Isoflurane induced eNOS signaling and cardioprotection

Baotic, Ines

Chapter 2

Isoflurane Favorably Modulates Guanosine Triphosphate Cyclohydrolase-1 and Endothelial Nitric Oxide Synthase during Myocardial Ischemia and Reperfusion Injury in Rats

Ines Baotic, M.D., Dorothee Weihrauch, Ph.D., Jesse Procknow, Ph.D., Jeanette Vasquez-Vivar, Ph.D., Zhi-Dong Ge, M.D., Ph.D., Shaan Sudhakaran, M.D., David C. Warltier, M.D., Ph.D., Judy R. Kersten, M.D.

From the Department of Anesthesiology (I.B., D.W., J.P., Z.-D.G., S.S., D.C.W., J.R.K.), Department of Biophysics, Redox Biology Program (J.V.-V.), and Department of Pharmacology and Toxicology (D.C.W., J.R.K.), Medical College of Wisconsin, Milwaukee, Wisconsin.

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ABSTRACT

Background: The authors investigated the hypothesis that isoflurane modulates nitric oxide (NO) synthesis and protection against myocardial infarction through time-dependent changes in expression of key NO regulatory proteins, guanosine triphosphate cyclohydrolase (GTPCH)-1, the rate-limiting enzyme involved in the biosynthesis of tetrahydrobiopterin and endothelial nitric oxide synthase (eNOS).

Methods: Myocardial infarct size, NO production (ozone-mediated chemiluminescence), GTPCH-1, and eNOS expression (real-time reverse transcriptase polymerase chain reaction and western blotting) were measured in male Wistar rats with or without anesthetic preconditioning (APC; 1.0 minimum alveolar concentration isoflurane for 30 min) and in the presence or absence of an inhibitor of GTPCH-1, 2,4-diamino-6-hydroxypyrimidine.

Results: NO^-_2 production (158 ± 16 and 150 ± 13 pmol/mg protein at baseline in control and APC groups, respectively) was significantly ($P < 0.05$) increased 1.5 ± 0.1 and 1.4 ± 0.1 fold by APC (n = 4) at 60 and 90 min of reperfusion, respectively, concomitantly, with increased expression of GTPCH-1 (1.3 ± 0.3 fold; n = 5) and eNOS (1.3 ± 0.2 fold; n = 5). In contrast, total NO (NO^-_2 and NO^-_3) was decreased after reperfusion in control experiments. Myocardial infarct size was decreased (43 ± 2% of the area at risk for infarction; n = 6) by APC compared with control experiments (57 ± 1%; n = 6). 2, 4-Diamino-6-hydroxypyrimidine decreased total NO production at baseline (221 ± 25 and 175 ± 31 pmol/mg protein at baseline in control and APC groups, respectively), abolished isoflurane-induced increases in NO at reperfusion, and prevented reductions of myocardial infarct size by APC (60 ± 2%; n = 6).

Conclusion: APC favorably modulated a NO biosynthetic pathway by up-regulating GTPCH-1 and eNOS, and this action contributed to protection of myocardium against ischemia and reperfusion injury.

A GROWING body of evidence implicates endothelial nitric oxide synthase (eNOS)–derived nitric oxide (NO) as a critical mediator of anesthetic preconditioning (APC)[1] and also suggests that an NO biosynthetic path-way is importantly modulated by disease states.[2] Three distinct nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and eNOS, contribute to NO production in the heart[3] ; however, eNOS, but not nNOS or iNOS, seems to play a major role during APC.[1,4] We previously demonstrated that the trigger and mediator phases of delayed pre-

conditioning with isoflurane were blocked by the nonselective NOS inhibitor, l-NG-nitroarginine methyl ester, whereas selective inhibitors of nNOS or iNOS had no effect.[4] Isoflurane increases the phosphorylation of serine 1177 on eNOS and stimulates NO production in human coronary artery endothelial cells and preconditions myocardium against infarction through an eNOS-sensitive pathway.[1] However, the precise mechanisms whereby isoflurane modulates NO biosynthesis are incompletely understood. eNOS activity is regulated by intracellular localization, posttranslational modifications, protein–protein interactions, and tetra-hydrobiopterin (BH4) cofactor availability.[2] The current investigation examined the hypothesis that isoflurane protects myocardium against ischemia and reperfusion injury by a time-dependent modulation of NO biosynthetic pathway gene and protein expression.

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 (2011).

In vivo Myocardial Infarction Model

Adult male Wistar rats (n = 254; 8 to 12 weeks old), weighing 300 to 360 g were anesthetized with thiobutabarbital sodium (100 mg/kg, intraperitoneal) and instrumented for the measurement of systemic hemodynamics as described previously.[5] Briefly, heparin- filled catheters were inserted into the right jugular vein and the right carotid artery for fluid administration and measurement of arterial blood pressure, respectively. A tracheotomy was performed, and rats were ventilated with positive pressure ventilation using an air and oxygen mixture. A left thoracotomy was performed in the fifth intercostal space, and the pericardium was opened. A 6-0 prolene ligature was placed around the proximal left anterior descending coronary artery and vein in the area immediately below the left atrial appendage for the production of coronary artery occlusion and reperfusion.

The experimental protocol is illustrated in figure 1. Following instrumentation and after 30 min of stabilization, rats were randomly assigned to 30-min preconditioning with isoflurane (1.3%; 1 minimum alveolar concentration) followed by washout for 15 min, or no treatment with volatile agent (control group), and in the presence or absence of an inhibitor of guanosine triphosphate cyclohydrolase (GTPCH)-1, 2,4-diamino-6-hydroxypy-rimidine (DAHP; 200 mg/kg, intraperitoneal) in separate experimental groups. Myocardial infarct size was measured by triphenyltetrazolium chloride staining and expressed as a percentage of the area at risk (AAR) for infarction as described previously. [5] In additional groups of rats, left ventricular (LV) tissue samples were collected at the following time points: before coronary artery occlusion; immediately before reperfusion; and after 30, 60, and 90 min of reperfusion.

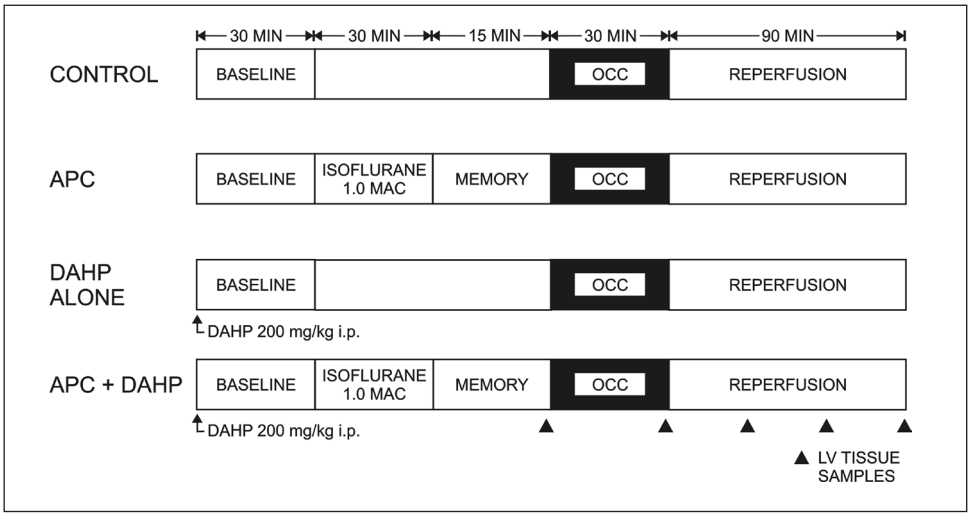

Fig. 1. Schematic diagram depicting the experimental protocols used to determine myocardial infarct size and modulation of nitric oxide in rats *in vivo*.

APC = anesthetic preconditioning; DAHP = 2,4-diamino-6-hydroxypyrimidine; LV = left ventricle; MAC = minimum alveolar concentration; OCC = coronary artery occlusion.

Tissue NO^-_2 and NO_x Analysis

NO^-_2 and total NO (NO_x: NO^-_2 and NO^-_3) concentrations from LV samples were quantified by ozone-mediated chemiluminescence. Tissue samples were rinsed, snap frozen in liquid nitrogen, pulverized and homogenized in buffer containing (150 mM NaCl, 20 mM Tris,1 mM EDTA,1 mM EGTA,1% Triton v/v, pH 7.5) followed by homogenization. Homogenates were centrifuged, and 250 µg of supernatant was filtered (Amicon® Ultra Centrifugal Filter, 10,000 MWCO, Millipore Corporation, USA) by centrifugation for 30 min at 12,000 rpm and 4°C (Microfuge R 22R Centrifuge, Beckman Coulter, USA). Samples (30 µl) were refluxed in reaction solution (50 mg KI in 1 ml of double-distilled water) mixed with glacial acetic acid (4 ml), and NO^-_2 was quantified by a chemiluminescence detector (Sievers 280 model NO analyzer, GE Analytical Instruments, USA) as described previously.[6] For NOx measurement, a 20-µl sample was injected into the reaction chamber of the NO analyzer containing a heated (95°C) solution of vanadium chloride and hydrochloric acid, which reduces NO^-_2 and NO^-_3 to NO, as described previously.[7] Each sample was analyzed in triplicate. NO^-_2 and NOx concentrations were calculated after subtraction of background levels and normalized to protein content (Bradford method).

eNOS and GTPCH-1 Expression

Gene expression in LV samples was quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) at five selected time points. Tissue was homogenized using a TissueLyser LT (Qiagen, USA). Total RNA was isolated using RNeasy Mini Kit (Qiagen) and treated with RNase-free DNAse (Qiagen) to remove residual DNA contamination. The quality and quantity of RNA was determined by UV-vis spectrophotometry (NanoDrop® ND-1000, Nano-Drop Technologies, USA). Only samples with 260/280-nm absorbance ratios between 1.8 and 2 were used for further analysis. Immediately after the quality control assessment, reverse transcription of total RNA samples to cDNA was performed using iScript cDNA synthesis Kit (Bio-Rad, USA). RT-PCR was performed using SYBR Green chemistry (iQ SYBR Green Supermix; Bio-Rad) and analyzed by an iCycler iQ5 (Bio-Rad). The reaction conditions consisted of initial template denaturation at 95°C for 3 min, followed by 35 cycles of amplification (95°C for 10 s and 60°C for 30s). Amplification was followed by a melting curve analysis, ranging from 55° to 95°C, with increasing steps of 0.5°C every 10

s. Expression of mRNA levels was normalized to β-glucuronidase. Samples were run in duplicate. The RT-PCR reaction was performed in a 25-µl reaction volume. A single PCR master mix was used for each set of samples to minimize errors. Integrated DNA Technologies (USA) primers (0.5 µl forward and 0.5 µl reverse primers) were used; 12.5 µl of iQ SYBR Green (Bio-Rad), 9.5 µl of nuclease-free water, and 2 µl of cDNA samples were added. The primers used are shown in table 1.

Table 1. RT-PCR Primers

	Forward	Reverse
GTPCH-1	TGC TTA CTG GTC CAT TCT G	TCC TTC ACA ATC ACC ATC TC
eNOS	AGC CCG GGA CTT CAT CAA TCA G	GCC CCA AAC ACC AGC TCA CTC TC
β-Glucuronidase	GTG GGG ATA ATG ACT TGCA G	GGA ACC CCT GGT AGA ACA GT

eNOS = endothelial nitric oxide synthase; GTPCH-1 = guanosine triphosphate cyclohydrolase 1; RT-PCR = reverse transcription polymerase chain reaction.

Comparative cycle threshold (Ct) method for RT-PCR data analysis was used to calculate the relative change in expression of the target gene: the Ct values of the replicates were averaged and adjusted for the β-glucuronidase by taking the difference in Ct (ΔCt). The difference in expression between the APC and control groups for each gene and each time point was calculated (ΔΔCt between groups), as well as comparing the values of the occlusion and reperfusion time points to pre-occlusion within each group and gene (ΔΔ Ct within group).

GTPCH- 1 and eNOS protein expression levels in LV samples were examined by western blot analysis. Briefly, 100 µg of tissue proteins were loaded onto a Criterion Precast Gel (10% Tris-HCl, Bio-Rad), and proteins were separated by electrophoresis. The separated proteins were transferred overnight to a polyvinylidene fluoride membrane, and the membrane was blocked with 5% bovine serum albumin (Sigma-Aldrich, USA) containing 5% nonfat dry milk. The blots were then incubated with primary antibody against GTPCH-1 (the antibody was kindly provided by Gregory Kapatos, Ph.D., Wayne State University Medical School, Detroit, Michigan) prepared with 5% milk in Tris-buffered saline and Tween 20 (TBS-T) at 1:1000 dilution. The blots were washed five times with 5-min rinse intervals with

TBS-T (0.1%) and then incubated with the secondary antibody (goat anti rat, IgG -HRP, Santa Cruz Biotechnology, USA) prepared in 5% milk in TBS-T in 1:5000 dilution. For eNOS identification, the blots were incubated with primary antibody against eNOS (Santa Cruz, sc-654) prepared in 3% bovine serum albumin in TBS-T at 1:1000 dilution, and after wash, the blots were incubated with the secondary antibody (GE Healthcare Biosciences, USA; ECL-Antirabbit IgG–Horseradish #45000682, Fisher HealthCare, USA) prepared in 5% milk in TBS-T at 1:5000 dilution. Image J (National Institutes of Health, USA) was used to analyze and quantify immunoreactive bands of target proteins.

Statistical Analysis

Data were expressed as mean ± SEM, unless otherwise specified. Comparison of several means was performed using one-way (infarct size) or two-way (hemodynamics) ANOVA, when appropriate, and the *post hoc* test used was the Newman–Keuls test. Hemodynamic data were analyzed with repeated measures. Changes in gene expression, gene product, and NO were analyzed using mixed-effects modeling with a random effect accounting for within-day or within-blot correlation, and treatment group and measurement time as crossed fixed effects. For significance testing, only the preplanned pairwise comparisons of the two treatment groups for each time point and the comparison of each time point to baseline within both treatment groups were considered. The familywise type I error rate was controlled at 5% for each location using the single-step adjustment method based on the multivariate normal distribution.[8] Sample size was selected based on the previous experience of the known biological variability within the model. All tests were two tailed, and analyses were performed using 2.12.0 (R Foundation for Statistical Computing, Austria) with the nlme 3.1–97 and multcomp 1.2–4 packages.

Results

Systemic Hemodynamics

Twenty-four animals were instrumented to obtain 24 successful infarct size experiments. There were no significant differences in hemodynamics between experimental groups at baseline (table 2). Isoflurane caused

similar decreases ($P < 0.05$) in heart rate, mean arterial pressure, and rate pressure product in the presence or absence of DAHP. Hemodynamics returned to baseline values after discontinuation of the anesthetic. DAHP alone produced slight decreases in mean arterial pressure before coronary artery occlusion, although there were no differences in hemodynamics between groups during coronary artery occlusion and reperfusion.

Table 2. Hemodynamics

					Reperfusion (h)	
	Baseline	**Isoflurane**	**Preocclusion**	**30-min OCC**	**1**	**2**
HR (beats × min^{-1})						
CON	323 ± 27	—	315 ± 21	342 ± 35	331 ± 36	325 ± 29
APC	371 ± 63	293 ± 82*	339 ± 54	371 ± 64	363 ± 46	363 ± 41
DAHP	348 ± 32	—	353 ± 44	360 ± 42	342 ± 47	333 ± 42
DAHP + APC	368 ± 40	293 ± 24*	363 ± 37	370 ± 35	359 ± 21	354 ± 14
MAP (mmHg)						
CON	112 ± 17	—	115 ± 12	123 ± 16	103 ± 20	97 ± 16*
APC	118 ± 13	68 ± 14*	108 ± 9	114 ± 4	88 ± 16*	87 ± 10*
DAHP	117 ± 26	—	101 ± 20*	100 ± 21*	82 ± 10*	81 ± 16*
DAHP + APC	118 ± 21	68 ± 11*	100 ± 15*	107 ± 16	89 ± 14*	80 ± 7*
RPP (beats × min^{-1} × mmHg × 10^3)						
CON	44.4 ± 8.2	—	44.6 ± 5.3	48.1 ± 10.9	40.8 ± 11.1	38.3 ± 8.2
APC	51.5 ± 12.8	27.4 ± 11.7*	44.4 ± 8.9*	48.4 ± 7.9	38.6 ± 6.0*	39.2 ± 7.1*
DAHP	47.9 ± 6.1	—	46.7 ± 10.7	45.2 ± 10.5	40.2 ± 9.9*	36.6 ± 8.2*
DAHP + APC	53.5 ± 11.6	28.1 ± 4.8*	48.6 ± 8.8	48.3 ± 10.8	41.4 ± 6.7*	37.9 ± 3.4*

Data are mean ± SD.

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

APC = anesthetic preconditioning; CON = control; DAHP = 2,4-diamino-6-hydroxpyrimidine; HR = heart rate; MAP = mean arterial blood pressure; OCC = coronary artery occlusion; RPP = rate pressure product.

Inhibition of GTPCH-1 Abolished APC

The AAR expressed as a percentage of LV weight was similar among groups (control, 40 ± 1; APC, 38 ± 3; DAHP, 39 ± 2; and APC + DAHP, 41 ± 2%). APC significantly ($P < 0.05$) decreased myocardial infarct size (fig. 2: 43 ± 2% of AAR; n = 6) compared with control experiments (57 ± 1%; n = 6). DAHP alone had no effect on infarct size (57 ± 1%; n = 6), but completely abolished the protection afforded by APC (60 ± 2%; n = 6).

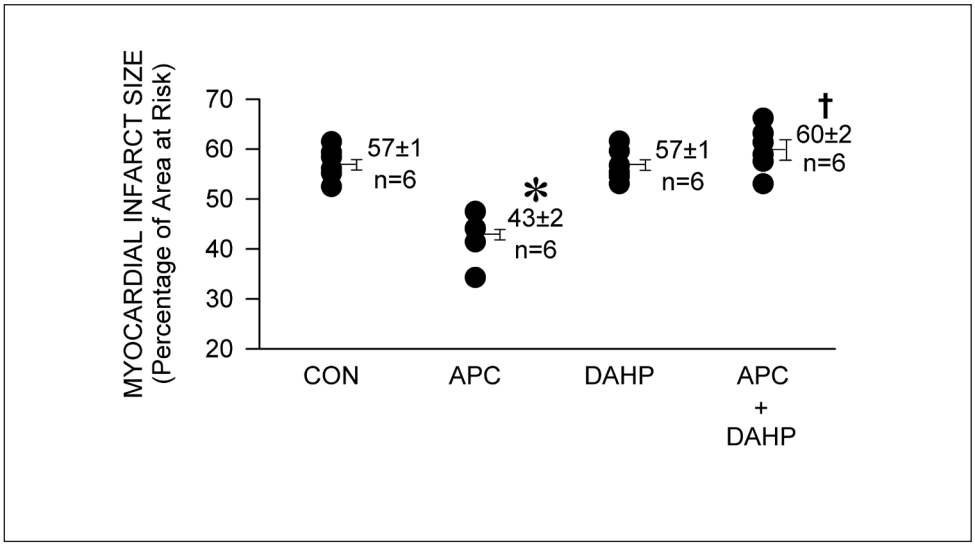

Fig. 2. Myocardial infarct size depicted as a percentage of the area at risk for infarction in CON rats and rats subjected to APC in the absence or presence of DAHP.

*Data are expressed as mean ± SE. *P < 0.05 versus control; †P < 0.05 versus APC alone. APC = anesthetic preconditioning; CON = control; DAHP = 2,4-diamino-6-hydroxypyrimidine.*

APC Produced Time-dependent Increases in Myocardial NO after Ischemia and Reperfusion

There were no differences in production of NO^-_2 or NO_x before coronary artery occlusion in control (158 ± 16 [n = 4] and 1,010 ± 58 pmol/mg protein [n = 4]) or APC groups (150 ± 13 [n = 4] and 909 ± 47 [n = 3]), respectively.

NO production (figs. 3 and 4) was unchanged by coronary artery occlusion in either group. In the APC group, NO^-_2 was significantly ($P < 0.05$) increased after 60 (1.5 ± 0.1 fold [n = 4]) and 90 min of reperfusion (1.4 ± 0.1 fold [n = 4]). NO^-_2 and NO_x production was significantly higher in the APC group after reperfusion, whereas NO_x production was decreased after reperfusion (0.3 ± 0.1 fold [n = 5] and 0.3 ± 0.1 fold [n = 5], respectively) in control experiments. DAHP pretreatment decreased NO production (NO^-_2 and NO_x, respectively) to a similar extent in both groups before coronary artery occlusion (control: 73 ± 6 [n = 4] and 221 ± 25 [n = 3]; and APC: 73 ± 4 [n = 4] and 175 ± 31 pmol/mg protein [n = 3]), and inhibition of GTPCH-1 abolished increases in NO production by APC during reperfusion.

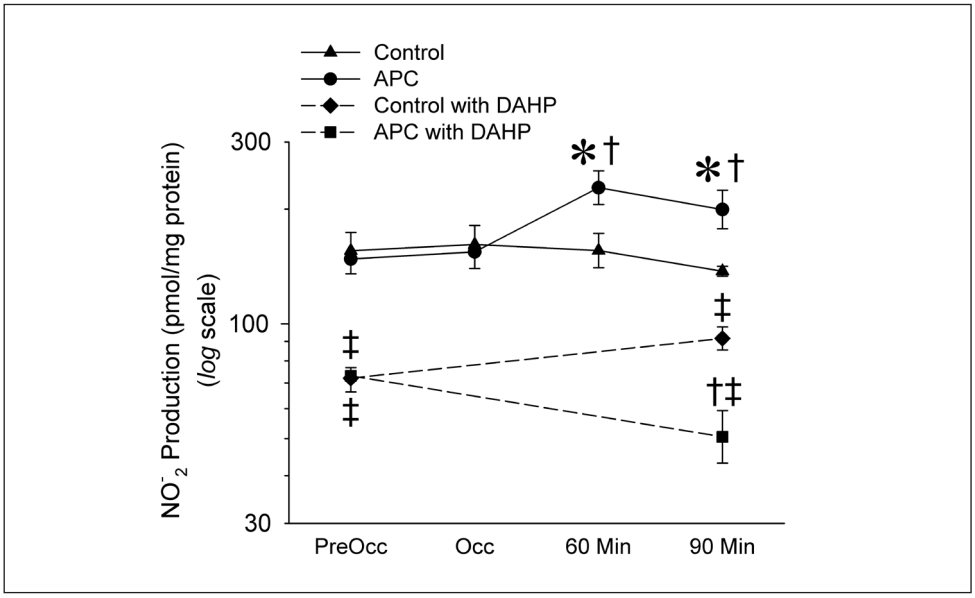

Fig. 3. Time-dependent changes in NO^-_2 production in control rats and in rats subjected to APC with or without DAHP, before (PreOcc) and during Occ, and after reperfusion (60 and 90 min).

*Data are expressed as mean ± SE. *P < 0.05 versus PreOcc baseline; †P < 0.05 versus control at the same time point;‡P < 0.05 versus respective control without DAHP. APC = anesthetic preconditioning; DAHP = 2,4-diamino-6-hydroxypyrimidine; Occ = coronary artery occlusion; PreOcc = before Occ.*

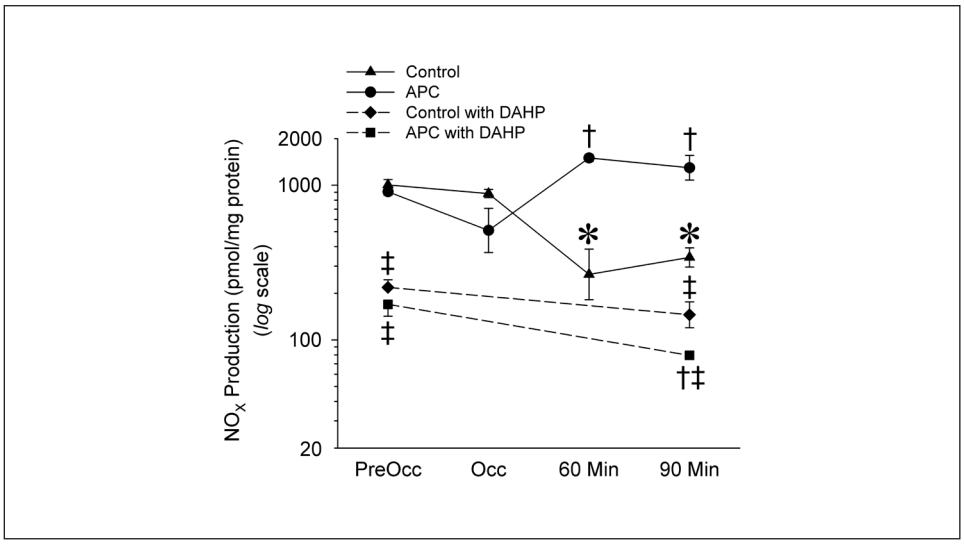

Fig. 4. Time-dependent changes in total nitric oxide (NO) (NO_x = NO^-_2 and NO^-_3) production in control rats and in rats subjected to APC with or without DAHP, before (PreOcc) and during Occ, and after reperfusion (60 and 90 min).

*Data are expressed as mean ± SE. *P < 0.05 versus PreOcc baseline; †P < 0.05 versus control at the same time point; ‡P < 0.05 versus respective control without DAHP. APC = anesthetic preconditioning; DAHP = 2,4-diamino-6-hydroxypyrimidine; Occ = coronary artery occlusion; PreOcc = before Occ.*

APC Favorably Modulated GTPCH-1 and eNOS Expression after Myocardial Ischemia and Reperfusion

GTPCH-1 mRNA abundance (fig. 5, A and B) was unchanged by coronary artery occlusion compared with baseline; however, gene expression was significantly ($P < 0.05$) increased after 60 min of reperfusion in both the control (3.5 ± 1.1 fold [n = 4]) and APC (11.0 ± 3.6 fold [n = 4]) groups. In contrast, eNOS gene expression (fig. 5, C and D) was substantially down-regulated in control animals (n = 4) during coronary artery occlusion and after 60 and 90 min of reperfusion; and this effect was mitigated by APC (n = 4). In the APC group, eNOS expression returned to baseline values at 60 min of reperfusion and was significantly greater than that observed in control experiments.

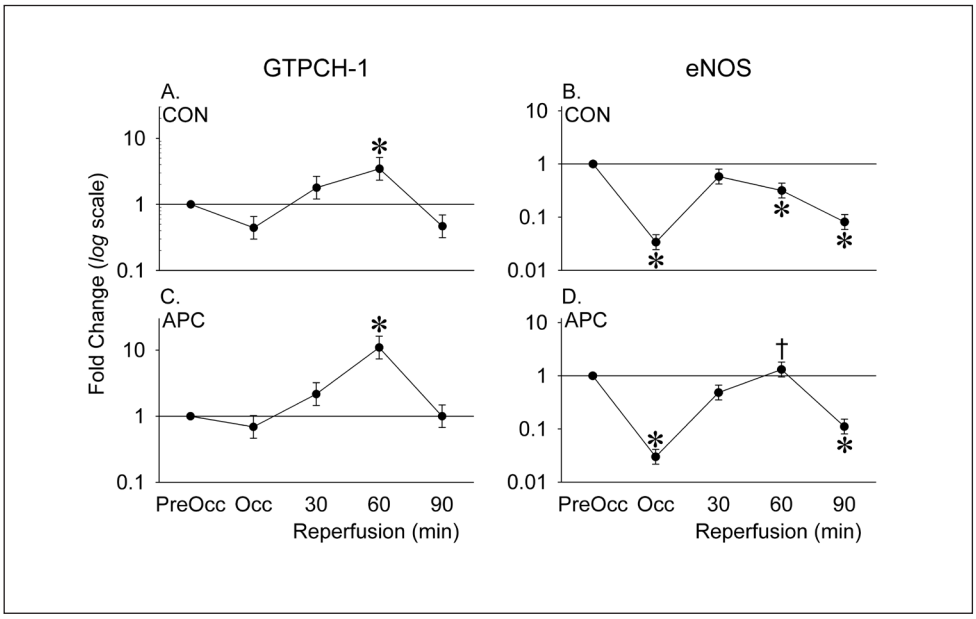

Fig. 5. Time-dependent changes in GTPCH-1 (A) and eNOS (B) gene expression in CON (A, B) rats and in rats subjected to APC (C, D), before (PreOcc) and during Occ, and after reperfusion (30, 60, and 90 min).

*Data are expressed as mean ± SE. *P < 0.05 versus PreOcc baseline; †P < 0.05 versus control at the same time point. APC = anesthetic preconditioning; CON = control; eNOS = endothelial nitric oxide synthase; GTPCH-1 = guanosine triphosphate cyclohydrolase; Occ = coronary artery occlusion; PreOcc = before Occ.*

GTPCH-1 and eNOS protein were unchanged by coronary artery occlusion (fig. 6) in control or APC groups. However, APC increased both GTPCH-1 and eNOS protein expression (by 1.3 ± 0.3 [n = 5] and 1.3 ± 0.2 arbitrary units [n = 5], respectively) at 60 min of reperfusion, and this increase was significantly greater than that observed in control experiments. Increases in eNOS expression were also sustained after 90 min of reperfusion in the APC group (n = 5).

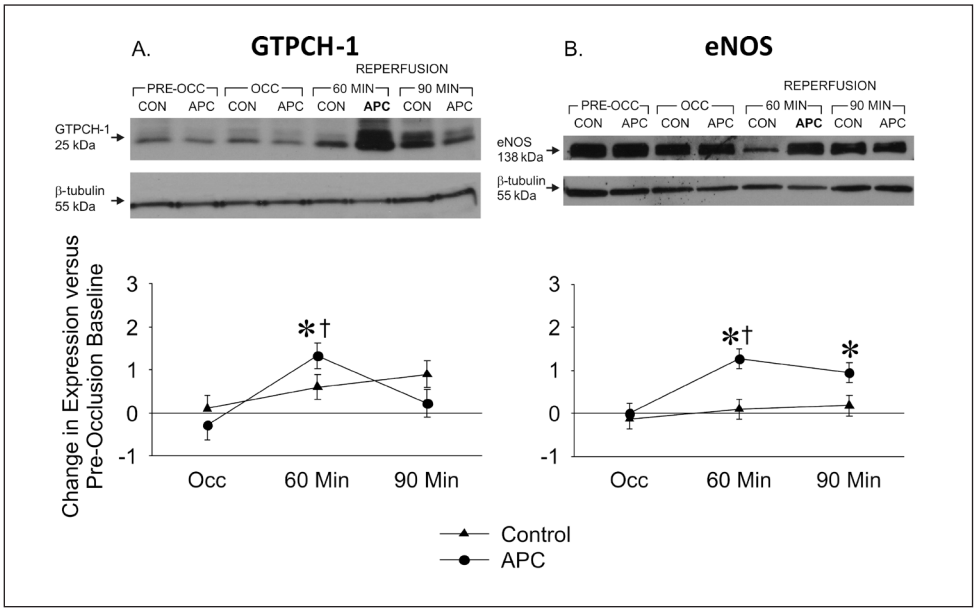

Fig. 6. Time-dependent changes in GTPCH-1 (A) and eNOS (B) protein expression in CON rats and in rats subjected to APC, before PreOcc and during Occ, and after reperfusion (60 and 90 min).

*Representative western blots are shown above the summary data. Data are expressed as mean ± SE. *P < 0.05 versus PreOcc baseline; †P < 0.05 versus control at the same time point. APC = anesthetic preconditioning; CON = control; eNOS = endothelial nitric oxide synthase; GTPCH-1 = guanosine triphosphate cyclohydrolase; Occ = coronary artery occlusion; PreOcc = before Occ*

Discussion

The results of this investigation demonstrated that isoflurane administered before ischemia effectively reduced myocardial ischemia and reperfusion injury by a GTPCH-1–dependent mechanism. APC increased the expression of GTPCH-1 and eNOS and stimulated the production of NO in myocardium after reperfusion. The cardioprotective effects of APC were blocked by DAHP, a pharmacologic antagonist of GTPCH-1, and this inhibitor also abolished APC-induced increases in NO. Taken together with previous evidence, the findings suggest that isoflurane stimulates a NO biosynthetic pathway, and this action represents an important mechanism contributing to the cardioprotective effects of volatile anesthetics.

eNOS-derived NO has been repeatedly implicated as a central mediator of anesthetic cardioprotection.[1,4,9–11] We have previously demonstrated

that isoflurane increased the production of NO by human coronary artery endothelial cells *in vitro*,[12] and this effect was dependent on interactions between eNOS and its physiologic binding partner, heat shock protein 90.[1] Isoflurane enhanced the association between heat shock protein 90 and eNOS and increased the phosphorylation (activation) of eNOS at serine 1177.[1,11,12] In contrast to these favorable effects, nonselective NOS antagonists, specific inhibitors of heat shock protein 90,[1] and hyperglycemia[2] all abolished anesthetic-induced reductions of myocardial infarct size in experimental models. Interestingly, hyperglycemia decreased isoflurane-stimulated colocalization of heat shock protein 90 with eNOS, the ratio of phosphorylation to total eNOS, and NO production in endothelial cells, and the findings suggested that modulation of a NO biosynthetic pathway may play a critical role in the pathogenesis of myocardial ischemia and reperfusion injury. This contention was corroborated by the findings that isoflurane-stimulated endothelial cells protected cardiomyocytes in co-culture against hypoxia and reoxygenation injury (decreased lactate dehydrogenase release and delayed opening of the mitochondrial permeability transition pore). Interestingly, the beneficial paracrine effects of endothelial cells on cardiomyocytes were NO dependent and blocked by NOS inhibition[12] or by culturing endothelial cells, but not cardiomyocytes, in high glucose media.[13]

The deleterious effects of myocardial ischemia on eNOS activity have been reported previously.[14] However, the present results extend these observations. eNOS gene expression was markedly decreased after coronary artery occlusion and reperfusion, but this action was mitigated by APC. APC increased eNOS protein during reperfusion accounting, in part, for increased NO production in myocardium of animals subjected to APC. In addition, isoflurane enhanced the compartmentalization of eNOS within endothelial caveolae, an action that increased serine 1177 phosphorylation and NO production. [11] Thus, the regulation of NO production by anesthetics is complex and mediated by interactions with numerous proteins involved in NO biosynthesis.

eNOS activity is regulated by its cofactor BH4, a pteridine, that is synthesized de novo by GTPCH-1, the first and rate limiting step in this synthetic pathway.[15] GTPCH-1 is constitutively expressed in cardiomyocytes, coronary vascular endothelial cells, and vascular smooth muscle cells,[16–18] and the expression of this NO biosynthetic pathway protein is also inducible by oxidative stress.[15] The current findings demonstrated that GTPCH-1 mRNA was increased at 60 min of reperfusion in both control myocardi-

um and animals subjected to APC. However, APC significantly increased GTPCH-1 protein at 60 min of reperfusion, whereas the expression of GTPCH-1 was unchanged in control experiments. These findings suggested that the cardioprotective effects of isoflurane were dependent on GTPCH-1 and regulation of eNOS during reperfusion, because infarct size reduction and enhanced NO production during APC were abolished by an inhibitor of GTPCH-1. The findings confirm and extend previous evidence that targeted overexpression of GTPCH-1 in endothelial cells[13] or myocardium[19] protects against ischemia and reperfusion injury *in vivo* and *in vitro*. GTPCH-1 expression and NO production were increased in Brown Norway rats that are resistant to myocardial infarction compared with the ischemia-sensitive Dahl S rats, [20] and human genetic variants of GTPCH-1 may predict cardiovascular risk.[21] Thus, modulation of GTPCH-1 may be adaptive against myocardial injury.

Isoflurane has been previously shown to increase the ratio of reduced (BH4) to oxidized (BH2) biopterin[2] in endothelial cells, concomitantly with increased NO production. In contrast, hyperglycemia produced deleterious effects on BH4 and a loss of cardioprotection during APC *in vivo*. Conversely, increased BH4 content by administration of a metabolic precursor, sepiapterin, restored the protective effects of APC to reduce myocardial infarct size[2] and reestablished protective endothelial cell–cardiomyocyte interactions during hyperglycemia.[13] Overexpression of human GTPCH-1 gene profoundly increased BH4 content in myocardium of transgenic mice and restored the protective effects of ischemic preconditioning during hyperglycemia.[19] During preliminary experiments, BH4 concentrations in reperfused rat myocardium were below the limits of detection, a finding consistent with previous reports that BH4 levels were depleted in ischemic hearts.[14] Although we were able to detect BH4 in transgenic mouse hearts[19] and endothelial cells,[2] these measurements were made in the absence of prolonged coronary artery occlusion and reperfusion as performed in the current investigation.

eNOS generates NO under normal conditions, but it is capable of producing superoxide anion when electron transfer within the enzyme's active site becomes uncoupled from l-arginine oxidation.[22] Uncoupling of eNOS occurs in the presence of low concentrations of intracellular BH4. In contrast, isoflurane has been previously demonstrated to increase eNOS dimerization, [11] an index of „coupled" enzyme activity. Taken together, the current and previous findings indicated that APC protects ischemic myo-

cardium against injury through important actions to maintain NO biosynthesis by

1. increasing eNOS and GTPCH-1 protein expression and
2. maintaining eNOS in a coupled state through cofactor (BH4) availability, eNOS compartmentalization in caveolae, and enhanced chaperone function (heat shock protein 90).

Diabetes, hyperglycemia, and other disease states have been shown to decrease the bioavailability of NO and contributed to increased cardiovascular risk.[23,24] Our findings confirmed the central role of NO during myocardial ischemia and reperfusion injury and suggested that isoflurane favorably modulated NO biosynthesis. However, the clinical benefits of volatile anesthetics have been incompletely realized, in part, because of interactions among pharmacologic therapies and disease states that differentially impact cardio-protective signaling pathways. For example, we have demonstrated that APC is blocked by diabetes and hyperglycemia,[2] but the beneficial effects of APC can be restored by statins,[25] sepiapterin,[2] overexpression of GTPCH-1,[19] and apoliporotein A-1 mimetic,[11] and all these interventions seem to be dependent on modulation of NO. Although as yet unproven in patients, the current and previous findings may support the use of a multimodal approach to target NO as a means of decreasing cardiovascular risk.[24]

The current findings should be interpreted within the constraints of several potential limitations. Isoflurane produced brief hemodynamic effects, although there were no differences in hemodynamics between groups during coronary artery occlusion and reperfusion that could account for the observed results. However, myocardial oxygen consumption was not directly measured. The activity of eNOS was assessed by determining the concentrations of NO^-_2 or total NO_x (the sum of NO^-_2 and NO^-_3) as indices of bioavailable NO, and hence, coupled eNOS activity. NO is converted to NO^-_2, which is further oxidized to NO^-_3. Both analyses were performed because high background concentrations of NO^-_3 , combined with a long half-life in comparison with NO^-_2 , may reduce the sensitivity of NO_x as a sole indicator of eNOS activity.[26] The current results suggested that isoflurane may have enhanced coupled activity of eNOS; however, we did not assess this action in myocardium directly. Previous evidence indicated that volatile anesthetics increased coupled eNOS activity in endothelial cells [11] and decreased production of reactive oxygen species in myocardium after reperfusion injury. [27] Multiple enzymatic sources of reactive

oxygen species are present in myocardium such as the mitochondrial electron transport chain, in addition to uncoupled eNOS, and thus, the precise contributions of different enzymes to the redox balance in myocardium may be difficult to identify.

In conclusion, the results of this investigation demonstrated that APC enhanced gene and protein expression of an NO biosynthetic pathway that includes eNOS and GTPCH-1. Activation of this pathway by volatile anesthetics resulted in protection against myocardial infarction and may be a major determinant of sensitivity versus resistance to myocardial ischemia and reperfusion injury.

Acknowledgments

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Competing Interests

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Correspondence

Address correspondence to Dr. Kersten: Department of Anesthesiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. jkersten@mcw.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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