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Role of Endothelial Nitric Oxide Synthase as a Trigger and Mediator of Isoflurane-induced Delayed Preconditioning in Rabbit Myocardium

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Background: **Isoflurane produces delayed preconditioning *in vivo*. The authors tested the hypothesis that endothelial, inducible, or neuronal nitric oxide synthase (NOS) is a trigger or mediator of this protective effect.**

Methods: **In the absence or presence of exposure to isoflurane (1.0 minimum alveolar concentration) 24 h before experimentation, pentobarbital-anesthetized rabbits (n = 128) instrumented for hemodynamic measurement received 0.9% saline (control), the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (10 mg/kg), one of two of the selective inducible NOS antagonists aminoguanidine (300 mg/kg) or 1400W (0.5 mg/kg), or the selective neuronal NOS inhibitor 7-nitroindazole (50 mg/kg) administered before exposure to isoflurane (trigger; day 1) or left anterior descending coronary artery occlusion (mediator; day 2). All rabbits underwent 30 min of coronary occlusion followed by 3 h of reperfusion. Tissue samples for reverse-transcription polymerase chain reaction and immunohistochemistry were also obtained in the presence or absence of *N*-nitro-L-arginine methyl ester with or without isoflurane pretreatment.**

Results: **Isoflurane significantly ($P < 0.05$) reduced infarct size (23 ± 5% [mean ± SD] of the left ventricular area at risk; triphenyltetrazolium chloride staining) as compared with control (42 ± 7%). *N*-nitro-L-arginine methyl ester administered before isoflurane or coronary occlusion abolished protection (49 ± 7 and 43 ± 10%, respectively). Aminoguanidine, 1400W, and 7-nitroindazole did not alter infarct size or affect isoflurane-induced delayed preconditioning. Isoflurane increased endothelial but not inducible NOS messenger RNA transcription and protein translation immediately and 24 h after administration of the volatile agent. Pretreatment with *N*-nitro-L-arginine methyl ester attenuated isoflurane-induced increases in endothelial NOS expression.**

Conclusions: **The results suggest that endothelial NOS but not inducible or neuronal NOS is a trigger and mediator of delayed preconditioning by isoflurane *in vivo*.**

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ISCHEMIC preconditioning (IPC) describes the process by which a brief period of ischemia imposed before a more prolonged coronary artery occlusion protects myocardium against infarction.[1] IPC is characterized by an early phase (limited to 1–3 h after the brief ischemic stimulus[2]) and a late, "delayed," or "second window" phase of preconditioning that occurs after 24 h and persists for as long as 72 h after the ischemic episode. Nitric oxide (NO) donors,[3] selective δ-opioid agonists,[4] adenosine receptor agonists,[5] and mitochondrial adenosine triphosphate–sensitive potassium (K_{ATP}) channel openers[6,7] mimic the protective effects of delayed IPC. Several protein kinases,[8–13] cyclooxygenase 2,[14,15] and nitric oxide synthase (NOS)[16,17] have been implicated in the signaling responsible for delayed IPC. Exposure to isoflurane 24 h before prolonged coronary occlusion and reperfusion reduced myocardial infarct size in rabbits.[18] This isoflurane-induced delayed preconditioning was mediated by cyclooxygenase 2. Inducible nitric oxide synthase (iNOS) regulates cyclooxygense-2 activity during delayed IPC,[19] and NO functions as both a trigger and a mediator of delayed IPC as well.[16] Recent evidence further suggests that neuronal nitric oxide synthase (nNOS) may also mediate the late phase of delayed ischemic preconditioning.[20] Acute isoflurane-induced preconditioning has been shown to be sensitive to modulation of NO signaling pathways.[21] Therefore, we tested the hypotheses that delayed preconditioning by isoflurane is NO dependent and investigated whether iNOS, nNOS, or endothelial nitric oxide synthase (eNOS) mediates delayed preconditioning by isoflurane.

Materials and 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 (Milwaukee, Wisconsin). Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals.* [22] of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.[23]

Administration of Isoflurane

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were placed in an induction chamber 24 h before acute experimentation as previously described.[24] After inhalational induction, anesthesia was maintained

with 1.0 minimum alveolar concentration (MAC) isoflurane (2.1%) in an air–oxygen mixture during spontaneous ventilation. The concentration of isoflurane inside the induction chamber was continuously measured with an infrared anesthetic analyzer that was calibrated with known standards before and during experimentation. Arterial blood gas tensions were obtained by sampling from an ear artery 1 h after anesthetic induction. Anesthesia was discontinued after 2 h, and emergence was allowed to occur. Each rabbit was then housed overnight before experimentation on the following day.

Surgical Instrumentation

Rabbits with or without previous exposure to isoflurane were anesthetized with intravenous sodium pentobarbital (30 mg/kg).[18] Briefly, a tracheostomy was performed through a ventral midline incision, and the trachea was cannulated. The rabbits were ventilated with positive-pressure using an air–oxygen mixture. Heparin-filled catheters were inserted into the right carotid artery and the left jugular vein for measurement of heart rate and mean arterial blood pressure, and fluid or drug administration, respectively. A left thoracotomy was performed at the fourth intercostal space. A prominent branch of the left anterior descending coronary artery (LAD) was identified, and a silk ligature was placed around this vessel approximately halfway between the base and 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 in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamics were continuously recorded on a polygraph throughout each experiment.

Experimental Protocols

The experimental design is illustrated in (fig. 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 3 h of reperfusion. In 18 separate experimental groups (n = 6–8/group), rabbits with or without previous exposure to isoflurane were randomly assigned to receive 0.9% saline (control), the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg; Sigma Aldrich, St. Louis, MO), the selective iNOS antagonists aminoguanidine hydrochloride (AG, 300 mg/kg; Sigma Aldrich) or 1400W dihydrochloride (0.5 mg/kg; Cayman, Ann Arbor, MI), or the selective nNOS inhibitor 7-nitroindazole[25] (7-NI, 50 mg/kg; Sigma) administered on either day 1 to examine whether NOS acts as a trigger (T) or day 2 to test the hypothesis that NOS acts as a mediator (M) of isoflurane-induced preconditioning. L-NAME was dissolved in 0.9% saline and administered as an intravenous infusion over 10 min beginning

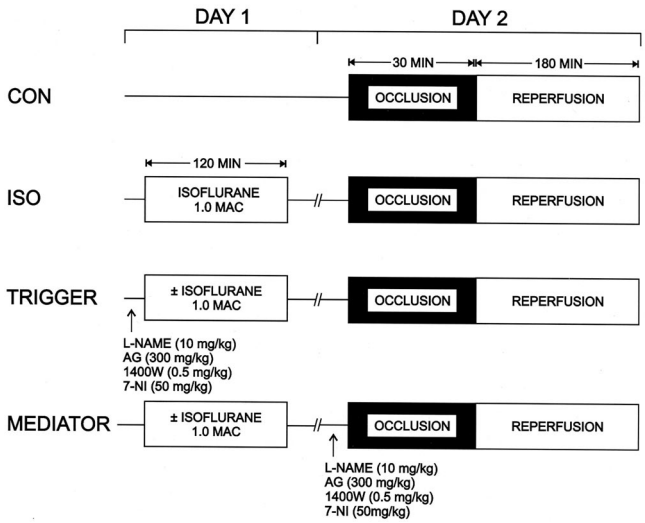


Fig. 1. Schematic illustration depicting the experimental protocol. AG = aminoguanidine; CON = control; ISO = isoflurane; L-NAME = *N*-nitro-L-arginine methyl ester; MAC = minimum alveolar concentration; 7-NI = 7-nitroindazole.

20 min before isoflurane exposure on day 1 or LAD occlusion on day 2. AG was dissolved in 0.9% saline, the pH of the solution was adjusted to 7.4 with 0.1 N NaOH, and the mixture then injected subcutaneously 1 h before isoflurane exposure on day 1 or coronary occlusion on day 2. 1400W was dissolved in dimethyl sulfoxide and administered intravenously 10 min before isoflurane exposure on day 1 or coronary occlusion on day 2. 7-NI was dissolved in dimethyl sulfoxide and administered into the peritoneum 1 h before isoflurane exposure on day 1 or coronary occlusion on day 2. In four additional groups of experiments (n = 5/group), rabbits with or without isoflurane pretreatment received dimethyl sulfoxide alone 10 min before isoflurane exposure on day 1 or coronary occlusion on day 2.

Determination of Myocardial Infarct Size

Myocardial infarct size was measured as previously described.[26] Briefly, the LAD was reoccluded, and 3 ml patent blue dye was injected intravenously at the completion of each experiment. The left ventricular (LV) 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 M phosphate buffer adjusted to pH 7.4. After overnight storage in 10% formaldehyde, infarcted and noninfarcted myocardium within the AAR were carefully separated and weighed. Myocardial infarct size was expressed as a percentage of the AAR. Rabbits that had intractable ventricular fibrillation and those with an AAR less than 15% of total LV mass were excluded from subsequent analysis.

Immunohistochemistry

Left ventricular tissue samples were obtained from separate groups of rabbits (n = 5/group) in the presence

or absence of L-NAME with or without previous administration of isoflurane. Hearts were rapidly excised before, immediately after, or 24 h after isoflurane exposure, and the left ventricle was isolated and frozen in liquid nitrogen (−70°C) for subsequent analysis. LV tissue samples were also obtained from rabbits (n = 5) 6 h after pretreatment with 6 brief (4-min) episodes of LAD occlusion interspersed with 4 min of reperfusion as a positive control for iNOS expression.[27] 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 1:50 dilutions of mouse monoclonal primary antibodies for eNOS (Biomol, Plymouth Meeting, PA) or iNOS (BD, San Diego, CA) in phosphate-buffered saline at 37°C for 30 min. Sections were washed three times for 5 min with phosphate-buffered saline. Sections were subsequently incubated with 1:200 dilution of biotinylated anti-mouse secondary antibody (Amersham Pharmacia, Piscataway, NJ) 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. Nuclear staining was achieved with 1 μM TO-PRO-3 (Molecular Probes, Eugene, OR) at room temperature for 2 min. Images were obtained using 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 488 and 633 nm, and the emitted fluorescence was determined after long pass filtering at corresponding wavelengths of 520 and 661 nm for fluorescein isothiocyanate and TO-PRO, respectively. Approximately 50 images were obtained from each rabbit heart. eNOS- and iNOS-positive structures were counted using the MetaMorph Imaging program (Universal Imaging, Downingtown, PA).

RT-PCR Analysis

Left ventricular tissue samples were obtained from separate groups of rabbits (n = 3/group) as described above for immunohistochemistry experiments. Hearts were rapidly excised before, immediately after, or 24 h after isoflurane exposure. LV tissue was also obtained from rabbit hearts (n = 3) exposed to repetitive brief ischemic stimuli as described above. LV tissue was homogenized and RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA). Genomic DNA contamination was prevented by treatment DNAase I (Quiagen). The concentration of total RNA was determined spectrophotometrically at a wavelength of 260 nm. Two micrograms total RNA was reverse-transcribed to complementary DNA (cDNA) using a RETROscript reverse-transcription polymerase chain reaction (RT-PCR) kit (Ambion, Austin, TX). Genomic DNA contamination was excluded by testing samples carried through the RT-PCR procedure in the absence of reverse transcriptase. A 1-μl aliquot of cDNA was used as a template for the polymerase chain reaction (PCR) amplification reaction containing two sets of primer sequences for the eNOS (forward: 5′-CAGTGTCCAACATGCTGCTGGAAATTG-3′; reverse: 5′-TAAAGGTCTTCTTCCTGGTGATGCC-3′) or iNOS gene (forward: 5′-GAGTGTCAGTGGCTTCCAGC-3′; reverse: 5′-CTGCAGGATGTCTTGAACG-3′) and 18S ribosomal RNA (rRNA) (QuantumRNA Universal [for eNOS] or Classic II [for iNOS] 18S internal standards). The PCR reaction was performed in a 50-μl reaction volume with 0.4 μM forward and reverse primers, 200 μM nucleotides, 2 μl mixed 18S primers with competimers (2:8 for eNOS, 1:9 for iNOS), and 0.5 U *Taq* Polymerase (Roche, Indianapolis, IN). The thermocycler profile was used in the linear range of amplification (28 cycles for eNOS, 36 cycles for iNOS) at 94°C for 15 s, 65°C for 30 s, and 72°C for 45 s. A single PCR master mix was used for each set of samples to minimize errors. The linear range of the PCR and the optimal ratio between 18S primers and their competimers were established in preliminary experiments to attenuate the amplification rate of abundant 18S rRNA. Amplified products were visualized and quantified by agarose gel electrophoresis using SYBR-Gold (Molecular Probes). Gels were photographed using a digital camera. Quantitative analysis of the band densities was performed using AlphaImager 2000 software (Alpha Innotech Corporation, San Leandro, CA). Band intensities of the eNOS or iNOS PCR products were normalized against those of the 18S rRNA product coamplified in the same reaction. Values were expressed relative to the intensity of bands obtained from rabbits that were not exposed to isoflurane. Data are presented as percent of control.

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. Changes were considered statistically significant when P was less than 0.05 (two tailed). All data are expressed as mean ± SD.

Results

One hundred thirty-four rabbits were instrumented to obtain 128 successful infarct size experiments. Two rabbits were excluded from the AG(T) group because the LV AAR was less than 15% of the LV mass. Four rabbits were excluded because intractable ventricular fibrillation occurred during coronary occlusion (one isoflurane plus L-NAME(T); two L-NAME(M) alone; two 1400W(T)). Arterial blood gas tensions were maintained within the physiologic range during pretreatment with isoflurane in all groups (data not shown).

Systemic Hemodynamics

There were no differences in baseline hemodynamics between groups (table 1). Infusion of L-NAME before LAD occlusion (L-NAME(M)) significantly ($P < 0.05$) decreased heart rate and rate–pressure product in the absence or presence of isoflurane pretreatment. Decreases in heart rate and rate–pressure product occurred during reperfusion in all experimental groups. There were no significant differences in hemodynamics among groups during and after coronary occlusion.

Myocardial Infarct Size

Body weight, LV mass, AAR weight, and the ratio of AAR to LV mass were similar between groups (table 2). Administration of isoflurane reduced infarct size (23 ± 5% of the LV AAR) as compared with control (42 ± 7% (fig. 2). Infusion of L-NAME alone on day 1 (L-NAME(T)) or day 2 (L-NAME(M)) did not alter infarct size (45 ± 5 and 43 ± 10%, respectively) but abolished isoflurane-induced protection (49 ± 7 and 43 ± 10%, respectively). Administration of AG on day 1 (AG(T)) or day 2 (AG(M)) had no effect on infarct size (41 ± 7 and 41 ± 7%, respectively; fig. 3). In contrast to the findings with L-NAME, administration of AG on day 1 or 2 did not alter the protection produced by isoflurane (28 ± 8 and 22 ± 7%, respectively). Similarly, administration of 1400W alone on day 1 (1400W(T)) or day 2 (1400W(M)) did not affect infarct size (42 ± 5 and 40 ± 5%, respectively; fig. 2), and did not modify the protection caused by isoflurane (20 ± 5 and 20 ± 5%, respectively). Administration of 7-NI alone on day 1 (7-NI(T)) or day 2 (7-NI(M)) also did not alter infarct size or affect isoflurane-induced cardioprotection (fig. 2). Dimethyl sulfoxide did not alter infarct size when administered on day 1 or day 2 (46 ± 5 and 44 ± 5%, respectively) or affect isoflurane-induced protection (24 ± 3 and 25 ± 7%, respectively).

Immunohistochemistry

Isoflurane significantly ($P < 0.05$) increased eNOS protein in LV tissue samples from rabbits obtained immediately or 24 h after 2 h administration of the volatile agent (trigger and mediator, respectively) as compared with tissue from rabbits that did not receive isoflurane (fig. 3). Six cycles of brief LAD occlusion and reperfusion enhanced iNOS protein in LV myocardium 6 h after the brief ischemic episodes (fig. 4). In contrast, 2 h administration of isoflurane did not affect iNOS translation in LV tissue samples immediately (data not shown) or 24 h after exposure to the agent (fig. 4).

RT-PCR Analysis

The amplification products obtained using primers for eNOS and iNOS cDNA matched the expected sizes of 485 and 298 base pairs (bp), respectively. Amplification did not occur in the absence of reverse transcriptase. 18S rRNA (315 and 489 bp for amplification with eNOS and iNOS primers, respectively) served as the internal standard for calculation of the relative abundance of target gene DNA in each PCR reaction. Twofold and 2.5-fold increases in eNOS gene expression were observed in LV tissue samples from rabbits obtained immediately and 24 h after 2 h of exposure to isoflurane, respectively, as compared with LV myocardium from rabbits that did not receive isoflurane (fig. 5). Ischemic preconditioning enhanced transcription of iNOS messenger RNA (mRNA) by 2.5-fold, but iNOS gene expression was unaffected by 2 h of exposure to isoflurane (fig. 6).

Discussion

The current results confirm and extend previous findings and indicate that isoflurane produces delayed protection against myocardial infarction in rabbits.[18] The late phase of ischemic preconditioning has been extensively studied by Bolli,[28] and a central role for NO metabolism has been identified. Administration of L-NAME before the ischemic stimulus blocked delayed protection, indicating that NO triggers delayed ischemic preconditioning.[29] The mechanism by which NO produces this effect is unclear, but generation of reactive intermediates, including $ONOO^-$ and $\dot{O}H$, has been implicated in this process.[3,30] The current results with L-NAME indicate that NO is also a trigger of isoflurane-induced delayed preconditioning. Administration of L-NAME before exposure to isoflurane abolished decreases in infarct size produced by the volatile agent. In addition, administration of either the selective iNOS antagonists AG or 1400W or the selective nNOS inhibitor 7-NI before isoflurane did not inhibit late preconditioning by the volatile agent. These data provide strong pharmacologic evidence that eNOS and not iNOS or nNOS is the enzyme responsible for the formation of NO that triggers this protection. These findings are supported by the RT-PCR and immunohistochemistry results demonstrating that the increases in eNOS transcription and translation occurred in rabbits receiving isoflurane 24 h before prolonged coronary occlusion. In contrast, isoflurane pretreatment 24 h before coronary occlusion did not alter iNOS gene or protein expression, whereas repetitive brief ischemic stimuli produced a marked augmentation of iNOS transcription and translation. The current results with isoflurane are also consistent with previous findings demonstrating that eNOS triggers delayed preconditioning against myocardial stunning because administration of iNOS inhibitors before the preconditioning stimulus did not attenuate this beneficial effect.[16]

In contrast to the role of eNOS as a trigger, iNOS has been shown to function as a mediator of delayed ischemic preconditioning.[28] Administration of L-NAME or AG immediately before prolonged coronary occlusion

Table 1. Systemic Hemodynamics

	Baseline	Before Occlusion	LAD Occlusion	Reperfusion 1 h	Reperfusion 2 h	Reperfusion 3 h
HR, min−1						
Saline	266 ± 31	264 ± 30	252 ± 28	238 ± 18*	233 ± 22*	225 ± 29*
ISO	260 ± 31	249 ± 29	243 ± 33	232 ± 20*	214 ± 21*	203 ± 21*
L-NAME(T)	259 ± 25	254 ± 32	246 ± 30	224 ± 28*	223 ± 23*	209 ± 32*
ISO + L-NAME(T)	258 ± 34	247 ± 35	231 ± 27*	216 ± 22*	214 ± 25*	209 ± 25*
L-NAME(M)	261 ± 15	211 ± 23*	221 ± 19*	184 ± 33*	186 ± 21*	188 ± 23*
ISO + L-NAME(M)	278 ± 23	233 ± 19*	234 ± 34*	213 ± 20*	204 ± 21*	198 ± 23*
AG(T)	266 ± 16	264 ± 17	259 ± 17	244 ± 20*	239 ± 26*	236 ± 30*
ISO + AG(T)	271 ± 19	266 ± 20	264 ± 19	234 ± 22*	223 ± 31*	222 ± 26*
AG(M)	270 ± 15	264 ± 17	253 ± 16	229 ± 20*	228 ± 22*	221 ± 10*
ISO + AG(M)	256 ± 19	257 ± 20	250 ± 18	231 ± 19*	219 ± 16*	219 ± 19*
1400W(T)	271 ± 11	268 ± 6	236 ± 36*	234 ± 26*	226 ± 19*	218 ± 24*
ISO + 1400W(T)	274 ± 16	273 ± 20	239 ± 44*	240 ± 21*	232 ± 20*	217 ± 22*
1400W(M)	261 ± 29	246 ± 27	248 ± 26	234 ± 19*	224 ± 26*	218 ± 22*
ISO + 1400W(M)	274 ± 19	267 ± 20	256 ± 27	243 ± 15*	224 ± 15*	214 ± 24*
7-NI(T)	249 ± 26	243 ± 23	213 ± 40*	217 ± 32*	188 ± 21*	190 ± 29*
ISO + 7-NI(T)	240 ± 29	227 ± 29	212 ± 19	202 ± 24*	190 ± 25*	187 ± 26*
7-NI(M)	263 ± 33	247 ± 29	242 ± 21	227 ± 19*	218 ± 15*	208 ± 18*
ISO + 7-NI(M)	225 ± 20	215 ± 14	212 ± 17	198 ± 21	192 ± 19	185 ± 27*
MAP, mmHg						
Saline	82 ± 8	78 ± 10	67 ± 7*	69 ± 11*	71 ± 10*	68 ± 10*
ISO	86 ± 12	85 ± 8	68 ± 12*	71 ± 11*	65 ± 13*	68 ± 10*
L-NAME(T)	84 ± 9	84 ± 11	74 ± 11	74 ± 19	75 ± 12	75 ± 9
ISO + L-NAME(T)	91 ± 7	90 ± 8	63 ± 16*	73 ± 16*	71 ± 13*	78 ± 12*
L-NAME(M)	82 ± 8	90 ± 10	72 ± 12	65 ± 15*	67 ± 11*	72 ± 11
ISO + L-NAME(M)	83 ± 13	92 ± 10	74 ± 15	76 ± 13	71 ± 17	71 ± 17
AG(T)	78 ± 6	76 ± 5	70 ± 11	71 ± 7	69 ± 12	72 ± 10
ISO + AG(T)	80 ± 5	77 ± 8	70 ± 12	72 ± 11	71 ± 13	72 ± 14
AG(M)	89 ± 11	86 ± 15	81 ± 11	80 ± 11	80 ± 9	76 ± 11*
ISO + AG(M)	93 ± 11	92 ± 10	84 ± 9	77 ± 11*	74 ± 12*	73 ± 6*
1400W(T)	82 ± 10	84 ± 6	68 ± 16*	69 ± 6*	65 ± 6*	63 ± 8*
ISO + 1400W(T)	86 ± 5	89 ± 7	65 ± 20*	72 ± 11*	70 ± 7*	70 ± 12*
1400W(M)	80 ± 11	76 ± 9	69 ± 7*	68 ± 10*	65 ± 7*	64 ± 8*
ISO + 1400W(M)	87 ± 7	89 ± 12	73 ± 10*	75 ± 7*	68 ± 7*	68 ± 11*
7-NI(T)	70 ± 5	72 ± 7	45 ± 17*	40 ± 16*	50 ± 9*	48 ± 6*
ISO + 7-NI(T)	72 ± 3	70 ± 11	55 ± 19*	56 ± 21*	56 ± 12*	59 ± 10*
7-NI(M)	76 ± 7	70 ± 8	56 ± 3*	56 ± 12*	53 ± 10*	51 ± 2*
ISO + 7-NI(M)	66 ± 10	67 ± 10	62 ± 13	51 ± 14*	51 ± 13*	53 ± 11*
RPP, min−1 · mmHg · 103						
Saline	24.9 ± 4.0	23.9 ± 3.6	19.9 ± 3.2*	19.3 ± 3.1*	19.3 ± 2.5*	17.5 ± 3.5*
ISO	25.5 ± 6.1	23.7 ± 4.6	19.3 ± 5.8*	19.2 ± 3.9*	16.7 ± 2.8*	16.4 ± 2.7*
L-NAME(T)	23.8 ± 3.2	23.6 ± 4.3	20.5 ± 5.0	19.1 ± 6.9*	18.9 ± 4.7*	17.5 ± 4.2*
ISO + L-NAME(T)	26.1 ± 3.8	24.7 ± 3.6	17.5 ± 5.3*	18.5 ± 5.5*	17.6 ± 4.4*	18.2 ± 4.3*
L-NAME(M)	24.0 ± 2.4	20.7 ± 2.6*	17.2 ± 2.8*	12.8 ± 1.8*	13.5 ± 1.1*	14.6 ± 1.9*
ISO + L-NAME(M)	26.3 ± 5.6	23.6 ± 2.9*	19.4 ± 4.8*	17.6 ± 3.5*	16.2 ± 3.8*	15.8 ± 4.2*
AG(T)	23.4 ± 0.6	23.1 ± 1.9	21.3 ± 2.1	20.2 ± 2.7	19.9 ± 3.7	19.6 ± 4.1*
ISO + AG(T)	24.3 ± 2.1	22.8 ± 2.1	21.3 ± 3.7	20.1 ± 3.5*	18.5 ± 4.5*	18.6 ± 4.5*
AG(M)	26.7 ± 3.6	25.6 ± 5.2	22.6 ± 2.9*	20.8 ± 2.6*	20.6 ± 2.9*	18.9 ± 2.6*
ISO + AG(M)	26.3 ± 3.8	26.1 ± 3.9	23.4 ± 2.6*	20.0 ± 2.7*	18.7 ± 3.2*	18.4 ± 2.1*
1400W(T)	24.7 ± 2.7	25.2 ± 1.7	18.7 ± 5.4*	19.1 ± 3.3*	17.5 ± 1.9*	16.3 ± 2.6*
ISO + 1400W(T)	26.5 ± 1.8	27.6 ± 2.5	18.8 ± 6.4*	20.3 ± 4.1*	19.3 ± 2.6*	17.7 ± 4.2*
1400W(M)	24.0 ± 5.0	21.4 ± 4.4	19.5 ± 3.4*	18.3 ± 3.1*	17.1 ± 3.1*	16.2 ± 2.6*
ISO + 1400W(M)	26.3 ± 3.3	26.4 ± 4.3	20.8 ± 3.9*	20.5 ± 2.5*	17.6 ± 2.5*	17.4 ± 4.3*
7-NI(T)	19.9 ± 2.4	20.1 ± 2.3	11.9 ± 4.5*	10.6 ± 3.5*	11.5 ± 2.3*	11.5 ± 2.5*
ISO + 7-NI(T)	19.8 ± 4.6	18.3 ± 4.5	13.6 ± 3.3*	13.4 ± 3.7*	13.1 ± 3.8*	13.0 ± 3.1*
7-NI(M)	22.9 ± 3.7	20.2 ± 3.1	16.1 ± 2.1*	15.5 ± 3.1*	14.2 ± 2.0*	12.7 ± 0.9*
ISO + 7-NI(M)	17.4 ± 3.4	16.9 ± 3.3	15.3 ± 3.7	12.1 ± 3.4*	11.8 ± 3.4*	11.7 ± 3.6*

Data are presented as mean ± SD. Saline (n = 8), ISO (n = 8), L-NAME(T) (n = 8), ISO + L-NAME(T) (n = 7), L-NAME(M) (n=7), ISO + L-NAME(M) (n=8), AG(T) (n=7), ISO + AG(T) (n=7), AG(M) (n = 7), ISO + AG(M) (n = 8), 1400W(T) (n = 7), ISO + 1400W(T) (n = 7), 1400W(M) (n = 7), ISO + 1400W(M) (n = 7), 7-NI(T) (n = 7), ISO + 7-NI(T) (n = 6), 7-NI(M) (n = 6), ISO + 7-NI(M) (n = 6).

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

AG = aminoguanidine; HR = heart rate; ISO = isoflurane; LAD = left anterior descending coronary artery; L-NAME = *N*-nitro-L-arginine methyl ester; M = mediator; MAP = mean arterial pressure; RPP = rate–pressure product; 7-NI = 7-nitroindazole; T = trigger.

Table 2. Left Ventricular Area at Risk

	Body Weight, g	LV, g	AAR, g	AAR/LV, %
Saline	2,657 ± 278	3.18 ± 0.26	1.08 ± 0.25	34 ± 7
ISO	2,706 ± 362	3.17 ± 0.45	1.07 ± 0.23	34 ± 5
L-NAME(T)	2,650 ± 305	2.95 ± 0.37	1.06 ± 0.26	36 ± 5
ISO + L-NAME(T)	2,618 ± 217	2.88 ± 0.27	1.08 ± 0.21	38 ± 8
L-NAME(M)	2,676 ± 197	2.52 ± 0.38	0.88 ± 0.28	34 ± 8
ISO + L-NAME(M)	2,682 ± 274	2.87 ± 0.29	0.96 ± 0.42	33 ± 13
AG(T)	2,715 ± 231	3.23 ± 0.43	1.09 ± 0.23	34 ± 5
ISO + AG(T)	2,670 ± 513	3.31 ± 0.41	1.17 ± 0.22	36 ± 7
AG(M)	2,619 ± 422	3.45 ± 0.49	1.18 ± 0.53	34 ± 13
ISO + AG(M)	2,628 ± 332	3.28 ± 0.33	1.10 ± 0.27	34 ± 9
1400W(T)	2,660 ± 106	2.92 ± 0.50	1.03 ± 0.30	35 ± 8
ISO + 1400W(T)	2,652 ± 122	3.24 ± 0.55	1.13 ± 0.13	35 ± 5
1400W(M)	2,643 ± 129	2.94 ± 0.36	0.98 ± 0.31	33 ± 9
ISO + 1400W(M)	2,702 ± 105	3.06 ± 0.08	1.03 ± 0.17	34 ± 5
7-NI(T)	2,743 ± 98	3.41 ± 0.55	1.18 ± 0.19	35 ± 3
ISO + 7NI(T)	2,683 ± 183	3.62 ± 0.36	1.25 ± 0.11	35 ± 2
7-NI(M)	2,700 ± 179	3.85 ± 0.65	1.48 ± 0.22	39 ± 4
ISO + 7-NI(M)	2,800 ± 179	3.88 ± 0.43	1.14 ± 0.31	29 ± 6

Data are presented as mean ± SD. Saline (n = 8), ISO (n = 8), L-NAME(T) (n = 8), ISO + L-NAME(T) (n = 7), L-NAME(M) (n = 7), ISO + L-NAME(M) (n = 8), AG(T) (n = 7), ISO + AG(T) (n = 7), AG(M) (n = 7), ISO + AG(M) (n = 8), 1400W(T) (n = 7), ISO + 1400W(T) (n = 7), 1400W(M) (n = 7), ISO + 1400W(M) (n = 7), 7-NI(T) (n = 7), ISO + 7-NI(T) (n = 6), 7-NI(M) (n = 6), ISO + 7-NI(M) (n = 6).

AG = aminoguanidine; AAR = area at risk; ISO = isoflurane; L-NAME = *N*-nitro-L-arginine methyl ester; LV = left ventricle; M = mediator; 7-NI = 7-nitroindazole; T = trigger.

blocked the protective effect of a remote series of brief ischemic stimuli.[17] Delayed ischemic preconditioning also did not occur in iNOS gene-knockout mice.[31] Despite these data suggesting clearly defined roles for NO derived from eNOS and iNOS as the trigger and mediator, respectively, of delayed ischemic preconditioning, controversy continues to exist regarding whether delayed pharmacologic preconditioning occurs through a similar mechanism. Adenosine-induced delayed cardioprotection was abolished in iNOS gene-knockout mice.[32] In direct contrast, another study conducted using an identical experimental model did not confirm this finding and instead proposed the hypothesis that eNOS masquerades as iNOS during delayed preconditioning produced by adenosine.[33] Selective iNOS inhibition immediately before prolonged coronary occlusion also did not alter the protection produced by remote administration of adenosine in rabbits.[34] The current results with isoflurane support these latter observations because selective iNOS inhibitors did not attenuate protection produced by remote administration of isoflurane. In addition, iNOS gene and protein expression was unaffected by exposure to isoflurane.

Other data have implicated eNOS as the critical source of NO during delayed preconditioning by ischemia and pharmacologic agonists. Anoxia-reoxygenation–induced delayed preconditioning occurred in iNOS- but not eNOS-deficient myocytes, and eNOS but not iNOS mRNA transcription increased in wild-type myocytes.[35] Overexpression of eNOS also attenuated myocardial ischemia–reperfusion injury in transgenic mice.[36,37] Increases in iNOS mRNA transcription were observed in rat myocardium after infarction but not hypoxia–reoxygenation.[38] A late up-regulation of coronary vascular NO production resulting from enhanced eNOS activity also occurred after a brief coronary occlusion in dogs.[39] More recently, eNOS was shown to mediate diazoxide-induced late preconditioning.[40] Phosphatidylinositol-3-kinase–mediated phosphorylation of the protein kinase Akt, an upstream enzyme that activates eNOS in this prosurvival signaling cascade, contributed to this protective effect.[40] The current results with L-NAME and selective iNOS and nNOS inhibitors support these findings and indicate that eNOS also plays a central role in delayed cardioprotection by remote exposure to isoflurane. We recently demonstrated that phosphatidylinositol-3-kinase signaling also mediates the protective effects of isoflurane during early reperfusion.[41] These latter data suggest the intriguing hypothesis that isoflurane may activate eNOS and produce NO through this phosphatidylinositol-3-kinase pathway during delayed preconditioning as well. Additional investigation will be required to confirm this contention.

The current results must be interpreted within the constraints of several potential limitations. AG and 1400W have been shown to be selective iNOS antagonists at the doses used in the current investigation.[19,34,42] The *in vitro* results using AG and 1400W were also consistent with the findings of the *in vivo* experiments. Nevertheless, dose–response relations to AG and 1400W were not performed, and the possibility that AG or 1400W may have affected other protein kinases involved in myocardial protection cannot be completely excluded from the analysis. Myocardial infarct size is determined primarily by the size of the AAR and extent of coronary collateral perfusion. The AAR expressed as a percentage

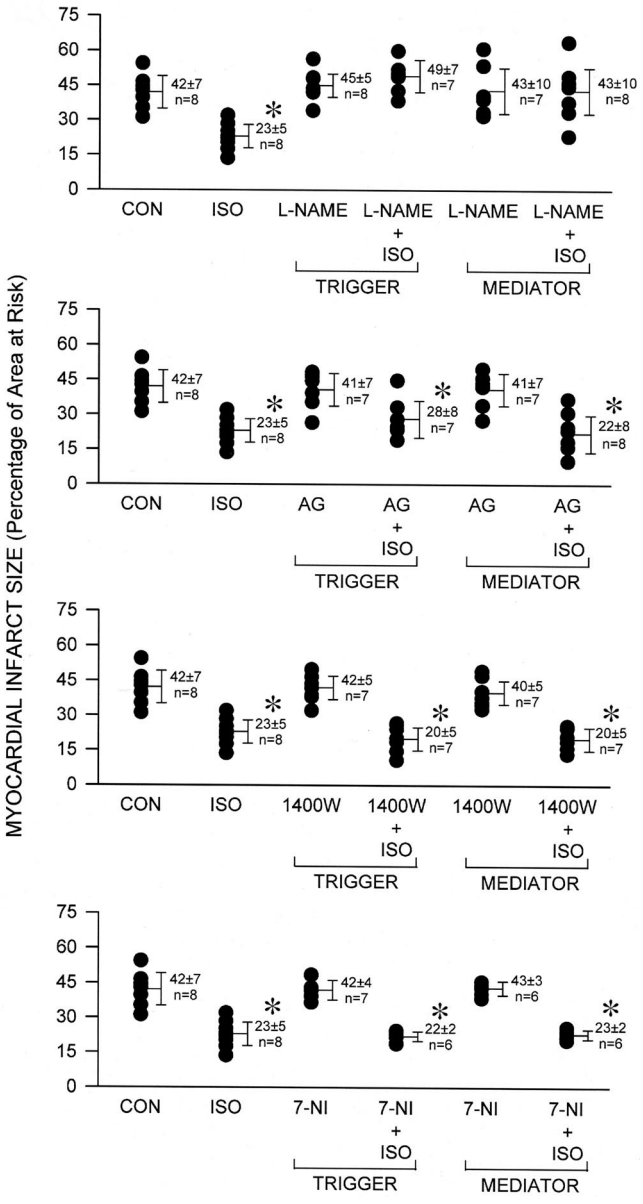


Fig. 2. Myocardial infarct size depicted as a percentage of the left ventricular area at risk in rabbits receiving *N*-nitro-L-arginine methyl ester (L-NAME; *top panel*), aminoguanidine (AG; *second panel*), 1400W (*third panel*), or 7-nitroindazole (7-NI; *bottom panel*) before (trigger [T]) administration of isoflurane (ISO) or prolonged coronary artery occlusion (mediator [M]) or at corresponding time points in the absence of isoflurane pretreatment. Each point represents a single experiment. * Significantly ($P < 0.05$) different from control (CON).

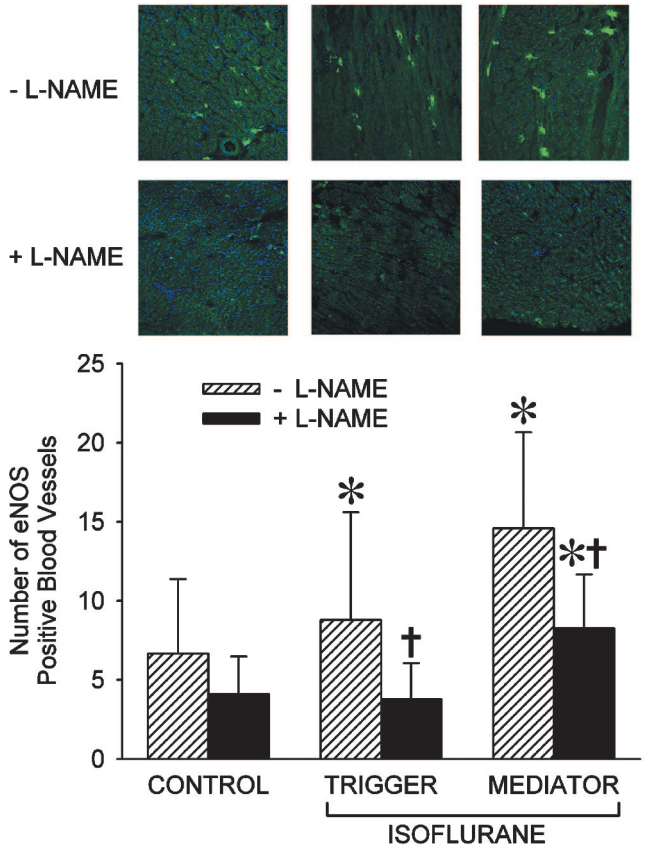
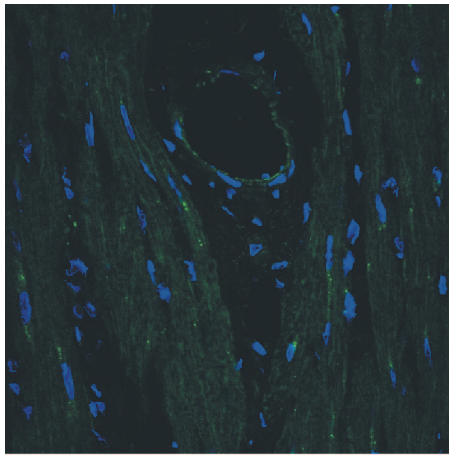


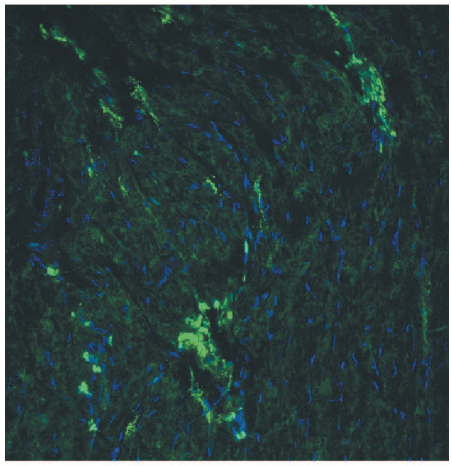
Fig. 3. Representative photomicrographs of immunofluorescent staining for endothelial nitric oxide synthase (eNOS; *top panel*) and histograms illustrating eNOS expression (*bottom panel*) in left ventricular tissue samples obtained from rabbits before (control), immediately after (trigger), or 24 h after (mediator) prolonged (2 h) administration of 1.0 minimum alveolar concentration isoflurane in the absence (−) and presence (+) of *N*-nitro-L-arginine methyl ester (L-NAME). * Significantly ($P < 0.05$) different from control. † Significantly ($P < 0.05$) different from (−) L-NAME.

of total LV mass was similar between groups. Rabbits have also been shown to possess little if any coronary collateral bloodflow.[43] Therefore, it seems unlikely that differences in collateral perfusion between groups account for the observed results. Nevertheless, coronary collateral blood flow was not specifically quantified in the current investigation. The reductions in myocardial infarct size produced by isoflurane-induced late preconditioning occurred independent of changes in major determinants of myocardial oxygen consumption. However, the current results should be qualified because coronary venous oxygen tension was not directly measured, and myocardial oxygen consumption was not calculated in the current investigation.

Neuronal NOS has been identified in the myocardium[44,45] and was recently shown to play a role in the late phase of ischemic preconditioning by Wang *et al.* [20] Our findings indicate that nNOS does not mediate isoflurane-induced delayed preconditioning because the selective nNOS inhibitor 7-NI did not affect reductions in infarct size produced by exposure to this volatile agent 24 h before coronary occlusion. Neuronal NOS was shown to mediate late ischemic preconditioning 72 h but not 24 h before prolonged coronary occlusion in conscious rabbits.[20] Whether exposure to volatile anesthetics 72 h before ischemia is associated with myocardial protection is unknown, and a role for nNOS in such a process also remains unexplored. Examination of these hypotheses represents an important goal for future research in our laboratory. The current immunohistochemistry and RT-PCR data also suggest that eNOS plays a role in delayed



ISOFLURANE (MEDIATOR)



REPETITIVE ISCHEMIA

Fig. 4. Representative photomicrographs of immunofluorescent staining for inducible nitric oxide synthase in left ventricular tissue samples obtained from rabbits 24 h (mediator) after prolonged (2 h) administration of 1.0 minimum alveolar concentration isoflurane (*top*) or 6 h after exposure to six cycles of brief (4 min) coronary artery occlusion interspersed with 4 min of reperfusion (repetitive ischemia; *bottom*). Prolonged administration of isoflurane did not affect inducible nitric oxide synthase expression immediately (data not shown) or 24 h after exposure, in contrast to brief repetitive ischemic stimuli.

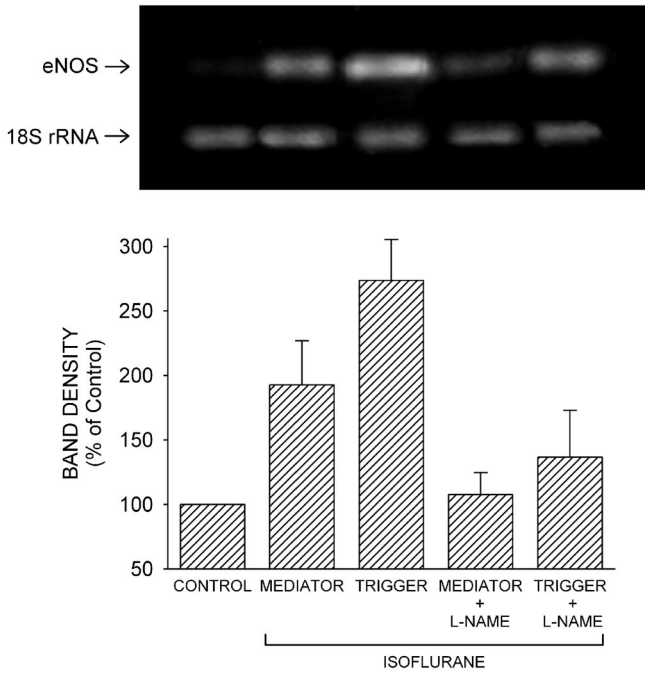


Fig. 5. Detection of endothelial nitric oxide synthase (eNOS) transcripts (485 base pairs [bp]) by reverse-transcription polymerase chain reaction in messenger RNA extracted from left ventricular tissue samples obtained from rabbits before (control; *lane 1*), immediately after (trigger; *lane 3*), or 24 h after (mediator; *lane 2*) prolonged (2-h) administration of 1.0 minimum alveolar concentration isoflurane in the absence or presence of *N*-nitro-L-arginine methyl ester (L-NAME) (*lanes 4* and *5*). 18S ribosomal RNA (rRNA; 315 bp) served as internal standard for each sample. Products were analyzed on a 2% agarose gel stained with SYBR gold (*top panel*). Histograms illustrating eNOS band density are depicted in the *bottom panel*. Values are expressed relatively to the intensity of bands obtained from rabbits that were not exposed to isoflurane.

cardioprotection by isoflurane. Currently available eNOS antagonists may also affect other NOS isoforms; therefore, we did not conduct experiments with these drugs. In addition, the immunohistochemistry results demonstrated that eNOS protein expression seems to be enhanced primarily in vascular endothelial cells. Whether eNOS protein also increases in myocardium in response to isoflurane-induced delayed preconditioning will require further study to ascertain. Nevertheless, eNOS transcription increased in LV myocardium exposed to isoflurane. The current results should also be qualified because we did not specifically measure NO production by eNOS, iNOS, or nNOS, nor did we determine the activity of these NOS isoenzymes.

N-nitro-L-arginine methyl ester inhibited the up-regulation of eNOS protein and mRNA observed during isoflurane-induced delayed preconditioning. This observation was somewhat surprising because L-NAME blocks NOS activity and NO production. However, Yuhanna *et al.* [46] previously demonstrated that inhibition of NO production with L-NAME produces a reduction in eNOS protein expression in rabbit pulmonary arterial endothelial cells *in vitro*. These data suggest that a positive feedback regulation of eNOS expression by endogenous or exogenous NO may occur in rabbits. In contrast, other species seem to regulate NOS expression by NO through a negative feedback mechanism. Therefore, it seems likely that the L-NAME–induced reduction in eNOS protein and mRNA expression observed in the current investigation

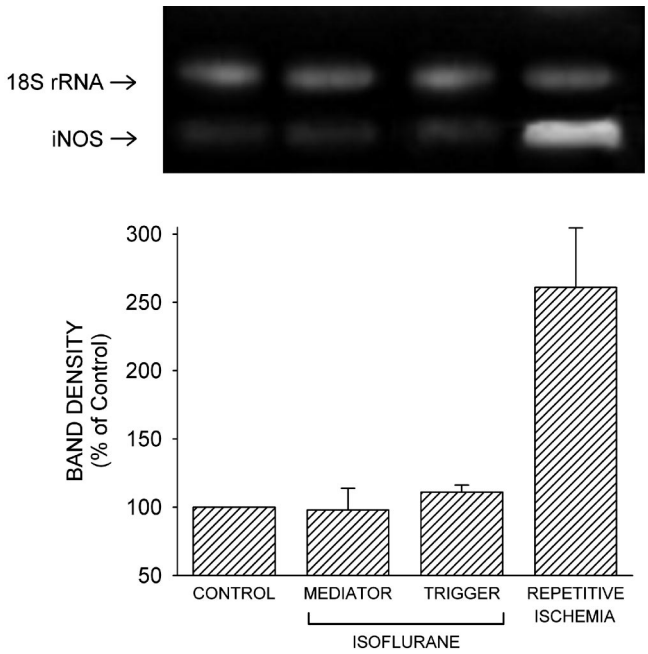


Fig. 6. Detection of inducible nitric oxide synthase (iNOS) transcripts (298 base pairs [bp]) by reverse-transcription polymerase chain reaction in messenger RNA extracted from left ventricular tissue samples obtained from rabbits before (control; *lane 1*), immediately after (trigger; *lane 3*), or 24 h after (mediator; *lane 2*) prolonged (2-h) administration of 1.0 minimum alveolar concentration isoflurane or from rabbits exposed to six cycles of brief (4-min) coronary artery occlusion and reperfusion (repetitive ischemia; *lane 4*). 18S ribosomal RNA (rRNA; 489 bp) served as internal standard for each sample. Products were analyzed on a 2% agarose gel stained with SYBR gold (*top panel*). Histograms illustrating iNOS band density are depicted in the *bottom panel*. Values are expressed relatively to the intensity of bands obtained from rabbits that were not exposed to isoflurane.

may be related to this unique rabbit specificity. Anesthetic-induced preconditioning has been previously reported in human myocardium,[47] but species differences in this phenomenon are known to exist that also require us to qualify our conclusions. For example, two recent studies[48,49] demonstrated that eNOS mRNA was reduced or did not increase after prolonged administration of isoflurane in rat myocardium. These data stand in contrast to our findings that eNOS transcription increases in response to a 2-h exposure to isoflurane in rabbit myocardium. Finally, the current results should be qualified because aging modulates the preconditioning process,[50] and the current investigation did not specifically use rabbits from a preselected age range. Nevertheless, rabbits of similar body weight were used in the current investigation.

In summary, the current results confirm that remote exposure to isoflurane produces delayed protection against myocardial infarction in rabbits. Inhibition of NO production by L-NAME before isoflurane exposure or prolonged coronary occlusion abolished reductions in infarct size produced by this volatile agent, indicating that NO acts as a trigger and mediator of this cardioprotective process. The current results suggest that eNOS but not iNOS or nNOS mediates delayed preconditioning by isoflurane.

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