

# Isoflurane Produces Delayed Preconditioning against Myocardial Ischemia and Reperfusion Injury

## Role of Cyclooxygenase-2

Katsuya Tanaka, M.D.,\* Lynda M. Ludwig, B.S.,† John G. Krolikowski, B.A.,‡ Dunbar Alcindor, B.S.,§ Phillip F. Pratt, Ph.D.,|| Judy R. Kersten, M.D.,# Paul S. Pagel, M.D., Ph.D.,\*\* David C. Wartier, M.D., Ph.D.††

**Background:** Whether volatile anesthetics produce a second window of preconditioning is unclear. The authors tested the hypothesis that isoflurane causes delayed preconditioning against infarction and, further, that cyclooxygenase (COX)-2 mediates this beneficial effect.

**Methods:** Rabbits (n = 43) were randomly assigned to receive 0.9% intravenous saline, the selective COX-2 inhibitor celecoxib (3 mg/kg intraperitoneal) five times over 2 days before coronary artery occlusion and reperfusion, or isoflurane (1.0 minimum alveolar concentration) 24 h before acute experimentation in the absence or presence of celecoxib pretreatment. Two additional groups of rabbits received a single dose of celecoxib either 30 min before or 21.5 h after administration of isoflurane. Rabbits were then instrumented for measurement of hemodynamics and underwent 30 min of coronary occlusion followed by 3 h of reperfusion. Myocardial infarct size was measured using triphenyltetrazolium staining. Western immunoblotting to examine COX-1 and COX-2 protein expression was performed in rabbit hearts that had or had not been exposed to isoflurane.

**Results:** Isoflurane significantly ( $P < 0.05$ ) reduced infarct size ( $22 \pm 3\%$  of the left ventricular area at risk) as compared with control ( $39 \pm 2\%$ ). Celecoxib alone had no effect on infarct size ( $36 \pm 4\%$ ) but abolished isoflurane-induced cardioprotection ( $36 \pm 4\%$ ). A single dose of celecoxib administered 2.5 h before coronary occlusion and reperfusion also abolished the delayed protective effects of isoflurane ( $36 \pm 4\%$ ), but celecoxib given 30 min before exposure to isoflurane had no effect ( $22 \pm 4\%$ ). Isoflurane did not alter COX-1 and COX-2 protein expression.

**Conclusions:** The results indicate that the volatile anesthetic isoflurane produces a second window of preconditioning against myocardial ischemia and reperfusion injury. Furthermore, COX-2 is an important mediator of isoflurane-induced delayed preconditioning.

A BRIEF period of myocardial ischemia imposed shortly before a more prolonged coronary artery occlusion pro-

ducts myocardium against infarction.<sup>1</sup> This process is known as *ischemic preconditioning* (IPC). IPC is characterized by an acute early phase limited to 1–3 h after the brief ischemic stimulus,<sup>2</sup> and a delayed late phase (*i.e.*, a second window) that emerges after 24 h and may persist for up to 72 h after the ischemic episode. The protective effects of delayed IPC are mimicked by a variety of drugs, including nitric oxide donors,<sup>3</sup> selective  $\delta$ -opioid agonists,<sup>4</sup> adenosine receptor agonists,<sup>5</sup> and mitochondrial adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel openers.<sup>6,7</sup> Volatile anesthetics have been shown to protect myocardium against reversible and irreversible ischemic injury.<sup>8–11</sup> This anesthetic-induced preconditioning (APC) is mediated by many of the same signal transduction elements involved in acute and delayed IPC. Whether volatile anesthetics produce delayed preconditioning remains unclear. Our laboratory recently demonstrated that isoflurane did not produce a second window of preconditioning in dogs,<sup>12</sup> but the effects of volatile agents in other species is unknown.

Delayed IPC is characterized by complex signal transduction cascades in which several protein kinases,<sup>13–18</sup> nuclear factor  $\kappa B$ ,<sup>19</sup> nitric oxide synthase,<sup>20,21</sup> and cyclooxygenase (COX)-2<sup>22,23</sup> have been implicated. In the current investigation, we tested the hypothesis that exposure to isoflurane 24 h before ischemia and reperfusion produces a second window of myocardial protection *in vivo*. In addition, we tested the hypothesis that COX-2 is involved in isoflurane-induced delayed preconditioning and, further, examined whether COX-2 functions as a trigger or mediator of this delayed protective effect.

## Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin). Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals*<sup>24</sup> of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.<sup>25</sup>

### Administration of Isoflurane

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were placed in an induction chamber 24 h

\* Research Fellow, † Graduate Student, ‡ Research Technologist, § Medical Student, || Assistant Professor, Pharmacology and Toxicology, # Professor of Anesthesiology, Pharmacology and Toxicology, \*\* Professor of Anesthesiology and Biomedical Engineering and Director of Cardiac Anesthesia, †† Professor of Anesthesiology, Biomedical Engineering, Pharmacology and Toxicology, and Medicine (Division of Cardiovascular Diseases), and Senior Vice Chairman of Anesthesiology, the Medical College of Wisconsin.

Received from the Departments of Anesthesiology, Pharmacology and Toxicology, and Medicine (Division of Cardiovascular Diseases), the Medical College of Wisconsin, Milwaukee, Wisconsin, and the Clement J. Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin. Submitted for publication August 26, 2003. Accepted for publication November 19, 2003. Supported in part by National Institutes of Health grants HL 63705 (to Dr. Kersten), HL 54820 (to Dr. Wartier), GM 08377 (to Dr. Wartier), and GM 066730 (to Dr. Wartier) from the United States Public Health Service, Bethesda, Maryland.

Address reprint requests to Dr. Wartier: Medical College of Wisconsin, MEB-M4280, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. Address electronic mail to: cknapp@mcw.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

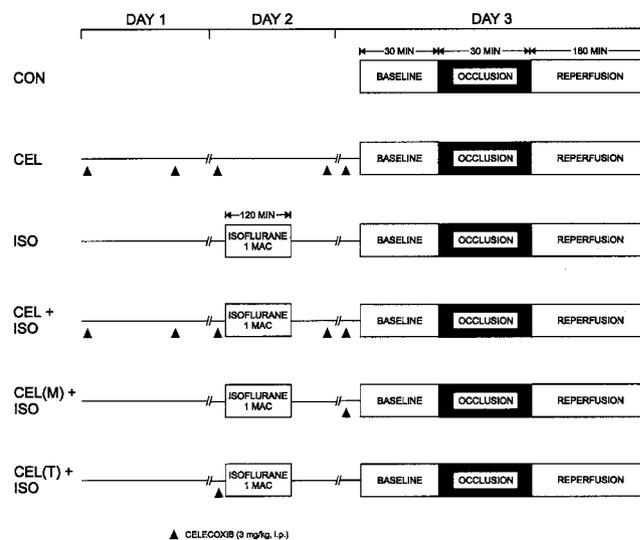
before acute experimentation. After inhalational induction, anesthesia was maintained with 1.0 minimum alveolar concentration (MAC) isoflurane (2.1%)<sup>26</sup> in 100% oxygen during spontaneous ventilation. The concentration of isoflurane inside the induction chamber was continuously measured by an infrared anesthetic analyzer that was calibrated with known standards before and during experimentation. Ten rabbits were randomly chosen for arterial blood gas analysis during administration of isoflurane. A 22-gauge catheter was inserted into a middle ear artery approximately 15 min after anesthetic induction, and arterial blood gas tensions were measured at selected intervals during administration of isoflurane. Anesthesia was discontinued after 2 h, and emergence was allowed to occur. Each rabbit was then housed overnight before experimentation on the next day.

### General Preparation

Rabbits with and without previous exposure to isoflurane were anesthetized with intravenous sodium pentobarbital (30 mg/kg) as previously described.<sup>10</sup> Briefly, a tracheostomy was performed through a ventral midline incision, and the trachea was cannulated. The rabbits were ventilated with positive pressure using an air-and-oxygen mixture. Heparin-filled catheters were inserted into the right carotid artery and the left jugular vein for measurement of arterial blood pressure and fluid or drug administration, respectively. A left thoracotomy was performed at the fourth intercostal space. A prominent branch of the left anterior descending coronary artery (LAD) was identified, and a silk ligature was placed around this vessel approximately halfway between the base and the apex for the production of coronary artery occlusion and reperfusion. Each rabbit was anticoagulated with 500 U heparin immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamic data were continuously recorded on a polygraph throughout experimentation.

### Experimental Protocol

The experimental design is depicted in figure 1. Baseline systemic hemodynamic data were recorded 30 min after instrumentation was completed. All rabbits underwent 30 min of LAD occlusion followed by 3 h of reperfusion. In four separate groups, rabbits were randomly assigned to receive only coronary artery occlusion and reperfusion (control), the selective COX-2 inhibitor celecoxib (3 mg/kg in 20% dimethyl sulfoxide, intraperitoneal) five different times at 12 h intervals for 2 days before LAD occlusion (48, 36, 24, 12, and 2.5 h before coronary occlusion and reperfusion), or exposure to 1.0 MAC isoflurane 24 h before acute experimentation in the absence or presence of celecoxib pretreatment. In two additional groups, rabbits received a single dose of cele-



**Fig. 1.** Schematic illustration of the experimental protocol used during myocardial infarct size experiments. Celecoxib (CEL; 3 mg/kg intraperitoneal) was administered five times over 2 days at 48, 36, 24, 12, and 2.5 h before left anterior descending coronary artery occlusion. CON = control; ISO = isoflurane; M = administration of a single dose of celecoxib during the mediator phase (2.5 h before left anterior descending coronary artery occlusion); MAC = minimum alveolar concentration; T = administration of a single dose celecoxib during the trigger phase (30 min before exposure to isoflurane).

coxib either 30 min before administration of isoflurane or 2.5 h before LAD occlusion after exposure to isoflurane on the previous day. Rabbits that developed intractable ventricular fibrillation and those with a left ventricular (LV) area at risk (AAR) for infarction less than 15% of the total LV mass were excluded from subsequent analysis.

### Determination of Myocardial Infarct Size

At the end of each experiment, myocardial infarct size was measured by means of a histochemical staining technique as previously described.<sup>27</sup> Briefly, the LAD was reoccluded, and 3 ml intravenous patent blue dye was injected. The LV AAR for infarction was separated from surrounding normal areas (stained blue), and the two regions were incubated at 37°C for 30 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to a pH of 7.4. After overnight storage in 10% formaldehyde, infarcted (unstained) and noninfarcted (stained red) myocardium within the AAR were carefully separated and weighed. Infarct size was expressed as a percentage of the AAR.

### Protein Isolation

Left ventricular tissue samples were also obtained from rabbits with (n = 3) and without (n = 3) previous exposure to isoflurane 24 h before anesthesia with intravenous sodium pentobarbital (30 mg/kg). After surgical instrumentation as described above, hearts were quickly excised, and the LV was isolated, quickly frozen in liquid

nitrogen, and stored at  $-70^{\circ}\text{C}$ . Tissue samples were homogenized in cold lysis buffer (pH 7.4) composed of 50 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 200  $\mu\text{M}$  sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 ml/20 g tissue protease inhibitor cocktail (Sigma P-8340; St. Louis, MO). The homogenate was centrifuged at 700g for 10 min to remove cellular debris and isolate total protein. Total protein was used because previous studies have indicated that COX-1 and COX-2 are membrane proteins contained within both the endoplasmic reticulum and the nuclear envelope.<sup>28,29</sup> All procedures were performed at  $4^{\circ}\text{C}$ , and protein concentration was determined by the Bradford method (BIO-RAD, Hercules, CA).

#### Western Immunoblotting

Equivalent amounts (50  $\mu\text{g}$ ) of protein samples were mixed with loading buffer (125 mM Tris, 4% sodium dodecyl sulfate, 5% glycerol, 0.03% bromophenol blue, and 1% 2-mercaptoethanol; pH 6.8) and heated at  $95^{\circ}\text{C}$  for 10 min. Samples were loaded onto a 12% polyacrylamide gel (BIO-RAD) and electrophoretically size separated at 180 V for approximately 1 h. After size separation, proteins were electrophoretically transferred to a nitrocellulose membrane (BIO-RAD) at 100 V for 1 h and then blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature for 2 h. Subsequently, nitrocellulose membranes were incubated overnight at  $4^{\circ}\text{C}$  in TBS-T containing 1% bovine serum albumin and a 1:1,000 dilution of goat primary antibodies for COX-1 or COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed four times with TBS-T for 10 min before a 30-min incubation with a 1:5,000 dilution of horseradish peroxidase-labeled donkey anti-goat immunoglobulin G (Santa Cruz Biotechnology) in TBS-T containing 1% bovine serum albumin. Bound antibody was detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ) on radiograph film. Ponceau staining of nitrocellulose membranes was used to verify equal protein loading. Quantitative analysis of the band densities was performed using AlphaImager 2000 software (Alpha Innotech Corporation, San Leandro, CA).

#### Statistical Analysis

Statistical analysis of data within and between groups was performed with analysis of variance for repeated measures followed by the Student-Newman-Keuls test. Statistical significance was defined as  $P < 0.05$ . All data are expressed as mean  $\pm$  SEM.

## Results

Forty-nine rabbits were instrumented to obtain 43 successful experiments. One rabbit was excluded because

the LV AAR was less than 15% of the LV mass (single dose of celecoxib 2.5 h before LAD occlusion + isoflurane). Five rabbits were excluded because of intractable ventricular fibrillation (1 control; 1 celecoxib alone; 1 isoflurane alone; 1 celecoxib + isoflurane; and 1 single dose of celecoxib 2.5 h before LAD occlusion + isoflurane).

#### Hemodynamics

No differences in baseline hemodynamics were observed between groups (table 1). Coronary artery occlusion and reperfusion produced similar decreases in mean arterial pressure and rate-pressure product in each group. No differences in hemodynamics were observed between groups. Arterial blood gas tensions and acid-base status were maintained within the normal range during pretreatment with isoflurane in all rabbits (data not shown).

#### Infarct Size

Body weight, LV weight, AAR weight, and the ratio of AAR to total LV mass were similar between groups (table 2). Isoflurane significantly ( $P < 0.05$ ) reduced infarct size ( $22 \pm 3\%$  of the LV AAR) as compared with control experiments ( $39 \pm 2\%$ ; fig. 2). Administration of celecoxib alone five times over 2 days before LAD occlusion had no effect on infarct size ( $36 \pm 4\%$ ), but this celecoxib pretreatment protocol abolished the protective effect of exposure to isoflurane ( $36 \pm 4\%$ ). Administration of a single dose of celecoxib 2.5 h before LAD occlusion also eliminated the delayed protective effects of isoflurane ( $36 \pm 4\%$ ). In contrast, a single dose of celecoxib administered 30 min before exposure to isoflurane did not affect infarct size ( $22 \pm 4\%$ ).

#### Protein Expression

Isoflurane did not alter COX-1 or COX-2 protein expression in rabbit myocardium 24 h after exposure to the volatile anesthetic as compared with control hearts (fig. 3).

## Discussion

Delayed IPC was recently shown to be mediated by COX-2 in rabbits<sup>22</sup> and mice.<sup>23</sup> Activation of  $\delta$ -opioid receptors also produced delayed preconditioning in rabbits<sup>30</sup> and rats.<sup>31</sup> These beneficial effects were abolished by selective COX-2 inhibition. IPC produced COX-2 transcription and translation and also enhanced the myocardial concentration of the prostaglandins PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  24 h after the initial ischemic stimulus.<sup>22</sup> Similar results were also observed during the second window of  $\delta$ -opioid-induced protection in rabbits<sup>30</sup> but not rats.<sup>31</sup> For example, prostacyclin (PGI<sub>2</sub>) synthase expression and PGI<sub>2</sub> production but not COX-2 in-

**Table 1. Systemic Hemodynamics**

	Baseline	30 min CAO	Reperfusion		
			1 h	2 h	3 h
HR, min <sup>-1</sup>					
Control	249 ± 8	243 ± 9	237 ± 10	236 ± 13	230 ± 14
Celecoxib	251 ± 9	236 ± 10*	226 ± 9*	210 ± 10*	199 ± 9*
Isoflurane	232 ± 7	226 ± 8	217 ± 8	203 ± 8*	195 ± 8*
Celecoxib + isoflurane	252 ± 10	239 ± 10	231 ± 11	229 ± 13	215 ± 12*
Celecoxib (M) + isoflurane	244 ± 10	239 ± 14	224 ± 10	215 ± 10	208 ± 10*
Celecoxib (T) + isoflurane	226 ± 14	226 ± 13	207 ± 9	197 ± 9*	199 ± 8*
MAP, mmHg					
Control	84 ± 3	68 ± 3*	70 ± 4*	70 ± 3*	70 ± 3*
Celecoxib	85 ± 2	72 ± 5*	69 ± 2*	70 ± 3*	74 ± 3*
Isoflurane	88 ± 4	75 ± 5	79 ± 3	76 ± 3	77 ± 3*
Celecoxib + isoflurane	88 ± 4	68 ± 5*	77 ± 3	78 ± 4	80 ± 5
Celecoxib (M) + isoflurane	93 ± 5	78 ± 4*	78 ± 4	81 ± 3	81 ± 5*
Celecoxib (T) + isoflurane	80 ± 4	60 ± 6*	65 ± 4*	67 ± 5*	71 ± 5*
RPP, min <sup>-1</sup> · mmHg · 10 <sup>3</sup>					
Control	23.5 ± 1.1	19.1 ± 1.2*	19.1 ± 1.5*	18.6 ± 1.4*	17.7 ± 1.6*
Celecoxib	23.4 ± 1.1	18.7 ± 1.7*	17.1 ± 0.6*	16.2 ± 0.7*	16.3 ± 0.7*
Isoflurane	23.1 ± 1.3	19.3 ± 1.6*	19.4 ± 1.3*	17.4 ± 1.0*	16.6 ± 1.0*
Celecoxib + isoflurane	25.1 ± 1.2	18.8 ± 1.8*	19.9 ± 1.4*	20.1 ± 1.8*	19.5 ± 2.1*
Celecoxib (M) + isoflurane	25.0 ± 1.9	20.5 ± 2.0*	19.4 ± 1.5*	19.1 ± 0.8*	18.2 ± 0.7*
Celecoxib (T) + isoflurane	20.7 ± 1.5	15.8 ± 1.9*	15.3 ± 1.3*	14.9 ± 1.5*	15.8 ± 1.4*

Data are presented as mean ± SEM.

\* Significantly ( $P < 0.05$ ) different from baseline.

CAO = coronary artery occlusion; HR = heart rate; MAP = mean arterial blood pressure; M and T = administration of a single dose of celecoxib during the mediator and trigger phases, respectively (see text); RPP = rate-pressure product.

creased 24 h after administration of the selective  $\delta$ -opioid agonist BW373U86 in rat hearts.<sup>31</sup> The second window of preconditioning precipitated by heat stress was associated with an increase in COX-2 protein in rat myocardium,<sup>32</sup> but adenosine A<sub>1</sub> or A<sub>3</sub> receptor-induced late preconditioning did not seem to involve a COX-2-dependent mechanism.<sup>33</sup> Therefore, COX-2 has been previously implicated in ischemic and pharmacologic delayed preconditioning, but a role for this enzyme has yet to be uniformly established and may also be species dependent.

Our laboratory has previously demonstrated that isoflurane protects the myocardium against irreversible ischemic injury and, further, that this protection is characterized by an acute memory phase in rats,<sup>11</sup> rabbits,<sup>10</sup> and dogs.<sup>8</sup> The results of the current investigation indicate that isoflurane reduces myocardial damage when administered 24 h before coronary artery occlusion in rabbit hearts *in vivo*, a protective effect that is equal in

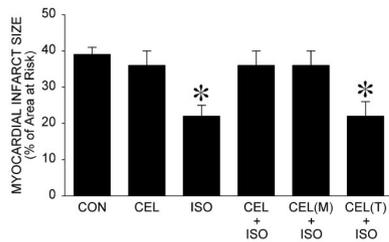
magnitude to acute preconditioning.<sup>10</sup> The current findings also show that this isoflurane-induced delayed preconditioning is abolished by administration of celecoxib after but not before exposure to isoflurane. These data suggest that COX-2 serves as a mediator but not a trigger for isoflurane-induced delayed preconditioning. The current results also indicate that COX-1 and COX-2 protein expression is not affected by exposure to isoflurane 24 h before coronary artery occlusion and reperfusion. These data show that enhanced COX-2 expression is not the mechanism for the delayed protective effect of the volatile agent and indirectly suggest that an increase in COX-2 activity may instead play a role in this process. Whether the activity of preexisting COX-2 was enhanced or prostaglandin synthesis was increased was not ascertained in the current study. The cellular specificity of COX expression and activity within the heart were also not identified. However, recent evidence indicates that cardiac myocytes are the major source of increased in-

**Table 2. Left Ventricular Area at Risk**

	No.	Body Weight, g	LV Weight, g	AAR Weight, g	AAR/LV, %
Control	8	2,880 ± 80	3.05 ± 0.12	0.92 ± 0.14	30 ± 4
Celecoxib	7	2,780 ± 70	3.03 ± 0.13	1.04 ± 0.08	35 ± 3
Isoflurane	7	2,890 ± 80	3.08 ± 0.13	0.87 ± 0.11	28 ± 3
Celecoxib + isoflurane	7	2,660 ± 70	3.07 ± 0.15	0.85 ± 0.09	28 ± 3
Celecoxib (M) + isoflurane	7	2,720 ± 70	2.88 ± 0.11	0.77 ± 0.08	27 ± 2
Celecoxib (T) + isoflurane	7	2,610 ± 80	3.05 ± 0.18	0.86 ± 0.11	28 ± 3

Data are presented as mean ± SEM.

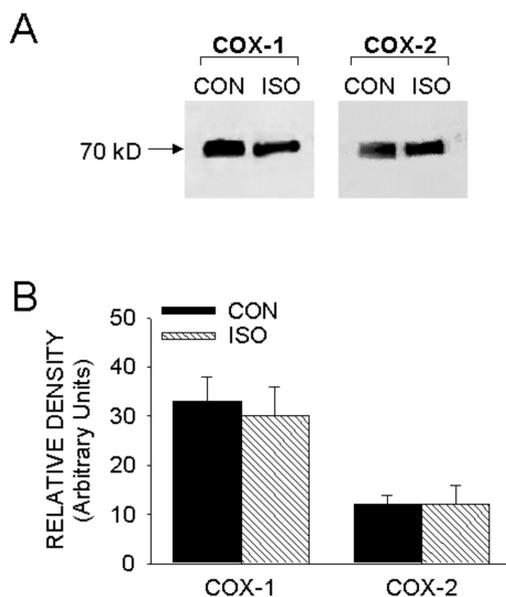
AAR = area at risk; LV = left ventricular; M and T = administration of a single dose of celecoxib during the mediator and trigger phases, respectively (see text).



**Fig. 2.** Myocardial infarct size expressed as a percentage of the left ventricular area at risk. CEL = celecoxib; CON = control; ISO = isoflurane; M = administration of celecoxib during mediator phase (2.5 h before left anterior descending coronary artery occlusion); T = administration of a single dose celecoxib during the trigger phase (30 min before exposure to isoflurane). \* Significantly ( $P < 0.05$ ) different from control.

ducible nitric oxide synthase (iNOS) expression in delayed IPC,<sup>34</sup> a crucial signaling element that likely modulates COX-2 expression and activity.<sup>35</sup>

The signaling mechanisms that regulate COX-2 expression and the functional involvement of COX-2-dependent prostaglandin synthesis during the second window of preconditioning remain to be fully elucidated. COX is the rate-limiting enzyme in the conversion of arachidonic acid to  $\text{PGH}_2$ .<sup>36</sup> In contrast with the constitutive COX-1 isoform, COX-2 is normally absent from cells and is induced in response to stress. iNOS and nitric oxide produced by this enzyme are also mediators of delayed preconditioning<sup>21</sup> and are frequently coinduced with COX-2.<sup>36</sup> IPC enhances iNOS expression in cardiac myocytes.<sup>34</sup> NO may act as both a trigger and a mediator of delayed IPC<sup>20</sup> through a COX-2-dependent mecha-



**Fig. 3.** (A) Western immunoblots illustrating total cyclooxygenase (COX)-1 and COX-2 myocardial protein expression under control conditions (CON) and 24 h after exposure to 1.0 minimum alveolar concentration isoflurane (ISO). (B) Histograms representing the relative band densities for COX-1 and COX-2 under control conditions (open bars) and 24 h after exposure to isoflurane (hatched bars).

nism.<sup>37</sup> IPC-induced increases in  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  are attenuated by iNOS inhibitors, but blockade of COX-2 does not affect iNOS activity.<sup>35</sup> These data suggest that COX-2 is located downstream of iNOS in this signaling pathway. In addition, iNOS, reactive oxygen species, and soluble guanylate cyclase do not seem to be involved in regulating increases in COX-2 expression during the second window of preconditioning,<sup>35</sup> despite observations indicating that these molecules play important signaling roles in delayed protection.<sup>20,38,39</sup> These data strongly suggest that other autocooids are involved in mediating the second window of preconditioning through COX-2.

COX-2 up-regulation in delayed IPC is dependent on protein kinase C (PKC), protein tyrosine kinase, and nuclear factor  $\kappa\text{B}$ .<sup>35</sup> The nitric oxide donor nitroglycerin produced a second window of protection in rabbits that was abolished by the PKC inhibitor chelerythrine.<sup>13</sup> In fact, the  $\epsilon$  isoform of PKC has been shown to play a critical role in delayed IPC.<sup>14</sup> A role for PKC activation and translocation in APC has also been strongly implied in several studies, but whether PKC mediates delayed protection associated with volatile agents remains to be identified. Trigger and mediator functions have been suggested for several families of protein tyrosine kinase during the second window of preconditioning. Protein tyrosine kinase subtypes also seem to be essential modulators of iNOS activity.<sup>15</sup> Our laboratory has recently demonstrated that Src protein tyrosine kinase is involved in APC,<sup>40</sup> but how this kinase species modulates delayed preconditioning by volatile anesthetics is also currently unknown. The mitogen-activated protein kinase family has been implicated as a critical regulator of protein expression in the delayed protection elicited by  $\delta$ -opioid agonists<sup>18</sup> and adenosine.<sup>41</sup> In fact, the p38 mitogen-activated protein kinase subfamily activator anisomycin mimics late preconditioning by enhancing both nuclear factor  $\kappa\text{B}$  and iNOS activity.<sup>42</sup> The janus tyrosine kinases and the signal transducers and activators of transcription also have been shown to play essential roles in COX-2 up-regulation that occurs during the second window of preconditioning.<sup>43</sup> Whether these other previously identified signal transduction molecules are also involved in delayed APC remains to be determined.

The results of the current investigation also show that celecoxib administered five times over 2 days abolishes the second window of protection produced by isoflurane. Although these initial experiments were conducted to determine whether COX-2 was involved in isoflurane-induced delayed preconditioning, this drug regimen was also chosen on the basis of the controversy that COX-2 inhibitors may increase cardiovascular events.<sup>44</sup> The incidence of myocardial ischemic episodes is increased during the perioperative period. Therefore, many patients taking COX-2 inhibitors before and after surgery may be at a higher risk for development of

cardiovascular complications. Our current data, although preliminary, support the contention that COX-2 inhibition may magnify the occurrence of cardiovascular events.

The current findings should be interpreted within the constraints of several potential limitations. The LV AAR for infarction and coronary collateral blood flow represent major determinants of myocardial infarct size. The LV AAR values were similar between experimental groups, and minimal coronary collateral blood flow has been previously reported in rabbits.<sup>45</sup> Therefore, it seems unlikely that the current results were substantially affected by these variables. There were no differences in systemic hemodynamics between groups before and after coronary artery occlusion. Therefore, it seems highly likely that the observed reductions in myocardial infarct size produced by remote exposure to isoflurane occurred independent of many of the hemodynamic determinants of myocardial oxygen consumption. Values of the rate-pressure product, an indirect index of myocardial oxygen consumption, were also similar between experimental groups. Nevertheless, myocardial oxygen consumption was not directly calculated, nor was coronary collateral blood flow measured during the current investigation.

Our laboratory previously reported that isoflurane administered 24 h before coronary artery occlusion and reperfusion did not protect against myocardial infarction in dogs.<sup>12</sup> Differences between the current and previous findings in rabbits and dogs may be due to some degree to variation in experimental design (*i.e.*, 2 *vs.* 6 h of isoflurane pretreatment, spontaneous *vs.* controlled ventilation, respectively) but more likely resulted because the late window of protection seems to occur at distinct times in different species.<sup>46</sup> In addition, the current investigation did not determine myocardial infarct size in rabbits at any time interval other than 24 h after isoflurane administration. Future research is needed to characterize the time and duration of the early and second windows of APC.

In summary, the current results indicate that exposure to isoflurane 24 h before coronary artery occlusion and reperfusion reduces experimental myocardial infarct size in rabbits. This isoflurane-induced delayed preconditioning was abolished by celecoxib when the selective COX-2 inhibitor was administered after but not before exposure to the volatile agent. COX-2 protein expression in rabbit myocardium 24 h after administration of isoflurane was unchanged and thus not responsible for the delayed protective effects of isoflurane. However, eicosanoids directly produced by this enzyme complex or others synthesized from intermediate products produced by COX-2 seem to be key elements of the signal transduction in APC. The results suggest that COX-2 is an essential mediator but not a trigger of the intracellular

signaling cascade responsible for isoflurane-induced delayed preconditioning *in vivo*.

The authors thank David A. Schwabe, B.S.E.E. (Senior Research Technologist, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin), for technical assistance. The authors also thank Mary Lorence-Hanke, A.A. (Administrative Assistant, Department of Anesthesiology, Medical College of Wisconsin), for assistance in preparation of the manuscript.

## References

- Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124-36
- Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M: Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993; 72:1293-9
- Takano H, Tang XL, Qiu Y, Guo Y, French BA, Bolli R: Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism. *Circ Res* 1998; 83:73-84
- Fryer RM, Hsu AK, Eells JT, Nagase H, Gross GJ: Opioid-induced second window of cardioprotection: Potential role of mitochondrial K<sub>ATP</sub> channels. *Circ Res* 1999; 84:846-51
- Takano H, Bolli R, Black RG Jr, Kodani E, Tang XL, Yang Z, Bhattacharya S, Auchampach JA: A<sub>1</sub> or A<sub>3</sub> adenosine receptors induce late preconditioning against infarction in conscious rabbits by different mechanisms. *Circ Res* 2001; 88:520-8
- Takashi E, Wang Y, Ashraf M: Activation of mitochondrial K<sub>ATP</sub> channel elicits late preconditioning against myocardial infarction via protein kinase C signaling pathway. *Circ Res* 1999; 85:1146-53
- Ockaili R, Emami VR, Okubo S, Brown M, Krottapalli K, Kukreja RC: Opening of mitochondrial K<sub>ATP</sub> channel induces early and delayed cardioprotective effect: Role of nitric oxide. *Am J Physiol Heart Circ Physiol* 1999; 277:H2425-34
- Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K<sub>ATP</sub> channels: Reduction of myocardial infarct size with an acute memory phase. *ANESTHESIOLOGY* 1997; 87:361-70
- Toller WG, Gross ER, Kersten JR, Pagel PS, Gross GJ, Warltier DC: Sarcolemmal and mitochondrial adenosine triphosphate-dependent potassium channels: Mechanism of desflurane-induced cardioprotection. *ANESTHESIOLOGY* 2000; 92:1731-9
- Tanaka K, Weihrauch D, Kehl F, Ludwig LM, LaDisa JF Jr, Kersten JR, Pagel PS, Warltier DC: Mechanism of preconditioning by isoflurane in rabbits: A direct role for reactive oxygen species. *ANESTHESIOLOGY* 2002; 97:1485-90
- Ludwig LM, Patel HH, Gross GJ, Kersten JR, Pagel PS, Warltier DC: Morphine enhances pharmacological preconditioning by isoflurane: Role of mitochondrial K<sub>ATP</sub> channels and opioid receptors. *ANESTHESIOLOGY* 2003; 98:705-11
- Kehl F, Pagel PS, Krolikowski JG, Gu W, Toller W, Warltier DC, Kersten JR: Isoflurane does not produce a second window of preconditioning against myocardial infarction *in vivo*. *Anesth Analg* 2002; 95:1162-8
- Banerjee S, Tang XL, Qiu Y, Takano H, Manchikalapudi S, Dawn B, Shirk G, Bolli R: Nitroglycerin induces late preconditioning against myocardial stunning *via* a PKC-dependent pathway. *Am J Physiol Heart Circ Physiol* 1999; 277:H2488-94
- Qiu Y, Ping P, Tang XL, Manchikalapudi S, Rizvi A, Zhang J, Takano H, Wu WJ, Teschner S, Bolli R: Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that epsilon is the isoform involved. *J Clin Invest* 1998; 101:2182-98
- Dawn B, Xuan YT, Qiu Y, Takano H, Tang XL, Ping P, Banerjee S, Hill M, Bolli R: Bifunctional role of protein tyrosine kinases in late preconditioning against myocardial stunning in conscious rabbits. *Circ Res* 1999; 85:1154-63
- Dawn B, Takano H, Tang XL, Kodani E, Banerjee S, Rezazadeh A, Qiu Y, Bolli R: Role of Src protein tyrosine kinases in late preconditioning against myocardial infarction. *Am J Physiol Heart Circ Physiol* 2002; 283:H549-56
- Tang XL, Kodani E, Takano H, Hill M, Shinmura K, Vondriska TM, Ping P, Bolli R: Protein tyrosine kinase signaling is necessary for NO donor-induced late preconditioning against myocardial stunning. *Am J Physiol Heart Circ Physiol* 2003; 284:H1441-8
- Fryer RM, Hsu AK, Gross GJ: ERK and p38 MAP kinase activation are components of opioid-induced delayed cardioprotection. *Basic Res Cardiol* 2001; 96:136-42
- Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R: Nuclear factor-κB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. *Circ Res* 1999; 84:1095-109
- Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK: The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase: Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res* 1997; 81:1094-107

21. Takano H, Manchikalapudi S, Tang XL, Qiu Y, Rizvi A, Jadoon AK, Zhang Q, Bolli R: Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 1998; 98:441-9
22. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R: Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A* 2000; 97:10197-202
23. Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL, Bolli R: Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol* 2000; 95:479-84
24. World Medical Association, American Physiological Society: Guiding principles for research involving animals and human beings. *Am J Physiol Regul Integr Comp Physiol* 2002; 283:R281-3
25. Institute of Laboratory Animal Resources: Guide for the Care and Use of Laboratory Animals, 7th edition. Washington, D.C., National Academy Press, 1996
26. Drummond JC: MAC for halothane, enflurane, and isoflurane in the New Zealand white rabbit: And a test for the validity of MAC determinations. *ANESTHESIOLOGY* 1985; 62:336-8
27. Warltier DC, Zyvoloski MG, Gross GJ, Hardman HF, Brooks HL: Determination of experimental myocardial infarct size. *J Pharmacol Methods* 1981; 6:199-210
28. Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL, Smith WL: Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J Biol Chem* 1995; 270:10902-8
29. Spencer AG, Woods JW, Arakawa T, Singer II, Smith WL: Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J Biol Chem* 1998; 273:9886-93
30. Kodani E, Xuan YT, Shinmura K, Takano H, Tang XL, Bolli R: Delta-opioid receptor-induced late preconditioning is mediated by cyclooxygenase-2 in conscious rabbits. *Am J Physiol Heart Circ Physiol* 2002; 283:H1943-57
31. Shinmura K, Nagai M, Tamaki K, Tani M, Bolli R: COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 2002; 283:H2534-43
32. Arnaud C, Joyeux-Faure M, Godin-Ribuot D, Ribuot C: COX-2: an *in vivo* evidence of its participation in heat stress-induced myocardial preconditioning. *Cardiovasc Res* 2003; 58:582-8
33. Kodani E, Shinmura K, Xuan YT, Takano H, Auchampach JA, Tang XL, Bolli R: Cyclooxygenase-2 does not mediate late preconditioning induced by activation of adenosine A1 or A3 receptors. *Am J Physiol Heart Circ Physiol* 2001; 281:H959-68
34. Wang Y, Guo Y, Zhang SX, Wu WJ, Wang J, Bao W, Bolli R: Ischemic preconditioning upregulates inducible nitric oxide synthase in cardiac myocyte. *J Mol Cell Cardiol* 2002; 34:5-15
35. Shinmura K, Xuan YT, Tang XL, Kodani E, Han H, Zhu Y, Bolli R: Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. *Circ Res* 2002; 90:602-8
36. Vane JR, Bakhle YS, Botting RM: Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 1998; 38:97-120
37. Li Q, Guo Y, Xuan YT, Lowenstein CJ, Stevenson SC, Prabhu SD, Wu WJ, Zhu Y, Bolli R: Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. *Circ Res* 2003; 92:741-8
38. Tang XL, Takano H, Rizvi A, Turrens JF, Qiu Y, Wu WJ, Zhang Q, Bolli R: Oxidant species trigger late preconditioning against myocardial stunning in conscious rabbits. *Am J Physiol Heart Circ Physiol* 2002; 282:H281-91
39. Kodani E, Xuan YT, Takano H, Shinmura K, Tang XL, Bolli R: Role of cyclic guanosine monophosphate in late preconditioning in conscious rabbits. *Circulation* 2002; 105:3046-52
40. Ludwig LM, Weihrauch D, Kersten JR, Pagel PS, Warltier DC: Protein kinase C translocation and Src protein tyrosine kinase activation mediate isoflurane-induced preconditioning *in vivo*: Potential downstream targets of mitochondrial adenosine triphosphate-sensitive potassium channels and reactive oxygen species. *ANESTHESIOLOGY* 2003; 100:532-9
41. Carroll R, Yellon DM: Delayed cardioprotection in a human cardiomyocyte-derived cell line: The role of adenosine, p38MAP kinase and mitochondrial KATP. *Basic Res Cardiol* 2000; 95:243-9
42. Zhao TC, Taher MM, Valerie KC, Kukreja RC: p38 Triggers late preconditioning elicited by anisomycin in heart: Involvement of NF- $\kappa$ B and iNOS. *Circ Res* 2001; 89:915-22
43. Xuan YT, Guo Y, Zhu Y, Han H, Langenbach R, Dawn B, Bolli R: Mechanism of cyclooxygenase-2 upregulation in late preconditioning. *J Mol Cell Cardiol* 2003; 35:525-37
44. Mukherjee D, Nissen SE, Topol EJ: Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA* 2001; 286:954-9
45. Maxwell MP, Hearse DJ, Yellon DM: Species variation in the coronary collateral circulation during regional myocardial ischaemia: A critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc Res* 1987; 21:737-46
46. Schwartz LM, Sebbag L, Jennings RB, Reimer KA: Duration and reinstatement of myocardial protection against infarction by ischemic preconditioning in open chest dogs. *J Mol Cell Cardiol* 2001; 33:1561-70