discrepancy between myocardial 6-keto-PGF₁α levels and COX2 activity is unclear. It may be related to PGL₂ synthase activity or a better coupling between COX2 and PGI₂ synthase. The fact that COX2 is activated by Pio without a detectable increase in iNOS in the WT and eNOS⁻/⁻ mice and in the iNOS⁻/⁻ mice suggests a different, yet undefined, posttranslation modification of COX2 in addition to the described S-nitrosylation. It might be that in the WT mice, COX2 is activated by both S-nitrosylation and NOS-independent pathways. This is a possible explanation to the greater increase in COX2 activity by Pio in the WT mice.

Pio is a thiazolidinedione with PPARγ agonist activity. AP2 and CD36 are PPARγ-dependent genes encoding proteins involved in lipid transport and storage (72). We measured the expression of these genes in response to Pio as a measure of PPARγ activation. Indeed, Pio activated PPARγ in the WT, eNOS⁻/⁻, and iNOS⁻/⁻ mice. The upregulation of CD36, and especially AP2 by Pio, was lower in the eNOS⁻/⁻ than in the WT and iNOS⁻/⁻ mice (Fig. 5). This correlates with the magnitude of IS limitation by Pio in the various mice strains (Fig. 1). However, it has not been previously reported that the
activation of PPARγ by Pio is dependent on eNOS. In contrast, it has been shown that eNOS−/− mice have insulin resistance and an impaired exocrine pancreatic secretion function. Pio improves both the exocrine secretion function and insulin resistance in eNOS−/− mice (45). Using human umbilical vein endothelial cells, our laboratory has shown that Pio increases the production of PGD2 and 15-deoxy-Δ12,14-PGJ2, which is formed by the nonenzymatic conversion of PGD2 (72). The
effect of Pio is attenuated by Sc58125, a selective COX2 inhibitor and small-interfering RNA to PGD2 synthase, but not by GW9662, a PPARγ antagonist, suggesting that the upregulation of PGD2 synthase is independent of PPARγ activation (72). We are currently investigating whether Pio limits IS and augments myocardial cPLA2 and COX2 in a cardiac-targeted PPARγ−/− mice.

**Limitations**

In the present study, we assessed IS after 30 min of ischemia and 4 h of reperfusion. It might be that the protective effect could be different if longer reperfusion periods were used. It should be further investigated whether the IS-limiting effects of pretreatment with Pio last with longer reperfusion periods (days and/or weeks) and whether the continuation of treatment is needed to sustain the effect after the induction of myocardial infarction.

As in our previous studies, we used ketamine and xylazine to induce anesthesia and isoflurane to maintain it. We added buprenorphine for analgesia before chest closure. One study suggested that ketamine preconditions isolated human right atrial myocardial cells by opening ATP-sensitive channel and stimulation of α- and β-adrenergic receptors (25). However, others suggested that myocardial norepinephrine levels were not increased by ischemic preconditioning in rabbits anesthetized with xylazine-ketamine, whereas they were increased in rabbits anesthetized with pentobarbital sodium (40) and that ketamine deactivates myocardial ATP-sensitive potassium channels (35). Most investigators report that ketamine may attenuate preconditioning (24, 33, 41–43). Isoflurane (13, 56) and opioid agonists (23) have been shown to protect against ischemia-reperfusion injury and reduce IS. Although we added all agents after the beginning of ischemia, recent studies have suggested that morphine has a postconditioning effect (11). We observed a significant effect of Pio on IS using the above-mentioned anesthetic regimen. However, others have reported that Pio limits IS in animals anesthetized with pentobarbital sodium (29, 64). Moreover, we have recently shown, using an identical model with the same regimen of anesthesia, that atorvastatin failed to limit IS in eNOS−/− and iNOS−/− mice (71). Thus we do not think that the anesthetic regimen had a significant effect on our results.

It should be remembered that Pio might exacerbate heart failure (38); however, β-blockers, the other class of drugs approved for perioperative treatment for the reduction of cardiovascular events in high-risk patients (21), may also induce/exacerbate heart failure.
In conclusion, the myocardial protective effect of Pio is iNOS independent and may be only partially dependent on eNOS. It seems that the magnitude of myocardial protection correlates with the magnitude of activation of PPAR\gamma, at least as assessed by AP2 levels. Pio increases myocardial 6-keto-PGF\(_1\alpha\) levels by upregulating and activating cPLA\(_2\), COX2, and PG\(_\text{II}\) synthase by an eNOS- and iNOS-independent mechanism. The partial independence of eNOS may have potential clinical implications since it has been shown that eNOS activity decreases with age, diabetes, and advanced atherosclerosis (50). Thus Pio may be particularly beneficial in these high-risk subsets. Further studies are needed for comparing the effects of statins and Pio on myocardial protection in aged animals, as well as in animals with diabetes and advanced atherosclerosis.

GRANTS

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