Acute and conditioned hypoxic tolerance augmented by endothelial nitric oxide synthase inhibition in mice

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1College of Literature, Science and the Arts, University of Michigan, Ann Arbor, Michigan; 2Department of Biology, Dickinson College, Carlisle, Pennsylvania; 3Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor; and 4Department of Surgery, William Beaumont Hospital, Royal Oak, Michigan

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Song MY, Zwemer CF, Whitesall SE, D’Aleyc LG. Acute and conditioned hypoxic tolerance augmented by endothelial nitric oxide synthase inhibition in mice. J Appl Physiol 102: 610–615, 2007. First published October 26, 2006; doi:10.1152/japplphysiol.00894.2006.—To identify a possible role for nitric oxide (NO) in acute hypoxic tolerance (HT) we measured hypoxic survival time (HST), effect of hypoxic conditioning (HC), and survival following hypoxic conditioning while blocking or mimicking the action of nitric oxide synthase (NOS). To inhibit NOS, CD-1 mice were given supplemental endogenous NOS inhibitor asymmetrical dimethylarginine (ADMA) or a synthetic NOS inhibitor Nω-nitro-L-arginine (L-NNA), both of which nonselectively inhibit three of the isoforms of NOS [inducible (iNOS), neuronal (nNOS), and endothelial NOS (eNOS)]. ADMA (10 mg/kg ip) or saline vehicle was given 5 min before HST testing. L-NNA was given orally at 1 g/l in drinking water with tap water as the control for 48 h before testing. Both ADMA and L-NNA significantly increased HST and augmented the HC effect on HT. Neither the nNOS selective inhibitor 7-nitroindazole (7-NI) nor the iNOS selective inhibitor N-[3-(aminomethyl)phenyl][methyl]-enthanimidamide (1400W) had a statistically significant effect on HST or HT. The NO donor, 3-morpholinosydnoeimine, when given alone did not significantly decrease HT, but it did mitigate the increased HT effect of L-NNA. These data confirm that acute NOS inhibition and the decreased ability to compensate during severe acute hypoxic hypoxia (34–37). To identify a possible role for NO in HT, we measured HST, HC, and survival following hypoxic conditioning while blocking or mimicking the action of NOS.

Currently, there are four known isoforms of NOS, each of which exists as homodimers with molecular masses between 130 and 160 kDa (38). The three best-characterized isoforms are neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (38). A newly discovered, albeit controversial, fourth isoform is mitochondrial NOS (mtNOS) (4, 51). To inhibit NOS, CD-1 mice were given the naturally occurring endogenous NOS inhibitor asymmetrical dimethylarginine (ADMA) or a synthetic NOS inhibitor Nω-nitro-L-arginine (L-NNA), both of which have been reported to nonselectively inhibit all three primary isoforms of NOS. To help mechanistically define the response, we used relatively selective NOS inhibitors to determine which isoform, or isoforms, of NOS were responsible for any changes in HT observed in response to ADMA and L-NNA treatments. In addition, we used a NO donor to determine whether we could reverse the effects we had observed with NOS inhibitors.

We refuted our initial hypothesis, and the data strongly supported the exact opposite in that NOS inhibition caused a marked and statistically significant increase in HT and that the response is apparently due to inhibition of eNOS.

MATERIALS AND METHODS

Adult male mice (Mus musculus, Swiss CD-1), weighing 25–31 g, were hypoxically or sham conditioned (SC) or treated, and then they were tested to determine HST. All animals were housed five per cage in a temperature-controlled environment with an artificial light-dark cycle. All experiments were conducted during daylight hours between 9:00 AM and 5:00 PM. The University of Michigan Committee for Animal Care and Use approved all protocols that were used in this study (approval nos. 8693 and 07124-V).

In this study, we looked at three variables that, taken together, we refer to as HT (34–37, 46). HT includes survival during acute severe hypoxic hypoxia as measured by hypoxic survival time (HST), the protective effect of acute hypoxic conditioning (HC) on HST, and survival following the HC procedure. The combination of these variables has proven valuable as an overall measure of the animal’s ability to compensate during severe acute hypoxic hypoxia (34–37). To identify a possible role for NO in HT, we measured HST, HC, and survival following hypoxic conditioning while blocking or mimicking the action of NOS.

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NITRIC OXIDE (NO) can act as a neurotransmitter, inhibit platelet adhesion, modulate cell growth and death, and induce vasodilatation (2, 3, 8, 10, 14, 39, 40). During conditions of high oxygen demand, NO can mediate relaxation of vascular smooth muscle and thus increase oxygen delivery to a tissue in need. Inhibition of NO synthase (NOS) reduces the availability of NO, which in turn would decrease vasodilatation and effectively favor vasoconstriction (7, 14, 33, 39–42, 45). From this line of reasoning, NO inhibition and the decreased ability to vasodilate during a period of hypoxia should compromise an animal’s compensatory response to acute hypoxic hypoxia. Thus we hypothesized that NO inhibition would decrease overall hypoxic tolerance (HT).

Compound Preparation and Treatment

ADMA. The nonselective endogenous NOS inhibitor ADMA (D4268, Sigma Chemical, St. Louis, MO) was injected into the intraperitoneal space 5 min before HC or HST. ADMA was dissolved in distilled water and frozen until used at which time it was diluted with normal saline to 1 mg/ml. ADMA was injected at a dose of 10 mg/kg and a volume of 0.25 ml, and normal saline was used as the volume control. This dose was selected because it had been shown previously to increase blood pressure (22).

L-NNA. The nonselective synthetic NOS inhibitor l-NNA (N-5501, Sigma Chemical) was administered orally 48 h before HC or HST. Groups of experimental or control mice were housed five to a cage with free access to one community water bottle. l-NNA-treated mice received 1 g/l of l-NNA dissolved in tap water, and the control mice received tap water. This dose was selected because our laboratory has shown previously that it increased blood pressure as measured by radiotelemetry (30).

7-Nitroindazole. The selective nNOS inhibitor 7-nitroindazole (7-NI) (N-7778, Sigma Chemical) was injected (60 mg/kg) intraperitoneally 30 min before HC or HST. 7-NI was suspended in peanut oil and sonicated immediately before injection. Control groups of mice received a peanut oil intraperitoneal injection of equal volume. This dose and timing were selected because they have been shown previously that a similar dose (50 mg/kg) maximally inhibited brain NOS activity in rats (23).

N-[3-(aminomethyl)phenyl]methyl]-enthanimidamide. The selective iNOS inhibitor N-[3-(aminomethyl)phenyl]methyl]-enthanimidamide (1400W; no. 1415, Tocris, Avonmouth, Bristol, UK) was injected intraperitoneally 30 min before HC or HST. The 1400W was dissolved in saline, and the control was an equal volume of saline. 1400W was given at a dose of 1 mg/kg and a volume of 0.25 ml/mouse. This dose and timing were selected because they have been shown previously to inhibit pharmacologically induced cardiac-preconditioning effect of iNOS in mice (17).

3-Morpholinosydnoeimine. The NO donor 3-morpholinosydnoeimine (SIN-1; no. 0756, Tocris, Avonmouth, Bristol, UK) was injected intraperitoneally 30 min before HST in a cohort of nonconditioned L-NNA-treated mice. SIN-1 was dissolved in normal saline immediately before use and injected intraperitoneally at an approximate dose of 20 mg/kg in 0.25 ml saline. An equal volume of normal saline was used as the control. This dose was selected because it was close to an infusion dose (SIN-1 at 3 mg·kg\(^{-1}\)·h\(^{-1}\)) that has been shown to reverse i-NOS-induced increases in mean arterial blood pressure in rats (49).

HC

HC protocols were done as previously described (34–37, 46). Briefly two or three mice were placed in either of two 500-ml airtight flow-through chambers and subjected to HC or SC before HST. Both chambers were initially flushed with room air (20.93% \(O_2\)) at 1.3 l/min. HC mice received four sequential exposures of 0.75, 1.5, 2, and 2.5 min of 46% \(O_2\)-balance \(N_2\) separated by 5 min of room air, including 5 min after the final HC. SC mice received normoxic room air for the same duration (21.75 min) as the HC mice.

HST

HST testing protocols were done as previously described (34–37, 46). Briefly, groups of five (2 SC and 3 HC or 3 SC and 2 HC) mice were transferred to five individual airtight flow-through chambers (3.7 cm ID × 10.5 cm in length) arranged in parallel for simultaneous HST testing. Hypoxic hypoxia was induced by flushing the five-chamber system for 20 s at 1.71 l/min with premixed 8.6% \(O_2\)-balance \(N_2\) and then by a constant flow of 4.6% \(O_2\)-balance \(N_2\) at 1.71 l/min. HST, as used in this and all previous studies (11, 25–27, 34–37), is the time from onset of 46% \(O_2\) to the cessation of spontaneous ventilation.

Within 60–90 s of the onset of hypoxia, the animal falls into a state that is often reported in humans as euphoric (18), loses consciousness, undergoes seizures, and then ceases spontaneous ventilation. At the cessation of spontaneous ventilation, the heart is still beating and induction of artificial ventilation could resuscitate the animal. However, because we did not ventilate the mice, cessation of spontaneous ventilation in the unconscious mouse leads irrevocably to cardiac arrest and hence is a precise measurement of the time to death due to hypoxia. At the cessation of spontaneous ventilation, no signs of pulmonary effusion were detected, and at necropsy, the lungs demonstrated no signs of atelectasis. Hypoxic hypoxia is not asphyxia, and death by acute hypoxic exposure is humane, and approved, as an end point for use in this model by the university Institutional Animal Care and Use Committee, which fully embraces all the articles of the recently published Principles for Establishment of Humane End Points (9).

Statistical Analyses

Survival curves were created and analyzed using the Kaplan and Meier product-limit method that accounted for censored data (survivors), and the log rank test was used to test for differences between curves. The log rank test as applied in our analyses is equivalent to the Mantel-Haenszel test, and it generates a \(P\) value that tests the null hypothesis that the curves are identical in overall populations. Median survival time was calculated for each group with fewer than 50% survivors (lived past 2,700 s). Sample sizes were sufficient to give a significance of \(P \leq 0.05\) and power of 0.95 or greater. Data were analyzed using commercially available statistical software (Microsoft Excel and Graphpad Prism 4.0).

RESULTS

The primary result is that NOS inhibition increased HT rather than decreased it as originally hypothesized. The administration of the endogenous NOS inhibitor ADMA at 10 mg/kg increased HT in both SC and HC mice. SC mice given ADMA \((n = 12)\) had a median survival time of 108.5 s (Fig. 1A), and SC mice given saline \((n = 20)\) had a significantly lower \((P = \ldots)\)
Nos inhibition increases acute hypoxic tolerance

No statistically significant effect of the nNOS inhibitor 7-NI was detected on HT (Fig. 3, A and B). SC mice treated with 7-NI (n = 10; 89 s) showed no statistical difference (P = 0.287) from control animals given vehicle (n = 8, mean HST: 80.5 s). HC mice treated with 7-NI (n = 8, mean HST: 205.5 s) showed no statistical difference (P = 0.433) from control animals given vehicle (n = 5, mean HST: 250 s). There were no survivors in either of the groups involving 7-NI or their vehicle controls.

No statistically significant effect of the iNOS inhibitor 1400W was detected on HT (Fig. 4, A and B). SC mice treated with 1400W (n = 6, mean HST: 117 s) were no different (P = 0.81) from SC animals (n = 4, mean HST: 92 s). Similarly, HC mice treated with 1400W (n = 12, mean HST: 285 s) showed no statistical difference (P = 0.851) from control animals given vehicle (n = 8, mean HST: 263 s), and there were two survivors in each HC group.

DISCUSSION

Our initial hypothesis that NOS inhibition would decrease HT was not supported. The opposite was observed in that NOS inhibition greatly improved HST and augmented the HC effect on HT, thus improving overall HT. Despite this unexpected outcome these data do make it clear that NOS, and perhaps eNOS specifically, play a role in the systemic response to acute hypoxia in CD-1 mice. Ongoing NOS activity and hence NO apparently oppose the acute compensatory responses to hypoxia such that the inhibition of NOS improves HT in the setting of acute hypoxic hypoxia. This improved HT can be produced by administration of L-NNA, an exogenous, synthetic NOS inhibitor or perhaps more physiologically by the body’s endogenous inhibition of NOS by ADMA or N²-monomethyl-L-arginine (L-NMMA).

Nonspecific NOS inhibition by ADMA and L-NNA significantly increases HT in sham-conditioned CD-1 mice. ADMA is an endogenous, non-isoform-selective, NOS inhibitor, and L-NNA is a synthetic, non-isoform-selective, NOS inhibitor. The reason that both endogenous and synthetic NOS inhibitors were used in this study is so that we could be reasonably sure that it was the NOS inhibition property of these compounds that was responsible and less likely a nonspecific response to either of the compounds. Compared with the increased HT in response to ADMA, the increased HT in response to L-NNA was slightly greater during DC and dramatically greater during HC. This may be due to the differing routes of drug delivery (ip vs. po for 2 days) and/or the dose or perhaps to intrinsic differences in activity between the two compounds. A full dose-response relationship was not developed, but instead doses were selected based on published reports of expected cardiovascular effect of elevating arterial blood pressure (22, 30). Furthermore, ADMA, a naturally occurring NOS inhibitor, was used to probe for a possible physiological component of NOS inhibition in the acute conditioning responses.

Additional support for an antagonistic role of the NOS-NO system in systemic acute hypoxic adaptation is afforded by the counteracting effects of an NO donor (SIN-1) in mice tested for HT without HC or SC. SIN-1 attenuated the increased HT produced by L-NNA alone. With the use of our original logic, additional NO during hypoxia should have enhanced vasodilation and thus improved blood flow delivery to hypoxic tissues

Fig. 2. N⁶-nitro-L-arginine (L-NNA) increases SC (A) and HC (B) hypoxic survival time. In A and B, ■ represents L-NNA-treated (1 g/ml po dissolved in drinking water) mice, and the solid line represents drinking water control (H₂O). With HC, 4 of 6 L-NNA-treated mice survived beyond 2,700 s. In C, ■ indicates saline-injected L-NNA-treated mice, and □ indicates 3-morpholinosydnoeimine (SIN-1)-injected L-NNA-treated mice. The NO donor SIN-1 significantly reduced HST in L-NNA-treated mice. Significant differences in survival curves between groups were determined using log rank test.
and improved HT. The opposite occurred, and additional NO (by SIN-1) actually attenuated the increased HT in response to L-NNA (Fig. 2C). This supports the idea that perhaps, in response to HC, there could be a physiological inhibition of the NO pathway by ADMA that, in turn, is involved in the increased tolerance for hypoxia produced by HC. Clearly supplemental ADMA improved HT in both the SC and HC groups (Fig. 1, A and B).

The nNOS inhibitor, 7-NI, had no significant effect on HT in SC or HC settings (Fig. 3). Similarly, the iNOS inhibitor, 1400W, had no significant effect on HT in SC or HC settings (Fig. 4). Thus it is unlikely the nNOS or iNOS inhibition results in the observed increase in HT seen with ADMA or L-NNA. By elimination, it is reasonable to suggest that NOS inhibition effect on hypoxia is likely eNOS mediated. Given the endothelial ubiquity of the eNOS isoform, it may be that the ability of NOS inhibition to improve hypoxic tolerance is mediated by the reduced capacity for NO vasodilation, thus resulting in a net vasoconstriction. Such eNOS inhibition could be naturally occurring during HC perhaps by ADMA (or L-NMMA) or artificially induced by L-NNA.

During an acute hypoxic challenge, blood may be preferentially supplied to the brain and the heart at the expense of other tissues. This is done by peripheral vasoconstriction and is observed as the diving response in mammals (29). Peripheral vasoconstriction is protective in diving, and other conditions of low oxygen, because more blood flows to the brain thus delivering oxygen to the most essential tissues (28, 47, 48, 50). Peripheral vasoconstriction is often associated with an increase in systemic vascular resistance (SVR). In previous studies, our laboratory observed an increase in arterial blood pressure caused by NOS inhibition with L-NNA given orally at 1 g/ml (30) as used in the present study. In the face of the sustained bradycardia we observed, this increased blood pressure is most likely in response to an increase in SVR. The use of L-NNA or ADMA at doses shown in other studies to increase blood pressure suggests that a similar tendency to increase SVR may have occurred in this hypoxic hypoxia protocol. Therefore, it is reasonable to conclude that eNOS inhibition can contribute to increased HT though a net peripheral vasoconstriction and redistribution of blood flow to essential tissues. L-NNA has been previously shown to increase the time to cessation of breathing in rats gradually exposed to 11,000 m simulated altitude (31). Although obtained in a different species and a different time course of hypoxic exposure, our data do in principle confirm this observation. Also, NO is a key oxygen-sensing molecule and signals other response factors such as hypoxic-inducible factor-1α (43, 44) that could contribute to the modulation of HT in the setting of HC. An elegant and exhaustive review (32) of the role of NO in cardiovascular adaptation to intermittent hypoxia has recently been published and expands and integrates these and alternative candidate mechanisms for the interaction of the NO-NOS system and HT.

Emery et al. (12) showed that L-NAME, another synthetic NOS inhibitor, augmented pulmonary and systemic vasoconstriction in rats during hypoxia compared with similar treatments during normoxia. They concluded that NOS inhibition, when coupled with hypoxia, leads to significantly more vasoconstriction than NOS inhibition by itself. This may explain why we observed such large increases in HT following HC plus L-NNA or ADMA. The HC may act as a brief period of nonlethal hypoxia that allows the endogenous NOS inhibition to decrease NO and increase SVR to the level offering optimum protection, thus conditioning the animal to tolerate a previously lethal hypoxic challenge.

Jernigan et al. (20, 21) showed that NOS inhibition during acute hypoxia diminishes levels of reactive oxygen species,
and this is coincident with augmented NO-mediated pulmonary vasodilation. Pulmonary vasodilatation could increase uptake to oxygen by the lungs, and this in turn could increase HT. In our study, we did not measure oxygen uptake, so we can neither support nor refute this position. In addition, if NO inhibition decreased reactive oxygen species, it may have been beneficial simply by reducing oxidative damage to cells. However, we did not measure oxidant injury in this study.

There are other possible mechanisms by which the NOS-NO system could alter HST and the conditioning effect on HST. NO generated by mtNOS can regulate cellular metabolism by interacting with mitochondrial cytochrome c oxidase in a competitive manner with oxygen (5, 6). Thus increases in NO, regardless of its NOS source, could decrease oxygen consumption and thus oxidative phosphorylation. The most proximate NO source would be mtNOS. This mechanism is presumed to be local (intracellular) in nature and may regulate local ATP homeostasis (5, 6, 24). Applied at the organ level, modest increases in NO may improve blood flow to downstream tissue that were demanding more oxygen. By contrast, abnormally high concentrations of NO could potentially compromise function by inhibiting ATP production. It would follow then that the acute hypoxia (4.5% \( \text{O}_2 \)) faced by both our SC and HC mice may elicit a global and rapid, but moderate, increase in NO production as all tissues attempt to acutely increase blood flow locally. This multiphasic argument was perhaps first put forth by Malyshiev et al. (31) when they concluded that the mild increase in NO that they observed in acute hypoxia appeared “to be a compensatory response aimed at the dilatation of blood vessels to maintain oxygen supply.” They went on to speculate that at higher NO concentrations produced by using a NO donor the excess NO may inhibit mitochondrial oxidative phosphorylation (31).

It is possible that the initial exposure to hypoxia permits, or initiates, a rapid increase in endogenous NOS inhibitors (for example ADMA or L-NMMA) that attenuate the activity of eNOS. These naturally occurring NOS inhibitors may also attenuate the activity of mtNOS, thereby improving (or sustaining) ATP production during the next hypoxic exposure. Whether or not the endogenous or exogenous NOS inhibitors we used in this study actually inhibit mtNOS has yet to be reported. Thus far, only melatonin (13) and \( \text{L-NNMA} \) (15, 16) have been reported to inhibit mtNOS and thus should be evaluated in the acute HC-HST model.

Additionally, Barer et al. found that NOS inhibition with \( \text{L-NAME} \) during chronic hypoxia exposure in rats augmented cerebral angiogenesis (1). This effect could improve tolerance to ischemia simply by improving delivery and cannot be discounted as a potential mechanism at work in our model. However, because our study was limited to <20 total min of hypoxic exposure and because changes in gene expression are likely to have much longer time constants, it is unlikely that angiogenesis was involved in improving HST.

In conclusion, a counterintuitive linkage between the NO-NOS system and the systemic response to hypoxia has been further explored. The data and arguments presented suggest that eNOS inhibition (and possibly mtNOS inhibition), whether induced by 1) acute HC, 2) supplemental administration of the endogenous NOS inhibitor ADMA, or 3) the exogenous administration of the synthetic NOS inhibitor \( \text{L-NNA} \), increases the acute tolerance to hypoxia. That is, NOS inhibition increases tolerance for acute hypoxia in CD-1 mice. If this response is manifest in larger species, and ultimately in humans, a possible extrapolation of this finding would be the suggestion that eNOS or mtNOS inhibition should be evaluated as a potential therapeutic or prophylactic intervention in the setting of acute hypoxic hypoxia. Indeed such has been suggested in the extensive review by Hobbs et al. (19) in which NOS inhibition was suggested as a potential therapeutic target. Our data also suggest that NOS inhibition may be adaptive and part of a larger systemic homeostatic regulation of both cellular metabolic activity and blood delivery. Rather than viewing NOS inhibition strictly through a prism of obligate pathology, we submit that limited and controlled NOS inhibition may be part of a normal murine conditioned response to routine hypoxia.

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NOS INHIBITION INCREASES ACUTE HYPOXIC TOLERANCE