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# *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*

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***Background:*** **The authors tested the hypotheses that protein kinase C (PKC)–specific isoform translocation and Src protein tyrosine kinase (PTK) activation play important roles in isoflurane-induced preconditioning *in vivo*.**

***Methods:*** **Rats (n = 125) instrumented for measurement of hemodynamics underwent 30 min of coronary artery occlusion followed by 2 h of reperfusion and received 0.9% saline (control); PKC inhibitors chelerythrine (5 mg/kg), rottlerin (0.3 mg/kg), or PKC-εV1–2 peptide (1 mg/kg); PTK inhibitors lavendustin A (1 mg/kg) or 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1; 1 mg/kg); mitochondrial adenosine triphosphate–sensitive potassium channel antagonist 5-hydroxydecanote (10 mg/kg); or reactive oxygen species scavenger *N*-acetylcysteine (150 mg/kg) in the absence and presence of a 30-min exposure to isoflurane (1.0 minimum alveolar concentration) in separate groups. Isoflurane was discontinued 15 min before coronary occlusion (memory period). Infarct size was determined using triphenyltetrazolium staining. Immunohistochemistry and confocal microscopic imaging were performed to examine PKC translocation in separate groups of rats.**

***Results:*** **Isoflurane significantly ($P < 0.05$) reduced infarct size (40 ± 3% [n = 13]) as compared with control experiments (58 ± 2% [n = 12]). Chelerythrine, rottlerin, PKC-εV1–2 peptide, lavendustin A, PP1, 5-hydroxydecanote, and *N*-acetylcysteine abolished the anti-ischemic actions of isoflurane (58 ± 2% [n = 8], 50 ± 3% [n = 9], 53 ± 2% [n = 9], 59 ± 3% [n = 6], 57 ± 3% [n = 7], 60 ± 3% [n = 7], and 53 ± 3% [n = 6], respectively). Isoflurane stimulated translocation of the δ and ε isoforms of PKC to sarcolemmal and mitochondrial membranes, respectively.**

***Conclusions:*** **Protein kinase C-δ, PKC-ε, and Src PTK mediate isoflurane-induced preconditioning in the intact rat heart. Opening of mitochondrial adenosine triphosphate–sensitive potassium channels and generation of reactive oxygen species are upstream events of PKC activation in this signal transduction process.**

COMPLEX, often redundant and interrelated signal transduction pathways involving several types of protein kinases have been implicated in endogenous myocardial protection against ischemia and reperfusion injury. Activation of protein kinase C (PKC) has been repeatedly shown to be essential for eliciting the protective effects produced by ischemic preconditioning (IPC), adenosine subtype 1 ($A_1$)[1] or $\alpha_1$-adrenergic[2] receptor agonists, δ-opioid agonists,[3] and nitric oxide donors.[4] PKC also seems to play an important role in myocardial protection produced by volatile anesthetics. The anti-ischemic actions of halothane have been shown to be abolished by PKC inhibition.[5] Isoflurane-enhanced recovery of contractile function in canine stunned myocardium was also partially attenuated by a PKC inhibitor.[6] More recently, protein tyrosine kinases (PTK) have been shown to be crucial signaling elements required for protection against ischemic injury to occur.[7] Whether PTK mediates myocardial protection produced by volatile anesthetics is currently unknown.

Reactive oxygen species (ROS) also play a crucial role in volatile anesthetic–induced preconditioning.[8,9] Our laboratory recently demonstrated that isoflurane generates the production of small quantities of ROS *in vivo* that are capable of triggering myocardial protection.[9] This process was dependent on activation of mitochondrial adenosine triphosphate–sensitive potassium ($K_{ATP}$) channels.[10] Another intriguing recent study suggested that this volatile anesthetic–induced production of ROS occurred upstream of PKC activation to elicit beneficial effects.[11] In the current investigation, we tested the hypothesis that specific PKC isoforms and Src PTK mediate isoflurane-induced preconditioning *in vivo*. We further hypothesized that isoflurane stimulates the translocation of PKC-δ and -ε in rat myocardium and that this translocation occurs downstream of mitochondrial $K_{ATP}$ channel opening and ROS production.

## Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Ani-

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mal 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*[12] of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.[13]

*General Preparation*

Male Wistar rats weighing between 280 and 420 g were anesthetized with intraperitoneal thiobutabarbital sodium (100–150 mg/kg) and instrumented for the measurement of systemic hemodynamics as previously described.[14] Briefly, heparin-filled catheters were inserted into the right jugular vein and the right carotid artery for fluid or drug administration and measurement of arterial blood pressure, respectively. A tracheotomy was performed, and the trachea was cannulated. Rats were ventilated with positive end-expiratory pressure using an air-and-oxygen mixture. A left thoracotomy was performed in the fifth intercostal space, and the pericardium was opened. A 6-0 Prolene ligature was placed around the proximal left descending coronary artery and vein in the area immediately below the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was produced by clamping the snare onto the epicardial surface of the heart with a hemostat and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperemic response. Hemodynamic data were continuously recorded on a polygraph throughout experimentation.

*Experimental Protocol*

The experimental design used in the current investigation is illustrated in figure 1. All rats underwent 30 min of coronary artery occlusion followed by 2 h of reperfusion. In 16 separate experimental groups, rats were randomly assigned to receive 0.9% saline, the nonselective PKC inhibitor chelerythrine (5 mg/kg in 0.9% saline; Sigma, St. Louis, MO),[7] the selective PKC-δ inhibitor rottlerin (0.3 mg/kg in dimethyl sulfoxide; BIOMOL, Plymouth Meeting, PA),[3] the selective PKC-ϵ inhibitor myristoylated PKC-ϵV1–2 peptide (1 mg/kg in 1:3 ethanol–0.9% saline; BIOMOL),[15] the nonselective PTK inhibitor lavendustin A (1 mg/kg in 0.9% saline; BIOMOL),[16] the selective Src PTK inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1; 1 mg/kg in dimethyl sulfoxide; BIOMOL),[17] the selective mitochondrial $K_{ATP}$ channel antagonist 5-hydroxydecanoate (5-HD; 10 mg/kg in 0.9% saline; Sigma Research Biochemicals, Natick, MA),[14] or the reactive oxygen species scavenger *N*-acetylcysteine (NAC; 150 mg/kg in 0.9% saline; Sigma)[9] in the absence and presence of isoflurane (1.0 minimum alveolar concentration [MAC]). Chelerythrine, rottlerin, PKC-ϵV1–2 peptide, lavendustin A, and PP1 were administered 1 h before coronary artery occlusion. 5-HD and NAC were administered 50 and 75 min before occlusion, respectively. Isoflurane was administered for 30 min and discontinued 15 min (memory period) before coronary artery occlusion. End-tidal concentrations of isoflurane were measured at the tip of the tracheotomy tube with use of an infrared gas analyzer that was calibrated with known standards before and during experimentation.

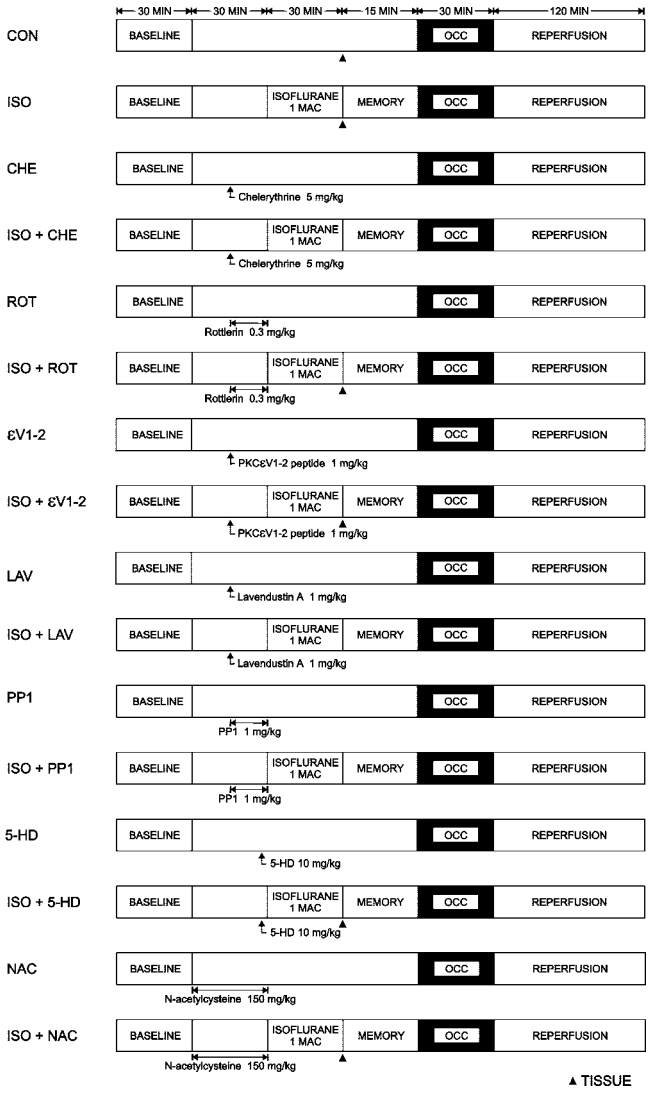


**Fig. 1. Schematic illustration of the experimental protocol used in infarct size experiments. 5-HD = 5-hydroxydecanoic acid; CHE = chelerythrine; ϵV1–2 = protein kinase C-ϵV1–2 peptide; ISO = isoflurane; LAV = lavendustin A; MAC = minimum alveolar concentration; NAC = *N*-acetylcysteine; OCC = occlusion; PP1 = 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; ROT = rottlerin.**

*Determination of Myocardial Infarct Size*

Myocardial infarct size was measured as previously described.[18] Briefly, at the end of each experiment, the coronary artery was reoccluded, and patent blue dye was injected intravenously to stain the normal region of

the left ventricle. The heart was rapidly excised, and the left ventricle was isolated. The left ventricular (LV) area at risk (AAR) was separated from surrounding blue-stained normal areas, and the two regions were incubated at 37°C for 15 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 samples within the AAR were carefully separated and weighed. Infarct size was expressed as a percentage of the LV AAR.

*Immunohistochemistry*

Left ventricular tissue samples were also obtained from separate groups of rats anesthetized with sodium thiobutabarbital that were pretreated with rottlerin, PKC-ϵV1–2 peptide, 5-HD, or NAC in the presence or absence of 1.0 MAC isoflurane. Hearts were rapidly excised after administration of pharmacologic antagonists or isoflurane, and the left ventricle was isolated and frozen in liquid nitrogen (−70°C) for subsequent analysis. Transverse cryostat sections (5 μm) of the left ventricle were mounted on positively charged Colorfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed for 20 min in 100% acetone at −20°C and rinsed with phosphate-buffered saline. Sections were incubated with 1:500 dilutions of rabbit polyclonal primary antibodies for PKC-δ or -ϵ (Santa Cruz Biotechnology, Santa Cruz, CA) or Src PTK (Upstate, Lake Placid, NY) in phosphate-buffered saline at 37°C for 1 h. Sections were washed three times for 5 min with phosphate-buffered saline. Sections were subsequently incubated with 1:1,000 dilutions of biotinylated anti-rabbit secondary antibodies (Santa Cruz Biotechnology) in phosphate-buffered saline at 37°C for 30 min. Sections were washed again three times for 5 min with phosphate-buffered saline before conjugation with 10 μg/ml streptavidin-labeled fluorescein isothiocyanate (Pierce, Rockford, IL) at 37°C for 15 min. To confirm PKC-ϵ translocation to mitochondria, sections were double stained using a 1:25 dilution of the mitochondrial marker prohibitin (Research Diagnostics, Flanders, NJ). A biotinylated anti-mouse secondary antibody (Amersham Pharmacia, Piscataway, NJ; 1:1000 dilution) was used to label prohibitin, followed by subsequent incubation with 10 μg/ml streptavidin R-phycoerythrin (Prozyme, San Leandro, CA). Nuclear staining was achieved with 1 μM TO-PRO-3 (Molecular Probes, Eugene, OR) at 37°C for 30 min. Images were obtained by means of a laser fluorescence imaging system and a confocal microscope. Use of the ×40 objective yielded a ×400 end magnification. A krypton–argon laser was used for excitation wavelengths of 490, 545, and 642 nm, and emitted fluorescence was determined after long-pass filtering at corresponding wavelengths of 520, 578, and 661 nm for fluorescein isothiocyanate, R-phycoerythrin, and TO-PRO, respectively. Approximately 5–10 images were obtained for each rat heart.

*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 ± SEM.

## Results

One hundred forty-three rats were instrumented to obtain 125 successful myocardial infarct size experiments. Eight rats were excluded as a result of technical difficulties with the experimental preparation. Malignant ventricular arrhythmias developed in 10 other rats before completion of the experiment, and these rats were excluded from further analysis.

*Hemodynamics*

No differences in baseline systemic hemodynamic results were observed between groups (table 1). Isoflurane significantly ($P < 0.05$) decreased heart rate, mean arterial pressure, and rate–pressure product in the presence or absence of all pharmacologic antagonists examined. Coronary artery occlusion and reperfusion produced similar decreases in mean arterial pressure and rate–pressure product in each experimental group.

*Infarct Size*

Body weight, LV weight, AAR weight, and ratio of AAR to total LV mass were similar among groups (data not shown). Isoflurane significantly reduced infarct size (40 ± 3% [n = 13] of the LV AAR; fig. 2) as compared with control experiments (58 ± 2% [n = 12]). Chelerythrine, rottlerin, PKC-ϵV1–2 peptide, lavendustin A, and PP1 abolished the anti-ischemic actions of isoflurane (58 ± 2% [n = 8], 50 ± 3% [n = 9], 53 ± 2% [n = 9], 59 ± 3% [n = 6], and 57 ± 3% [n = 7], respectively; fig. 2). Chelerythrine, rottlerin, PKC-ϵV1–2 peptide, lavendustin A, and PP1 alone had no effect on infarct size (54 ± 2% [n = 8], 64 ± 4% [n = 6], 60 ± 3% [n = 6], 56 ± 4% [n = 6], and 57 ± 4% [n = 9], respectively; fig. 3). 5-HD and NAC also eliminated the protection produced by isoflurane (60 ± 3% [n = 7] and 53 ± 3% [n = 6], respectively) but did not affect infarct size when administered alone (54 ± 4% [n = 7] and 52 ± 3% [n = 6], respectively; fig. 4).

*Immunohistochemistry*

Eighteen rats were instrumented for immunohistochemical analysis. Isoflurane (n = 3) stimulated translocation of PKC-δ and -ϵ to sarcolemmal and mitochondrial membranes, respectively (fig. 5). Control rat myocardium

**Table 1. Systemic Hemodynamics**

| | | | | | | Reperfusion | |
|---|---|---|---|---|---|---|---|
| | No. | Baseline | Isoflurane | Preocclusion | 30 min CAO | 1 h | 2 h |
| HR, $min^{-1}$ | | | | | | | |
| Control | 12 | 366 ± 15 | — | 368 ± 13 | 339 ± 10* | 309 ± 13* | 300 ± 12* |
| Isoflurane | 13 | 369 ± 11 | 275 ± 12 | 326 ± 13* | 340 ± 13* | 303 ± 11* | 302 ± 11* |
| Chelerythrine | 8 | 391 ± 12 | — | 359 ± 7* | 356 ± 14* | 329 ± 16* | 296 ± 14* |
| Isoflurane + chelerythrine | 8 | 354 ± 11 | 278 ± 12* | 328 ± 18 | 323 ± 17 | 274 ± 9* | 280 ± 8* |
| Rottlerin | 6 | 359 ± 15 | — | 336 ± 15* | 333 ± 19* | 317 ± 16* | 304 ± 17* |
| Isoflurane + rottlerin | 9 | 358 ± 10 | 286 ± 14* | 314 ± 14* | 336 ± 14 | 298 ± 7* | 289 ± 8* |
| εV1–2 | 6 | 352 ± 23 | — | 326 ± 12 | 350 ± 12 | 314 ± 16 | 303 ± 13* |
| Isoflurane + εV1–2 | 9 | 368 ± 9 | 284 ± 12* | 292 ± 21* | 331 ± 12* | 308 ± 12* | 296 ± 12* |
| LaVendustin A | 6 | 332 ± 19 | — | 329 ± 13 | 338 ± 16 | 303 ± 10 | 298 ± 12 |
| Isoflurane + lavendustin A | 6 | 387 ± 17 | 290 ± 20* | 357 ± 18 | 376 ± 21 | 325 ± 26* | 313 ± 15* |
| PP1 | 9 | 348 ± 11 | — | 303 ± 12* | 319 ± 10* | 291 ± 11* | 284 ± 7* |
| Isoflurane + PP1 | 7 | 353 ± 12 | 243 ± 17* | 284 ± 15* | 299 ± 16* | 293 ± 11* | 291 ± 10* |
| 5-HD | 7 | 372 ± 16 | — | 354 ± 18 | 372 ± 11 | 326 ± 14* | 320 ± 19* |
| Isoflurane + 5-HD | 7 | 382 ± 19 | 277 ± 13* | 338 ± 18* | 352 ± 16* | 297 ± 13* | 303 ± 8* |
| NAC | 6 | 325 ± 13 | — | 273 ± 18* | 276 ± 24* | 244 ± 21* | 238 ± 20* |
| Isoflurane + NAC | 6 | 363 ± 8 | 325 ± 5* | 241 ± 13* | 311 ± 12* | 309 ± 19* | 289 ± 13* |
| MAP, mmHg | | | | | | | |
| Control | 12 | 111 ± 5 | — | 107 ± 5 | 102 ± 6 | 79 ± 6* | 69 ± 7* |
| Isoflurane | 13 | 114 ± 3 | 60 ± 3* | 103 ± 5 | 104 ± 4 | 69 ± 6* | 63 ± 7* |
| Chelerythrine | 8 | 125 ± 6 | — | 125 ± 6 | 116 ± 8 | 88 ± 8* | 78 ± 9* |
| Isoflurane + chelerythrine | 8 | 119 ± 6 | 67 ± 5* | 104 ± 5* | 103 ± 6* | 58 ± 6* | 60 ± 5* |
| Rottlerin | 6 | 114 ± 3 | — | 108 ± 4 | 107 ± 6 | 81 ± 7* | 75 ± 4* |
| Isoflurane + rottlerin | 9 | 117 ± 6 | 87 ± 6* | 106 ± 8 | 113 ± 7 | 82 ± 6* | 74 ± 4* |
| εV1–2 | 6 | 120 ± 6 | — | 110 ± 4 | 123 ± 6 | 96 ± 6* | 89 ± 4* |
| Isoflurane + εV1–2 | 9 | 121 ± 7 | 67 ± 4* | 108 ± 6 | 119 ± 5 | 90 ± 5* | 73 ± 6* |
| Lavendustin A | 6 | 103 ± 5 | — | 111 ± 5 | 113 ± 6 | 76 ± 3* | 70 ± 4* |
| Isoflurane + lavendustin A | 6 | 118 ± 5 | 73 ± 5* | 113 ± 4 | 107 ± 6 | 76 ± 10* | 58 ± 8* |
| PP1 | 9 | 123 ± 3 | — | 145 ± 5* | 139 ± 5* | 103 ± 6* | 84 ± 4* |
| Isoflurane + PP1 | 7 | 112 ± 7 | 65 ± 5* | 119 ± 7 | 114 ± 6 | 83 ± 7* | 73 ± 6* |
| 5-HD | 7 | 108 ± 5 | — | 115 ± 7 | 110 ± 8 | 75 ± 6* | 67 ± 5* |
| Isoflurane + 5-HD | 7 | 112 ± 4 | 67 ± 4* | 105 ± 7 | 102 ± 9 | 72 ± 7* | 61 ± 6* |
| NAC | 6 | 114 ± 10 | — | 88 ± 5* | 105 ± 11 | 82 ± 11* | 81 ± 12* |
| Isoflurane + NAC | 6 | 140 ± 6 | 70 ± 6* | 113 ± 6* | 110 ± 9* | 98 ± 9* | 98 ± 7* |
| RPP, $min^{-1} \cdot mmHg \cdot 10^3$ | | | | | | | |
| Control | 12 | 46.4 ± 3.3 | — | 44.7 ± 4.0 | 39.0 ± 2.7 | 29.8 ± 2.3* | 25.6 ± 2.8* |
| Isoflurane | 13 | 50.2 ± 2.1 | 22.0 ± 1.6* | 41.0 ± 3.1* | 40.7 ± 2.6* | 26.8 ± 2.6* | 24.1 ± 2.5* |
| Chelerythrine | 8 | 59.0 ± 3.5 | — | 54.1 ± 3.1 | 49.2 ± 4.8* | 35.5 ± 4.0* | 29.4 ± 3.5* |
| Isoflurane + chelerythrine | 8 | 50.5 ± 3.3 | 24.7 ± 2.0* | 41.0 ± 3.3* | 38.7 ± 3.2* | 21.4 ± 2.2* | 22.6 ± 1.9* |
| Rottlerin | 6 | 49.1 ± 2.4 | — | 44.9 ± 2.9 | 42.3 ± 3.5* | 31.2 ± 2.1* | 28.5 ± 1.5* |
| Isoflurane + rottlerin | 9 | 50.9 ± 3.5 | 31.6 ± 2.4* | 41.3 ± 3.3* | 44.2 ± 3.1* | 30.4 ± 1.8* | 28.1 ± 1.3* |
| εV1–2 | 6 | 51.0 ± 5.4 | — | 42.6 ± 1.7 | 49.9 ± 2.6 | 36.3 ± 2.9* | 32.7 ± 2.5* |
| Isoflurane + εV1–2 | 9 | 55.0 ± 3.7 | 25.8 ± 1.7* | 39.5 ± 3.6* | 45.8 ± 3.2* | 34.4 ± 2.2* | 28.0 ± 2.7* |
| Lavendustin A | 6 | 41.2 ± 4.1 | — | 44.7 ± 3.2 | 44.3 ± 3.4 | 29.0 ± 1.0* | 26.6 ± 1.6* |
| Isoflurane + lavendustin A | 6 | 54.1 ± 3.8 | 27.3 ± 2.8* | 48.4 ± 3.8 | 45.6 ± 4.5 | 31.7 ± 5.5* | 23.8 ± 3.5* |
| PP1 | 9 | 51.6 ± 2.1 | — | 52.8 ± 2.7 | 50.6 ± 2.4 | 35.2 ± 3.0* | 29.5 ± 1.0* |
| Isoflurane + PP1 | 7 | 47.3 ± 4.3 | 21.4 ± 2.7* | 41.3 ± 4.7 | 38.2 ± 2.4 | 29.2 ± 2.4* | 26.8 ± 1.9* |
| 5-HD | 7 | 47.2 ± 2.7 | — | 48.3 ± 2.0 | 47.7 ± 2.7 | 31.4 ± 2.1* | 28.9 ± 2.3* |
| Isoflurane + 5-HD | 7 | 52.3 ± 3.8 | 25.5 ± 1.0* | 44.0 ± 4.1* | 43.0 ± 3.7* | 28.3 ± 2.7* | 25.1 ± 2.6* |
| NAC | 6 | 44.9 ± 4.8 | — | 30.1 ± 1.6* | 35.6 ± 6.1* | 26.1 ± 5.4* | 24.3 ± 4.7* |
| Isoflurane + NAC | 6 | 60.7 ± 3.8 | 22.7 ± 2.9* | 42.3 ± 3.6v | 39.9 ± 4.7* | 34.2 ± 3.9* | 32.4 ± 2.5* |

Data are presented as mean ± SEM.

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

CAO = coronary artery occlusion; 5-HD = 5-hydroxydecanoic acid; HR = heart rate; MAP = mean arterial pressure; NAC = *N*-acetylcysteine; PP1 = 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine; RPP = rate–pressure product; εV1–2 = PKCεV1–2 peptide.

(n = 3) exhibited a diffuse cytosolic staining for PKC-δ and -ϵ. Rottlerin (n = 3) and PKC-ϵV1–2 peptide (n = 3) prevented isoflurane-induced translocation of PKC-δ and -ϵ, respectively. Pretreatment with 5-HD (n = 3) and NAC (n = 3) also blocked isoflurane-induced PKC translocation. Isoflurane did not produce translocation of Src PTK.

## Discussion

Translocation and phosphorylation of protein kinases are involved in the signaling pathways that protect myocardium against ischemia and reperfusion injury. IPC stimulated translocation of PKC-δ and -ϵ in isolated rat

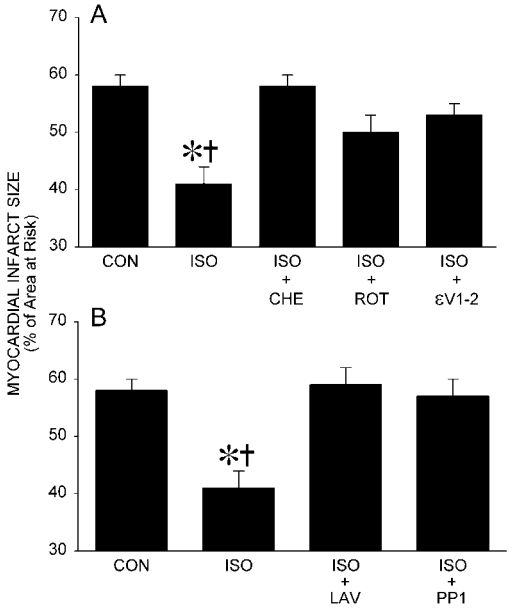


**Fig. 2. Histogram depicting myocardial infarct size expressed as a percentage of the left ventricular area at risk in rats receiving saline (CON) or 1.0 minimum alveolar concentration isoflurane (ISO) in the absence or the presence of chelerythrine (ISO + CHE), rottlerin (ISO + ROT), and protein kinase C-εV1–2 peptide (ISO + εV1–2) (*A*), or lavendustin A (ISO + LAV) and PP1 (ISO + PP1) (*B*). * Significantly ($P < 0.05$) different from control. † Significantly ($P < 0.05$) different from ISO + CHE, ISO + ROT, ISO + PKC-εV1–2 peptide, ISO + LAV, and ISO + PP1.**

hearts,[19,20] whereas the ϵ and η isoforms of PKC were translocated in rabbits.[21] Activation of $\delta_1$-opioid receptors selectively translocated PKC-α to sarcolemmal membranes, PKC-δ to mitochondrial membranes, and PKC-ϵ to mitochondria and intercalated disks.[3] Two recent investigations indicated that volatile anesthetics also produce selective translocation of various PKC isoforms.[20,22] Isoflurane caused selective translocation of PKC-δ to mitochondria and nuclei and PKC-ϵ to sarcolemmal membranes, nuclei, and intercalated disks in rat myocardium concomitant with myocardial protection.[20] Isoflurane also increased site-specific phosphorylation of PKC-δ in this model.[20] In contrast with these findings, sevoflurane stimulated translocation of PKC-δ to sarcolemma and PKC-ϵ to mitochondria, nuclei, and

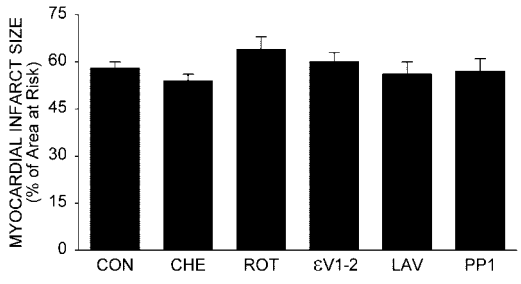


**Fig. 3. Histogram depicting myocardial infarct size expressed as a percentage of the left ventricular area at risk in rats receiving saline (CON), chelerythrine (CHE), rottlerin (ROT), protein kinase C-εV1–2 peptide (εV1–2), lavendustin A (LAV), or PP1.**

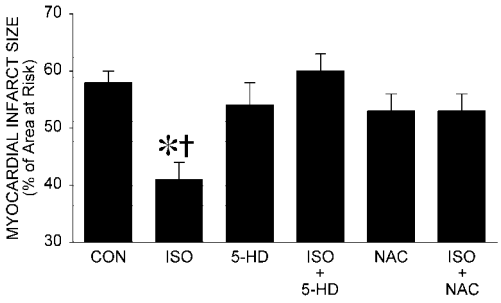


**Fig. 4. Histogram depicting myocardial infarct size expressed as a percentage of the left ventricular area at risk in rats receiving saline (CON) or 1.0 minimum alveolar concentration isoflurane (ISO) in the absence or the presence of 5-hydroxydecanoic acid (ISO + 5-HD) or *N*-acetylcysteine (ISO + NAC). * Significantly ($P < 0.05$) different from control. † Significantly ($P < 0.05$) different from 5-HD, ISO + 5-HD, NAC, and ISO + NAC.**

intercalated disks in human atrial tissue.[22] The results of another study[11] showed that sevoflurane induced activation of the PKC-ϵ isoform concomitant with ROS production and myocardial protection.

The current results confirm and extend these previous findings[11,20,22] by showing that the nonselective PKC antagonist chelerythrine, as well as the selective PKC-δ and -ϵ specific inhibitors rottlerin and PKC-εV1–2 peptide, respectively, eliminated reductions in infarct size produced by isoflurane *in vivo*. These data suggest that

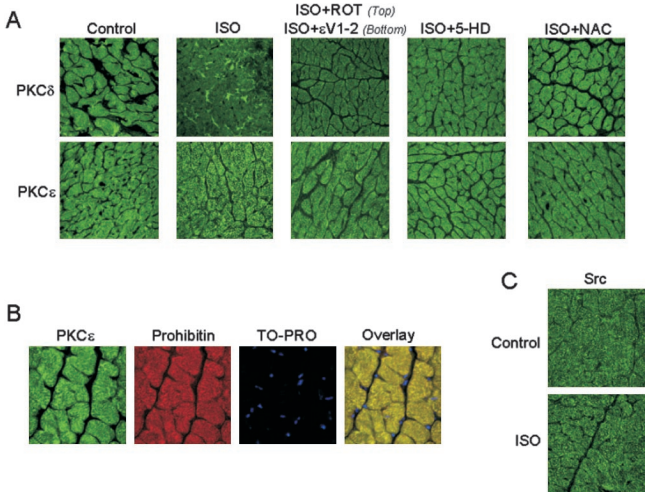


**Fig. 5. Representative photomicrographs of immunofluorescent staining for protein kinase C (PKC)-δ, PKC-ϵ, and Src protein tyrosine kinase. Immunofluorescence of PKC-δ and -ϵ was localized to sarcolemmal and mitochondrial membranes, respectively, in rat myocardium pretreated with 1.0 minimum alveolar concentration isoflurane (ISO) for 30 min (*A*). The selective PKC-δ and -ϵ inhibitors rottlerin and PKC-εV1–2 peptide, respectively, inhibited isoflurane-induced PKC-δ and -ϵ translocation, respectively. The selective mitochondrial adenosine triphosphate–sensitive potassium channel antagonist 5-hydroxydecanoic acid (5-HD) and the reactive oxygen species scavenger *N*-acetylcysteine (NAC) also inhibited PKC translocation produced by isoflurane. Costaining of PKC-ϵ with the mitochondrial marker prohibitin and the nucleic acid stain TO-PRO indicated that PKC-ϵ translocated to the mitochondrial and not the sarcolemmal membrane (*B*). Isoflurane did not produce translocation of Src protein tyrosine kinase.**

PKC-δ and -ϵ are both crucial mediators of isoflurane-induced protection against irreversible ischemic injury in the intact rat heart. However, in contrast with previous results in rat myocardium,[20] the current data indicate that isoflurane caused translocation of PKC-δ and -ϵ to sarcolemmal and mitochondrial membranes, respectively. Furthermore, the current results show for the first time that the nonselective PTK inhibitor lavendustin A and the Src-specific PTK inhibitor PP1 inhibited the anti-ischemic actions of isoflurane. These data indicate that Src PTK is another important signaling element of the signal transduction cascade responsible for anesthetic-induced preconditioning. The current findings also show that 5-HD and NAC inhibited both PKC translocation and myocardial protection produced by isoflurane. These latter data strongly suggest that PKC translocation occurs downstream of activation of mitochondrial $K_{ATP}$ channels and generation of ROS during isoflurane-induced preconditioning.

Small bursts of ROS generated by mitochondria have been strongly implicated as triggers of signal transduction that cause preconditioning phenomena.[23] ROS have been shown to activate PKC, restore myocardial contractility, and reduce infarct size in rabbit hearts.[24] Our laboratory recently demonstrated that opening of mitochondrial $K_{ATP}$ channels by isoflurane triggers the production of ROS during anesthetic-induced preconditioning.[10] Another recent study indicated that the anti-ischemic actions of sevoflurane were mediated in part by ROS-dependent PKC-ϵ activation.[11] These results suggested that PKC-ϵ translocation occurred downstream of ROS generation. The current results confirm and extend these previous findings.[11] ROS are also known to directly activate $G\alpha_i$ and $G\alpha_o$ proteins.[25,26] Notably, we have previously demonstrated that activation of $A_1$[27] and $\delta_1$-opioid receptors[14] mediated myocardial protection produced by isoflurane. Volatile anesthetic–induced preservation of ventricular myocyte viability during ischemia was also sensitive to inhibition by adenosine receptor antagonists and $G_i$ proteins.[28] Therefore, the current and previous findings suggest that activation of mitochondrial $K_{ATP}$ channels initiate the production of ROS that subsequently activate G proteins and several protein kinases, including PKC and PTK, that have been implicated in the signal transduction responsible for other forms of preconditioning against ischemia and reperfusion injury.

The current results indicate that mitochondrial $K_{ATP}$ channel opening represents an upstream event that facilitates PKC activation, but PKC translocation by volatile agents may also be necessary for $K_{ATP}$ channel activation to occur as well. Chelerythrine abolished sevoflurane-induced increases in mitochondrial $K_{ATP}$ activity in rat ventricular myocytes and prevented protection against ischemia.[28] Experiments conducted in a whole cell patch clamp preparation showed that isoflurane enhanced PKC-induced activation of sarcolemmal $K_{ATP}$ channel current in isolated cardiac myocytes.[29] These observations with volatile anesthetics are supported by other investigations showing that PKC increased $K_{ATP}$ channel activity in other forms of preconditioning.[30,31] In fact, another study suggested that PKC-ϵ translocation seems to precede mitochondrial $K_{ATP}$ channel opening during IPC.[32] These data indicate that the temporal relation between PKC activation and $K_{ATP}$ channel opening has not been clearly established in many preconditioning phenomena. The apparent discrepancy between these findings and the aforementioned results with volatile anesthetics may be related to differential regulation of sarcolemmal and mitochondrial $K_{ATP}$ channels that display distinct time- and spatial-dependent relations with other signaling molecules. It is also likely that mitochondrial $K_{ATP}$ channel opening and ROS production may initiate a feed-forward system to stimulate PKC activation that further enhances $K_{ATP}$ channel activity during anesthetic-induced preconditioning. Additional study will be needed to confirm this intriguing hypothesis of a signal amplification loop.[33]

Sequential activation of several proteins within a transduction cascade may facilitate signal amplification and interaction between other redundant systems to produce myocardial protection. PTK activation has been shown to occur downstream of PKC during IPC in isolated rabbit hearts.[34] In particular, PKC-dependent activation of the Src subfamily of PTK has been shown to mediate IPC.[35] Compelling evidence strongly suggests that the ϵ isoform of PKC forms a signaling complex with Src PTK, thereby enhancing enzymatic activity and producing protection against ischemia and reperfusion injury.[36] PKC-δ has also been shown to induce Src PTK activation.[37] Another investigation showed that Src PTK functioned as a trigger but not a mediator of IPC in rats.[17] In contrast, the nonselective PTK inhibitor genistein activated sarcolemmal $K_{ATP}$ channel activity in a whole cell patch clamp ventricular myocyte model.[38] This process was further enhanced by isoflurane.[38] As described above, it is highly likely that sarcolemmal and mitochondrial $K_{ATP}$ channels are modulated by distinct mechanisms, and the previous results may represent evidence that this is the case. Nevertheless, the current data indicate that Src PTK is an integral component of the transduction pathway responsible for volatile anesthetic–induced preconditioning *in vivo*. Future investigations are needed to delineate the relation among PKC-specific isoforms, Src PTK, and other potential downstream mediators of protection.

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 AAR was similar between experimental groups, and minimal coronary collateral blood flow has been previously

reported in rats.[39] Therefore, it seems unlikely that the current results were substantially affected by these variables. Isoflurane caused similar hemodynamic effects among experimental groups, but mean arterial pressure returned to baseline values after isoflurane had been discontinued before coronary occlusion. It seems unlikely that transient alterations in systemic hemodynamics produced by isoflurane were responsible for the reductions in myocardial infarct size associated with administration of this volatile anesthetic. Values of the rate–pressure product, an indirect index of myocardial oxygen consumption, were also similar between experimental groups. In addition, the specificity of 5-HD as a mitochondrial $K_{ATP}$ channel antagonist remains controversial.[40] Nonetheless, 5-HD has been extensively used to characterize the role of mitochondrial $K_{ATP}$ channels in preconditioning.

The current results show that isoflurane produced selective translocation of PKC-δ and -ϵ to the sarcolemmal and mitochondrial membranes, similar to previous observations made in sevoflurane-preconditioned human atrial tissue.[22] However, the current data contrast with the results of a previous study conducted in the isolated rat heart.[20] We speculate that the disparity in the observations between the current and previous[20] investigations may be related to differences in experimental design. Uecker *et al.*[20] examined isoform-selective PKC translocation 10 min after a 15-min exposure to isoflurane (1.5 MAC) and thus identified PKC localization during a memory period. In contrast, we excised rat hearts immediately after a 30-min pretreatment with isoflurane (1.0 MAC) *in vivo*. It is possible that isoflurane produces specific localization of PKC isoforms in isolated *versus in vivo* hearts or that a temporal redistribution of these protein kinases may occur after administration of the volatile anesthetic has been discontinued. Additional study is needed to clarify this latter hypothesis. Also, PKC-δ and -ϵ have recently been shown to exert opposing effects during ischemia and reperfusion injury.[41] PKC-ϵ activation produced a protective effect, whereas PKC-δ stimulation promoted myocardial damage.[41] However, findings from the current and previous[20,22] investigations support that both PKC-δ and -ϵ are involved in mediating volatile anesthetic–induced preconditioning. Further research is needed to distinguish the particular roles that PKC-δ and -ϵ play in this signaling pathway to elicit myocardial protection.

In summary, the current findings indicate that the nonselective PKC inhibitor chelerythrine and the selective PKC-δ and -ϵ inhibitors rottlerin and PKC-ϵV1–2 peptide abolished isoflurane-induced reductions in myocardial infarct size in rats. The PTK inhibitor lavendustin A and the selective Src PTK inhibitor PP1 also blocked the beneficial actions produced by isoflurane. Immunofluorescence studies revealed that isoflurane stimulated PKC-δ and -ϵ translocation to the sarcolemmal and mitochondrial membranes, respectively, in rat myocardium. The mitochondrial $K_{ATP}$ channel antagonist 5-HD and the ROS scavenger NAC eliminated reductions in infarct size and inhibited PKC translocation produced by administration of isoflurane. Therefore, the current results show that PKC-δ, PKC-ϵ, and Src PTK are crucial elements that mediate isoflurane-induced preconditioning *in vivo*. Mitochondrial $K_{ATP}$ channel opening and ROS occur upstream of PKC activation in this signal transduction process.

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