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Morphine reduces the threshold of helium preconditioning against myocardial infarction: the role of opioid receptors in rabbits

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Abstract

Objectives—Brief, repetitive administration of helium before prolonged coronary artery occlusion and reperfusion protects myocardium against infarction. Opioid receptors mediate the cardioprotective effects of ischemic pre- and postconditioning, but whether these receptors also play a role in helium preconditioning is unknown. We tested the hypotheses that opioid receptors mediate helium preconditioning and that morphine (a μ_1 -opioid receptor agonist with δ_1 -opioid agonist properties) lowers the threshold of cardioprotection produced by helium *in vivo*.

Design—Randomized, prospective study.

Setting—University research laboratory.

Participants—Male New Zealand white rabbits.

Interventions—Rabbits (n=56) were instrumented for measurement of systemic hemodynamics and subjected to a 30 min left anterior descending coronary artery (LAD) occlusion and 3 h reperfusion. In separate experimental groups, rabbits (n=6 or 7 per group) received 0.9% saline (control), one or three cycles of 70% helium-30% oxygen administered for 5 min interspersed with 5 min of an air-oxygen mixture, morphine (0.1 mg/kg, i.v.), or the nonselective opioid antagonist naloxone (6 mg/kg, i.v.) before LAD occlusion. Other groups of rabbits received three cycles of helium or one cycle of helium plus morphine (0.1 mg/kg) in the absence or presence of naloxone (6 mg/kg) before ischemia and reperfusion. Statistical analysis of data was performed with analysis of variance for repeated measures followed by Bonferroni's modification of Student's *t* test.

Measurements and Main Results—Myocardial infarct size was determined using triphenyltetrazolium chloride staining and presented as a percentage of the left ventricular area at risk. Helium reduced myocardial infarct size in an exposure-related manner [36 ± 6 ($P > 0.05$) and

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25±4% ($P<0.05$ versus control) for one and three cycles of helium, respectively; data are mean ±SD] compared with control (44±7%). Morphine and naloxone alone did not affect infarct size (45±2 and 40±8%, respectively). The combination of one cycle of helium and morphine reduced infarct size (24±5%, $P<0.05$ versus control) to an equivalent degree as three cycles of helium. Naloxone pretreatment abolished cardioprotection produced by three cycles of helium (47±2%) and the combination of one cycle of helium plus morphine (45±4%).

Conclusions—The results indicate that morphine lowers the threshold of helium preconditioning. Opioid receptors mediate helium preconditioning and its augmentation by morphine *in vivo*.

Keywords

Myocardial ischemia; preconditioning; helium; morphine; opioid receptor

Noble gases with or without anesthetic properties (e.g., xenon, helium) protect myocardial and neural tissue against irreversible ischemic injury^{1–3}. The mechanisms responsible for these beneficial effects remain incompletely characterized. The cardio- and neuroprotective actions of xenon have been known for nearly a decade and were initially attributed to the anesthetic effects of the “inert” gas⁴. However, it is now clear that brief exposure to any noble gas before prolonged coronary artery occlusion and reperfusion produces preconditioning independent of an anesthetic effect². Helium-induced reductions in myocardial infarct size *in vivo* were mediated by several components of the phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase (Erk1/2) signaling cascades^{5–7} known to reduce cellular necrosis and apoptosis⁸. These kinases converge upon and inhibit opening the mitochondrial permeability transition pore (mPTP; a nonspecific channel located on the inner mitochondrial membrane)^{2,9}, in part by maintaining intracellular acidosis during early reperfusion¹⁰. Production of reactive oxygen species (ROS), opening of mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}), and activation of mitochondrial calcium-activated potassium (K_{Ca}) channels have also been implicated in cardioprotection by helium^{7,11} in healthy, but not senescent, hearts¹¹. G protein-coupled receptor ligands (e.g., δ_1 -opioid, adenosine, bradykinin) have been shown to activate prosurvival signaling and play essential roles in classical ischemic pre- and postconditioning¹², but whether such receptor ligands mediate preconditioning by noble gases is currently unknown. This investigation tested the hypotheses that opioid receptors mediate helium preconditioning and further, that morphine, a μ_1 opioid receptor agonist with δ_1 agonist properties, lowers the threshold of cardioprotection produced by helium *in vivo*.

Methods

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

Experimental Preparation

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg) as previously described². Additional doses of sodium 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 inspired 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. Temperature was maintained using a heating blanket. Heparin-filled catheters were inserted 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.kg⁻¹.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. A silk ligature was placed around this vessel approximately halfway between the base and the apex for the production of coronary artery occlusion and reperfusion. Intravenous heparin (500 U) was administered immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Systemic hemodynamics were continuously recorded on a polygraph throughout each experiment.

Experimental Protocol

Baseline hemodynamics and arterial blood gas tensions were recorded 30 min after instrumentation was completed (figure 1). All rabbits underwent a 30 min LAD occlusion followed by 3 h of reperfusion. In eight separate experimental groups, rabbits (n= 7 to 8 per group) received 0.9% saline (control), one or three cycles of 70% helium-30% oxygen administered for 5 min interspersed with 5 min of the air-oxygen mixture before coronary occlusion, or intravenous morphine (0.1 mg/kg in 0.9% saline) or naloxone (6 mg/kg in 0.9% saline) administered 30 min before LAD occlusion. Other groups of rabbits received three cycles of helium or one cycle of helium plus morphine (0.1 mg/kg) in the absence or presence of naloxone (6 mg/kg) before ischemia and reperfusion. A previous study demonstrated that the doses of morphine (0.1 mg/kg) and naloxone (6 mg/kg) used in the current investigation did not affect hemodynamics and infarct size¹³. In contrast, a higher dose (0.3 mg/kg) of morphine administered before ischemia reduced myocardial infarct size¹³.

Measurement of Myocardial Infarct Size

Myocardial infarct size was measured as previously described¹⁴. Briefly, the LAD was reoccluded at the completion of each experiment and 3 ml of patent blue dye was injected intravenously. The left ventricular area at risk 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 less than 15% of total left ventricular (LV) mass were excluded from subsequent analysis.

Statistical Analysis

A power analysis indicated that a group size of $n = 6$ was required for a minimal difference in infarct size of 20% (α error < 0.05; β error < 20%) with a power of 95%. Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by Bonferroni's modification of Student's t test¹⁵. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean $\pm$ standard deviation (SD).

RESULTS

Fifty-six rabbits were instrumented to obtain 54 successful infarct size experiments. Two rabbits were excluded because intractable ventricular fibrillation occurred during coronary artery occlusion or reperfusion (1 helium + morphine; 1 helium + morphine + naloxone). Arterial blood gas tensions were maintained within the physiologic range during administration of helium in all groups (data not shown) similar to previously reported data¹⁰. No differences in baseline systemic hemodynamics were observed between groups (Table 1). Helium, morphine, and naloxone did not affect hemodynamics. Coronary artery occlusion reduced heart rate, mean arterial pressure, and rate-pressure product in most experimental groups. There were no differences in hemodynamics between groups during LAD occlusion. Declines in heart rate, mean arterial pressure, and rate-pressure product occurred during reperfusion in all experimental groups.

There were no differences in left ventricular (LV) mass and LV area at risk weight between groups (Table 2). The ratios of LV area at risk to total LV mass were similar between groups. Helium reduced myocardial infarct size in an exposure-related manner [36 ± 6 ($P > 0.05$) and $25 \pm 4\%$ ($P < 0.05$) for one and three cycles of helium, respectively; data are mean $\pm$ SD] compared with control ($44 \pm 7\%$). Morphine and naloxone alone did not affect infarct size (45 ± 2 and $40 \pm 8\%$, respectively). The combination of one cycle of helium and morphine reduced infarct size ($24 \pm 5\%$, $P < 0.05$) to an equivalent degree as three cycles of helium. Naloxone pretreatment abolished cardioprotection produced by three cycles of helium ($47 \pm 2\%$) and the combination of one cycle of helium plus morphine ($45 \pm 4\%$).

DISCUSSION

The current results confirm previous findings^{2,5-7} 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 further demonstrate for the first time that the nonselective opioid antagonist naloxone abolishes reductions in myocardial infarct size produced by this brief, intermittent exposure to helium. These data suggest that helium preconditioning reduces myocardial necrosis by activating opioid receptors. Similar to previous findings¹³,

pretreatment with morphine did not affect myocardial infarct size in the dose (0.1 mg/kg) used in the current investigation. However, the combination of this dose of morphine and a single 5 min cycle of 70% helium-30% oxygen reduced myocardial necrosis to a similar degree as three cycles of helium alone, indicating that morphine lowers the threshold of helium preconditioning. Naloxone also abolished these reductions in infarct size produced by the combination of subthreshold doses of morphine and helium, suggesting that the opioid receptors also mediate this additive effect.

Opioid agonists were previously shown to produce cardioprotection when administered before prolonged coronary artery occlusion^{13,16–20} or immediately before reperfusion^{21,22}. The δ_1 -opioid receptor was most often implicated in pre- and postconditioning^{16,21,23}, but roles for κ - and μ_1 -opioid receptors have also been more recently suggested^{24–26}. As reported with other G protein-linked receptor ligands (e.g., adenosine²⁷, bradykinin²⁸), the mechanisms by which opioid agonists produce cardioprotection have been extensively studied, and many of the components of the reperfusion injury salvage kinase (RISK)²⁹ pathway [including PI3K, Erk1/2, protein kinase C, glycogen synthase kinase 3 β (GSK-3 β) and mammalian target of rapamycin-70-kDa ribosomal protein s6 kinase (mTOR/p70s6K)] were shown to mediate these protective effects^{18,20,21,30}. Furthermore, noble gases^{1,2,5–7,10} protect myocardium against infarction by activating several enzymes in the RISK pathway that converge upon and inhibit mPTP. Thus, the current observations indicating that naloxone abolishes helium-induced reductions in infarct size and that a subthreshold dose of morphine reduces the threshold required for helium preconditioning are not entirely surprising considering the remarkable similarity between the signaling pathways responsible for cardioprotection produced by opioids and noble gases.

The precise opioid receptor subtype responsible for helium preconditioning and its augmentation by morphine remains to be determined. Morphine demonstrates a high degree of specificity for μ_1 -opioid receptors, but the selective δ_1 -opioid antagonist 7-benzylidenanoltroxone attenuated the morphine-induced cardioprotection in isolated ventricular myocytes^{31,32}. Thus, morphine may reduce the threshold of helium preconditioning through δ_1 -opioid receptor activation. While this hypothesis certainly appears to be highly plausible, we did not specifically investigate the role of δ_1 -opioid receptors in helium preconditioning using a selective δ_1 -opioid receptor antagonist, nor did we examine whether a selective δ_1 -opioid agonist (such as TAN-67 or BW373U86³³) may also be capable of enhancing helium-induced reductions in infarct size *in vivo*. In addition, we did not examine the role of the inhibitory guanine (G_i) nucleotide-binding proteins that are known to couple δ_1 -opioid receptors to intracellular signaling kinases. Previous data indicated that the G_i antagonist pertussis toxin abolished cardioprotection produced by isoflurane³⁴, but the role of G_i proteins in helium preconditioning has yet to be defined. These objectives represent important goals of future research.

The current results should be interpreted within the constraints of several other possible limitations. A formal dose-response relationship between morphine and myocardial infarct size was not conducted nor were plasma concentrations of morphine measured in the current study. A previous investigation demonstrated that pretreatment with morphine (0.3 but not 0.1 mg/kg) before prolonged coronary artery occlusion and reperfusion reduced the extent of

infarction in rats¹³. As observed in the current investigation, helium preconditioning was previously shown to be dependent on the number of brief exposure episodes conducted before prolonged coronary artery occlusion and reperfusion⁶. Whether exposure episodes shorter than 5 min or helium concentrations lower than 70% will also decrease myocardial necrosis in this rabbit model was not examined in the current investigation and remains to be defined. Infarct size is determined primarily by the size of the area at risk and the degree of coronary collateral perfusion. The area at risk to total left ventricular mass ratio was similar between experimental groups. Coronary collateral blood flow has also been shown to be minimal in rabbits³⁵. Thus, the current results were most likely not related to differences in collateral perfusion. Nevertheless, coronary collateral blood flow was not specifically quantified. The reductions in infarct size produced by helium in the absence or presence of morphine or naloxone 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 amongst groups before and during coronary artery occlusion that would account for differences in infarct size observed between groups. A 30 min coronary artery occlusion was used to produce myocardial infarction in rabbits. Whether helium preconditioning also reduces infarct size after greater periods of coronary artery occlusion is unknown. Finally, the current findings implicating a role for opioid receptors 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 results further indicate that morphine lowers the threshold of helium preconditioning and also demonstrate that opioid receptors mediate helium preconditioning and its augmentation by morphine *in vivo*.

Acknowledgments

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Figure 1.
Schematic illustration depicting the experimental protocols used in the current investigation.
Abbreviations: He = helium; MOR = morphine; NAL = naloxone

Figure 2.

Myocardial infarct size depicted as a percentage of left ventricular area at risk in rabbits receiving 0.9% saline (control, CON), pretreatment with one or three cycles of 70% helium-30% oxygen (He), intravenous morphine (0.1 mg/kg, MOR) in the absence or presence of one cycle of helium, or intravenous naloxone (6 mg/kg, NAL) in the absence or presence of three cycles of helium or the combination of morphine (0.1 mg/kg) and one cycle of helium. Each point represents a single experiment. All data are mean ± SD.

*Significantly ($P < 0.05$) different from CON, one cycle of He alone, and other interventions.

Table 1

Hemodynamics

	Baseline	Intervention	Occlusion	Reperfusion (min)		
				60	120	180
HR (min ⁻¹)						
CON	253±34	254±35	236±30	226±24 [*]	217±21 [*]	209±33 [*]
He (1 Cycle)	248±33	243±24	233±20	229±32	222±33 [*]	216±29 [*]
He (3 Cycles)	243±35	237±29	219±17 [*]	208±23 [*]	201±19 [*]	191±25 [*]
MOR (0.1 mg/kg)	259±22	253±21	228±25 [*]	233±24 [*]	213±17 [*]	206±17 [*]
He (1 Cycle) + MOR (0.1 mg/kg)	257±34	256±29	242±22	235±19	222±17 [*]	215±14 [*]
NAL (6 mg/kg)	265±18	232±18	216±30 [*]	206±20 [*]	200±18 [*]	194±16 [*]
He (3 Cycles) + NAL (6 mg/kg)	251±17	249±17	249±28	239±32	220±27 [*]	214±28 [*]
He (1 Cycle) + MOR (0.1 mg/kg) + NAL (6 mg/kg)	268±20	253±24	238±28 [*]	239±32 [*]	229±37 [*]	227±40 [*]
MAP (mmHg)						
CON	77±8	74±8	68±5	70±5	72±7	68±8
He (1 Cycle)	71±8	70±13	63±12	67±11	68±12	68±10
He (3 Cycles)	77±8	77±14	60±13	58±8 [*]	62±9 [*]	58±17
MOR (0.1 mg/kg)	75±8	71±5	66±7 [*]	65±6 [*]	64±10 [*]	64±7 [*]
He (1 Cycle) + MOR (0.1 mg/kg)	66±10	67±9	57±8	59±10	61±7	61±9
NAL (6 mg/kg)	73±9	69±10	63±18	68±5	65±9	62±10
He (3 Cycles) + NAL (6 mg/kg)	76±11	72±10	59±9 [*]	57±9 [*]	56±8 [*]	57±9 [*]
He (1 Cycle) + MOR (0.1 mg/kg) + NAL (6 mg/kg)	73±15	74±12	69±16	70±13	72±7	71±11
RPP (min ⁻¹ •mmHg•10 ⁻³)						
CON	23.4±5.0	22.5±5.5	18.9±3.3	19.1±3.2	18.3±3.1 [*]	16.8±4.3 [*]
He (1 Cycle)	19.5±2.8	19.1±3.6	16.7±3.8	17.4±4.6	17.2±4.8	16.6±3.3 [*]
He (3 Cycles)	21.3±4.3	22.4±5.7	15.5±3.5 [*]	14.2±3.2 [*]	14.7±2.2 [*]	13.1±5.0 [*]
MOR (0.1 mg/kg)	22.1±3.2	20.4±2.5	17.2±2.3 [*]	17.5±2.8 [*]	15.8±2.3 [*]	15.1±1.6 [*]
He (1 Cycle) + MOR (0.1 mg/kg)	19.4±4.1	19.6±3.2	16.2±2.2	16.2±2.2	15.8±3.5	15.2±1.8 [*]

	Reperfusion (min)			
	Baseline	Intervention	Occlusion	60 120 180
NAL (6 mg/kg)	22.4±3.6	18.9±2.6	16.0±5.3*	16.1±2.3* 15.3±3.0* 14.1±2.5*
He (3 Cycles) + NAL (6 mg/kg)	21.5±2.4	20.4±2.9	17.4±3.9*	16.2±3.8* 14.6±3.4* 14.5±3.7*
He (1 Cycle) + MOR (0.1 mg/kg) + NAL (6 mg/kg)	22.3±4.2	21.1±2.7	18.4±3.0	18.8±2.7 18.4±2.6* 18.2±3.0*

Data are mean±SD

* Significantly (P<0.05) different from baseline

Abbreviations: HR = heart rate; MAP = mean arterial pressure; RPP = rate pressure product; CON = control; He = helium; MOR = morphine; NAL = naloxone

Table 2

Left Ventricular Area at Risk

	N	LV (g)	AAR (g)	AAR/LV (%)
CON	7	3.26±0.43	1.20±0.41	36±9
He (1 Cycle)	7	3.46±0.28	1.25±0.11	36±4
He (3 Cycles)	7	3.84±0.28	1.30±0.08	34±2
MOR (0.1 mg/kg)	7	3.32±0.17	1.35±0.20	41±5
He (1 Cycle) + MOR (0.1 mg/kg)	6	3.30±0.22	1.23±0.14	37±3
NAL (6 mg/kg)	7	3.31±0.38	1.17±0.30	35±7
He (3 Cycles) + NAL (6 mg/kg)	7	3.22±0.63	1.36±0.40	42±5
He (1 Cycle) + MOR (0.1 mg/kg) + NAL (6 mg/kg)	6	3.27±0.38	1.39±0.20	42±5

Data are mean±SD

Abbreviations: LV = left ventricle; AAR = area at risk; CON = control; He = helium; MOR = morphine; NAL = naloxone