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This study was conducted in mongrel dogs to test the hypothesis that 20 d normobaric intermittent hypoxic conditioning (IHC) evokes cardioprotective adaptations of the myocardial nitric oxide synthase (NOS) system. Specifically, the proposal that IHC suppresses myocardial NOS activity sufficiently to dampen the cytotoxic burst of NO formation upon reperfusion of ischemic myocardium was tested. Mongrel dogs were conditioned by a 20 d program of IHC (FIO_2 9.5-10%; 5-10 min hypoxia/cycle, 5-8 cycles/d with intervening 4 min normoxia). On day 21, ventricular myocardium was sampled for measuring NOS activity (colorimetric assay) and endothelial NOS (eNOS) content (immunoblot). In separate experiments, myocardial nitrite (NO_2^-) release, an stable product of NO oxidation, was measured at baseline and during reperfusion following 1 h occlusion of the left anterior descending coronary artery (LAD). Values in IHC dogs were compared with respective values in non-conditioned, control dogs. IHC lowered left and right ventricular NOS activity by 60%, from 100-115 to 40-45 mU/g protein ($P < 0.01$), and decreased eNOS content by 30%. IHC dampened cumulative NO_2^- release during the first 5 min reperfusion from 32 ± 7 to 14 ± 2 μmol/g ($P < 0.05$), but did not alter hyperemic LAD flow (15 ± 2 *vs.* 13 ± 2 ml/g). Attenuation of the NOS/NO system may contribute to IHC-induced protection of myocardium from ischemia-reperfusion injury.

HYPOXIC CONDITIONING SUPPRESSES CYTOTOXIC NITRIC OXIDE PRODUCTION UPON MYOCARDIAL REPERFUSION

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INTRODUCTION

Synthesis of Nitric Oxide and regulation of nitric oxide synthase activity

Nitric oxide (NO) is produced from *L*-arginine by nitric oxide synthase (NOS) in a complex chemical reaction (Figure 1). NOS isoforms produce NO by catalyzing a five electron oxidation of the guanidino nitrogen of *L*-arginine. Oxidation of *L*-arginine to *L*-citrulline occurs via two successive monooxygenation reactions, with N^{ω} hydroxyl *L*-arginine as an intermediate (Figure 1).

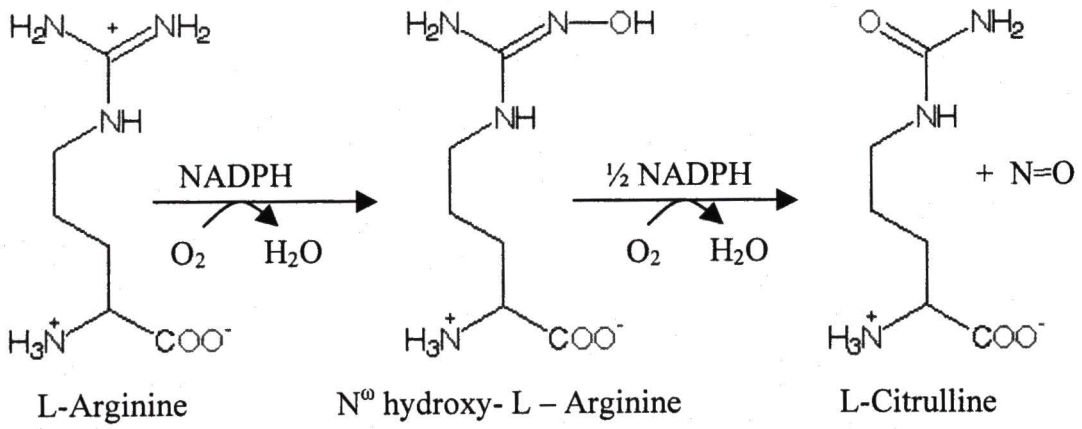


Figure 1. ***The pathway of NO synthesis from L-arginine***

Nitric oxide synthase isoenzymes, various substrates, and cofactors are required for NO synthesis. Substrates for the NOS reaction include *L*-arginine, molecular oxygen and NADPH. Obligatory or facilitatory cofactors include tetrahydrobiopterin (H4B), FAD, FMN, Mg^{2+} and Ca^{2+}.

Three NOS isoforms have been definitively identified: neuronal NOS or type I NOS (nNOS), inducible NOS or type II NOS (iNOS), and endothelial NOS or type III NOS e(NOS). NOS is composed of a flavin-containing C-terminal domain with binding sites for FAD, FMN, NADPH, and a catalytic N-terminal oxygenase domain with binding sites for *L*-arginine and 6-R-tetrahydrobiopterin (H4B). The Ca^{2+}/calmodulin binding region links these domains. NOS isoenzymes are only active as homodimers, as electrons pass from the reductase domain of one monomer to the oxidase domain of the other. There are multiple mechanisms of transcriptional and/or post-transcriptional regulation of NOS. Phosphorylation by various kinases is one of the critical post-transcriptional mechanisms regulating NOS activity. Considering NOS activation, phosphorylation of eNOS has been more enthusiastically examined. Table 1 summarizes eNOS phosphorylation sites and active kinases [10].

Table 1. ***Protein kinases proposed to regulate eNOS phosphorylation at various sites.***

SITE (Bovine sequence)	Phosphorylating Kinase(s)	Effect on eNOS
S1179	PKB, PKA, PKG, AMPK, CaMKII	Activates
S635	PKA	Activates
S617	PKB, PKA	Activates
T496	AMPK, PKC	Inhibits
S116	PKC	Activates

Phosphorylation is one of the widely studied regulatory mechanisms of eNOS activity. S1179 is a well-characterized site of bovine eNOS, which is activated by phosphorylation in response to various physiological stimuli including shear stress, VEGF, insulin-like growth factor-I, sphingosine-1 phosphate, estrogen, and hydrogen peroxide [10]. Bovine eNOS S635 is located within an auto-inhibitory element, where it is linked to the calmodulin(CaM) binding site [14]. Phosphorylation of bovine eNOS at S635, in the response to increased shear stress, impedes CaM binding to eNOS and interferes electron flux between two eNOS monomers [14]. This indicates that phosphorylation of bovine eNOS at S635 is a mechanism of eNOS regulation, although its functional importance remains to be clarified [14]. Bovine eNOS is also phosphorylated at S617 in response to bradykinin, ATP, and VEGF [54]. By using phosphomimicking eNOS constructs, Michell et al. demonstrated that phosphorylation of bovine eNOS at S617 increased Ca^{2+}/CaM sensitivity of the enzyme without altering its maximum activity [54]. Phosphorylation of bovine eNOS at T496 is known to suppress eNOS activity [15]. Lastly, phosphorylation of bovine eNOS at S116 has been proposed to enhance eNOS activity via an unknown mechanism [42].

Phosphorylation is also important in the regulation of nNOS and iNOS activity [2,57,64]. Phosphorylation of mouse nNOS at S847 by CaM-KII inhibits the enzyme [64]. Less is determined about phosphorylation sites of iNOS.

Although NOS isoforms are classified as either Ca^{2+}-dependent or Ca^{2+}-independent, all three NOS isoforms likely share a common mechanism for metabolizing

L-arginine to *L*-citrulline and NO. nNOS and eNOS are constitutively expressed and require a high concentrations of Ca^{2+} for enzyme activity [13,47,67]. On the other hand, iNOS is inducible and is classified as Ca^{2+} independent, because its high affinity for Ca^{2+}/calmodulin allows the enzyme to be active at the basal intracellular Ca^{2+} concentrations [16].

Physiological roles of NOS isoforms in cardiac function

NOS isoforms have 50-60% sequence homology (Figure 2) [3], and 90% homology for the same isoform between different species [41]. However, each NOS isoform functions in specific locations. nNOS and eNOS are constitutively expressed in various tissues including myocardium. nNOS is located in nerve endings, skeletal myocytes, and in cardiac sarcoplasmic reticulum (SR), sarcolemmal *L*-type Ca^{2+} channels [21]. The location of nNOS in sarcolemma and SR enables the enzyme to modulate sarcolemmal Ca^{2+} entry and SR Ca^{2+} sequestration [39]. Khan et al. demonstrated the function of nNOS in contractility, Ca^{2+} transients, and sarcomere shortening by studying wild type (WT) mice and nNOS -/- mice [39]. While high pacing of in situ heart (660-840 bpm) stimulated both contractility (51±5 %; $p<0.001$) and lusitropy (20.3±2 %; $p<0.05$), these responses were markedly attenuated in nNOS -/- mice [39]. Also, they proved that transient Ca^{2+} concentrations and sarcomere shortening were suppressed in isolated heart of nNOS -/- mice comparing to WT mice [39]. Acute inhibition or genetic deletion of eNOS or iNOS has little impact on basal cardiac contractile function; however, changes in nNOS activity profoundly affect mechanical function. Transgenetic deletion

or inhibition of nNOS increased L-type Ca^{2+} current and amplitude of contractile shortening in isolated murine heart and in vivo murine myocardium [5]. By altering intracellular Ca^{2+} concentration, NO can influence inotropic responses of the myocardium.

Endothelial NOS is constitutively expressed in various cell types including vascular endothelial and myocardial cells, and is closely localized to T-tubular caveolae where it is associated with caveolin-3, a myocyte-specific structural protein in skeletal muscle, diaphragm, and myocardium [51]. The eNOS-caveolin interaction is a potentially important form of NOS signaling. Caveolin serves as a structural protein, and also maintains eNOS in an inactive state through a direct inhibitory allosteric interaction [26]. Indeed, overexpression of caveolin produces tonic inhibition of eNOS and endothelial dysfunction [25].

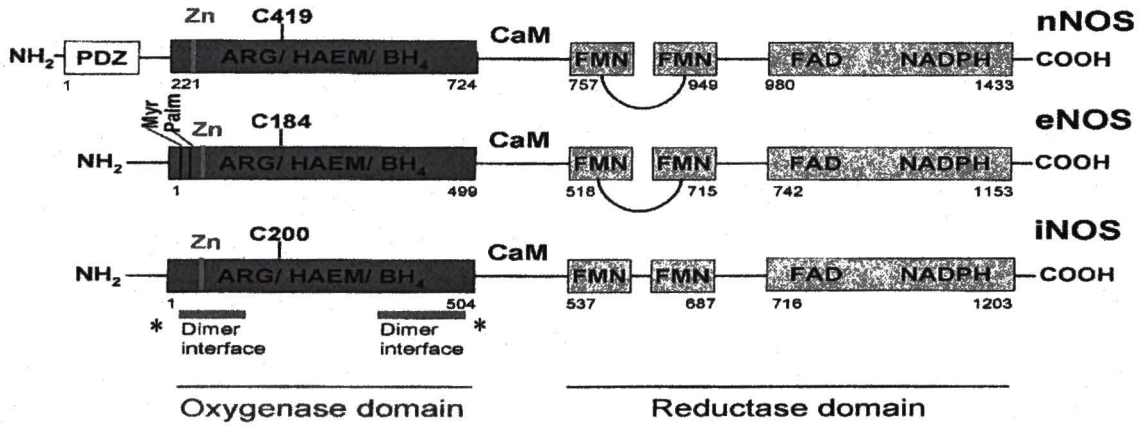


Figure 2. ***Domain structure of human nNOS, eNOS, and iNOS.*** Oxygenase, reductase and PDZ domains are denoted by solid boxes, and the amino acid residue number at the start/end of each domain is shown. The cysteine residue which ligates the heme and the CaM-binding site are indicated for each isoform, myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown. The location of the zinc-ligating cysteines are shown in gray in dark gray box. The autoinhibitory loop within the FMN regions of nNOS and eNOS are also shown. Grey bars(*) indicate the dimer interface in the oxygenase domain. [3]

Inducible NOS expression and activity are stimulated by immune reactions. Macrophages, monocytes and eosinophils harbor the iNOS isoform. Cardiomyocyte iNOS is induced by inflammatory cells infiltrating the myocardium in response to inflammatory cytokines under stressful conditions. The amount of NO produced by iNOS is potentially greater than that from nNOS or eNOS. Excessive NO formation by iNOS can cause toxic effects and even death of cardiomyocytes by generating its highly reactive derivatives, peroxynitrite ($ONOO^-$). Recently, a constitutive platelet NOS (cNOS) was identified [71], but its role is poorly understood.

Characteristics and biochemistry of NO

A small, electroneutral molecule, NO can readily traverse cell membranes and move among cellular compartments. The membrane permeability and solubility of NO is close to that of O_2, and NO can rapidly diffuse throughout cells or tissues. Because of NO's high diffusibility, when its concentration exceeds the physiological range, NO can bind to hemoglobin and form a heme-NO complex, thereby preventing heme from binding O_2. This competition by NO increases the Km for O_2 binding by heme [1]. Through this mechanism, high concentrations of NO could limit O_2 supply to tissues and deprive NOS of its O_2 substrate.

NO is uncharged molecule that possesses an unpaired electron in the outer orbital of its nitrogen atom, making it a free radical. Even though NO carries an unpaired electron, it is not as toxic as other reactive oxygen and nitrogen species. NO is rather

stable relative to other free radicals; its half life is several seconds to several minutes depending on the prevailing cellular conditions and NO concentration.

Most of the pathological actions of NO are inflicted by its more aggressive derivatives, such as peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH). NO can irreversibly combine with $\cdot O_2^-$ in diffusion limited, biradical condensation to form peroxynitrite (Figure 3: reaction 12). $ONOO^-$ and ONOOH can inflict lethal cellular injury by initiating a peroxidation cascade [23], modifying DNA bases [23], causing DNA single or double strand breaks [24,80], depleting cellular thiols [24], activating matrix metalloproteinases [63,84], nitrosating aromatic compounds [8,24,69], and inactivating components of the mitochondrial respiratory chain [11]

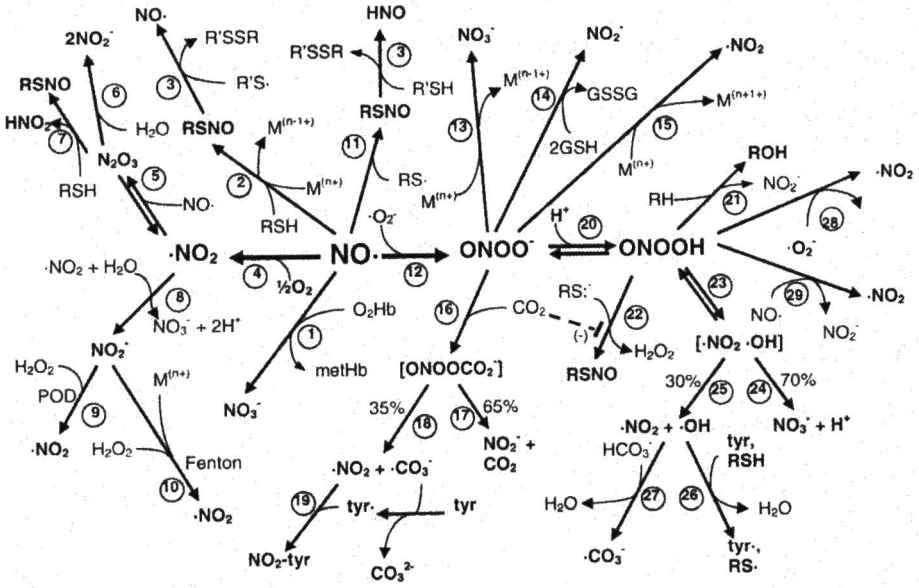


Figure 3. ***Biological chemistry of NO and its derivatives.*** NO metabolism produces a variety of potentially cytotoxic reactive oxygen and nitrogen species. Irreversible condensation of NO and precursor of many of these harmful substances. From ref. [49]. See [49] for details on the numerous reactions.

Physiological functions of NO

Since the 1980s, NO has been widely recognized as a powerful vasodilator generated by endothelial cells [29,35,65]. In addition, NO can suppress platelet aggregation [30] and neurotransmission [30], regulate cell survival [49] and functions of various cell types including many of those involved in immunity and inflammation [30], and reduce myocardial O_2 consumption [74, 75]. Because of these multiple physiological functions, NO is considered a critical regulator of cellular function.

Normal cardiac function requires adequate delivery of O_2 and fuels to sustain myocardial ATP production. Systemic hypoxia can compromise O_2 delivery and threaten myocardial ATP synthesis. Under hypoxic conditions, the vasodilatory function of NO can help restore normal O_2 delivery to the tissue. Also, Setty et al. reported that NO reduces right ventricular myocardial O_2 consumption (MVO_2) in canine [74] and contributes to adaptation to hypoperfusion by restraining MVO_2 and by enhancing vasodilation with less reduction in myocardial PO_2 [75]. By reducing myocardial O_2 requirement during hypoxia, NO can protect the myocardium from insufficient O_2 supply. This finding is supported by several previous in vitro studies [43, 66, 77, 79, 87]. However, NO's effectiveness depends on its concentration [49]. Unfortunately, the NO concentration in vivo is unclear at present partly due to lack of direct methods to measure. Using a porphyrinic-based NO-selective electrode, NO within membrane bilayers can be measured between 10 nM and 5 μM [48]. In general, physiological concentration of NO (< 5 μM) are beneficial, but excessive NO formation during ischemia can injure ischemic myocardium by activating proapoptotic transcription factors and/or producing

cytotoxic peroxynitrite upon reperfusion. For example, administration of NO or a NO donor prior to ischemia evokes pharmacological preconditioning, which is cardioprotective against I/R injury [9], but high NO concentration during reperfusion, when massive ROS is produced and reacts with NO, is toxic and induces necrosis and apoptosis in myocardium [34, 81].

Nitric oxide also attenuates β-adrenergic activation of myocardium [7, 95, 96]. In animal models of aging, increased nNOS activity [17] further reduces the inotropic effects of catecholamines. Endogenous NO decreases cardiac contraction in response to activation of the autonomic nervous system through β_3 receptor [71]. Involvement of β_3 adrenoceptors in negative inotropic effect of NO was further substantiated by using BRL 37344, a β_3 adrenoceptor agonist [71]. The activation of β_3-receptor can activate eNOS, which sequentially activates guanylate cyclase, cGMP formation, and cGMP-dependent protein kinase G (PKG). PKG can suppress cAMP and PKA activation, respectively, and thereby produce a negative inotropic effect. At concentration above 5μM, NO hyperactivats PKG, which then suppresses voltage-dependent Ca^{2+} channels and phosphorylation of troponin I decreases myofilament Ca^{2+} responsiveness [76]. Even during β-adrenergic stimulation, high concentrations of NO can inhibit ryanodine receptors and dampen myocardial function [57].

The multifarious functions of NO have become extremely important in several physiological and pathological phenomena such as vasodilation, platelet inhibition, bronchodilation, modulation of intestinal motility, neural transmission, endothelial

permeability, smooth muscle proliferation, insulin secretion, calcium transportation and redistribution, immunological function and metabolism [30, 56].

NO in ischemia/reperfusion injury

As noted above, NO is a "dual-edged sword," exerting beneficial effects at physiological concentrations, but inflicting injury at higher, pathological concentration, especially under conditions of myocardial ischemia/reperfusion (I/R). I/R injury is defined as damage to tissue caused when blood supply is restored to the tissue after an ischemic period. Ischemia can modify the chemical milieu within the tissue such that the restoration of tissue perfusion and O_2 delivery upon reperfusion results in inflammation and oxidative damage rather than recovery of normal function. During the reperfusion, high concentration of NO interacts with massively produced ROS, then forms $ONOO^-$. As described earlier, increased $ONOO^-$ formation cause serious detrimental effects [34, 81] on I/R injury patients. Clinical examples of I/R occur as a result of successful balloon angioplasty, tissue plasminogen activator (tPA) induced thrombolysis, and organ transplantation [9]. In organ transplantation, I/R injury is routinely observed following reperfusion of the transplanted organ.

Hypoxia-reoxygenation, NO formation and expression of NOS

Several studies have reported that continuous hypoxia and prolonged intermittent hypoxia conditioning of adult rats and neonatal rabbits evoked mRNA expression [1, 22, 32], and synthesis and accumulation of NOS isoforms in myocardium [19, 70, 78, 86].

However, intermittent hypoxia conditioning led to decreased NOS content and activity in guinea-pig heart [55] and mouse brain [45], and also dampened expression of eNOS in vascular endothelial cells [44, 52]. Therefore, the intensity and/or duration of hypoxic stimulation may determine its impact on NOS expression and activity. Increased coronary vascular shear stress during hypoxia-induced hyperemia could produce bursts of NO production during brief cycles of intermittent hypoxia. In addition to enzymatic production, NO can be produced from nitrite (NO_2^-) at low pH under reducing conditions, such as those prevailing in the stomach, in the epidermis, or in the hypoxia or ischemic heart. During hypoxia, met-hemoglobin functions as an oxygenase and reduces NO_2^- to NO [31]. Either enzymatic or non-enzymatic production of NO suppresses expression of NOS genes [33], which could suppress NOS activity enough to dampen excess NO formation during myocardial ischemia-reperfusion. In addition, moderate generation of NO during intermittent hypoxia could increase myocardial NO stores, in form of *S*-nitrosothiol and *S*-nitrosohemoglobin; accumulation of these NO stores is thought to be an adaptive mechanism to intermittent hypoxia [83]. NO alone or in collaboration with other transcriptional factors could suppress expression of eNOS [33, 50] leading to progressive restraint of excessive nitric oxide formation over the 20 d of normobaric intermittent hypoxia conditioning (IHC) program described by Zong et al [97].

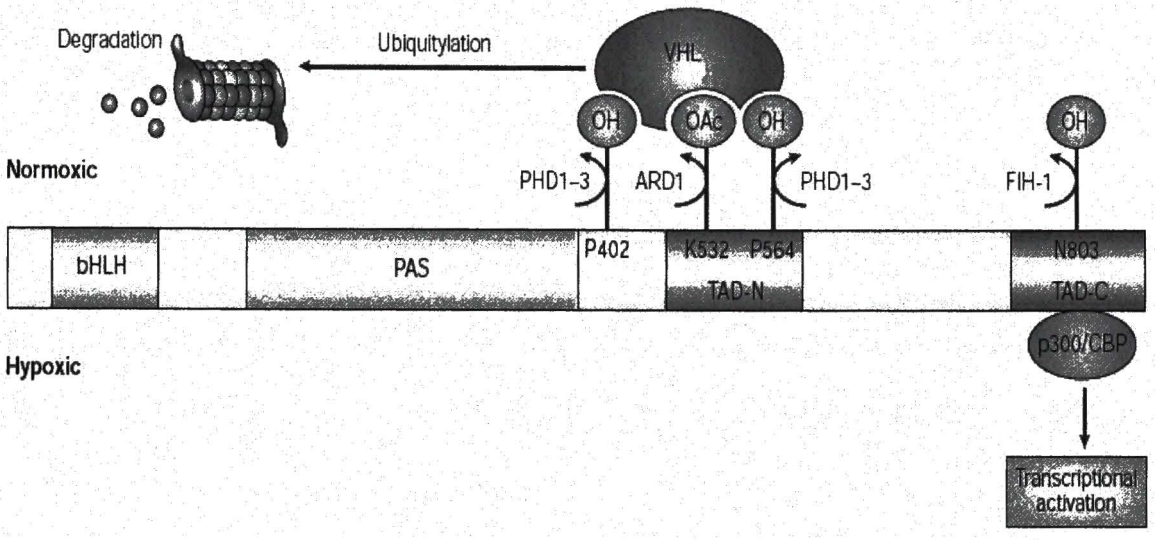
The current study tested the proposal that intermittent hypoxia conditioning suppressed myocardial NOS activity and eNOS content, and thereby minimize the cytotoxic burst of NO formation upon reperfusion of occluded coronary arteries.

Hypoxia inducible factor-1 and NOS

The transcription factor, hypoxia inducible factor-1 (HIF-1) regulates a spectrum of proteins related to angiogenesis, glucose/energy metabolism, tumour development and ischemic/hypoxic diseases [69, 73]. HIF-1 is a heterodimer of two subunits, HIF-1α and HIF-1β which contain basic-helix-loop-helix PAS domains that bind to a DNA core sequence (G/ACGTG) in the hypoxia responsive elements (HRE) of target genes [82]. HIF-1β subunits are constitutively expressed and O_2-independent, whereas HIF-α subunits are degraded in the presence of normal O_2 concentrations and stabilized by hypoxia. Three HIF-α subunits have been identified in mammals: HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α appear closely related in their ability to induce transcriptional activity of hypoxia responsive genes [82, 85]. On the other hand, HIF-3α is involved in negative regulation of the response via an alternately spliced transcript coding the inhibitory PAS domain protein [46].

HIF-1α subunits are regulated by multiple steps, including mRNA splicing and subcellular localization. The analysis of post-translational modification of these proteins revealed that a series of non-heme, iron-dependent oxygenases hydroxylate specific HIF-α residues in an O_2-dependent manner [12, 36, 37, 90]. Hydroxylation of two prolyl residues, Pro 402 and 564 in human HIF- 1α, mediates interaction with the von Hippel-Lindau (VHL): E3 ubiquitin ligase complex that targets HIF-α for proteosomal destruction (Figure 4) [28, 53, 72]. Under normoxic conditions (FIO_2 21%), HIF-1α is tagged for proteosomal degradation by O_2-dependent proline hydroxylation [38]. The half life of HIF- 1 is extremely short, less than 1 min, so within a few min of

reoxygenation HIF-1 decomposes, the HIF-1α subunit is proteolytically degraded, and expression of HIF-1 responsive genes is silenced.



Firgure 4. ***O_2-dependent regulation of HIF-1 activity.*** O_2 regulates the rate at which HIF-1α protein is degraded. In normoxic conditions, O_2-dependent hydroxylation of proline (P) residues 402 and 564 in HIF-1α by the enzymes PHD (prolylhydroxylase-domain protein) 1-3 is required for the binding of the von Hippel-Lindau (VHL) tumour-suppressor protein, which is the recognition component of an E3 ubiqutin-protein ligase. VHL binding is also promoted by acetylation of lysine (K)residue 532 by the ARD1 acetyltransferase. Ubiquitinylation of HIF-1α targets the protein for degradation by the 26S proteasome. O_2 is also regulates the interaction of HIF-1α with transcriptional coactivators. O_2-dependent hydroxylation of asparagine (N) residue 803 in HIF-1α by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α, and thereby inhibits HIF-1 mediated gene transcription. Under hypoxic conditions, the rate of asparagine and proline hydroxylation decreases. VHL cannot bind to HIF-1α that is not prolyl-hydroxylated, resulting in a deceased rate of HIF-1α degradation. By contrast, p300 and CBP can bind to HIF-1α that is not asparaginyl-hydroxylated, allowing transcriptional activation of HIF-1 target genes. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; TAD-C, carboxy-terminal transactivation domain; TAD-N, amino-terminal transactivation domain. [73].

Genes regulated by HIF-1 contain hypoxia responsive elements (HRE), transcriptional regulatory motifs that include one or more HIF-1 binding sites [72]. A HRE has been identified in eNOS [53] and in nNOS genomic sequence [28]. eNOS mRNA abundance was increased during chronic hypoxic stress suggesting that eNOS gene contains HRE. However, eNOS can be inhibited indirectly by HIF-1 through activation of erythropoietin (EPO)[88]. Prolonged exposure to hypoxia causes circulating EPO to increase a hundred-fold [61]. EPO, which increases erythropoiesis, triggers eNOS via increased shear stress on endothelial cells. However, when coronary endothelial cells were cultured without alteration in shear stress on endothelium, EPO caused down-regulated basal NO production and NOS activity [88]. The regulation of eNOS activity by HIF-1 is still unclear.

Rationale for this study

Our previous finding [97] of IHC induced cardioprotection led us to explore the mechanisms of this remarkably protective phenomenon. Since intermittent hypoxia stimulates HIF-1 [49], we focused on the end products of HIF-1 induction of hypoxia-responsive genes. eNOS is an end product of HIF-1 transcriptional activation. Increased eNOS expression leads to enhanced formation of NO. Excessive NO can rapidly and irreversibly condense with $\cdot O_2^-$ to generate $ONOO^-$, the precursor of a host of highly reactive, cytotoxic ROS (Figure 2) [49, 60]. $ONOO^-$ may contribute to cardiac sympathovagal imbalances in the brainstem and cardiomyocytes, which may predispose

to arrhythmia [18]. NO also reacts with bicarbonate to form carbonate radial (CO_3^-). These toxic derivatives chemically modify proteins, lipids, and DNA.

Collectively, physiological amounts of NO may protect myocardium. In contrast, excessive NO could injure myocardium by producing $ONOO^-$, by direct attack of respiratory chain components and metabolic enzymes, and by activating PKG [49]. Accordingly this study was designed to test the hypothesis that intermittent, normobaric hypoxia conditioning dampens NOS activity and cytotoxic formation of NO and its derivatives during reperfusion following coronary artery occlusion. The study also tested whether these changes in formation of vasodilator NO affected reperfusion reactive hyperemia.

Significance

Aside from early reperfusion, interventions to prevent ischemia/reperfusion (I/R) injury were unavailable until 20 years ago, when Murry et al. [58] first described ischemic preconditioning in dogs. Ischemic preconditioning is accomplished by temporarily occluding a coronary artery for a few minutes, which alone does not injure the tissue but instead makes it more resistant to subsequent, severe ischemic insult [89]. Even though preconditioning is a powerful cardioprotective phenomenon, its clinical use is extremely limited. Preconditioning requires coronary artery occlusion, which is invasive, inherently risky and impractical in conscious patients. Can the heart be protected from I/R injury without imposing such an invasive procedure on the patient?

We previously demonstrated that a 20 day program of normobaric, intermittent hypoxic conditioning produced powerful cardioprotection against ischemia/reperfusion injury [97]. IHC produced a dramatic reduction in myocardial infarct and lethal arrhythmias during coronary artery occlusion-reperfusion experiments conducted one day after completing the IHC program [97]. Unlike ischemic preconditioning, IHC is a noninvasive process which doesn't require dangerous coronary artery occlusions. Moreover, our IHC protocol is distinguished by its intermittent, 4 min reoxygenation episodes, in contrast to conditioning programs of several h/d of continuous hypoxia applied to smaller mammals (Table 2). Although several of these studies provided information on mechanisms of hypoxia-induced cardioprotection, these results should be extrapolated to larger mammals with caution, especially considering the high metabolic rates of rodent myocardium. Moreover, IHC was far more cardioprotective than prolonged hypoxia programs, which produced only modest reductions in infact size [97] and reperfusion arrhythmia [97].

Specific aim

The specific aim of this study was to test the hypothesis that intermittent hypoxic conditioning suppresses myocardial nitric oxide synthase activity and thereby dampens explosive, cytotoxic NO formation upon reperfusion of occluded coronary arteries.

To accomplish this aim mongrel dogs were conditioned by the 20d IHC program previously shown to confer robust cardioprotection [97]. One day after completion of the IHC program, the left anterior descending coronary artery was occluded for 1h, followed

by 5 h reperfusion. Nitrite, a stable product of NO, was measured as an index of NO formation [40, 52]. NO_2^- was measured in coronary sinus and systemic arterial plasma. Myocardial NO_2^- formation was computed as coronary flow (ml/g tissue) simultaneously measured with an electromagnetic flow probe placed around LAD adjacent to the occlusion site. NOS activity was measured in protein extracts of myocardial biopsies, and eNOS content and phosphorylation were measured by immunoblot.

Table2. ***Studies in small mammals of hypoxia conditioning-induced cardioprotection against ischemia-reperfusion injury.***

Citation	Species	FIO_2 (%)	h/d	Days	Outcome
Asemu *et al.*, 1999[4]	rat	11*	4	25-30	64% reduction in ventricular tachyarrhythmias
Baker *et al.*, 1999[6]	rabbit†	12	24	9	Improved post-ischemic LV contractile recovery
Eells *et al.*, 2000[20]	rabbit†	12	24	7-10	Improved post-ischemic LV contractile recovery
Forkel *et al.*, 2004[27]	rat	10.5	24	14	Improved post-ischemic RV but not LV function
Manukhina *et al.*, 2000[50]	rat	11*	5	40	Less aortic hypotension during coronary occlusion
Neckár *et al.*, 2002[63]	rat	11*	8	24-32	15% smaller infarct; increased post-ischemic dP/dt
Neckár *et al.*, 2004[61]	rat	11*	8	24-32	31% smaller infarct
Zhong *et al.*, 2000[91]	rat	11*	6	14-42	Decreased cardiac arrhythmias
Zhong *et al.*, 2002[92]	rat	11*	6	28-42	Improved recovery of LV systolic, diastolic function
Zhu *et al.*, 2003[93]	rat†	11*	6	42	Delayed contracture; improved LV systolic recovery
Zhu *et al.*, 2004[94]	rat†	11*	6	60	Improved LV recovery; decreased LDH release

LDH: lactate dehydrogenase; LV: left ventricular; RV: right ventricular; dP/dt: rate of change of LV pressure. *:These studies used hypobaric hypoxia; FIO_2 values are normobaric equivalents of the reduced atmospheric O_2 content. †Neonate.

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CHAPTER II

HYPOXIC CONDITIONING SUPPRESSES NITRIC OXIDE PRODUCTION UPON MYOCARDIAL REPERFUSION

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ABSTRACT

Background: Physiologically modulated concentrations of nitric oxide (NO) are generally beneficial, but excessive NO can injure myocardium by producing cytotoxic peroxynitrite. Recently we reported that intermittent, normobaric hypoxia conditioning (IHC) produced robust cardioprotection against infarction and lethal arrhythmias in a canine model of coronary occlusion-reperfusion. Hypothesis: IHC suppresses myocardial nitric oxide synthase activity and thereby dampens explosive, cytotoxic NO formation upon reperfusion of occluded coronary arteries. Methods: Mongrel dogs were conditioned by a 20 d program of IHC (FIO_2 9.5-10%; 5-10 min hypoxia/cycle, 5-8 cycles/d with intervening 4 min normoxia). One day later, ventricular myocardium was sampled for measuring nitric oxide synthase (NOS) activity (colorimetric assay) and endothelial NOS (eNOS) content (immunoblot). In separate experiments, myocardial nitrite (NO_2^-) release, an index of NO formation, was measured at baseline and during reperfusion following 1 h occlusion of the left anterior descending coronary artery (LAD). Values in IHC dogs were compared with respective values in non-conditioned, control dogs. Results: IHC lowered left and right ventricular NOS activity by 60%, from 100-115 to 40-45 mU/g protein ($P < 0.01$), and decreased eNOS content by 30%. IHC dampened cumulative NO_2^- release during the first 5 min reperfusion from 32 ± 7 to 14 ± 2 μmol/g ($P < 0.05$), but did not alter hyperemic LAD flow (15 ± 2 *vs.* 13 ± 2 ml/g). Conclusions: IHC suppressed myocardial NOS activity, eNOS content and NO formation upon reperfusion

without compromising reactive hyperemia. Attenuation of the NOS/NO system may contribute to IHC-induced protection of myocardium from ischemia-reperfusion injury.

Key words: nitric oxide synthase, cardioprotection, intermittent hypoxia, dogs, myocardial ischemia

Abbreviations: BL: baseline; CK: creatine kinase; eNOS: endothelial nitric oxide synthase; G6PDH: glucose 6-phosphate dehydrogenase; HIF-1: hypoxia-inducible factor 1; IHC: intermittent hypoxia conditioning; I/R: ischemia-reperfusion; LAD: left anterior descending coronary artery; LCX: left circumflex coronary artery; LDH: lactate dehydrogenase; NOS: nitric oxide synthase; PFK: phosphofructokinase; RV: right ventricular myocardium

INTRODUCTION

Restoration of coronary blood flow is the most effective means of salvaging ischemic myocardium, but reperfusion can paradoxically exacerbate ischemic injury [4]. Ischemia alters the chemical composition of cells, creating an environment in which the reintroduction of oxygen upon reperfusion precipitates inflammation and oxidative stress, compromising recovery of cellular function and even causing cell death. With its high metabolic demand, myocardium ranks among the tissues most susceptible to ischemia/reperfusion (I/R) injury. Myocardial I/R injury can occur in a variety of clinical settings including balloon angioplasty, tissue plasminogen activator induced thrombolysis, coronary artery bypass grafting, and heart transplantation.

Recent studies in our laboratory [56, 30] demonstrated the cardioprotective potential of intermittent, normobaric hypoxia conditioning (IHC). Dogs were conditioned by a 20 day program of brief (5-10 min) hypoxia exposures interspersed with periods of reoxygenation. IHC produced dramatic reductions in myocardial infarct and ventricular arrhythmias during coronary artery occlusion-reperfusion experiments conducted one day after completing the IHC program [56]. This potent cardioprotection developed progressively over the course of the 20 d IHC program, since a single IHC session imparted no cardioprotection against infarction or arrhythmias, and the protection afforded by a 10 d IHC program, although appreciable, was less complete than that provided by the full 20 d program [30]. The progressive development of the

cardioprotection suggested that changes in gene expression and protein content may have produced the cardioprotected phenotype.

Myocardial nitric oxide (NO) production increases during ischemia [32]. Moderate NO production can be beneficial: it relaxes smooth muscle to increase tissue perfusion, suppresses platelet aggregation and leukocyte adherence to endothelium, and dampens fibrinogen formation. However, excessive NO formation can injure ischemic myocardium by activating pro-apoptotic transcription factors [44], inhibiting metabolic enzymes [34] and/or producing cytotoxic peroxynitrite upon reperfusion [31]. Administration of NO or NO donors prior to acute myocardial ischemia has been demonstrated to evoke cardioprotection against I/R injury [5], but excessive NO concentrations induce necrosis and apoptosis in myocardium [21, 46, 48].

This study was conducted in mongrel dogs to test the hypothesis that IHC evokes cardioprotective adaptations of the myocardial NOS system. Specifically, the proposal that IHC suppresses myocardial NOS activity sufficiently to dampen the cytotoxic burst of NO formation upon reperfusion of ischemic myocardium was tested.

METHODS

Animals. Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and was conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1996). Healthy adult mongrel dogs were assigned to IHC and non-conditioned groups. The IHC dogs completed a 20 day IHC program [1] by breathing hypoxic atmospheres in a 270 liter acrylic chamber. Daily sessions consisted of 5-8 cycles of hypoxia (FIO_2 9.5-10%), each 5-10 min, with intervening 4 min exposures to room air. Dogs were maintained on a 12:12 h light/dark cycle and received standard chow diet and water *ad libitum* throughout the conditioning program. The dogs were fasted overnight before the terminal experiment.

Surgical preparation and myocardial ischemia-reperfusion protocol. Coronary artery occlusion-reperfusion experiments were conducted in IHC dogs (n = 6) one day after completing the IHC program, and in non-IHC, control dogs (n = 5). After an overnight fast, dogs were anesthetized with sodium pentobarbital (30 mg/kg, *iv*), intubated, and mechanically ventilated with room air. A femoral vein was cannulated to administer $NaHCO_3$ and supplemental pentobarbital. A saline filled catheter was introduced into the thoracic aorta via a femoral artery to measure aortic pressure and to sample arterial blood for measuring blood gases and nitrite (NO_2^-). Arterial PO_2, PCO_2,

and pH were maintained within normal limits by ventilating with supplemental O_2, adjusting tidal volume and respiratory rate, and by administering $NaHCO_3$.

The heart was exposed through a left thoracotomy in the fifth intercostal space. The left anterior descending coronary artery (LAD) was isolated distal to its first major diagonal branch. An electromagnetic flow probe (Transonic Systems model 2SB, Ithaca, NY) was placed on the LAD to monitor coronary flow, and a silk suture was passed around the LAD immediately distal to the flow probe, forming a snare. After heparin administration (500 U/kg) the interventricular coronary vein, which selectively drains the LAD perfusion territory [49], was cannulated to sample coronary venous blood. Body temperature was measured with an intramuscular needle probe placed in the *quadriceps femoris* and maintained at 36.5 – 37.5°C with a circulating H_2O heating pad positioned under the dog.

The LAD occlusion-reperfusion protocol commenced after completion of surgery and stabilization of hemodynamic and blood gas variables. The LAD was occluded for 1 h by tightening the snare, and then reperfused for 5 h by releasing the snare [56]. Ventricular fibrillation occurred in two control dogs following reperfusion; direct current countershocks (10 J) were applied to the epicardium within 15 s, by use of internal paddles, to restore sinus rhythm [30]. Systemic arterial and coronary venous blood samples for measuring NO_2^- were collected just before LAD occlusion and during the first 40 min of reperfusion.

Nitrite determination. Myocardial release of NO_2^-, a stable product of NO oxidation [7], was measured as an index of NO formation [24, 28]. NO_2^- concentrations in aortic and coronary venous plasma were measured by the Griess reaction, using a commercially available kit (Endogen, Rockford, IL). Plasma samples obtained after centrifugal sedimentation of formed elements were mixed with 1 vol reagent diluent and filtered (10 kD molecular weight cut off) by centrifugation at 14,000 g for 30 min at room temperature. 50 µl aliquots of filtrate were pipetted onto a 96 well plate. Griess reagents I and II were added, the plate was incubated for 10 min at room temperature, and then absorbance at 540 nm was measured in a Power Wave XS plate reader (Bio Tek, Winooski, VT). NO_2^- release was calculated by multiplying LAD flow times the arteriovenous difference in NO_2^- concentration, and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$.

Extraction of myocardial enzymes. Enzymes were measured in myocardial biopsies taken from 8 IHC dogs one day after completing the IHC program, and from 4 control dogs. The dogs were anesthetized as described above, and the heart exposed through a left thoractomy. Transmural biopsies of LAD- and left circumflex (LCX)-perfused left ventricle and right ventricle were excised, quickly rinsed in cold saline, blotted and flash-frozen in liquid nitrogen. The frozen tissues were pulverized under liquid nitrogen using a mortar and pestle. Powdered tissue was extracted by homogenization in 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM ADP, 10 mM glutathione and 10 mM ethylenediaminetetraacetic acid (EDTA). Extract was

centrifuged at 100,000 x g for 20 min. The pellet was resuspended in phosphate buffer and re-extracted twice. The three supernatant fractions were combined, divided into aliquots, and stored at -80 °C.

Measurement of NOS and myocardial enzymes. Total NOS activity in tissue extracts was determined from the Griess reaction, with a commercially available kit (Oxis International, Portland, OR). Tissue extract was combined with assay buffer on 96-well plates. 1 mM NADPH solution and nitrate reductase were added sequentially according to kit instructions. After 60 min of incubation, 10 μl aliquots of cofactor and lactate dehydrogenase solutions were added to each well. 20 min later, Griess Reagents I and II were added. After another 10 min incubation, the plate was read at 540 nm. Other myocardial enzymes (lactate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphofructokinase, creatine kinase) were measured by standard colorimetric assays [2]. Extract protein concentration was measured according to Bradford [6] for normalization of enzyme activity.

Immunoblot assessment of eNOS content and phosphorylation. Endothelial nitric oxide synthase (eNOS) in myocardial extracts was analyzed. Samples containing 20 μg protein were resolved by electrophoresis (100 V for 2 h) on 10% polyacrylamide gels. Proteins were electrophoretically transferred (350 V for 3 h) from the gels to nitrocellulose membranes. Source gels were stained with Coomassie blue to confirm electrophoretic transfer. Membranes were incubated in 5% nonfat milk for 2 h to block

non-specific binding sites.

Rabbit anti-eNOS polyclonal antibody (Stressgen, Victoria, BC) was used to detect eNOS. Mouse anti-$P^{1177}S$ eNOS and anti-$P^{495}T$ eNOS antibodies (BD Bioscience) were used to detect eNOS phosphorylation. Immune complexes were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) upon exposure to X-ray film. Human endothelial cell lysates provided a positive control for eNOS. Protein band density was quantified with analytical software (AlphaEaseFC 4.0, Alpha Innotech, San Leandro, CA). Band densities of total eNOS, $P^{1177}S$ eNOS and $P^{495}T$ eNOS were normalized to actin density, and band densities of phosphorylated eNOS were normalized to total eNOS band densities.

Statistical analyses. Data are expressed as mean ± standard error. Single comparisons of means between control and IHC groups were performed by applying two-tailed unpaired Student's *t*-tests. Repeated measurements of nitrite release and LAD flow were compared by one-way analysis of variance (ANOVA). When ANOVA detected statistically significant differences, Tukey's test was applied to identify specific differences between mean values. P values < 0.05 were taken to indicate statistical significance.

RESULTS

Activities of myocardial enzymes. Four enzymes known to be induced by sustained hypoxia, lactate dehydrogenase (LDH), glucose 6-phosphate dehydrogenase (G6PDH), creatine kinase (CK), and phosphofructokinase (PFK) [15, 41, 42, 37], were measured in LAD- and left circumflex coronary artery perfused regions of left ventricular myocardium, and in right ventricular myocardium of IHC and control dogs. No statistically significant treatment effects on these enzyme activities were detected in any perfusion territory, although a trend toward increased G6PDH activity in the LAD region of IHC vs. control dogs was noted (Figure 1).

NO production during myocardial reperfusion. Nitrite (NO_2^-) was measured to assess NO formation in the LAD-perfused myocardium before and after LAD occlusion (Figure 2A). Pre-ischemic NO_2^- release did not differ among the IHC *vs.* control groups ($P = 0.41$). Following release of the LAD occlusion, NO_2^- release increased sharply and peaked at 2 min reperfusion in the control group, and then subsided (Figure 2A). In contrast, there was not a statistically significant surge in NO_2^- release upon reperfusion in the IHC group. During the initial 5 min of reperfusion, the cumulative myocardial NO_2^- release of the IHC group was less than half that of the control group (14 ± 2 *vs* 32 ± 7 $\mu mol \times g^{-1}$; $P = 0.017$; Figure 2B).

A robust hyperemia occurred following LAD reperfusion, which gradually subsided between 5 and 15 min reperfusion (Figure 3A). IHC did not attenuate the initial

reperfusion hyperemia; indeed, cumulative LAD flow during the first 5 min reperfusion in the IHC group was comparable to that of the control group (Figure 3B). IHC did tend to lower LAD flow at 5-10 min reperfusion (Figure 3A), although this effect was not statistically significant and occurred after the period in which IHC dampened NO_2^- release (Figure 2A).

Nitric oxide synthase activity. IHC lowered NOS activity in the LAD and left circumflex artery perfusion territories of the left ventricle, and in right ventricular myocardium by 55-63% (Figure 4). These IHC-induced reductions in NOS activity paralleled the 56% reduction in NO_2^- formation during reperfusion hyperemia (Figure 2B).

Endothelial NOS content and phosphorylation. Immunoblotting revealed an IHC-induced 30% decrease in left ventricular myocardial eNOS content, the principal constitutive NOS isoform in myocardium (Figure 5A). eNOS activity is modulated by phosphorylation at ^{1177}S, which increases eNOS activity, or at ^{495}T, which inactivates the enzyme. When normalized to total eNOS content, ^{1177}S phosphorylation was sharply increased in the IHC *vs.* control group, but ^{495}T phosphorylation was unaltered by IHC (Figure 5B).

DISCUSSION

Recently we demonstrated remarkable cardioprotection evoked by a 20 d IHC program [30, 56]. 1 h occlusion and 5 h reperfusion of the LAD infarcted nearly 40% of the ischemic myocardium in control dogs, but only 1% in the IHC dogs. Moreover, IHC prevented ventricular tachycardia and fibrillation that occurred upon LAD reperfusion in 82% of the non-hypoxic dogs [30]. The gradual development of this potent cardioprotection over the course of the 20 d IHC program [30] suggested that progressive changes in myocardial contents of potentially protective or adverse proteins may have contributed to the protection.

Since IHC might stimulate hypoxia inducible factor (HIF-1)-dependent transcription, we examined a panel of potentially cardioprotective enzymes expressed in response to HIF-1 [41, 42]. In contrast to chronic hypoxia, which decreases activities of CK [37] and increases G6PDH, LDH and PFK activities in rat myocardium [15, 41], IHC did not alter these enzyme activities in canine myocardium. These divergent adaptive responses to chronic and intermittent hypoxic conditioning could conceivably be related to differences in hypoxic 'dose', *i.e.* intensity of hypoxia x duration of exposure, which correlates with the extent of HIF-1 activation [29, 31]. In any case these results indicate that IHC, as employed in the present study, does not induce cardioprotection by mechanisms mediated by HIF-1.

Favorable vs. detrimental effects of nitric oxide. A physiological vasodilator and trigger of ischemic and pharmacological preconditioning [3], NO is another putative mediator of IHC induced cardioprotection. Administration of NO or NO donors prior to severe ischemia has been found to reduce I/R injury, including cardiomyocyte apoptosis [27, 36]. NO increases cardiomyocyte function by inhibiting phosphodiesterase III, thereby increasing cyclic AMP concentration and activating protein kinase A [25]. Moreover, physiological concentrations of NO might increase myocardial function by directly activating voltage gated sarcolemmal Ca^{2+} channels or Ca^{2+} dependent Ca^{2+} release channels in sarcoplasmic reticulum [33]. Other potential cardioprotective actions of NO include coronary vasodilation [17], suppression of inflammatory neutrophil infiltration [55], preservation of coronary endothelial function [51], and reduction of myocardial O_2 demand [20, 43, 45]. NO can decrease mitochondrial Ca^{2+} uptake by activating mitochondrial ATP dependent K^+ channels [1, 38], thereby ameliorating mitochondrial Ca^{2+} overload.

At high concentrations, NO and its derivatives [31] can injure myocardium. NO rapidly and irreversibly condenses with $\cdot O_2^-$ [35] to generate peroxynitrite ($ONOO^-$), the precursor of a host of highly reactive, cytotoxic ROS and reactive nitrogen species [31]. NO and $ONOO^-$ may directly attack catalytic Fe·S-centers of respiratory chain components and the Krebs cycle enzyme aconitase, and thereby impair ATP production [7, 18, 47, 54]. High NO concentrations also initiate apoptotic cell death [21, 46] by activating mitogen-activated protein kinases and pro-apoptotic transcription factors [46].

Reactive nitrogen species including NO and $ONOO^-$ are thought to contribute to cardiac sympathovagal imbalances in the brainstem and activation of cardiomyocytes, which may predispose to arrhythmia [10]. $ONOO^-$ initiates peroxidation of membrane phospholipids [39].

Ischemia-reperfusion creates conditions that favor formation in myocardium of NO and its toxic derivative $ONOO^-$. The intense reactive hyperemia upon myocardial reperfusion produces shear stress, a major stimulus of NO formation by eNOS [8]. Moreover, changes in the intracellular milieu during ischemia lead to explosive formation of the superoxide radical ($\cdot O_2^-$) when O_2 is reintroduced upon reperfusion of myocardium [19, 50, 53]. Oxidative stress imposed by ischemia-reperfusion can uncouple eNOS, causing the enzyme to release $\cdot O_2^-$ instead of NO by disrupting the normal flow of electrons from NADPH to *L*-arginine [14]. On the other hand, decreased eNOS content would lower the potential for $\cdot O_2^-$ formation by the uncoupled enzyme. Thus, myocardial ischemia-reperfusion enhances formation of both NO and $\cdot O_2^-$, yet IHC-induced reductions in eNOS content and activity could dampen formation of NO from normally functioning eNOS and $\cdot O_2^-$ from the uncoupled enzyme, and thereby lessen $ONOO^-$ formation following reperfusion.

Mechanism of intermittent hypoxia suppression of myocardial NOS. In the present study the 20 day IHC program lowered myocardial NOS activity and eNOS content, and produced a commensurate dampening of nitrite release during the first 5 min

of reperfusion, indicating decreased NO formation. Notably, the reduction in NO formation did not attenuate reactive hyperemia; thus, it appears that high concentrations of NO are not mandatory for full coronary vasodilation upon reperfusion.

Chronic and intermittent hypoxic exposures produce directionally opposite changes in eNOS activity. Thus, chronic hypoxia increased eNOS activity of rat myocardium [13, 40], but intermittent hypoxia dampened rat myocardial eNOS activity [26]. Chronic hypoxia increases expression of erythropoietin via HIF-1 activation [23]. By increasing hematocrit, erythropoietin enhances intravascular shear stress on endothelium, thereby activating eNOS. However, when chronic hypoxia did not increase hematocrit, erythropoietin lowered eNOS activity [52]. The 20 d IHC program tested here does not increase hematocrit [30, 56]. Conceivably, HIF-1 activation in this study might indirectly have lowered eNOS activity via sub-erythropoietic concentrations of erythropoietin.

A second possible mechanism of IHC suppression of eNOS may involve hypoxic enhancement of the nitrite reductase activity of deoxyhemoglobin. In the IHC paradigm, arterial hemoglobin O_2 saturation falls to roughly 70-75% during each hypoxia cycle [30]. Deoxyhemoglobin catalyzes reduction of nitrite to NO, which escapes the erythrocytes in sufficient quantities to activate the endothelium-dependent vasodilatory mechanism during hypoxia [9, 11, 22]. Conceivably, deoxyhemoglobin may have generated sufficient NO during the cyclic bouts of hypoxia to suppress eNOS expression [16],

causing myocardial eNOS content to progressively decline over the course of the 20 d IHC program.

Endothelial NOS activity is modulated by phosphorylation. Although total myocardial NOS content fell, IHC increased fractional eNOS phosphorylation at ^{1177}S, a post-translational modification that enhances eNOS activity [12]. There was no difference in ^{495}T phosphorylation, a repressor of the enzyme's activity [12]. Although ^{1177}S phosphorylation would activate eNOS, it appears this mechanism was not enough to overcome the decrement of eNOS content.

Summary. A 20 d IHC program suppressed canine myocardial NOS activity, eNOS content and reperfusion NO release without compromising reactive hyperemia. Other, potentially cardioprotective enzymes were not altered by IHC. Collectively, these results suggest that dampening NO production upon reperfusion may protect the heart from the ravages of myocardial ischemia-reperfusion.

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Figure 1

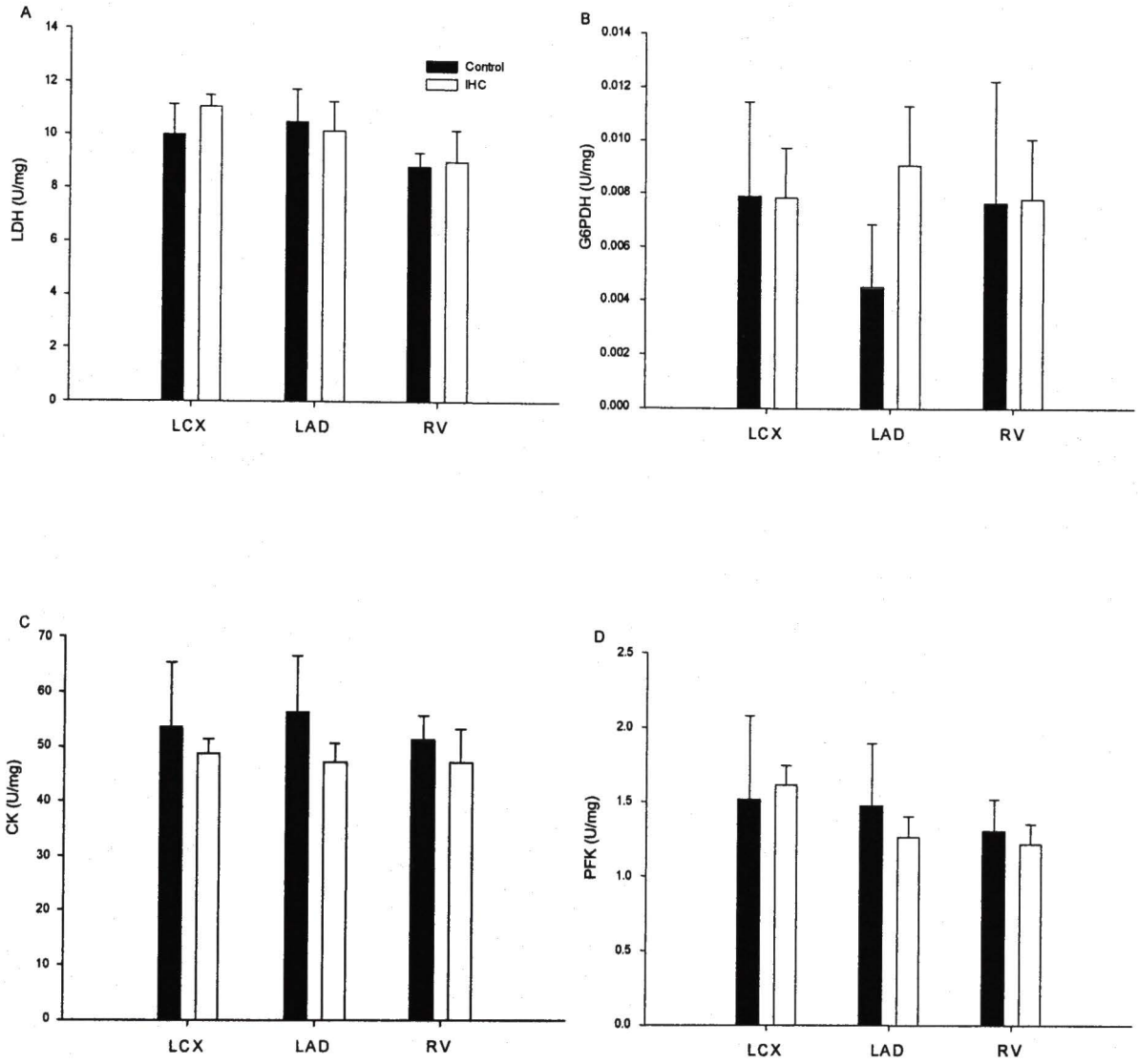


Figure 1. *IHC did not alter activities of hypoxia-inducible myocardial enzymes.* Lactate dehydrogenase (LDH), glucose-6-phophate dehydrogenase (G6PDH), creatine kinase (CK), and phosphofructokinase (PFK) activities were measured in transmural biopsies taken from left ventricular myocardium perfused by the left anterior descending (LAD) and left circumflex (LCX) coronary arteries, and from right ventricular myocardium (RV) of non-hypoxic controls (filled bars) and dogs conditioned by 20d IHC (open bars). Values are means ± SEM from 4 control and 8 IHC dogs. No statistically significant between-group differences were detected.

Figure 2

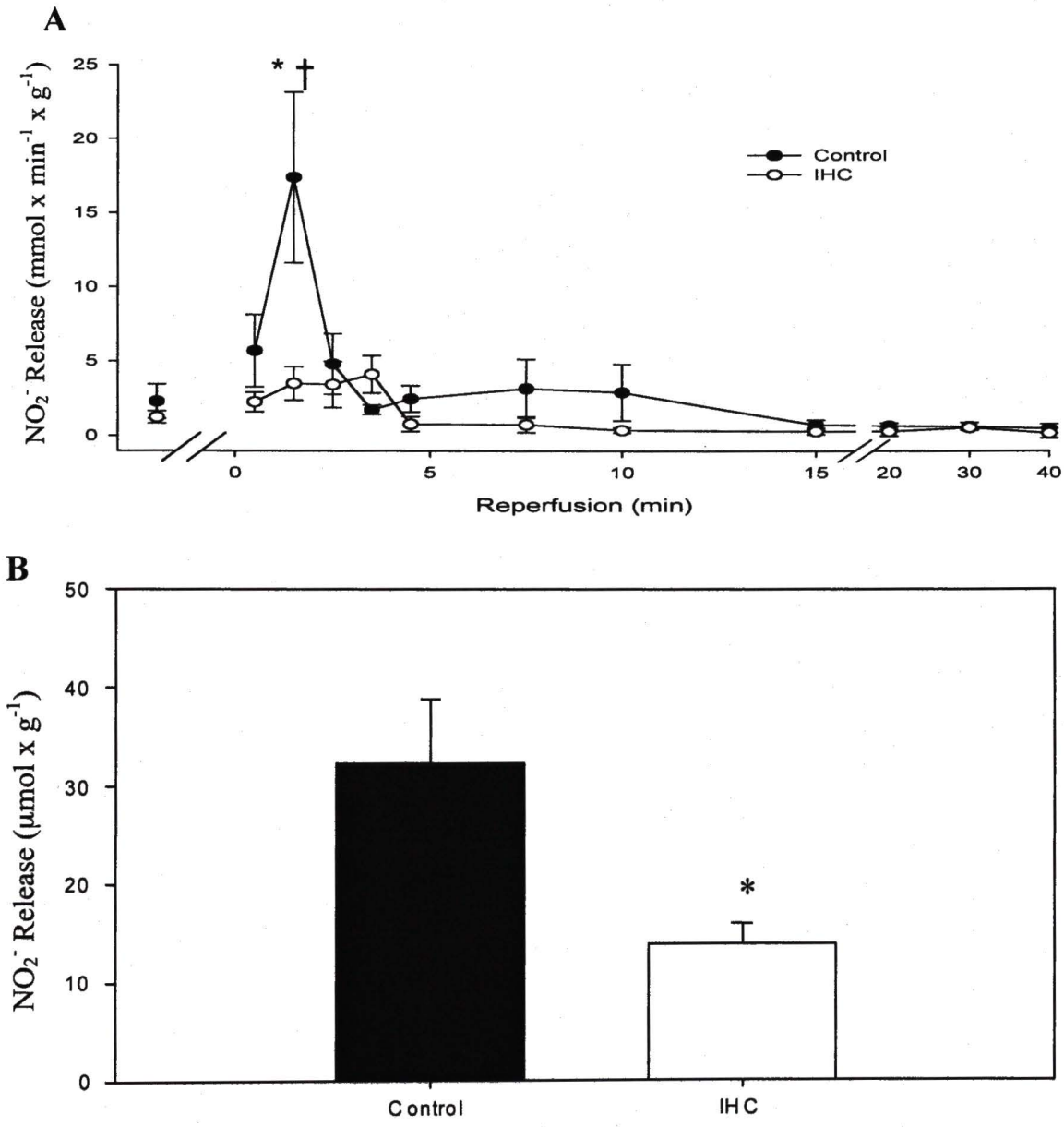


Figure 2. *Nitrite release during reperfusion reactive hyperemia.* NO_2^- release peaked within 2 min reperfusion in non-hypoxic control dogs (Panel A). IHC dampened this reperfusion NO_2^- burst, and lowered cumulative NO_2^- release during the first 5 min reperfusion by 56 % (Panel B). Means ± SEM from 6 IHC and 5 control experiments. * $P < 0.05$ *vs.* non-hypoxic control, † $P < 0.05$ *vs.* pre-occlusion baseline (BL).

Figure 3

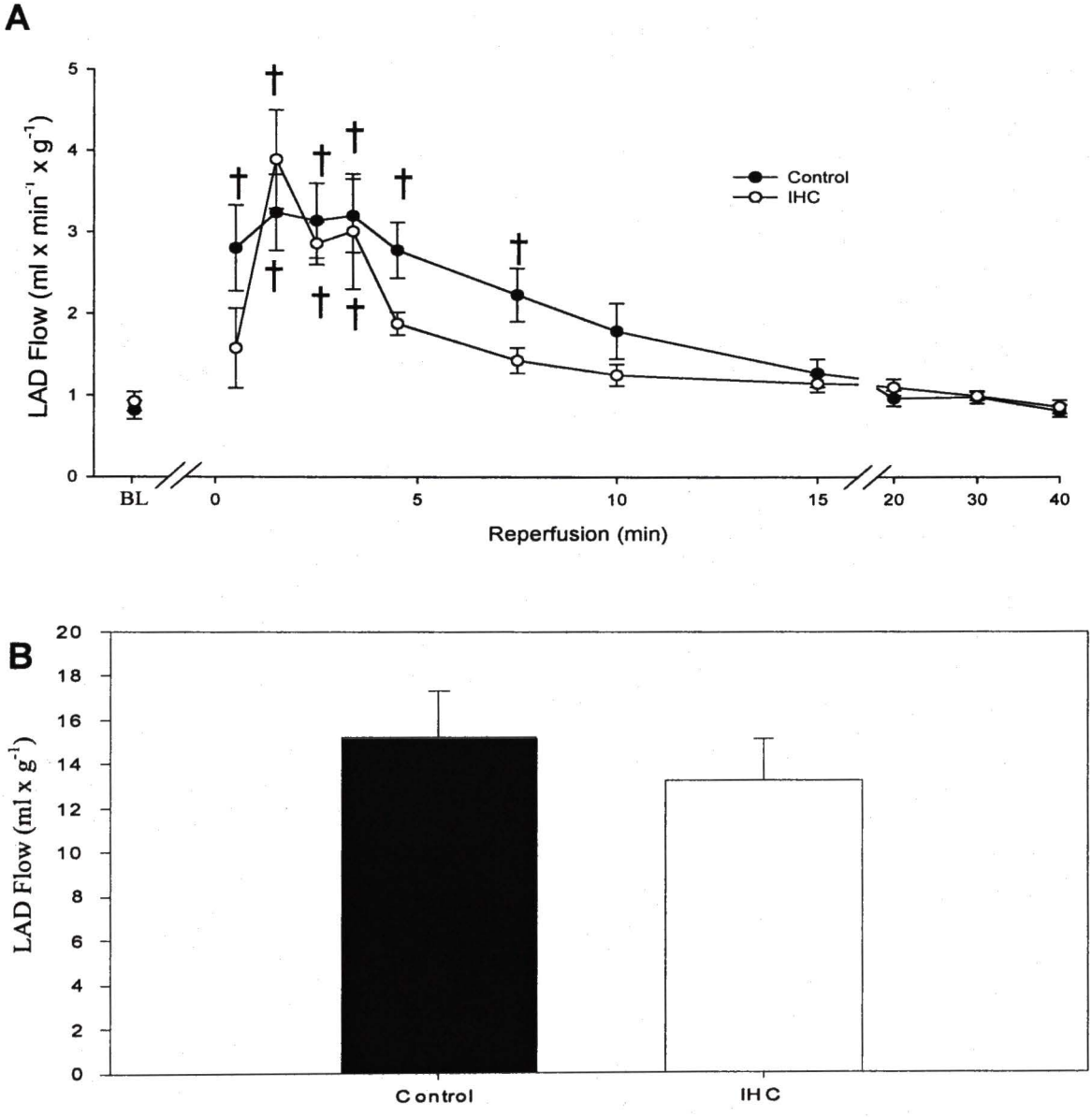


Figure 3. *LAD blood flow during reperfusion.* No significant differences were detected in LAD flow during pre-occlusion baseline or following reperfusion (Panel A). Cumulative LAD flow during the first 5 min reperfusion did not differ in non-hypoxic *vs.* IHC dogs (Panel B). Means ± SEM from the same experiments as in Figure 2. † $P < 0.05$ *vs.* baseline (BL).

Figure 4

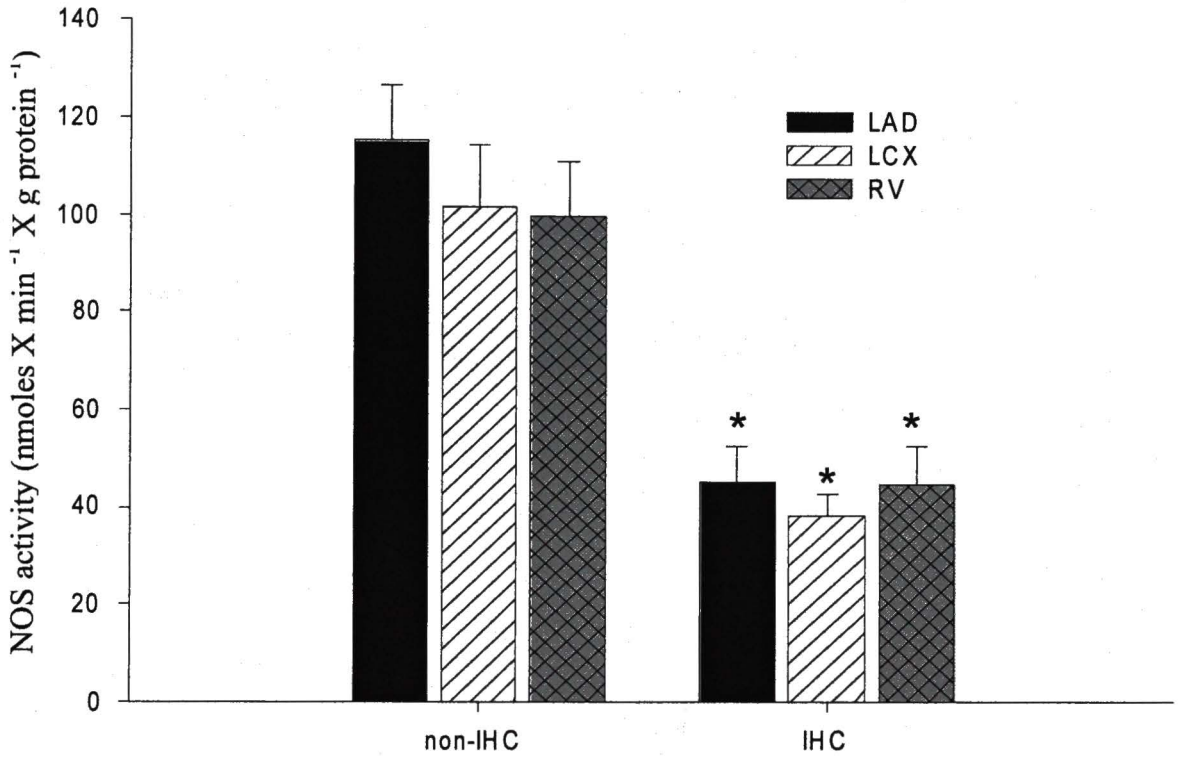
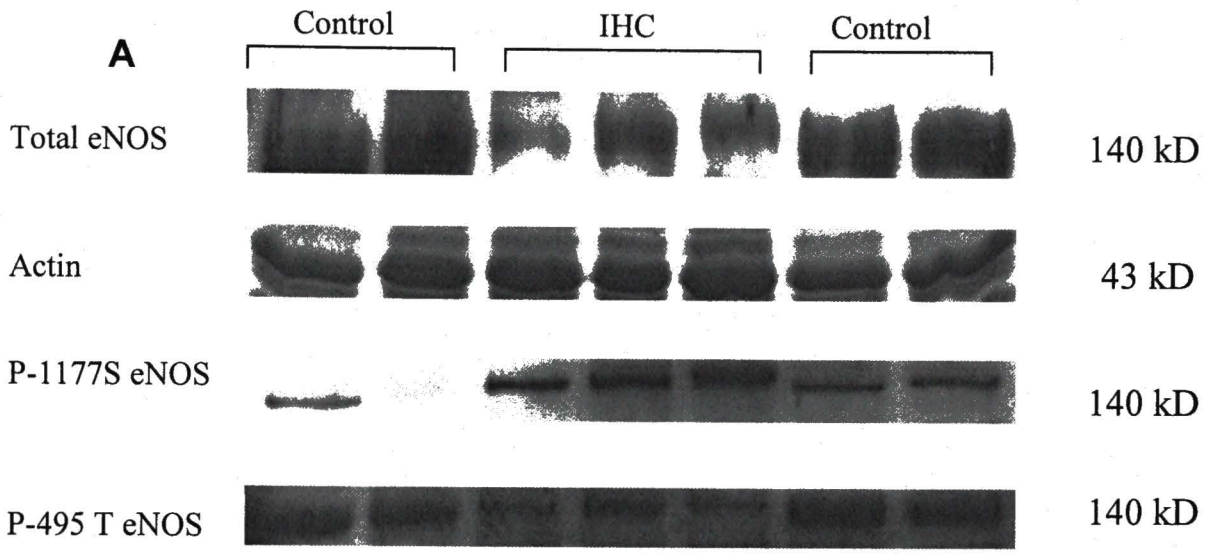


Figure 4. *Intermittent hypoxia conditioning decreases nitrite oxide synthase activities in eft and right ventricular myocardium.* NOS activities were measured in the same nyocardial biopsies as in Figure 1. Abbreviations as in Figure 1. * $P<0.05$ vs. non-hypoxic control.

Figure 5



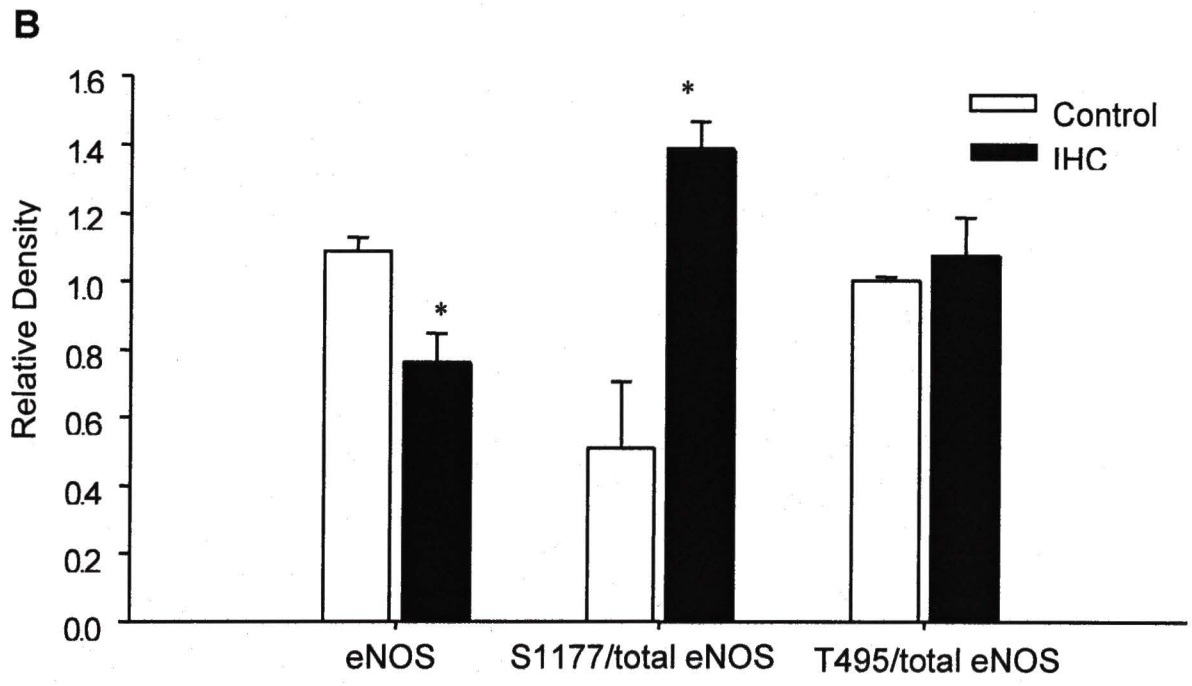


Figure 5. *Immunoblot of eNOS in left ventricular myocardium of non-conditioned and intermittent hypoxia-conditioned dogs.* Densities of the bands for total eNOS, P-^{1177}S eNOS, and P-^{495}T eNOS are normalized to densities of the actin bands, which served as loading controls. Values in panel B are means ± SEM from 3 IHC and 4 non-hypoxic control experiments per group. *P<0.05 vs. control.

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