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Hyperoxic and hyperbaric-induced cardioprotection: Role of nitric oxide synthase 3

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Abstract

Objective: The relative contributions of the fraction of inspired oxygen (F_IO_2) and atmospheric pressure (ATM) to cardioprotection are unknown. We determined whether the product of $F_IO_2 \times$ ATM (oxygen partial pressure) controls the extent of hyperoxic+hyperbaric-induced cardioprotection and involves activation of nitric oxide synthase (NOS).
Methods: Adult Sprague Dawley rats ($n=10$/gp) were treated for 1 h with (1) normoxia+normobaria (21% O_2 at 1 ATM), (2) hyperoxia+normobaria (100% O_2 at 1 ATM), (3) normoxia+hyperbaria (21% O_2 at 2 ATM) and (4) hyperoxia+hyperbaria (100% O_2 at 2 ATM).
Results: Infarct size following 25 min ischemia and 180 min reperfusion was decreased following hyperoxia+normobaria and normoxia+hyperbaria compared with normoxia+normobaria and further decreased following hyperoxia+hyperbaria treatment. L-NAME (200 μM) reversed the cardioprotective effects of hyperoxia+hyperbaria. Nitrite plus nitrate content was increased 2.2-fold in rats treated with normoxia+hyperbaria and hyperoxia+hyperbaria. NOS3 protein increased 1.2-fold and association of hsp90 with NOS3 four-fold in hyperoxic+hyperbaric rats.
Conclusions: Cardioprotection conferred by hyperoxia+hyperbaria is directly dependent on oxygen availability and mediated by NOS.

Keywords: Hyperbaric oxygen; Nitric oxide; Ischemia; Infarction

1. Introduction

Cardiovascular disease remains a major health care problem in the United States affecting the outcome of both cardiac and non-cardiac surgery. Pharmacological or physical preconditioning protects the heart against subsequent lethal ischemia. However, there are no current therapies that have proven to directly protect the heart against the deleterious effects of ischemia in humans. Therefore other strategies to protect the heart against ischemia that can be applied clinically need to be evaluated. Pretreatment of the heart with hyperbaria (increased atmosphere pressure) prior to ischemia limits post-ischemic infarct size [1]. In addition, pretreating hearts with hyperoxia (increased fraction of inspired oxygen) prior to ischemia improves post-ischemic cardiac function and reduces infarct size [2]. The product of F_IO_2 and atmospheric pressure determines the partial pressure of oxygen in a given volume as defined in the equation:

$$\text{Partial pressure of oxygen (ATM)} = F_IO_2 \times \text{atmospheric pressure (ATM)}.$$

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However the relative contribution of F_IO_2 and atmospheric pressure to cardioprotection and the underlying mechanisms are unknown.

Hyperbaric oxygenation (HBO) is a mainstay in the treatment of several conditions including carbon monoxide poisoning, decompression sickness and impaired oxygen delivery [3]. Other benefits of clinical HBO therapy include defibrillation, enhancement of the efficacy of various drugs used in conjunction with HBO therapy, reduction of anginal attacks, relief of dyspnea, lowered cholesterol levels, normalized ECGs, antiarrhythmic effects, stabilization of impaired fat metabolism and improvement of liver function [4,5]. However repeated exposure to hyperbaria+hyperoxia (5 ATM at 100% O_2) increases sensitivity to seizures in rats [6]. Nitric oxide (·NO) may be responsible for the signaling mechanism involved in brain toxicity resulting from treatment with hyperbaria+hyperoxia because NOS inhibitors are able to delay the onset of seizures. Elevations in oxygen tension at atmospheric pressures between 2.0 and 2.5 above ambient air also result in an increase in ·NO production by pulmonary endothelial cells, lungs and the cerebral cortex. Angiogenesis and downregulation of intercellular adhesion molecule (ICAM) expression caused by hyperbaric oxygen treatment have been attributed to changes in ·NO concentration [7]. In human umbilical vein endothelial cells and bovine aortic endothelial cells, HBO treatment for 90 min following hypoxia/hypoglycemia abolished ICAM protein expression, polymorphonuclear adhesion and increased ·NO production [7]. Hyperoxic-induced cardioprotection is mediated in part through a nuclear factor κB pathway [8] which may activate nitric oxide synthase (NOS).

Several studies indicate that ·NO plays a central role in cardioprotection [9,10]. Regulation of ·NO production from NOS occurs by multiple mechanisms including interaction with heat shock protein 90 (hsp90). Increases in hsp90 association with NOS3 play a critical role in increasing cardioprotection [11]. In rat cerebral cortex exposed to 2.8 ATM an elevation in ·NO concentration occurred after 45 min of exposure. The amount of NOS protein in brain remained unchanged but the association of NOS1 with hsp90 was increased two-fold [12]. Taken together these studies suggest hyperbaria increases ·NO production which then plays a role in ischemia/reperfusion injury in major organ systems. Heme oxygenase-1 (HO-1) belongs to the heat shock protein family. HO-1 converts heme into bilirubin iron and carbon monoxide, and is known to have cell-protective properties [13,14]. Therefore hyperoxia+hyperbaria may activate HO-1 and confer cardioprotection. However the role of NOS–hsp90 and HO-1 in mediating hyperbaric- and hyperoxic-induced cardioprotection at pressures that do not result in seizures is unknown. We hypothesized that the partial pressure of oxygen determines the extent of immediate cardioprotection with increased resistance to ischemia mediated by nitric oxide synthase and heme oxygenase.

The objectives of our study were to determine (i) whether the product of F_IO_2 and ATM determine the extent of hyperoxic- and hyperbaric-induced cardioprotection and (ii) the role of NOS and HO-1 in the mechanism by which hyperoxia and hyperbaria confers cardioprotection.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats, *Rattus norvegicus*, at 8–9 weeks of age maintained on a customized 3075S rodent diet (TEKLAD, Wilmington, DE) were used in these studies. The animals were kept on a 12 h light/dark cycle. All animal work was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Institutional Animal Care and Use at the Medical College of Wisconsin.

2.2. Cardioprotection with hyperoxia+hyperbaria

We performed the following experiments in random order to investigate whether the product of F_IO_2 and atmospheric pressure determines the extent of cardioprotection. Rats were exposed to hyperoxia (100% O_2)+normobaria (1 ATM), normoxia (21% O_2)+hyperbaria (2 ATM) or hyperoxia (100% O_2)+hyperbaria (2 ATM) for 1 h. Rats exposed to normoxia (21% O_2)+normobaria (1 ATM) for 1 h served as untreated controls. The animals were then allowed to recover in room air for 10 min. Resistance to myocardial ischemia was then determined using an isolated perfused heart model [15].

2.2.1. Normoxia+normobaria treatment (21% O_2, 1 ATM)

All animals were placed inside the experimental hyperbaric chamber at the Medical College of Wisconsin (756.6 L volume capacity). The interior of the chamber was illuminated to maintain rats in the light portion of the light/dark cycle. Atmospheric pressure measurements were obtained each day from the National Weather Service for the city of Milwaukee, WI and the oxygen concentration of room air was measured using an oxygen sensor located in the exhaust valve of the hyperbaric chamber. Carbon dioxide levels in the chamber were measured using a catheter placed inside the hyperbaric chamber and exteriorized for measurement using a POET gas analyzer. Control animals underwent sham treatment for 1 h in the chamber by remaining at ambient atmospheric pressure and oxygen concentration. The amount of time spent in the chamber for the control animals was the same as that for the paired treated animals. Following sham treatment animals were then removed from the chamber for a 10-min recovery period. During this recovery period, heparin (150 U/kg) and sodium pentobarbital (50 mg/kg) were administered intraperitoneally. The chest was then opened by a median sternotomy, the heart excised and perfused.

2.2.2. Hyperoxia+normobaria treatment (100% O_2, 1 ATM)

Rats were placed in the hyperbaric chamber. Once sealed, 750 L of 100% oxygen was added over a 6-min period with

the exhaust valve kept open to prevent any increases in chamber pressure within the chamber. After this volume was added, a final measurement of the oxygen concentration was taken to confirm hyperoxic conditions were present. All intake and exhaust valves were then closed and environmental conditions maintained for 60 min. After 66 min the door to the chamber was then opened and the animals were allowed to recover for 10 min.

2.2.3. Normoxia + hyperbaria treatment (21% O_2, 2 ATM)

Rats were placed in the hyperbaric chamber and an additional 750 L of 21% oxygen was introduced into the chamber over a 2-min period to raise atmospheric pressure to 2 ATM. This elevated pressure was confirmed by the pressure gauge located outside the hyperbaric chamber. The intake valve was sealed with atmospheric pressure monitored every 15 min for 1 h. When the pressure fell below 1.95 ATM, additional oxygen was added to the chamber *via* the intake valve to restore atmospheric pressure to 2 ATM. After 66 min, the chamber was decompressed to a pressure of 1.45 ATM over 35 s. This pressure was maintained for 3 min. A final decompression to atmospheric pressure was performed over an additional 35 s so that the total decompression time equaled 4 min and 10 s. The chamber door was opened and the rats were allowed to recover for 10 min before excision of the heart.

2.2.4. Hyperoxia + hyperbaria treatment (100% F_IO_2, 2 ATM)

Rats were placed in the hyperbaric chamber and 750 L of 100% oxygen was introduced into the chamber over a 2-min period to raise atmospheric pressure to 2 ATM. Rats were then treated in an identical manner to that described for the normoxia + hyperbaria group.

2.3. Isolated heart perfusion

Isolated rat hearts were perfused retrogradely with bicarbonate buffer at constant perfusion pressure (98 mmHg) with a balloon inflated in the left ventricle. End-diastolic pressure was set to 5 mmHg and developed pressure was recorded during steady-state conditions [15]. Hearts were allowed to beat spontaneously. Hearts were kept in temperature-controlled chambers to maintain myocardial temperature at 37 °C during periods of perfusion and ischemia.

2.4. Infarct size determination

Following 3 h of reperfusion, 1.0% (w/v) 2,3,5 Triphenyl-Tetrazolium Chloride (TTC) was delivered to the isolated heart *via* a syringe attached to the 3-way stopcock. TTC was administered at a rate of 1 ml/min for 10 min. The left ventricle was then isolated and sliced into 1 mm cross-sectional segments. The tissues were then incubated for 15 min in a 1% TTC solution in 100 mM phosphate buffer (pH 7.4) at 37°C. Tissues were then stored in 10% formaldehyde overnight and the extent infarcted myocardium was measured. The infarcted tissue was then separated from the non-infarcted tissue and measured as a percentage (% Infarct = Infarct Area / Area at Risk) × 100 using a fluorescent, digital, color camera with a

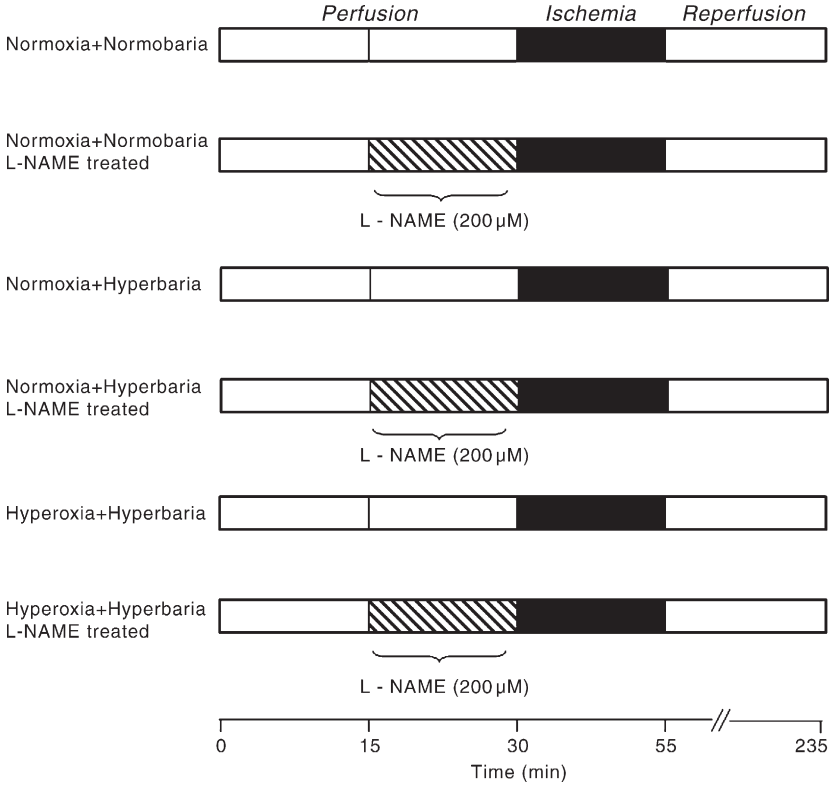

Fig. 1. Experimental protocol used to study the role of nitric oxide synthase in acute cardioprotection by hyperbaria plus hyperoxia.

55 mm lens (Nikon). Imaging analysis software (Imaging Research, St. Catharines, ON) was programmed to recognize infarcted tissue (scan area) in proportion to entire heart (total target area) (Fig. 2) [15].

2.5. Role of NOS

To determine whether cardioprotection induced by hyperoxia+hyperbaria is mediated by ·NO, isolated hearts from rats treated with normoxia+normobaria, normoxia+hyperbaria and hyperbaria+hyperoxia were perfused with L-NAME, a general NOS inhibitor (200 μM) for 15 min prior to 25 min global ischemia and 3 h reperfusion. Resistance to myocardial ischemia was then determined. The experimental protocol is shown in Fig. 1.

2.6. Generation of nitrite plus nitrate

Hearts from rats treated with normoxia+normobaria, normoxia+hyperbaria and hyperoxia+hyperbaria were perfused with bicarbonate buffer for 15 min. Nitrite and nitrate content of the coronary effluent and heart tissue were determined as previously described using an ozone chemiluminescence method [15,16].

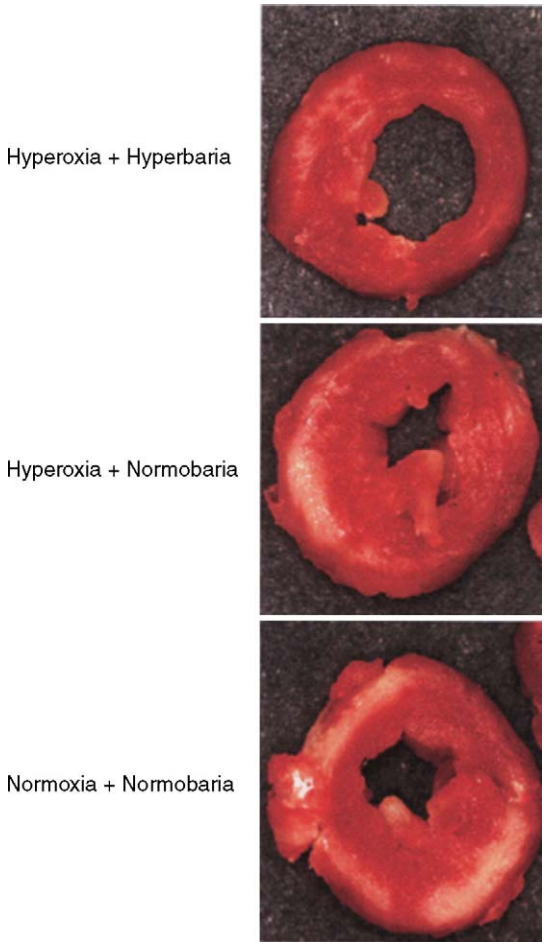

Fig. 2. Infarct size in hearts from rats treated with hyperoxia+hyperbaria, hyperoxia+normobaria, or normoxia+normobaria. Viable tissue stains dark red when triphenyltetrazolium chloride reacts with intracellular dehydrogenases to form an insoluble red formazan dye. Infarcted tissue remains pale due to lack of staining of viable tissue.

2.7. Western analysis and immunoprecipitation studies

Western analysis and immunoprecipitation of heart homogenates from rats treated with normoxia+normobaria and hyperoxia+hyperbaria for NOS1, NOS2, NOS3, hsp90 and HO-1 were performed using the method we described previously [11]. Antibodies used were polyclonal rabbit NOS1, NOS2 and NOS3 (Santa Cruz Biotechnology, Cat #SC-648, Cat #SC-651 and Cat #SC-654, monoclonal hsp90 (BD Transduction, Cat #610419) and monoclonal HO-1 (Stressgen, Cat #OSA110), respectively. For the immunoprecipitation studies we used an H32 NOS3 monoclonal antibody raised in mouse (Biomol, Plymouth Meeting, PA, Cat #SA246). The secondary antibody used for NOS3 was goat anti-rabbit IgG Horseradish peroxidase conjugate (Bio-Rad Laboratories, Cat #170-6515). The secondary antibody used for hsp90 was goat anti-mouse IgG Horseradish peroxidase conjugate (Sigma, Cat #A-8924).

2.8. Statistical analysis

Statistical analysis was performed by use of repeated-measures analysis of variance with the Greenhouse–Geisser adjustment used to correct for the inflated risk of a type I error. If significant, the Mann–Whitney test was used as a second step to confirm which groups were significantly different [15]. Significance was set at $p<0.05$.

3. Results

3.1. Oxygen and cardioprotection studies

To investigate whether the product of F_IO_2 and ATM determines the extent of hyperbaric- and hyperoxic-induced cardioprotection, rats were treated for 1 h with (1) normoxia+normobaria (21% O_2 at 1 ATM), (2) hyperoxia+normobaria (100% O_2 at 1 ATM), (3) normoxia+hyperbaria (21% O_2 at 2 ATM) or (4) hyperoxia+hyperbaria (100% O_2 at 2 ATM). Following the addition of oxygen to the hyperbaric chamber, carbon dioxide levels fell from 0.03% to levels that were undetectable. Over the subsequent 60 min treatment period, carbon dioxide levels rose to a maximum value of 0.001%. We did not observe seizures in any of the four experimental groups. Immediately following treatment and recovery the heart was excised and perfused. Aerobic cardiac function prior to ischemia was unaffected by treatment with hyperoxia+normobaria, normoxia+hyperbaria and by hyperoxia+hyperbaria compared with normoxia+normobaria (heart rate=260±10 beats/min; coronary flow rate=12±2 ml/min/g; left ventricular developed pressure=118±20 mmHg). Thus there were no changes in pre-ischemic function as a consequence of

hyperoxic/hyperbaric treatment that were predictive of post-ischemic recovery. Hearts were then subjected to 25 min global no flow ischemia followed by 3 h of reperfusion. Recovery of left ventricular developed pressure (LVDP) and cumulate lactate dehydrogenase leakage over the first hour of reperfusion and infarct size/area at risk (% of left ventricle) at 3 h reperfusion was used to assess resistance to ischemia. Increasing the product of $F_IO_2 \times$ ATM conferred cardioprotection in a linear manner. In untreated hearts (normoxia+normobaria), infarct size (mean±SD, %LV) was 13±2% and percent recovery of LVDP was 34±7%. Treatment with hyperoxia+normobaria reduced infarct size by 31% to 9±2% and increased recovery of LVDP to 40±11% (Figs. 2 and 3). Treatment with normoxia+hyperbaria reduced infarct size by 46% to 7±3% and increased recovery of LVDP to 38±4% (Fig. 3). Treatment with hyperoxia+hyperbaria further reduced infarct size by 62% to 5±2% and increased recovery of LVDP to 45±8% (Figs. 2 and 3) compared with untreated rats. Coronary flow was re-established within 10–15 s of reperfusion and recovered to 80–90% of pre-ischemic values in all experimental groups. We then subjected hearts to 45 min of ischemia to determine if hyperoxic+hyperbaric treatment protects the heart against injury from a more severe ischemic insult. In normoxic+normobaric treated rats, infarct size and recovery of LVDP were 56±4% and 15±5%, respectively. Hyperoxic+hyperbaric treatment decreased infarct size to 46±4% and increased recovery of LVDP to 24±4%. Our results suggest the product of F_IO_2 and ATM determines the extent of cardioprotection.

Hyperbaric+hyperoxic and normobaric+normoxic treated rats subjected to 25 min of myocardial ischemia demonstrated a low level of leakage of lactate dehydrogenase prior to ischemia (0.20±0.06 IU/g and 0.15±0.3 IU/g, respectively). Post-ischemic enzyme leakage was increased

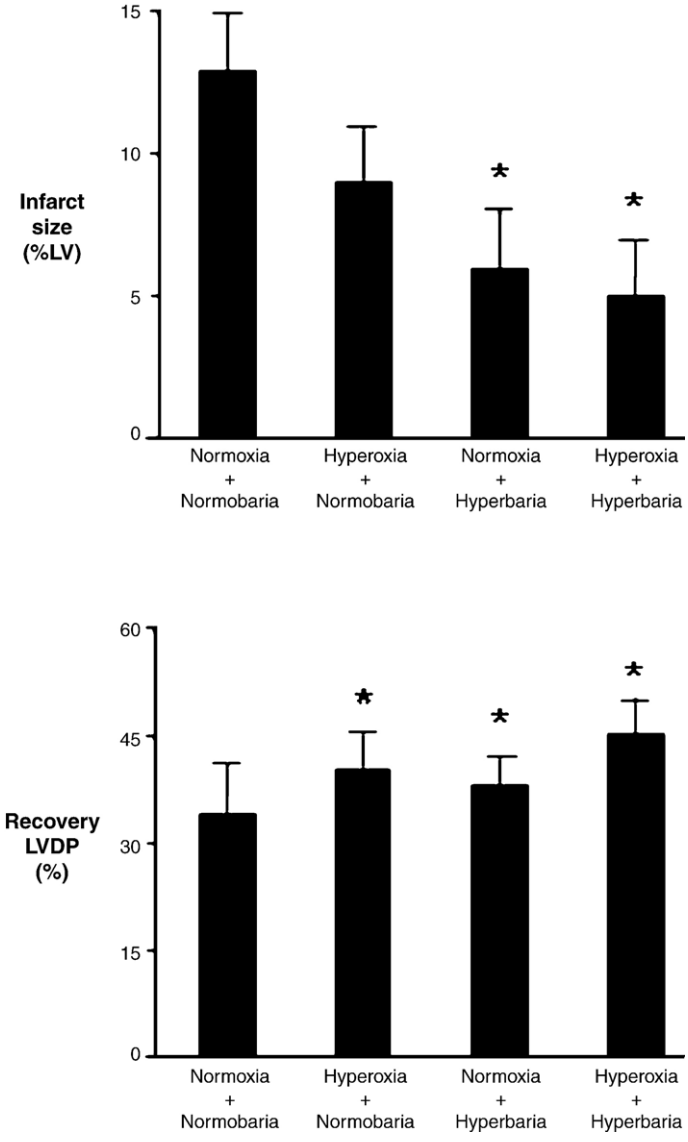

Fig. 3. Acute cardioprotection conferred by hypoxia and hyperbaria. Data are mean±SD, $n=10$ hearts/group. *$p<0.05$, vs normoxia+normobaria.

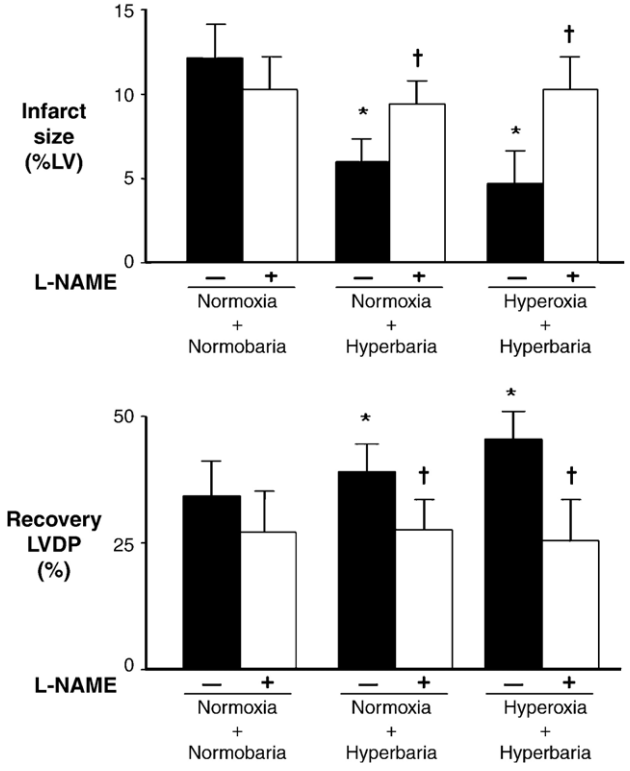

Fig. 4. Nitric oxide generation and acute cardioprotection conferred by hyperoxia+hyperbaria. Data are mean±SD, $n=10$ hearts/group. L-NAME (200 μM). *$p<0.05$, vs normoxia+normobaria, †$p<0.05$, with L-NAME vs without L-NAME.

in both hyperbaric+hyperoxic and normobaric+normoxic groups (5.1±1.3 IU/g and 5.9±1±7 IU/g, respectively) compared with pre-ischemic values. However there were no differences in post-ischemic enzyme leakage between treatment groups.

3.2. Role of NOS in oxygen-induced cardioprotection

To determine whether enhanced ·NO production from NOS is responsible for increased resistance to ischemia in hearts from animals treated with normoxia+hyperbaria and hyperoxia+hyperbaria, isolated hearts were perfused with a general NOS inhibitor (L-NAME, 200 μM) for 15 min prior to 25 min global ischemia. L-NAME decreased coronary flow rate from 13±2 ml/min/g to 9±2 ml/min/g in hearts treated with normoxia+hyperbaria and from 12±2 ml/min/g to 9±2 ml/min/g in hearts treated with hyperoxia+hyperbaria. Hearts were reperfused for 3 h. L-NAME increased infarct size in hearts from rats treated with normoxia+hyperbaria (6±2%) and hyperoxia+hyperbaria (11±2%) to that observed in hearts from untreated (normoxia+normobaria) rats (13±2%), but did not alter infarct size in hearts from normoxic+normobaric rats (11±2%) (Fig. 4). L-NAME decreased recovery of LVDP in hearts from rats treated with hyperoxia+hyperbaria (25±8%) and normoxia+hyperbaria (26±6%) but had no effect on hearts from rats treated with normoxia+normobaria (27±4%) (Fig. 4). Thus resistance to ischemia in hearts from rats treated with normoxia+hyperbaria and hyperoxia+hyperbaria is decreased by inhibition of NOS to levels present in hearts from untreated (normoxia+normobaria) rats.

3.3. Nitrite plus nitrate production

The data from the NOS inhibition studies suggest that hearts from rats treated with normoxia+hyperbaria and hyperoxia+hyperbaria produce more ·NO than hearts from untreated rats (normoxia+normobaria). To determine the extent to which normoxia+hyperbaria and hyperoxic+hyperbaric treatment increased ·NO production, hearts were analyzed for nitrite plus nitrate content and nitrite plus nitrate leakage [15]. Nitrite plus nitrate levels in the coronary perfusates were approximately 0.2 μM. We found that in hearts previously treated with normoxia+hyperbaria and hyperoxia+hyperbaria tissue levels for nitrite plus nitrate, and leakage of nitrite plus nitrate from the heart were increased 2.2- and 2.3-fold respectively compared with normoxic+normobaric treated hearts (Fig. 5). Our observation suggests that nitrite plus nitrate production by the heart is increased more than 2-fold in rats treated with normoxia+hyperbaria and hyperoxia+hyperbaria.

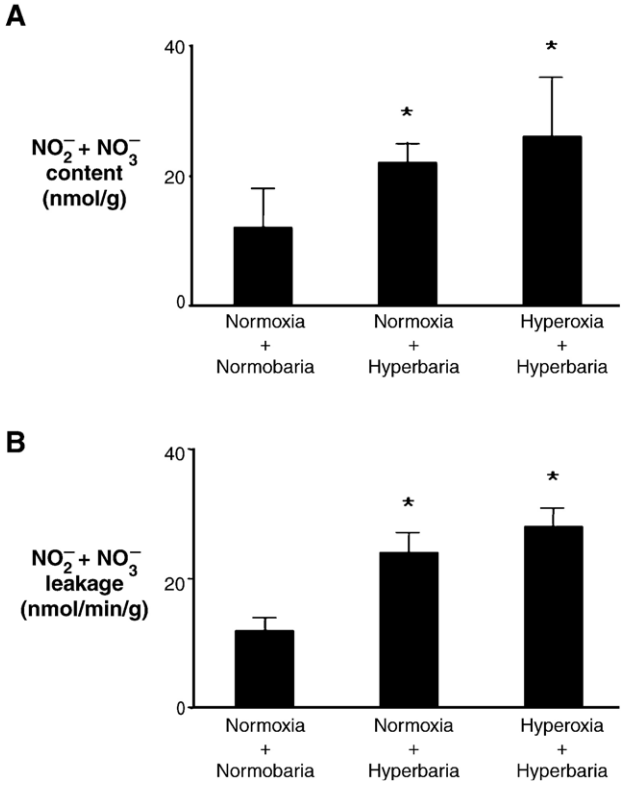

Fig. 5. Hyperoxia+hyperbaria induced $NO_2^-+NO_3^-$ formation in heart. Panel A shows $NO_2^-+NO_3^-$ content in heart tissue. Panel B shows $NO_2^-+NO_3^-$ leakage from heart into coronary effluent. Data are mean±SD, $n=8$ hearts/group. *$p<0.05$, normoxia+normobaria vs hyperoxia+hyperbaria.

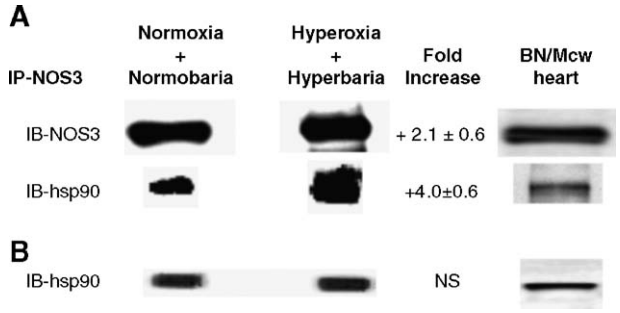

Fig. 6. Western analysis of NOS3 and hsp90 expression and association in heart homogenates from rats treated with normoxia+normobaria and hyperoxia+hyperbaria. (A) Western analysis for NOS3 content and hsp90 associated with NOS3 by immunoprecipitating NOS3 and probing for NOS3 and hsp90. (B) Western analysis for hsp90 content in homogenates of hearts from rats treated with normoxia+normobaria and hyperoxia+hyperbaria. IP, immunoprecipitation; and IB, immunoblot. Heart from BN/Mcw rat used as positive controls. N=4–6/group.

3.4. Protein levels for NOS1, NOS2 and NOS3

To determine whether increased nitrite plus nitrate production in hearts treated with hyperoxia+hyperbaria was related to increased expression of NOS, we examined protein levels for NOS1, NOS2 and NOS3. Using standard Western analysis, the only NOS isoform we were able to detect was NOS3. NOS1 and NOS2 could not be detected by Western analysis. Hearts from BN/Mcw rats were used as positive controls for the Western analysis for NOS3. Treatment of rats with hyperoxia+hyperbaria increased NOS3 protein expression by 22% compared with untreated (normoxia+normobaria) rat hearts (Fig. 6A). Our data suggest that the increase in ·NO production in hearts treated with hyperoxia+hyperbaria was due to increased NOS3 protein expression.

3.5. Hsp90 and its association with NOS3 protein

·NO production from NOS3 may be regulated by a protein–protein interaction. Since hsp90 increases ·NO generation from NOS3 [17], we then determined whether increased resistance to ischemia in hearts treated with hyperoxia+hyperbaria was related to the extent of hsp90 association with NOS3 in comparison with hearts from normoxic+normobaric-treated rats. The extent of hsp90 association with NOS3 was measured immediately prior to the onset of ischemia. Hyperoxia+hyperbaria increased the association of hsp90 with NOS3 compared with normoxic+normobaric hearts by 4-fold (Fig. 6A). To determine if the increase in association of hsp90 with NOS3 was due to changes in hsp90 content, Western analysis of hsp90 in total heart homogenate was performed. Hyperoxic+hyperbaric treatment did not change the total content of hsp90 in the heart (Fig. 6B). This increased association of hsp90 with NOS3 supports the notion that hsp90 helps NOS3 produce ·NO, which is responsible for the observed cardioprotection against ischemia/reperfusion injury seen in hyperoxic+hyperbaric treated rats.

3.6. Protein levels for HO-1

The protein content of HO-1 in the heart was determined by SDS-PAGE and standard Western analysis using a monoclonal antibody specific for the protein. Purified rat HO-1 protein (Stressgen, SPP730) was used as a positive control. We did not detect the expression of HO-1 protein in either experimental group (data not shown). Thus HO-1 does not appear to play a role in cardioprotection conferred by hyperoxia+hyperbaria in our model.

4. Discussion

Our study demonstrates that the product of $F_IO_2 \times$ ATM determines the extent of cardioprotection conferred by elevation of the partial pressure of oxygen. Furthermore increased resistance to myocardial ischemia immediately conferred by hyperoxic+hyperbaric treatment is mediated by a mechanism involving increased ·NO production from NOS3.

During hyperoxic+hyperbaric treatment, the vascular supply of oxygen to the heart exceeds demand. Upon withdrawal from the hyperoxic+hyperbaric environment pO_2 reverts to normoxic+normobaric levels immediately. Increased resistance to myocardial ischemia is observed immediately after treatment with hyperoxia+hyperbaria, suggesting that induction of new genes is not necessary for the cardioprotective effects of hyperoxia+hyperbaria to be manifested. Indeed HO-1 was not induced by treatment with hyperoxia+hyperbaria. Therefore the myocardium must utilize existing proteins as the protective mechanism in its response to the increased partial pressure of oxygen. Our results indicate that the heart appears to adapt to short-term increases in oxygen availability by a mechanism involving an increased protein–protein interaction of hsp90 with NOS3 to increase ·NO levels, which confers increased resistance to ischemia. Increased cardioprotection could not be attributed to elevated levels of carbon dioxide as the concentration of this gas did not exceed 0.001% during treatment of rats with hyperoxia. Our findings may enhance understanding of the cardioprotective mechanisms resulting from adaptation to hyperbaria+hyperoxia in the adult heart, and may lead to the development of new strategies to protect hearts of patients undergoing surgical repair for acquired heart disease.

Our results confirm and extend previous studies of exposure of rats and mice to increased F_IO_2 to decrease infarct size and increase recovery of developed pressure following comparable periods of global ischemia in the isolated perfused heart model [2,18,19]. In a study of the response to hyperoxic exposure between rats and mice [19], 1 h of exposure to 80% or >95% oxygen resulted in greater cardioprotection as manifested by increased recovery of LVDP and a reduction of infarct size in the rat. Even 24 h after the hyperoxic exposure, isolated rat hearts still displayed the beneficial effects of treatment. In contrast, cardioprotection in the mouse occurred after a shorter exposure time and yielded reduced beneficial effects in infarct size (35% vs 51% in rats) when compared

with the rat model [19]. In general, tolerance to hyperoxia is species-dependent with smaller animals less resistant to this environmental stress, which may partly explain the varying cardioprotective effects. Hyperoxia alone is an attractive mode of treatment for patients with ischemic heart disease in that breathing hyperoxic gas produces the same result as ischemic pre-conditioning without subjecting the patient to transient ischemic conditions, which may cause additive injury.

Our study demonstrates that hyperoxia (100% O_2) alone confers cardioprotection as evidenced by a 31% reduction in post-ischemic infarct size and an 18% increase in recovery of LVDP. Furthermore the combination of hyperoxia+hyperbaria (2.0 ATM) confers increased cardioprotection with a further reduction in infarct size by 62% and an increase in recovery of LVDP by 32%. However there were no differences in LDH leakage between treated and untreated groups. Reperfusion of the heart after global ischemia leads to areas of "no reflow." This may underestimate the extent of enzyme leakage from irreversibly injured myocardium to account for the absence of differences between groups. The underlying mechanism appears to involve ·NO as its oxidation product (nitrite plus nitrate) is increased 2-fold after hyperoxic+hyperbaric oxygen treatment compared with normoxic+normobaric controls. Also when L-NAME (a general NOS inhibitor) was administered, the observed cardioprotective effects following treatment with hyperoxia+hyperbaria were lost. In addition, NOS3 expression and NOS3 association with hsp90 was increased after 1 h treatment with hyperoxia+hyperbaria. We suggest the mechanism for cardioprotection conferred by hyperoxic+hyperbaric treatment might involve an increase in NOS3 protein expression, which results in increased ·NO levels in the heart to confer protection against subsequent ischemia/reperfusion injury. ·NO has to interact with a cellular target for its physiological effect to be manifested. The heme site of guanylyl cyclase is one such target for ·NO and this interaction results in the conversion of guanosine-5′-triphosphate to the intracellular second messenger, cyclic guanosine-3′, 5′-monophosphate (cGMP). Soluble guanylyl cyclase plays a pivotal role in the transduction of intercellular messages conveyed by ·NO. ATP-dependent potassium (K_{ATP}) channels are important mediators of protection against ischemia–reperfusion injury in myocardium [20]. Thus hyperoxia+hyperbaria-induced increases in ·NO levels may activate K_{ATP} channels by a cGMP-dependent mechanism [21] to confer cardioprotection.

Under conditions of normoxia+normobaria, oxygen supply to the heart equals oxygen demand. We previously showed that under conditions of normobaric hypoxia, where oxygen supply becomes limiting despite increases in hematocrit to increase oxygen delivery, the heart increases production of ·NO from NOS3 through increased association with hsp90, which confers increased resistance to ischemia [11]. Our current study reveals that under conditions of hyperoxia+hyperbaria, the heart also increases production of ·NO from NOS3 by increasing NOS3 association with hsp90 to confer increased resistance to subsequent myocardial ischemia. Taken together our studies suggest that NOS3 in the heart responds to changes in oxygen availability as an adaptive response that confers protection against a subsequent ischemic insult. Increased protein–protein association between hsp90 and NOS3 may represent a universal mechanism for controlling ·NO production from NOS3.

Thom and colleagues showed that hyperbaria causes an increase in ·NO synthesis in brain and abdominal great vessels as a stress response mechanism [12,22]. Badr and associates [23] also reported that hyperbaric oxygen therapy within 6 h of reperfusion injury exerts a protective effect on rats with cerebral infarction. Our study extends these two previous observations to the heart and suggests a role for NOS in mediating cardioprotection conferred by hyperoxia+hyperbaria. Furthermore, our study suggests that an elevation in the partial pressure of oxygen increases the association of hsp90 with NOS3 in the heart. Our findings contrast with the studies in the brain by Thom et al. [12] who demonstrated that the association between hsp90 and NOS1 was increased in brain following an elevation in the partial pressure of oxygen. However hyperbaria did not change the association of NOS3 with hsp90. We did not observe a role for hsp90 association with NOS1 in heart following hyperoxia+hyperbaria treatment.

Cardioprotection conferred by increased partial pressure of oxygen suggests this diatomic molecule acts more as a pharmaceutical rather than a support for cellular metabolism. Indeed under high pressure, oxygen has been suggested to behave as a drug [3]. Coronary artery bypass surgery and coronary angioplasty remain as clinical treatments to treat myocardial ischemia. The ability to pharmacologically precondition the heart against ischemia with diatomic oxygen may offer a more practical way of harnessing protective mechanisms endogenous to the heart. Oxygen is already used in humans, so safety and toxicity profiles are established. In patients with angina pectoris, treatment with HBO and tissue plasminogen activator (TPA) alleviated myocardial infarct symptoms 6.5 h faster than in patients who received TPA alone [5]. These patients also reported relief from chest pain within 10 min of exposure to hyperbaria. The underlying mechanism of action may be due to increased oxygen dissolved in the plasma, which helps the damaged heart in oxygenating the body, while at the same time promotes increased collateral circulation. The potential benefit afforded to patients undergoing bypass surgery and transplantation seems to be logical and feasible since the donor or recipient hearts are available for HBO treatment prior to these surgical procedures.

As delineated by Kindwall [3] and the Undersea and Hyperbaric Medical Society website [24], there exist a variety of clinical applications for HBO therapy. However in many cases HBO therapy remains clinically marginalized because of the lack of large prospective randomized outcome studies and convincing basic science to support the subcellular mechanism of action. In particular, cardiac surgery has not been an area known to take advantage of HBO therapy. By

delineating the physiology, biochemistry and subcellular mechanism of the cardioprotective actions of HBO therapy in the rat heart, a natural extension of our work would eventually be a clinical application in the arena of cardiac surgery in humans.

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