Anesthesiology 2010; 112:73–85

Isoflurane Postconditioning Protects against Reperfusion Injury by Preventing Mitochondrial Permeability Transition by an Endothelial Nitric Oxide Synthase–dependent Mechanism

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ABSTRACT

Background: The role of endothelial nitric oxide synthase (eNOS) in isoflurane postconditioning (IsoPC)-elicited cardioprotection is poorly understood. The authors addressed this issue using *eNOS*$^{-/-}$ mice.

Methods: *In vivo* or Langendorff-perfused mouse hearts underwent 30 min of ischemia followed by 2 h of reperfusion in the presence and absence of postconditioning produced with isoflurane 5 min before and 3 min after reperfusion. Ca^{2+}-induced mitochondrial permeability transition (MPT) pore opening was assessed in isolated mitochondria. Echocardiography was used to evaluate ventricular function.

Results: Postconditioning with 0.5, 1.0, and 1.5 minimum alveolar concentrations of isoflurane decreased infarct size from 56 ± 10% (n = 10) in control to 48 ± 10%, 41 ± 8% (n = 8, $P < 0.05$), and 38 ± 10% (n = 8, $P < 0.05$), respectively, and improved cardiac function in wild-type mice. Improvement in cardiac function by IsoPC was blocked by N^G-nitro-L-arginine methyl ester (a nonselective nitric oxide synthase inhibitor) administered either before ischemia or at the onset of reperfusion. Mitochondria isolated from postconditioned hearts required significantly higher *in vitro* Ca^{2+} loading than did controls (78 ± 29 μM *vs.* 40 ± 25 μM $CaCl_2$ per milligram of protein, n = 10, $P < 0.05$) to open the MPT pore. Hearts from *eNOS*$^{-/-}$ mice displayed no marked differences in infarct size, cardiac function, and sensitivity of MPT pore to Ca^{2+}, compared with wild-type hearts. However, IsoPC failed to alter infarct size, cardiac function, or the amount of Ca^{2+} necessary to open the MPT pore in mitochondria isolated from the *eNOS*$^{-/-}$ hearts compared with control hearts.

Conclusions: IsoPC protects mouse hearts from reperfusion injury by preventing MPT pore opening in an eNOS-dependent manner. Nitric oxide functions as both a trigger and a mediator of cardioprotection produced by IsoPC.

What We Already Know about This Topic

- Isoflurane protects the heart from ischemia reperfusion injury, when given on reperfusion
- Nitric oxide synthases are important to this effect but their mechanisms are unclear

What This Article Tells Us That Is New

- In mice, isoflurane protection depended on activation of the endothelial nitric oxide synthase subtype and inhibited the mitochondrial permeability transition pore opening
- Nitric oxide served as both a trigger and a mediator of myocardial protection in this setting

MYOCARDIAL injury is attenuated in hearts subjected to brief episodes of ischemia/reperfusion (I/R) that interrupt the initial period of sustained reperfusion (ischemic postconditioning).[1,2] This cardioprotective effect can also be achieved by pharmacological interventions instituted at the onset of reperfusion.[3–5] We and others have shown that the volatile anesthetic isoflurane reduces myocardial damage when administered during the early phase of reperfusion (isoflurane postconditioning [IsoPC]).[6–8] Whether cardioprotection produced by IsoPC is modified by genetic factors, such as deficiency of the nitric oxide synthase (NOS) gene, remains unknown.

Pharmacological studies suggest that nitric oxide derived from L-arginine is involved in IsoPC-induced cardioprotection against I/R injury in rabbits and rats.[9,10] Under physiologic conditions, nitric oxide is predominantly produced by endothelial NOS (eNOS) in vascular endothelium.[11] How-

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Received from Departments of Anesthesiology and of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin. Submitted for publication May 18, 2009. Accepted for publication September 30, 2009. Supported in part by National Institutes of Health research grants HL 063705 (to J.R.K.), HL 054820 (to D.C.W.), and GM 066730 (to J.R.K. and D.C.W.) from the United States Public Health Services, Bethesda, Maryland.

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ever, during myocardial I/R, nitric oxide may be produced from three distinct NOS isoforms expressed in cardiac myocytes: neuronal NOS, inducible NOS, and eNOS.[11,12] Because pharmacological inhibitors usually nonselectively inhibit all isoforms of NOS, it is unknown which isoform is responsible for IsoPC-induced cardioprotection. Gene knockout mice for the eNOS isoform (*eNOS*$^{-/-}$ mice) have recently been used as a tool to examine the role of eNOS in physiologic and pathologic states such as ischemic preconditioning.[13] Whether *eNOS*$^{-/-}$ mice are also useful for establishing the role of the eNOS isoform in IsoPC-induced cardioprotection remains unclear. Furthermore, the targets of nitric oxide in IsoPC are not yet identified. Growing evidence suggests that among potential candidates, the mitochondrial permeability transition (MPT) pore is a key target for nitric oxide-induced protection against I/R injury.[14–17] Accordingly, we investigated whether IsoPC protects the myocardium against I/R injury by modulating MPT in wild-type (WT) and *eNOS*$^{-/-}$ mice.

Materials and Methods

Animals

Male C57BL/6J WT mice and *eNOS*$^{-/-}$ mice (weight: 25.3 ± 1.3 g; age: 9–12 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were kept on a 12-h light–dark cycle in a temperature-controlled room. All experimental procedures used in this study were approved by the Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, WI) and conformed to the Guide for the Care and Use of laboratory Animals (NIH Publication No. 85–23, revised 1996).

Acute Myocardial I/R Injury In Vivo

Surgical Preparation. We have previously described the mouse *in vivo* model of myocardial I/R injury.[18,19] Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg) and ventilated with room air supplemented with 100% oxygen at a rate of approximately 104 breaths/min with a tidal volume of approximately 0.5 ml using a rodent ventilator (Harvard Apparatus, South Natick, MA). Arterial blood gas tensions and acid-base status were maintained within a physiologic range (pH between 7.35 and 7.45, Pa_{CO_2} between 35 and 45 mmHg, and Pa_{O_2} between 120 and 180 mmHg) by adjusting the respiratory rate or tidal volume. Myocardial ischemia was produced by occluding the left anterior descending coronary artery, and reperfusion was initiated by loosening the suture. Body temperature was maintained between 36.8° and 37.5°C throughout the experiment by using a heating pad (Model TC-1000, CWE Inc., Ardmore, PA). The infarct area was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride *via* the aortic root, and the area at risk was delineated by perfusing phthalo blue dye (Heucotech Ltd., Fairless Hill, PA) into the aortic root after tying the coronary artery at the site of previous occlusion. As a result of these procedures, the nonischemic portion of the left ventricle (LV) was stained dark blue. Viable myocardium within the area at risk was stained bright red, and infarcted tissue was light yellow.

Experimental Protocol. *In vivo* IsoPC experiments followed two different protocols (fig. 1). The concentration-dependent effects of IsoPC were determined in C57BL/6J mice randomly assigned to four experimental groups (8–10 mice/group): control, $ISO_{0.5}$, $ISO_{1.0}$, and $ISO_{1.5}$ (fig. 1A). After instrumentation was completed, all mice were stabilized for 30 min and subjected to 30 min of coronary occlusion followed by 2 h of reperfusion. Isoflurane was administered through an isoflurane-specific vaporizer (Ohio Medical Instruments, Madison, WI). Postconditioning was produced with 0.5, 1.0, or 1.5 minimum alveolar concentrations (MAC) of isoflurane (1.0 MAC = 1.40% in the mouse) administered during the last 5 min of coronary occlusion and first 3 min of reperfusion. Control mice received no isoflurane. Arterial blood pressure was measured with a Millar pressure catheter (Model SPR-1000, Millar Instruments, Houston, TX) inserted into the right carotid artery and connected to a pressure transducer (ADInstruments, Sydney, Australia), as described previously.[20] Heart rate was monitored by using the electrocardiogram. Area at risk and infarct size were determined after each experiment. The effects of disruption of the eNOS gene on infarct size were measured in C57BL/6J or *eNOS*$^{-/-}$ mice with and without IsoPC (fig. 1B).

Transthoracic Echocardiography

C57BL/6J and *eNOS*$^{-/-}$ mice were sedated by the inhalation of isoflurane (1.50%) and oxygen and anchored to a warming platform in the supine position. Transthoracic echocardiography was performed with a VisualSonics Vevo 770 high-resolution imaging system (Toronto, Canada) equipped with a 30-MHz transducer (Scanhead RMV 707). As described previously,[21] LV dimensions and fractional shortening were measured by a two-dimensionally guided M-mode method. Pulsed Doppler waveforms recorded in the apical four-chamber view were used for measurements of the peak velocities of mitral E (early mitral inflow) and A (late mitral inflow) waves, E wave acceleration velocity, E wave acceleration time, E wave deceleration velocity, and E wave deceleration time, isovolumic contraction time, ejection time, and isovolumic relaxation time of LV. Myocardial performance index was calculated with the following formula: myocardial performance index = (isovolumic contraction time + isovolumic relaxation time)/ejection time.

I/R Injury in Isolated Hearts

Langendorff-perfusion of the Heart. The hearts were perfused at a constant pressure of 55 mmHg with Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, and 11 mM glucose.[19,22] The buffer was continuously bubbled with a mixture of 95% oxygen/5% carbon dioxide through an in-line filter (5-μm pore size). A fluid-filled plastic balloon was inserted into the chamber of LV

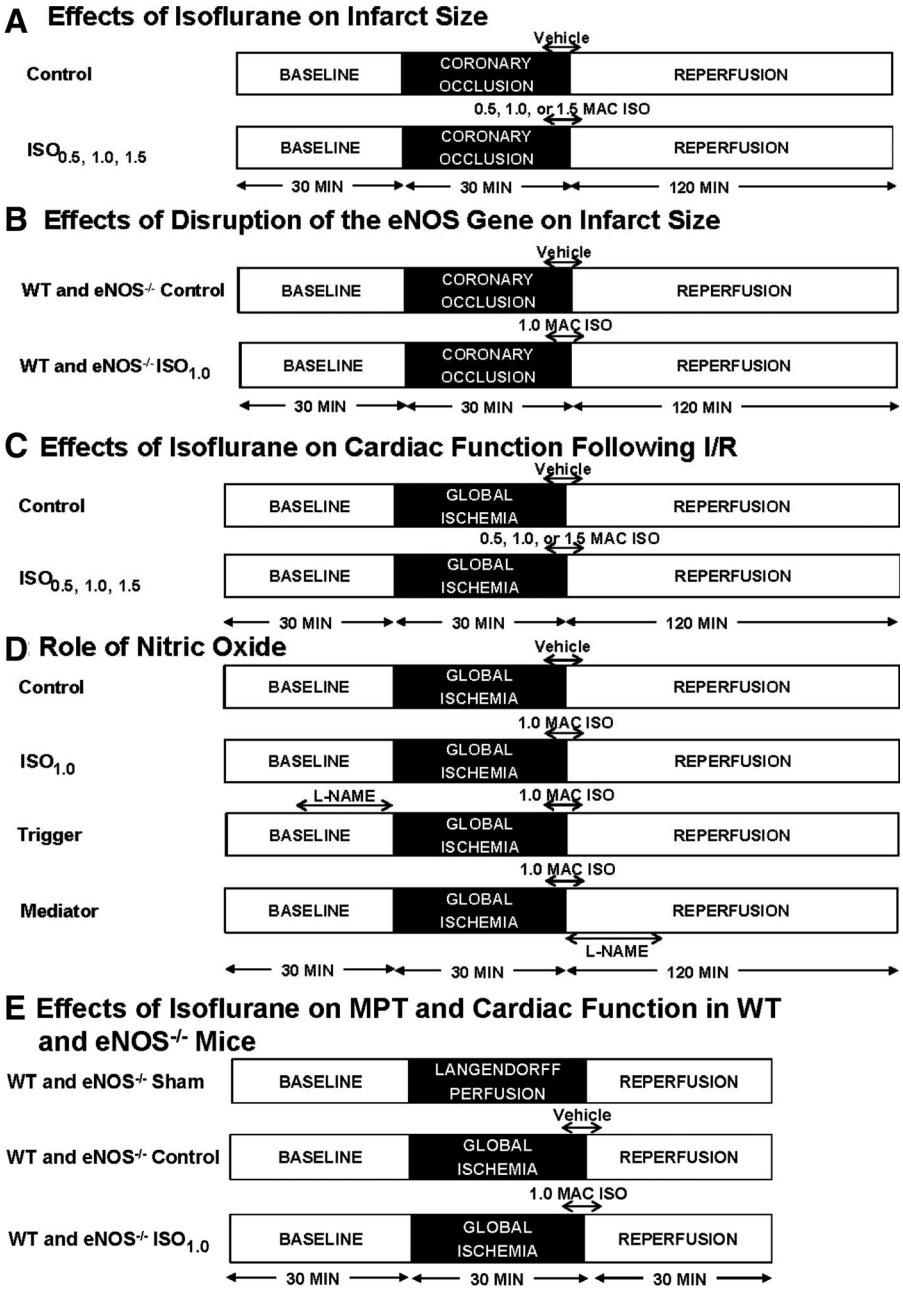

Fig. 1. Schematic diagram depicting the experimental protocols. (*A*) E ffects of different concentrations of isoflurane on myocardial infarct size in WT mice after I/R; (*B*) effects of disruption of the *eNOS* gene on infarct size; (*C*) effects of different concentrations of isoflurane on cardiac function in Langendorff-perfused WT hearts after I/R; (*D*) role of nitric oxide in the cardioprotective effect produced by isoflurane; (*E*) effects of isoflurane on MPT and cardiac function in WT and $eNOS^{-/-}$ mice. $eNOS^{-/-}$ = endothelial nitric oxide synthase gene knockout; I/R = ischemia/reperfusion; $ISO_{0.5}$ = 0.5 MAC isoflurane; $ISO_{1.0}$ = 1.0 MAC isoflurane; $ISO_{1.5}$ = 1.5 MAC isoflurane; L-NAME = N^G-nitro-L-arginine methyl ester; MAC = minimum alveolar concentration; MPT = mitochondrial permeability transition; WT = wild type.

through the mitral valve and connected to a pressure transducer for continuous measurement of LV pressure. The hearts were immersed in perfusate maintained at 37.2° ± 0.3° C, and the balloon was inflated to a diastolic pressure of approximately 5–10 mmHg. Coronary flow was monitored by an in-line flow probe connected to a flow meter (Transonics Systems Inc., Ithaca, NY). The LV pressure signal was monitored to obtain heart rate and LV dP/dt. The LV developed pressure (LVDP) was calculated as the difference between the systolic and end-diastolic LV pressure. Global I/R was produced by cessation of perfusion followed by reperfusion at a designated time.

Experimental Protocol. Langendorff-perfused hearts were used in three different protocols (fig. 1). The effects of isoflurane on cardiac function after I/R were measured in C57BL/6J mice (fig. 1C). All hearts were perfused for 30 min for stabilization, and baseline LV contraction and coronary flow were recorded. Hearts were then randomly assigned

Table 1. Systemic Hemodynamics during *In Vivo* Experiments

Group	Baseline	Coronary Occlusion	Reperfusion 2 min	Reperfusion 30 min	Reperfusion 60 min	Reperfusion 120 min
Effects of isoflurane on heart rate and blood pressure during ischemia/reperfusion in the wild-type mice						
Heart rate (beats/min)						
Control	402 ± 69	374 ± 60	429 ± 63	409 ± 53	398 ± 41	395 ± 56
$ISO_{0.5}$	411 ± 56	390 ± 34	400 ± 36	396 ± 31	382 ± 28	388 ± 17
$ISO_{1.0}$	416 ± 61	389 ± 48	402 ± 23	393 ± 39	374 ± 28	395 ± 31
$ISO_{1.5}$	406 ± 76	369 ± 31	431 ± 62	420 ± 42	389 ± 31	398 ± 55
Mean arterial blood pressure (mmHg)						
Control	74 ± 15	57 ± 15*	58 ± 15*	60 ± 9*	57 ± 12*	55 ± 12*
$ISO_{0.5}$	78 ± 11	59 ± 14*	58 ± 11*	59 ± 14*	60 ± 14*	61 ± 14*
$ISO_{1.0}$	76 ± 14	58 ± 11*	59 ± 11*	61 ± 11*	61 ± 12*	60 ± 11*
$ISO_{1.5}$	73 ± 14	50 ± 17*	49 ± 16*	52 ± 16*	53 ± 16*	55 ± 14*
Effects of $ISO_{1.0}$ on heart rate during ischemia/reperfusion in the wild-type and *eNOS*$^{-/-}$ mice						
WT control	403 ± 42	384 ± 41	391 ± 40	397 ± 37	401 ± 29	402 ± 52
WT $ISO_{1.0}$	424 ± 61	392 ± 50	399 ± 22	393 ± 42	379 ± 27	399 ± 29
eNOS$^{-/-}$ control	397 ± 50	384 ± 39	416 ± 54	434 ± 32	400 ± 25	424 ± 34
eNOS$^{-/-}$ $ISO_{1.0}$	391 ± 55	383 ± 45	428 ± 46	403 ± 34	397 ± 31	392 ± 30

* $P < 0.05$ *versus* baseline (n = 7–10 mice/group).

eNOS$^{-/-}$ = endothelial nitric oxide synthase gene knockout mice; $ISO_{0.5}$ = 0.5 minimum alveolar concentration of isoflurane; $ISO_{1.0}$ = 1.0 minimum alveolar concentration of isoflurane; $ISO_{1.5}$ = 1.5 minimum alveolar concentration of isoflurane.

to four groups: control, $ISO_{0.5}$, $ISO_{1.0}$, and $ISO_{1.5}$ (7–8 hearts/group) and subjected to 30 min of no-flow global ischemia and 2 h of reperfusion. Isoflurane was bubbled into the Krebs-Henseleit solution using an agent-specific vaporizer placed in the 95% oxygen/5% carbon dioxide gas mixture line, and isoflurane concentration was determined by gas chromatography in the coronary effluent. In the control group, oxygen/carbon dioxide-bubbled buffer was administered 5 min before and 3 min after reperfusion, whereas in the other groups, the buffer containing 0.5, 1.0, or 1.5 MAC isoflurane was administered 5 min before and 3 min after reperfusion. During global ischemia, buffer with isoflurane was perfused into the aorta by an infusion pump at a rate of 0.4 ml/min. LV contraction and coronary flow were continuously recorded during I/R. The role of nitric oxide as a trigger or mediator of IsoPC was evaluated in C57BL/6J mice (n = 7 mice/group) (fig. 1D). The hearts treated with 1.0 MAC isoflurane to produce postconditioning were perfused with 30 μm N^G-nitro-L-arginine methyl ester (L-NAME, a nonselective NOS inhibitor) for 20 min either before ischemia (trigger group) or at the onset of reperfusion (mediator group). LVDP, +dP/dt (maximum rate of increase of LVDP), and −dP/dt (maximum rate of decrease of LVDP) at baseline and 30 and 60 min after reperfusion were determined. To evaluate the effects of IsoPC on MPT and cardiac function (fig. 1E), C57BL/6J and *eNOS*$^{-/-}$ mice were randomly assigned to one of the following six groups: (1) WT sham, (2) WT control, (3) WT $ISO_{1.0}$, (4) *eNOS*$^{-/-}$ sham, (5) *eNOS*$^{-/-}$ control, and (6) *eNOS*$^{-/-}$ $ISO_{1.0}$. After the stabilization for 30 min, control and $ISO_{1.0}$ hearts were subjected to 30-min global ischemia and 30-min reperfusion with or without 1.0 MAC isoflurane. Sham hearts were not subjected to ischemia. Mitochondria were isolated from the LV for determination of Ca^{2+}-induced MPT pore opening, as described later.

Detection of the Permeability Transition Pore Opening in Isolated Mitochondria

Preparation of Isolated Mitochondria. LVs were minced in ice-cold isolation buffer containing 50 mM sucrose, 200 mM mannitol, 5 mM KH_2PO_4, 1 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (Sigma-Aldrich, St. Louis, MO), 5 mM 3-(*N*-morpholino)propanesulfonic acid (Sigma-Aldrich), and 0.1% bovine serum albumin (pH 7.3) and gently homogenized in the same buffer with T 25 disperser (IKA-Werke, Staufen, Germany) in the presence of protease from *Bacillus licheniformis* (0.5 mg/ml). The homogenate was differentially centrifuged, and the final mitochondrial pellet was resuspended in isolation buffer without ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Ca^{2+}-induced MPT Pore Opening. Opening of the MPT pore after *in vitro* Ca^{2+} overload was assessed by following changes in the membrane potential ($\Delta\psi_m$) by using the

fluorescent dye rhodamine 123 (50 nM; Invitrogen, Carlsbad, CA) in the presence of pyruvate and malate (5 mM).[23,24] Fluorescence was monitored with a PTI spectrofluorometer (Photon Technology International Inc., Birmingham, NJ). Excitation and emission wavelengths were set to 503 and 527 nm, respectively. Isolated mitochondria (0.5 mg/ml) were suspended in 1.0-ml recording buffer containing 220 mM sucrose, 10 mM 4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid (Sigma-Aldrich), and 10 mM KH_2 PO_4, pH = 7.3. At the end of the preincubation period, pulses of 10 μM $CaCl_2$ were administered in 60-s intervals. After sufficient Ca^{2+} loading, MPT pore opening results in a sudden collapse of $\Delta\psi_m$. To achieve complete mitochondrial depolarization, 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (Sigma-Aldrich) was added into the buffer at the end of the experiment. The amount of Ca^{2+} necessary to trigger this sudden collapse of $\Delta\psi_m$ was used as an indicator of the susceptibility of the MPT pore to Ca^{2+} overload.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis of echocardiographic data was performed with two-way repeated-measures analysis of variance. Repeated-measures analysis of variance followed by Bonferroni multiple comparison test was used to evaluate differences in heart rate, mean arterial blood pressure, and coronary flow over time between groups. One-way analysis of variance followed by Bonferroni *post hoc* test was used to analyze area at risk, infarct size, LVDP, +dP/dt, −dP/dt, LV end-diastolic pressure, and the amount of Ca^{2+} loading necessary to open the MPT pore. A value of $P < 0.05$ was considered statistically different.

Results

IsoPC Protected Against Myocardial I/R Injury in WT Mice

Heart rate at baseline was not different among the four experimental groups (table 1). Coronary artery occlusion significantly decreased mean arterial blood pressure in all groups in comparison with baseline blood pressure. No significant differences in arterial blood pressure and heart rate were observed between groups during coronary occlusion and reperfusion.

Area at risk and myocardial infarct size are shown in figure 2. There were no significant differences in area at risk among the four experimental groups (fig. 2A). Coronary occlusion followed by reperfusion resulted in an infarct size of 56 ± 10% of area at risk (n = 10) in WT mice, which was significantly decreased to 41 ± 8% ($P < 0.05$, n = 8) and 38 ± 10% ($P < 0.05$, n = 8) by 1.0 and 1.5 MAC isoflurane administered 5 min before and 3 min after reperfusion, respectively. Infarct size was not altered by 0.5 MAC isoflurane treatment (48 ± 10%, not significant, n = 8, fig. 2B).

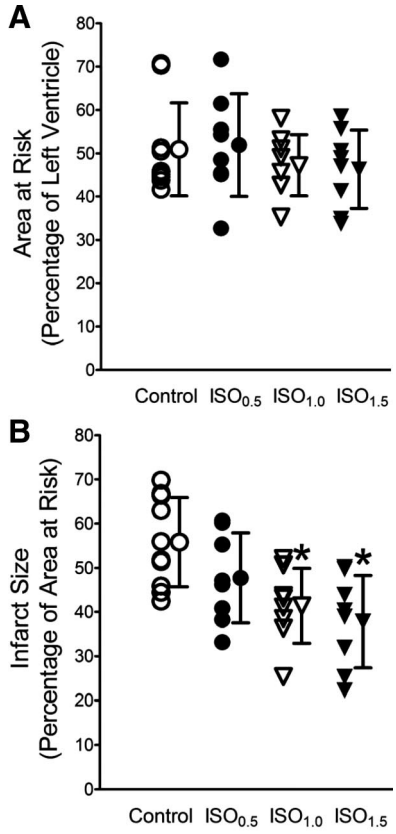

Fig. 2. Concentration-dependent decreases in myocardial infarct size by isoflurane postconditioning (IsoPC) in wild-type mice subjected to 30 min of coronary occlusion followed by 2 h of reperfusion. (*A*) Area at risk expressed as a percentage of left ventricle area; (*B*) myocardial infarct size expressed as a percentage of area at risk. IsoPC was produced by 0.5, 1.0, or 1.5 minimum alveolar concentration of isoflurane ($ISO_{0.5}$, $ISO_{1.0}$, or $ISO_{1.5}$) administered during the last 5 min of ischemia and first 3 min of reperfusion. * $P < 0.05$ *versus* control (n = 8–10 mice/group).

Disruption of eNOS Gene Abolished IsoPC-elicited Decrease in Infarct Size

The effects of disruption of *eNOS* gene on area at risk and myocardial infarct size are shown in figure 3. There were no significant differences in area at risk among groups. Myocardial infarct size was decreased in the WT $ISO_{1.0}$ group compared with the WT control group (40 ± 8% *vs.* 55 ± 10% respectively, n = 7, $P < 0.05$) but was not significantly decreased by IsoPC in the $eNOS^{-/-}$ group (53 ± 8%; n = 7), suggesting that eNOS is obligatory for the cardioprotective effect of IsoPC. Furthermore, to investigate whether loss of the cardioprotective effect of IsoPC in the $eNOS^{-/-}$ mice arose from the changes in cardiac structure or function, we assessed the hearts of $eNOS^{-/-}$ mice and WT mice with echocardiography. There were no significant differences between the $eNOS^{-/-}$ mice and WT mice in multiple cardiac parameters, including heart rate, LV internal diameters in diastole and systole, fractional shortening, peak velocity of mitral E and A waves, mitral E/A ratio, isovolumic contraction time, ejection time, isovolumic relaxation time, myocardial performance index, mitral E acceleration, E wave acceleration time, mitral E deceleration, and E wave decelera-

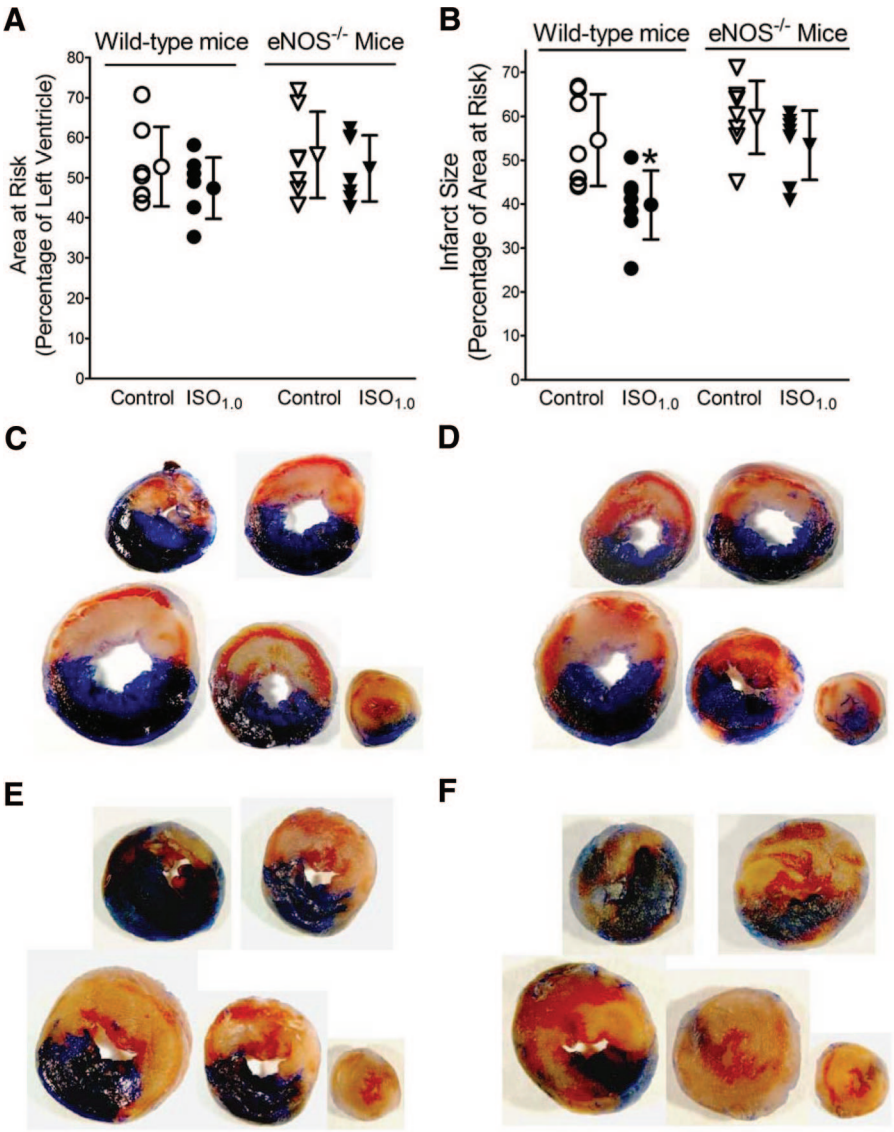

Fig. 3. Myocardial infarct size was decreased by isoflurane postconditioning (IsoPC) in wild-type mice but not in *endothelial nitric oxide synthase* knockout (*eNOS*$^{-/-}$) mice subjected to 30 min of coronary occlusion followed by 2 h of reperfusion. IsoPC was produced by 1.0 minimum alveolar concentration of isoflurane ($ISO_{1.0}$) administered during the last 5 min of ischemia and first 3 min of reperfusion. (*A*) area at risk; (*B*) infarct size; (*C*) representative heart slices from a wild-type control mouse; (*D*) heart slices from a wild-type $ISO_{1.0}$ mouse; (*E*) heart slices from an *eNOS*$^{-/-}$ control mouse; (*F*) heart slices from an *eNOS*$^{-/-}$ $ISO_{1.0}$ mouse. The hearts were stained with 2,3,5-triphenyltetrazolium chloride and phthalol blue dye to delineate area at risk (red plus light yellow areas) and infarct size (light yellow areas). * $P < 0.05$ *versus* control (n = 7 mice/group).

tion time. Only LV wall thickness in diastole was found to be greater in *eNOS*$^{-/-}$ mice than in WT mice (table 2).

IsoPC Improved Functional Recovery in Perfused WT Hearts

The effects of different concentrations of isoflurane on cardiac function in Langendorff-perfused WT hearts are shown in figure 4. Baseline values of LVDP, +dP/dt, −dP/dt, LV end-diastolic pressure, coronary flow, and heart rate were comparable among groups. During reperfusion, LVDP, +dP/dt, −dP/dt, coronary flow, and heart rate in all groups gradually increased, and LV end-diastolic pressure decreased. At all time points, there were no differences between the $ISO_{0.5}$ group and control group. However, LVDP, +dP/dt, and −dP/dt were significantly increased from 30 min to 2 h after reperfusion in the $ISO_{1.0}$ and $ISO_{1.5}$ groups in comparison with the control group. In addition, LV end-diastolic pressure was decreased in the $ISO_{1.5}$ group.

Nitric Oxide Functioned as Both a Trigger and a Mediator of IsoPC in WT Hearts

The importance of nitric oxide in cardioprotection by IsoPC is shown in figure 5. No differences in the baseline values of LVDP, +dP/dt, and −dP/dt were observed between the experimental groups. At 30 and 60 min after reperfusion, increases in LVDP, +dP/dt, and −dP/dt produced by IsoPC

Table 2. Echocardiographic Parameters in Wild-Type and *eNOS*$^{-/-}$ Mice

	Wild-Type Mice	*eNOS*$^{-/-}$ Mice
Heart rate (beats/min)	473 ± 49	454 ± 45
AWd (mm)	0.81 ± 0.12	0.98 ± 0.14*
AWs (mm)	1.28 ± 0.24	1.34 ± 0.15
PWd (mm)	0.88 ± 0.10	1.04 ± 0.16*
PWs (mm)	1.34 ± 0.30	1.37 ± 0.18
LVIDd (mm)	3.57 ± 0.29	3.35 ± 0.23
LVIDs (mm)	2.16 ± 0.40	2.22 ± 0.27
Fractional shortening (%)	39 ± 8	34 ± 8
Peak E (cm/s)	79 ± 16	75 ± 12
Peak A (cm/s)	48 ± 13	55 ± 11
Mitral E/A ratio	1.72 ± 0.37	1.40 ± 0.29
IVCT (ms)	14.1 ± 4.4	15.4 ± 3.4
Ejection time (ms)	42.2 ± 4.2	46.4 ± 4.0
IVRT (ms)	16.4 ± 2.3	15.9 ± 1.3
MPI	0.73 ± 0.14	0.68 ± 0.11
Mitral E acceleration (cm/ms)	8,303 ± 2,145	7,763 ± 1,325
Eat (ms)	9.1 ± 1.8	9.9 ± 0.6
Mitral E deceleration (cm/ms)	4,382 ± 1,656	4,962 ± 1,782
Edt (ms)	16.2 ± 6.9	16.0 ± 4.2
LV mass (g)	91 ± 15	99 ± 18

* $P < 0.05$ *versus* wild-type (n = 8 mice/group).

AWd = anterior wall (interventricular septum) at end diastole; AWs = anterior wall at end systole; Eat = mitral E wave acceleration time; Edt = E wave deceleration time; *eNOS*$^{-/-}$ mice = endothelial nitric oxide synthase gene knockout mice; fractional shortening = 100 × (LVIDd-LVIDs)/LVIDd; IVCT = isovolumic contraction time of LV; IVRT = isovolumic relaxation time of LV; LV = left ventricle; LVIDd = LV internal diameter at end diastole; LVIDs = LV internal diameter at end systole; MPI = myocardial performance index (the ratio of the sum of isovolumic contraction and relaxation times to ejection time); Peak A = peak velocity of mitral A wave; Peak E = peak velocity of mitral E valve; PWd = posterior wall at end diastole; PWs = posterior wall at end systole.

were significantly attenuated by L-NAME administered either before ischemia or at the onset of reperfusion, suggesting that nitric oxide functions as both a trigger and a mediator of IsoPC cardioprotection. The effects of isoflurane and L-NAME on heart rate and coronary flow are shown in table 3. There were no significant differences among the four groups.

IsoPC Prevented MPT Pore Opening by an eNOS-dependent Mechanism

The impact of IsoPC on Ca^{2+}-induced MPT pore opening of mitochondria is summarized in figure 6. In the WT sham group, the concentration of *in vitro* Ca^{2+} loading necessary to open the MPT pore was 148 ± 47 μM $CaCl_2$ per milligram of protein (n = 10) . This concentration was reduced to 40 ± 25 μM $CaCl_2$ per milligram of protein (n = 10, $P < 0.05$) in mitochondria isolated from Langendorff-perfused WT hearts subjected to 30 min of global ischemia followed by 30 min of reperfusion (fig. 6). IsoPC significantly increased the amount of Ca^{2+} overload required for MPT pore opening to 78 ± 29 μM $CaCl_2$ per milligram of protein (n = 10, $P < 0.05$ *vs.* I/R group), indicating that IsoPC delayed MPT pore opening. The concentration of Ca^{2+} required to trigger MPT pore opening was comparable between *eNOS*$^{-/-}$ (126 ± 38 μM $CaCl_2$ per milligram of protein, n = 10) and the WT sham groups (not significant) and between *eNOS*$^{-/-}$ (33 ± 19 μM $CaCl_2$ per milligram of protein, not significant, n = 10) and the WT control groups. IsoPC did not significantly alter the amount of Ca^{2+} overload required for MPT pore opening (41 ± 19 μM $CaCl_2$ per milligram of protein) compared with *eNOS*$^{-/-}$ control group, suggesting that isoflurane-induced inhibition of MPT pore opening is eNOS dependent.

IsoPC Improved Cardiac Function in an eNOS-dependent Manner

The effects of IsoPC on cardiac function in WT and *eNOS*$^{-/-}$ hearts are shown in figure 7. There were no significant differences in the baseline values of LVDP, +dP/dt, and −dP/dt among groups. Global ischemia for 30 min followed by reperfusion for 30 min caused significant decreases in LVDP, +dP/dt, and −dP/dt in WT and *eNOS*$^{-/-}$ hearts. IsoPC significantly increased LVDP, +dP/dt, and −dP/dt in WT hearts but not in *eNOS*$^{-/-}$ hearts subjected to I/R insult, suggesting that eNOS plays an obligatory role in the cardioprotective effect produced by IsoPC. Heart rate and coronary flow during I/R are shown in table 3. There were no significant differences at all time points between WT sham and *eNOS*$^{-/-}$ sham, WT control and *eNOS*$^{-/-}$ control, and WT $ISO_{1.0}$ and *eNOS*$^{-/-}$ $ISO_{1.0}$ groups.

Discussion

The data of this study demonstrates the dose-dependent effects of IsoPC on acute myocardial I/R injury in intact mice and in isolated hearts. Isoflurane administered during the last 5 min of ischemia and the first 3 min of reperfusion reduced infarct size and was associated with improved recovery of LV systolic contractility and diastolic relaxation. These findings confirm other reports by Chiari *et al.*[6] and Feng *et al.*,[7] demonstrating that IsoPC decreases myocardial infarct size in intact rabbits and improves LV contractility in isolated rat hearts. Myocardial reperfusion after acute ischemia is a necessary process to salvage viable myocardium and limit infarct size. However, restoration of coronary flow initiates a series of adverse events that also cause myocardial stunning, apoptosis, and necrosis[25] known as reperfusion injury.[26–29] The results of this study indicate that isoflurane produces potent cardioprotective effects against acute reperfusion injury in mice.

IsoPC was produced in this investigation by administration of isoflurane 5 min before and 3 min after reperfusion.

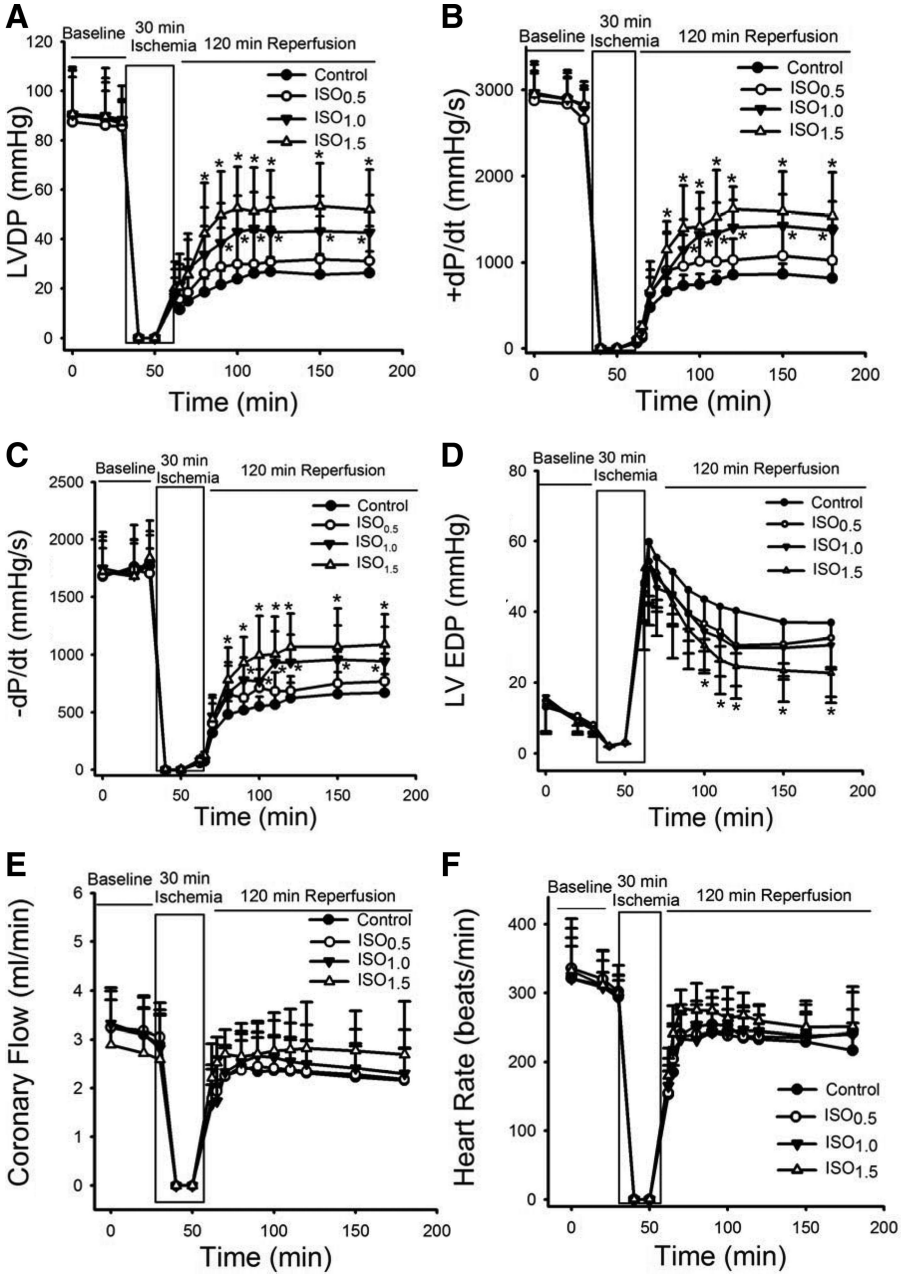

Fig. 4. Isoflurane postconditioning produced concentration-dependent increases in cardiac function in Langendorff-perfused wild-type hearts subjected to 30 min of global ischemia followed by 2 h of reperfusion. (*A*) Left ventricular developed pressure (LVDP); (*B*) +dP/dt (maximum rate of increase of LVDP); (*C*) −dP/dt (maximum rate of decrease of LVDP); (*D*) LV end-diastolic pressure (LVEDP); (*E*) coronary flow; (*F*) heart rate. $ISO_{0.5}$ = 0.5 minimum alveolar concentration of isoflurane; $ISO_{1.0}$ = 1.0 minimum alveolar concentration of isoflurane; $ISO_{1.5}$ = 1.5 minimum alveolar concentration of isoflurane. * $P < 0.05$ *versus* control (n = 7–8 hearts/group).

Our laboratory has previously shown that 1.0 MAC isoflurane administered upon reperfusion limited infarct size in rabbits.[6] Using this same protocol in pilot experiments, we found that 1.0 MAC isoflurane did not significantly reduce myocardial infarct size in intact WT mice. Slightly longer durations of administration of isoflurane in mice *versus* rabbits were required to elicit a decrease in infarct size. This difference may be caused by smaller ventilatory volumes, which delayed isoflurane to enter the myocardium in the mouse *in vivo* model.

Pharmacological studies suggest that nitric oxide generated by NOS may contribute to a IsoPC-elicited cardioprotective effect in rabbits and rats.[9,10] To examine whether eNOS is obligatory for IsoPC, we studied the effects of disruption of the *eNOS* gene on cardioprotection produced by IsoPC. Myocardial infarct size was similar in *eNOS*$^{-/-}$ mice and WT mice subjected to a 30-min coronary occlusion followed by 2-h reperfusion. This result is in agreement with a recent study by Xuan *et al.*[13] but is inconsistent with a report by Jones *et al.*[30] which states that myocardial infarct

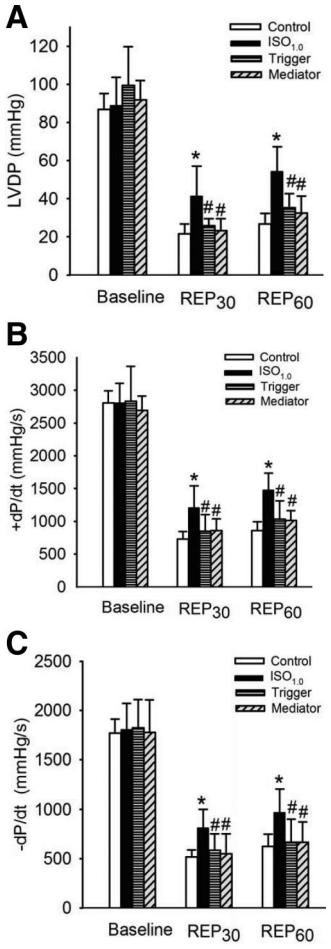

Fig. 5. Nitric oxide is both a trigger and a mediator of isoflurane postconditioning-induced protection in wild-type hearts subjected to 30 min of ischemia followed by 60 min of reperfusion. (*A*) LVDP (left ventricular developed pressure); (*B*) +dP/dt (maximum rate of increase of LVDP); (*C*) −dP/dt (maximum rate of decrease of LVDP). REP_{30} = 30 min after reperfusion; REP_{60} = 60 min after reperfusion. Buffer containing 1.0 minimum alveolar concentration of isoflurane ($ISO_{1.0}$) was administered in the $ISO_{1.0}$, trigger and mediator groups. To inhibit nitric oxide synthesis before and after myocardial exposure to isoflurane, the hearts were perfused with 30 μm N^G-nitro-L-arginine methyl ester (a nonselective endothelial nitric oxide synthase inhibitor) for 20 min either before ischemia (trigger group) or at the onset of reperfusion (mediator group). * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* $ISO_{1.0}$ (n = 7 hearts/group).

size was larger in *eNOS*$^{-/-}$ mice than in the WT mice. Nonetheless, myocardial infarct size was significantly decreased by 1.0 MAC IsoPC in WT mice but not in the *eNOS*$^{-/-}$ mice. Furthermore, cardiac function was significantly improved by 1.0 MAC IsoPC in isolated WT hearts but not in *eNOS*$^{-/-}$ hearts. Our data provides direct evidence that nitric oxide derived from eNOS (rather than other NOS isoforms) is critical for cardioprotection by isoflurane against acute I/R injury. This is the first study to demonstrate the critical dependency of IsoPC on the eNOS isoform using *eNOS*$^{-/-}$ mice.

WT hearts treated with isoflurane to produce postconditioning were perfused with 30 μM L-NAME for 20 min either before ischemia or at the onset of reperfusion to inhibit nitric oxide synthesis before or after myocardial exposure to isoflurane, respectively. IsoPC-induced improvement in the recovery of cardiac function after I/R was blocked by L-NAME in both groups. This result suggests that nitric oxide functions as both a trigger and a mediator of IsoPC. Recently, we found that isoflurane could directly stimulate vascular endothelial cells to produce nitric oxide.[31] In normal cardiac tissues, nitric oxide is predominantly derived from eNOS.[11] Therefore, it is possible that nitric oxide derived from eNOS in coronary endothelial cells acts as a trigger to initiate intracellular events leading to IsoPC. Conversely, during I/R cardiac myocytes express eNOS, and nitric oxide production is increased.[11,32–35] We speculate that eNOS-dependent nitric oxide, as a critical mediator of cardioprotection produced by IsoPC, might be derived from both cardiac myocytes and coronary endothelial cells.

The MPT pore, a large and nonspecific pore spanning both inner and outer mitochondrial membranes, remains closed during myocardial ischemia but opens due to Ca^{2+} overload and excessive production of reactive oxygen species during reperfusion.[36,37] Opening of the MPT pore results in collapse of the mitochondrial membrane potential, uncoupling of the respiratory chain, efflux of Ca^{2+} and cytochrome c, and matrix swelling. These events are thought to be pathognomonic for myocardial reperfusion injury.[37–39] A study by Feng *et al.*[7] reported that NAD^+ content in WT rat myocardium was reduced by IsoPC, suggesting that IsoPC may prevent MPT pore opening because mitochondria possess 72–92% of the total cellular contents of NAD^+.[37] In this study, we used a quantitative potentiometric approach to address the susceptibility of the MPT pore to open after Ca^{2+} loading in purified mitochondria that were directly isolated from *ex vivo* injured myocardium. Although the procedures for mitochondrial isolation might induce additional mitochondrial damage, isolated mitochondria are a unique tool to identify molecular entities that are modified by physiological, biochemical, or pharmacological processes.[40–43] Mitochondria isolated from WT hearts after IsoPC required significantly higher *in vitro* Ca^{2+} loading than did mitochondria without IsoPC to open the MPT pore. This suggests that IsoPC protects mitochondria by delaying MPT pore opening. It is very possible, therefore, that the beneficial actions of IsoPC on hearts subjected to I/R are related to mitochondrial protection. Interestingly, the protective effects of IsoPC on mitochondria were abolished in *eNOS*$^{-/-}$ mice, suggesting that mitochondria are a target of eNOS-derived NO.[14,16] Physiologic levels of nitric oxide have shown to be capable of inhibiting MPT pore opening.[15] Although the exact mechanisms by which nitric oxide inhibits MPT pore opening are unclear, eNOS-derived nitric oxide is involved in an indirect (through signaling pathways) and/or direct modulation of MPT pore opening in IsoPC.

The results of this investigation should be interpreted within the constraints of several potential limitations. The

Table 3. Hemodynamics in the Langendorff-Perfused Hearts

	Baseline	Reperfusion			
		2 min	10 min	30 min	60 min
Effects of L-NAME on coronary flow and heart rate during ischemia/reperfusion in the WT hearts					
Heart rate (beats/min)					
Control	294 ± 29	153 ± 32*	240 ± 26	254 ± 37	233 ± 34*
$ISO_{1.0}$	290 ± 26	167 ± 42*	251 ± 45	264 ± 43	250 ± 56*
Trigger	284 ± 30	187 ± 56*	203 ± 34*	222 ± 35*	247 ± 19*
Mediator	288 ± 34	155 ± 56*	190 ± 43*	220 ± 52*	248 ± 55*
Coronary flow (ml/min)					
Control	2.90 ± 0.63	1.79 ± 0.55*	2.25 ± 0.61	2.34 ± 0.66	2.31 ± 0.63
$ISO_{1.0}$	2.92 ± 0.65	1.62 ± 0.42*	2.37 ± 0.52	2.65 ± 0.77	2.48 ± 0.85
Trigger	2.78 ± 0.71	1.54 ± 0.35*	1.83 ± 0.52*	1.97 ± 0.51*	1.81 ± 0.55*
Mediator	3.11 ± 0.76	1.30 ± 0.63*	1.94 ± 0.73*	2.28 ± 0.44*	2.34 ± 0.45*
Effects of $ISO_{1.0}$ on heart rate and coronary flow during ischemia/reperfusion in the WT and $eNOS^{-/-}$ hearts					
Heart rate (beats/min)					
WT sham	295 ± 30	287 ± 29	284 ± 28	289 ± 21	—
WT control	297 ± 32	158 ± 33*†	220 ± 32*†	241 ± 28*†	—
WT $ISO_{1.0}$	290 ± 25	170 ± 40*†	221 ± 53*†	244 ± 35†	—
$eNOS^{-/-}$ sham	300 ± 29	292 ± 28	283 ± 27	278 ± 32	—
$eNOS^{-/-}$ control	290 ± 22	168 ± 45*†	243 ± 38*	267 ± 33	—
$eNOS^{-/-}$ $ISO_{1.0}$	293 ± 25	160 ± 35*†	233 ± 51*	247 ± 38	—
Coronary flow (ml/min)					
WT sham	2.93 ± 0.65	2.75 ± 0.53	2.77 ± 0.42	2.66 ± 0.46	—
WT control	2.81 ± 0.67	1.60 ± 0.57*†	2.08 ± 0.67	2.36 ± 0.64	—
WT $ISO_{1.0}$	3.06 ± 0.55	1.63 ± 0.58*†	2.44 ± 0.37	2.60 ± 0.44	—
$eNOS^{-/-}$ sham	2.85 ± 0.57	2.79 ± 0.53	2.75 ± 0.41	2.70 ± 0.43	—
$eNOS^{-/-}$ control	2.87 ± 0.46	1.75 ± 0.58*†	2.16 ± 0.69	2.37 ± 0.57	—
$eNOS^{-/-}$ $ISO_{1.0}$	2.72 ± 0.48	1.78 ± 0.47*†	2.04 ± 0.45*	2.26 ± 0.46	—

The hearts were treated with 1.0 minimum alveolar concentration of isoflurane ($ISO_{1.0}$) with or without L -NAME (trigger and mediator groups) in wild-type (WT) or endothelial nitric oxide synthase knock-out ($eNOS^{-/-}$) mice, as described in figures 5 and 7.

* $P < 0.05$ *versus* baseline.

† $P < 0.05$ *versus* WT sham (n = 7–10 hearts/group).

L-NAME = N^G-nitro-L -arginine methyl ester.

$eNOS^{-/-}$ mice are known to have a higher systemic blood pressure than WT mice.[13,44] Because hypertension may initiate changes in cardiac structure and function, we used echocardiography to evaluate the hearts of $eNOS^{-/-}$ and WT C57BL/6J mice at baseline conditions. Our results suggest that there is concentric hypertrophy of the LV in $eNOS^{-/-}$ mice in comparison with WT mice. This is consistent with a previous investigation by Yang *et al.*,[45] whereas another study by Ruetten *et al.*[46] reported that LV morphology and function were similar between $eNOS^{-/-}$ mice and WT mice. The increased LV wall thickness observed in $eNOS^{-/-}$ mice in our study may be attributable to increased afterload. LV hypertrophy may alter cardiac function and therefore change myocardial I/R injury. However, no significant differences in multiple indices of LV function including systolic contractility (fractional shortening) and diastolic relaxation (ratio of mitral E/A and MVDT) were identified in $eNOS^{-/-}$ mice compared with WT mice (table 3). Therefore, it is unlikely that loss of the cardioprotective effect of isoflurane in $eNOS^{-/-}$ mice can be attributed to changes in hemodynamics or cardiac function.

Five protocols of experiments were performed to examine the cardioprotective effect produced by IsoPC and the role of eNOS in IsoPC in this study. A large number of comparisons were conducted, and the error rate within each family of comparisons was meticulously controlled. One-way analysis of variance followed by Bonferroni *post hoc* test was used to analyze infarct size, LVDP, +dP/dt, and −dP/dt. A global

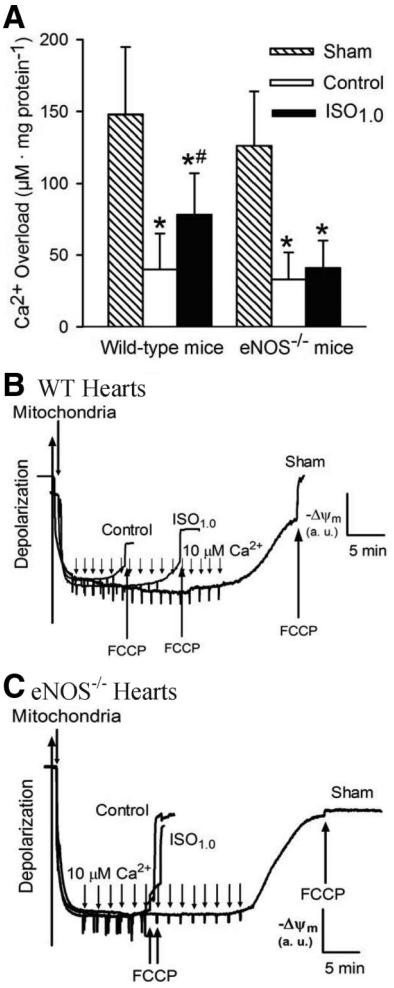

Fig. 6. Inhibition of the mitochondrial permeability transition pore opening by isoflurane postconditioning in wild-type (WT) hearts but not in *endothelial nitric oxide synthase* knockout (*eNOS*$^{-/-}$) hearts subjected to 30 min of ischemia followed by 30 min of reperfusion. (*A*) The amount of *in vitro* Ca^{2+} overload necessary to open the mitochondrial permeability transition pore in WT hearts and in *eNOS*$^{-/-}$ hearts; (*B*) representative tracings showing the changes in membrane potential ($\Delta\psi_m$) of mitochondria isolated from WT hearts after *in vitro* Ca^{2+} loading; (*C*) tracings showing the changes in $\Delta\psi_m$ of mitochondria isolated from the *eNOS*$^{-/-}$ hearts after *in vitro* Ca^{2+} loading. Opening of the mitochondrial permeability transition pore was assessed after *in vitro* Ca^{2+} overload by following changes in $\Delta\psi_m$ using the fluorescent dye rhodamine 123. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added at the arrows to depolarize mitochondria. * $P < 0.05$ *versus* sham; # $P < 0.05$ *versus* control (n = 10/group).

adjustment of error rate was not performed, because these parameters were correlated with one another, rather than being independent.

In summary, this investigation demonstrates that brief administration of isoflurane during the late phase of ischemia and early phase of reperfusion reduces myocardial damage and improves cardiac function by preventing MPT pore opening. This cardioprotective effect is dependent on eNOS. Nitric oxide produced by eNOS serves not only as a trigger but also as a mediator of cardioprotection against I/R injury produced by IsoPC.

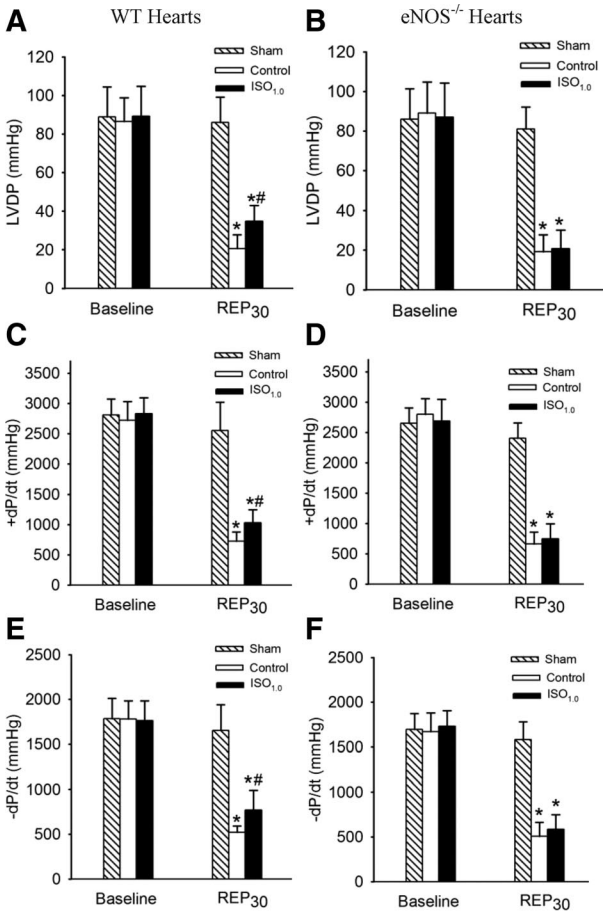

Fig. 7. Isoflurane postconditioning improved cardiac function in wild-type (WT) hearts but not in *endothelial nitric oxide synthase* knockout (*eNOS*$^{-/-}$) hearts subjected to 30 min of global ischemia followed by 30 min of reperfusion. (*A*) L eft ventricular developed pressure (LVDP) in WT hearts; (*B*) LVDP in *eNOS*$^{-/-}$ hearts; (*C*) +dP/dt (maximum rate of increase of LVDP) in WT hearts; (*D*) +dP/dt in *eNOS*$^{-/-}$ hearts; (*E*) −dP/dt (maximum rate of decrease of LVDP) in WT hearts; (*F*) −dP/dt in *eNOS*$^{-/-}$ hearts. $ISO_{1.0}$ = 1.0 minimum alveolar concentration of isoflurane; REP_{30} = 30 min after reperfusion. * $P < 0.05$ *versus* sham; # $P < 0.05$ *versus* control (n = 10/group).

The authors thank John G. Krolikowski, B.A., Amie R. Billstrom, B.A. (Research Technologists), David Schwabe, B.S. (Engineer), John Tessmer, B.A. (Manager), and Chiaki Kwok, B.A. (Research Associate), all from the Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin, for excellent technical assistance.

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