Published in final edited form as:
Anesthesiology. 2015 September ; 123(3): 582–589. doi:10.1097/ALN.0000000000000778.

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

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Abstract

Background—We investigated the hypothesis that isoflurane modulates 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 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, 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 as compared to 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).

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Conflict of Interest: Dr. Kersten has received honoraria from Ikaria, Inc., Hampton, New Jersey. All other authors have no conflicts of interest.

Conclusions—APC favorably modulated a NO biosynthetic pathway by upregulating GTPCH-1 and eNOS, and this action contributed to protection of myocardium against ischemia and reperfusion injury.

Introduction

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 pathway is importantly modulated by disease states.[2] Three distinct NOS isoforms, neuronal (nNOS), inducible (iNOS), and eNOS all contribute to NO production in the heart,[3] however, eNOS, but not nNOS or iNOS, appears to play a major role during APC.[1,4] We previously demonstrated that the trigger and mediator phases of delayed preconditioning with isoflurane were blocked by the non-selective 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, post-translational modifications, protein-protein interactions, and tetrahydrobiopterin (BH_4) co-factor 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; 8–12 weeks old), weighing 300–360 g were anesthetized with thiobutabarbital sodium (100 mg/kg, intraperitoneal) and instrumented for the measurement of systemic hemodynamics as previously described.[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-hydroxypyrimidine (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 previously described.[5] In additional groups of rats, left ventricular (LV) tissue samples were collected at the following time points: prior to coronary artery occlusion; immediately prior to reperfusion; and after 30, 60, and 90 min of reperfusion.

Tissue NO^-_2 and NO_x Analysis

Nitrite (NO^-_2) and total NO (NO_x: NO^-_2 and nitrate (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, Billerica, MA) by centrifugation for 30 min at 12,000 rpm and 4°C (Microfuge R 22R Centrifuge, Beckman Coulter, Brea, CA). 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 nitrite was quantified by a chemiluminescence detector (Sievers 280 model NO analyzer, GE Analytical Instruments, Boulder, CO) as described previously.[6] For NO_x 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 previously described.[7] Each sample was analyzed in triplicate. Nitrite and NO_x 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, Valencia, CA). 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, NanoDrop Technologies, Wilmington, DE). Only samples with 260/280nm absorbance ratios between 1.8 and 2 were used for further analysis. Immediately following the quality control assessment, reverse transcription of total RNA samples to cDNA was performed using iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). 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 10s, 60°C for 30s). Amplification was followed by a melting curve analysis, ranging from 55°C to 95°C, with increasing steps of 0.5°C every 10s. Expression of mRNA levels was normalized to beta-glucuronidase (β-Gluc). 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 (IDT, Coralville, IA) primers: 0.5uL forward and 0.5uL reverse primers were used. 12.5uL of iQ SYBR Green (Bio-Rad), 9.5uL of Nuclease Free water and 2uL of cDNA samples were added. The primers used are shown in Table 1.

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-Gluc 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 (ΔΔCt 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 CriterionTM Precast Gel (10% Tris-HCl, Bio-Rad) and proteins were separated by electrophoresis. The separated proteins were transferred overnight to a PVDF membrane and the membrane was blocked in 5% bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO) containing 5% nonfat dry milk. The blots were then incubated with primary antibody against GTPCH-1 (the antibody was kindly provided by Gregory Kapatos, PhD of Wayne State University Medical School, Detroit, MI) prepared with 5% milk in TBS-T at 1:1000 dilution. The blots were washed 5 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, Dallas, TX) 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% BSA in TBS-T at 1:1000 dilution, and after wash, the blots were incubated with the secondary antibody (GE Healthcare Biosciences; ECL-Antirabbit IgG – Horseradish #45000682, Fisher HealthCare, Houston, TX) prepared in 5% milk in TBS-T at 1:5000 dilution. Image J (NIH, Bethesda, Maryland) was used to analyze and quantify immunoreactive bands of target proteins.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SE), unless otherwise specified. Comparison of several means was performed using one-way (infarct size) or two-way (hemodynamics) analysis of variance, 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 pre-planned 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 family-wise 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 previous experience of the known biological variability within the model. All tests were two-tailed and analyses performed using 2.12.0 (R Foundation for Statistical Computing, Vienna, 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 prior to coronary artery occlusion, although, there were no differences in hemodynamics between groups during coronary artery occlusion and reperfusion.

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; APC+DAHP: 41±2%). APC significantly (P < 0.05) decreased myocardial infarct size (Figure 2: 43±2% of AAR; n=6) compared to 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).

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 1010±58 pmol/mg protein (n=4)] or APC groups [150±13 (n=4) and 909±47 (n=3)], respectively. NO production (Figures 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)], respectively. NO^{-}_{2} and NO_x production were significantly higher in the APC group after reperfusion, whereas, NO_x 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 by APC during reperfusion.

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

GTPCH-1 mRNA abundance (Figure 5A and 5B) was unchanged by coronary artery occlusion compared to 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 (Figure 5C and 5D) 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.

GTPCH-1 and eNOS protein were unchanged by coronary artery occlusion (Figure 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).

Discussion

The results of this investigation demonstrated that isoflurane administered prior to 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 production of NO in myocardium after reperfusion. The cardioprotective effects of APC were blocked by DAHP, a pharmacological 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 physiological 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, non-selective 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 co-localization of heat shock protein 90 with eNOS, the ratio of phospho- to total eNOS, and NO production in endothelial cells, findings that suggested 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 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 previously been reported.[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. Additionally, 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 co-factor BH_4, 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 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 myocardium and in 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, since 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 as compared to 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 previously been shown to increase the ratio of reduced (BH_4) to oxidized (BH_2) biopterin[2] in endothelial cells, concomitantly with increased NO production. In contrast, hyperglycemia produced deleterious effects on BH_4 and a loss of cardioprotection during APC *in vivo*. Conversely, increased BH_4 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 BH_4 content in myocardium of transgenic mice and restored the protective effects of ischemic preconditioning during hyperglycemia.[19] During preliminary experiments, BH_4 concentrations in reperfused rat myocardium were below the limits of detection, a finding consistent with previous reports that BH_4 levels were depleted in ischemic hearts.[14] Although we were able to detect BH_4 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, 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 BH_4. In contrast, isoflurane has previously been demonstrated to increase eNOS dimerization,[11] an index of "coupled" enzyme activity. Taken together, the current and previous findings indicated that APC protects ischemic myocardium 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 co-factor (BH_4) 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, due to interactions among pharmacological therapies and disease states that differentially impact cardioprotective 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] over-expression of GTPCH-1,[19] and apolipoprotein A-1 mimetic,[11] and all of these interventions appear to be dependent on modulation of NO. Although as yet unproven in patients, the current and previous findings may support the use of a multi-modal 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 concentrations of nitrite or total NO_x (the sum of nitrite and nitrate) as indices of bioavailable NO, and hence, coupled eNOS activity. NO is converted to nitrite which is further oxidized to nitrate. Both analyses were performed since high background concentrations of nitrate, combined with a long half-life in comparison to nitrite, 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

The authors would like to thank David Schwabe, B.S. (Engineer), Amie Bilstrom B.S. (Research Technologist) and John Tessmer B.A. (Research Technologist) all from the Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin for assistance in completion of this project, and Aniko Szabo PhD from the Institute for Health and Society, Division of Biostatics, Medical College of Wisconsin, for statistical analysis.

This work was supported by the National Institutes of Health, Bethesda, MD (PO1GM066730 and 2T32GM089586 to Dr. Kersten).

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Final Box Summary Statement

What we already know about this topic

- Nitric oxide (NO) is a protective molecule and an important mediator of anesthetic preconditioning (APC).
- Further understanding of the basic mechanisms involved in NO biology in APC is critical to advancing the field.

What this article tells us that is new

- APC favorably enhances gene expression in the NO biosynthetic pathway by upregulation of GTPCH-1 and eNOS.
- The work identifies potential key regulatory points in NO biosynthesis that may determine clinically relevant sensitivity vs. resistance to APC.

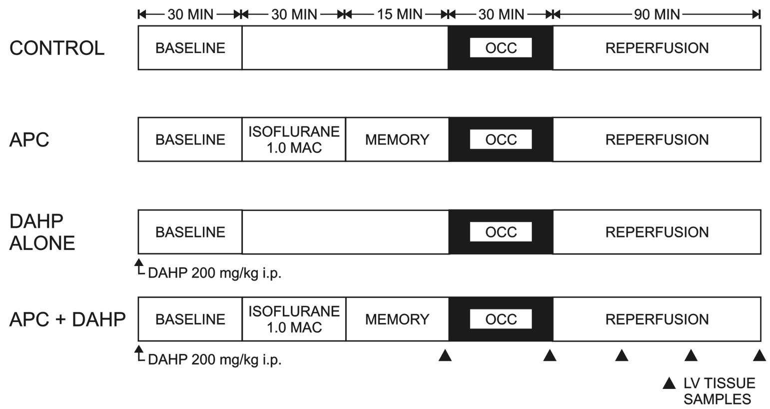

Figure 1.

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.

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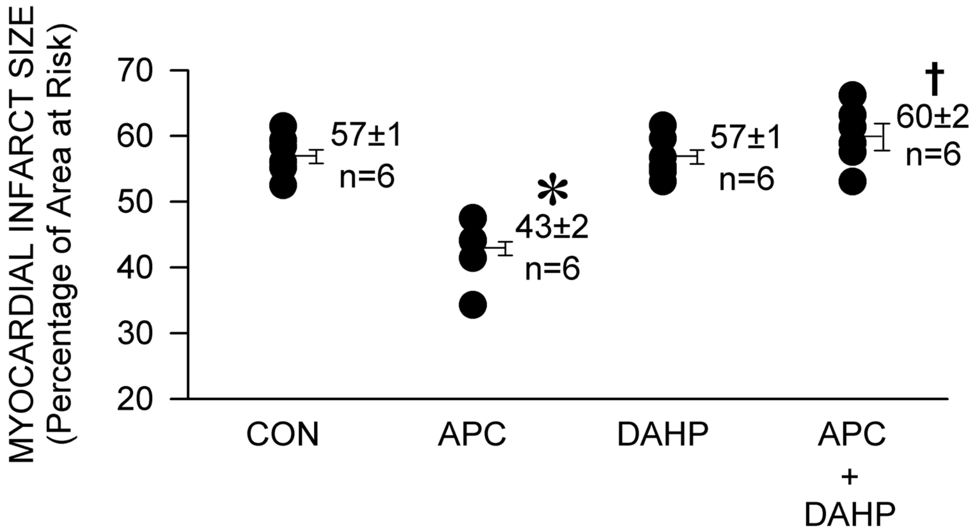

Figure 2.

Myocardial infarct size depicted as a percentage of the area at risk (% of AAR) for infarction in control (CON) rats and in rats subjected to anesthetic preconditioning (APC) in the absence or presence of 2,4-diamino-6-hydroxypyrimidine (DAHP). Data are expressed as mean ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. APC alone.

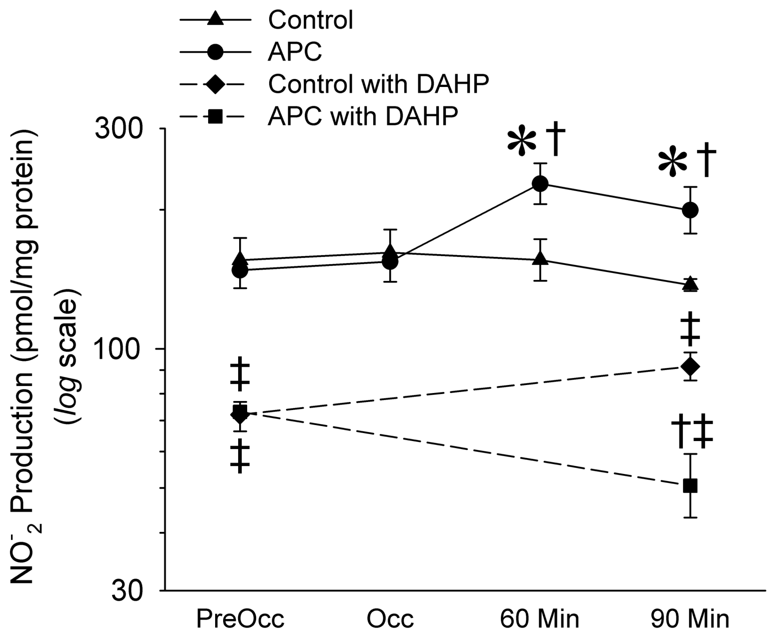

Figure 3.

Time-dependent changes in NO^-_2 production in control rats and in rats subjected to anesthetic preconditioning (APC) with or without 2,4-diamino-6-hydroxypyrimidine (DAHP), before (PreOcc) and during coronary artery occlusion (Occ), and after reperfusion (60 and 90 min). Data are expressed as mean ± SE. *$P < 0.05$ vs. PreOcc baseline; †$P < 0.05$ vs. control at the same time point; ‡$P < 0.05$ vs. respective control without DAHP.

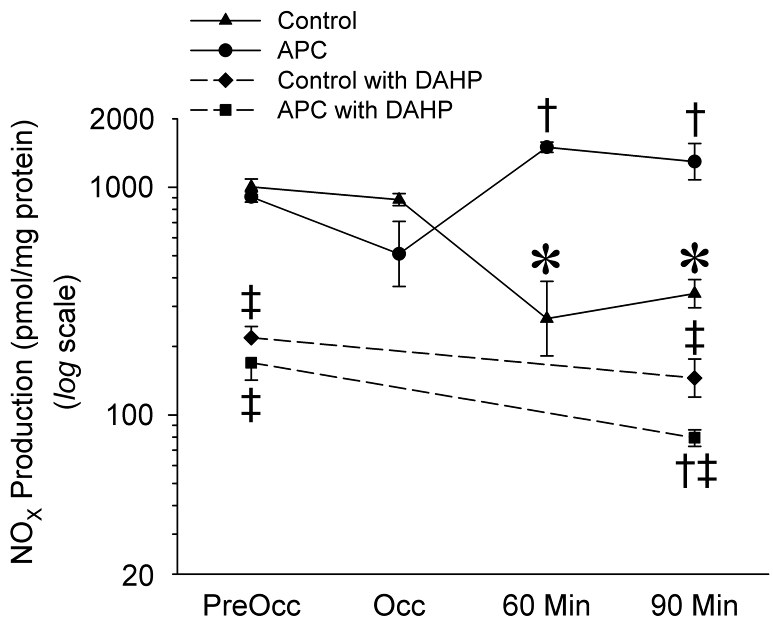

Figure 4.

Time-dependent changes in total NO (NO^{-}_{2} and NO^{-}_{3}: NO_x) production in control rats and in rats subjected to anesthetic preconditioning (APC) with or without 2,4-diamino-6-hydroxypyrimidine (DAHP), before (PreOcc) and during coronary artery occlusion (Occ), and after reperfusion (60 and 90 min). Data are expressed as mean ± SE. **P* < 0.05 vs. PreOcc baseline; †*P* < 0.05 vs. control at the same time point; ‡*P* < 0.05 vs. respective control without DAHP.

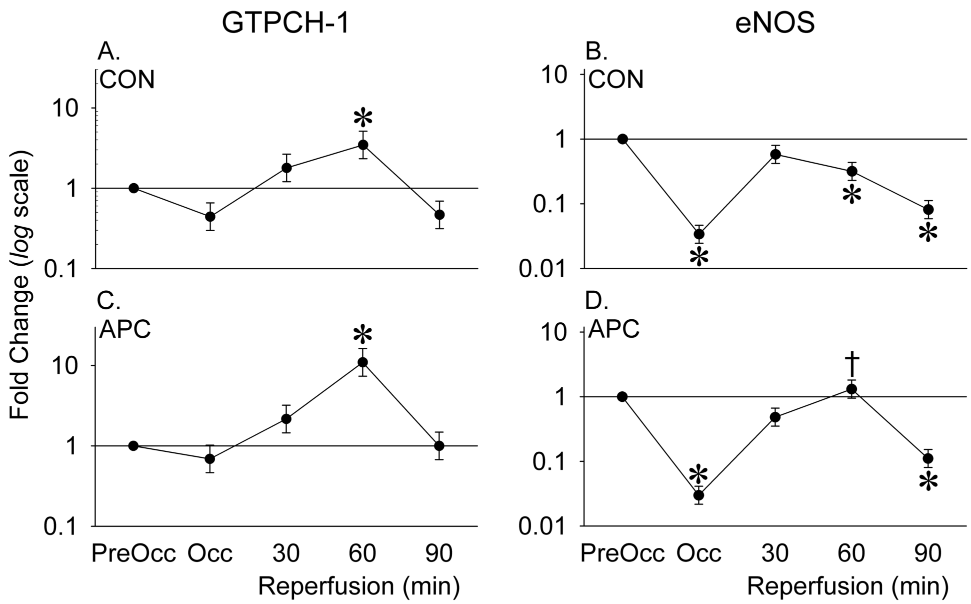

Figure 5.

Time-dependent changes in guanosine triphosphate cyclohydrolase (GTPCH)-1 (panel A) and endothelial nitric oxide synthase (eNOS; panel B) gene expression in control (CON: panels A and B) rats and in rats subjected to anesthetic preconditioning (APC: panels C and D), before (PreOcc) and during coronary artery occlusion (Occ), and after reperfusion (30, 60 and 90 min). Data are expressed as mean ± SE. $*P < 0.05$ vs. PreOcc baseline; $\dagger P < 0.05$ vs. control at the same time point.

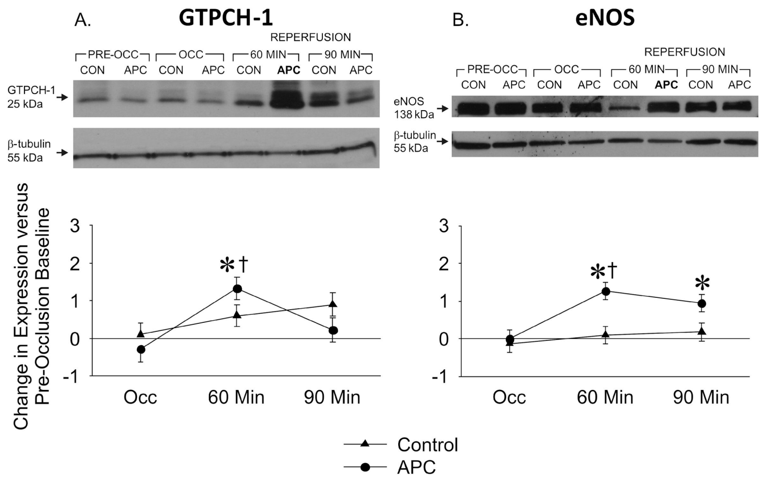

Figure 6.

Time-dependent changes in guanosine triphosphate cyclohydrolase (GTPCH)-1 (panel A) and endothelial nitric oxide synthase (eNOS; panel B) protein expression in control (CON) rats and in rats subjected to anesthetic preconditioning (APC), before (PreOcc) and during coronary artery occlusion (Occ), and after reperfusion (60 and 90 min). Representative western blots are shown above the summary data. Data are expressed as mean ± SE. *$P < 0.05$ vs. PreOcc baseline; †$P < 0.05$ vs. control at the same time point.

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-Gluc	GTG GGG ATA ATG ACT TGCA G	GGA ACC CCT GGT AGA ACA GT

Table 2

Hemodynamics

	Baseline	Isoflurane	Preocclusion	30 min Occ	Reperfusion (h) 1	Reperfusion (h) 2
HR beats x 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 x min^{-1} x mmHg x 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.