

# Role of Heat Shock Protein 90 and Endothelial Nitric Oxide Synthase during Early Anesthetic and Ischemic Preconditioning

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**Background:** Nitric oxide is known to be essential for early anesthetic preconditioning (APC) and ischemic preconditioning (IPC) of myocardium. Heat shock protein 90 (Hsp90) regulates endothelial nitric oxide synthase (eNOS) activity. In this study, the authors tested the hypothesis that Hsp90-eNOS interactions modulate APC and IPC.

**Methods:** Myocardial infarct size was measured in rabbits after coronary occlusion and reperfusion in the absence or presence of preconditioning within 30 min of isoflurane (APC) or 5 min of coronary artery occlusion (IPC), and with or without pretreatment with geldanamycin or radicicol, two chemically distinct Hsp90 inhibitors, or N<sup>G</sup>-nitro-L-arginine methyl ester, a nonspecific nitric oxide synthase NOS inhibitor. Isoflurane-dependent nitric oxide production was measured (ozone chemiluminescence) in human coronary artery endothelial cells or mouse cardiomyocytes, in the absence or presence of Hsp90 inhibitors or N<sup>G</sup>-nitro-L-arginine methyl ester. Interactions between Hsp90 and eNOS, and eNOS activation, were assessed with immunoprecipitation, immunoblotting, and confocal microscopy.

**Results:** APC and IPC decreased infarct size (by 50% and 59%, respectively), and this action was abolished by Hsp90 inhibitors. N<sup>G</sup>-nitro-L-arginine methyl ester blocked APC but not IPC. Isoflurane increased nitric oxide production in human coronary artery endothelial cells concomitantly with an increase in Hsp90-eNOS interaction (immunoprecipitation, immunoblotting, and immunohistochemistry). Pretreatment with Hsp90 inhibitors abolished isoflurane-dependent nitric oxide production and decreased Hsp90-eNOS interactions. Isoflurane did not increase nitric oxide production in mouse cardiomyocytes, and eNOS was below the level of detection.

**Conclusion:** The results indicate that Hsp90 plays a critical role in mediating APC and IPC through protein-protein interactions, and suggest that endothelial cells are important contributors to nitric oxide-mediated signaling during APC.

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GROWING evidence indicates that heat shock protein 90 (Hsp90) regulates endothelial nitric oxide synthase (eNOS) phosphorylation (Serine 1177)<sup>1</sup> and modulates subsequent nitric oxide production.<sup>1-5</sup> eNOS activity is regulated by protein-binding partners such as Hsp90,<sup>2,6,7</sup> and the association between Hsp90 and eNOS maintains the enzyme in a coupled state in which eNOS produces nitric oxide and not superoxide anion.<sup>6</sup> Activation of eNOS is associated with phosphorylation of Serine 1177, and nitric oxide production is proportional to the extent of eNOS phosphorylation.<sup>8</sup> Certain pathophysiological stimuli, such as hypoxia or angiotensin-1, have been demonstrated to enhance Hsp90-eNOS association and eNOS phosphorylation in porcine coronary artery endothelial cells, concomitantly with increased nitric oxide release.<sup>3,4</sup> This action was blocked by specific Hsp90 inhibitors.<sup>3,4,7</sup>

Early myocardial preconditioning is a cellular adaptive phenomenon whereby brief episodes of myocardial ischemia interspersed with reperfusion (ischemic preconditioning [IPC]) or exposure to certain pharmacological agents such as volatile anesthetics (anesthetic preconditioning [APC]), decreases the extent of ischemia and reperfusion injury after a subsequent prolonged period of coronary artery occlusion. IPC and APC activate phosphatidylinositol-3-kinase which stimulates extracellular signal-regulated kinase 1/2 and downstream serine/threonine protein kinase Akt that contributes to the activation of eNOS through phosphorylation of Serine 1177.<sup>9,10</sup> The abolition of eNOS activity blocks the cardioprotective effect of upstream proteins such as extracellular signal-regulated kinase 1/2 or 70-kDa ribosomal protein s6 kinase activated by isoflurane.<sup>11</sup> Nitric oxide has previously been demonstrated to enhance adenosine triphosphate-regulated potassium channel activation that mediates cardioprotection,<sup>10,12-14</sup> and this molecule has been suggested to be a trigger of IPC<sup>15</sup> and APC.<sup>16</sup> At least three nitric oxide synthase (NOS) isoforms contribute to nitric oxide production in the heart, although eNOS appears to play a major role in early myocardial preconditioning.<sup>12,14</sup> Unlike eNOS, the involvement of Hsp90 in APC or IPC has not been elucidated. Thus, we tested the hypothesis that Hsp90 and eNOS are critical triggers of APC and IPC by activating nitric oxide-dependent cardioprotective signaling.

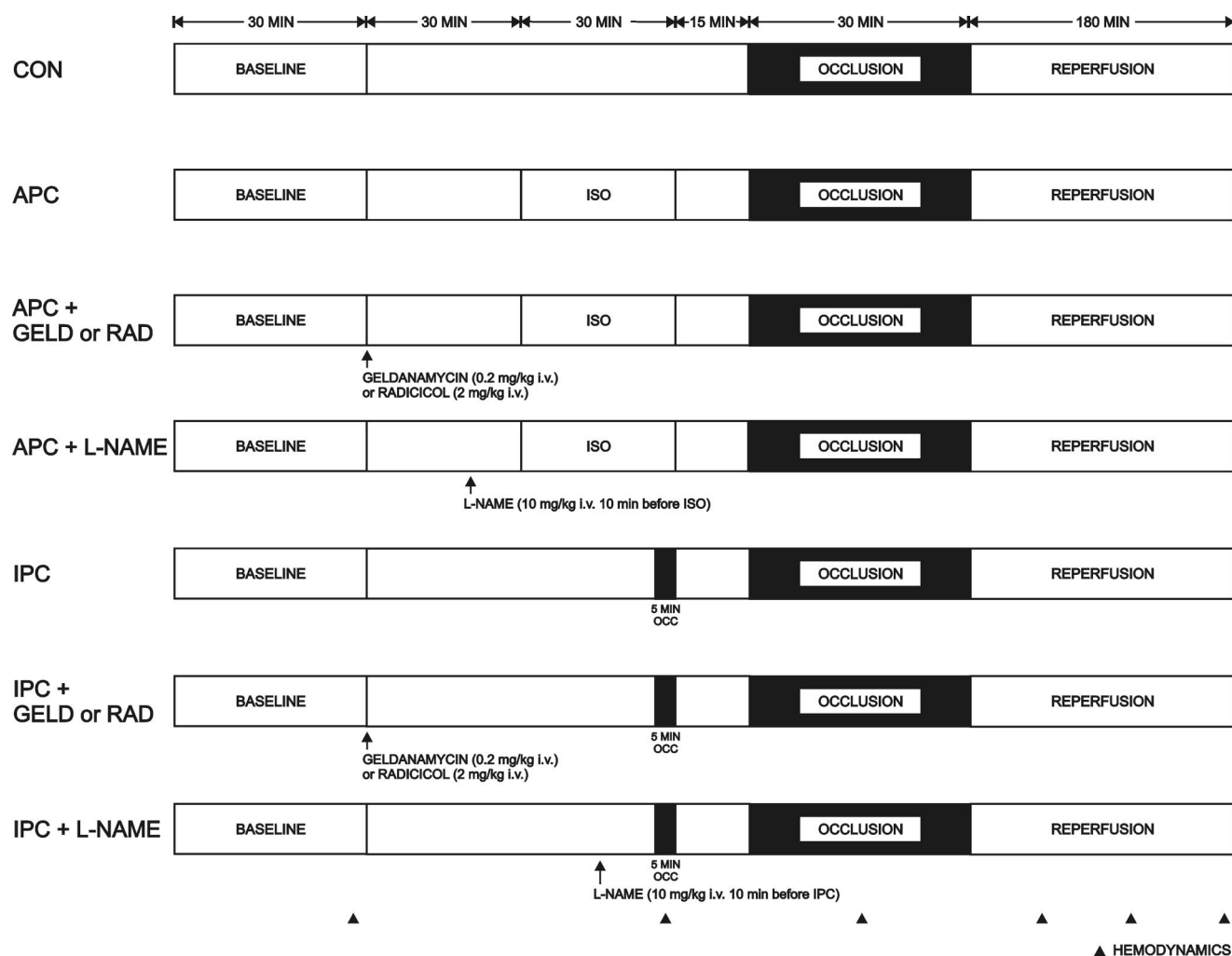


Fig. 1. Schematic illustration depicting the experimental protocols used to determine myocardial infarct size in rabbits *in vivo*. Additional experiments were completed with geldanamycin, radicicol, and L-NAME alone. APC = anesthetic preconditioning; CON = control; GELD = geldanamycin; IPC = ischemic preconditioning; ISO = isoflurane; L-NAME = *N*-nitro-L-arginine methyl ester; RAD = radicicol.

## Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional 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* of the American Physiologic Society, and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

### In Vivo Myocardial Infarction Model

Male New Zealand white rabbits were anesthetized with intravenous sodium pentobarbital (30 mg/kg) and instrumented as previously described.<sup>17</sup> Briefly, a tracheotomy was performed and rabbits were ventilated with positive pressure using an air-oxygen mixture (30% fractional inspired oxygen concentration). Arterial blood gas tensions and acid-base status were maintained within a normal physiologic range by adjusting the respiratory rate or tidal volume throughout the experiment. Hepa-

rin-filled catheters were positioned in the right carotid artery and the left jugular vein for continuous measurement of arterial blood pressure, and fluid and drug administration ( $0.9\%$  saline;  $15 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ), respectively. After a thoracotomy, a silk ligature was placed around the left anterior descending coronary artery, approximately halfway between the base and the apex, for the production of coronary artery occlusion and reperfusion. 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.

The experimental protocol is illustrated in figure 1. All rabbits underwent a 30-min left anterior descending coronary artery occlusion followed by 3 h of reperfusion. The rabbits were randomly assigned to receive 30 min of isoflurane  $2.1\%$  (1 minimal alveolar concentration, APC) or a single 5-min left anterior descending coronary artery occlusion (IPC), ending 15 min before prolonged coronary artery occlusion and reperfusion, in the presence or

absence of pretreatment with intravenous 0.9% saline (control), geldanamycin 0.2 mg/kg (Sigma-Aldrich, Saint Louis, MO)<sup>18</sup> or radicicol 2 mg/kg (A.G. Scientific, Inc., San Diego, CA),<sup>18</sup> two chemically distinct specific Hsp90 inhibitors or *N*-nitro-L-arginine methyl ester (L-NAME) 10 mg/kg (Sigma-Aldrich),<sup>19</sup> a non selective NOS inhibitor.

Myocardial infarct size was measured as previously described,<sup>20</sup> and expressed as a percentage of the left ventricular area at risk. Rabbits that developed intractable ventricular fibrillation and those with an area at risk less than 15% of total left ventricular mass were excluded from subsequent analysis.

#### *Human Coronary Artery Endothelial Cells and HL-1 Cardiomyocytes*

Human coronary artery endothelial cells isolated from healthy coronary arteries (Cell Applications, Inc., San Diego, CA) were cultured without cryopreservation, propagated to 5th passage in growth medium (Cell Applications, Inc.), and used for experiments between the 4th and 5th passage. HL-1 cardiomyocytes, derived from the AT-1 mouse atrial myocyte immortalized lineage (a gift from William C. Claycomb, Ph.D., Professor of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana) were maintained according to described protocols.<sup>21</sup> Cells were used for experiments when approximately 70 to 80% confluent. Human coronary artery endothelial cells and HL-1 cardiomyocytes were seeded on plastic dishes (100 mm) and pretreated at 37°C in growth medium with geldanamycin 17.8  $\mu$ M or radicicol 20  $\mu$ M for 3 h, or with NG-monomethyl-L-arginine monoacetate 1 mM (Sigma-Aldrich) for 12 h<sup>6,18</sup> before exposure to isoflurane or air (control). Cell growth medium was replaced with physiologic saline solution buffer (NaCl 137.0 mM, KCl 5.4 mM, MgCl<sub>2</sub> 1.0 mM, CaCl<sub>2</sub> 2.0 mM, HEPES 10.0 mM, D-Glucose 5.0 mM, pH 7.40) during ozone chemiluminescence experiments, because growth medium contains nitrite that would increase the background signal. The cells were exposed to isoflurane 0.42 mM (1 minimal alveolar concentration), continuously monitored by a gas analyzer (Poet IQ, Criticare Systems Inc., Waukesha, WI) at continuous air flow (0.7 l/min) in a specific incubator chamber (Billups-Rozenburg, Del-Mar, CA) maintained at 37°C. Because gas flow can induce a shear-stress-dependent nitric oxide release by itself,<sup>22</sup> the control group was exposed to air alone at the same flow rate.

#### *Ozone Chemiluminescence*

Nitrite concentration corresponding to the stable breakdown product of nitric oxide in aqueous solution was assessed by ozone chemiluminescence. Previous evidence suggests that nitric oxide release in response to stimuli in coronary endothelial cells peaks at 60 min.<sup>3</sup> Therefore, nitric oxide measurements were performed 60 min after isoflurane exposure. Samples (20  $\mu$ l) were refluxed in glacial acetic acid containing sodium iodide

and nitrite quantified in a nitric oxide chemiluminescence analyzer (Sievers Instruments Inc., Boulder, CO) as previously described.<sup>23</sup> Nitrite concentrations were calculated after subtraction of background levels and normalized to protein content (Bradford method).

#### *Immunoblotting and Coimmunoprecipitation*

Human coronary artery endothelial cells were lysed in 500  $\mu$ l of lysis buffer (20.0 mM 3-(N-morpholino)propanesulfonic acid, 2.0 mM EGTA, 5.0 mM EDTA, protease inhibitor cocktail (1:100; Sigma-Aldrich), phosphatase inhibitors cocktail (1:100; Calbiochem, San Diego, CA), 0.5% detergent (Nonidet P-40 detergent pH 7.4, Sigma-Aldrich) 20 min after the beginning of isoflurane exposure, because this time period corresponds to the peak of eNOS phosphorylation in coronary endothelial cells in response to various stimuli.<sup>3,4</sup> Fifteen to 25  $\mu$ g of proteins were loaded on to precast gel 7.5% tris-HCl gels (Criterion, Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene fluoride membranes. After blocking the membranes in 5% milk in tris-buffered saline, immunoblots were performed with rabbit monoclonal antiphospho-eNOS (1:1,000; Cell Signaling Technology, Inc., Danvers, MA), rabbit polyclonal anti-eNOS (1:5,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and mouse monoclonal anti-Hsp90 (1:1,000; Santa Cruz Biotechnology, Inc.), and were incubated overnight at 4°C. The membrane was then washed and incubated with secondary antibodies horseradish peroxidase-conjugated donkey antirabbit immunoglobulin G for eNOS (1:10,000; Santa Cruz Biotechnology, Inc.), goat antirabbit for phospho-eNOS (1:10,000; Bio-Rad) and goat antimouse for Hsp90 (1:8,000; Bio-Rad Laboratories). Coimmunoprecipitation was performed by incubating the cell lysates (200  $\mu$ g) with eNOS monoclonal antibody (2  $\mu$ g/mg of total cell protein) for 16 h at 4°C, followed by a 2-h incubation with a 1:1 protein A-to-protein G ratio of sepharose leads. After centrifugation, the immunoprecipitates were washed in PBS-T (Sigma-Aldrich), resuspended in Laemmli buffer, boiled for 5 min, loaded onto SDS-PAGE (Sigma-Aldrich) 7.5% gels, and transferred to a polyvinylidene fluoride membrane. The primary antibody used for immunoblotting was anti-eNOS (1:5,000, Santa Cruz Biotechnology, Inc.) while the secondary antibody was anti-Hsp90 (1:8,000; Bio-Rad Laboratories). The membranes were developed using the ECLplus Western blot chemiluminescence detection reagent (Bio-Rad Laboratories), and densitometric analysis was carried out by using image acquisition and analysis software (Scion Image; Scion Corp., Frederick, MD).

#### *Confocal Microscopy*

To visualize colocalization of Hsp90 and eNOS, human coronary artery endothelial cells were cultured on gelatin-coated slides, as previously described.<sup>19</sup> After 60 min of isoflurane exposure, human coronary artery endothe-



lial cells were fixed in 1% paraformaldehyde, permeabilized in 0.5% Triton  $\times$  100 (Sigma-Aldrich), and incubated for 30 min at 37°C with primary monoclonal antibody anti-eNOS (1:100; Biomol International, Plymouth, PA) in phosphate-buffered saline (PBS). Incubations with corresponding biotinylated secondary antibodies Alexa 488 conjugated (1:1,000; Invitrogen, Eugene, OR) were conducted for 30 min at 37°C. After washing with PBS, the cells were incubated for 15 min at 37°C with monoclonal antibody anti-Hsp90 (1:50; Santa Cruz Biotechnology, Inc.). Incubations with corresponding biotinylated secondary antibodies Alexa 546 conjugated (1:1,000; Invitrogen) were conducted for 30 min at 37°C followed by 1:1000 TO-PRO-3 (nuclear stain; Molecular Probes, Eugene, OR) for 5 min at room temperature and washed again with PBS. Images were visualized using confocal microscopy (Eclipse TE 200-U microscope with EZ C1 laser scanning software; Nikon, Melville, NY) using excitation wavelengths of 488 nm, 546 nm, and 663 nm; and emission wavelengths of greater than 520 nm, 578 nm, and 661 nm for eNOS, HSP90, and TO-PRO-3, respectively. The number of double-stained cells indicating colocalization of Hsp90 and eNOS were counted and expressed as a percentage of the total cell count.

#### High-Performance Liquid Chromatography

Superoxide radical anion measurements have been performed using a hydroethidine probe and high-performance liquid chromatography-based quantitation of 2-hydroxyethidium in cell lysates as described elsewhere.<sup>24</sup> Briefly, hydroethidine (10  $\mu$ M, Sigma-Aldrich) was added immediately before isoflurane exposure. After 60 min of isoflurane exposure, the cells were washed twice with ice-cold Dulbecco's PBS (Sigma-Aldrich). The cells were pelleted and then lysed in ice-cold Dulbecco's PBS containing 0.1% Triton  $\times$  100, and lysate aliquots mixed (1:1) with 0.2 M perchloric acid in methanol. After initial centrifugation (30 min  $\times$  20,000 g at 4°C) the supernatants were mixed (1:1) with 1 M potassium phosphate buffer pH 2.6 and centrifuged again (15 min  $\times$  20,000 g at 4°C). The supernatants were obtained for high-performance liquid chromatography analysis. The quantitation of hydroethidine and its oxidation products was carried out *via* high-performance liquid chromatography (ESA Biosciences Inc., Chelmsford, MA) with electrochemical detection using a Synergi Polar RP column (250 mm  $\times$  4.6 mm, 4  $\mu$ m; Phenomenex, Torrance, CA). The results were normalized to protein concentration in the lysates, as analyzed using Bradford reagent. All the reagents, additions, and incubations were protected from exposure to light.

#### Statistics

Data were expressed as mean  $\pm$  SD. Comparison of two means was performed using the Student *t* test. Comparison of several means was performed using one-

way (one factor tested) or two-way (two factors tested) analysis of variance, when appropriate, and the *post hoc* test used was the Newman-Keuls test. Hemodynamic data were analyzed with repeated measures ANOVA. All *P* values were two-tailed, and a *P* value  $< 0.05$  was considered significant. Statistical analysis was performed using NCSS 2007 software (Statistical Solutions Ltd., Cork, Ireland).

## Results

### *Involvement of Hsp90 and eNOS in APC and IPC in Vivo*

One hundred and four rabbits were instrumented to obtain 96 successful experiments in which infarct size was measured. Eight rabbits were excluded, because intractable ventricular fibrillation occurred during coronary artery occlusion (two in the control group, two in the geldanamycin group, three in the radicicol group, and one in the L-NAME group, respectively). Arterial blood gas tensions were maintained within the physiologic range in each group (data not shown). Systemic hemodynamics were similar at baseline among groups. L-NAME caused a brief increase in mean arterial pressure and decrease in heart rate (table 1), as compared with the control experiments. Left ventricular mass, area-at-risk mass, and the ratio of area at risk to left ventricular mass were not different between groups (table 2). APC and IPC decreased myocardial infarct size ( $23 \pm 6\%$  and  $19 \pm 7\%$  of the left ventricular area at risk, respectively), as compared with control experiments ( $46 \pm 2\%$ ,  $P < 0.05$ ) (fig. 2). Geldanamycin, radicicol, or L-NAME alone did not affect infarct size ( $42 \pm 6\%$ ,  $47 \pm 2\%$ , and  $45 \pm 10\%$ , respectively), but geldanamycin and radicicol abolished APC ( $46 \pm 2\%$  and  $46 \pm 3\%$ , respectively) and IPC ( $47 \pm 3\%$  and  $45 \pm 2\%$ , respectively). L-NAME prevented infarct size reduction with APC ( $46 \pm 2\%$ ) but not IPC ( $24 \pm 4\%$ ).

### *Isoflurane-dependent Nitric Oxide Production in Human Coronary Artery Endothelial Cells and HL-1 Cardiomyocytes*

Isoflurane significantly increased nitric oxide production in human coronary artery endothelial cells, as compared with control experiments ( $141 \pm 19$  vs.  $50 \pm 6$  nmoles/mg of protein, respectively;  $P < 0.05$ ; fig. 3). Pretreatment with geldanamycin, radicicol, and *N*-G-monomethyl-L-arginine monoacetate abolished isoflurane-dependent nitric oxide production. In contrast, isoflurane did not increase nitric oxide production in HL-1 cardiomyocytes, as compared with the control ( $190 \pm 28$  vs.  $181 \pm 27$  nmoles/mg of protein, respectively; not significant).

**Table 1. Hemodynamics**

	Baseline	Intervention	Occlusion	Reperfusion (min)		
				60	120	180
HR (min <sup>-1</sup> )						
CON	256 ± 37	258 ± 30	251 ± 30	233 ± 20	227 ± 21	219 ± 25*
APC	259 ± 26	261 ± 22	240 ± 25	232 ± 27	226 ± 31	223 ± 34*
IPC	255 ± 18	237 ± 17	246 ± 21	223 ± 20*	211 ± 28*	197 ± 23*
GELD	263 ± 24	245 ± 22	230 ± 25*	229 ± 21*	212 ± 22*	206 ± 26*
APC + GELD	246 ± 23	241 ± 19	225 ± 12	213 ± 14*	203 ± 15*	201 ± 16*
IPC + GELD	251 ± 17	238 ± 28	229 ± 35	223 ± 27	211 ± 31*	199 ± 26*
RAD	251 ± 23	233 ± 18	234 ± 29	233 ± 27	223 ± 23	218 ± 21
APC + RAD	245 ± 27	245 ± 24	248 ± 19	239 ± 27	226 ± 22	219 ± 23
IPC + RAD	253 ± 26	233 ± 22	241 ± 22	223 ± 23*	213 ± 22*	212 ± 23*
L-NAME	246 ± 23	206 ± 22*†	213 ± 19*	188 ± 26*†	186 ± 17*	187 ± 13*
APC + L-NAME	250 ± 18	219 ± 25*†	223 ± 21	222 ± 21‡	220 ± 21	215 ± 23*
IPC + L-NAME	259 ± 22	229 ± 15	231 ± 27	226 ± 41	212 ± 44*	200 ± 33*
MAP (mmHg)						
CON	74 ± 7	70 ± 5	59 ± 6*	60 ± 10*	62 ± 11*	63 ± 10
APC	78 ± 14	60 ± 13	67 ± 15	69 ± 17	68 ± 13	69 ± 14
IPC	76 ± 7	64 ± 9	61 ± 7*	65 ± 11	70 ± 8	70 ± 14
GELD	72 ± 12	74 ± 5	60 ± 10*	64 ± 8	64 ± 8	63 ± 8
APC + GELD	60 ± 7	61 ± 13	53 ± 6	53 ± 6	56 ± 7	56 ± 6
IPC + GELD	69 ± 8	65 ± 8	61 ± 16	60 ± 7	59 ± 9	64 ± 12
RAD	60 ± 5	67 ± 13	56 ± 15	58 ± 10	55 ± 11	56 ± 11
APC + RAD	71 ± 17	84 ± 14‡	68 ± 12	62 ± 8*	60 ± 10*	63 ± 10*
IPC + RAD	69 ± 16	76 ± 12	61 ± 8	61 ± 10	64 ± 6	63 ± 6
L-NAME	81 ± 8	88 ± 14†	62 ± 20	64 ± 17	63 ± 13	69 ± 13
APC + L-NAME	65 ± 10	54 ± 20‡	63 ± 5‡	55 ± 15	65 ± 10	59 ± 11
IPC + L-NAME	66 ± 6	75 ± 14‡	69 ± 17‡	69 ± 20	75 ± 18	78 ± 17

Data are mean ± SD; n=8 per group.

\*  $P < 0.05$  vs. baseline; †  $P < 0.05$  vs. corresponding control; ‡  $P < 0.05$  vs. corresponding drug alone (GELD, RAD, L-NAME).

APC = anesthetic preconditioning (isoflurane, 1 minimal alveolar concentration); CON = control; GELD = geldanamycin; HR = heart rate; IPC = ischemic preconditioning; L-NAME = *N*-nitro-L-arginine methyl ester; MAP = mean arterial pressure; RAD = radicolol.

### *Hsp90/eNOS Association and eNOS Activation in Human Coronary Artery Endothelial Cells and HL-1 Cardiomyocytes*

Isoflurane increased the ratio of phospho-eNOS to total eNOS twofold ( $0.56 \pm 0.26$  vs.  $0.28 \pm 0.08$  in isoflurane vs. control cells, respectively) (fig. 4), and this occurred concomitantly with increased association between Hsp90 and total eNOS, as evaluated with immunopre-

cipitation, immunoblotting (fig. 5), and colocalization assessed by confocal microscopy (fig. 6). Pretreatment with geldanamycin, radicolol, and *N*-G-monomethyl-L-arginine monoacetate abolished isoflurane-induced eNOS activation (phospho-eNOS to total eNOS). In HL-1 cardiomyocytes, total eNOS and phospho-eNOS were below detection levels of Western blot analysis (data not shown).

**Table 2. Left Ventricular Area at Risk**

	Body Weight (g)	LV (g)	AAR (g)	AAR/LV (%)
CON	2,367 ± 310	3.35 ± 0.29	1.41 ± 0.18	42 ± 5
APC	2,534 ± 422	3.07 ± 0.39	1.09 ± 0.11	36 ± 5
IPC	2,555 ± 211	3.16 ± 0.59	1.16 ± 0.41	36 ± 9
GELD	2,504 ± 217	3.14 ± 0.36	1.11 ± 0.16	36 ± 7
APC + GELD	2,210 ± 97	3.28 ± 0.32	1.44 ± 0.23	44 ± 4
IPC + GELD	2,500 ± 120	3.60 ± 0.51	1.36 ± 0.38	37 ± 8
RAD	2,185 ± 57	3.12 ± 0.25	1.39 ± 0.15	44 ± 3
APC + RAD	2,160 ± 106	3.22 ± 0.29	1.32 ± 0.23	41 ± 5
IPC + RAD	2,569 ± 169*	3.80 ± 0.49	1.46 ± 0.25	39 ± 4
L-NAME	2,749 ± 147†	3.14 ± 0.66	1.24 ± 0.41	39 ± 8
APC + L-NAME	2,288 ± 113*	3.43 ± 0.35	1.37 ± 0.23	40 ± 5
IPC + L-NAME	2,238 ± 169*	3.15 ± 0.18	1.11 ± 0.23	35 ± 6

Data are mean ± SD, n = 8 per group.

\*  $P < 0.05$  vs. corresponding drug alone (GELD, RAD, L-NAME); †  $P < 0.05$  vs. Control.

AAR = area at risk; APC = anesthetic preconditioning (isoflurane, 1 minimal alveolar concentration); CON = control; GELD = geldanamycin; IPC = ischemic preconditioning; L-NAME = *N*-Nitro-L-arginine methyl ester; LV = left ventricle; RAD = radicolol.

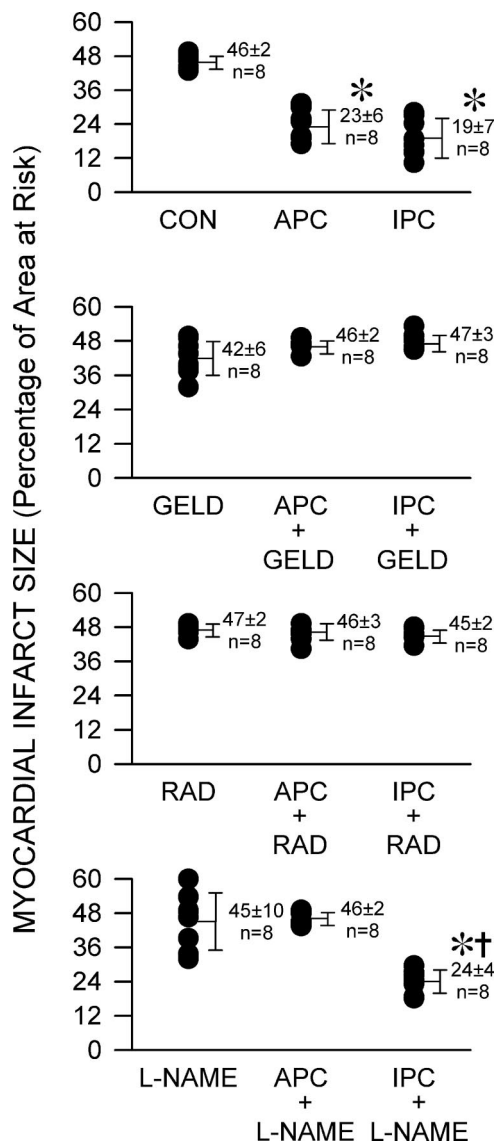


Fig. 2. Myocardial infarct size depicted as a percentage of left ventricular area at risk in rabbits in the absence (CON) or presence of anesthetic (APC) or ischemic preconditioning (IPC), and in the presence or absence of pretreatment with geldanamycin (GELD, 0.2 mg/kg), radicicol (RAD, 2 mg/kg), or *N*-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg). Each point represents a single experiment. Data are mean  $\pm$  SD; n = 8 per group. \*  $P < 0.05$  versus CON; †  $P < 0.05$  versus L-NAME alone. CON = control.

#### Superoxide Anion Production by Isoflurane

Superoxide anion production in human coronary artery endothelial cells was not altered in control experiments, as compared with isoflurane treatment ( $0.14 \pm 0.08$  vs.  $0.16 \pm 0.07$  nmoles/mg of protein, respectively; n = 13 in each group; not significant).

## Discussion

The present results demonstrate the importance of Hsp90 as a modulator of eNOS in APC and IPC *in vivo*. Isoflurane preconditioning and IPC significantly de-

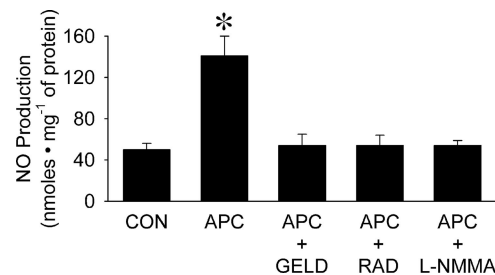


Fig. 3. Effect of isoflurane (anesthetic preconditioning [APC], 0.42 mm or 1 minimal alveolar concentration) on nitric oxide (NO) production, as compared with control experiments in the absence of volatile anesthetic (CON) in human coronary artery endothelial cells with or without pretreatment with geldanamycin (GELD, 17.8  $\mu$ M), radicicol (RAD, 20  $\mu$ M), or *N*-G-mono-methyl-L-arginine monoacetate (L-NMMA, 1 mM). Data are mean  $\pm$  SD. \*  $P < 0.05$  versus CON. n = 6 per group.

creased myocardial infarct size, as compared with control experiments, and this protection was abolished by the specific Hsp90 inhibitors geldanamycin or radicicol. The findings further indicate that Hsp90 association with eNOS is increased by isoflurane in human coronary artery endothelial cells and that enhanced protein-protein interactions contribute to eNOS activation and increased nitric oxide production during APC.

A growing body of evidence implicates eNOS-derived nitric oxide as a critical component of APC signal transduction. We have previously demonstrated that the non-selective NOS inhibitor L-NAME blocked the trigger and mediator phase of delayed APC, whereas specific inhibitors of inducible NOS or neuronal NOS had no effect.<sup>19</sup> Endothelial NOS expression (message and protein) was increased immediately, and 24 h after exposure to isoflu-

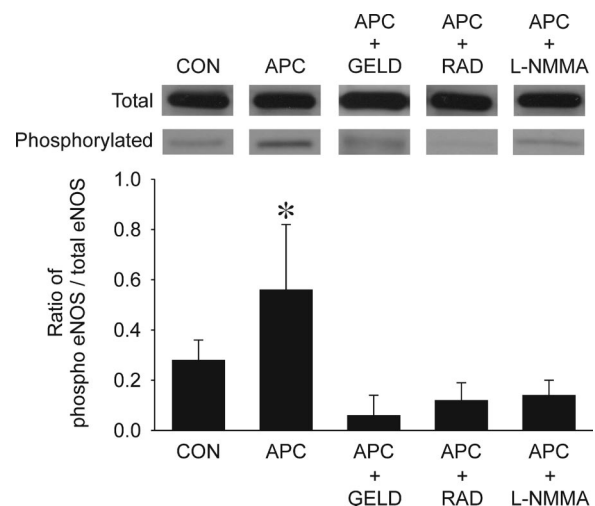
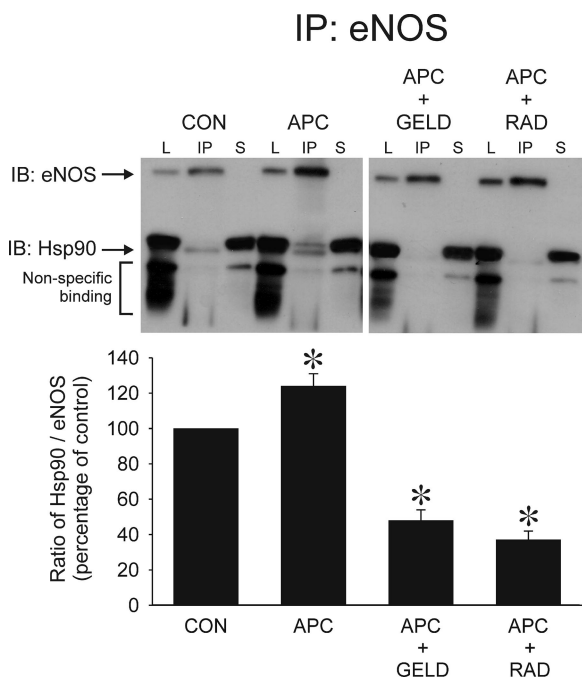


Fig. 4. Representative Western blot of total and phosphorylated endothelial nitric oxide synthase (eNOS) in human coronary artery endothelial cells exposed to isoflurane (anesthetic preconditioning [APC], 0.42 mm or 1 minimal alveolar concentration), as compared with control (CON) experiments in the absence of volatile anesthetic with or without pretreatment with geldanamycin (GELD, 17.8  $\mu$ M), radicicol (RAD, 20  $\mu$ M), or *N*-G-mono-methyl-L-arginine monoacetate (L-NMMA, 1 mM). Histograms show the ratio of phospho-eNOS to total-eNOS in each group. Data are mean  $\pm$  SD. \*  $P < 0.05$  versus CON; n = 6 per group.

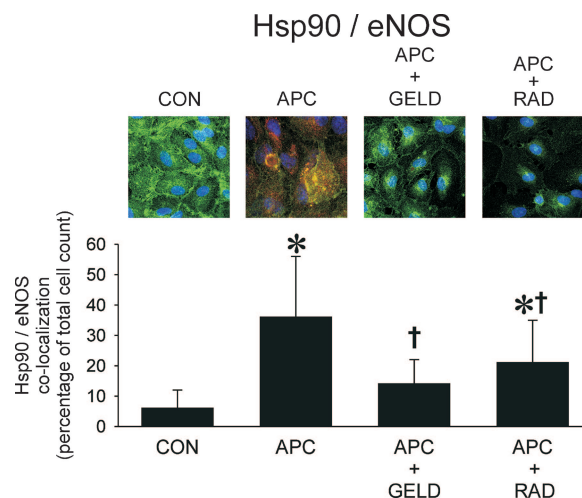




**Fig. 5.** Immunoprecipitation (IP) of endothelial nitric oxide synthase (eNOS) and immunoblotting (IB) for eNOS and heat shock protein 90 (Hsp90) in human coronary artery endothelial cells exposed to isoflurane (anesthetic preconditioning [APC], 0.42 mm or 1 minimal alveolar concentration), as compared with control experiments (CON) with or without pretreatment with geldanamycin (GELD, 17.8  $\mu$ M) or radicicol (RAD, 20  $\mu$ M). Histograms show the ratio of Hsp90/eNOS in each group. eNOS and Hsp90 bands are indicated by arrows. Immunoreactive bands that appear below Hsp90 indicate nonspecific binding and were not included in the analysis. Lysate (L) represents total protein before immunoprecipitation. Supernatant (S) represents protein after immunoprecipitation of eNOS. Data are mean  $\pm$  SD. \*  $P < 0.05$  versus CON;  $n = 5$  per group.

rane<sup>19</sup> and postconditioning with isoflurane was mediated through eNOS-sensitive regulation of the pro-survival phosphatidylinositol-3-kinase serine/threonine protein kinase Akt signaling cascade.<sup>11</sup> APC with the volatile anesthetic desflurane was also shown to be mediated by eNOS-derived nitric oxide.<sup>25</sup> APC increased eNOS activity measured in left ventricular myocardium and inhibition of eNOS abolished infarct size reduction when a NOS inhibitor was administered either before or after desflurane.

The current results confirm and extend these previous findings, and indicate that APC is critically dependent on both eNOS and Hsp90. Hsp90 is an important physiologic regulator of eNOS, and modulation of APC or IPC by Hsp90 has not been previously elucidated. Hsp90 is a highly conserved, mostly cytosolic protein expressed in all eukaryotic cells,<sup>26</sup> and is likely to modulate APC and IPC through an evolutionary conserved cellular response.<sup>27</sup> This highly abundant protein is a molecular chaperone involved in protein folding and maturation. Recent evidence indicates that Hsp90 has an integral role in cell signal transduction pathways, and its critical importance in the cell is illustrated by the lethality of its



**Fig. 6.** Colocalization of endothelial nitric oxide synthase (eNOS) with heat shock protein 90 (Hsp90) in human coronary artery endothelial cells exposed to isoflurane (anesthetic preconditioning [APC], 0.42 mm or 1 minimal alveolar concentration), as compared with control experiments (CON) with or without pretreatment with geldanamycin (GELD, 17.8  $\mu$ M) or radicicol (RAD, 20  $\mu$ M). The eNOS/Hsp90 colocalization is demonstrated by yellow staining. Data are mean  $\pm$  SD. \*  $P < 0.05$  versus CON; †  $P < 0.05$  versus APC; 11–18 fields per group.

homozygous disruption in *Drosophila*.<sup>28</sup> Hsp90 is a physiologic binding partner and regulator of eNOS,<sup>29,30</sup> and impairment of eNOS/Hsp90 interactions disrupts nitric oxide-dependent signaling. Endothelial NOS and Hsp90 form complexes in endothelial and smooth muscle cells in response to a variety of eNOS-activating stimuli. This action enhances phosphorylation of eNOS by serine/threonine protein kinase Akt,<sup>31</sup> increases nitric oxide release,<sup>2</sup> and facilitates cyclic guanosine monophosphate production.<sup>26</sup> In contrast, specific inhibitors of Hsp90, such as geldanamycin and radicicol, affect the conformational state of Hsp90 by binding to its unique adenosine triphosphate binding site<sup>32</sup>, and prevent agonist-induced eNOS-Hsp90-serine/threonine protein kinase Akt association and eNOS phosphorylation.<sup>31</sup>

Hsp90/eNOS association may be of importance in ischemic myocardium. Chronic hypoxia increases resistance to myocardial ischemia and reperfusion injury through a nitric oxide-mediated mechanism. This protection is dependent on enhancement of Hsp90/eNOS association and increased nitric oxide production that is blocked by geldanamycin.<sup>7</sup> Hsp90 appears to confer a benefit in chronically hypoxic myocardium by promoting nitric oxide generation and limiting superoxide anion production.<sup>7</sup> Targeted overexpression of Hsp90 in myocardium reduces infarct size and enhances the association between Hsp90, eNOS, and Akt, resulting in increased phosphorylation of eNOS (at Serine 1177).<sup>1</sup> In contrast to these beneficial effects, disrupting the interactions between Hsp90 and eNOS in endothelial cells uncouples enzyme activity. As a result of eNOS uncoupling, the enzyme produces superoxide anion and not nitric oxide,

and increased superoxide anion production is inhibitable with L-NAME.<sup>6,18,33</sup>

The present results demonstrate that Hsp90 is a crucial element in IPC and APC *in vivo*. Furthermore, experiments conducted in human coronary artery endothelial cells show that isoflurane increases eNOS activity, and that this action is dependent on enhanced association between Hsp90 and eNOS as demonstrated with coimmunoprecipitation and immunohistochemistry. In contrast, isoflurane failed to increase eNOS activity or nitric oxide production in cardiomyocytes. While isoflurane increased nitric oxide concentration in human coronary artery endothelial cells, the concentration of superoxide anion was unaltered. The function of eNOS to generate nitric oxide or superoxide anion is thought to be regulated by site-specific phosphorylation,<sup>34</sup> tetrahydrobiopterin concentrations,<sup>35</sup> and Hsp90.<sup>6</sup> Isoflurane did not change superoxide anion concentrations under baseline conditions, but it is possible that isoflurane might decrease superoxide generation under conditions where eNOS is uncoupled (e.g., during hyperglycemia), and in a manner that is Hsp90-dependent. This hypothesis remains to be tested, however. Isoflurane has been shown to increase the production of signaling reactive oxygen species from mitochondria of cardiomyocytes.<sup>36</sup> In contrast, isoflurane had no effect on superoxide anion production from endothelial cells. Thus, it is unlikely that endothelial cells are a source of signaling reactive oxygen species during APC.

The role of eNOS during classic IPC is controversial.<sup>14</sup> Prolonged ischemia and reperfusion is associated with the loss of cardiac endothelial NOS protein, and IPC completely prevents loss of NOS protein and increases NOS activity and cyclic guanosine monophosphate levels.<sup>37</sup> Genetic models demonstrate a role for eNOS and nitric oxide during early IPC. Myocardial ischemia and reperfusion injury is attenuated in mice with myocyte-specific overexpression of eNOS<sup>38</sup> and infarct size is reduced in transgenic mice overexpressing either bovine or human eNOS.<sup>39</sup> Conversely, myocardial infarct size is markedly increased in eNOS knockout mice.<sup>40</sup> In the present investigation, infarct size reduction by APC was blocked by L-NAME, but this NOS inhibitor did not abolish the protection of IPC. IPC may be a more robust stimulus for eliciting cardioprotective signaling than APC, and it is possible that a higher concentration of L-NAME might be necessary to block IPC, as compared with APC. There may also be greater redundancy of signaling pathways in IPC, only one of which includes nitric oxide as a critical intermediate. In contrast to results with L-NAME, inhibitors of Hsp90 blocked both APC and IPC. These results could suggest that Hsp90 may have binding partners in addition to or as an alternative to eNOS during cardioprotection, and that these depend on the specific mechanical (IPC) or pharmacological (APC) stimulus that induces protection. In addition, the role of APC or IPC to enhance Hsp90 association with NOS isoforms in cardiomyocytes is unknown, and is the subject of

ongoing investigations in our laboratory. Nonetheless, the results indicate that Hsp90 plays an important role to modulate cardioprotection during diverse preconditioning stimuli. Other heat shock proteins, such as Hsp70, may also contribute to cardioprotection.<sup>41</sup> Cooverexpression of Hsp70 and Hsp90 increases eNOS protein in endothelial cells; however, unlike Hsp90, there is no evidence to indicate that Hsp70 or other heat shock proteins regulate eNOS coupling.<sup>42</sup>

The current results should be interpreted within the constraints of several potential limitations. L-NAME produced brief hemodynamic effects during *in vivo* experiments that may have theoretically contributed to alterations in myocardial infarct size. However, L-NAME produced similar hemodynamic effects during IPC and APC, yet infarct size reduction was blocked only in the APC group. Thus, it is unlikely that hemodynamic changes alone substantially contributed to the results. However, myocardial oxygen consumption was not directly measured in the current investigation. Experiments were also completed in normal animals, and results might be different in diseased<sup>43</sup> or aged myocardium.<sup>44</sup> The area at risk for infarction is an important determinant of myocardial infarct size in rabbits, but there were no differences in this variable among groups that could account for the current findings. Experiments were completed with two chemically distinct Hsp90 inhibitors. A limitation of the use of geldanamycin in the current investigation is that this drug undergoes oxidation and reduction cycling, and this cycling can produce superoxide anion. In contrast, radicicol does not demonstrate redox cycling, nor does this drug produce reactive oxygen species. Thus, the results cannot be explained by redox-sensitive effects of pharmacological inhibitors. The role of Hsp90 and eNOS during anesthetic preconditioning was investigated using isoflurane. A recent study confirmed the cardioprotective effects of sevoflurane, another halogenated anesthetic, in human endothelial cells.<sup>45</sup> Whether Hsp90 is involved in cardioprotection produced by other volatile anesthetic agents is unknown. The effects of isoflurane to modulate Hsp90 interactions and nitric oxide production were examined in human endothelial cells and in mouse HL-1 cardiomyocytes *in vitro*. Results may be different in adult rabbit or human cardiomyocytes. Interactions between endothelial cells and cardiomyocytes during cardioprotection are unknown and are an important focus for future investigations.

In conclusion, the current results indicate that Hsp90 has an important function to modulate APC and IPC *in vivo*. APC enhances the association between Hsp90 and eNOS, resulting in activation of this enzyme and enhanced production of nitric oxide in endothelial cells, but not in cardiomyocytes. The results demonstrate that protein-protein interactions are essential to the process of cardioprotection and further suggest a potential role for endothelial cell-cardiomyocyte coupling during APC and IPC.



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