COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts

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Shinmura, Ken, Maiko Nagai, Kayoko Tamaki, Masato Tani, and Roberto Bolli. COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts. Am J Physiol Heart Circ Physiol 283: H2534–H2543, 2002. First published August 8, 2002; 10.1152/ajpheart.00209.2002.—Opioids confer biphasic (early and late) cardioprotection against myocardial infarction by opening mitochondrial ATP-sensitive K+ channels. It is unknown whether cyclooxygenase-2 (COX-2), which mediates ischemia-induced late preconditioning, also mediates opioid-induced cardioprotection. Isolated perfused rat hearts were subjected to 20 min of global ischemia followed by 20 min of reperfusion. BW-373U86 (BW), a δ-opioid receptor agonist, was administered 1, 12, or 24 h before death. Recovery of left ventricular developed pressure (LVDP) after ischemia-reperfusion improved when BW was administered 1 or 24 h before ischemia (control: 57 ± 8, BW 1 h: 75 ± 5, BW 24 h: 85 ± 6%) but not when it was administered 12 h before (60 ± 5%). Levels of 6-keto-PGF1α (a stable metabolite of PGI2) in coronary effluent after 20 min of reperfusion were higher with 24-h BW pretreatment than in controls (1,053 ± 92 vs. 724 ± 81 pg/ml), whereas 6-keto-PGF1α levels at baseline did not differ. Administration of a selective COX-2 inhibitor, NS-398, abolished the late phase of cardioprotection (recovery of LVDP, 53 ± 8%) and attenuated the increase in PGI2 (706 ± 138 pg/ml) but did not block the early phase of cardioprotection. The selective COX-1 inhibitor SC-560 did not affect either phase of protection. Western immunoblotting revealed upregulation of PGI2 synthase protein 24 h after BW administration without changes in COX-1 and COX-2 protein levels. In conclusion, the late (but not the early) phase of δ-opioid receptor-induced preconditioning is mediated by COX-2. A functional coupling between COX-2 and upregulated PGI2 synthase appears to underlie this cardioprotective phenomenon in the rat.

myocardial ischemia; opioid; prostaglandin; reperfusion injury

OPIOIDS HAVE BEEN SHOWN TO confer biphasic (early phase and late phase) cardioprotection against myocardial infarction (12–16, 28, 31, 32) similar to ischemic preconditioning (PC) (4, 41). Opioid-induced cardioprotection was first described by Schultz et al. (31), who demonstrated that opioids mimic the early phase of ischemic PC via the activation of the δ-opioid receptors and a Gia protein-mediated mechanism. Fryer et al. (12) reported that stimulation of δ-opioid receptors 24–48 h before an ischemic insult also induces a late phase of cardioprotection against myocardial infarction. Their recent studies (13–15, 21, 28) have revealed that activation of PKC-δ and subsequent activation of the p44 isoform of extracellular signal-regulated kinase and tyrosine kinases are essential in the development of opioid-induced cardioprotection. In contrast to the intense research related to the signaling pathways that lead to δ-opioid-dependent cardioprotection, little is known regarding the effectors (mediators) of this phenomenon. Gross and his colleagues (12, 21, 26, 32) demonstrated that both phases of opioid-induced cardioprotection are abolished by the administration of 5-hydroxydecanoic acid, indicating that opening of mitochondrial ATP-sensitive K+ (KATP) channels is involved. However, the cardioprotective protein(s) that mediate the beneficial effects of opioids remain to be identified.

Ischemic PC is a biphasic phenomenon (4, 41). The rapid nature of the early phase suggests that it involves the modification of proteins that are already present. In contrast, the late phase of ischemic PC requires the synthesis of cardioprotective proteins that are the effectors (mediators) of protection 12–72 h after ischemic PC (4). Pharmacological and genetic evidence indicates that upregulation of inducible nitric oxide synthase (iNOS) is essential for late PC (3, 5, 18). In addition to iNOS, we recently found that cyclooxygenase-2 (COX-2) mediates the protective effects of ischemia-induced late PC in rabbits and mice (17, 33, 34). Analysis of COX byproduct levels suggests that COX-2 mediates the late phase of cardioprotection via increased production of cytoprotective prostanooids, mainly PGI2 and PGE2 (33, 34). In contrast, COX-2 does not mediate late PC induced pharmacologically by activation of adenosine A1 or A3 receptors (23). Discrepancy between these findings suggests that differ-
ences in the signaling pathways exist between ischemic and pharmacological PC. Recent findings suggest that COX-2 mediates opioid receptor-induced late PC in rabbits (24). However, whether COX-2 or any prostanoid is involved in opioid-induced cardioprotection in other species has not been examined. Furthermore, it remains unknown whether opioid-induced late PC is mediated by an increase in the expression of COX-2 itself or in one of the PG synthases that operates downstream of COX-2 (36).

The aims of this study were 1) to determine whether COX-1 or COX-2 mediates opioid-induced cardioprotection in rats and 2) to determine the mechanism(s) whereby COX-2 is involved in cardioprotection. With the use of a potent nonpeptide δ-opioid receptor agonist, BW-373U86, we demonstrated that the opioid-induced late phase of cardioprotection is mediated by COX-2. In addition, we found evidence for a novel, heretofore unrecognized functional coupling between COX-2 and upregulated PGI2 synthase during opioid-induced late PC.

MATERIALS AND METHODS

Materials

We purchased (±)-1(S*),2α,5β]-4-{[2,5-dimethyl-4-(2-propanyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl}-N,N-diethylbenzamide hydrochloride (BW-373U86) from Sigma-RBI (St. Louis, MO). NS-398, valeryl salicylate, and monoclonal antibodies against PGI2 synthase were purchased from Cayman Chemical (Ann Arbor, MI). PGE2, 6-keto-PGF1a, and thromboxane (Tx)B2, enzyme immunoassay kits and vistra enhanced chemiluminescence Western blotting kit were purchased from Amersham Pharmacia biotech (Buckinghamshire, UK). Monoclonal antibodies against COX-2 were purchased from Alexis (San Diego, CA). We purchased (∓)-1(S*),2α,5β]-4-{[2,5-dimethyl-4-(2-propanyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl}-N,N-diethylbenzamide hydrochloride (BW-373U86) from Sigma-RBI (St. Louis, MO). NS-398, valeryl salicylate, and monoclonal antibodies against PGI2 synthase were purchased from Cayman Chemical (Ann Arbor, MI). PGE2, 6-keto-PGF1a, and thromboxane (Tx)B2, enzyme immunoassay kits and vistra enhanced chemiluminescence Western blotting kit were purchased from Amersham Pharmacia biotech (Buckinghamshire, UK). Monoclonal antibodies against COX-2 were purchased from Alexis (San Diego, CA).

Langendorff Perfusion of the Hearts

All procedures in the present study conformed to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals (DHEW Publication No. 85-23, Revised 1996, Office of Science and Health Reports DRR/NIH, Bethesda, MD 20205).

One hundred and eight, 12-wk-old male Fischer 344 rats weighing 210–250 g were anesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Hearts were excised quickly and perfused with modified Krebs-Henseleit buffer [containing (in mmol/l) 118 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 1.75 CaCl2, 0.5 EDTA, 11 glucose, and 5 pyruvate] gassed with 95% O2-5% CO2 at 37°C. Isolated hearts were allowed to stabilize for 30 min before the commencement of perfusion. Perfusion pressure was maintained at 70 mmHg.

Measurement of Left Ventricular Function

A plastic catheter with a latex balloon was inserted into the left ventricle through the left atrium. Before the induction of ischemia, hearts were paced at 5 Hz, and the left ventricular (LV) end-diastolic pressure (LVEDP) was adjusted to 10 mmHg by filling the balloon with water. Pacing was turned off during global ischemia and turned on 10 and 20 min after reperfusion to measure the recovery of LV function. To measure LV pressure, at 10 min of reperfusion the hearts that were in ventricular fibrillation were converted to sinus rhythm by tapping. The balloon was also deflated during global ischemia and during the first 10 min of reperfusion. Indexes of LV function [LV systolic pressure (LVSP); LV developed pressure (LVEDP); LV peak and negative rate of change of LVSP (dP/dt)] were recorded as described previously (37).

Experimental Protocols

Rats were assigned to twelve groups (Fig. 1). All groups received 10 min of initial perfusion in a recirculating mode, and the isolated perfused hearts were then subjected to 20 min of global ischemia followed by 20 min of reperfusion.

Dose response and time course of BW-373U86-induced cardioprotection. An initial dose response for BW-373U86 was established to determine the optimal dose for inducing late-phase cardioprotection (Fig. 1, A). Group I (control) received vehicle (500 μg/kg sterile water) injected subcutaneously and underwent 20 min of global ischemia followed by 20 min of reperfusion 24 h later. In groups II–IV (BW-373U86 (BW) 0.1, BW 0.33, BW 1.0 (BW 24 h)), different doses of BW-373U86 were administered subcutaneously (0.1, 0.33, or 1.0 mg/kg) 24 h before death. BW-373U86 was dissolved in sterile water just before injection. We used the dose of BW-
373U86 (1.0 mg/kg) that produced the greatest recovery of LV function after ischemia-reperfusion for subsequent groups. The second series of rats were used to define the time course of opioid-induced cardioprotection (Fig. 1B). Groups V and VI (BW 1 h, BW 12 h) received 1.0 mg/kg of BW-373U86 injected 1 h or 12 h before death and underwent 20 min of global ischemia followed by 20 min of reperfusion.

Effect of COX-selective inhibitors on BW-373U86-induced cardioprotection. The third series of the experiments used either selective COX-1 or COX-2 inhibitor (Fig. 1, C and D). In group VII (NS-398 (NS) + BW 1 h), rats were pretreated with an intraperitoneal injection of the selective COX-2 inhibitor NS-398 (5 mg/kg) 30 min before BW-373U86 injection and were killed 1 h after injection. Isolated hearts were subjected to 20-min global ischemia followed by the 20-min reperfusion protocol. In group VIII (SC-560 (SC) + BW 1 h), rats were pretreated with an intraperitoneal injection of the selective COX-1 inhibitor SC-560 (10 mg/kg) instead of NS-398, received the BW-373U86 injection, and were killed 1 h later. Rats were treated with NS-398 (5 mg/kg, group IX; BW 24 h + NS) or SC-560 (10 mg/kg, group X: BW 24 h + SC) intraperitoneally 30 min before death. Hearts were then subjected to the ischemia-reperfusion protocol. Groups XI (NS) and XII (SC) were the drug control groups. Rats received an intraperitoneal injection of NS-398 (5 mg/kg) or SC-560 (10 mg/kg) without BW-373U86 pretreatment and were killed 30 min later. NS-398 and SC-560 were dissolved in DMSO (10/20 mg/ml, respectively) and diluted twice with sterile water (final volume 1.0 ml/kg, DMSO 50%). This dose of NS-398 has previously been shown to block COX-2 activity 24 h after ischemic PC in rabbits (17, 33). This dose of SC-560 has previously been reported to reduce serum TxB2 levels, which reflect COX-1 activity, by >90% in doxorubicin-treated rats (9).

Measurement of Creatine Kinase and Lactate Dehydrogenase Activities

Perfusate was collected at the end of reperfusion to measure the activity of creatine kinase (CK) and lactate dehydrogenase (LDH) released during the 20 min of reperfusion. The volume of recirculating coronary perfusate was 50 ml/heart, and CK and LDH activity was measured by standard enzymatic methods. Total amount of CK and LDH released in the perfuse are expressed as IU/g wet weight of ventricle.

Measurement of PGE2, 6-Keto-PGF1α, and TxB2 Levels

Perfusate was collected after 10 min of preischemic perfusion and after 20 min of reperfusion to measure prostaglandin (PG) levels in the coronary effluent. The PGE2, 6-keto-PGF1α, (a stable metabolite of PGI2) and TxB2 (a stable metabolite of TxA2) levels were determined using immunoassay kits.

Western Immunoblotting

Eight rats from groups I (control), V (BW 1 h), VI (BW 12 h), and IV (BW 24 h) were euthanized without an ischemic insult 24 h after injection. The heart was excised quickly, and the left ventricle was stored at −140°C until use. Tissue samples were homogenized in buffer A (containing in mM) 25 Tris-HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 PMSF, 1 DTT, 25 NaF, and 1 Na3VO4 and 25 μg/ml leupeptin) and centrifuged at 1,000 g for 10 min. The supernatant (the cytosolic fraction) was carefully taken off and recentrifuged at 16,000 g for 15 min to eliminate any contaminating pellet. The initial pellet was resuspended in a lysis buffer (buffer A + 1% Triton X-100) and incubated at 4°C for 2 h. Samples were centrifuged at 16,000 g for 15 min. The resulting supernatants were collected as membranous fractions (30, 33, 40). Standard SDS-PAGE Western immunoblotting techniques assessed the expressions of COX-2, COX-1, and PGI2 synthase protein. Briefly, proteins (100 μg) were electrophoresed on a 10% denaturing gel and then electrophoretically transferred onto nitrocellulose membranes overnight at 4°C. Gel transfer efficiency was determined by making photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining (30). The membranes were incubated in 5% nonfat dry milk in a washing buffer (10 mM Tris-HCl, (pH 7.2), 0.15 M NaCl, and 0.05% Tween-20), followed by incubation with specific monoclonal antibodies (1:500 dilution) at 35°C for 2 h. After being rinsed with washing buffer, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (1:3,000 dilution) at room temperature for 1.5 h and developed using the vistra enhanced chemiluminescence Western blotting kit. Protein signals and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated by an image scanning densitometer, and each protein signal was normalized to the corresponding Ponceau stain signal (30). Protein content was expressed as a percentage of the corresponding protein in group I (control group).

Statistical Analysis

Data are reported as means ± SE. For intragroup comparisons, hemodynamic variables were analyzed by a two-way repeated-measures ANOVA (time and group), followed by Student’s t-tests for paired data with the Bonferroni correction. For intergroup comparisons, data were analyzed by either a one-way or a two-way repeated-measures ANOVA (time and group) as appropriate followed by Student’s t-tests for paired data with the Bonferroni correction. All statistical analyses were performed using the SAS software system.

RESULTS

BW-373U86-Induced Cardioprotection Against Ischemia-Reperfusion Injury

Pretreatment with BW-373U86 24 h before ischemia improved the recovery of LVSP, LVDP, and peak positive and negative dP/dt at doses of 0.33 and 1.0 mg/kg (groups III and IV) (Fig. 2, Tables 1 and 2). In contrast, 0.1 mg/kg BW-373U86 failed to induce appreciable protection (group II) (Fig. 2, Tables 1 and 2). Because the duration of global ischemia was short, only small amounts of CK and LDH were released and there was no difference among the control and BW groups (groups I–IV) (Table 3).

Whereas recovery of LV function after ischemia-reperfusion improved when BW-373U86 was administered 1 h or 24 h before the ischemic insult (groups IV and V) (Fig. 2, Tables 1 and 2), pretreatment with BW-373U86 12 h before the ischemic insult was not effective (group VI) (Fig. 2, Tables 1 and 2), confirming the biphasic nature of opioid-induced PC. These results are consistent with the report by Fryer et al. (12) in which infarct size was compared between the control and opioid-pretreated groups. CK and LDH release
during reperfusion was similar in the control and BW groups (groups I, V, and VI) (Table 3).

**Effect of Selective COX Inhibitor on BW-373U86-Induced Cardioprotection**

Pretreatment with the selective COX-2 inhibitor NS-398 30 min before the administration of BW-373U86 did not block the BW-373U86-induced early phase of cardioprotection (group VII) (Fig. 3, Tables 1 and 2). In contrast, the administration of NS-398 30 min before the induction of prolonged ischemia abolished BW-373U86-induced late phase of cardioprotection (groups VIII and X) (Fig. 3, Tables 1 and 2). Neither NS-398 nor SC-560 in itself affected the recovery of LV function after ischemia-reperfusion (groups XI and XII) (Fig. 3, Tables 1 and 2).

There was no difference in CK and LDH release between the control and selective COX inhibitor-treated hearts (groups XI and XII), indicating that NS-398 and SC-560 in itself did not exacerbate ischemia-reperfusion injury (Table 3). CK and LDH release during reperfusion was similar in the control and all COX inhibitor-treated groups (groups VII–XII) (Table 3).

**Effect of BW-373U86 on Myocardial Prostanoid Levels**

Changes in the levels of PGE₂, TxB₂, and 6-keto-PGF₁α in the coronary effluent after the administration of BW-373U86 were evaluated (Figs. 4 and 5). 6-keto-PGF₁α levels after 10 min of preischemic perfusion (an index of 6-keto-PGF₁α levels at baseline) were similar among the control and all BW-373U86-treated groups (groups I, and IV–VI) (Fig. 4). However, 6-keto-PGF₁α.

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**Table 1. LV pressure**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>10 min After Reperfusion</th>
<th>20 min After Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>LVSP (mmHg)</td>
<td>LVEDP (mmHg)</td>
</tr>
<tr>
<td>Group I, control</td>
<td>10</td>
<td>79 ± 6</td>
<td>10.3 ± 0.4</td>
</tr>
<tr>
<td>Group II, BW 0.1 (mg/kg)</td>
<td>9</td>
<td>79 ± 9</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Group III, BW 0.33 (mg/kg)</td>
<td>9</td>
<td>69 ± 6</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>Group IV, BW 1.0 (mg/kg) = BW 24 h</td>
<td>9</td>
<td>75 ± 8</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td>Group V, BW 1 h</td>
<td>9</td>
<td>79 ± 4</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Group VI, BW 12 h</td>
<td>9</td>
<td>82 ± 2</td>
<td>10.3 ± 0.2</td>
</tr>
<tr>
<td>Group VII, NS + BW 1 h</td>
<td>10</td>
<td>77 ± 4</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Group VIII, SC + BW 1 h</td>
<td>9</td>
<td>86 ± 7</td>
<td>10.4 ± 0.2</td>
</tr>
<tr>
<td>Group IX, BW 24 h + NS</td>
<td>8</td>
<td>80 ± 9</td>
<td>10.5 ± 0.3</td>
</tr>
<tr>
<td>Group X, BW 24 h + SC</td>
<td>8</td>
<td>85 ± 6</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>Group XI, NS</td>
<td>8</td>
<td>75 ± 5</td>
<td>10.5 ± 0.3</td>
</tr>
<tr>
<td>Group XII, SC</td>
<td>8</td>
<td>89 ± 7</td>
<td>10.3 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE (in mmHg)(LV); n, no. of rats. LVSP, left ventricular systolic pressure; LVEDP, LV end-diastolic pressure; LVDP, LV developed pressure (LVSP – LVEDP); BW; BW-373U86; NS, NS-398; SC, SC-560. *P < 0.05 vs. group I (control), †P < 0.05 vs. group IV (BW 24 h), ‡P < 0.05 vs. group XII (SC).
levels after 20 min of reperfusion were higher in group IV (BW 24 h) than in group I (control). The PGE2 and TxB2 levels were similar after 10 min of preischemic perfusion and after 20 min of reperfusion among the control and all BW-373U86-treated groups (Fig. 5). These results indicate that the increase in PGI2 observed in group IV was independent of the production of other prostanoids. Increase in PGI2 production results from upregulation of PGI 2 synthase protein levels among groups I (control), V (BW 1 h), and VI (BW 12 h) (Fig. 7). However, PGI2 synthase protein expression increased significantly (+56% in the membranous fraction, +49% in the cytosolic fraction) 24 h after the administration of BW-373U86 (group IV) (P < 0.05).

**DISCUSSION**

This study provides three major findings: 1) COX-2 does not contribute to BW-373U86-induced early PC, 2) COX-2 mediates BW-373U86-induced late PC by increasing PGI2 production, and 3) the increase in PGI2 production results from upregulation of PGI2 synthase rather than COX-2.

**BW-373U86-Induced Cardioprotection in Isolated Perfused Rat Hearts**

Gross and colleagues (12, 29, 31, 32) have demonstrated the biphasic nature of opioid-induced cardioprotection against myocardial infarction in rats. Similar cardioprotective effects of opioids have been observed in mice (16), rabbits (28), and even human myocytes (1). Recent advances in pharmacological technology have made it possible to develop drugs with high selectivity for the 6-opioid receptor, minimizing the risk of side effects, such as addiction (8). Therefore, opioids have the potential for development as therapeutic cardioprotective agents. This study demonstrates that BW-373U86 can induce both an early and a late phase of cardioprotection in isolated perfused rat hearts, suggesting that opioid-induced cardioprotection is independent of circulating factors and neural modulation. Although opioids can protect myocytes directly during simulated ischemia-reperfusion (21, 26), opioid-induced late PC has been demonstrated only in in vivo experiments (12, 16), and thus a role of indirect (neural) mechanisms cannot be ruled out. Our results show that the myocardium itself acquires tolerance against ischemia-reperfusion injury after the administration of BW-373U86. The dosage of

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Table 2. LV peak positive and negative dP/dt

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>20 min After Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peak positive dP/dt, mmHg/s</td>
<td>Peak negative dP/dt, mmHg/s</td>
</tr>
<tr>
<td>Group I, control</td>
<td>10</td>
<td>1850 ± 140</td>
<td>960 ± 100</td>
</tr>
<tr>
<td>Group II, BW 0.1 (mg/kg)</td>
<td>9</td>
<td>2000 ± 230</td>
<td>980 ± 130</td>
</tr>
<tr>
<td>Group III, BW 0.33 (mg/kg)</td>
<td>9</td>
<td>1710 ± 150</td>
<td>870 ± 80</td>
</tr>
<tr>
<td>Group IV, BW 1.0 (mg/kg) = BW 24 h</td>
<td>9</td>
<td>1810 ± 200</td>
<td>870 ± 100</td>
</tr>
<tr>
<td>Group V, BW 1 h</td>
<td>9</td>
<td>2000 ± 120</td>
<td>920 ± 70</td>
</tr>
<tr>
<td>Group VI, BW 12 h</td>
<td>9</td>
<td>2040 ± 110</td>
<td>1030 ± 70</td>
</tr>
<tr>
<td>Group VII, NS + BW 1 h</td>
<td>10</td>
<td>1920 ± 120</td>
<td>890 ± 60</td>
</tr>
<tr>
<td>Group VIII, SC + BW 1 h</td>
<td>9</td>
<td>2110 ± 230</td>
<td>1060 ± 120</td>
</tr>
<tr>
<td>Group IX, BW 24 h + NS</td>
<td>8</td>
<td>1810 ± 250</td>
<td>1000 ± 140</td>
</tr>
<tr>
<td>Group X, BW 24 h + SC</td>
<td>9</td>
<td>2160 ± 210</td>
<td>1020 ± 100</td>
</tr>
<tr>
<td>Group XI, NS</td>
<td>8</td>
<td>1780 ± 120</td>
<td>880 ± 90</td>
</tr>
<tr>
<td>Group XII, SC</td>
<td>9</td>
<td>2170 ± 190</td>
<td>1070 ± 130</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. *P < 0.05 vs. group I (control); †P < 0.05 vs. group IV (BW 24 h).

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Table 3. Creatine kinase and LDH release in the perfusate

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Creatine kinase, IU/g wt</th>
<th>LDH, IU/g wt</th>
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<tbody>
<tr>
<td>Group I, control</td>
<td>10</td>
<td>15 ± 3</td>
<td>10 ± 2</td>
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<tr>
<td>Group II, BW 0.1 (mg/kg)</td>
<td>9</td>
<td>13 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Group III, BW 0.33 (mg/kg)</td>
<td>9</td>
<td>15 ± 3</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Group IV, BW 1.0 (mg/kg) = BW 24 h</td>
<td>9</td>
<td>14 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Group V, BW 1 h</td>
<td>9</td>
<td>20 ± 6</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Group VI, BW 12 h</td>
<td>9</td>
<td>17 ± 2</td>
<td>10 ± 1</td>
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<tr>
<td>Group VII, NS + BW 1 h</td>
<td>10</td>
<td>18 ± 6</td>
<td>12 ± 4</td>
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<td>Group VIII, SC + BW 1 h</td>
<td>9</td>
<td>18 ± 4</td>
<td>14 ± 2</td>
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<td>Group IX, BW 24 h + NS</td>
<td>8</td>
<td>15 ± 5</td>
<td>10 ± 3</td>
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<td>Group X, BW 24 h + SC</td>
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<td>17 ± 5</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Group XI, NS</td>
<td>8</td>
<td>14 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Group XII, SC</td>
<td>9</td>
<td>21 ± 7</td>
<td>14 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. *P < 0.05 vs. group I (control); †P < 0.05 vs. group IV (BW 24 h).
BW-373U86 that we used (1 mg/kg) was higher than that used by Patel et al. (29), who administered BW-373U86 intravenously at 0.1 mg/kg. The different route of administration may account for the differences in the sensitivity of hearts to BW-373U86 in that study and ours.

Role of COX-2 in Opioid-Induced Early PC

Opioids can mimic the early phase of ischemic PC by opening mitochondrial K_{ATP} channels (12, 21, 26, 32), but it has not been determined whether COX-2 is involved in opioid-induced early PC. Because expression of COX-2 protein is detectable even in the normal heart (33, 34), it is theoretically conceivable that COX-2 may play a role.

Li and Kloner (25) found that preadministration of 10 mg/kg of aspirin before repetitive cycles of brief ischemia did not abolish the early phase of ischemic PC and concluded that the cardioprotective effects of ischemic PC are not mediated by prostanooids. Camitta et al. (6) demonstrated that targeted disruption of the COX-1 or COX-2 gene did not affect the early phase of ischemic PC, although myocardial ischemia/reperfusion injury was exacerbated in COX-1^{−/−} or COX-2^{−/−} mice compared with control wild-type mice. In accordance with these studies, we found that BW-373U86-induced early PC was not abolished by 5 mg/kg NS-398, which completely blocked late PC. In addition, there was no difference in the PG levels either at baseline or during reperfusion between the control and BW-373U86-treated hearts (Figs. 4 and 5). These findings suggest that fundamentally different mechanisms are responsible for opioid-induced early and late PC, and further corroborate the notion that prostanooids do not contribute to early PC.
Role of COX-2 in Opioid-Induced Late PC

COX-2 mediates the protective effects of ischemia-induced late PC in rabbits and mice (17, 33), but does not mediate late PC induced by activation of adenosine A1 or A2 receptors in rabbits (23). In the present study, the selective COX-2 inhibitor NS-398 completely abolished the late PC induced by BW-373U86 in rats. These results are congruent with our recent finding that COX-2 mediates δ-opioid receptor-induced late PC in rabbits (24), indicating that the contribution of COX-2 to δ-opioid late PC is not species specific. Furthermore, our data suggest that COX-2 plays a key role at least in some types of pharmacological PC.

Involvement of COX-2 in Opioid-Induced Late PC

The next important question was how COX-2 mediates opioid-induced cardioprotection in isolated perfused hearts. We have found that COX-2 is upregulated after repetitive episodes of brief ischemia, leading to increased synthesis of PGE$_2$ and PGI$_2$ in the preconditioned myocardium (33, 34). Both PGI$_2$ and the PGE family have been reported to have cardioprotective properties (11, 20, 22, 35). Thus COX-2 dependent production of these prostanoids might protect the myocardium from ischemia-reperfusion injury during the late phase of ischemic PC. In recent studies in rabbits, we found that COX-2 protein expression was increased 24 h after BW-373U86 (24). Surprisingly, in this study, we found that COX-2 protein was not upregulated during opioid-induced late PC in rats, although opioid-induced cardioprotection was COX-2 dependent. Analysis of prostanoids released into the perfusate demonstrated that 24 h after the administration of BW-373U86, PGI$_2$ production was increased but PGE$_2$ production was not (Figs. 4 and 5). This is consistent with our finding that PGI$_2$ synthase protein content was increased by 56% 24 h after the administration of BW-373U86 (Fig. 7).

COX-1 and COX-2 are located upstream of respective prostanoid synthases and regulate the synthesis of prostanoids by supplying PGH$_2$, the common precursor of bioactive prostanoids (39). COX-1 is responsible for constitutive PG formation, whereas COX-2 is induced in response to stress, but is also constitutively ex-
pressed (39). Increasing evidence indicates that constitutive COX-2 does not contribute to the basal levels of PG production in the myocardium (6, 33, 34). However, it is unknown whether constitutive COX-2 produces PGH2, which each prostanoid synthase could utilize under pathological conditions. Every prostanoid synthase is known to utilize COX-1-derived PGH2; recently, a functional coupling between COX-2 and specific prostanoid synthases has also been proposed (7, 38). Ueno et al. (38) demonstrated that the perinuclear enzymes thromboxane synthase and PGI2 synthase generate their respective products via COX-2 rather than COX-1 in HEK-293 cells cotransfected with COX and prostanoid synthase. They also found that the COX selectivity of these lineage-specific prostanoid synthases was affected by the concentration of arachidonic acid. Although it is unknown whether the interaction between COX-2 and prostanoid synthases in rat myocardium is similar to that in transfected cultured cells, we propose that a functional coupling between COX-2 and upregulated PGI2 synthase is established during opioid-induced late PC. Our finding that the PGI2 levels at baseline did not change in opioid-treated hearts suggests that this coupling becomes functionally active during ischemia-reperfusion, possibly as a result of the release of arachidonic acid during this condition (10). Indeed, the fact that opioid-induced protection was abolished by the administration of a selective COX-2 inhibitor, but not a selective COX-1 inhibitor, suggests that COX-2-derived PGH2 plays an important role on the development of opioid-induced late PC. Recent evidence that COX-2 is the major source of systemic biosynthesis of PGI2 in healthy volunteers (27) and in patients with atherosclerosis (2) supports this hypothesis. The precise reason why PGI2 synthase couples preferentially to COX-2 as opposed to COX-1 during opioid-induced late PC is unknown. Nevertheless, our data identify, for the first time, upregulation of PGI2 synthase as a critical element in late PC, thereby revealing a mechanism of delayed cardioprotection that was heretofore unknown. These findings warrant further studies aimed at defining the preferential functional coupling between upstream COX and specific prostanoid synthesis in the cardiovascular system.

Our present finding that opioid-induced late PC is associated with upregulation of PGI2 synthase, but not COX-2 itself, differs from our recent findings in rabbits, in which COX-2 expression was increased (24), and reveals possible species-specific mechanisms underlying the functional involvement of COX-2-dependent prostanoid synthesis in opioid-induced cardioprotection. Mechanism(s) whereby PGI2 synthase is upregulated after stimulation of opioid receptors is unknown. It has been reported that TNF-α and IL-1 upregulate PGI2 synthase mRNA (7, 19, 42). The promoter of the PGI2 synthase gene contains several response elements, including NF-κB, NF-IL6, and Sp1 (42). NF-κB has been shown to play an essential role in ischemia-induced late PC (40), but its role in opioid-induced late PC is unknown. Study of the signaling pathways that lead to upregulation of PGI2 synthase is potentially a fruitful one, because regulation of PGI2 synthase has important clinical implications.

In conclusion, this study offers novel insights into the mechanism of opioid-induced late PC and late PC in general. Our findings show that COX-2 does not mediate opioid-induced early PC, whereas it does mediate late PC by increasing PGI2 production. Surprisingly, however, the increased COX-2-dependent biosynthesis of PGI2 cannot be explained by increased expression of COX-2 protein but appears instead to be the result of upregulated expression of PGI2 synthase, suggesting that a functional coupling between COX-2 and PGI2 synthase plays an important role in opioid-induced late PC. To our knowledge, this is the first time that PGI2 synthase has been shown to be upregulated during late PC and that an interaction between
COX and a specific prostanoid synthase has been shown in the heart. We propose that COX-2-dependent synthesis of PGI2 via preferential coupling of COX-2 with upregulated PGI synthase is a previously unrecognized mechanism for cardioprotection and a new pathway whereby opioid receptors protect the ischemic myocardium during late PC. The concept that cardiac PGI2 production is affected by stimulation of opioid receptors, suggests novel therapeutic strategies aimed at enhancing the production of cardioprotective prostanoids in the ischemic myocardium using selective opioid receptor agonists.

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REFERENCES


