Role of Erk1/2, p70s6K, and eNOS in isoflurane-induced cardioprotection during early reperfusion *in vivo*

[Le rôle des Erk1/2, p70s6K et eNOS dans la cardioprotection induite par l'isoflurane pendant la reperfusion in vivo précoce]

John G. Krolikowski BA,* Dorothee Weihrauch DVM PhD,* Martin Bienengraeber PhD,*† Judy R. Kersten MD,*† David C. Warltier MD PhD,*†‡§ Paul S. Pagel MD PhD*§

Purpose: Administration of isoflurane during early reperfusion after prolonged coronary artery occlusion decreases myocardial infarct size by activating phosphatidylinositol-3-kinase (PI3K) signal transduction. The extracellular signal-related kinases (Erk1/2) represent a redundant mechanism by which signaling elements downstream from PI3K, including 70-kDA ribosomal protein s6 kinase (p70s6K) and endothelial nitric oxide synthase (eNOS), may be activated to reduce reperfusion injury. We tested the hypothesis Erk1/2, p70s6K, and eNOS mediate isoflurane-induced postconditioning in rabbit myocardium *in vivo*.

Methods: Barbiturate-anesthetized rabbits ($n = 78$) instrumented for measurement of systemic hemodynamics were subjected to a 30–min coronary occlusion followed by three hours reperfusion. Rabbits were randomly assigned to receive 0.9% saline (control), the Erk1/2 inhibitor PD 098059 (2 $mg \cdot kg^{-1}$), the p70s6K inhibitor rapamycin (0.25 $mg \cdot kg^{-1}$), the nonselective nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 10 $mg \cdot kg^{-1}$), the selective inducible NOS antagonist aminoguanidine hydrochloride (AG, 300 $mg \cdot kg^{-1}$), or the selective neuronal NOS inhibitor 7-nitroindazole (7-NI, 50 $mg \cdot kg^{-1}$) in the presence or absence of 1.0 minimum alveolar concentration isoflurane administered for three minutes before and two minutes after reperfusion.

Results: Brief exposure to 1.0 minimum alveolar concentration isoflurane reduced ($P < 0.05$) infarct size (21 ± 4% [mean ± SD] of left ventricle area at risk, respectively; triphenyltetrazolium staining) as compared to control (41 ± 5%). PD 098059, rapamycin, and L-NAME, but not AG nor 7-NI, abolished the protection produced by isoflurane.

Conclusion: The results suggest that the protective effects of isoflurane against infarction during early reperfusion are mediated by Erk1/2, p70s6K, and eNOS *in vivo*.

***Objectif** : L'administration d'isoflurane pendant la reperfusion précoce qui suit une occlusion prolongée de l'artère coronaire diminue la taille de l'infarctus myocardique en activant la transduction du signal de la phosphatidylinositol-3-kinase (PI3K). Les kinases extracellulaires reliées au signal (Erk1/2) représentent un mécanisme redondant par lequel le signalement des éléments en aval à partir de PI3K, incluant la s6 kinase de protéines ribosomales 70-kDA (p70s6K) et l'oxyde nitrique synthase endothéliale (eNOS), peuvent être activés pour réduire la lésion de reperfusion. Nous avons testé l'hypothèse que les Erk1/2, p70s6K et eNOS assuraient la médiation du postconditionnement induit par l'isoflurane dans des myocardes de lapin in vivo.*

***Méthode** : Des lapins anesthésiés aux barbituriques ($n = 78$), instrumentés pour la mesure de l'hémodynamique générale, ont été soumis à une occlusion coronaire de 30 min, suivie de trois heures de reperfusion. Répartis au hasard, ils ont reçu une solution salée à 0,9 % (témoin), l'inhibiteur de Erk1/2, PD 098059 (2 $mg \cdot kg^{-1}$), l'inhibiteur de p70s6K, la rapamycine (0,25 $mg \cdot kg^{-1}$), l'inhibiteur non sélectif de l'oxyde nitrique synthase (NOS) l'ester méthylique N-nitro-L-arginine (L-NAME ; 10 $mg \cdot kg^{-1}$), l'antagoniste sélectif de la NOS inductible, le chlorhydrate d'aminoguanidine (AG, 300 $mg \cdot kg^{-1}$) ou l'inhibiteur sélectif de NOS neuronal, le 7-nitro-indazole (7-NI, 50 $mg \cdot kg^{-1}$) en présence ou non d'une concentration alvéolaire minimale de 1,0 d'isoflurane administrée pendant trois minutes avant et deux minutes après la reperfusion.*

From the Departments of Anesthesiology,* Pharmacology and Toxicology,† and Medicine (Division of Cardiovascular Diseases),‡ the Medical College of Wisconsin and the Clement J. Zablocki Veterans Affairs Medical Center, and the Department of Biomedical Engineering,§ Marquette University, Milwaukee, Wisconsin, USA.

Address correspondence to: Dr. Paul S. Pagel, Medical College of Wisconsin, MEB-M4280, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, USA. Phone: 414-456-5728; Fax: 414-456-6507; E-mail: pspagel@mcw.edu

This work was supported in part by American Heart Association Greater Midwest Affiliate grant AHA 0265259Z (to Dr. Weihrauch) and National Institutes of Health grants HL 063705 (to Dr. Kersten), HL 054820 (to Dr. Warltier), GM 008377 (to Dr. Warltier), and GM 066730 (to Dr. Warltier) from the United States Public Health Service (Bethesda, MD).

Accepted for publication August 9, 2005.

Revision accepted September 14, 2005.

***Résultats** : L'exposition brève à une concentration alvéolaire minimale de 1,0 d'isoflurane a réduit (P < 0,05) la taille de l'infarctus (21 ± 4 % [moyenne ± ET] de l'aire du ventricule gauche à risque, respectivement ; coloration au triphényltétrazolium) comparativement au témoin (41 ± 5 %). Les PD 098059, rapamycine et L-NAME, mais non les AG ou 7-NI, ont aboli la protection produite par l'isoflurane.*

***Conclusion** : Le résultat suggère que les effets protecteurs de l'isoflurane contre l'infarctus pendant la reperfusion précoce dépendent de la médiation des Erk1/2, p70s6K et eNOS in vivo.*

VOLATILE anesthetics produce pharmacological preconditioning against myocardial ischemia-reperfusion injury,[1] and also exert protective effects when administered during early reperfusion following prolonged coronary artery occlusion.[2–5] This "anesthetic-induced postconditioning" produces reductions in myocardial infarct size that are very similar to those observed during preconditioning with these agents[1,5] and may be clinically relevant because the precise timing of coronary artery occlusion is unknown in the vast majority of patients with acute myocardial infarction. We recently demonstrated that isoflurane-induced postconditioning is mediated by activation of the prosurvival phosphatidylinositol-3-kinase (PI3K)-Akt signaling cascade.[5,6] This pathway has also been implicated in myocardial protection during early reperfusion produced by brief, repetitive ischemic stimuli[5,7–9] (termed "ischemic postconditioning")[8] and other drugs including bradykinin,[10] adenosine receptor agonists,[10] statins,[11] and opioids.[12]

The extracellular signal-related kinases (Erk1/2) are mitogen-activated protein kinases that play important roles in cell differentiation, proliferation, and survival.[13] Activation of Erk1/2 has been proposed as a redundant mechanism by which downstream elements of the PI3K-Akt cascade may be stimulated to favourably modulate reperfusion injury.[13] The extracellular signal-related kinases mediate ischemic[14] and pharmacological postconditioning,[10,15] and also play important roles in preconditioning produced by the volatile anesthetic desflurane.[16] Thus, the current investigation tested the hypothesis isoflurane-induced postconditioning is dependent on activation of Erk1/2. Both PI3K-Akt and Erk1/2 will phosphorylate 70-kDA ribosomal protein s6 kinase (p70s6K), an important regulator of protein translation.[13] A selective inhibitor of this enzyme (rapamycin) abolished cardioprotection during ischemic[9] and pharmacological postconditioning.[10,12] The phosphatidylinositol-3-kinase-Akt also activates endothelial nitric oxide (NO) synthase (eNOS), and a central role for NO has also been implicated in postconditioning by ischemia,[9] an adenosine agonist,[10] and bradykinin.[10] Thus, we also tested the hypothesis that p70s6K and eNOS mediate the protective effects of isoflurane during early reperfusion after prolonged coronary artery occlusion *in vivo*.

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the Guiding Principles in the Care and Use of Animals of the American Physiologic Society and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with *iv* sodium pentobarbital (30 mg·kg^{-1}) as previously described.[5,17] Briefly, a tracheostomy was performed through a midline incision, and each rabbit was ventilated with positive pressure using an air-oxygen mixture (fractional inspired oxygen concentration = 0.33). Heparin-filled catheters were inserted into the right carotid artery and the left jugular vein for measurement of mean arterial blood pressure and fluid or drug administration, respectively. A thoracotomy was performed at the left fourth intercostal space, and the heart was suspended in a pericardial cradle. 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. Intravenous heparin (500 U) was administered immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamics were continuously recorded on a polygraph throughout each experiment.

The experimental design is illustrated in Figure 1. Baseline hemodynamics and arterial blood gas tensions were recorded 30 min after instrumentation was completed. All rabbits underwent a 30-min LAD occlusion followed by three hours of reperfusion. In 12 separate experimental groups, rabbits (n = 6–7 per group) were randomly assigned to receive 0.9% saline (control), the MEK-1 inhibitor PD 098059 (2 mg·kg^{-1}; Erk1/2 is activated by phosphorylation via the upstream kinase MEK-1),[18] the p70s6K inhibitor rapamycin (0.25 mg·kg^{-1}), the nonselective NO synthase (NOS) inhibi-

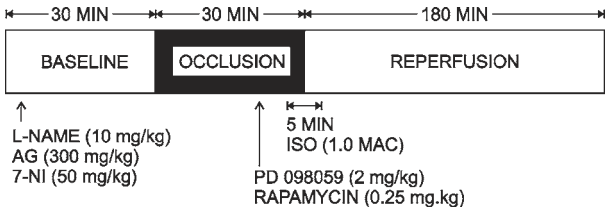

FIGURE 1 Schematic illustration depicting the experimental protocol. L-NAME = *N*-nitro-L-arginine methyl ester; AG = aminoguanidine; 7-NI = 7-nitroindazole; ISO = isoflurane.

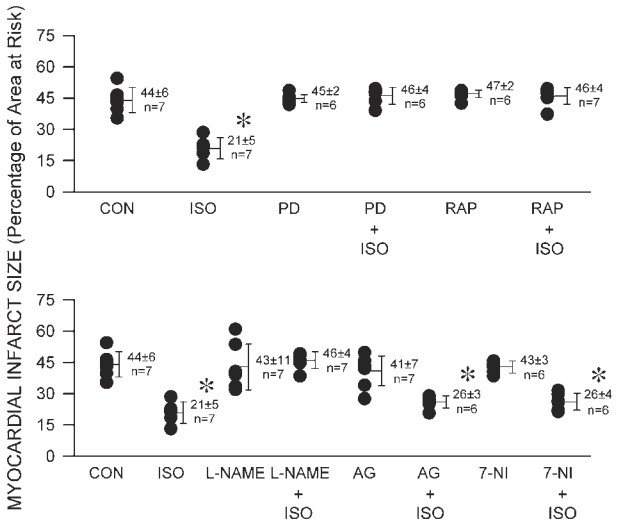

FIGURE 2 Myocardial infarct size depicted as a percentage of the left ventricular area at risk in rabbits receiving 0.9% saline control (CON) or 1.0 minimum alveolar concentration (MAC) isoflurane (ISO) in the presence or absence of pretreatment with selective Erk1/2 inhibitor PD 098059 (PD) or selective p70s6K inhibitor rapamycin (RAP; top panel), and in the presence and absence of pretreatment with the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME), the selective inducible nitric oxide (NO) synthase (iNOS) inhibitor aminoguanidine (AG), or the selective neuronal NO synthase (nNOS) inhibitor 7-nitroindazole (7-NI; bottom panel). Each point represents a single experiment. All data are mean ± SD. *Significantly ($P < 0.05$) different from CON.

tor *N*-nitro-L-arginine methyl ester (L-NAME; 10 $mg \cdot kg^{-1}$),[19] the selective inducible NOS (iNOS) antagonist aminoguanidine hydrochloride (300 $mg \cdot kg^{-1}$),[19] or the selective neuronal NOS (nNOS) inhibitor 7-nitroindazole (7-NI, 50 $mg \cdot kg^{-1}$)[19] in the presence or absence of 1.0 minimum alveolar concentration (MAC) isoflurane (1.0 MAC = 2.05% in the rabbit) administered for three minutes before and two minutes after reperfusion. Isoflurane was administered for three minutes before reperfusion in order to establish a blood concentration of the volatile agent when the coronary blood flow was restored. We have previously demonstrated that 1.0 MAC isoflurane administered using this technique produces reductions in myocardial infarct size that are similar to those observed during preconditioning with this volatile anesthetic.[1,5] We have also previously shown that the sample size used in the current investigation is adequate to provide statistically significant differences in infarct size between interventions.[1,5] PD 098059 and rapamycin were dissolved in dimethylsulfoxide and administered intravenously ten minutes before reperfusion. *N*-nitro-L-arginine methyl ester was dissolved in 0.9% saline and administered as an *iv* infusion over ten minutes beginning 30 min before LAD occlusion. Aminoguanidine hydrochloride was dissolved in 0.9% saline, the pH of the solution was adjusted to 7.4 with 0.1N NaOH, and the mixture then injected subcutaneously one hour before coronary occlusion. Seven-NI was dissolved in dimethylsulfoxide and administered into the peritoneum one hour before coronary occlusion.

Myocardial infarct size was measured as previously described.[20] Briefly, the LAD was reoccluded at the completion of each experiment and 3 mL of patent blue dye was injected intravenously. The left ventricular area at risk (AAR) for infarction was separated from surrounding normal areas (stained blue), and the two regions were incubated at 37°C for 20 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 $mol \cdot L^{-1}$ phosphate buffer adjusted to pH 7.4. Infarcted and noninfarcted myocardium within the AAR were separated and weighed after storage overnight in 10% formaldehyde. Myocardial infarct size was expressed as a percentage of the AAR. Rabbits that developed intractable ventricular fibrillation and those with an AAR less than 15% of total left ventricular mass were excluded from subsequent analysis.

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by the Student-Newman-Keuls test. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean ± standard deviation .

Results

Eighty-six rabbits were instrumented to obtain 78 successful experiments. Two rabbits were excluded

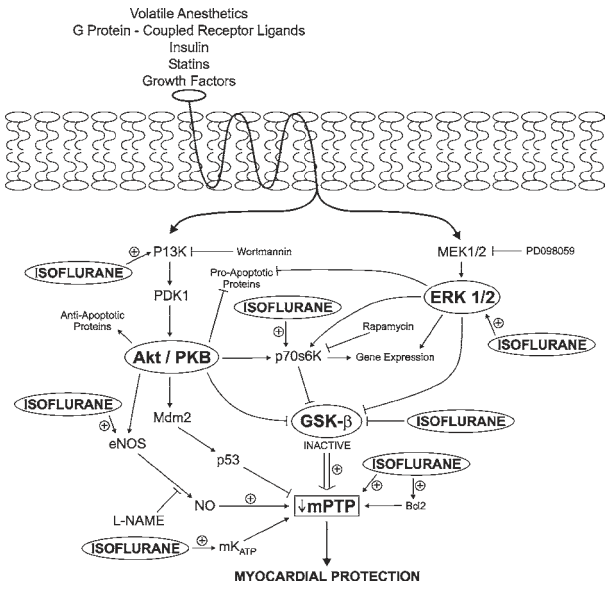

FIGURE 3 Schematic illustration of potential sites of action of isoflurane on cardioprotective signaling during early reperfusion. G protein-coupled receptor ligands, insulin, statins, growth factors, and volatile anesthetics activate parallel phosphoinositol-3-kinase (PI3K) or extracellular signal regulated kinase (Erk1/2) cascades to produce protection. PI3K phosphorylates phosphoinositol dependent kinase 1 (PDK1) which activates Akt (also known as protein kinase B [PKB]). This moiety stimulates the activity of anti-apoptotic proteins and simultaneously inhibits activity of pro-apoptotic proteins. Akt/PKB also inhibits the beta isoform of glycogen synthase kinase (GSK-β) and activates endothelial nitric oxide (NO) synthase (eNOS), 70-kDA ribosomal protein s6 kinase (p70s6K), and murine double minute two (Mdm2) protein. p70s6K also inhibits GSK-β activity. Nitric oxide produced by eNOS inhibits whereas Mdm2-induced phosphorylation of the pro-apoptotic protein p53 opens the mitochondrial permeability transition pore (mPTP). Inhibition of GSK-β and activation of mitochondrial adenosine triphosphate-regulated potassium (mK_{ATP}) channels also act to close mPTP. The transition state of the mitochondrial pore is critical for the preservation of mitochondrial integrity and cell viability during ischemia and reperfusion. Activation of Erk1/2 also stimulates p70s6K, inhibits GSK-β, and blocks the formation of pro-apoptotic proteins. Wortmannin, rapamycin, PD 098059, and *N*-nitro-L-arginine methyl ester (L-NAME) block the activity of PI3K, p70s6K, Erk1/2, and eNOS, respectively. Isoflurane has been proposed to enhance the activity of PI3K-Akt, p70s6k, Erk1/2, and eNOS. Isoflurane has also been suggested to directly inhibit GSK-β and mPTP concomitant with enhanced expression of the anti-apoptotic protein B cell lymphoma 2 (Bcl2). Lastly, isoflurane-induced activation of mK_{ATP} inhibits mPTP. These actions of isoflurane may contribute to the cardioprotective effects of the volatile anesthetic during early reperfusion.

because of technical problems during instrumentation. Six rabbits were excluded because intractable ventricular fibrillation occurred during LAD occlusion (1 rapamycin alone; 1 7-NI alone; 2 PD 098059 + isoflurane; 1 L-NAME + isoflurane; 1 rapamycin + isoflurane). There were no differences in baseline hemodynamics between groups (Table I). Coronary artery occlusion significantly ($P < 0.05$) decreased rate-pressure product in most experimental groups. Decreases in heart rate, mean arterial pressure, and rate-pressure product were observed during reperfusion in all experimental groups.

Body weight, left ventricular mass, AAR weight, and the ratio of AAR to left ventricular mass were similar between groups (Table II). Brief exposure to 1.0 MAC isoflurane during early reperfusion reduced infarct size (21 ± 5% of the left ventricular AAR, respectively) as compared to control (42 ± 5%; Figure 2). PD 098059, rapamycin, and L-NAME alone did not affect infarct size (45 ± 2, 47 ± 3, and 43 ± 11%, respectively), but abolished the protective effects of isoflurane during early reperfusion (46 ± 4, 46 ± 4, and 46 ± 4%, respectively; Figure 2). In contrast to the findings with L-NAME, the selective iNOS and nNOS inhibitors did not affect isoflurane-induced myocardial protection (26 ± 3 and 26 ± 4%, for AG and 7-NI, respectively).

Discussion

The current results confirm our previous findings[5,6] demonstrating that brief exposure to 1.0 MAC isoflurane immediately before and during early reperfusion protects against myocardial infarction in rabbits. The findings demonstrate for the first time that the selective MEK-1 antagonist PD 098059 abolishes this protective effect. These data provide pharmacological evidence suggesting that activation of Erk1/2 mediates isoflurane-induced postconditioning. The current results support the findings of several previous studies demonstrating that Erk1/2 plays an important role in myocardial protection against ischemia and reperfusion injury (Figure 3). Fryer *et al.* showed that PD 098059 blocked myocardial necrosis produced by ischemic and δ_1-opioid-induced preconditioning concomitant with inhibition of Erk isoform phosphorylation in rats.[18] Erk1/2 was very recently also implicated in ischemic postconditioning.[14] Toma *et al.* recently showed that Erk1/2 mediates desflurane-induced preconditioning.[16] The adenosine A_1/A_2 subtype receptor agonist 5'-(*N*-ethylcardoxamido) adenosine (NECA) and bradykinin administered during early reperfusion reduced infarct size by activation of Erk1/2 in isolated rabbit hearts.[10] *N*-ethylcardoxamido adenosine and bradykinin-induced phosphorylation of Erk1/2

TABLE I Systemic hemodynamics

	n	*Baseline*	*LAD occlusion*	*Reperfusion (min)* 2	60	120	180
HR (min^{-1})							
Control	7	265 ± 34	249 ± 33	236 ± 19*	240 ± 22*	231 ± 25*	218 ± 25*
ISO (1.0 MAC)	7	257 ± 26	253 ± 21	252 ± 11	238 ± 20*	234 ± 34*	225 ± 35*
PD	6	233 ± 35	215 ± 28*	208 ± 21*	199 ± 17*	189 ± 9*	187 ± 14*
ISO (1.0 MAC)+PD	6	233 ± 28	209 ± 19*	198 ± 31*	193 ± 29*	182 ± 8*	173 ± 6*
RAPA	6	242 ± 25	209 ± 26*	207 ± 41*	197 ± 33*	204 ± 20*	198 ± 16*
ISO (1.0 MAC)+RAPA	7	251 ± 29	236 ± 30	239 ± 29	238 ± 26	229 ± 23*	221 ± 20*
L-NAME	7	211 ± 25	221 ± 21	184 ± 36*	184 ± 36*	186 ± 23*	188 ± 25*
ISO (1.0 MAC)+L-NAME	7	236 ± 36	221 ± 48	230 ± 43	225 ± 43*	228 ± 29	223 ± 25*
7NI	6	247 ± 29	242 ± 21	240 ± 19	227 ± 19*	218 ± 15*	208 ± 18*
ISO (1.0 MAC)+7NI	6	247 ± 26	236 ± 32	238 ± 21	235 ± 21	210 ± 9*	200 ± 17*
AG	7	264 ± 18	253 ± 18	229 ± 21*	229 ± 21*	228 ± 24*	221 ± 11*
ISO (1.0 MAC)+AG	6	265 ± 41	239 ± 24*	238 ± 19*	249 ± 46	237 ± 45*	226 ± 35*
MAP (mmHg)							
Control	7	75 ± 8	64 ± 4*	64 ± 5*	65 ± 7*	69 ± 9	65 ± 8*
ISO (1.0 MAC)	7	83 ± 10	70 ± 14*	54 ± 11*	67 ± 13*	65 ± 11*	70 ± 9*
PD	6	61 ± 15	53 ± 18	56 ± 24	51 ± 18	44 ± 15*	46 ± 17*
ISO (1.0 MAC)+PD	6	63 ± 18	47 ± 16	44 ± 15*	44 ± 17*	61 ± 18	51 ± 7*
RAPA	6	76 ± 8	50 ± 11*	51 ± 15*	48 ± 15*	52 ± 7*	58 ± 10*
ISO (1.0 MAC)+RAPA	7	76 ± 9	62 ± 11*	65 ± 13	65 ± 10	60 ± 10	59 ± 9*
L-NAME	7	90 ± 10	72 ± 13*	65 ± 17*	65 ± 16*	67 ± 12*	72 ± 11*
ISO (1.0 MAC)+L-NAME	7	75 ± 9	57 ± 22*	55 ± 20*	61 ± 18	62 ± 14	65 ± 14
7NI	6	70 ± 8	56 ± 3*	55 ± 9*	56 ± 12*	53 ± 10*	51 ± 2*
ISO (1.0 MAC)+7NI	6	71 ± 15	58 ± 9*	59 ± 14*	64 ± 11*	59 ± 10*	62 ± 9*
AG	7	86 ± 16	81 ± 11	80 ± 12	80 ± 12	80 ± 10	76 ± 11*
ISO (1.0 MAC)+AG	6	72 ± 11	69 ± 7	56 ± 15*	69 ± 15	69 ± 16	68 ± 16
RPP (min^{-1}·mmHg·10^3)							
Control	7	23.5 ± 4.0	19.2 ± 3.0*	18.4 ± 2.3*	19.3 ± 3.0*	18.7 ± 2.3*	16.3 ± 1.6*
ISO (1.0 MAC)	7	24.0 ± 3.5	20.6 ± 3.7*	18.5 ± 3.5*	19.4 ± 3.5	18.3 ± 3.6*	18.1 ± 1.4*
PD	6	17.4 ± 5.5	14.0 ± 5.3*	13.8 ± 5.6*	12.3 ± 4.6*	10.2 ± 2.3*	10.7 ± 2.7*
ISO (1.0 MAC)+PD	6	17.0 ± 5.7	11.3 ± 3.8*	10.4 ± 3.6*	10.3 ± 3.6*	12.7 ± 2.9*	10.4 ± 0.6*
RAPA	6	21.0 ± 3.5	12.8 ± 3.7*	13.0 ± 5.5*	11.7 ± 4.8*	12.9 ± 2.6*	13.5 ± 2.6*
ISO (1.0 MAC)+RAPA	7	21.4 ± 3.3	17.6 ± 4.4*	18.2 ± 5.4	18.0 ± 4.0	16.4 ± 3.0*	15.3 ± 2.2*
L-NAME	7	20.7 ± 2.8	17.2 ± 3.0*	12.8 ± 2.0*	12.6 ± 1.6*	13.5 ± 1.2*	14.6 ± 2.0*
ISO (1.0 MAC)+L-NAME	7	19.7 ± 3.4	14.9 ± 6.3*	15.2 ± 6.2*	16.0 ± 5.1	16.1 ± 2.5*	16.5 ± 2.0*
7NI	6	20.2 ± 3.1	16.1 ± 2.1*	16.3 ± 2.9*	15.5 ± 3.1*	14.2 ± 2.0*	12.7 ± 0.9*
ISO (1.0 MAC)+7NI	6	20.2 ± 4.6	16.0 ± 3.5*	16.7 ± 3.5*	17.3 ± 3.6*	14.5 ± 2.0*	14.4 ± 2.7*
AG	7	25.6 ± 5.6	22.6 ± 3.2	20.8 ± 2.8*	20.8 ± 2.8*	20.6 ± 3.2*	18.9 ± 2.8*
ISO (1.0 MAC)+AG	6	21.7 ± 4.9	18.5 ± 2.3*	15.9 ± 3.1*	19.8 ± 6.1	18.9 ± 5.8	17.3 ± 4.2*

HR = heart rate; ISO = isoflurane; PD = PD098059; RAPA = rapamycin; L-NAME = *N*-nitro-L-arginine methyl ester; 7NI = 7-nitroindazole; AG = aminoguanidine; MAC = minimum alveolar concentration; LAD = left anterior descending coronary artery. Data are mean ± SD. *Significantly ($P < 0.05$) different from baseline.

TABLE II Left ventricular area at risk

	Body weight (g)	*LV (g)*	*AAR (g)*	*AAR/LV (%)*
Control	2,628 ± 308	3.21 ± 0.29	1.11 ± 0.28	34 ± 8
ISO (1.0 MAC)	2,569 ± 265	3.43 ± 0.40	1.18 ± 0.41	34 ± 11
PD	2,717 ± 299	3.71 ± 0.36	1.44 ± 0.24	39 ± 5
ISO (1.0 MAC)+PD	2,767 ± 121	3.86 ± 0.41	1.35 ± 0.25	35 ± 9
RAPA	2,850 ± 164	3.62 ± 0.58	1.35 ± 0.22	37 ± 3
ISO (1.0 MAC)+RAPA	2,786 ± 234	3.97 ± 0.53	1.61 ± 0.29	41 ± 7
L-NAME	2,676 ± 213	2.52 ± 0.41	0.88 ± 0.30	34 ± 8
ISO (1.0 MAC)+L-NAME	2,771 ± 125	3.51 ± 0.35	1.36 ± 0.31	38 ± 6
7NI	2,700 ± 179	3.85 ± 0.65	1.48 ± 0.22	39 ± 4
ISO (1.0 MAC)+7NI	2,600 ± 110	3.20 ± 0.25	1.27 ± 0.21	40 ± 4
AG	2,619 ± 456	3.45 ± 0.53	1.18 ± 0.58	34 ± 14
ISO (1.0 MAC)+AG	2,617 ± 41	3.75 ± 0.84	1.24 ± 0.06	34 ± 7

AAR = area at risk; LV = left ventricle; ISO = isoflurane; PD = PD098059; RAPA = rapamycin; L-NAME = *N*-nitro-L-arginine methyl ester; 7NI = 7-nitroindazole; AG = aminoguanidine; MAC = minimum alveolar concentration; Data are mean ± SD.

isoforms observed in this study[10] were abolished by PD 098059 pretreatment. Another adenosine A_1/A_2 receptor agonist (AMP579) also exerted protective effects during reperfusion in rabbits[15] via an Erk1/2-dependent mechanism.[21]

The current results further demonstrate that administration of rapamycin during coronary artery occlusion abolishes reductions in infarct size produced by isoflurane during early reperfusion. These data suggest that p70s6K mediates isoflurane-induced postconditioning. The current findings support previous results implicating p70s6K in cardioprotection against reperfusion injury, as ischemic postconditioning occurred concomitant with p70s6K phosphorylation in isolated perfused rat hearts.[9] Rapamycin also blocked decreases in the extent of myocardial infarction produced by δ_1-opioid receptor agonists when administered during early reperfusion.[12] The current results further demonstrate that administration of L-NAME before ischemia and brief exposure to isoflurane abolished decreases in infarct size produced by the volatile agent. In addition, pretreatment with either the selective iNOS antagonist AG or the selective nNOS inhibitor 7-NI did not inhibit postconditioning by isoflurane. These data provide pharmacological evidence that eNOS and not iNOS or nNOS mediates cardioprotection by isoflurane during early reperfusion. The current results support the findings of previous studies implicating a role for NO metabolism in postconditioning phenomena. Activation of Erk1/2 and an increase in NO production was initially proposed to mediate ischemic postconditioning,[22] and a subsequent study demonstrated that PI3K-mediated phosphorylation of eNOS was central to this process.[9] Cardioprotection produced by NECA and bradykinin during reperfusion was also mediated by stimulation of NO synthase metabolism, although eNOS was not specifically identified as the enzymatic source of NO in this study.[10] We have also recently demonstrated that eNOS triggers and mediates delayed preconditioning by isoflurane in a similar rabbit model of ischemia-reperfusion injury.[19] These latter data indicated that eNOS appear to play an important role in other forms of myocardial protection produced by volatile anesthetics as well.

The precise mechanisms by which Erk1/2, p70s6K, and eNOS produce postconditioning by isoflurane remain to be completely elucidated. Activation of the PI3K-Akt signaling cascade mediated the cardioprotective effect of isoflurane during early reperfusion in rabbit myocardium.[5,6] This pathway has been shown to play a major role in cell survival during reperfusion by activating p70s6K and eNOS, favourably affecting the balance between pro- and anti-apoptotic proteins, and inhibiting caspase formation and glycogen synthase kinase-3β activity.[13,23] The extracellular signal-related kinases also facilitate cell survival by stimulating p70s6K, inactivating several proapoptotic proteins known to produce mitochondrial damage (e.g., Bad, Bax, Bim), and blocking the formation of the apoptotic enzyme caspase 3.[13] Thus, it appears highly likely based on evidence accumulated to date that PI3K-Akt and Erk1/2 represent redundant prosurvival mechanisms by which downstream signaling elements are activated to provide protection against cellular injury during reperfusion. Phosphatidylinositol-3-kinase- or Erk1/2-induced activation of p70s6K may contribute to preservation of myocardial integrity during reperfusion by inactivating glycogen synthase kinase 3β.[24] and inhibiting apoptotic cell death.[22] In particular, glycogen synthase kinase 3β has recently been shown to mediate convergence of cardioprotective signaling, including Akt and p70s6K, to inhibit the mitochondrial permeability transition pore (mPTP) in isolated cardiac myocytes *in vitro*.[24] It has become increasing clear that prevention of mPTP opening plays a critical role as an end-effector in myocardial protection against ischemia-reperfusion injury.[25–29] Whether isoflurane inhibits mPTP by attenuating glycogen synthase kinase-3β activity through Erk1/2-p70s6K signaling is presently unknown. This hypothesis is being actively investigated by our laboratory, and certainly appears to be very plausible based on recent findings indicating that desflurane-induced preconditioning is mediated by inhibition of mitochondrial permeability transition.[30] Phosphatidylinositol-3-kinase-Akt has also been previously demonstrated to phosphorylate eNOS, and the NO produced as a result of activation of this enzyme has been shown to mediate cellular protection.[31] This NO-induced protective effect may be related to inhibition of mPTP opening concomitant with reperfusion.[32] Thus, the current results suggesting that eNOS plays a role in isoflurane-induced postconditioning may again be related to inhibition of mitochondrial permeability transition through enhanced NO production. Further study will also be required to confirm this intriguing hypothesis.

The current results must be interpreted within the constraints of several potential limitations. PD 098059, rapamycin, AG, and 7-NI have been shown to be selective inhibitors of Erk1/2, p70s6K, iNOS, and nNOS, respectively, at the doses used in the current investigation. Nevertheless, dose-response relationships to these selective inhibitors were not performed, and the possibility that these drugs may have inhibited other protein kinases involved in myocardial protection cannot be completely excluded

from the analysis. Currently available eNOS antagonists may affect other NOS isoforms, and thus, we chose not to conduct experiments with these drugs. Myocardial infarct size is determined primarily by the size of the AAR and extent of coronary collateral perfusion. The AAR expressed as a percentage of total left ventricular mass was similar between groups in the current investigation. Rabbits have also been shown to possess little if any coronary collateral blood flow.[33] Thus, it appears unlikely that differences in collateral perfusion between groups account for the observed results. However, coronary collateral blood flow was not specifically quantified in the current investigation. The reductions in myocardial necrosis produced by the brief administration of isoflurane during early reperfusion occurred independent of changes in major determinants of myocardial oxygen consumption. Nevertheless, the current results require qualification because coronary venous oxygen tension was not directly measured, and myocardial oxygen consumption was not calculated in the current investigation. The results also require qualification because we did not specifically examine the biochemical actions of isoflurane on Erk1/2, p70s6K, and eNOS phosphorylation nor did we measure the activities of these enzymes in rabbit myocardium exposed to ischemia and reperfusion. Nevertheless, our pharmacological data strongly suggest a central role for Erk1/2, p70s6K, and eNOS in isoflurane-induced postconditioning against infarction because inhibitors of these enzymes blocked protection produced by the volatile agent. The current results should also be qualified because we did not specifically measure NO production by eNOS.

We used a 30-min coronary artery occlusion in order to produce myocardial infarction in rabbits. Whether brief exposure to isoflurane before and during early reperfusion also produces cardioprotection after more prolonged periods of coronary artery occlusion is unknown and will require further study to ascertain. Anesthetic pre- and postconditioning have recently been shown to produce opposing genomic responses during cardioprotection.[34] These data suggest that signal transduction cascades during postconditioning by volatile anesthetics may be different from those responsible for preconditioning. Thus, the possibility exists that Erk1/2, p70s6K, and eNOS do not play substantial roles in preconditioning by isoflurane. However, a role for Erk1/2 has been previously demonstrated in desflurane-induced preconditioning,[16] and we have recently shown the eNOS plays an important role in delayed preconditioning by isoflurane.[19] Nevertheless, further research will be required in order to determine the relative specificity of signal elements in pre- as compared to postconditioning by volatile agents. The current results require qualification because aging has recently been shown to modulate cardioprotection,[35] and we did not specifically use rabbits from a preselected age range. Nevertheless, rabbits of similar body weight were used in the current investigation. Finally, the current findings implicating a role for Erk1/2, p70s6K, and eNOS in cardioprotection by isoflurane were obtained in barbiturate-anesthetized rabbits. Whether similar results occur in other animal species or humans is unknown.

In summary, the current results confirm that brief exposure to isoflurane immediately before and during early reperfusion reduces myocardial infarct size in barbiturate-anesthetized, acutely instrumented rabbits. The findings indicate that selective pharmacological inhibitors of Erk1/2 or p70s6K abolish this isoflurane-induced postconditioning against infarction. In addition, inhibition of NO production by L-NAME before the administration of isoflurane abolished reductions in infarct size produced by this volatile agent, indicating that NO mediates this protective effect. The current results further suggest that eNOS but not iNOS or nNOS mediates postconditioning by isoflurane *in vivo*. The current findings are potentially important from a clinical perspective. The ability to briefly administer a volatile anesthetic as a therapeutic agent immediately before or during early reperfusion may contribute to the salvage of viable myocardium in patients with acute coronary artery occlusion, but further research will be required in order to test this hypothesis.

Acknowledgements

The authors thank David A. Schwabe BSEE for technical assistance and Mary Lorence-Hanke AA for assistance in preparation of the manuscript.

References

1 *Tanaka K, Ludwig LM, Kersten JR, Pagel PS, Warltier DC.* Mechanisms of cardioprotection by volatile anesthetics. Anesthesiology 2004; 100: 707–21.

2 *Schlack W, Preckel B, Stunneck D, Thamer V.* Effects of halothane, enflurane, isoflurane, sevoflurane and desflurane on myocardial reperfusion injury in the isolated rat heart. Br J Anaesth 1998; 81: 913–9.

3 *Siegmund B, Schlack W, Ladilov YV, Balser C, Piper M.* Halothane protects cardiomyocytes against reoxygenation-induced hypercontracture. Circulation 1997; 96: 4372–9.

4 *Varadarajan SG, An J, Novalija E, Stowe DF.* Sevoflurane before or after ischemia improves contrac-

tile and metabolic function while reducing myoplasmic Ca^{2+} loading in intact hearts. Anesthesiology 2002; 96: 125–33.

5 *Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR, Warltier DC.* Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: evidence for anesthetic-induced postconditioning in rabbits. Anesthesiology 2005; 102: 102–9.

6 *Weihrauch D, Krolikowski JG, Bienengraeber M, Kersten JR, Warltier DC, Pagel PS.* Morphine enhances isoflurane-induced postconditioning against myocardial infarction: the role of phosphatidylinositol-3-kinase and opioid receptors in rabbits. Anesth Analg 2005; 101: 942–9.

7 *Kin H, Zhao ZQ, Sun HY, et al.* Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004; 62: 74–85.

8 *Zhao ZQ, Corvera JS, Halkos ME, et al.* Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol 2003; 285: H579–88.

9 *Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM.* Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004; 95: 230–2.

10 *Yang XM, Krieg T, Cui L, Downey JM, Cohen MV.* NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK, and NO. J Mol Cell Cardiol 2004; 36: 411–21.

11 *Bell RM, Yellon DM.* Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. J Am Coll Cardiol 2003; 41: 508–15.

12 *Gross ER, Hsu AK, Gross GJ.* Opioid-induced cardioprotection occurs via glycogen synthase kinase inhibition during reperfusion in intact rat hearts. Circ Res 2004; 94: 960–6.

13 *Hausenloy DJ, Yellon DM.* New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res 2004; 61: 448–60.

14 *Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K.* Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. Am J Physiol Heart Circ Physiol 2005; 289: H1618–26.

15 *Xu Z, Yang XM, Cohen MV, Neumann T, Heusch G, Downey JM.* Limitation of infarct size in rabbit hearts by the novel adenosine receptor agonist AMP 579 administered at reperfusion. J Mol Cell Cardiol 2000; 32: 2339–47.

16 *Toma O, Weber NC, Wolter JI, Obal D, Preckel B, Schlack W.* Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular-signal regulated kinase 1 and 2 in the rat heart in vivo. Anesthesiology 2004; 101: 1372–80.

17 *Tanaka K, Weihrauch D, Kehl F, et al.* Mechanism of preconditioning by isoflurane in rabbits: a direct role for reactive oxygen species. Anesthesiology 2002; 97: 1485–90.

18 *Fryer RM, Pratt PF, Hsu AK, Gross GJ.* Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. J Pharmacol Exp Ther 2001; 296: 642–9.

19 *Chiari PC, Bienengraeber MW, Weihrauch D, et al.* Role of endothelial nitric oxide synthase as a trigger and mediator of isoflurane-induced delayed preconditioning in rabbit myocardium. Anesthesiology 2005; 103: 74–83.

20 *Warltier DC, Zyvoloski MG, Gross GJ, Hardman HF, Brooks HL.* Determination of experimental myocardial infarct size. J Pharmacol Methods 1981; 6: 199–210.

21 *Kis A, Baxter GF, Yellon DM.* Limitation of myocardial reperfusion injury by AMP579, an adenosine A_1/A_{2A} receptor agonist: role of A_{2A} receptor and Erk1/2. Cardiovasc Drug Ther 2003; 17: 415–25.

22 *Yang XM, Downey JM, Cohen MV.* Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by activation of ERK and production of nitric oxide. Circulation 2003; 108(Suppl): IV745 (abstract).

23 *Murphy E.* Inhibit GSK-3 or there's heartbreak dead ahead. J Clin Invest 2004; 113: 1526–8.

24 *Juhaszova M, Zorov DB, Kim SH, et al.* Glycogen synthase kinase-3 mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004; 113: 1535–49.

25 *Weiss JN, Korge P, Honda HM, Ping P.* Role of mitochondrial permeability transition in myocardial disease. Circ Res 2003; 93: 292–301.

26 *Argaud L, Gateau-Roesch O, Chalabreysse L, et al.* Preconditioning delays Ca^{2+}-induced mitochondrial permeability transition. Cardiovasc Res 2004; 61: 115–22.

27 *Argaud L, Gateau-Roesch O, Muntean D, et al.* Specific inhibition of mitochondrial permability transition prevents lethal reperfusion injury. J Mol Cell Cardiol 2005; 38: 367–74.

28 *Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M.* Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111: 194–7.

29 *Downey JM, Cohen MV.* We think we see a pattern emerging here (Editorial). Circulation 2005; 111: 120–1.

30 *Piriou V, Chiari P, Gateau-Roesch O, et al.* Desflurane-induced preconditioning alters calcium-induced mitochondrial permeability transition. Anesthesiology 2004; 100: 581–8.
31 *Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM.* Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999; 399: 601–5.
32 *Balakirev MY, Khramtsov VV, Zimmer G.* Modulation of the mitochondrial permeability transition by nitric oxide. Eur J Biochem 1997; 246: 710–8.
33 *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.
34 *Lucchinetti E, da Silva R, Pasch T, Schaub MC, Zaugg M.* Anaesthetic preconditioning but not postconditioning prevents early activation of the deleterious cardiac remodelling programme: evidence of opposing genomic responses in cardioprotection by pre- and postconditioning. Br J Anaesth 2005; 95: 140–52.
35 ***Sniecinski R, Liu H.*** Reduced efficacy of volatile anesthetic preconditioning with advanced age in isolated rat myocardium. Anesthesiology 2004; 100: 589–97.