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The Mechanism of Helium-Induced Preconditioning: A Direct Role for Nitric Oxide in Rabbits

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Abstract

BACKGROUND—Helium produces preconditioning against myocardial infarction by activating prosurvival signaling, but whether nitric oxide (NO) generated by endothelial NO synthase plays a role in this phenomenon is unknown. We tested the hypothesis that NO mediates helium-induced cardioprotection *in vivo*.

METHODS—Rabbits ($n = 62$) instrumented for hemodynamic measurement were subjected to a 30-min left anterior descending coronary artery occlusion and 3 h reperfusion, and received 0.9% saline (control) or three cycles of 70% helium–30% oxygen administered for 5 min interspersed with 5 min of an air–oxygen mixture before left anterior descending coronary artery occlusion in the absence or presence of pretreatment with the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg), the selective inducible NOS inhibitor aminoguanidine hydrochloride (AG; 300 mg/kg), or selective neuronal NOS inhibitor 7-nitroindazole (7-NI; 50 mg/kg). In additional rabbits, the fluorescent probe 4,5-diaminofluroscein diacetate (DAF-2DA) and confocal laser microscopy were used to detect NO production in the absence or presence of helium with or without L-NAME pretreatment.

RESULTS—Helium reduced ($P < 0.05$) infarct size (24% ± 4% of the left ventricular area at risk; mean ± SD) compared with control (46% ± 3%). L-NAME, AG, and 7-NI did not alter myocardial infarct size when administered alone. L-NAME, but not 7-NI or AG, abolished helium-induced cardioprotection. Helium enhanced DAF-2DA fluorescence compared with control (26 ± 8 vs 15 ± 5 U, respectively). Pretreatment with L-NAME abolished these helium-induced increases in DAF-2DA fluorescence.

CONCLUSIONS—The results indicate that cardioprotection by helium is mediated by NO that is probably generated by endothelial NOS *in vivo*.

Brief exposure to a noble gas before prolonged coronary artery occlusion and reperfusion protects myocardium against irreversible ischemic injury.[1,2] The mechanisms responsible for these cardioprotective effects have not been thoroughly characterized. Reductions in myocardial infarct size produced by the nonanesthetic noble gas helium *in vivo* were mediated by the phosphotidylinositol-3-kinase (PI3K) signaling kinase pathway[3] through inhibition of the mitochondrial permeability transition pore (mPTP).[1] PI3K and its downstream target Akt (protein kinase B) activate endothelial nitric oxide (NO) synthase (eNOS) by phosphorylation of the Ser^{1177} residue, thereby increasing the formation of NO.[4,5] A central role for NO has

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been implicated in several forms of ischemia-induced[6–10] and volatile anesthetic-induced cardio-protection. [11–14] NO promotes translocation of the ε isoform of protein kinase C (PKC-ε),[15,16] and directly activates mitochondrial adenosine triphosphate-regulated potassium (K_{ATP}) channels,[17] thereby protecting myocardium against ischemic injury. NO also regulates apoptosis (programmed cell death),[18,19] which, along with necrosis, is an important contributor to permanent myocardial damage after prolonged ischemia and reperfusion.[20] Whether NO is also responsible for reductions in extent of ischemic injury produced by brief, repetitive exposure to helium has yet to be defined. We tested the hypothesis that NO generated by eNOS mediates helium-induced preconditioning in barbiturate-anesthetized, acutely instrumented rabbits. We further tested the hypothesis that brief administration of helium directly increases NO production independent of subsequent ischemia and reperfusion in rabbit ventricular myocardium *in vivo* using the NO-specific fluorescent probe 4,5-diaminofluroscein diacetate (DAF-2DA)[21–23] and confocal laser microscopy.

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*.

Experimental Preparation

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with IV sodium pentobarbital (30 mg/kg) as previously described.[1] Additional doses of pentobarbital were titrated as required to assure that pedal and palpebral reflexes were absent throughout the experiment. Briefly, a tracheostomy was performed through a midline incision, and each rabbit was ventilated with positive pressure using an air–oxygen mixture (fractional in-spired oxygen concentration = 0.30). Arterial blood gas tensions and acid–base status were maintained within a normal physiological range by adjusting the respiratory rate or tidal volume throughout the experiment. A pulse oximeter was placed on the right hindpaw of each rabbit for measurement of continuous arterial oxygen saturation. Heparin-filled catheters were positioned in the right carotid artery and the left jugular vein for measurement of arterial blood pressure and fluid or drug administration, respectively. Maintenance fluids (0.9% saline; $15\ mL \cdot kg^{-1} \cdot min^{-1}$) were continued for the duration of each experiment. 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. IV 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. Systemic hemodynamics were continuously recorded on a polygraph through-out each experiment.

Experimental Protocol

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 3 h of reperfusion. In 8 separate groups, rabbits (n = 7–8 per group) were randomly assigned to receive 0.9% saline (control), 3 cycles of 70% helium–30% oxygen administered for 5 min interspersed with 5 min of 70% nitrogen–30% oxygen before coronary artery occlusion, the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg), the selective inducible NOS (iNOS) inhibitor aminoguanidine

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hydrochloride (AG; 300 mg/kg), or the selective neuronal NOS (nNOS) inhibitor 7-nitroindazole (7-NI; 50 mg/kg) in the absence or presence of helium pretreatment. L-NAME was dissolved in 0.9% saline and administered as an IV infusion over 10 min beginning 30 min before LAD occlusion. AG was dissolved in 0.9% saline, the pH of the solution was adjusted to 7.4 with 0.1 N sodium hydroxide, and the mixture was then injected subcutaneously 1 h before coronary occlusion. 7-NI was dissolved in dimethylsulfoxide and administered into the peritoneum 1 h before LAD occlusion. The doses of L-NAME, AG, and 7-NI used in the current investigation did not produce hemodynamic effects nor affect infarct size when administered alone in an identical rabbit model.[13] Dimethylsulfoxide also did not affect myocardial infarct size in rabbits.[24]

Measurement of Myocardial Infarct Size

Myocardial infarct size was measured as previously described. Briefly, the LAD was reoccluded at the completion of each experiment and 3 mL of patent blue dye was injected IV. The left ventricular area at risk 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. Infarcted and noninfarcted myocardium within the area at risk were carefully separated and weighed after storage overnight in 10% formaldehyde. Myocardial infarct size was expressed as a percentage of the area at risk. Rabbits that developed intractable ventricular fibrillation and those with an area at risk <15% of total left ventricular mass were excluded from subsequent analysis.

Detection of NO

In four additional experimental groups, rabbits instrumented as described above ($n = 3$ per group) were randomly assigned to receive 0.9% saline or L-NAME (10 mg/kg) in the absence or presence of helium pretreatment, and DAF-2DA (Daiichi Pure Chemicals, Tokyo, Japan) was used to detect NO production.[21–23] DAF-2DA (1 mg dissolved in 280 µL dimethylsulfoxide) was injected as an IV bolus immediately before a single 5 min cycle of 70% helium–30% oxygen or at a corresponding time point in rabbits that were not exposed to the noble gas (Fig. 1). Rabbits assigned to receive IV L-NAME were pretreated with the drug 30 min before administration of helium. Rabbits were killed with an overdose of pentobarbital 5 min after discontinuation of helium during administration of 70% nitrogen–30% oxygen. The heart was rapidly excised and immediately frozen in liquid nitrogen. The fresh, frozen left ventricle was isolated and divided into four sections of equal size. Cryostat sections (20 µm) of the left ventricle were mounted on standard microscope slides and examined using confocal laser microscopy without delay. Using a laser fluorescence imaging system mounted on a confocal microscope, images were recorded and stored for subsequent off-line analysis on a computer workstation equipped with image analysis software. Use of the 40× objective yielded a 400× end magnification on a 292 × 195 μm^2 digital image (768 × 512 pixels). The signal-to-noise ratio was enhanced using the Kalman method. Excitation was produced using a krypton–argon laser at a wavelength of 488 nm, and emitted fluorescence was measured at 550 nm after long pass filtering. Background was identified as an area with-out cells or with minimal cytosolic fluorescence. In each rabbit, 20 Kalman-averaged images were obtained, and approximately 8–10 DAF-2DA-stained cells were analyzed in each image by subtraction of background fluorescence from the pixel intensity of each cell.

Statistical Analysis

Statistical analysis of data within and among groups was performed with analysis of variance for repeated measures followed by Bonferroni's modification of Student's *t*-test. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean ± SD.

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RESULTS

Sixty-two rabbits were instrumented to obtain 59 successful infarct size experiments. Two rabbits were excluded because the left ventricular area at risk was <15% of the total left ventricular mass. One rabbit was excluded because intractable ventricular fibrillation occurred during coronary artery occlusion. Arterial blood gas tensions were maintained within the physiologic range during administration of helium in all groups (data not shown). Arterial oxygen saturation remained at 100% during and after administration of helium with or without other drug interventions (data not shown). Baseline systemic hemodynamics were similar among groups (Table 1). Helium did not affect hemodynamics. A significant ($P < 0.05$) decrease in heart rate and an increase in mean arterial blood pressure were observed in L-NAME-pretreated rabbits in the absence of helium compared with control before LAD occlusion and reperfusion, but no difference in rate–pressure product was observed between these groups. Coronary artery occlusion reduced rate–pressure product in most experimental groups. Declines in heart rate and rate–pressure product occurred during reperfusion in all experimental groups.

Body weight, left ventricular mass, area at risk weight, and the ratio of area at risk to left ventricular mass were similar between groups (Table 2). Brief, intermittent exposure to 70% helium–30% oxygen before LAD occlusion reduced myocardial infarct size (24% ± 4% of the left ventricular area at risk) when compared with control (46% ± 3%; Fig. 2). Administration of L-NAME, AG, or 7-NI alone did not affect infarct size (46% ± 10%, 41% ± 7%, and 43% ± 3%, respectively). L-NAME, but not AG or 7-NI, abolished helium-induced cardioprotection (45% ± 2%, 21% ± 3%, and 24% ± 1%, respectively). A single 5-min exposure to 70% helium–30% oxygen increased DAF-2DA fluorescence (26 ± 8 U) compared with control (15 ± 5 U; Fig. 3). L-NAME did not affect DAF-2DA fluorescence (15 ± 4 U) when administered alone, but pretreatment with L-NAME abolished helium-induced increases in DAF-2DA fluorescence (14 ± 5 U).

DISCUSSION

The current results confirm previous findings[1] demonstrating that three cycles of 5 min 70% helium–30% oxygen preconditioning interspersed with 5 min washout periods of an air–oxygen mixture reduce myocardial infarct size after prolonged coronary artery occlusion and reperfusion *in vivo*. The results demonstrate for the first time that NO mediates this helium-induced preconditioning, and further indicate that the nonanesthetic noble gas directly enhances NO production in a NOS-dependent manner independent of ischemia and reperfusion. Whereas pretreatment with the nonselective NOS inhibitor L-NAME abolished reductions in infarct size produced by brief, intermittent exposure to helium, neither the iNOS antagonist AG nor the nNOS inhibitor 7-NI affected the cardioprotective effects of the helium. These data provide pharmacological evidence that eNOS but not iNOS or nNOS mediates helium preconditioning in rabbits. PI3K-Akt has been shown to phosphorylate eNOS, and the NO produced as a result of this activation mediated cellular protection.[4,5] Other evidence also indicated that NO plays a crucial role in cardioprotection.[25] NO activated PKC-ε and promoted its intracellular translocation in rabbit ventricular myocytes.[15,16] NO also directly opened mitochondrial K_{ATP} channels *in vitro*.[17] PKC-ε and mitochondrial K_{ATP} channels have been shown to be essential components of the signaling responsible for ischemic, pharmacologic, and anesthetic preconditioning.[26,27] NO nitrosated and inactivated several caspases known to execute apoptosis,[28] preserved extracellular signal-regulated kinase (Erk1/2) activity,[29] and blocked metabolism of the antiapoptotic protein B cell lymphoma protein-2 (Bcl-2), thereby preventing the mitochondrial disruption and cytochrome c release that initiate the cell suicide program.[30] This NO-induced protective effect may be related to inhibition of mPTP opening concomitant with reperfusion.[31]

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Helium was previously shown to attenuate irreversible ischemic injury by activating PI3K-Akt and Erk1/2 signal transduction and inhibiting mPTP opening *in vivo*.[1] Preliminary data also suggested that mitochondrial K_{ATP} channels mediate cardioprotection by brief, repetitive administration of helium.[32] Thus, the current results suggesting that eNOS plays a role in helium-induced preconditioning may be related to inhibition of mitochondrial permeability transition or activation of mitochondrial K_{ATP} channels through enhanced NO production. Whether NO-induced PKC-ε activation also mediates cardioprotection by helium remains unknown, but previous studies indicated that the anesthetic noble gas xenon produces preconditioning in rat heart *in vivo* concomitant with PKC-ε phosphorylation and translocation[33] through PI3K signaling and mitochondrial K_{ATP} channel opening. [34] These data with xenon strongly suggest that helium may also protect myocardium against irreversible ischemic injury by activating PKC-ε through a NO-dependent mechanism. Our laboratory will be conducting experiments designed to test this hypothesis in the near future.

The current results should be interpreted within the constraints of several potential limitations. AG and 7-NI have been shown to be selective inhibitors of iNOS and nNOS, respectively, at the doses and routes of administration used in the current investigation. [35–37] Nevertheless, dose–response relationships to these inhibitors and L-NAME were not performed, and the possibility that these drugs may have inhibited other protein kinases involved in cardioprotection cannot be completely excluded from the analysis. Unlike the selective iNOS and nNOS inhibitors used in the current investigation, commercially available antagonists of eNOS are not highly selective, and thus, we chose not to perform experiments with these drugs. We did not specifically examine the biochemical actions of helium on eNOS phosphorylation nor did we measure the activity of this enzyme in rabbit myocardium. Nevertheless, our pharmacological data strongly suggest a central role for eNOS in heliuminduced preconditioning against infarction because selective inhibitors of iNOS and nNOS failed to block the protection produced by the nonanesthetic noble gas. Key modulators of apoptosis regulated by NO,[28–31] including caspases, Bcl-2, and mPTP, were not specifically examined during helium preconditioning in the current investigation. However, the current results demonstrated that helium directly increases NO production in a NOS-dependent fashion using DAF-2DA staining and confocal laser microscopy. Helium preconditioning was abolished by a selective inhibitors of PI3K-Akt and Erk1/2 in an identical rabbit model.[1] PI3K and Erk1/2 signaling pathways are major regulators of pro- compared with antiapoptotic protein balance. [3,38] Thus, it appears likely that the cardioprotective effects of helium are due, at least in part, to an attenuation of programmed cell death mediated by the actions of NO, but further study will also be needed to test this hypothesis.

The results should also be qualified because the route and duration of administration of L-NAME, AG, and 7-NI were heterogeneous, and these pharmacokinetic factors may have influenced the results. Plasma concentrations of these NOS inhibitors were also not determined. Myocardial infarct size is determined primarily by the size of the area at risk and the extent of coronary collateral perfusion. The area at risk expressed as a percentage of total left ventricular mass was similar among groups in the current investigation, and coronary collateral blood flow is minimal in rabbits.[39] Thus, differences in collateral perfusion among groups probably did not account for the observed results, but coronary collateral blood flow was not specifically quantified. The reductions in infarct size produced by helium in the absence or presence of other drug interventions occurred independent of changes in major determinants of myocardial oxygen consumption. Nevertheless, coronary venous oxygen tension was not directly measured nor was myocardial oxygen consumption calculated. Notably, no significant differences in hemodynamics were observed among groups before and during coronary artery occlusion that would account for differences in infarct size observed among groups. We used a 30-min coronary artery occlusion to produce myocardial infarction in rabbits. Whether brief repetitive exposure to helium also produces cardioprotection after more prolonged periods of

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coronary artery occlusion is unknown. Finally, the current findings implicating a role for eNOS in cardioprotection by helium were obtained in barbiturate-anesthetized rabbits, and whether similar results occur in other animal species or humans is unknown.

In summary, the current results confirm that brief, intermittent administration of helium before prolonged coronary artery occlusion and reperfusion protects myocardium against infarction in rabbits. The findings further indicate that helium directly increases NO production in a NOS-dependent manner independent of ischemia and reperfusion, and this NO, most likely derived from eNOS, but not iNOS or nNOS, mediates helium-induced cardioprotection *in vivo*.

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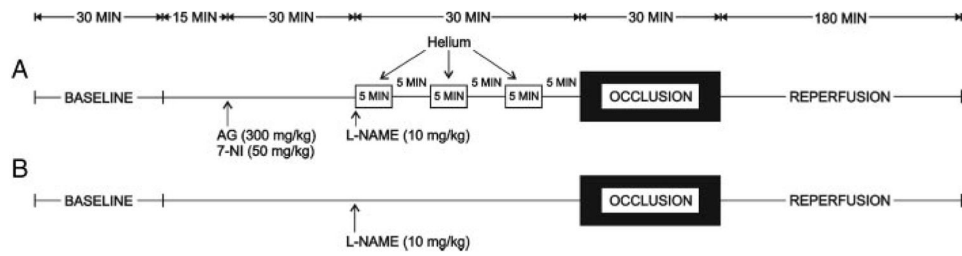

Figure 1.
Schematic illustration depicting the experimental protocols used to determine myocardial infarct size (panel A, top) and nitric oxide production (panel B, bottom). AG = aminoguanidine hydrochloride; 7-NI = 7-nitroindazole; L-NAME = *N*-nitro-L-arginine methyl ester.

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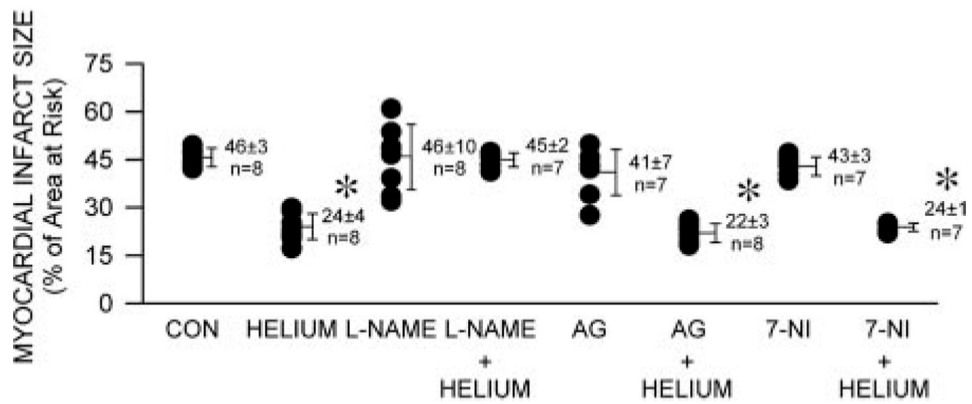

Figure 2.
Myocardial infarct size depicted as a percentage of left ventricular area at risk in rabbits receiving 0.9% saline (control, CON) or three cycles of 70% helium–30% oxygen administered for 5 min interspersed with 5 min of 70% nitrogen–30% oxygen in the absence or presence of pretreatment with the nonselective nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME), the selective inducible NOS inhibitor aminoguanidine hydrochloride (AG), or the selective neuronal NOS inhibitor 7-nitroindazole (7-NI). Each point represents a single experiment. All data are mean ± SD *Significantly ($P < 0.05$) different from CON.

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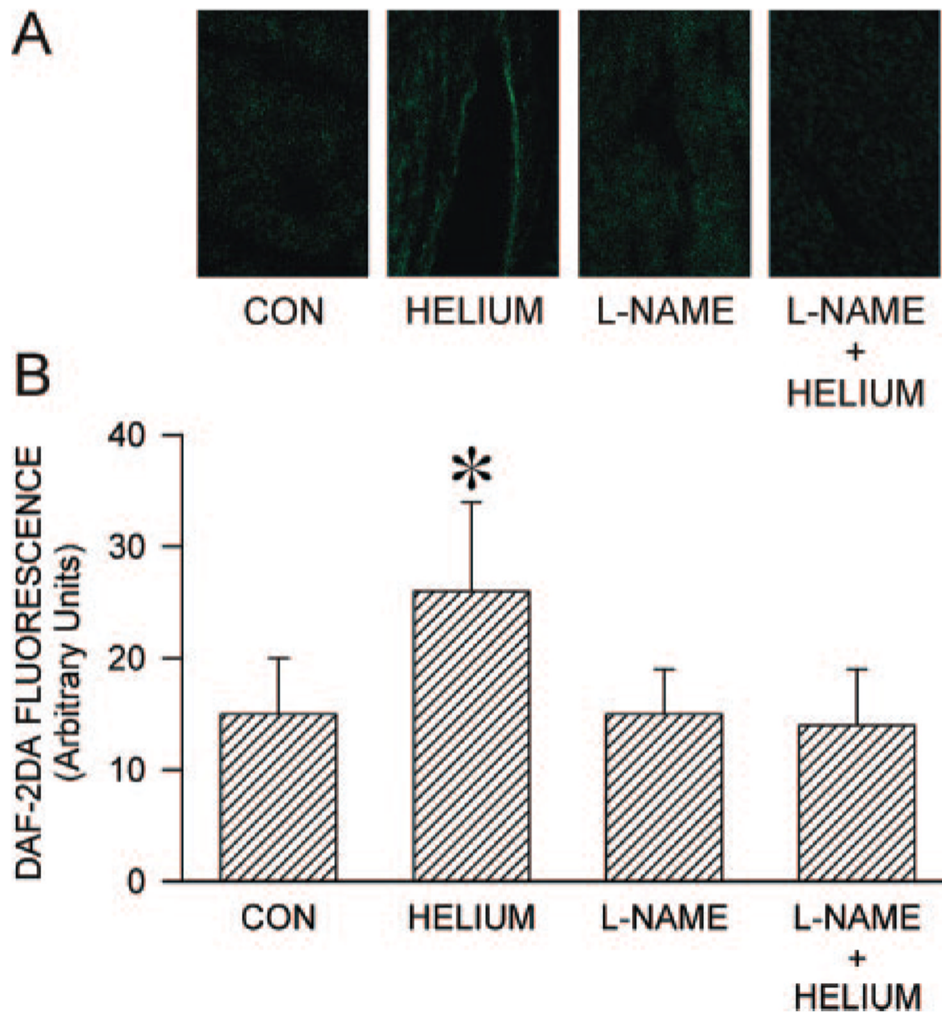

Figure 3.
Representative photomicrographs (top panels) depicting nitric oxide production as detected by 4,5-diaminofluroscein diacetate (DAF-2DA) fluorescence and confocal laser microscopy in rabbit ventricular myocardium pretreated with 0.9% saline control (CON) or nonselective nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg) in the absence or presence of a single 5 min exposure to 70% helium–30% oxygen. Histograms summarizing the results of DAF-2DA fluorescence experiments are depicted in the bottom panel. *Significantly ($P < 0.05$) different from CON.

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Table 1

Hemodynamics

	Baseline	Intervention	Occlusion	Reperfusion (min)		
				60	120	180
HR (min^{-1})						
CON	247 ± 35	238 ± 33	246 ± 37	224 ± 26*	219 ± 27*	214 ± 28*
He	253 ± 29	237 ± 28	223 ± 16*	216 ± 27*	206 ± 23*	198 ± 25*
L-NAME	246 ± 23	206 ± 22*	213 ± 19*	188 ± 26*	186 ± 17*	187 ± 13*
L-NAME + He	237 ± 11	218 ± 20	222 ± 29	225 ± 39	218 ± 18*	208 ± 27*
AG	259 ± 17	254 ± 18	243 ± 18	229 ± 21*	228 ± 24*	221 ± 11*
AG + He	221 ± 20	216 ± 16	211 ± 17	198 ± 16*	189 ± 12*	186 ± 10*
7-NI	261 ± 30	247 ± 27	241 ± 20	226 ± 17*	217 ± 14*	207 ± 16*
7-NI + He	251 ± 25	217 ± 20*	209 ± 12*	201 ± 19*	196 ± 21*	193 ± 18*
MAP (mm Hg)						
CON	73 ± 9	68 ± 7	60 ± 8*	58 ± 10*	59 ± 12*	58 ± 10*
He	74 ± 10	78 ± 16	63 ± 14	64 ± 11	65 ± 9	66 ± 17
L-NAME	81 ± 8	88 ± 14†	62 ± 20	64 ± 17	63 ± 13*	69 ± 13*
L-NAME + He	64 ± 7	74 ± 9	59 ± 18	58 ± 11	63 ± 10	63 ± 11
AG	84 ± 12	81 ± 16	76 ± 11	75 ± 12	75 ± 10	71 ± 11
AG + He	67 ± 7	72 ± 11	66 ± 13	70 ± 11	71 ± 6	68 ± 8
7-NI	73 ± 10	70 ± 7	57 ± 5*	58 ± 13*	53 ± 9*	52 ± 3*
7-NI + He	68 ± 9	64 ± 6	62 ± 11	63 ± 8	66 ± 8	63 ± 10
RPP ($min^{-1} \cdot mm\ Hg \cdot 10^{-3}$)						
CON	20.6 ± 4.2	18.7 ± 3.8	17.2 ± 3.5	15.1 ± 2.6*	15.4 ± 3.7*	14.5 ± 3.4*
He	21.2 ± 4.4	21.3 ± 6.4	16.5 ± 3.6*	16.1 ± 3.2*	15.7 ± 2.3*	15.0 ± 4.1*
L-NAME	22.6 ± 3.3	19.9 ± 3.5	14.9 ± 4.7*	12.9 ± 1.7*	13.0 ± 1.4*	14.2 ± 2.2*
L-NAME + He	17.6 ± 1.6	18.0 ± 2.1	15.0 ± 4.0	14.8 ± 3.4*	15.4 ± 2.3*	14.9 ± 3.0*
AG	21.8 ± 3.9	20.6 ± 5.6	18.5 ± 3.2	17.2 ± 2.8	17.1 ± 3.2	15.7 ± 2.8*
AG + He	16.9 ± 1.9	17.7 ± 2.1	16.0 ± 2.9	15.6 ± 1.9	15.3 ± 1.1	14.5 ± 1.2*
7-NI	21.9 ± 4.2	20.1 ± 2.9	16.4 ± 2.0*	15.8 ± 2.9*	14.1 ± 1.8*	12.7 ± 0.8*
7-NI + He	19.7 ± 3.7	15.2 ± 1.9	15.1 ± 2.5*	14.8 ± 2.6*	14.9 ± 2.3*	14.1 ± 2.9*

Data are mean ± SD.

HR = heart rate; MAP = mean arterial pressure; RPP = rate pressure product; CON = control; He = helium; L-NAME = *N*-nitro-L-arginine methyl ester; AG = aminoguanidine hydrochloride; 7-NI = 7-nitroindazole.

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

† Significantly ($P < 0.05$) different from corresponding control.

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Table 2

Left Ventricular Area at Risk

	N	**Body weight (g)**	**LV (g)**	**AAR (g)**	**AAR/LV (%)**
CON	8	2543 ± 305	3.78 ± 0.51	1.51 ± 0.18	40 ± 2
He	8	2668 ± 205	3.87 ± 0.24	1.28 ± 0.08	33 ± 2
L-NAME	7	2749 ± 147	3.14 ± 0.66	1.24 ± 0.41	39 ± 8
L-NAME + He	7	2414 ± 90	3.36 ± 0.19	1.44 ± 0.15	43 ± 3
AG	7	2619 ± 256	3.45 ± 0.53	1.18 ± 0.48	34 ± 11
AG + He	8	2501 ± 146	3.24 ± 0.18	1.17 ± 0.05	36 ± 3
7-NI	7	2634 ± 239	3.88 ± 0.60	1.48 ± 0.20	38 ± 4
7-NI + He	7	2467 ± 134	3.28 ± 0.43	1.14 ± 0.09	35 ± 4

Data are mean ± SD.

LV = left ventricle; AAR = area at risk; CON = control; He = helium; L-NAME = *N*-nitro-L-arginine methyl ester; AG = aminoguanidine hydrochloride; 7-NI = 7-nitroindazole.