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HIGHLIGHTED TOPIC | *The Physiology and Pathophysiology of the Hyperbaric and Diving Environments*

Two faces of nitric oxide: implications for cellular mechanisms of oxygen toxicity

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Allen BW, Demchenko IT, Piantadosi CA. Two faces of nitric oxide: implications for cellular mechanisms of oxygen toxicity. *J Appl Physiol* 106: 662–667, 2009. First published October 9, 2008; doi:10.1152/japplphysiol.91109.2008.— Recent investigations have elucidated some of the diverse roles played by reactive oxygen and nitrogen species in events that lead to oxygen toxicity and defend against it. The focus of this review is on toxic and protective mechanisms in hyperoxia that have been investigated in our laboratories, with an emphasis on interactions of nitric oxide (NO) with other endogenous chemical species and with different physiological systems. It is now emerging from these studies that the anatomical localization of NO release, which depends, in part, on whether the oxygen exposure is normobaric or hyperbaric, strongly influences whether toxicity emerges and what form it takes, for example, acute lung injury, central nervous system excitation, or both. Spatial effects also contribute to differences in the susceptibility of different cells in organs at risk from hyperoxia, especially in the brain and lungs. As additional nodes are identified in this interactive network of toxic and protective responses, future advances may open up the possibility of novel pharmacological interventions to extend both the time and partial pressures of oxygen exposures that can be safely tolerated. The implications of a better understanding of the mechanisms by which NO contributes to central nervous system oxygen toxicity may include new insights into the pathogenesis of seizures of diverse etiologies. Likewise, improved knowledge of NO-based mechanisms of pulmonary oxygen toxicity may enhance our understanding of other types of lung injury associated with oxidative or nitrosative stress.

superoxide; superoxide dismutase 3; neurogenic pulmonary edema

If all evil were prevented, much good would be absent from the universe.

Thomas Aquinas (c.1265 C.E.)

THE AVIDITY OF OXYGEN (O_2) for electrons is the chemical foundation of aerobic metabolism but also creates the potential to injure cells and tissues, especially in the presence of endogenous organometallic coordination compounds that catalyze its incomplete reduction. Examples of such harmful products include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). Thus, as oxygen passes from the atmosphere to the mitochondria, it traverses tissues that would be vulnerable to oxidative damage, but for powerful biochemical defenses that evolved as the primordial, reductive atmosphere was transformed, ~2.4 billion years ago (19). However, if oxygen partial pressures are raised sufficiently above normal atmospheric levels, for enough time, those defenses are breeched, and oxygen toxicity occurs.

Historically, reports of harmful effects of oxygen followed soon after the discovery and purification of the gas in the late 18th century, which permitted scientists to expose animals to oxygen-enriched atmospheres. But the identification of pathophysiological mechanisms awaited the development and maturation of cell biology as a free-standing discipline in the late 1800s. It was then that independent studies of oxygen toxicity in the central nervous system (CNS) (5) and in the lungs (38) initiated the search for discrete mechanisms affecting particular cell types. The practical demands imposed by the development of deep diving and the therapeutic use of normobaric [$P_{O_2} = 1$ atmosphere absolute (ATA)] as well as hyperbaric oxygen ($P_{O_2} > 1$ ATA; HBO_2), provided further impetus for this research. Beginning in the 1940s, the worldwide occurrence of many cases of retrolental fibroplasia, a toxic effect of normobaric oxygen on the retinal vasculature of premature infants, imposed a new urgency on the investigation of cellular and

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molecular events involved in oxygen toxicity (40). In the 1950s, a crucial discovery was that oxidative injury shared a common factor with that caused by X-rays and other types of ionizing radiation: the formation of chemically reactive species, including ions and free radicals (18, 21).

In the 1960s, the discovery and characterization of the antioxidant enzyme superoxide dismutase (SOD) opened a broad vista on the complex biochemical systems that defend air-breathing organisms from toxic effects of oxygen. And the 1980s and 1990s saw the discovery of physiological and pathological roles for endogenously produced nitric oxide (NO) and its active metabolites (6), permitting additional advances. Our understanding of the intra- and extracellular mechanisms of oxygen toxicity has progressed as the biochemistry of protection, by a variety of antioxidant enzymes and low-molecular-weight antioxidants, has been elucidated.

REACTIVE OXYGEN SPECIES AND NO

In 1991, investigators at Duke University tested the hypothesis that a specific isoform of SOD, extracellular SOD or SOD3 (32), as an important extracellular antioxidant enzyme, could confer protection from CNS oxygen toxicity (34). Quite unexpectedly, they found that the overexpression of this enzyme in transgenic mice aggravated rather than alleviated CNS O_2 toxicity. A possible clue to this paradox was unearthed in the literature: the superoxide anion ($\cdot O_2^-$), the substrate for SOD, inactivates the gaseous vasodilator NO in hyperoxia (36). The mechanism of this inactivation was identified as a diffusion-limited reaction, resulting in the formation of the strong oxidant peroxynitrite (3, 4):

$$NO\cdot + \cdot O_2^- \rightarrow ONOO^- \quad (1)$$

where $ONOO^-$ is peroxynitrite anion. These were the first important clues that NO and its metabolites might play a role in CNS oxygen toxicity. To further test this idea, mice were pretreated with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a general inhibitor of NO synthesis, and the difference in sensitivity to CNS O_2 toxicity between the transgenic mice and wild-type (WT) controls was abolished.

NO AND CEREBRAL BLOOD FLOW

Because NO is a powerful vasodilator and, therefore, a regulator of cerebral blood flow (CBF), efforts were made to measure changes in regional cerebral flow (rCBF) in HBO_2. The first attempt to do this, using laser-Doppler flowmetry, a technique originally developed to measure the velocity of particles in moving fluids (43), gave negative results. When used to assess blood flow in vivo, this method is only approximate, because it detects the velocity of red blood cells, which may, in fact, increase in vasoconstriction. Thus this initial study found no decrease in brain cortical blood flow in rats exposed to 3 ATA O_2 (44). Other laboratories have also employed this method with conflicting results (8, 41). A more responsive method uses the clearance of biologically inert gases, such as xenon or hydrogen, for assessing blood flow in vivo (25). In particular, hydrogen clearance electrodes, although invasive, provide a more sensitive and direct measure of blood flow than does laser-Doppler flowmetry. In our hands, this approach has revealed that exposure of anesthetized rats to 5 ATA O_2, when sufficiently prolonged to induce EEG responses of CNS O_2 toxicity, is associated with a pronounced biphasic rCBF response: transient vasoconstriction followed by hyperemia (11). These effects are less pronounced and more difficult to delineate at the lower levels of HBO_2 used in therapeutic exposures, generally 2–3 ATA O_2.

The initial cerebral vasoconstriction of hyperoxia had been known for many years, long before its mechanism was identified, and its protective function is easily appreciated, since it keeps brain P_{O_2} from rising steeply in normobaric hyperoxia (28). The secondary increase in rCBF seen in hyperbaric hyperoxia, as well as the HBO_2-related EEG spikes that follow it, were not observed after NO synthase (NOS) inhibition with L-NAME, but did appear subsequently after treatment with L-arginine, a substrate (along with O_2) for NO production. Furthermore, the CBF responses to the NO-based vasodilators *S*-nitrosoglutathione and *S*-nitrosohemoglobin were abolished by HBO_2 at 3 ATA. Additionally, at least two separate components of the pathway from hyperoxia to seizures were teased apart by inhibiting excitatory synaptic transmission using MK-80, a blocker of the *N*-methyl-D-aspartate receptor; this eliminated EEG spiking but did not alter rCBF responses to HBO_2 (12).

CEREBRAL NO LEVELS IN HBO_2

In 2001, it was further discovered that HBO_2 exposure at 5 ATA is associated with increased levels in brain of NO and its metabolites (collectively NOx), and these levels correlate positively with the increases in rCBF that precede the EEG discharges of CNS O_2 toxicity (13). It was confirmed that L-NAME protects against the secondary increase in rCBF and inhibits EEG discharges at 5 ATA, and that L-arginine abolishes this protective effect (12). It was proposed that elevated NOx in the brain during prolonged exposure to extremes of HBO_2 might be due to the fact that HBO_2 increases the levels of both substrates required for NO synthesis, O_2 and L-arginine (33, 45).

SOD REGULATES CEREBRAL NO

In 2002, the role of SOD3 in regulating vascular responses in the brain to oxygen was investigated using genetically altered mice expressing different levels of this enzyme. Predictable alterations in rCBF responses to high P_{O_2} in these mice (WT animals and two mutant strains, one lower and one higher in SOD3 activity than the WT) confirmed that scavenging of $\cdot O_2^-$ by SOD3 is critical to vascular function in the brain (14). Because inactivation of NO by $\cdot O_2^-$ disrupts NO-dependent basal tone, the (strategic location of cerebrovascular SOD3 between endothelium and smooth muscle preserves and regulates the endogenous dilator function of NO·. And SOD3 is present in high concentration in those vessels in which NO· is particularly important for vascular relaxation (14).

These findings have broad implications for vascular diseases in which extracellular $\cdot O_2^-$ production exceeds SOD3 function, as, for instance, when the enzyme is released from its heparan binding domain by oxidative or proteolytic stress. Since the binding of SOD3 to heparan sulfate proteoglycans on cell surfaces and in the extracellular matrix allows it to persist in relatively high concentrations in specific regions (23), its cleavage not only increases extracellular oxidative stress, due to clearance of SOD3 from the tissues, but the concomitant

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increase in $\cdot O_2^-$ accelerates the production of peroxynitrite, resulting in nitrosative stress (31).

SOURCES OF THE NO THAT MODULATE CEREBRAL VASCULAR TONE

The three known isoforms of NOS are named on the basis of their principal anatomical locations: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III). nNOS is found primarily in the central and peripheral nervous systems, iNOS in inflammatory/immune cells, and eNOS in the vascular endothelium. However, nNOS has also been identified in the microvasculature, in addition to eNOS, and is known to contribute to vascular function (24). This finding is consistent with the role of these two isoforms in the secondary, hyperemic response of the cerebral vasculature to HBO_2. Thus rCBF in WT mice, after 60 min in HBO_2 at 5 ATA, increases more than in $eNOS^{-/-}$ or $nNOS^{-/-}$ mice, suggesting that both eNOS and nNOS are sources for the NO that mediates the escape from hyperoxic vasoconstriction and thus initiates the hyperemic phase. Also, WT and $nNOS^{-/-}$ mice exposed to 5 ATA O_2 show the expected initial decrease in rCBF, but $eNOS^{-/-}$ mice do not, suggesting that other compensatory mechanisms, not mediated by NO and, therefore, not inhibited by superoxide, maintain basal vascular tone in the brains of those animals. Furthermore, brain NO metabolites (NOx) decrease in WT and $eNOS^{-/-}$ mice within 30 min of HBO_2 but rise minutes later above control levels, whereas they do not change in $nNOS^{-/-}$ mice. Moreover, 3-nitrotyrosine (3-NT), a common product of the action of reactive nitrogen species, especially of peroxynitrite, on proteins, increases in the cerebrospinal fluid during HBO_2 in WT and $eNOS^{-/-}$, but not in $nNOS^{-/-}$ mice, an important finding that is discussed further below. These results suggest that the initial cerebral vasoconstriction seen in HBO_2 is due to inactivation by the $\cdot O_2^-$ of NO synthesized by eNOS. However, the subsequent HBO_2-induced vasodilation and hyperemia depend on NO synthesized by both eNOS and nNOS (2).

Our present understanding of cellular mechanisms by which NO participates in the manifestations of oxygen toxicity, or in the countervailing processes that defend against it, is incomplete. The information we have, however, can be mapped in some detail, especially for the guanylate cyclase-dependent tone of the cerebral vasculature as it is influenced by eNOS and nNOS (see Fig. 1). Additional pathways need to be fully delineated, particularly those that link the brain, autonomic nervous system, lungs, and heart into an integrated physiological system.

ROLE OF nNOS AND PEROXYNITRITE IN HBO_2

Studies using knockout mice lacking either nNOS or eNOS provided the first in vivo evidence that an HBO_2-induced increase in brain peroxynitrite is associated with the development of CNS O_2 toxicity. Peroxynitrite production was assessed by measuring its by-product 3-NT using intracerebral microdialysis in the striatum. In mice expressing eNOS (WT and $nNOS^{-/-}$ animals), rCBF decreased over 30 min of HBO_2 at 5 ATA, followed by a rise to above preexposure levels. In $eNOS^{-/-}$ mice, however, HBO_2 did not reduce rCBF, and the secondary elevation in rCBF was attenuated. These measurements provide evidence that HBO_2 causes cerebral vasoconstriction by interfering with eNOS-derived NO. Since $eNOS^{-/-}$ and $nNOS^{-/-}$ mice show a less robust hyperemic phase than do WT mice after prolonged HBO_2, CBF in this secondary phase depends on NO derived from both eNOS and nNOS. Because the perivascular nerves are a major source of nNOS-derived NO (20), and nNOS does not appear to modulate basal vascular

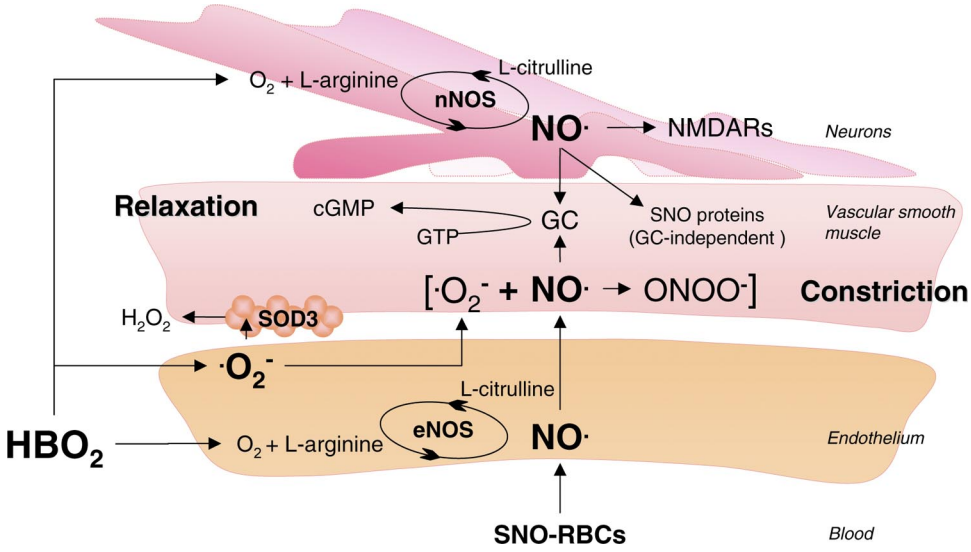

Fig. 1. Hyperbaric oxygen (HBO_2) alters the basal state of the nitric oxide (NO) signaling via guanylate cyclase (GC) in the cerebral vasculature in several ways. Levels of superoxide anion ($\cdot O_2^-$) increase, elevating the production of hydrogen peroxide (H_2O_2) through extracellular superoxide dismutase (SOD3) activity; another reaction produces peroxynitrite ($ONOO^-$) at the expense of NO (NO·) bioactivity, resulting in contraction: the initial cerebral vasoconstriction. NO bound to thiols (SNO) on hemoglobin in red blood cells (RBCs) is a source for NO in the brain that is independent of local synthesis, but its delivery to the brain requires the allosteric release of O_2 by hemoglobin, which is absent in extreme HBO_2. However, since oxygen is a substrate for NO production by the NO synthases, a higher Po_2 accelerates NO production by endothelial NO synthase (eNOS) in the endothelium and by neuronal NO synthase (nNOS) in neurons, eventually resulting in escape from vasoconstriction: the secondary hyperemia. Elevated levels of NO bioactivity in neurons also act directly on *N*-methyl-D-aspartate receptors (NMDARs), leading to excitation and shortened seizure latency.

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tone, this isoform can be implicated in the secondary vasodilation seen in HBO_2.

In mice that express nNOS (WT and $eNOS^{-/-}$ animals), interstitial 3-NT in the striatum increases progressively throughout the entire period of HBO_2 exposure, whereas, in $nNOS^{-/-}$ mice, striatal 3-NT does not change significantly. These results imply that nNOS-derived NO generates the bulk of brain $ONOO^-$, because the production of NO by nNOS far outweighs that derived from eNOS. Therefore, eNOS-deficient and nNOS-deficient mice have different cerebrovascular responses and different degrees of tolerance to HBO_2. Thus, when NO bioactivity derived from eNOS is suppressed by reaction with $\cdot O_2^-$, protective vasoconstriction is invoked, and later, when NO production by eNOS is increased by prolonged exposure to HBO_2, secondary hyperemia occurs. Under these conditions, NO derived from nNOS may mediate a direct toxic effect of HBO_2 on the brain by its reaction with superoxide to generate peroxynitrite (10).

NO, superoxide, and peroxynitrite, termed "the good, the bad and the ugly" by one of the pioneer investigators of their pathophysiology (4), do not stand in static relationship with one another; for, although *Eq. 1* is straightforward, it does not show the feedback loops, both positive and negative, that connect its reactants and product with other active components of its biochemical milieu. Many other NO-related biochemical pathways exist; particularly interesting are those that involve cGMP-independent actions of NO in the *S*-nitrosylation of proteins (39), including ion-channel proteins, an effect that can alter excitability in the CNS (1). Many such pathways and their biological effects await further investigation.

NORMOBARIC VS. HBO_2 TOXICITY

When exposure to hyperoxia is limited to ~1 ATA O_2, the lung rather than the brain appears to be the primary target of O_2 toxicity. A well-defined pattern of diffuse pulmonary damage gradually develops, characterized by an extensive inflammatory response and destruction of the alveolar-capillary barrier, leading to edema, impaired gas exchange, respiratory failure, and death (9). The severity of these effects increases over several days, and overt CNS O_2 toxicity does not occur under these conditions. This is in contrast to exposures at higher inspired O_2 pressures, 2–3 ATA, in which pulmonary injury is greatly accelerated and is of a substantially different character: less inflammation is manifest, and events in the brain are a prelude to injury in the lung. The CNS-mediated component of this lung injury can be attenuated by selective inhibition of nNOS or by unilateral transection of the vagus nerve. Thus, in HBO_2, extrapulmonary, neurogenic events predominate in the pathogenesis of this more acute form of pulmonary oxygen toxicity, as nNOS activity drives lung injury by modulating the output of central autonomic pathways (15).

The essential change in the lung pathology as hyperoxia increases from normobaric to hyperbaric levels is that the responses shift from direct inflammatory injury to CNS-mediated noninflammatory injury, and, although the differences are qualitative and graded, the direct effect of oxygen on the lung predominates in normobaric hyperoxia, whereas CNS effects prevail in hyperbaric conditions. Although the pattern of injury changes as the balance between these two phenotypes shifts, there does not appear to be a condition in which one or the other effect is totally lacking. The former develops slowly, and, because the entire surface of the lung is directly exposed to the hyperoxic environment for many hours, the diffuse inflammatory response destroys the alveolar-capillary barrier and leads to respiratory failure (9). However, at hyperbaric exposures, pulmonary damage develops more rapidly, is more heterogeneous, and is presaged by events in the brain. This injury is driven by extrapulmonary mechanisms in which NO derived from nNOS may link elevated P_{O_2} in brain to acute damage in the lung via central autonomic pathways (15).

The role of peroxynitrite, the product of NO degradation by superoxide, is also different in normobaric hyperoxia than in HBO_2. In HBO_2 at 3 ATA, the initial result of the reaction represented in *Eq. 1* is a transient vasoconstriction due to loss of NO, and the brain is protected by the limitation in blood flow and regulation of the delivery of oxygen (34). However, in normobaric hyperoxia, the production of peroxynitrite by the reaction of NO with $\cdot O_2^-$ causes pulmonary oxidative damage that may ultimately contribute to fatal pulmonary edema (7).

The specific mechanisms by which modulation of neuronal NO leads to lung injury in hyperbaric hyperoxia are unknown, but it has been shown that NO signaling along CNS-mediated adrenergic/cholinergic pathways contributes to lung injury. Lung innervation is complex and includes not only autonomic adrenergic and cholinergic fibers, but also nonadrenergic noncholinergic (NANC) systems, which perform defensive, regulatory, and immunomodulary functions (22, 27, 29, 30, 35, 37). In the brain, the inhibitory NANC system involving NO opposes cerebral vasoconstriction (42), but, in the lung, nNOS does not seem to be important for normal function, since nNOS knockout mice have intact endothelium-dependent vasodilation (16). Also, although epinephrine and acetylcholine are major neurotransmitters involved in regulating pulmonary airway and vascular function, NANC neurotransmitters, including vasoactive intestinal peptide and NO, also have roles. Immunohistochemical studies reveal the existence of nNOS in nerve terminals, supplying lung vessels and lower airways (17, 26). The role of CNS-mediated effects on pulmonary vascular and cardiovascular function hyperbaric hyperoxia is a fruitful area for further study. Thus, in addition to its effects on the lung and brain, HBO_2 is known to increase systemic vascular resistance and reduce cardiac output. These responses to HBO_2 could shift systemic blood to the pulmonary circulation, raising pulmonary blood volume and pulmonary arterial or venous pressure. Even though these hemodynamic displacements may be rapid and transient, structural disruption of pulmonary capillaries would cause protein-rich pulmonary edema and persistent damage to the blood-gas barrier (15).

SUMMARY AND FUTURE DIRECTIONS

A new paradigm is emerging in place of the 19th century dichotomy in which oxygen toxicity is manifested as either a pulmonary insult in normobaric exposures, the Lorraine Smith effect (38), or CNS injury in hyperbaric exposures, the Paul Bert effect (5). It is now apparent that the effects of oxygen on lung and brain are part of a pathophysiological continuum in which a common factor is NO and its interactions with other reactive species. Thus, just as acute lung injury is now known to occur in HBO_2 exposures along with CNS effects, it seems likely that further work will demonstrate that subtle CNS

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effects occur in normobaric hyperoxia, along with pulmonary injury.

The discovery that NO-dependent neurogenic pathways link brain and lung in the pathophysiology of pulmonary injury in hyperbaric hyperoxia (15), with the associated hypothesis that this may involve pulmonary hypertension, elevated left atrial pressure, or both, suggests a similarity between pulmonary injury in HBO_2 and pulmonary hypertension of altitude and certain other etiologies. This might be particularly important for the study of pulmonary injury due to traumatic head injury. If this connection is valid and its mechanisms can be elucidated, the significance of research into the mechanisms of HBO_2 toxicity could be greatly enhanced.

The fact that NO production may either exacerbate or mitigate the toxic effects of oxygen, depending on the particular NOS isoform that produces it, affords new opportunities for pharmacological interventions to prevent or allay the toxic effects of normobaric and HBO_2. For example, the CNS-mediated component of this lung injury can be delayed and attenuated by selective inhibition of nNOS. In addition, selective promotion of eNOS activity in the pulmonary vasculature, without stimulating eNOS in the cerebral vasculature, could protect both the brain and the lung. Such precisely targeted interventions at specific points in the NO signaling systems could increase the safety and efficacy of hyperbaric therapy and improve the operational capabilities of military, commercial, and recreational divers.

GRANTS

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