Intermittent hypoxia induced opioidergic protection of the heart

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INTERMITTENT HYPOXIA INDUCED OPIOIDERIC PROTECTION OF THE HEART

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
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In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY

By
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ABSTRACTS


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INTRODUCTION

“Therapeutic Hypoxia” vs “Detrimental Hypoxia”

In humans chronic intermittent hypoxia has been associated with impairments in cardiovascular, respiratory and cognitive function [52]. Consequently, from a clinical standpoint it is generally felt that intermittent hypoxia causes perturbations that result only in maladaptations to hypoxia, such as the severe hypertension that occurs with sleep apnea, and that hypoxia of any sort is harmful and should be avoided. These maladaptations are primarily mediated by the oxygen sensitive transcription factors, hypoxia inducible factors 1 and 2 [64].

The view of hypoxia as detrimental has been challenged by a number of reports demonstrating that intermittent hypoxia is also capable of eliciting beneficial cardiovascular, respiratory and cognitive adaptations [52]. Indeed, patients with coronary artery disease [8] and chronic obstructive pulmonary disease [33] benefitted from programs of cyclic, moderate hypoxia. Intermittent hypoxic conditioning (IHC) produces cardioprotective anti-infarct and anti-arrhythmic effects in dogs [92], and, in rats, elicits vasoprotective effects in rats [49], enhances function and plasticity of respiratory and non-respiratory motoneurons [16], promotes hippocampal neurogenesis & anti-depressant effects [90] and, remarkably, reduces long-term memory impairments when applied 7 days after focal cerebral ischemia [73]. The therapeutic effects of intermittent hypoxia thus extend beyond the treatment of cardiovascular disease and are directly related to the hypoxia “dose” [57], i.e. the intensity, duration and number of episodes, and cumulative duration of hypoxia, as well as arterial carbon dioxide and other variables.

Moderate hypoxia, defined as exposure to 9-16% inspired O₂ and low numbers of hypoxic cycles in the range of 3-15 episodes per day, seems to offer the most therapeutic benefit without detrimental consequences [57]. This therapeutic range of intermittent hypoxia is concordant with the finding that programs of moderately severe intermittent hypoxia (e.g. 12%
O$_2$) of a few (e.g., 5 or 6) cycles of brief duration (e.g. 5 min hypoxia, 5 min reoxygenation) are optimal for inducing metabolic adaptations in mitochondria [70]. Research into the mechanisms that mediate detrimental vs beneficial IHC will help define the biological and medical consequences of hypoxia. Furthermore, delineating the precise mechanisms of therapeutic IHC may lead to the development of drugs that enhance the benefits of IHC while further minimizing the potential for maladaptation.

**Cardioprotective mechanisms for intermittent hypoxia conditioning**

The pathological cardiovascular adaptations elicited by intermittent hypoxia protocols modeling sleep apnea are in large part mediated by the hypoxia inducible transcription factors [64]. Intermittent hypoxia is also known to result in beneficial adaptations, driven in part by metabotropic G-protein coupled receptors (GPCR) and tyrosine kinases, which could possibly be harnessed for therapeutic intervention, athletic training, and neurocognitive enhancement [16]. In addition, metabolic substrate shifts towards increased NADH-dependent oxidation and enhanced efficiency of mitochondrial oxidative phosphorylation are observed in rat liver and heart following IHC [70]. Moreover, it was previously demonstrated that IHC affords robust protection of canine myocardium against ischemia/reperfusion injury as evidenced by its powerful anti-infarct and anti-arrhythmic effects following 1 h coronary artery occlusion and 5 h reperfusion [92]. Additional studies in the canine model demonstrated that the cardioprotection afforded by IHC was dependent on β$_1$-adrenergic receptor stimulation [48] suggesting a role for the sympathetic nervous system in the induction of cardioprotection. Indeed, hypoxia activates the sympathetic nervous system via carotid body chemoreception [34], and chronic intermittent hypoxia evokes sympathetic reflex activity [61]. The same IHC program was similarly cardioprotective in rats, reducing infarct size as well as preserving vasomotor responses following ischemia/reperfusion [49].
The myocardial and vasoprotective effects of IHC may in part be explained by modulation of nitric oxide production. In dogs IHC dampens endothelial nitric oxide synthase (NOS) expression and NOS activity, and furthermore suppresses nitrite release, a surrogate measure of nitric oxide formation, during post-ischemic reactive hyperemia. However, it was suggested that the reduced amounts of nitric oxide generated upon myocardial reperfusion might still be sufficient to afford some degree of cellular protection while avoiding the deleterious impact of excessive nitric oxide generation [67].

The cellular mechanisms giving rise to cardioprotection during IHC induced β₁-adrenergic receptor stimulation have not been delineated. β-adrenergic signaling activates multiple kinases such as protein kinase A and C, p38 MAPK, and ERK, all of which are capable of activating the cardioprotective transcription factor CREBP in isolated rat hearts [50], implicating one or more of these kinases in IHC cardioprotection. β₁-adrenergic signaling can also induce cytoprotective genes such as heat shock proteins 20 [23] and 27 [50]. β₁-adrenergic activation also evokes pro-survival signaling by epidermal growth factor receptor (EGFR) transactivation, although chronic β₁-adrenergic activity eventually impairs cardiomyocyte function due in part to transcriptional changes in pro-inflammatory cytokine expression [32]. Thus, other cardioprotective mechanisms must be active during IHC to counteract the potential for deleterious cardiac remodeling due to β₁-adrenergic activation.

**DOR-mediated mechanisms of cardioprotection**

It is well established that the delta opioid receptor (DOR) antagonizes the actions of β-adrenergic receptors [62], making DOR an attractive candidate to contribute to IHC induced cardioprotection. Furthermore, DORs are markedly expressed on the post-ganglionic parasympathetic nerve fibers [19] ideally positioning them to antagonize the sympathetic nervous system and the activity of adrenergic receptors.
Exercise induced opioidergic cardioprotection has been demonstrated to be DOR and not KOR specific [22; 6]. Myocardial enkephalin bioavailability and activity are altered by cyclic occlusion and reperfusion of the sinoatrial nodal artery in canines [38], global ischemic preconditioning in isolated rat hearts [84] and by post-conditioning [86] and hypoxic conditioning [51] in rats. Thus, cardioprotective maneuvers including SA nodal preconditioning and exercise are associated with increased delta opioidergic signaling.

Classical preconditioning mechanisms of DOR mediated cardioprotection involve adenosine, phosphoinositol 3-kinase (PI3K) and reactive oxygen species (ROS) signaling as demonstrated in isolated rat hearts [60]. PI3K activation occurs downstream of DOR through transactivation of EGFR in rabbit hearts [27]. ROS are emitted by mitochondria downstream of EGFR/PI3K activation in a matrix metalloproteinase-dependent mechanism in isolated rabbit cardiomyocytes [46]. Hypobaric hypoxic conditioning induced cardioprotection was previously demonstrated to be ROS-dependent in rats, the infarct sparing effects being abolished by antioxidant N-acetylcysteine (NAC) treatment during the hypoxia regimen [44]. Redox sensitive/dependent transcription factors such as AP-1, SRF [47], PPARβ/δ [3], and Nrf-2 [11] are thus likely involved in IHC induced cardioprotection. Together these findings implicate DOR and ROS signaling as mediators of IHC cardioprotection. Using the canine model of IHC, the ability of DOR antagonizes such as naltrindole or antioxidants such as NAC to blunt IHC-induced cardioprotection when administered during daily IHC sessions support the involvement of DOR and ROS in the evolving cardioprotection. The potential interplay between DOR, ROS and β₁-adrenergic receptors during IHC also merits investigation.

DOR mechanisms of vagal enhancement

The heart is a major site of enkephalin synthesis [83] and opiate receptors within the heart moderate vagal bradycardia [25]. In addition, brief SA nodal arterial occlusion raises interstitial endogenous enkephalins and augments vagal bradycardia during subsequent
occlusions in a manner reversed by the DOR antagonist naltrindole [39]. This observation led to studies examining the factors that result in enhanced vagal bradycardia, and initial observations demonstrated that although exogenous administration of enkephalins blunt vagal bradycardia, subtype specific pharmacological activation of DOR could enhance vagal bradycardia [26]. This finding prompted additional studies that demonstrated the modulation of vagal bradycardia by DOR to be dependent on enkephalin concentrations. Thus low concentrations of enkephalins enhanced and high concentrations blunted the vagal bradycardia. Furthermore, repeated occlusions of the SA nodal artery enhance exogenous-MEAP induced vagal bradycardia in a manner reversed by DOR-1 specific antagonism [24]. Moreover, it was demonstrated that repeated exposure of the sinoatrial node to the DOR-1 agonist TAN-67 blunted the ability of the DOR-2 agonist deltorphin to lessen vagally-induced bradycardia [21]. Together these findings indicated the existence of vagotonic DOR-1 and vagolytic DOR-2 subtypes in the canine heart.

Vagal mechanisms of cardioprotection

The therapeutic anti-inflammatory effects of vagal nerve stimulation have received much attention due to its efficacy in treating inflammatory disorders such as rheumatoid arthritis [30] and obesity related inflammation and metabolic disorders [76]. Stimulating the vagus for treatment of cardiovascular disorders such as heart failure [18] and in the setting of ischemia-reperfusion injury [10] also produced favorable outcomes and clinical utility. The principal neurotransmitter of the vagus, acetylcholine, has anti-apoptotic effects on cardiomyocytes in vitro during hypoxia-reoxygenation [88]. Additionally, the underlying mechanisms associated with vagally mediated cardioprotection may also include augmenting defenses against ROS. Muscarinic receptor (M₂) activation by acetylcholine converges with DOR activation on the EGFR/PI3K transactivation pathway to induce mitochondrial ROS formation and cardioprotection [46]. Moreover, the M₂/PI3K/endothelial NOS pathway may also play a protective role during hypoxic stress [54], perhaps preserving vasomotion. This concept would
be consistent with the vasoprotective effects of IHC in rats [4]. More importantly, mitochondrial ROS formation may lead to cytoprotective protein activation and gene transcription of antioxidant defense systems. For example, vagal stimulation upregulates all three (Cu^{2+}, Zn^{2+} and Mn^{2+}) superoxide dismutase isozymes and reduces oxidative and nitrosative stress in rat hearts [45].

In a cardiomyocyte cell line M_{2} activation dampens pro-oxidative stress originating from mitochondria, xanthine oxidase, and NADPH-oxidase emitted ROS [54]. Furthermore, acetylcholine treatment following hypoxia and activated cytoprotective kinases and activation of transcription factors PGC1α, Nrf2, and MtfA [71], culminating in decreased death of the cardiomyocytes. The resulting mitochondrial biogenesis was revealed by increased mitochondrial density, mass, and mitochondrial DNA. The non-selective muscarinic receptor antagonist atropine abolished these effects. Collectively, these findings suggest that the powerful cardioprotective pathways mobilized by IHC include a neural parasympathetic component that may explain the gradual development of near complete cardioprotection over the 20 day IHC program. The cardiac phenotype may adapt, up-regulating anti-oxidant defenses, mitochondrial biogenesis, preserving cardioprotective NO formation, and reducing oxidative stress.

**Enhancing vagal bradycardia by opiodergic IHC**

The metabolic challenge of exercise has favorable effects on autonomic function and vagal activity [41]. Moreover, adenosine, a known cardioprotective agent [60], potentiates canine vagal activity in isolated atria [74] suggesting that metabolic challenges, including hypoxia, can augment vagal function. Thus, vagal tone is enhanced in Tibetans living at high altitudes [91], and two weeks of intermittent hypoxia training has been shown to increase the high-frequency vagal component of heart rate variability in humans [87]. Additionally, chronic intermittent hypobaric hypoxia conditioning augments acetylcholine-induced aortic relaxation in
a dose-dependent manner in rats [85]. This increased responsiveness to acetylcholine was sensitive to non-specific opioid receptor blockade, implicating opioidergic signaling in mediating the adaptation.

The observation that repeated 5 min cycles of sinoatrial nodal artery occlusion and reperfusion raised nodal enkephalins and promoted vagal bradycardia [39] raises the possibility that metabolic perturbations could be used to harness beneficial opioidergic activity as outlined in the preceding paragraphs. To explore this possibility ischemic preconditioning by repeated SA nodal artery occlusions were employed to test whether an enhanced vagotonic DOR-1 response paralleled the previously observed [39] increases in interstitial enkephalins and to define the role of competing vagolytic DOR-2 responses. It was demonstrated that the repeated occlusions progressively augmented vagotonic transmission and that the vagotonic-DOR-2 effect was dampened following the ischemic preconditioning SA nodal artery occlusions [20]. Interestingly, the potent cardioprotective neural vagal pathways can be recruited by other metabolic perturbations such as remote ischemic preconditioning in humans [31], which is sensitive to DOR blockade and requires DOR/adenosine receptor interaction in isolated rabbit cardiomyocytes [72]. Thus, stimulating the innate cardioprotective effects of the vagus through opioidergic IHC represents a potential mechanism of IHC induced cardioprotection.

Functional Regulation of DOR Activity

DOR activation is known to elicit both excitatory and inhibitory responses [40] by coupling to select G-proteins. This observation, known as functional selectivity/agonist-directed trafficking/conformational-specific agonism, is not uncommon among GPCRs [89]. Pharmacological agonists have been designed to activate specific DOR subtypes, DOR-1 and DOR-2. Excitatory DOR activation couples to Gsα and stimulates adenylyl cyclase resulting in cyclic adenosine monophosphate (cAMP) accumulation. Inhibitory DOR activation couples to Giα and inhibits adenylyl cyclase resulting in reduced cellular cAMP concentrations. The
precise mechanisms responsible for DOR G-protein switching, which fundamentally changes vagal DOR phenotype have not been determined. Although a polymorphism of the DOR has been described [75] it does not appear to alter DOR G-protein coupling but rather compromises calcium signaling, and no evidence for splice variants of the DOR has been found.

It has been proposed that DOR function is determined in part by the number of cell surface receptors [29]. Electron microscopy [13], c-Myc fusion labeling [78], and photoaffinity labeling [59] have demonstrated that most of the DOR protein is located in the intracellular compartment and few DORs are found on the surface in the opioid naïve state [2]. As a result, recruitment of DOR to the cell surface may result in rapid, efficient upregulation of receptor function.

DOR function appears to be cell type specific. For example, most of the labeled DOR-eEGP are localized to the intracellular compartment in striatal grey matter cholinergic interneurons [4] as opposed to the cell surface as reported in dorsal root ganglion neurons [69]. In the former study, Pavlovian conditioning induced translocation of DORs to the membrane surface in the cholinergic interneurons [4] suggesting that cell surface translocation increases receptor function. These findings lend support for recruitment of DORs to the surface as a mechanism to enhance vagal transmission, such as during repeated SA nodal artery occlusions [20]. An increase in DOR expression could also result in enhanced vagotonic DOR-1 responses once receptors are recruited to the surface. Exercise [22] and increased sympathetic activity [5] induce DOR transcription. Conceivably IHC challenge may similarly increase neuronal DOR protein content contributing to the enhanced vagotonic effect of IHC.

Delta opioid receptors contain an amino acid sequence in the transmembrane domain that has high affinity for the monosialoganglioside GM1 [81]. Infusion of exogenous GM1 into the SA node enhances vagal bradycardia and results in attrition of vagolytic DOR-2 responses [17]. Interactions between GM1 and the DOR could perhaps alter the functional selectivity of the receptor upon agonist binding or result in G-protein biased signaling. Such alterations in
functional responses are observed in DOR/mu-opioid receptor (MOR) heterodimers which have been proposed to have DOR-1 activity, thereby opposing DOR-2 responses [77]. The latter findings would be consistent with attrition of the DOR-2 activity by repeated DOR-1 activation [21], although it is reported that MOR are not expressed in adult mammalian hearts [36]. Thus, the heterodimer proposition for a vagotonic DOR-1 phenotype can be questioned.

Collectively, these findings suggest that IHC may alter DOR function through receptor trafficking and alterations in the membrane environment, such as increased GM1, that result in G-protein switching and vagal phenotype change. An enhanced vagotonic DOR-1 response and parasympathetic GM1 content following IHC would support this possibility.

**Opioidergic IHC stimulates neuritogenic and neurotrophic GM1 synthesis**

The monosialoganglioside GM1 has cardioprotective [43] and neurotrophic effects most evident during axon process outgrowth and synaptogenesis [80]. Studies using sialidase or KCl+dibutryl cAMP to mimick physiological upregulation of GM1 synthesis in vitro resulted in axon outgrowth, whereas high pharmacological concentrations of GM1 provoked dendritic outgrowth [80]. Axon outgrowth as seen during axotomy induced Nue3 sialidase activation and GM1 synthesis which also activates signaling that regulates axon growth and peripheral nerve regeneration [42]. As occurs with DOR, GM1 binds with high affinity to tyrosine kinase receptors TrkA [56] and TrkB [63]. These associations are required for neurotrophic receptor signaling and trafficking [55]. Thus, GM1 facilitates nerve growth and survival by multiple mechanisms.

An increase in parasympathetic nerve growth accompanying enhanced vagal control of the heart could be mediated by DOR stimulation during IHC. In support of this mechanism, chronic exposure of a neuronal cell line to opioids results in opioid receptor supersensitivity, increased cAMP formation, and GM1 synthesis, suggesting a positive feedback mechanism whereby excitatory opioid receptor signaling (cAMP/PKA activation) activates GM1 synthesis.
leading to enhanced receptor sensitivity [79]. Chronic stimulation of DOR during IHC may then induce a physiological increase in GM1, facilitating parasympathetic nerve growth in the fibers with high DOR content.

**Functional links between DOR and acetylcholinergic activity**

Acetylcholine is the main neurotransmitter in the vagus and peripheral parasympathetic nerves. Under most conditions the rate limiting step in acetylcholine synthesis is intracellular choline import by the high affinity choline transporter (ChT-1) [15] as physiological choline acetyltransferase (ChAT) operates well below maximum capacity. DORs are recruited to the membrane surface by chronic stimulation, Ca\(^{2+}\), nerve growth factor, and repeated opioid exposure [9], such as may occur during IHC. The vesicular acetylcholine transporter (VACHT), which pumps acetylcholine into synaptic vesicles, co-localizes with DOR in synaptosomal/vesicular membrane fractions [19] along with ChT-1[9]. The colocalization of these proteins within intracellular vesicular membranes could functionally link these proteins by facilitating acetylcholinergic activity in parallel with the recruitment and trafficking of vagotonic DOR-1 receptors. Moreover, since DOR and ChT-1 activity can be modulated by GM1 [17; 15] translocation of DOR and ChT-1 to cell membrane GM1 lipid microdomains would be expected to promote vagotonic DOR-1 activity and/ enhanced choline uptake, respectively. An enhanced vagal bradycardia, increased cardiac autonomic nerve GM1, and parallel changes in the distribution of these proteins would support this concept.

**Hypoxia enhancement of acetylcholinergic protein expression**

Long term hypoxia exposure reportedly induces changes in parasympathetic neurochemical markers such as ChAT and muscarinic receptor density in guinea pig hearts.
Parasympathetic neurochemical markers such as the aforementioned ChT-1 and VACHT are also modulated by nerve activity [65]. ChAT and VACHT are transcribed from the same gene although their expression can be differentially regulated by signaling pathways such PI3K and PKA [12]. ChT-1 transcription is also modulated by cAMP-dependent pathways [7]. Transcriptional modulation of these genes appears require the availability of downstream effector proteins. An enhanced vagotonic delta opioidergic profile may thus regulate these presynaptic-acetylcholinergic proteins directly through DOR-1/cAMP-dependent pathways or indirectly through pathways activated by increased presynaptic nerve fiber activity during IHC. Regulation of the ChT-1, VACHT, and ChAT in cardiomyocytes mediated by myocardial DOR signaling may also occur to maintain homeostasis [66], thereby counteracting the maladaptive effects of chronic β-adrenergic stimulation [28] during IHC. IHC provoked changes in acetylcholinergic proteins blocked by DOR antagonism would lend support to these scenarios.

SUMMARY

- Hypoxia, particularly chronic, severe hypoxia, can elicit maladaptations driven primarily by hypoxia inducible factors.
- Therapeutic intermittent hypoxia can be administered by applying a few cycles of short duration, moderately intense hypoxia. Therapeutic intermittent hypoxia induced cardioprotection may be driven by metabotropic GPCR signaling.
- The cardioprotective effects of IHC are mediated at least in part by β₁-adrenergic receptor signaling. The cellular mechanisms may involve expression of genes and subsequent synthesis of cytoprotective proteins.
• IHC may be mediated by DOR and mitochondrial-derived ROS as well. The cellular mechanisms may involve activation of redox sensitive transcription factors and bolstering of anti-oxidant defenses.

• Cardioprotective vagal activity may be enhanced by IHC. The cellular mechanisms may include anti-ischemic protection, mitochondrial biogenesis, anti-oxidant enzyme regulation, and reduction in pro-oxidative stress.

• DOR-1 enhances vagal bradycardia and vagotonic DOR-1 is regulated by ischemic preconditioning and possibly, IHC.

• Preconditioning and repeated DOR-1 stimulation may attenuate vagolytic DOR-2 activity and enhance vagotonic DOR-1 activity.

• Vagal DOR phenotype change may be dependent on DOR expression, increased GM-1 synthesis and concomitant nerve growth. IHC may modulate the vagal DOR phenotype by increasing GM1 synthesis or upregulating DOR protein expression.

• DOR and presynaptic cholinergic proteins ChT-1 and VACHT are colocalized within the vesicular membrane fractions and within GM-1 microdomains, functionally linking these proteins and their distribution within parasympathetic nerves.

• Transcription of presynaptic acetylcholinergic proteins could be regulated by DOR signaling during IHC.

SIGNIFICANCE

Although basic strategies aimed at exploiting the cardioprotective potential of opioids are under investigation [36] the precise mechanisms by which opioids afford therapeutic effects are as yet unknown. Vagal function declines with age and metabolic disease, so strategies to improve or enhance vagal activity are being examined as potential treatments [35]. Approaching these problems by targeted stimulation of the delta opioid receptor system has
received scant attention. Targeted applications could aid in disease prevention and therapy for the aging global population. To this end, IHC is a cost-effective tool to deliver the therapeutic benefits of opioidergic activity on vagal function. In conjunction with the application of technology affording early detection of ischemic coronary events, prophylactic treatment with IHC could ease the financial and disease burden for at-risk individuals. Thus, information on the basic mechanisms underlying IHC cellular protection resulting from this study will thereby provide the empirical foundation necessary to foster clinical translation of IHC therapy. Defining the cellular mechanisms of IHC will enable repair of signaling pathways that are compromised by the effects of ischemia-reperfusion, aging or advanced disease, such as disruption of lipid membrane composition and membrane structure [1].

HYPOTHESES

- Hypothesis 1. DOR activation mediates cardioprotection and augmented parasympathetic influence afforded by IHC.
- Hypothesis 2. IHC and DOR mobilize mechanisms that result in ROS generation, vagal-DOR phenotype modulation, regulation of DOR expression, GM1 synthesis, parasympathetic nerve growth, and enhanced acetylcholinergic systems. Thus, blockade of these signaling mechanisms disables IHC-induced cardioprotection.
SPECIFIC AIMS

Specific Aim #1: To demonstrate the impact of δ-opioidergic signaling in mediating the cardioprotection afforded by IHC.

Hypothesis 1A: To assess the impact of IHC induced DOR activity on cardioprotection, mongrel dogs will be subjected to a 20 d IHC program as previously described (Figure 1) [92]. DOR specific antagonism during intermittent hypoxia conditioning will be achieved by subcutaneous naltrindole administration to a subset of dogs 15 min before the start of daily intermittent hypoxia treatment. Because crosstalk between DOR signaling and ROS [60] may occur during IHC, NAC will be administered in the chow diet of a subset of dogs 2 h before the start of IHC. Dogs conditioned by a modified hypoxia program of the same duration and intensity as IHC, but lacking intervening reoxygenation episodes, will be used to assess the importance of intermittent reoxygenation episodes to the evolving cardioprotection.
Figure 1. *Intermittent hypoxia conditioning (IHC) program and coronary artery occlusion-reperfusion protocol.* Panel A: Daily IHC sessions consisted of 5-8 exposures, each 5-10 min, to 9.5-10% inspired O$_2$, with intervening 4 min exposures to room air. The room air exposures were omitted in the uninterrupted hypoxia program. Panel B: N-acetylcysteine (NAC) was taken orally in chow diet 2 h before each IHC session. Naltrindole was administered by sc injection 15 min before IHC. Panel C: 24 h after completing the IHC program, the left anterior descending coronary artery (LAD) was occluded for one hour, and then abruptly reperfused for 5 h before masses of infarcted myocardium (IS) and ischemic area at risk (AAR) were determined.

**Hypothesis 1B:** To determine whether IHC improves vagal bradycardia and cardioprotective vagotonic-DOR1 activity, dogs will be subjected to IHC and DOR antagonism as described in Figure 1. A subset of dogs will receive daily injections of subcutaneous naltrindole 15 min before the start of intermittent hypoxia conditioning. Another group of dogs will undergo non-hypoxic sham conditioning as a control using 21% O$_2$ gas exposure. Following the conditioning programs MEAP will be infused through a microdialysis probe into the SA node [38]. Vagal stimulations before and during MEAP infusion will assess vagal and DOR function. Heart rate will be monitored by standard lead II electrocardiography.

**Specific Aim#2:** To test whether IHC induced opioidergic signaling regulates cardiac DOR content, GM1 synthesis, and nerve growth, cardiac tissue (atrium, left ventricle, SA node) GM1 content will be analyzed by immunohistochemistry, and DOR content will be assessed by immunoblotting and immunohistochemistry in dogs subjected to IHC with and without naltrindole, and in non-hypoxic sham dogs. Mechanistic insight on vagal and DOR function and distribution following intermittent hypoxia will be gained. The study will also include the
development and implementation of a novel immunohistochemical approach to evaluate tissue GM1 labelled fibers and co-localization with parasympathetic fibers (see Appendix 1).

**Specific Aim#3**: To further examine the underlying mechanisms responsible for IHC cardioprotection and to determine whether IHC induced opioidergic signaling regulates acetylcholinergic systems, immunoblotting and immunohistochemistry of the choline transporter and vesicular acetylcholine transporter will be performed in biopsies of SA node, atrium and left ventricular myocardium to assess protein content and distribution.
REFERENCES


50. Marais E, Genade S, Lochner A. CREB activation and ischaemic preconditioning.  
*Cardiovasc Drugs Ther* 22:3-17, 2008.


60. **Peart JN, Gross GJ.** Cardioprotection following adenosine kinase inhibition in rat hearts. *Basic Res Cardiol* 100: 328-336, 2005.


77. van Rijn RM, Whistler JL. The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. Biol Psychiatry 66:777-84, 2009.


Figure Legends

**Figure 1.** *Intermittent hypoxia conditioning (IHC) program and coronary artery occlusion-reperfusion protocol.* Panel A: Daily IHC sessions consisted of 5-8 exposures, each 5-10 min, to 9.5-10% inspired O₂, with intervening 4 min exposures to room air. The room air exposures were omitted in the uninterrupted hypoxia program. Panel B: *N*-acetylcysteine (NAC) was taken orally in chow diet 2 h before each IHC session. Naltrindole was administered by sc injection 15 min before IHC. Panel C: 24 h after completing the IHC program, the left anterior descending coronary artery (LAD) was occluded for one hour, and then abruptly reperfused for 5 h before masses of infarcted myocardium (IS) and ischemic area at risk (AAR) were determined.
**FIGURES**

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δ-Opioid Receptor (DOR) Signaling and Reactive Oxygen Species (ROS) Mediate Intermittent Hypoxia Induced Protection of Canine Myocardium

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Running Title: DOR, ROS and Cardioprotection

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ABSTRACT

Intermittent, normobaric hypoxia conditioning (IHC) confers robust cardioprotection against ischemia-induced myocardial infarction and lethal ventricular arrhythmias. δ-Opioid receptor (DOR) signaling and reactive oxygen species (ROS) have been implicated in cardioprotection afforded by ischemic pre- and post-conditioning. This study examined the contributions of DOR and ROS mechanisms in mediating IHC-induced cardioprotection. Mongrel dogs completed a 20 day IHC program consisting of 5-8 daily 5-10 min cycles of moderately severe hypoxia (FIO₂ 0.095-0.10), with intervening exposures to 4 min of room air, and were compared with non-IHC controls. Subsets of dogs received the DOR antagonist naltrindole (200 µg/kg, sc) or antioxidant N-acetylcysteine (250 mg/kg, po) before each IHC session. Twenty-four hours after the last session, the left anterior descending coronary artery was occluded for 1h and then reperfused for 5 h. Arrhythmias detected by lead II electrocardiography were scored according to the Lambeth conventions. Left ventricles were sectioned and stained with 2,3,5-triphenyl-tetrazolium chloride, and infarcts were expressed as infarct size as a % of the area at risk (IS/AAR). IHC sharply decreased IS/AAR from 41±5 (n=12) to 1.8±0.9% (n=9; P<0.001) and arrhythmia score from 4.1±0.3 to 0.7±0.2 (P<0.001). Naltrindole (n=6) abrogated IHC mediated cardioprotection with an IS/AAR of 35±5% and an arrhythmia score of 3.7±0.7 (P<0.001 vs. untreated IHC). N-acetylcysteine (n=6) interfered to a similar degree with an IS/AAR of 42±3% and arrhythmia score of 4.7±0.3 (P<0.001 vs. untreated IHC). These results suggest that both DOR and ROS signaling are integral parts of a common mechanism responsible for the robust cardioprotection afforded by IHC.
New and Noteworthy

Intermittent, normobaric hypoxia conditioning exerts an unusually robust degree of myocardial protection during ischemia/reperfusion, but the cardioprotective mechanisms are not fully understood. This study demonstrated the obligatory participation of δ-opioid receptors, reactive oxygen species and regular reoxygenation in the gradually evolving cardioprotection in an integral, all-or-none relationship.

Keywords: cardiac arrhythmias, enkephalins, myocardial infarction, N-acetylcysteine, naltrindole

Abbreviations: DOR: delta opioid receptor; IHC: intermittent hypoxia conditioning; IS/AAR: mass of myocardial infarct/mass of ischemic area at risk; LAD: left anterior descending coronary artery; NAC: N-acetylcysteine; NEP: neutral endopeptidase; Nrf2: nuclear factor E2-related factor 2; PKA: protein kinase A; PKC: protein kinase C; ROS: reactive oxygen species; UHC: uninterrupted hypoxia conditioning.
INTRODUCTION

Intermittent, normobaric hypoxic conditioning (IHC) is a powerful stimulus which over the course of a few weeks progressively increases myocardial resistance to ischemia and reperfusion injury in dogs (33, 56). The cardioprotection imparted by IHC is more extensive than that afforded by a wide variety of ischemic and pharmacological preconditioning protocols (52). IHC proved safe and effective in human patients (37): it increased exercise tolerance and aerobic capacity in 50-70 year old men with a history of myocardial infarction (7), restored baroreflex sensitivity and hypercapnic ventilator responses in adults with chronic obstructive pulmonary disease (19), and lowered systolic and diastolic blood pressures in young adult men with stage 1 arterial hypertension (32). The precise mechanisms underlying IHC warrant further investigation given the potential clinical impact and non-invasive, non-pharmacological character of the therapy.

Cardioprotection can be achieved by targeting a variety of G-protein-coupled receptors, including β1-adrenergic and δ-opioid receptors (DOR) (52). In this regard, β1-adrenergic receptor blockade applied during the IHC regimen abolished the anti-infarct and anti-arrhythmic cardioprotection in dogs (33). Although untested in IHC, DOR (18, 21, 41, 42) and reactive oxygen species (ROS) signaling (12, 48) are both widely implicated in myocardial preconditioning. The well documented role of DOR signaling in the cardioprotective responses to preconditioning (21, 52), and the myocardium’s high enkephalin content (2, 54) suggests that myocardial opioids are well positioned to play a pivotal role in IHC mediated cardioprotective effects. ROS also may be central to the evolving IHC phenotype. Myocardial ROS, generated during repeated hypoxia and reoxygenation, may recruit adaptive cellular responses by modulating redox-sensitive proteins, such as protein kinase C (41) and transcription factor Nrf-2 (9), to induce cytoprotective responses and gene programs that render the myocardium resistant to ischemia-reperfusion injury.
This study was designed to test the hypothesis that DOR and/or ROS signaling are integral to the development of the IHC mediated cardioprotective phenotype. The ability of DOR blockade with naltrindole or anti-oxidant treatment with N-acetylcysteine (NAC) during daily IHC to blunt the development of the ischemia resistant cardiac phenotype was tested. Both naltrindole and NAC blocked the anti-infarct and anti-arrhythmic effects of IHC, thus suggesting the repetitive activation of ROS and DOR systems during IHC are integral parts of the evolving robust cardioprotection.
METHODS

Animal experimentation was conducted in accordance with the Guide to the Care and Use of Laboratory Animals (U.S. National Research Council Publication 85–23, revised 2011), and was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. Forty-five mongrel dogs (31 males, 14 females; body mass 25-35 kg) were assigned to the following protocols and treatments: non-hypoxic/non-IHC controls (n=13); intermittent, normobaric hypoxia conditioning (IHC; n=12); IHC with daily naltrindole injection (IHC + NAL; n=10); IHC with daily NAC ingestion (IHC + NAC; n=6). An additional four dogs were subjected to uninterrupted hypoxia conditioning (UHC) with daily, continuous hypoxic exposures equal to the daily, interrupted hypoxic exposures of the IHC dogs. The dogs exhibited no signs of distress during the conditioning sessions. Data from six of the non-hypoxic and six of the IHC dogs were reported previously (33). The non-IHC group included six dogs that completed a sham conditioning program (33, 56) and seven cage-rested, untreated dogs. Previous reports demonstrated no differences in study endpoints between these non-IHC subgroups (33, 56), so they were combined in this study. Dogs were maintained on a 12:12 h light:dark cycle, consumed a standard chow diet and water ad libitum, and were fasted overnight before terminal experiments.

Intermittent, normobaric hypoxia conditioning

Dogs were conditioned by a previously described (56), 20 d IHC regimen. Daily sessions consisted of 5–8 exposures to hypoxia (FIO₂ 9.5–10%) for 5–10 min in an acrylic chamber, with intervening 4 min room air exposures (Figure 1A). The number and duration of hypoxic exposures were adjusted to progressively increase the cumulative exposure from 25 min on day 1 to 70 min on days 11-20 (56). Inspired O₂ during hypoxia was 10% on days 1-5, and 9.5% on days 11–20 (56), and was continuously monitored with an AlphaOmega Instruments series
2000 O₂ analyzer (Cumberland, RI, USA). The sham protocol was identical to the IHC protocol, except the chamber was ventilated with compressed air (20.5–21% FIO₂) instead of N₂ gas.

**Naltrindole and N-acetylcysteine treatment**

Naltrindole hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in sterile 0.9% NaCl to a concentration of 2 mg/ml, passed through a 0.45 μm pore diameter filter, and injected (200 μg/kg, sc) 15 min before the start of the daily IHC protocol (Figure 1B). In a trial experiment this dosage of naltrindole prevented the vagolytic effect of the DOR agonist methionine-enkephalin-arg-phe from 15 min to 2 h post-injection (25), an interval which exceeded the longest daily IHC session during the 20 day program. The membrane permeable, aminothiol antioxidant, N-acetylcysteine (NAC; Sigma), was added to the chow diet and ingested (250 mg/kg) 2 h before the daily IHC session (Figure 1B) to ensure the antioxidant's absorption into the systemic circulation. The time course of plasma NAC concentration (Figure 2) was examined in 5 dogs. Blood was sampled (2 ml) from the lateral saphenous vein via an indwelling catheter immediately before feeding, hourly for 5 h and again 24 h post-feeding. After centrifugation, plasma proteins were precipitated with an equal volume of 0.6 N HClO₄ and then the sample was centrifuged, neutralized with KOH, chilled on ice for 30 min, and re-centrifuged. NAC in the supernatant was measured by HPLC with fluorescence detection of sulphydryl compounds (LC10AT, Shimadzu Instruments, Kyoto, Japan) (36). The NAC peaks were identified and quantified by comparison with authentic NAC standards. After oral intake, the NAC concentration in the systemic circulation increased over 2 h to a plateau which persisted for another 2 h before subsiding (Figure 2). The daily IHC sessions were conducted during this NAC plateau.

**Coronary artery occlusion-reperfusion protocol**
Terminal, coronary artery occlusion-reperfusion experiments were conducted 24 h after completion of the IHC program. Dogs were anesthetized with sodium pentobarbital (25 mg/kg iv), intubated and mechanically ventilated. Catheters were placed for blood sampling (right femoral artery), administering medications (right femoral vein), and injecting radioactive microspheres (left atrium). Two catheters were inserted in the left femoral artery and threaded into the thoracic aorta to collect reference blood samples for assessing collateral flow. Needle electrodes were inserted subcutaneously for lead II electrocardiography. Arterial PO$_2$, PCO$_2$, and pH were kept within respective normal limits by supplemental O$_2$, adjusting ventilation frequency, and iv NaHCO$_3$ administration.

The heart was accessed via a left thoracotomy and suspended in a pericardial cradle. A silk suture was passed around the left anterior descending coronary artery (LAD) immediately distal to its first diagonal branch and threaded through a length of polyethylene tubing, forming a snare. The LAD was occluded by tightening the snare, and the occlusion was confirmed by the appearance of cyanosis and dyskinesis in the LAD perfusion territory. The snare was released after 60 min of occlusion, and the LAD was reperfused for 5 h (Figure 1C). When ventricular fibrillation occurred ($n=15$ dogs), direct current countershocks (10 J) were applied to the epicardium within 15 s. No more than three countershocks were required to restore spontaneous rhythm in these fifteen dogs. Collateral blood flow to the inner two-thirds of the left ventricular wall of the ischemic territory and non-ischemic, left circumflex-perfused myocardium was assessed (47) by injecting radioactive microspheres ($^{46}$Sc; Perkin Elmer, Boston, MA, USA) into the left atrium at 30 min LAD occlusion. In eight dogs (one non-hypoxic, three intermittent hypoxia, four intermittent hypoxia + naltrindole) the mean collateral blood flow exceeded 0.25 ml · min$^{-1}$ · g$^{-1}$. Since this degree of collateral flow limited the severity of ischemia (47, 56) these dogs were excluded from analyses of infarction and arrhythmias (Table 1).

Infarct size and ischemic area at risk
Masses of ischemic area at risk and infarcted myocardium were measured by standard techniques (47, 56). At 5 h of reperfusion, the heart was arrested, and the aorta and LAD were cannulated and simultaneously perfused at 85 mmHg with 0.9% NaCl containing 2.5% Evans blue dye and dye-free 0.9% NaCl, respectively, to demarcate the area at risk. The left ventricle and interventricular septum were divided into 6-7 transverse 8 mm-thick sections which were weighed and then incubated at 37°C for 30 min in 100 mM phosphate buffer, pH 7.4, containing 1% (w/v) 2,3,5-triphenyl tetrazolium chloride, imparting a deep red color to non-infarcted myocardium. Masses of non-ischemic, non-infarcted and infarcted tissue were computed by multiplying the respective planimetrically determined fractional volumes by the masses of each section.

Analysis of arrhythmias

Left ventricular arrhythmias were analyzed according to the Lambeth II conventions (11) and arrhythmias scored as previously described (33). Arrhythmia scores (1: isolated ventricular premature beats; 2: bigeminy/salvos; 3: ventricular tachycardia; 4: transient ventricular fibrillation with spontaneous resumption of sinus rhythm; 5: ventricular fibrillation requiring countershocks to restore sinus rhythm) were assigned based on the most severe arrhythmia detected during the first 30 min reperfusion, when the arrhythmias were the most intense.

Statistical analyses

Analysis of variance (ANOVA) was performed for between-group comparisons of infarct size/area at risk (IS/AAR), arrhythmia scores, regional myocardial blood flow, left ventricle mass/body mass, hematocrit and hemoglobin content. Holms-Sidak post-hoc tests were applied when ANOVA detected statistically significant differences of mean values. These statistical analyses were performed with SigmaPlot for Windows v. 11.0 (Systat Software, San Jose, CA, USA). Linear regression analysis was used to define relationships between IS/AAR and arrhythmia score vs. collateral flow, and analysis of covariance was applied for statistical
comparison of these regressions. Regression analyses were performed with IBM SPSS Statistics for Windows v. 21, 64-bit edition (SPSS Inc., Chicago, IL, USA). $P$ values $<0.05$ were taken to indicate statistically significant treatment effects.
RESULTS

Left ventricular mass, arterial hematocrit and hemoglobin content

No statistically significant differences in the ratio of left ventricle mass to body mass, hematocrit or blood hemoglobin content were detected among the treatment groups at the conclusion of the respective IHC or non-hypoxic, non-IHC programs (Table 1). Thus, neither the IHC nor uninterrupted hypoxia programs, or IHC in combination with the DOR antagonist, naltrindole, or the antioxidant, NAC, produced appreciable erythropoiesis or left ventricular hypertrophy.

Intermittent hypoxia conditioning exerted robust cardioprotection against ischemia-reperfusion, manifest by >90% reduction in left ventricular infarct size (P<0.001; Figure 3A) and a similarly robust decrease (P<0.001) in reperfusion ventricular arrhythmias (Figure 3B) in dogs subjected to subsequent 60 min occlusion and 5 h reperfusion of the LAD. These results extend previous reports of IHC-induced cardioprotection in dogs (33, 56).

Hypoxia-induced cardioprotection requires intermittent reoxygenation

The contribution of the cyclic room air exposures during the IHC sessions to the development of cardioprotection was examined by conditioning dogs by a modified 20 d program in which the daily hypoxia exposure was of the same duration and intensity as that of the IHC program, but without the intermittent reoxygenations. Unlike IHC, the uninterrupted hypoxia conditioning program failed to produce any reduction in infarct size (Figure 3A) or ventricular arrhythmias (Figure 3B; P<0.001 vs. respective IHC values). Thus, the intermittent reoxygenations during the IHC sessions are essential for induction of the cardioprotected phenotype.

Antioxidant NAC blocked development of IHC cardioprotection

Cyclic hypoxia-reoxygenation provokes reactive oxygen species (ROS) formation in heart (20, 50), and ROS have been implicated in hypoxia-induced cardioprotection (28). Thus, the failure of uninterrupted hypoxia conditioning to decrease myocardial infarct or ventricular arrhythmias
suggested ROS may be essential for development of IHC cardioprotection. To interrogate further the contributions of ROS to the cardioprotection afforded by IHC, the antioxidant NAC was administered prior to each IHC session throughout the program. IHC sessions were conducted between 2 and 4 h after NAC intake to coincide with the plateau of circulating NAC concentration (Figure 3). The anti-infarct (Figure 3A) and anti-arrhythmic (Figure 3B) cardioprotection afforded by 20 days IHC was prevented by coincident NAC treatment (P<0.001 vs. respective IHC values).

DOR receptor antagonist naltrindole abrogated IHC cardioprotection

To investigate the role of DOR activation in IHC induced cardioprotection, the DOR specific antagonist, naltrindole, was administered 15 min before each daily IHC session. Naltrindole completely abrogated the reductions in infarct size (Figure 3A) and arrhythmias (Figure 3B) afforded by IHC (P<0.001 vs. respective IHC values). Thus, DOR signaling appeared to be essential for the cardioprotection.

IHC induced cardioprotection and its blockade by naltrindole and N-acetylcysteine were independent of collateral blood flow during coronary artery occlusion

Coronary collateral blood flow during coronary artery occlusion can profoundly influence the extent of ischemia-reperfusion injury (47, 56). Collateral flow varies appreciably among mongrel dogs, and hypoxia is a well-known stimulus of coronary angiogenesis (44). Accordingly, regional coronary collateral flow was measured at 30 min LAD occlusion by the use of radioactive microspheres (22). Within the ischemic territory, neither the mean collateral flow (Figure 4A), or the minimum collateral flow (Figure 4B) differed among the treatment groups. Likewise, coronary flow to the non-ischemic left ventricular myocardium in the left circumflex perfusion territory (Figure 4C) was similar among the treatment groups. Thus, the daily bouts of cyclic hypoxia-reoxygenation did not provoke appreciable coronary angiogenesis, nor did daily administration of the DOR antagonist or antioxidant affect coronary collateral blood flow.
To examine further the relationships between coronary collateral blood flow and myocardial ischemia-reperfusion injury, infarct and arrhythmias were assessed as functions of mean collateral blood flow in the non-hypoxic and hypoxia-conditioned dogs. IHC produced substantial downward shifts both in the relationships between coronary collateral flow and myocardial infarct size (Figure 5A) and between collateral flow and arrhythmia score (Figure 5B), relative to non-hypoxic dogs (ANCOVA: P<0.001 vs. non-hypoxic dogs). However, naltrindole and NAC treatment prevented the IHC-induced shifts in these relationships. Thus, differences in coronary collateral flow during LAD occlusion cannot explain the cardioprotection afforded by IHC or its abrogation by DOR blockade, antioxidant treatment or the omission of intermittent reoxygenations.
DISCUSSION

This study extends previous reports (33, 56) that intermittent, normobaric hypoxia conditioning increases myocardial resistance to severe coronary ischemia, exemplified by dramatic decreases in left ventricular myocardial infarct size and arrhythmia score. The study’s new findings provide information on the mechanisms that impart this remarkable cardioprotection. Specifically, the protection was abrogated by daily administration of the selective DOR antagonist naltrindole, the broad spectrum antioxidant NAC, and by omitting the cyclic exposures to room air during the hypoxia sessions. The cardioprotection and its blockade cannot be ascribed to differences in collateral blood flow during ischemia. These results implicate endogenous DOR agonists (e.g., enkephalins) and ROS as essential mediators of IHC cardioprotection, and complement the previous finding that the β₁-adrenergic receptor antagonist metoprolol severely attenuated the cardioprotection (33). Thus, the remarkable protection afforded by IHC appears to arise from the integrated contributions of ROS, β₁-adrenergic and δ-opioidergic signaling (43) acting in concert, as outlined in Figure 6.

The findings of this study are important because (1) they support a novel method of cardioprotection, i.e. intermittent, normobaric hypoxia and (2) they identify simultaneous contributions of δ-opioid receptor (DOR) and reactive oxygen species (ROS) signaling in the slowly evolving (33) cardioprotection. This novel approach to protecting the heart is practical, powerful, and has proven to be safe in the clinical laboratory (7, 32). The clinical implications for a broader population of patients at risk of heart disease should be compelling. The current findings provide a foundation for further investigation of the mechanisms responsible for this novel cardioprotection.

δ-Opioid receptor activity has been implicated in ischemic pre- (46) and post-conditioning (26, 54), as well as in exercise-induced (3, 13, 38) and pharmacologically mediated cardioprotection (1), although the precise mechanisms are still under investigation (21). Ischemic
preconditioning augments myocardial content of the endogenous DOR agonist enkephalins (53), and repeated brief occlusions of the sinoatrial nodal artery increased nodal enkephalin concentrations, enhancing cardioprotective vagal bradycardia (25). Furthermore, a continuous hypoxic conditioning program in rats increased myocardial enkephalin content that was further induced by ischemia/reperfusion and afforded anti-infarct but not anti-arrhythmia protection (35). These findings suggest that hypoxia has the potential to increase myocardial enkephalins and subsequent DOR activity.

The finding that uninterrupted hypoxia does not produce cardioprotection suggests mitochondrial derived ROS generated during repeated reoxygenation (30) may contribute to IHC. ROS released from mitochondria following hypoxia and simulated ischemia and reperfusion conferred preconditioning-like protection in isolated chick cardiomyocytes that was blocked with anti-oxidant treatment (49). Likewise, NAC treatment abolished the anti-infarct adaptation to severe intermittent hypobaric hypoxia conditioning in rats (28). This severe hypoxia protocol elicited both anti-arrhythmic and anti-infarct cardioprotection, while less intense hypobaric hypoxic exposure afforded only anti-arrhythmia protection (29), indicating the cardioprotective effects of hypoxia-induced ROS are dose-dependent.

Reactive oxygen species may promote IHC activation of DOR-mediated pathways by reducing enkephalin degradation by Zn-dependent proteases. Hypoxia and oxidative stress suppressed the expression of one such peptidase, neutral endopeptidase (NEP), in nerve cell cultures (16). NEP is also inactivated by hypoxia (39) and by receptor-mediated ROS formation (17). The hypoxia-enhanced peptide accumulation can alter physiological responses (31), making a similar DOR mediated mechanism plausible in the heart, where enkephalins are abundant. NAC may abolish IHC induction of ischemia resistance through a similar mechanism, whereby the antioxidant maintains enkephalin degradation and blunts adaptive DOR signaling during the IHC regimen. Thus, the suppression of local myocardial peptide degradation by ROS may
promote DOR stimulation. It would be most interesting to determine whether IHC or ROS alter the even more abundant myocardial Zn-dependent enkephalin aminopeptidase activity.

Catecholamines and their O-methylated metabolites directly suppressed the enzymatic degradation of methionine-enkephalin in rat brain homogenates (8). In this manner, IHC-induced catecholamine release may contribute to the stabilization of endogenous enkephalins and enhancement of DOR activity.

Conversely, DOR activity induces ROS formation (41). Ischemic and ligand-activated preconditioning both activate phosphatidylinositol-3-kinase-dependent pathways that trigger mitochondrial ROS generation. ROS mobilize multiple cardioprotective effectors, including protein kinase C (PKC) and nuclear factor E2-related factor 2 (Nrf2), the latter a key ROS-responsive transcription factor that activates expression of a host of antioxidant enzymes (55). Interestingly, a recent report (9) identifies a cytoprotective DOR/PKC/Nrf2 pathway in HEK293t cells which is triggered by sequential ligand activation and hypoxia-reoxygenation. The protection was blocked by naltrindole or Nrf2 siRNA, implicating a direct link between ROS and DOR activity. Buelna-Chontal et al. (6) demonstrated that PKC-catalyzed Nrf2 phosphorylation was required for the postconditioning induction of Nrf2-regulated antioxidant gene expression in rat heart. IHC likely harnesses PKC signaling, given PKC’s central role in cardioprotection (10, 52). Indeed, intermittent hypoxia increased PKC-δ expression/translocation in parallel with anti-infarct cardioprotection in rats (28). NAC treatment during this hypoxia regimen abolished PKC-δ expression and translocation as well as cardioprotection (28).

Hypoxia itself up-regulates adrenal enkephalin synthesis and subsequent release into the circulation (23, 34). The preproenkephalin promoter contains two cyclic AMP responsive elements, and cyclic AMP-dependent signal transduction upregulates preproenkephalin mRNA (51); thus, IHC may modulate DOR activity by potentiating cyclic AMP production. Indeed,
metoprolol blunted isoproterenol induction of preproenkephalin mRNA in C6 rat glioma cells (14). DOR activation augments hypoxia/reoxygenation elicited neurotransmitter release from intrinsic cardiac adrenergic cells and cardiomyocyte PKA activation (24). The sequential relationship between β-adrenergic stimulation (4), PKA activation and ROS formation (40) could in turn potentiate DOR activation of PKC. Thus there are multiple reciprocal interactions among all key IHC evoked signaling elements (Figure 6).

Reactive oxygen species have been implicated in adrenergically-mediated cardioprotection. In isolated working rat hearts, myocardial infarction was attenuated by pre-ischemic treatment with isoproterenol in a manner reversed in part by co-treatment with NAC (45). In a similar fashion, anti-oxidant treatment abolishes PKA-dependent hypothermia-induced cardioprotection in rats (27). Conversely, H$_2$O$_2$ provokes formation of disulfide bonds between PKA’s regulatory subunits, thereby permitting subcellular translocation and activation of PKA (5). Given the pivotal role that ROS play in orchestrating adaptive responses to oxidative stress, such as occurs during hypoxia and reoxygenation (30, 49, 50), it is reasonable to propose that ROS may potentiate cardioprotective signal transduction. Collectively, these findings argue that adrenergic, opioid, and ROS dependent pathways interact to elicit expression of an extraordinary ischemia-resistant myocardial phenotype.

**Clinical Perspective**

In this study, coronary occlusion-reperfusion was imposed 24 h after the final IHC session, thus, the maximal duration of the IHC-induced cardioprotection is unknown. Means to maintain or improve this cardioprotection merit investigation. Could preemptive IHC protocols improve the outcome of or recovery from elective coronary bypass surgery or other procedures in patients at risk to acute myocardial infarction? Opioid antagonists are increasingly prescribed within smoking cessation and weight loss programs. Do these antagonists pose an additional risk for vulnerable cardiac populations? One might predict that patients receiving antioxidant, DOR
and/or β-adrenoceptor antagonist therapies might be resistant to IHC or require more intense or prolonged IHC programs to reach a given cardioprotective threshold. Neither the specific ROS mediating the cardioprotection, the processes that generate them in response to IHC, nor the cardioprotective effectors, e.g. cytoprotective proteins, have been identified. In most clinical settings, myocardial ischemic events cannot be anticipated; the important question of whether IHC can prevent ischemic injury or promote post-ischemic recovery when administered after the ischemic event has not been tested. Unraveling IHC’s cardioprotective mechanisms would also foster development of pharmacological interventions that harness IHC pathways and mitigate concerns about the safety of hypoxia exposure in potentially vulnerable populations.

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**Author Contributions:** JAE, HFD, JLC, and RTM conceived the investigation and designed the experiments; JAE, AGW, JS and LG conducted the experiments and analytical procedures; JAE and JS analyzed the data and prepared the figures; JAE and RTM drafted the manuscript; JAE,
AGW, HFD, JLC and RTM edited and revised the manuscript; RTM approved the final version of the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. *Intermittent hypoxia conditioning (IHC) program and coronary artery occlusion-reperfusion protocol.* Panel A: Daily IHC sessions consisted of 5-8 exposures, each 5-10 min, to 9.5-10% inspired O₂, with intervening 4 min exposures to room air. The room air exposures were omitted in the uninterrupted hypoxia program. Panel B: *N*-acetylcysteine (NAC) was taken orally in chow diet 2 h before each IHC session. Naltrindole was administered by sc injection 15 min before IHC. Panel C: 24 h after completing the IHC program, the left anterior descending coronary artery (LAD) was occluded for one hour, and then abruptly reperfused for 5 h before masses of infarcted myocardium (IS) and ischemic area at risk (AAR) were determined.

Figure 2. *Arterial plasma N-acetylcysteine (NAC) concentrations after NAC intake.* NAC was measured in plasma sampled from the saphenous vein immediately before (0 h) and at defined times after the dog consumed 250 mg NAC/kg body mass in chow. Intermittent hypoxia conditioning (IHC) was conducted 2-4 h after NAC intake to coincide with the period of maximal plasma NAC concentration. Mean values ± SEM from 5 dogs.

Figure 3. *Cardioprotection afforded by intermittent hypoxia conditioning requires δ-opioid receptor (DOR) activity and reactive oxygen species.* Dogs received daily treatments of DOR antagonist naltrindole (NAL) or antioxidant *N*-acetylcysteine (NAC) before intermittent hypoxia conditioning (IHC) sessions, or completed a modified hypoxia protocol lacking intermittent reoxygenations (uninterrupted hypoxia: UHC). One day after completing the conditioning regimen, the left anterior descending coronary artery was occluded for 60 min and then reperfused. Panel A: Masses of infarcted and ischemic at-risk myocardium were measured at 5 h reperfusion, and infarct mass (IS) was expressed as a percentage of at-risk myocardial mass (AAR). Panel B: Arrhythmias at 0-30 min reperfusion were evaluated and scored according to the Lambeth conventions (11). Mean values ± SEM from 12 non-hypoxic, 9 IHC, 6 IHC + naltrindole, 6 IHC + NAC, and 4 UHC experiments. *P<0.001 vs. all other groups.
Figure 4. *Collateral blood flow during coronary artery occlusion.* The mean flow to the inner two-thirds of the left ventricular myocardial ischemic area at risk (AAR; left), the minimum flow at the core of the AAR (middle) and the flow to the non-ischemic left circumflex (LC) artery perfusion territory (right) were assessed from myocardial distribution of radioactive microspheres injected at 30 min coronary artery occlusion. Mean values ± SEM from 12 non-hypoxic, 9 intermittent hypoxia conditioned (IHC), 6 IHC + naltrindole (NAL), 6 IHC + N-acetylcysteine (NAC), and 4 uninterrupted hypoxia (UHC) experiments. No statistically significant between-group differences were detected by ANOVA.

Figure 5. *Infarct size (panel A) and arrhythmia score (panel B) as functions of collateral blood flow during coronary artery occlusion.* Each point represents a single experiment. Lines in panel A represent least-squares linear regressions of infarct mass (% of mass of ischemic area at risk) vs. mean collateral flow to the inner two-thirds of the ischemic left ventricular free wall. Experiments in which collateral flow exceeded 0.25 ml·g⁻¹·min⁻¹ are excluded. IHC: intermittent hypoxia conditioned; NAL: naltrindole treated; NAC: N-acetylcysteine treated; UHC: uninterrupted hypoxia conditioned; IS/AAR: myocardial infarct size as a percentage of ischemic area at risk.

Figure 6. *Proposed cardioprotective mechanisms mobilized by intermittent, normobaric hypoxia.* Intermittent hypoxia elicits β₁-adrenergic receptor activation, δ-opioid receptor activation and formation of reactive oxygen species (ROS), which in turn evoke protein kinase-mediated signaling and expression of cytoprotective gene programs, culminating in the development of robust cardioprotection over the 20 day intermittent hypoxia conditioning program. Inhibition of any one of these mechanisms is sufficient to blunt the cardioprotection. Evidence for these mechanisms and possible interactions between them are discussed in the text.
Table 1. Characteristics of treatment groups.

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<td>Body mass (kg)</td>
<td>26.8 ± 1.8</td>
<td>26.2 ± 2.1</td>
<td>31.3 ± 1.2</td>
<td>28.2 ± 1.7</td>
<td>28.2 ± 0.3</td>
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<tr>
<td>LV mass (g)</td>
<td>135 ± 6</td>
<td>139 ± 8</td>
<td>166 ± 11*</td>
<td>158 ± 10</td>
<td>158 ± 3</td>
</tr>
<tr>
<td>LV/body mass (g/kg)</td>
<td>5.1 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td>5.6 ± 0.4</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>41.4 ± 0.8</td>
<td>40.6 ± 1.9</td>
<td>38.5 ± 1.2</td>
<td>38.0 ± 1.4</td>
<td>44.5 ± 2.0</td>
</tr>
<tr>
<td>Hgb (g/dl)</td>
<td>13.9 ± 0.3</td>
<td>13.7 ± 0.6</td>
<td>14.3 ± 0.8</td>
<td>13.1 ± 0.5</td>
<td>15.5 ± 0.2</td>
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</table>

Values are mean ± SEM. †Animals with mean collateral blood flow > 0.25 ml · kg⁻¹ · min⁻¹ were excluded from analyses of infarct and arrhythmias. Hct: hematocrit; Hgb: blood hemoglobin content; IHC: intermittent hypoxia conditioning; NAC: N-acetylcysteine; NAL: naltrindole; UHC: uninterrupted hypoxia conditioning. *P < 0.05 vs. non-hypoxic, non-IHC value (ANOVA and Holms-Sidak post hoc analysis).
Figure 1. Intermittent hypoxia conditioning (IHC) program and coronary artery occlusion-reperfusion protocol. Panel A: Daily IHC sessions consisted of 5-8 exposures, each 5-10 min, to 9.5-10% inspired O₂, with intervening 4 min exposures to room air. The room air exposures were omitted in the uninterrupted hypoxia program. Panel B: N-acetylcysteine (NAC) was taken orally in chow diet 2 h before each IHC session. Naltrindole was administered by sc injection 15 min before IHC. Panel C: 24 h after completing the IHC program, the left anterior descending coronary artery (LAD) was occluded for one hour, and then abruptly reperfused for 5 h before masses of infarcted myocardium (IS) and ischemic area at risk (AAR) were determined.
Figure 2. *Arterial plasma N-acetylcysteine (NAC) concentrations after NAC intake.* NAC was measured in plasma sampled from the saphenous vein immediately before (0 h) and at defined times after the dog consumed 250 mg NAC/kg body mass in chow. Intermittent hypoxia conditioning (IHC) was conducted 2-4 h after NAC intake to coincide with the period of maximal plasma NAC concentration. Mean values ± SEM from 5 dogs.
Figure 3. **Cardioprotection afforded by intermittent hypoxia conditioning requires δ-opioid receptor (DOR) activity and reactive oxygen species.** Dogs received daily treatments of DOR antagonist naltrindole (NAL) or antioxidant N-acetylcysteine (NAC) before intermittent hypoxia conditioning (IHC) sessions, or completed a modified hypoxia protocol lacking intermittent reoxygenations (uninterrupted hypoxia: UHC). One day after completing the conditioning regimen, the left anterior descending coronary artery was occluded for 60 min and then reperfused. Panel A: Masses of infarcted and ischemic at-risk myocardium were measured at 5 h reperfusion, and infarct mass (IS) was expressed as a percentage of at-risk myocardial mass (AAR). Panel B: Arrhythmias at 0-30 min reperfusion were evaluated and scored according to the Lambeth conventions (11). Mean values ± SEM from 12 non-hypoxic, 9 IHC, 6 IHC + naltrindole, 6 IHC + NAC, and 4 UHC experiments. *$P<0.001$ vs. all other groups.
Figure 4. **Collateral blood flow during coronary artery occlusion.** The mean flow to the inner two-thirds of the left ventricular myocardial ischemic area at risk (AAR; left), the minimum flow at the core of the AAR (middle) and the flow to the non-ischemic left circumflex (LC) artery perfusion territory (right) were assessed from myocardial distribution of radioactive microspheres injected at 30 min coronary artery occlusion. Mean values ± SEM from 12 non-hypoxic, 9 intermittent hypoxia conditioned (IHC), 6 IHC + naltrindole (NAL), 6 IHC + N-acetylcysteine (NAC), and 4 uninterrupted hypoxia (UHC) experiments. No statistically significant between-group differences were detected by ANOVA.
Figure 5. Infarct size (panel A) and arrhythmia score (panel B) as functions of collateral blood flow during coronary artery occlusion. Each point represents a single experiment. Lines in panel A represent least-squares linear regressions of infarct mass (% of mass of ischemic area at risk) vs. mean collateral flow to the inner two-thirds of the ischemic left ventricular free wall. Experiments in which collateral flow exceeded 0.25 ml·g⁻¹·min⁻¹ are excluded. IHC: intermittent hypoxia conditioned; NAL: naltrindole treated; NAC: N-acetylcysteine treated; UHC: uninterrupted hypoxia conditioned; IS/AAR: myocardial infarct size as a percentage of ischemic area at risk.
Figure 6. Proposed cardioprotective mechanisms mobilized by intermittent, normobaric hypoxia. Intermittent hypoxia elicits β1-adrenergic receptor activation, δ-opioid receptor activation and formation of reactive oxygen species (ROS), which in turn evoke protein kinase-mediated signaling and expression of cytoprotective gene programs, culminating in the development of robust cardioprotection over the 20 day intermittent hypoxia conditioning program. Inhibition of any one of these mechanisms is sufficient to blunt the cardioprotection. Evidence for these mechanisms and possible interactions between them are discussed in the text.
CHAPTER 3

Intermittent Hypoxic Conditioning Augments Parasympathetic Influence Over the Canine Heart

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Running Title: DOR, GM1, parasympathetic, cholinergic, adrenergic

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ABSTRACT

Background: Intermittent hypoxia conditioning (IHC) produces remarkable anti-arrhythmic and anti-infarct effects during myocardial ischemia/reperfusion in dogs, in a manner dependent on a combination of β₁-adrenergic, δ-opioid receptor (DOR) and reactive oxygen signaling. The vagotonic DOR-1 subtype expressed on myocardial post-ganglionic parasympathetic neurons is well positioned to moderate the innate cardioprotective effects of the vagus. Methods: Mongrel dogs completed 20 days IHC or sham conditioning programs. Sinoatrial node, atrium and left ventricle were biopsied for immunoblotting and immunohistochemistry of DOR, cholinergic and adrenergic markers, and the DOR-1 potentiating monosialoganglioside, GM1. Results: IHC significantly enhanced vagal bradycardia relative to sham dogs (P<0.05). This effect persisted in the presence of vagolytic concentrations of the DOR agonist, met-enkephalin-arg-phe (MEAP, P<0.05). Paradoxically, DOR content was unchanged and DOR-labeled fibers declined following IHC. DOR blockade with naltindole during daily hypoxia conditioning produced a robust compensatory increase in DOR (atria and ventricle) and vesicular acetylcholine transporter (VACHT, atria) protein contents (P<0.05) relative to both sham and IHC groups. IHC reduced the ventricular content of tyrosine hydroxylase (P<0.05) relative to shams. IHC produced an increase in low intensity GM1 positive fiber staining (P<0.05) which almost completely overlapped with fibers containing cholinergic markers. IHC increased low and high intensity VACHT positive fibers in the sinoatrial node (P<0.05) collectively suggesting an increase in parasympathetic arborization. The increased ratio of cholinergic/adrenergic labeling suggests a cardioprotective shift in autonomic balance. Surprisingly, IHC reduced the number of low intensity DOR labeled fibers in both the atria and SA node (P<0.05) perhaps reflecting a selective loss of the vagolytic DOR-2 subtype. Summary: IHC improves autonomic balance in favor of vagal control. The role of this adaptation in mediating the IHC-induced cardioprotection remains to be determined.
1. INTRODUCTION

Although sustained hypoxia is harmful, a program of moderate, normobaric, cyclic hypoxia and reoxygenation has been used to successfully treat clinically resistant arrhythmias in patients [5] and evolves remarkable cardioprotection against ischemia and reperfusion in dogs [56]. This intermittent hypoxia conditioning (IHC) produces a gradually evolving [33] resistance to ischemia-reperfusion injury in dogs manifest as robust reductions in infarct size and lethal arrhythmias [41]. The protection afforded by IHC appears to require regular β-adrenergic receptor, δ-opioid receptor (DOR) and reactive oxygen signaling because the protection is abrogated by the coincident daily administration of the β₁-adrenergic receptor antagonist metropolol, the DOR antagonist naltrindole or the antioxidant N-acetyl-cysteine [Estrada et al., in review]. Collectively these findings suggested that IHC, like exercise training, may protect the myocardium from ischemia-reperfusion injury by enhancing cholinergic and opioid influence over the heart.

Delta opioid receptor (DOR) signaling has been implicated in a variety of cardiac preconditioning protocols [22; 37; 43]. In heart, DOR immunolabeling is primarily co-localized with markers of parasympathetic innervation [13] and DOR signaling can modify vagal transmission in either direction [16; 18]. Extended hypoxic conditioning augments DOR expression in brain [20]. In rats, hypoxic conditioning increased myocardial enkephalin content and afforded protection against myocardial infarction [35], and preserved arterial vasomotor responses to acetylcholine following ischemia-reperfusion [34]. Repeated preconditioning-like occlusions of the SA node artery improved vagal transmission [14] in parallel with local accumulation of the DOR agonist, met-enkephalin-arg-phe (MEAP). Repeated DOR-1 stimulation [15], and exposure to the monosialoganglioside GM1 [12], produce similar improvements in vagal transmission. DOR-1 signaling in vitro elevates cAMP and then GM1, neurite formation and more DOR-1 in an excitatory positive feedback loop [9]. Application of
GM1 exposure in the SA node improves vagal transmission by reducing vagolytic DOR-2 signaling while preserving vagotonic DOR-1 signaling [12]. Thus, the protective phenotype associated with IHC might well include convergent structural and functional adjustments in both opioid and cholinergic systems.

Accordingly, this study tested the hypothesis that IHC increases cholinergic and opioid influence in the heart by increasing myocardial GM1 and DOR contents, parasympathetic fiber densities and, thus, a net increase in the efficiency of vagal transmission. To test this hypothesis, the bradycardic response to vagal stimulation was assessed over a broad range of sinoatrial nodal MEAP concentrations in intermittent hypoxia-conditioned and non-hypoxic control dogs. Parasympathetic and sympathetic markers, DOR and GM1 were measured in sinoatrial node, atrium and left ventricle, and colocalization of GM1 and parasympathetic markers was assessed in atrial tissue of hypoxia conditioned and control dogs.
2. METHODS

2.1 Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and were conducted in compliance with the Laboratory Animals (U.S. National Research Council Publication 85-23, revised 2011). Mongrel dogs were assigned to 3 groups: non-hypoxic sham, IHC, or IHC + daily sc naltrindole (IHC+N). Dogs were conditioned in acrylic chambers for 20 consecutive days using either compressed nitrogen gas or compressed air (21% O2) to displace chamber room air for IHC conditioning [5-8 cycles of 5-10 min hypoxia (9.5-10% FIO2) + 4 m intervening normoxia] or for sham conditioning with 21% O2. On day 21 animals were physiologically evaluated and cardiac tissue was flash-frozen for biochemical and fixed for histochemical analyses.

2.1.1 Surgical preparation and physiological evaluation of vagal DOR phenotypes

Surgical preparations and physiological evaluation procedures were performed as previously described [17]. Briefly, sodium pentobarbital (32.5 mg/kg) anesthetized dogs were intubated and ventilated mechanically with room air at 225 mL/kg/min. A 0.9% NaCl-filled catheter was inserted into the right femoral vein and forwarded into the inferior vena cava. An infusion port Millar Mikro-Tip transducer was inserted into the right femoral artery and advanced into the aorta above the L5 region to measure heart rate and sample arterial blood, and perfuse the target L5 sympathetic ganglion with experimental agents. The venous line was attached to a Statham P23ID pressure transducer to measure continuous central venous pressure in real time (PowerLab; ADI Instruments, Colorado Springs, CO). Surface electrodes were placed and lead II electrocardiogram (ECG) was continuously recorded. An electro-magnetic flow probe (10 mm) was installed around the isolated left femoral artery to measure hindlimb blood flow. Anesthesia was regularly evaluated and supplemental pentobarbital was administered as required. Acid-base balance and blood gases in arterial samples were measured with an
Instrumentation Laboratories blood gas analyzer (Lexington, MA). The pO₂ (90-120 mmHg), pH (7.35-7.45), and pCO₂ (30-40 mmHg) were adjusted to normal by administering supplemental oxygen or bicarbonate or by modifying the tidal volume.

The right and left cervical vagus nerves were isolated through a ventral midline incision and double ligated with umbilical tape to abort afferent nerve traffic. The nerves were then returned to the prevertebral compartment for later retrieval. The carotid arteries were isolated and fitted with adjustable plastic snares to evaluate opioid interactions during sympathetic activation via bilateral carotid occlusion (BCO) as previously described [1]. A single dose of succinylcholine (1 mg/kg) was intravenously administered to inhibit muscle movement for the 10 min required for electrosurgical incision of the right lateral chest. Thoracotomy of the right side was performed to expose the heart. The pericardium was opened and pericardial margins were sutured to the body wall to support the heart.

2.1.3 Nodal microdialysis
Dialysis probes were made from 1 cm lengths of dialysis fiber (200 μm i.d. x 220 μm o.d.) obtained from a Clirans TAF 08 (Asahi Medical, Northbrook, IL) artificial kidney as previously described [18]. The probe was fixed with glass fiber inflow and outflow lines and inserted into sinoatrial nodal tissue using a 25g needle. Transmural passage of molecules with masses greater than 36 kDa were impeded by the probe. A micro infusion pump fitted with the inflow line was used to equilibrate the probe with vehicle (saline) at 5ul/min for one hour.

2.1.4 Assessment of vagal bradycardia and vagal-DOR responses
Following 1-hr equilibration, the right vagus nerve was stimulated at a supramaximal voltage (e.g., 15 V) at 3 Hz for 15 s. Heart rate was recorded when it reached a new low steady state during the 15-s stimulus. Heart rate was allowed 105 s for complete recovery. Increasing doses
of MEAP were then added to the nodal perfusate and a dose response was constructed between $5 \times 10^{-12}$ to $15 \times 10^{-15}$ mol/min. Each new dose was infused for 5 min at 5 μl/min before testing each vagal response. The probe was then perfused with saline until the baseline vagal response was reconfirmed.

2.1.5 Assessment of gangliolytic DOR-2 responses on hind limb conductance

After 30 min to restore normal nodal function, increasing doses of met-enkephalin ($5 \times 10^{-8}$ to $1 \times 10^{-5}$ mol/kg) were introduced into the descending aorta to construct a DOR-2 dose response using increasing femoral conductance as the outcome measure. Bolus doses (1 ml) were injected just proximal to the final segmental arteries providing direct access to the L5 sympathetic ganglia controlling femoral arterial conductance [1]. Each dose was introduced with a 5 ml saline flush. The hemodynamic effect of each dose was recorded and allowed to return to baseline for 5–10 min before applying the next dose.

2.2 Biopsy preparation

The heart was stopped and biopsies of sinoatrial node, atrium and left ventricle were taken. Biopsies were subdivided; one portion was snap-frozen with liquid nitrogen and stored at -80°C, and the other portion was immersion-fixed in 4% paraformaldehyde for 8 h. Fixed tissues were cryoprotected with serial sucrose solutions (10–30%), then stored at -80°C.

2.3 Tissue homogenization and handling

Snap-frozen atrial and left ventricular biopsies were pulverized in a liquid N$_2$-cooled mortar [49] and extracted by homogenization in 10 vol (v/w) radioimmunoprecipitation assay extraction buffer (1% IGEPAL CA-630, 0.5% cholate, 0.5% SDS, 150mM NaCl, 10mM Tris-Cl, 1mM EDTA, 1Mm PMSF, pH 7.6; Sigma-Aldrich, USA) with added protease and phosphatase inhibitors (Cell Signaling Technology, Boston MA) at 4°C. Homogenates were then sequentially
drawn through 18, 21 and 23 ga needles to decrease sample viscosity by shearing nucleic acids. Cellular debris was sedimented by 15 min centrifugation at 15,000 g in a refrigerated (4°C) centrifuge (Eppendorf, Hauppage, NY, USA). The supernatant was aliquoted. Protein concentrations were measured by BCA assay (Pierce, Grand Island, NY USA) in a microplate reader (Bio-Rad, Hercules, CA, USA)

2.4 Protein electrophoresis & immunoblotting

SDS-polyacrylamide (4% bis-acrylamide stacking, 10% bis-acrylamide resolving) gels (1.0 mm) were prepared from stock reagents (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. SDS running and transfer buffers were prepared as originally described with addition of SDS to the transfer buffer at 0.02% w/v [47]. RIPA lysates were combined with concentrated sample loading buffer yielding a final buffer concentration of 250mM Tris-HCl, pH 8.5, 2% w/v lithium dodecyl sulphate, 100mM DTT, 0.4mM EDTA, 10% v/v glycerol, 0.2mM phenol red, and 0.2mM Brilliant Blue G [10] and a final protein concentration of 1 mg/mL. Samples were heated at 70°C for 10 min, then cooled on ice. Five or 10 µg of protein were loaded into each well. Proteins were electrophoresed at 60 V through the stacking gel and at 100 V through the resolving gel until the loading buffer dye reached the gel edge. Resolved proteins were then electroblotted onto vinyl membranes (Bio-Rad) at 70 V for 60-90 min, rinsed with deionized water to remove buffer salts, and then dried 1 h at room temperature.

2.5 Immunoblotting, ECL imaging and protein quantification

Membranes were reactivated with a brief rinse in methanol, rehydrated with optimized washing buffer (KPL Inc, Gaithersberg, MD USA), incubated with a synthetic blocking buffer (KPL Inc) for 1 h, and then incubated with primary antibodies diluted in blocking buffer for 3 h. After three 5 min rinses in washing buffer, membranes were incubated for 1 h with HRP-conjugated secondary antibodies diluted in synthetic blocking buffer, and then rinsed for 5 min four times in
washing buffer and once in deionized water. Membranes were then incubated with an extended range ECL reagent (Advansta, Menlo Park, CA USA) optimized for use in digital imagers for 2 min before being examined with a G-Box CCD imager (Syngene, Frederick, MD USA). Gene Tools imaging software was set to capture unsaturated images for band density quantification. Band densities were normalized to a single data point; β-tubulin density served as loading control.

2.6 Nerve fiber analysis

An overview of the novel nerve fiber analysis procedure is summarized in Figure 1.

2.6.1 GM-1 immunohistochemistry optimization
Sections (12 µm) were cut from OCT tissue blocks using a cryostat at -20°C, mounted onto gel coated slides with frosted ends (Electron Microscopy Sciences, Hatfield, PA, USA), air dried for 1 h and then stored at -80°C. Frozen slides were brought to room temperature before further processing. In situ GM1 preservation was achieved by cold (-20°C) anhydrous acetone (Sigma Aldrich, St. Louis, MO, USA) fixation and permeabilization for 3 min followed by drying for 15 min [23; 24; 39; 43] in coplin jars. Individual wells were formed for each section with a pap-pen (Sigma Aldrich). Sections were then blocked with ice cold PBS Plus (10 mg/mL protease-free BSA, 5% normal goat serum, 0.01% NaN₃: Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at 4°C. CTxB-594 was diluted in PBS Plus and sections were then incubated in CTxB solution for 6 h at 4°C, followed by rinsing with cold PBS Plus for ten times for 5 min each on an orbital shaker. Slides were then incubated with DAPI (1 mg/mL: Life Technologies) in PBS for 1 min then sequentially rinsed with deionized water and immunofluorescence mounting solution (Electron Microscopy Sciences, Hatfield, PA, USA) before applying fresh mounting medium. Cover slips were placed and sealed with nail varnish at room temperature. Sections were kept wet throughout the procedure to avoid immunofluorescence artifacts.
2.6.2 Immunohistochemistry

Sections were treated and blocked as described above, and incubated overnight at 4°C with primary antibodies diluted in cold PBS Plus. Sections were rinsed with cold PBS Plus ten times for 5 min on an orbital shaker, and then incubated for 3 h at 4°C with Alexafluor-488 conjugated secondary antibodies diluted in cold PBS Plus. Sections were then rinsed with cold PBS Plus 4 times for 5 min, followed by incubation with DAPI in PBS, deionized water and mounting medium as described above before applying fresh mounting medium and applying coverslips.

2.6.3 Image acquisition, image reconstruction and line scan analysis

Color micrographs were captured and scaled with an epifluorescence microscope (Olympus BX-41) at 10X magnification with ImagePlusPro software. Exposure times were optimized for contrast between signal and noise. Computer images were processed with NIH ImageJ and the Morphology plug-in. Micrographs were first converted to 8-bit grayscale images, then, using the Domes algorithm intensity data from the structural markers were extracted from images with uneven backgrounds. The images were then reconstructed providing a strong 1st approximated background adjustment. Three vertical and 3 horizontal line scans, equally spaced and spanning the micrograph were then taken. Line scan data was imported into MATLAB to analyze fluorescence intensities [42]. The data were then readjusted for baseline estimation using the “msbackadj” function. The “mspeak” function was then applied to detect peaks. The latter command was modified to preserve peak heights and record peaks above threshold, as determined by the average peak intensity of a control. Line intensity scans of photomicrographs obtained using confocal microscopy demonstrate that peak intensity magnitude and width are correlated with fiber size, although differentiating fibers based on peak width measurements are not valid using images obtained by epifluorescence [42]. Thus, peaks in atrial and SA nodal tissue sections were assigned to bins based on fluorescence intensity,
and counted digitally. Because fluorescence intensities varied across structural markers, peaks were empirically assigned to intensity ranges corresponding to neurites/smaller and larger fibers. (See Appendix 1 for method details).

2.7 Sources of antibodies

Rabbit polyclonal antibody raised against C-terminal DOR sequence was obtained from Research and Diagnostic Antibodies (Las Vegas, NV, USA). Rabbit polyclonal antibody raised against synthetic peptide from vesicular acetylcholine transporter (VACHT) and rabbit monoclonal antibodies raised against synthetic peptides from choline transporter-1 (ChT-1) and tyrosine hydroxylase-1 (TH) were obtained from Abcam (Cambridge, MA, USA). Rabbit monoclonal antibodies raised against synthetic peptides from synapsin-1 and alpha-tubulin were acquired from Cell Signaling Technology (Danvers, MA, USA). Cholera-toxin subunit B (CtxB) conjugated to Alexafluor 594 (CTXB-594) was acquired from Life Technologies (Grand Island, NY, USA) for GM-1 detection. Preabsorbed goat anti-rabbit secondary antibodies conjugated to Alexafluor 488 were acquired from Jackson ImmunoResearch (Hatfield, PA, USA), and preabsorbed goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were acquired from Cell Signaling Technology. Antibody dilutions are reported in Table 1.

2.8 Statistical analysis

Student’s t-test was used to compare immunoblot and immunohistochemistry data in sham and IHC animals. Immunoblot data from the 3 groups were analyzed by single-factor ANOVA followed by Holms-Sidak’s post-hoc analysis. P values <0.05 were taken to indicate statistically significant treatment effects. Statistical analyses were performed with SigmaStat software.
3. RESULTS

3.1 IHC, HR and vagal bradycardia

Resting heart rates in anesthetized dogs were not different after IHC (133 ± 5) vs sham, (124 ± 10) (P=0.536). Vagal stimulations were conducted to test the hypothesis that IHC augments vagal bradycardia. Vagal transmission at 3 Hz produced a sharp decline in heart rate. The magnitude of the decline was consistently greater in the IHC animals than in sham conditioned dogs (P<0.05) (Figure 2). At higher doses (5 x 10^{-13} to 15 x 10^{-10} moles/min) MEAP generally produces a progressive DOR-2 vagolytic effect; however, that effect was modest in this case and not different between IHC and sham dogs.

3.2 Femoral conductance

Increasing doses of ME produced a clear and progressive increase in femoral arterial blood flow and conductance (Figure 3). This DOR-2 mediated response was virtually identical in both treatment groups indicating IHC did not affect ganglionic DOR-2 responsiveness.

3.3 DOR content in the atria and left ventricle following IHC

The hypothesis that IHC increases DOR protein expression was tested in myocardial tissue extracts of atrial and left ventricular myocardium. Quantitative ECL immunoblot densitometry was used for analysis of cardiac tissue lysates (Figure 4A). There was no change in atrial DOR content following IHC, but a near doubling in DOR atrial expression following IHC with naltrindole relative to sham (P<0.05) and IHC (P<0.05) (Figure 4B). Left ventricular tissue lysates yielded similar results (Figure 4C). Naltrindole + IHC increased DOR expression 2.5- and 2.9-fold relative to sham (P<0.01) and IHC (P<0.01), respectively. There was no difference in ventricular DOR expression following IHC relative to sham animals (Figure 4C). These results suggest that DOR stimulation and blockade may respectively down and up-regulate DOR expression.
3.4 VACHT, ChT-1, and TH content in the atria and left ventricle following IHC

The hypothesis that cholinergic (VACHT & ChT-1) protein content increases and adrenergic tyrosine hydroxylase (TH) content decreases following IHC was tested (Figure 4). Cardiac tissue lysates were analyzed by quantitative ECL immunoblot densitometry. In atrium, VACHT content was essentially unchanged following IHC relative to sham animals, but there was a marked increase in VACHT content following IHC + naltrindole vs sham (1.9-fold) (P<0.05) and IHC (2.3-fold) (P<0.01) (Figure 4D). This result may represent a compensation for the lack of DOR stimulation during IHC, indicating a role for DOR signaling in regulating cardioprotective cholinergic protein expression. Immunoblots of left ventricle lysates did not demonstrate any change in VACHT content following IHC vs sham or IHC + naltrindole animals (Figure 4E).

Collectively, the cardiac VACHT analyses suggest that DOR signaling during IHC differentially regulates transcription at the “cholinergic gene locus”, consistent with findings in the literature, although these results were discordant with the proposed hypothesis.

Atrial and left ventricular ChT-1 contents were not altered by IHC vs sham or IHC + naltrindole, indicating that the rate controlling protein in acetylcholine synthesis is not altered by IHC or DOR signaling (Figure 4F, G). IHC and DOR signaling may also attenuate adrenergic influence by decreasing TH expression. Immunoblotting of atrial lysates demonstrated no significant changes in TH content following IHC relative to sham and IHC plus naltrindole animals (Figure 4H). Similarly, TH expression in left ventricular myocardium did not change (P=0.066) following IHC relative to the other groups (Figure 4I). Although the three group ANOVA did not achieve sufficient power to detect treatment effects. TH was shown to fall approximately 30% following IHC vs sham conditioning (P=0.03) when Student’s t-test was applied to these 2 groups, indicating that IHC attenuates adrenergic activity.
3.5 Immunohistochemistry of parasympathetic and sympathetic markers and GM1

Dual labeling experiments were performed to test the hypothesis that IHC modifies the distribution of autonomic fibers (Figure 5). The extents of CtxB⁺ nerve fiber intensity overlap with synapsin⁺, VACHT⁺, ChT-1⁺, ChAT⁺, and TH⁺ structures were quantified. As expected, CtxB⁺ and synapsin⁺ intensities overlapped extensively. Also, the overlap between CtxB⁺ and the parasympathetic markers (VACHT⁺, ChT-1⁺, and ChAT⁺) was also extensive. CtxB⁺ intensities were well correlated with DOR⁺ intensities, as well. In contrast, the intensity overlap between GM-1 and the sympathetic marker (TH⁺) was limited.

3.6 Atrial & sinoatrial nerve fiber line scan intensity analysis using GM1 as structural marker

To test the hypothesis that IHC may induce GM1 synthesis and nerve growth, line scan intensity analysis was conducted using the GM-1 specific fluorescent labeled CtxB-594 (Figure 6). Sample line intensity scans over two contrasting regions of interest demonstrate peak detection of background noise vs low and high intensity fibers (Figure 7). Analysis of atrial CtxB⁺ nerves demonstrate a significant change in CtxB⁺ structures corresponding to low intensity fibers (peak intensities 5-14) following IHC vs sham conditioning (P<0.05) (Table 3). This result is consistent with the hypothesis that IHC enhances vagal bradycardia by inducing GM1 synthesis. The increase in moderate size fibers suggests a possible increase in nerve fiber arborization.

Analyses of sinoatrial CtxB⁺ nerves did not demonstrate an increase in lower intensity fibers (peak intensities 5-9), although there was an apparent increase in CtxB⁺ structures corresponding to high intensity fibers (peak intensity >19). Thus IHC appeared to increase myocardial GM-1 positive nerve fibers.

3.7 Atrial & SA nodal nerve fiber line scan intensity analysis using DOR as structural marker

To test the hypothesis that IHC may induce the development of DOR containing nerve fibers, DOR positive fibers in SA node and atrial biopsies were analyzed by line intensity scans.
Analysis of atrial DOR⁺ nerves demonstrated an unexpected decrease in DOR⁺ structures corresponding to low intensity fibers (peak intensity 5-9) following IHC vs sham dogs (P<0.05) (Table 2). A similar decrease was observed in low intensity (peak intensities 5-9) and perhaps in a broader range of higher intensity fibers as well (peak intensities 5-14) in the sinoatrial node vs IHC relative to sham animals (both intensity ranges, P<0.05) (Table 3).

3.8 Atrial & SA nodal parasympathetic and sympathetic nerve fiber line scan intensity analysis using VACHT, ChT-1, and TH as structural markers

Line scan intensity analyses of immunohistochemistry images were conducted to test the hypothesis that IHC alters the autonomic fiber balance within the SA node and atrial tissues. Line scan intensity analysis of VACHT positive atrial fibers identified no statistically significant IHC increase in the number of VACHT⁺ fibers, although an upward trend was apparent in the higher intensity fibers (>9) (Table 2). Analysis of SA nodal I tissues demonstrate an increase in VACHT⁺ innervation corresponding to low intensity fibers (peak intensities 5-9) following IHC relative to sham animals (P<0.05) animals (Table 3), suggesting that IHC may be inducing de novo parasympathetic innervation. IHC also produced a significant increase in VACHT⁺ structures corresponding with high intensity nodal fibers (peak range >4) (P<0.05 vs sham).

Line scan intensity analysis of ChT-1 positive fibers in atrial (Table 2) and SA nodal tissue (Table 3) sections showed no significant changes in ChT-1⁺ innervation in any of the intensity ranges. Line scan intensities of TH positive nerves in atrial sections were unchanged by IHC although there appeared to be a decline in low intensity TH⁺ innervation (peak intensity 5-14) following IHC relative to sham animals (Table 2). Changes in TH⁺ innervation in SA node were likewise not significant (Table 3).

The divergent effects of IHC on the relative numbers of parasympathetic and sympathetic fibers suggested an IHC-induced shift in autonomic balance. To examine this possibility, the ratios of
total VACHT+ fibers /TH+ fibers at intensities >4 were computed (Figure 8) and a trend, albeit not statistically significant, emerged in the atria and SA node favoring increased parasympathetic influence. Similar upward trends were seen in comparisons at other intensity ranges.
4. DISCUSSION

Recent studies in this laboratory extended the list of known mediators of IHC cardioprotection from β₁-adrenergic activity to include DOR and ROS signaling (Estrada et al., in review). The current study specifically examined IHC-induced cardioprotective mechanisms in dogs. The results demonstrate that IHC consistently augments vagal transmission, suggestive of structural and/or functional improvements in the vagal/myocardial interface and could reflect an increased vagotonic DOR-1 influence. These overall findings are consistent with the hypothesis that IHC enhances vagal function. Furthermore, compared with prior work [18], the enhanced response to vagal stimulation persisted over a broad MEAP dose spectrum, and the expected DOR-2 vagolytic response was much reduced and often absent following IHC. The apparent shift of DOR in favor of the DOR-1 subtype was not however observed for the gangliolytic DOR-2 dose response relationship where femoral conductances in IHC and sham dogs were identical over a broad met-enkephalin concentration range. These findings demonstrate divergent effects of IHC on cardiac vs. peripheral autonomic DOR interactions.

Although IHC enhanced vagal bradycardia, the response to vagal stimulation during MEAP treatment does not convincingly demonstrate vagal DOR phenotype changes following IHC. Specifically, although the sustained and elevated vagally mediated bradycardia in the face of high dose MEAP is very unusual, a clear vagolytic effect of MEAP was not observed in the sham animals. The results are consistent with an increase in DOR-1 influence following IHC. Proving this IHC enhancement of DOR-1 would require a demonstration that the enhanced vagal influence is reversed with a selective DOR-1 antagonist [18]. The vagolytic DOR-2 activity is robust and predominates with increasing agonist dosages [26; 17]. Here, immunoblotting revealed no appreciable IHC effect on DOR protein content in atrium or left ventricle. However, repeated exposure to MEAP or GM-1 functionally down-regulates DOR-2 responsiveness and exposes the underlying vagotonic DOR-1 activity [12; 9]. This shift in DOR
character may represent the selective down-regulation of DOR-2 activity secondary to repeated daily hypoxic challenges, similar to that observed acutely during repeated arterial occlusion [14]. However, the studies’ small sample size precludes definitive conclusions about the fate of the opposing DOR-2 activity. Thus the findings are consistent with an increase in DOR-1 influence in the SA node but ambiguous about the fate of DOR-2 receptors.

An alternative explanation for the apparent enhancement of the vagotonic DOR-1 response could involve functional interactions between the kappa opioid receptor (KOR) and DOR. The KOR colocalizes with TH positive sympathetic fibers and VACH-T positive parasympathetic fibers [29]. Repetive stimulation of parasympathetic KOR by adrenergic receptor induced dynorphins [4] during IHC may upregulate DOR-1 function [28].

Intermittent hypoxia conditioning did not produce the anticipated elevations of DOR content in either the atria or left ventricle. This outcome differs from extended hypoxia conditioning which elevates brain enkephalin content as well as DOR mRNA abundance and content for several days [20]. Moreover, DOR antagonism during IHC, which abrogated the IHC mediated cardioprotection (Estrada et al., in review), resulted in a compensatory increase in atrial and ventricular DOR content. In addition, VACH-T content increases in the atria, but not the left ventricle, following IHC with DOR antagonism. Together these results suggest that DOR signaling during IHC may regulate transcriptional activity of the cholinergic gene locus and DOR. This interpretation warrants further investigation, given the small sample size and the recognized role of DOR signaling in antagonizing β1-adrenergic activity [38].

During heart failure sympathetic overactivity and chronic β1-adrenergic stimulation provoke compensatory DOR expression [48]. Antagonizing DOR during IHC may elicit similar cellular responses where unopposed β1-adrenergic receptor activity increases DOR expression. Conversely, naltrindole treatment could result in increased DOR cycling, distribution or
maturation through post-translational pathways. Stimulation of DOR activity results in efficient down-regulation and degradation of receptors through ubiquitination dependent and independent pathways [25]. Receptor occupancy with naltrindole during IHC could alter trafficking of DOR, increasing the pool of functional receptors [31]. Moreover, approximately 50% of newly synthesized DOR are shunted to proteasomal degradation [31]. On the other hand naltrindole, which is membrane permeable, could act as a molecular chaperone [32], sequestering receptors away from degradation pathways.

The cholinergic gene locus and ChT-1 genes are regulated by PI3K and PKA [3; 7], two kinases potentially activated by IHC and DOR. An enhancement in cholinergic transmission due to improved acetylcholinergic protein synthesis and processing following IHC could contribute to the observed augmentation of vagal bradycardia. Although IHC did not increase atrial or left ventricular VACHT or ChT-1 contents, IHC with DOR blockade did increase atrial VACHT content. These results suggest that augmentation of the cholinergic machinery is not responsible for the IHC-induced improvements in vagal transmission.

Lack of receptor signaling during DOR blockade may result in differential regulation of the cholinergic gene locus and elevation of VACHT expression. This result may indicate an adaptive mechanism to counteract sympathetic activity. Alternatively, an increase in atrial cardiomyocyte VACHT may facilitate autocrine acetylcholinergic activity to offset hypertrophic signals [40] otherwise antagonized by myocardial inhibitory DOR activity. Additionally, while IHC did not elevate VACHT content, it decreased left ventricular TH content suggesting attenuation of adrenergic influence over the heart. The effect of IHC on cardiac TH content appears to be independent of DOR activity, but inversely related to β1-adrenergic receptor activity [8], enhancing adrenergic responsiveness and cardiac efficiency. Moreover, activation of the KOR in canine hearts is sympatholytic [46] and perhaps may also modulate TH expression through proteosomal degradation pathways in synaptic terminals [25]. Furthermore,
contraction induced production of myokines, such as the leukocyte inhibitory factor (LIF) [4] during IHC may alter sympathetic nerve phenotype. β1-adrenergic activity during IHC may upregulate LIF expression, resulting in reduced expression of TH and adrenergic character and increased expression of ChAT and cholinergic character within sympathetic nerves [29]. IHC may thus favor a shift of autonomic balance in favor of a cholinergic phenotype through receptor mediated mechanisms.

GM1+ low intensity fibers were increased following IHC in the atria but not in the SA nodal nerve fibers suggesting that IHC alters fiber content but that the distribution is heterogeneous. GM-1 labeling strongly colocalizes with markers for parasympathetic structures, suggesting that changes in GM1 content should correlate with changes in parasympathetic innervation and/or function. The increase in VACHT-labeled low and high intensity fibers in the sinoatrial node following IHC argues that parasympathetic innervation and influence over the heart are enhanced by IHC. The decrease in sinoatrial and atrial DOR labeling in these same low and high intensity fibers following IHC may result from stimulation induced trafficking [21] and redistribution of receptor sub-types, giving the appearance of a decrease in fiber innervation.

Although cholinergic protein expression is not uniformly increased following IHC, opioids may modulate innervation of cardiac tissues to enhance cardiac efficiency. IHC induced opioidergic activity may mediate the increase in VACHT+ parasympathetic innervation relative to TH+ sympathetic innervation in atrium and SA node. The increase in sinoatrial parasympathetic labeling, and its apparent increase relative to sympathetic labeling supports the hypothesis that the relative cholinergic influence over the heart following IHC may be augmented with respect to the falling adrenergic influence. Alternatively, the overall increase in parasympathetic VACHT intensity could represent accumulation of synaptic vesicles [53]. The activity dependent enrichment of VACHT positive within synaptic specializations [19, 57] following IHC could enhance acetylcholine transmission.
GM1 modulates an array of cellular functions including ion transport, neuronal differentiation, G protein coupled receptor signaling, neuroprotection, and immunosensitivity [30]. The finding that opioid exposure of cultured neuroblastoma cells increases endogenous GM1 concentrations and, thereby, augments calcium influx and axonogenesis in vitro [52] exemplifies the potent neurotrophic properties of GM1. IHC could induce parasympathetic neurite growth by inducing GM1 synthesis. Interestingly, in a feline model of GM1 gangliosidosis, acetylcholine synthesis is increased in brain slices, especially the cortex [27]. Furthermore, ChT-1 activity is promoted by the GM1 lipid raft environment in vitro and in forebrain nerve terminals [11]. Thus, the increase in GM1 positive parasympathetic fibers may enhance cholinergic activity through multiple hierarchical and inter-related mechanisms, consistent with the pleiotrophic nature of this molecule [30].

In conclusion, IHC improves the efficacy of vagal transmission which may result in part from increases in GM-1 and parasympathetic fibers. The enhanced vagal bradycardia following IHC is facilitated by increased parasympathetic influence and decreased adrenergic influence over the heart. Although overall DOR content fell and the resulting DOR-1/DOR-2 distribution remains unclear, the results are compatible with greater DOR-1 influence on the intermittent hypoxia conditioned heart. This functional improvement in vagal transmission may well result from the down-regulation of vagolytic DOR-2 influences exposing vagotonic DOR-1. Additional data will be required to increase confidence in the hypothesized DOR-2 downregulation. The hyperemic effect of peripheral preganglionic DOR-2 activity appears unchanged. Antagonism of DOR during IHC may elicit compensatory mechanisms that facilitate cholinergic transmission. In contrast, IHC appears to induce a cardioprotective autonomic phenotype that favors greater parasympathetic control of the heart. Furthermore, by applying a novel an immunohistochemistry structural analysis of cardiac autonomic nerve GM1, we demonstrate extensive colocalization of GM1 with parasympathetic innervation.
REFERENCES


9. Crain SM, Shen KF. After chronic opioid exposure sensory neurons become 
  supersensitive to the excitatory effects of opioid agonists and antagonists as occurs after 

    Simultaneous electrophoretic analysis of proteins of very high and low molecular mass 

11. Cuddy LK, Winick-Ng W, Rylett RJ. Regulation of the high-affinity choline transporter 
    activity and trafficking by its association with cholesterol rich lipid-rafts. J Neurochem 

12. Davis S, Deo SH, Barlow M, Yoshishige D, Farias M, Caffrey JL. The monosialosyl 
    ganglioside GM-1 reduces the vagolytic efficacy of delta2-opioid receptor stimulation. 

13. Deo SH, Barlow MA, Gonzalez L, Yoshishige D, Caffrey JL. Cholinergic location of 
    delta-opioid receptors in canine atria and SA node. Am J Physiol Heart Circ Physiol 

14. Deo SH, Barlow MA, Gonzalez L, Yoshishige D, Caffrey JL. Repeated arterial 
    occlusion, delta-opioid receptor (DOR) plasticity and vagal transmission within the 

15. Deo SH, Johnson-Davis S, Barlow MA, Yoshishige D, Caffrey JL. Repeated delta1- 
    opioid receptor stimulation reduces delta2-opioid receptor responses in the SA node. Am 

16. Farias M 3rd, Jackson K, Johnson M, Caffrey JL. Cardiac enkephalins attenuate 
    vagal bradycardia: interactions with NOS-1-cGMP systems in canine sinoatrial node. Am 


45. **Shen KF, Crain SM.** Cholera toxin-B subunit blocks excitatory effects of opioids on sensory neuron action potentials indicating that GM1 ganglioside may regulate Gs-linked opioid receptor functions. *Brain Res* 531:1-7, 1990.


Figure Legends

Figure 1: Overview of immunohistochemistry and data acquisition procedure for autonomic nerve fibers.

Figure 2. Assessment of vagal bradycardia and vagal-DOR interactions following IHC. The vagus was stimulated at 3 Hz and vagal bradycardia was induced in IHC and sham dogs. *P>0.05. Data are mean ± SEM; Sham, n=6; IHC, n=11; MEAP, Met-enkephalin-Arg(6) Phe(7); vehicle, saline.

Figure 3. Femoral arterial conductance (Panel A) and flow (Panel B) following IHC. Mean values ± SEM. Sham/control, n=6; IHC n=11; ME, met-enkephalin.

Figure 4. DOR and autonomic marker content in atria and left ventricular tissues. Panel A: representative immunoblots for sham, IHC, and IHC+N experiments for each target protein from atria and left ventricle lysates. Panels B-G: mean values ± SEM from atria (Panels B,D,F,H) and left ventricle (Panels C,E,G,I). Data are normalized to a sham animal and corrected for loading using β-tubulin. *P<0.05, **P<0.01; LV, left ventricle. Sham, n=3; IHC, n=7; IHC+N, n=2; 5-10μg of total protein were loaded onto each lane.

Figure 5. Colocalization of GM1 with cardiac autonomic nerve markers in SAN and atrial tissues. Dual immunolabelling in SAN and atrial tissues were used to determine the colocalization (yellow) of GM1 (CtxB, red channel) with autonomic nerve (synapsin, green channel), parasympathetic (VAChT, ChAT, ChT-1; green channel) sympathetic (TH, green channel), and DOR (green channel) positive nerve fibers. Nuclei were counterstained with DAPI (blue channel) in two experiments (Panels C and F).

Figure 6. Sample line scan intensities. CtxB-594 was used to illuminate GM1 positive nerve fibers (Panel A). A vertical line scan was taken over a region void of high intensity signal (arrow). A horizontal line scan was taken over a region passing through several nerve fibers (arrow head). The resulting line intensity scan from each sampled region are illustrated (Panels
C & D). The line scans are scaled up to facilitate viewing (Panels D & E). Scale Bars are 100 μm.

Figure 7. Line scan intensity analysis following image reconstruction using the Domes application and Morphology Plug-in for ImageJ. CtxB immunolabelling was used to detect GM1 (Panels A-D, 10X magnification) in an atrial section and immunofluorescence images were captured with an epifluorescence microscope. The image (Panel A) was converted into an 8-bit grey-scaled photomicrograph (Panel B). Data from the structural markers were extracted from photomicrographs and reconstructed using the Domes application (Panel C). The background fluorescence was effectively reduced. Immunofluorescence intensities were acquired from 3 horizontal and 3 vertical line scans (yellow lines in Panel C) equidistance apart. Data was imported into Microsoft Excel for arrangement into vertical and horizontal plots, then imported into MATLAB for further analysis. A representative 100μm line scan intensity profile demonstrates peak detection in MATLAB using the Bioinformatics Tool Kit (Panel D). Blue line represents original signal, green line denoised signal, and red marks peaks (Panel D). Scale bar: 200 μm.

Figure 8. Autonomic balance following IHC. Panels A-D: represent the ratio of total fibers >4 for parasympathetic (ChT-1, VAChT) vs. sympathetic (TH) markers. Autonomic balance appears to be enhanced following IHC in both atria and sinoatrial node tissues. Error bars, SEM. Sham, n=3 for atria and SAN; IHC, n=4 and n=5 for sinoatrial node and atria, respectively.
<table>
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Table 1. Antibody Dilution Table. CtxB-594, cholera toxin subunit B-594 conjugate; Syn, synapsin; ChAT, choline acetyltransferase; TH, tyrosine hydroxylase; DOR, delta opioid receptor; VAChT, vesicular acetylcholine transporter; ChT-1, choline transporter-1; β-tubulin-HRP, beta-tubulin horseradish peroxidase conjugate; RabMab, high affinity (K_d 10^{-10} – 10^{-13}) rabbit monoclonal antibody; RabPab, rabbit polyclonal antibody. Highly cross-absorbed goat anti-rabbit conjugated to Alexafluor 488 was used as the secondary antibody for all immunohistochemistry experiments. Goat anti-rabbit conjugated to horseradish peroxidase was used as the secondary antibody for all western blotting experiments.
Table 2. Comparison of fiber intensities between sham and IHC animals in the atria following line scan analysis of immunolabeled tissue sections. Data for analysis were collected from at least 5 photomicrographs each from different regions of two 12μm sections, at least 0.5 centimeter apart, and averaged together. Low and high intensity values were determined empirically for each target. Low intensity values are [5-9] and [5-14]. High intensity values are >4, >9, >14, and for DOR [5-14]. *P<0.05, relative to sham; **P<=0.01, relative to sham. Peaks per millimeter (Peaks / mm) correspond to fiber counts, ± SEM. Sham, n=3; IHC n=5, except for GM1 where n=4.
<table>
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<th>High Intensity Fibers (Peaks / mm)</th>
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<td>36.0 ± 1.2**</td>
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<td>VACHT [5-9]; &gt;4</td>
<td>31.5 ± 1.0</td>
<td>37.1 ± 1.1*</td>
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<tr>
<td>TH [5-9]; &gt;4</td>
<td>26.8 ± 4.0</td>
<td>28.2 ± 8.5</td>
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Table 3. Comparison of fiber intensities between sham and IHC animals in the SA node following line scan analysis of immunolabeled tissue sections. Low and high intensity values were determined empirically for each target. Due to the limited availability of SA nodal tissue, 3-5 photomicrographs from different regions of one 12μm section were used for the analysis. Low intensity values are [5-9]. High intensity values are >4, >19, and [5-14]. *P<0.05; **P<0.01. Peaks per millimeter (Peaks / mm) correspond to fiber counts, ± SEM. Sham, n=3; IHC, n=4.
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CHAPTER 4
SUMMARY & CONCLUSIONS

The results of this investigation suppose the following statements:

- IHC is dependent on DOR and ROS activity.
- IHC changes the cardiac phenotype by enhancing parasympathetic influence over the heart and decreasing adrenergic influence.
- IHC may enhance vagotonic-DOR1 receptors and vagal bradycardia, however the fate of vagolytic-DOR2 receptors remains uncertain.
- GM1 extensively colocalization with parasympathetic fibers.
- IHC increases GM1 positive fibers in the atria, and VACHT positive parasympathetic fibers in the SA node.
- IHC decreases DOR positive fibers in the SA node with parallel decreases in the atria, perhaps reflecting a redistribution of DOR1/DOR2 receptors.
- DOR antagonism during IHC results in compensatory increases in DOR and VACHT content.
- Collectively, IHC is dependent on opioidergic activity, which may interact with ROS, to induce a cardiac phenotype characterized in part by profound tolerance to ischemia and reperfusion injury, and enhanced autonomic balance.

Implications

IHC enhances parasympathetic influence over the heart, a beneficial adaptation that also occurs in response to exercise conditioning. As such, IHC may be an alternative conditioning strategy for persons who cannot exercise to reap similar cardiovascular benefits. Additionally, mechanistic insights gained by the current study will aid in the development of compounds to facilitate or repair IHC pathways. For example, pharmaceutical compounds such as LIGA-20
mimic the actions of GM1 and restore signaling in GM1 deficiencies associated with Parkinson’s disease [1], and could possibly be used to make IHC more efficacious for clinical settings.
REFERENCES

CHAPTER 5
FUTURE STUDIES

The following studies are proposed to determine with more certainty that IHC induces a change in the cardiac phenotype by repeated stimulation of DOR-1.

1. Include a sufficient number of IHC plus naltrindole animals to verify the results and strengthen the conclusionsof the study described in Chapter 3.
2. Determine if DOR-1 antagonism during IHC blocks cardioprotection and vagal bradycardia.
4. Determine the content of proteins that influence the formation of DOR-1 subtype, such as receptor transporting protein 4 [2].
5. Conduct a confocal analysis of autonomic nerves to confirm growth of parasympathetic nerves following IHC.
6. TH content decreased in the left ventricle following IHC. Determine β1- adrenergic receptor content in tissues. Adrenergic receptor content may increase to compensate for the reduction in TH, representing a beneficial adaption induced by IHC and DOR.
7. Determine how long IHC affords the degree of cardioprotection observed following 20 days, and whether there is coincident reversion of cholinergic and adrenergic protein profiles.

A series of studies should be performed to delineate the involvement of the proposed kinase pathways and the sequence in which these kinases may be potentially activated. These findings would give insight into the end effector proteins which IHC and DOR act upon, and which mediate the cardioprotection. Additionally, studies should be conducted to investigate the therapeutic potential of IHC when applied after [4], rather than in advance of, a coronary event.
One potential mechanism in this context would be the hypoxia-induced recruitment and infiltration of stem cells [3] to repair damaged myocardium. Additionally, these findings would lend support for the utilization of IHC in other contexts such as ischemic stroke, and for organ protection following cardiopulmonary arrest and resuscitation.
REFERENCES


APPENDIX

A1. GM1 IMMUNOHISTOCHEMISTRY AND NERVE FIBER ANALYSIS METHOD DEVELOPMENT

Recombinant cholera toxin subunit B (CtxB) binds with high affinity and specificity to GM1. CtxB is often utilized as a neural structural marker in conjunction with retrograde labeling techniques. However, to this investigators knowledge there are no reports of CtxB fluorescent conjugates being used to describe the distribution of GM1 within cardiac autonomic nerves in tissue sections. The lack of such reports likely is due to the technical problems with GM1 immunohistochemistry which this study addressed.

It is recommended that immunohistochemistry fixation methods for different gangliosides be optimized [6] and use of detergents be omitted [3]. Fixation with dry acetone at freezing temperature effectively preserves GM1 localization [5]. Additionally, anatomical analysis of cardiac tissue autonomic nerves in tissue sections can require tedious, labor-intensive manual counting of fibers [2]. Furthermore, cardiac tissue autofluorescence can produce a strong and heterogenous background. Manual background determination and subtraction often sacrifices positive fluorescence signal. An automated method to accurately count fibers is thus desirable and both labor- and cost-efficient. Immunofluorescent probes afford discrete labeling superior to that achieved by enzymatic methods, and are thus better suited for the fine analysis of nerve fibers and for the experimental reproducibility essential for this investigation. High affinity rabbit monoclonal antibodies (K\text{D} 10^{-10} to 10^{-13}) are ideal probes for quantitative immunohistochemistry in this respect and improve signal to noise ratio.

An image line scan intensity analysis method for automated analysis of nerve fibers [5] was modified to suit analysis of cardiac tissue. A standard epifluorescence microscope was used to capture images. Intensity data from greyscaled photomicrographs were extracted from nerve markers. The images were reconstructed with removal of background signal and camera noise.
using a specialized computer algorithm that extracts intensities from structural markers [4]. Differences between groups were determined empirically by organizing the data in discrete bins.

A limitation to this type of analysis is the assumption that intensity corresponds with the size of fibers. Indeed, intensities do correlate with fiber size when using confocal photomicrographs, but this correlation may not necessarily hold true for epifluorescence photomicrograph data due to light scattering. 12μm thick sections were used to minimize tissue autofluorescence and light scattering. Empirical examination of short line scans demonstrate that intensity appears to correlate well with fiber thickness of CtxB labeled nerves. Another limitation to this analysis is that image reconstruction algorithms are “specialized” and often work better for one set of data than another [1]. For example, the adopted analysis used an algorithm more suitable for “thick” sections, and resulted in skewed data in a relatively small proportion of line scans when applied to the CtxB labeled cardiac nerves. Empirical testing of various algorithms was only conducted on CtxB labeled nerves. It remains undetermined if other algorithms are better suited for the less abundant protein markers. However, the relative abundance of GM1 is an advantage when interpreting the data using the analysis developed here.
A1.2 Illustration of the effects of standard immunohistochemistry protocols on CtxB labeling. Standard buffers include detergents such as TX-100 to permeabilize tissues and facilitate the diffusion of probes. TX-100 extracted GM1 from 4% PFA fixed atria tissue sections and markedly decreased CtxB-594 fluorescence intensity at room temperature (A) and at 4°C (B). GM1 is effectively fixed in tissue sections with dry acetone at -20°C for 3 min. Other lipids are extracted by acetone and permeability of antibody probes with this procedure was satisfactory.
A1.3 IN SITU GM1 PRESERVATION PROCEDURE

1. Cool anhydrous acetone in air tight container @ -20°C for >30min
2. Set cryostat at -20°C
3. Cut OCT embedded tissue @ 12μm
4. Mount tissue sections onto gelatin coated slides
5. (Store slides in slide case wrapped in ziplock bag @ -80°C if necessary and allow case to return to room temperature before continuing to prevent moisture from accumulating on dried sections)
6. Remove OCT with tweezers
7. Place dry slide mounted sections into coplin jars with lids
8. Incubate sections with cold dry acetone @ -20°C for 3 min with lid closed
9. Air dry sections under air hood
10. Use pap-pen to create an incubation well for the section
11. Use PBS Plus buffer for diluting probes and as rinsing buffer [Burry et al]
12. Use PBS for diluting DAPI and as a wash buffer
13. Block sections with PBS Plus for 30 min @ 4°C
14. Incubate sections with diluted probes @ 4°C overnight (3 h when using CtxB-conjugate as the probe)
15. Rinse sections with cold PBS Plus on horizontal shaker 10 x 5 min each
16. Incubate sections with secondary probe for 3 h @ 4°C (proceed to PBS rinse step when using CtxB-conjugate as the probe)
17. Rinse sections with cold PBS Plus on horizontal shaker 4 x 5 min each
18. Incubate section with DAPI 1 min
19. Wash section with PBS 2 x 5 min each
20. Wash section with fluorescent mounting medium 1 x 5 min
21. Apply fluorescent mounting medium, coverslip, and seal with nail varnish

A1.4 PHOTOMICROGRAPH ACQUISITION

1. Optimize exposure times and camera settings to maximize signal to noise ratio
2. Use the 10X lens to capture images

A1.5 IMAGE RECONSTRUCTION

1. Convert to images to grayscale using ImageJ
2. Reconstruct images with the Domes application found in the ImageJ “Morphology” plug-in
3. Take the image with the highest intensity pixels and use this to set the peak value for reconstructing all images

A1.6 LINE SCAN DATA ACQUISITION
1. Set the scale to pixels/μm for selected image
2. Create a set of 3 horizontal and 3 vertical line scans equal distance apart and save in ROI manager (do not ‘Flatten’, close image and start line scans over if this button is pressed)
3. Apply the line scan set to the selected image
4. Be sure to press the ‘Deselect’ and ‘show all’ button to view all line scans
5. Press the ‘More’ tab and select ‘multiplot’
6. Copy and paste line scan XY output into Microsoft Excel
7. Discard all X output (line scan length) save one
8. Align all Y output (intensity data) into columns

A1.7 LINE SCAN INTENSITY ANALYSIS
1. Copy and paste line scan data from Excel into MATLAB
2. Name the variables (X and Y)
3. Use the “msbackadj” function from the MATLAB Bioinformatics Toolkit to apply a background correction, adjusting the algorithm to “preserve heights”
4. Use the “mspeaks” function to count peaks with the wavelet denoising option of the algorithm turned on, adjust threshold when necessary
5. Copy and paste the peak list output (vertical column) into Microsoft Excel
6. The number of peaks is the first number that appears in the “# peaks x 2” output
7. Use adjacent column to record the “#” value, this is the peak count for that particular line scan
8. Use Microsoft Excel or SigmaPlots to analyze the data
9. Peaks within a certain intensity range can be measured by binning the data
10. Apply statistics to data
REFERENCES


