Cyclooxygenase-1 and -2 Knockout Mice Demonstrate Increased Cardiac Ischemia/Reperfusion Injury but Are Protected by Acute Preconditioning

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Background—The purpose of this study was to examine the effects of cyclooxygenase (COX) deficiency on baseline functional characteristics and on recovery of left ventricular developed pressure (LVDP) after 20 minutes of global ischemia and 40 minutes of reperfusion in untreated and preconditioned hearts.

Methods and Results—Compared with hearts from wild-type (WT) and COX-2−/− mice, baseline cardiac prostaglandin (PG) E2 and 6-keto-PGF1α levels were significantly decreased in hearts from COX-1−/− mice. After ischemia, cardiac PGE2 levels increased in WT, COX-1−/−, and COX-2−/− mice (P<0.05). Recovery of function (LVDP) after global ischemia in hearts from COX-1−/− and COX-2−/− mice was significantly less than in WT hearts. Pretreatment of WT mice with indomethacin for 2 days before ischemia significantly decreased LVDP recovery; however, perfusion of WT hearts with indomethacin for 40 minutes before ischemia did not significantly alter LVDP recovery. Postischemic recovery of LVDP in COX-1−/− and COX-2−/− was unchanged by perfusion with 5 μmol/L PGE2, PGD2, PGF2α, or carboprostacyclin. Hearts from COX-2−/− mice showed an increase in ischemic contracture compared with hearts from WT and COX-1−/− mice; however, hearts did not differ in intracellular pH, ATP, or inorganic phosphate during ischemia. Ischemic preconditioning significantly improved postischemic LVDP recovery in COX-1−/−, COX-2−/−, and WT mice.

Conclusions—Genetic disruption or 2-day chemical inhibition of COX-1 and COX-2 decreases recovery of LVDP after ischemia; however, acute perfusion with indomethacin is not detrimental. These data are consistent with protection due to the altered expression of some protein that is modulated by COX or its metabolites. (Circulation. 2001;104:2453-2458.)

Key Words: ischemia ■ reperfusion ■ preconditioning ■ prostaglandin ■ cyclooxygenase

The cyclooxygenases (COX) COX-1 and COX-2 catalyze the key first enzymatic steps in the metabolism of arachidonic acid.1 COX-1 is constitutively expressed in most tissues,2 whereas COX-2 is induced in response to proinflammatory cytokines and stress. More recently, investigators have shown that COX-2 also plays an important role in basal organ and tissue homeostasis.3

Cyclooxygenase inhibitors, such as aspirin and the new COX-2–specific inhibitors, are widely used. Aspirin is used as a treatment at the onset of acute myocardial ischemia. The beneficial effects of aspirin are ascribed to its inhibition of platelet aggregation and inhibition of thromboxane generation by COX-1.4 A mounting body of data, however, suggests that inhibition of COX-2 enhances cell death, particularly apoptotic cell death.5,6 It has recently been suggested that apoptosis may play a role in ischemia/reperfusion injury.7 Despite these data suggesting that inhibition of COX-2 can increase cell injury, however, very few studies have investigated the effect of COX inhibition on myocyte response to ischemia-reperfusion injury.

A recent study showed that COX-2 is upregulated 24 hours after a brief ischemic episode (preconditioning) and that addition of COX-2–specific inhibitors, administered 24 hours after preconditioning, just before the sustained period of ischemia, blocks the infarct-limiting effects of “late preconditioning.”8 Another study using isolated cardiomyocytes showed that oxidative stress upregulated COX-2 and that addition of a COX-2–specific inhibitor enhanced oxidative stress–induced injury.5 These data suggest that upregulation of COX-2 may have beneficial effects and that inhibition of COX may be associated with enhanced ischemia/reperfusion injury.

Because of the widespread use of COX inhibitors and data suggesting that COX inhibition can enhance cell injury, we investigated the effects of genetic manipulation of COX-1 and COX-2 on ischemia/reperfusion injury. COX-1−/− and COX-2−/− mice have been phenotypically characterized, and it has been demonstrated that both isoforms have important roles in intestinal, renal, pulmonary, and reproductive homeostasis.3,9–11 Because chemical inhibitors are often non-
specific and genetically altered mice can have compensatory mechanisms, this study examined the effects of reduced COX activity on ischemia-reperfusion injury using both methods of reducing COX activity. We report here that long-term inhibition or genetic disruption of COX-1 and/or COX-2 enhances ischemic injury, but short-term inhibition does not.

Methods

Isolated Mouse Heart Preparation

Adult COX-1\(-/-\), COX-2\(-/-\), and wild-type (WT) littermate control mice (25 to 35 g) were used. Mice lacking the Pghs-1 and Pghs-2 genes were developed by Langenbach, Morham and colleagues as described previously.3,9 Mice were generation 15 to 20, bred on a C57BL6/129 hybrid background, and genotyped as described.10 All mice received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Hearts were cannulated and perfused in the Langendorff mode with Krebs-Henseleit buffer as previously described.12

Hearts were cannulated and perfused in the presence of 10\(^{-6}\) mol/L indomethacin.3,9 Mice were generation 15 to 20, bred on a C57BL6/129 hybrid background, and genotyped as described.10 All mice received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Hearts were cannulated and perfused in the Langendorff mode with Krebs-Henseleit buffer as previously described.12

Western Blot Analysis

Hearts were perfused with phosphate-free Krebs-Henseleit buffer, and changes in concentrations of phosphorus metabolites were measured with a Varian Unity 500-MHz NMR spectrometer as described previously.12

Measurement of PGE\(_2\) and 6-keto-PGF\(_{1\alpha}\)

Hearts from WT, COX-1\(-/-\), and COX-2\(-/-\) mice were snap-frozen in liquid nitrogen after perfusion, and COX-1 and COX-2 expression was determined as previously described.10

Experimental Protocols

WT, COX-1\(-/-\), and COX-2\(-/-\) hearts were perfused with Krebs-Henseleit buffer for a stabilization period of 20 minutes, followed by a 40-minute period of baseline measurements, 20 minutes of global no-flow ischemia, and 40 minutes of reperfusion: left ventricular developed pressure (LVDP) and heart rate were measured throughout. Recovery of contractile function was determined by measurement of the LVDP at the end of reperfusion expressed as a percentage of preischemic LVDP.

To determine whether indomethacin treatment had an effect on postischemic contractile functional recovery, hearts from WT mice were perfused with the presence of 10 \(\mu\)mol/L indomethacin (WT indom-perfused) starting at the beginning of baseline measurement and continuing throughout the protocol. Another group of mice (WT indo-pretreated) were preconditioned with indomethacin (5 mg/kg body wt) by gavage once daily for 2 days before heart removal. Hearts were then perfused with 10 \(\mu\)mol/L indomethacin.

To evaluate the effect of selected prostaglandins, COX-1\(-/-\) and COX-2\(-/-\) hearts were perfused with 5 \(\mu\)mol/L PGE\(_2\), PGD\(_2\), PGE\(_2\)ar, or carboxoprostacyclin (a stable analogue of PGL\(_2\)) starting at the baseline measurement and continuing throughout the protocol. The prostaglandins were dissolved in 100% EtOH and diluted 1:500 in Krebs-Henseleit buffer. This concentration of EtOH has no effect on contractile function (data not shown). Hearts were preconditioned with 4 cycles of 5 minutes of ischemia followed by 5 minutes of reperfusion, and postischemic recovery of contractile function was compared with that of nonpreconditioned hearts.

Statistics

Results are expressed as mean ± SEM. Data were analyzed with commercial software. When ANOVA demonstrated that significant differences existed, Fisher’s least significant difference post hoc test was performed. Results were considered to be significant if a value of \(P\leq0.05\) was obtained.

Results

COX-1 and COX-2 Protein Expression

Western blot analysis was performed to determine whether disruption of either Pghs-1 or Pghs-2, the genes coding for COX-1 and COX-2, resulted in compensatory changes in cardiac expression of COX-2 or COX-1 after ischemia-reperfusion. COX-1 protein, detected as a single \(\approx 70\)-kDa band, was present in WT and COX-2\(-/-\) hearts but was undetectable in COX-1\(-/-\) hearts (Figure 1). There was no significant difference in cardiac COX-1 expression between WT and COX-2\(-/-\) mice. COX-2 protein expression was not detectable in WT, COX-1\(-/-\), or COX-2\(-/-\) hearts. These data show that COX-1 is the major COX isoform expressed in WT mouse heart and that there is no compensatory upregulation of COX-1 protein in COX-2\(-/-\) mice after ischemia-reperfusion. COX-2 levels in heart are below the limits of detection of Western analysis, although low levels of COX-2 can be detected in hearts of rabbits.12

WT and COX-2\(-/-\) hearts had similar cardiac PGE\(_2\) levels at baseline (Figure 2). In contrast, compared with the WT or COX-2\(-/-\) hearts, COX-1\(-/-\) and WT indomethacin-pretreated hearts had significantly lower basal PGE\(_2\) levels. WT hearts had basal levels of 6-keto-PGF\(_{1\alpha}\) that were slightly but not significantly higher than COX-2\(-/-\) hearts; however, COX-1\(-/-\) hearts had significantly lower levels (Figure 3). These results suggest that COX-1 is the primary isomform responsible for basal PGE\(_2\) and 6-keto-PGF\(_{1\alpha}\) biosynthesis. WT, COX-1\(-/-\), and COX-2\(-/-\) hearts all demonstrated an increase in PGE\(_2\) levels after ischemia (Figure 2). WT hearts that were either perfused with indomethacin or treated with indomethacin for 2 days showed no significant increase in tissue levels of PGE\(_2\) after ischemia. Thus, induction of PGE\(_2\) synthesis by ischemic stress occurs in the presence of genetic COX deficiency, albeit to a lesser extent in...
COX-1$^{-/-}$ hearts, and is blocked in the presence of indomethacin. After ischemia, WT and COX-2$^{-/-}$ hearts exhibited an increase in 6-keto-PGF$_{1}$ levels (Figure 3). COX-1$^{-/-}$ hearts did not show an increase in 6-keto-PGF$_{1}$ levels after ischemia, suggesting that under conditions in which COX-2 is not upregulated, COX-1 is the primary isoform responsible for generation of 6-keto-PGF$_{1}$.

**Preischemic Hemodynamics**

During the preischemic period, LVDP, +dP/dt$_{max}$, −dP/dt$_{min}$, and heart rate were not statistically different between any of the genotypes or with addition of indomethacin (Table). These data demonstrate that genetic COX deficiency or chemical COX inhibition does not significantly alter baseline cardiac function and suggest that prostaglandins do not play a major role in basal cardiac contractility.

**Postischemic Contractile Function**

All hearts began ischemic contracture at ≈14 minutes and reached a maximum pressure at ≈17.5 minutes. Maximum contracture was significantly higher (P<0.05) in COX-2$^{-/-}$ (114.8±6.6 cm H$_{2}$O), WT indomethacin-perfused (100.5±7.5 cm H$_{2}$O), and WT indomethacin-prevented (104.2±2.1 cm H$_{2}$O) than in untreated WT (77.6±5.4 cm H$_{2}$O) or COX-1$^{-/-}$ (73.7±9.5 cm H$_{2}$O) hearts.

Recovery of contractile function was significantly lower in COX-1$^{-/-}$ (14.1%) and COX-2$^{-/-}$ (13.3%) hearts than WT (30.4%) hearts (P<0.05, Figure 4). Perfusion of WT hearts with indomethacin had no effect on LVDP recovery (29.0%); however, 2-day pretreatment of WT mice with indomethacin followed by perfusion of hearts with indomethacin led to a significant decrease in LVDP recovery (6.2%) compared with WT hearts (P<0.05).

**Intracellular pH and Phosphate Levels**

In all groups studied, ATP levels fell to 20% of baseline after 20 minutes of ischemia, then recovered to ≈30% of baseline during reperfusion (Figure 5A). There were no statistically significant differences between WT, COX-1$^{-/-}$, and COX-2$^{-/-}$ hearts at any time point during the experiments. After the onset of ischemia, phosphocreatine decreased to undetectable levels in all 3 experimental groups (Figure 5B). Phosphocreatine levels in all hearts increased to ≈50% of baseline after 5 minutes of reflow and remained relatively constant thereafter. There were no significant differences in phosphocreatine between the genotypes at any time point. Intracellular pH was ≈7.2 to 7.3 in all hearts before ischemia, then fell to 5.86 in WT hearts, trended lower to 5.63 in COX-1$^{-/-}$ hearts, and was significantly lower at 5.49 (P<0.05) in COX-2$^{-/-}$ hearts (Figure 5C). During reperfusion, intracellular pH recovered rapidly in all groups.

**Addition of Prostaglandins**

To investigate whether exogenous prostaglandins could reverse the decrease in postischemic LVDP recovery observed in COX-1$^{-/-}$ and COX-2$^{-/-}$ deficient mice, COX-1$^{-/-}$ and COX-2$^{-/-}$ hearts were perfused in the presence of PGE$_{2}$, PGD$_{2}$, PGF$_{2a}$, and carboprostacyclin at concentrations comparable to those found in hearts after ischemia. As shown in Figure 6A and 6B, addition of PGE$_{2}$, PGD$_{2}$, PGF$_{2a}$, and carboprostacyclin did not reverse the poorer recovery of postischemic LVDP that was observed in COX-1$^{-/-}$ and COX-2$^{-/-}$ hearts compared with WT hearts (P<0.05).

**Preconditioning**

Because it has been shown that COX-2 inhibitors block late preconditioning, we examined whether acute preconditioning is altered in COX-1$^{-/-}$ or COX-2$^{-/-}$ hearts (Figure 7). In WT hearts, preconditioning resulted in an improvement in postischemic LVDP (expressed as a percentage of initial preischemic LVDP), from 30.4% in nonpreconditioned hearts to 43.4% in preconditioned hearts. Preconditioning also...
improved postischemic LVDP in COX-1−/− hearts (14.1% in nonpreconditioned to 37.4% in preconditioned) and COX-2−/− hearts (13.3% in nonpreconditioned versus 42.4% in preconditioned). Recovery of contractile function after preconditioning was similar in all 3 groups of hearts studied, despite poorer recovery of function in nonpreconditioned COX-1−/− and COX-2−/− hearts. Thus, the COX-1−/− and COX-2−/− hearts showed a greater percentage recovery of function than the WT hearts (Figure 7). This is consistent with previous data showing that indomethacin perfusion enhanced the preconditioning-induced improvement in postischemic function.18

Discussion

Effects of COX Disruption/Inhibition at Baseline and After Ischemia/Reperfusion

COX-1 but not COX-2 protein was detected by Western blot in WT hearts, and there were no significant differences in COX-1 expression between COX-2−/− and WT hearts. Furthermore, compared with WT or COX-2−/− hearts, baseline PGE2 and 6-keto-PGF1α levels were significantly decreased in COX-1−/− hearts. PGE2 and 6-keto-PGF1α were present in COX-1−/− hearts, however, suggesting that even though COX-2 was undetectable by Western blot, sufficient COX-2 is present at baseline to generate these prostaglandins. Basal contractile function was not different between the groups, suggesting that chemical inhibition or genetic disruption of COX-1 and/or COX-2 and the resultant changes in prostaglandin levels does not alter this parameter.

Ischemia resulted in a similar increase in PGE2 and 6-keto-PGF1α levels in WT and COX-2−/− hearts. The levels of PGE2 and 6-keto-PGF1α attained after ischemia, however, were significantly lower in COX-1−/− hearts. These data suggest that after ischemia, PGE2 and 6-keto-PGF1α are generated primarily via COX-1. If COX-2 is upregulated, however, it can be a major contributor to the generation of PGE2 and 6-keto-PGF1α.8

Treatment with indomethacin for 2 days or genetic disruption of COX-1 or COX-2 had a detrimental effect on postischemic recovery of LVDP. On reperfusion after 20 minutes of ischemia, COX-1−/− and COX-2−/− hearts both showed recovery of LVDP that was ≈50% of that in WT hearts. Hearts from mice pretreated with indomethacin for 2 days showed postischemic

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Recovery of LVDP after ischemia as percent of baseline in WT, COX-1−/−, COX-2−/−, WT indo-perfused, and WT indo-pretreated mouse hearts (n=5). Values are mean±SEM. *P<0.05 vs WT.

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Changes in intracellular ATP (A), phosphocreatine (B), and intracellular pH (C) as determined by 31P NMR spectroscopy in WT (○), COX-1−/− (□), and COX-2−/− (●) mouse hearts (n=4). Values are mean±SEM. *P<0.05 vs WT.
recovery of LVDP that was only 25% of that in WT hearts. These data suggest that COX-1 and COX-2 are cardioprotective. Treatment with indomethacin for 40 minutes before ischemia, however, did not alter recovery of function. The observation that addition of indomethacin for 40 minutes before ischemia and continued indomethacin perfusion throughout reflow did not have a detrimental effect on recovery of function, whereas chronic inhibition of COX-1 and/or COX-2 was detrimental, suggests that protection is not mediated by the generation of a COX metabolite during ischemia or reperfusion and is not mediated by the presence of a short-lived metabolite at the start of ischemia. Furthermore, addition before ischemia and throughout reperfusion of either PGE₂, PGD₂, PGE₂(α), or carboprostacyclin did not rescue the COX-1⁻/⁻ or COX-2⁻/⁻ hearts from poor recovery of LVDP. It is likely that the disruption of the COX enzymes induces a subtle cardiac alteration, perhaps through altered gene expression. Long-term alteration in the profiles of prostaglandins could lead to altered gene expression via altered binding to peroxisomal proliferator-activated receptors, for example, which could result in an altered gene profile and an altered response to injury. It is also possible that a COX metabolite in some tissue other than heart mediates protection, most likely via release of a circulating factor.

The observation that loss of COX-2 enhances ischemia-reperfusion injury was somewhat surprising, because COX-2 was undetectable in WT or COX-1⁻/⁻ hearts and because COX-2⁺/⁻ hearts have levels of PGE₂ and 6-keto-PGF₁α that are not significantly different from those in WT hearts. One might expect that COX-1 would adequately substitute for the loss of COX-2. Although COX-1 and COX-2 are almost identical in structure, they are regulated differently by glutathione and can have different metabolite profiles in vivo.³,¹³ Furthermore, COX-1 cannot substitute for COX-2, as illustrated by the phenotype of the COX-2⁻/null mice. The COX-2⁻/null mice generated by Morham et al³ and used in this study developed severe nephropathy and are susceptible to peritonitis. Dinchuk et al⁴ generated COX-2⁻/null mice showing even more severe pathology: renal dysplasia (100% penetrance), cardiac fibrosis (50% penetrance), and female infertility. These studies show that even though COX-1 is present in these mice, it cannot compensate for the lack of COX-2, suggesting that COX-2, presumably via a prostaglandin metabolite, modulates function independently of COX-1. Interestingly, cardiac fibrosis was observed in the COX-2⁻/deficient mice generated by Dinchuk et al.⁴ Furthermore, the severity of the cardiac fibrosis did not correlate with the severity of renal dysplasia, suggesting that the fibrosis was not secondary to the renal pathology. We did not observe cardiac fibrosis in our COX-2⁻/null mice. The cardiac fibrosis in the mice generated by Dinchuk et al,⁴ however, is consistent with our observation that COX-2⁻/null hearts have enhanced ischemic injury.

COX-2⁻/⁻ and indomethacin-treated but not COX-1⁻/⁻ or WT hearts exhibited a significant increase in the maximum ischemic contracture. This suggests that a COX-2 metabolite mediates a decrease in ischemic contracture. Many factors can influence the development of ischemic contracture, including a decrease in ATP, an increase in cytosolic Ca²⁺, a higher intracellular pH, a lower level of inorganic phosphate, or an alteration in the Ca²⁺ sensitivity of a contractile protein.¹⁵ The lack of an alteration in baseline contractile function in COX-2⁻/⁻ hearts tends to argue against an alteration in contractile proteins. There were no significant differences in ATP or inorganic phosphate between

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**Figure 6.** Recovery of LVDP after ischemia as percentage of baseline in WT, COX-1⁻/⁻, and prostaglandin-perfused COX-1⁻/⁻ mouse hearts (A, n=3) and WT, COX-2⁻/⁻, and prostaglandin-perfused COX-2⁻/⁻ mouse hearts (B, n=3). Values are mean±SEM. *P<0.05 vs WT.

**Figure 7.** Recovery of LVDP after ischemia as percentage of baseline in preconditioned and nonpreconditioned WT, COX-1⁻/⁻, and COX-2⁻/⁻ hearts (n=5). Values are mean±SEM. *P<0.05 vs WT.
WT, COX-1−/−, or COX-2−/− hearts. Interestingly, COX-2−/− hearts had a significantly lower intracellular pH during ischemia than WT hearts. The lower intracellular pH in COX-2−/− hearts, however, would reduce rather than enhance contracture. Thus, the data do not support a role for ATP, inorganic phosphate, or intracellular pH in the increase in ischemic contracture observed in COX-2−/− hearts. This suggests a possible role for a COX-2–mediated alteration in cytosolic Ca2+ during ischemia as a possible mediator.

Effects of COX Disruption on Preconditioning

Preconditioning with brief, intermittent periods of ischemia has been shown to reduce subsequent cardiac ischemia–reperfusion injury.15 There is an initial immediate protection (acute preconditioning) and a second window of protection that occurs 24 hours later (late preconditioning). Studies of the effects of COX inhibitors on the protective effects of acute preconditioning have used only short treatment with COX inhibitors and have shown conflicting results.16–19 Our data suggest that neither COX-1 nor COX-2 is required for acute preconditioning. In fact, the preconditioning-induced improvement in posts ischemic LVDP observed in COX-1−/− and COX-2−/− hearts was greater (on a percentage basis compared with non preconditioned) than that observed in WT hearts. Thus, acute preconditioning was enhanced in COX-1−/− and COX-2−/− hearts. We previously observed that indomethacin slightly enhanced the protective effects of preconditioning,18 and we attributed this protection to shunting of arachidonic acid metabolites to lipoygenase metabolites, which have been shown to be protective.18,19

Shinmura et al8 showed that COX-2 inhibitors block late preconditioning. They showed that preconditioning on day 1 leads to upregulation of COX-2 on day 2 and that inhibition of upregulated COX-2 just before the sustained ischemia (on day 2) blocks the protection. Addition of a COX-2 inhibitor administered 40 minutes before the preconditioning stimulus (day 1), however, did not block the protective effects of late preconditioning measured 24 hours later, suggesting that COX-2 inhibition does not block the induction of preconditioning. Late preconditioning is mediated by new gene expression 24 hours after an initial preconditioning stimulus.5 Acute preconditioning does not require synthesis of new proteins,20 and COX-2 is not upregulated. Thus, it is not surprising that COX inhibition has different effects on acute preconditioning versus late preconditioning.

Summary

Although use of COX inhibitors is common, the effect of COX inhibition on heart, particularly during ischemia and reperfusion, is not well understood. Inhibitors of COX have been reported to enhance injury in many tissues.56 The data from this study suggest that even though we cannot detect COX-2 protein in WT hearts, COX-2 is important for the function of the heart during ischemia/reperfusion, because hearts from COX-2−/− mice show a significant decrease in posts ischemic recovery of LVDP. We find that long-term inhibition (2 days or longer) of COX-1 or COX-2 enhances ischemic injury. Addition of indomethacin for 40 minutes before ischemia was not detrimental, suggesting that the protection mediated by COX is due to altered expression of some protein that is modulated by COX metabolites. Thus, these data suggest that COX can reduce cell death in cardiac tissue, similar to its role in other tissues, and that long-term inhibition of COX can enhance ischemic injury. These data suggest caution in the use of COX inhibitors in patients with ischemic heart disease.

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