L-Arginine-NO pathway and CNS oxygen toxicity

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Bitterman, Noemi, and Haim Bitterman. L-Arginine-NO pathway and CNS oxygen toxicity. J. Appl. Physiol. 84(5): 1633–1638, 1998.—The involvement of the l-arginine-nitric oxide (NO) pathway in the pathogenesis of hyperoxia-induced seizures was studied by using agents controlling NO levels. We selected two inhibitors of nitric oxide synthase, the systemic inhibitor N-nitro-l-arginine methyl ester (l-NNAME) and the novel cerebral-specific inhibitor 7-nitroindazole, and two generators of NO, the NO donor S-nitroso-N-acetylpenicillamine and the physiological precursor l-arginine. Rats with chronic cortical electrodes were injected intraperitoneally with different doses of one of the agents or their vehicles before exposure to 0.5 MPa O2 and O2 with 5% CO2 at an absolute pressure of 0.5 MPa. The duration of the latent period until the onset of electrical discharges in the electroencephalogram was used as an index of central nervous system O2 toxicity. The two nitric oxide synthase inhibitors l-NNAME and 7-nitroindazole significantly prolonged the latent period to the onset of seizures on exposure to both hyperbaric O2 and to the hypercapnic-hyperoxic mixture. Pretreatment with the NO donor S-nitroso-N-acetylpenicillamine significantly shortened the latent period, whereas l-arginine, the physiological precursor of NO, significantly prolonged the latent period to onset of seizures. Our results suggest that the l-arginine-NO pathway is involved in the pathophysiology of hyperoxia-induced seizures via various regulating mechanisms.

EXPOSURE TO OXYGEN at high pressure is commonly employed in both military and professional (enriched air) diving and in the clinical field of hyperbaric medicine (11, 19).

Breathing oxygen at high partial pressures produces marked hemodynamic and neurological effects (8, 16). Among the most significant are cerebral vasocostriction (4, 16) and the hyperoxia-induced seizures, defined and classified as generalized, tonic-clonic (grand mal) seizures (16). At a certain point in time, an initial oxygen-induced vasocostriction is reported to be impaired and an increase in cerebral blood flow is observed (8, 16). It has been suggested (30) that this increase in cerebral blood flow might be correlated with the development of hyperoxia-induced seizures.

Exposure to hyperbaric oxygen (HBO) in combination with a low level of CO2, either from an external source or because of an internal buildup of CO2, is a common situation in underwater activity and certain pathological conditions. An increased level of CO2 in the breathing mixture leads to significant and dramatic shortening of the latent period preceding seizures in both humans and experimental animals (16). Although not fully proven, it is accepted that the increased susceptibility to hyperoxia-induced seizures in the presence of hypercapnia is due to the cerebral vasodilator effect of CO2 (8, 16).

The exact mechanism responsible for the hyperoxia-induced hemodynamic changes and neurological manifestations is still unknown. It is well accepted that reactive oxygen species, which are produced in excess on exposure to HBO and overwhelm the body’s normal antioxidant defense systems, mediate the hyperoxic insult (14, 29). However, the specific biological messengers responsible for the various expressions of the hyperoxic insult have not yet been identified.

Nitric oxide (NO), a multifunctional small radical molecule, is involved in a number of regulatory mechanisms (9, 21), including vasodilatation, neurotransmission and neuromodulation, inhibition of platelet aggregation, and modulation of leukocyte adhesion. On the basis of the growing evidence for the biological importance of NO and the l-arginine-NO pathway in neural and vasoregulatory mechanisms of the brain, we decided to investigate their role in the regulation of central nervous system (CNS) oxygen toxicity.

To evaluate the role of NO in the pathophysiology of hyperoxia-induced seizures, we selected some agents affecting NO levels. On one hand, we used two inhibitors of NO synthase (NOS): N-nitro-l-arginine methyl ester (l-NNAME), a central and vascular NOS inhibitor, and the novel 7-nitroindazole (7-NI), which is thought to inhibit NOS solely in the brain in vivo (22). On the other hand, we employed agents known to increase NO levels: l-arginine (a precursor of NO), d-arginine (the inactive d-isomer of l-arginine), and the NO donor S-nitroso-N-acetylpenicillamine (SNAP). Two hyperoxic mixtures were used: pure HBO at 0.5 MPa [5 atmospheres absolute (ata)] and HBO with 5% CO2 at the same pressure.

Our model was a freely moving rat with continuous monitoring of the electroencephalogram (EEG). Animals were exposed to one of the two hyperoxic mixtures after injection of different concentrations of the various agents, until the onset of typical hyperoxia-induced electrical discharges in the EEG.

MATERIALS AND METHODS

Animals. Experiments were carried out in male Sprague-Dawley rats, weighing 220–280 g and kept in a normal day-night cycle. The rats were fed ad libitum on commercial food and water.

Experimental procedures. Under equithensin anesthesia [0.2–0.3 ml/100 g body weight ip; 1% chloral hydrate (wt/vol), 4% nembutal (wt/vol), and 1.3% MgSO4 (wt/vol) dissolved in

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absolute alcohol, propylene glycol, and water], the animal's skull was exposed and four stainless steel screws were inserted into the bone bilaterally over the parietal and posterior cortex, two screws at each hemisphere. Soldered wires linked the screws to a miniature connector fixed to the bone with self-cure acrylic. Rats recovered from surgery for 4 days before testing.

One hundred sixty-four rats were assigned at random to 18 groups with the following agents before exposure to 0.5 MPa oxygen (n = 9–10 rats/group): 1) L-NAME at a dose range of 1–20 mg/kg; 2) 7-NI (50 mg/kg); 3) SNAP at doses from 250–1,000 µg/kg; 4) L-arginine at doses of 30–300 mg/kg; 5) D-arginine (300 mg/kg); 6) L-arginine (300 mg/kg) together with L-NAME (20 mg/kg); and 7) D-arginine (300 mg/kg) together with L-NAME (20 mg/kg). Additionally, 72 rats were treated with L-NAME (1–20 mg/kg) or 7-NI (50 mg/kg; n = 9 rats/group) before exposure to 0.5 MPa oxygen with 5% CO2.

RESULTS

Figure 1 presents a typical EEG tracing from a rat exposed to HBO: control recording made at atmospheric pressure (A), the appearance of typical spike and wave electrical discharges in the EEG on exposure to 0.5 MPa oxygen (B), and the EEG on return to atmospheric pressure immediately after the onset of seizures (C). There were no notable changes in the baseline EEG, in the pattern of the seizures or their severity, or in the behavior of the rats under the effect of any of the agents employed in our study in any of the experimental groups.

Figure 2 shows the reciprocal of the latent period to the onset of seizures (the speed to seizure appearance) after injection of various doses of L-NAME or 7-NI and their vehicles (normal saline and peanut oil, respectively). A significant prolongation of the latency to the onset of seizures (expressed by a decrease in the speed to seizure appearance) was observed after intraperitoneal injection of each NOS inhibitor on exposure to pure oxygen at 0.5 MPa. With L-NAME, the prolongation reached statistical significance compared with vehicle at doses of 5 mg/kg or greater (P < 0.05 by
Tukey’s test). 7-NI induced a marked prolongation of the latent period ($P < 0.01$) at 50 mg/kg.

Figure 3 presents the effect of the two studied NOS inhibitors on the onset of hyperoxia-induced seizures on exposure to hypercapnic-hyperoxic mixture. In vehicle-treated rats breathing hyperoxic mixture with 5% CO$_2$ at 0.5 MPa, the duration of the latent period was dramatically shorter compared with that on exposure to pure oxygen at the same pressure (5.7 ± 0.3 vs. 19.1 ± 4 min, respectively; $P < 0.001$ by t-test). As seen in Fig. 3, pretreatment with L-NAME decreased the speed to seizures (i.e., exerted a protective effect) in a dose-related manner, reaching statistical significance at a dose of 20 mg/kg ($P < 0.05$ by Tukey’s test). The specific cerebral NOS inhibitor 7-NI caused a highly significant, almost the maximal possible, prolongation of the latent period ($P < 0.0001$ by t-test).

The effect of agents that increase NO levels and their control on the latent period to HBO-induced seizures is presented in Figs. 4 and 5. Pretreatment with L-arginine, the natural physiological precursor of NO, postponed HBO toxicity, as presented by a dose-related decrease in the speed of onset of hyperoxic-induced seizures, reaching statistical significance at a dose of 300 mg/kg ($P < 0.05$ by Tukey’s test). The inactive isomer D-arginine (300 mg/kg), given as a control, had no effect on the latent period to HBO-induced seizures (Fig. 4). In contrast, the NO donor SNAP, given before HBO exposure, caused a significant dose-related decrease in the latent period, reaching a significant enhancement of toxicity at the dose of 1,000 µg/kg ($P < 0.05$) (Fig. 5).

Combined treatment with either L-arginine or D-arginine (300 mg/kg) given 10 min after L-NAME (20 mg/kg) provided a significant protection ($P < 0.05$ by Tukey’s test) against hyperoxia-induced seizures compared with control. However, the combined effect of the two protective drugs on the latent period was not significantly different from the protection achieved with L-NAME alone.

Figure 6 summarizes the relative effect of each NO-controlling agent on the mean latency period to seizures normalized against its respective control. As can be seen, all agents tested herein increased the time...
to onset of seizures, except for SNAP, which shortened the latent period.

**DISCUSSION**

In the present study, we evaluated the involvement of the L-arginine-NO pathway in the pathogenesis of hyperoxia-induced seizures by using various agents controlling NO levels. It has previously been suggested by Oury et al. (25) and Zhang et al. (33) that NO is an important mediator of the pathophysiology of oxygen toxicity by using N^\text{ω}-nitro-L-arginine as a NOS inhibitor. Both studies utilized mortality and the appearance of clinical convulsions as indexes of oxygen toxicity. We extended the previous observations by using the objective, well-defined parameter of typical electrical discharges in the EEG as a criterion for CNS oxygen toxicity. We also evaluated the role of NO on exposure to hypercapnic-hyperoxic mixture. To further evaluate the role of NO, we selected agents controlling NO levels in both directions: two NOS inhibitors, the commonly used systemic NOS inhibitor L-NAME and the novel cerebral NOS inhibitor 7-NI, as well as the two NO generators, the NO donor SNAP and the physiological precursor L-arginine.

L-NAME and 7-NI, two NOS inhibitors, postponed the appearance of hyperoxic seizures significantly, without any noticeable side effects on the EEG or on the animals' behavior.

There are a number of possible explanations for the protective effects of NOS inhibitors against hyperoxia-induced seizures that are related to the vasodilatory, prooxidative, and neuromodulatory functions of NO. Exposure to HBO is accompanied by cerebral vasorestriction (4, 16), which is subsequently followed by cerebral vasodilatation. It has been suggested that this breakdown of cerebral vasoregulation and the resultant increase in cerebral blood flow correlate with the development of hyperoxia-induced seizures (30). The basic mechanisms of the vasoregulatory effects of oxygen are not fully understood, and it has been suggested that NO, which is known to mediate cerebral vasodilation (13), may be involved. On the basis of this, the inhibition of NO production by pretreatment with NOS inhibitors such as L-NAME or 7-NI may postpone the breakdown of hyperoxic cerebral vasoconstriction, thus attenuating the dramatic preseizure increase in cerebral blood flow and the inflow of oxygen and reactive oxygen intermediates to the brain. It may therefore be suggested that the NOS inhibitors postponed HBO-induced seizures by diminishing cerebral vasodilatation.

The protective effect of both NOS inhibitors was also demonstrated in our study on exposure to a hyperoxic-hypercapnic mixture (oxygen with 5% CO_2 at a pressure of 0.5 MPa). As already known in human and animal models (8, 16), hypercarbia, which is common in diving and in various pathological clinical conditions, augments the neurotoxic effects of HBO by shortening the duration of the latent period to the onset of seizures and decreasing the threshold toxic pressure of oxygen. It is accepted that this increased toxicity on exposure to elevated concentrations of CO_2 is caused by cerebral vasodilatation (8, 16), thus increasing the inflow of reactive oxygen species or other humoral toxic mediators to the brain. In various studies NO has been implicated in the mediation of hypercapnic cerebral vasodilatation (5, 12), and NOS inhibitors were reported to reduce the increase in cerebral blood flow elicited by hypercapnic challenges (5, 27). We therefore suggest that the protective effect of NOS inhibitors given before exposure to HBO with 5% CO_2 might also be explained by decreased hypercapnia-induced NO production, reducing cerebral vasodilatation.
As seen in Fig. 6, there are considerable differences in the relative effectiveness of the two NOS inhibitors studied herein in protection against hyperoxia alone and against exposure to breathing a hyperoxic-hypercapnic mixture. L-NAME was more effective on exposure to pure HBO than on exposure to the hyperoxic-hypercapnic mixture (a 123% increase in the duration of the latent period to the onset of seizures compared with 49%, respectively). In contrast, 7-NI, a selective cerebral NOS inhibitor, was much more effective in hyperoxic-hypercapnic exposure than in pure oxygen (223% increase in the latent period vs. 71%, respectively). The cerebral NOS inhibitor 7-NI produced almost complete protection (P < 0.0001 compared with its vehicle) against the hypercapnic mixture, which is the best neuroprotection we have ever achieved with any compound tested against HBO seizures.

The dramatic protective effect of the cerebral NOS inhibitor in hyperoxic-hypercapnic exposure, as well as its superior protective effect compared with the systemic NOS inhibitor L-NAME, suggests that CO2 enhances CNS oxygen toxicity not only by inducing vasodilatation but also via direct neural mechanisms causing an increased sensitivity to HBO. The second explanation for the protective effect of NOS inhibitors may be related to the prooxidative actions of NO. It is well accepted that exposure to HBO increases the generation of reactive oxygen species (superoxide and hydroxyl radicals, hydrogen peroxide, and so on) (14, 29). Under these conditions of increased tissue levels of oxygen free radicals, NO may augment cytotoxicity. NO and superoxide radical react rapidly to form peroxynitrite anion (2). Peroxynitrite is directly cytotoxic and also decomposes into other toxic species in the tissues (31). Recent studies suggest that CO2 modulates most of the reactions of peroxynitrite in biological systems (26), acting as a true catalyst of the decomposition of peroxynitrite, a finding that can explain the increased sensitivity to HBO on exposure to hypercapnic-hyperoxic mixtures. It has also been shown that NO reacts with hydrogen peroxide to produce singlet oxygen, a highly reactive and toxic form of oxygen radical (23). Hence, treatment with NOS inhibitors such as L-NAME and 7-NI, leading to a decrease in tissue NO levels, may attenuate the generation of cytotoxic intermediates formed by the interaction of NO with oxygen-reactive species. We also cannot exclude direct antioxidant effects of NOS inhibitors, unrelated to NO production, such as have recently been shown with L-NAME, which reduce endothelial cell injury induced by hydrogen peroxide (28).

A third mechanism by which NOS inhibitors protect against HBO-induced seizures may be related to the neuromodulatory effects of NO, such as increasing the release of the potentiating neurotransmitters glutamate and aspartate (17). These effects of NO might explain the antiepileptic effect of L-NAME in other experimental models of epilepsy, such as pentylenetetrazol-induced seizures (24).

Increasing tissue NO levels is an alternative approach that can enlighten the role of the NO-L-arginine pathway in hyperoxia-induced seizures. We selected the NO donor SNAP and L-arginine, which is the physiological precursor for NO production. On the basis of our experiments with NOS inhibitors, we assumed that increasing tissue levels of NO by pretreatment with either L-arginine or the NO donor would enhance the development of HBO-induced toxicity because of the vasodilatatory, prooxidant, and neuromodulatory activity of NO. Pretreatment with the NO donor SNAP indeed confirmed our expectations. The significant NO-induced decrease in the duration of the latent period to the onset of seizures is expressed in Fig. 5 by an increase in the speed of development of seizures. In contrast, pretreatment with L-arginine induced a dose-related protective effect against hyperoxic seizures.

D-arginine, the inactive isomer of L-arginine, failed to induce any significant change in the latent period to convulsions, both when given alone and in combination with L-NAME. The rather unexpected specific effect of L-arginine can be explained by both NO-dependent and NO-independent mechanisms (10).

It is well known that NO exerts opposing effects, depending on the tissue redox state (18), on differences between species and on the concentrations and the source of NO.

By using L-NAME we probably inhibited the vasodilatory, epileptogenic, and prooxidative effects of NO. It is suggested that by giving L-arginine we may have uncovered the opposing effects of NO, namely, its antiepileptic (6), antioxidative (15), and cytoprotective (32) manifestations.

Another possibility that cannot be excluded is that L-arginine protected against hyperoxia-induced seizures via routes that are unrelated to the production of NO. In addition to the NOS pathway, L-arginine is also metabolized to L-ornithine and urea by arginase.

L-Ornithine is further metabolized to polyamines (spermine, putrescine, spermidine) and L-proline. There are reports suggesting possible involvement of L-arginine and polyamines in the pathophysiology of HBO toxicity (7, 20). In this regard, it has been demonstrated that polyamines inhibit the oxidation and peroxidation of unsaturated fatty acids both in vivo and in vitro, and it is assumed that their role in cellular defense mechanisms against oxygen toxicity is that of oxygen-reactive species scavengers (7). The differences between the effects of SNAP and L-arginine support the hypothesis that at least part of the effect of L-arginine might be mediated through protective mechanisms unrelated to NO.

Because in our study both L-NAME and L-arginine given alone protected rats against hyperoxia-induced seizures, it is not surprising that given together they had no opposing effects in the prevention of CNS oxygen toxicity. Another study in which the protective effect of L-NAME was not reversed by addition of L-arginine has been reported by Shimizu et al. (28) in an oxidative model of H2O2-induced endothelial cell injury.

Altogether, our findings can be explained by the ambiguous and dual face exhibited by NO, which acts...
both as a cytotoxic and a cytoprotective agent. A variety of direct and indirect cytotoxic and cytoprotective effects of NO have been reported in different models of cellular injuries (1, 10). Specific redox-based neuroprotective and neurodestructive effects of NO were reported by Lipton et al. (18). Furthermore, NO has also been shown to specifically exert cytoprotective effects in oxidative stress (32). The balance between the cytotoxic and cytoprotective effects of NO, and the net outcome of these effects in any given pathophysiological condition, seems to depend on myriad factors and mechanisms that are presently only partly elucidated in the specific case of CNS oxygen toxicity. Moreover, it is possible that the mechanisms underlying the protection against HBO-induced seizures may be partly NO dependent and partly NO independent, acting through other mechanisms. Our results support a role for the L-arginine-NO pathway in the pathophysiology of hyperoxia-induced seizures.

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This study was carried out in adherence with the National Institutes of Health guidelines for the use of experimental animals. The opinions and assertions presented are those of the authors and are not to be construed as the official views of the Israel Naval Medical Institute.

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