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ORIGINAL PAPER

Pressure-related Increase of Asymmetric Dimethylarginine Caused by Hyperbaric Oxygen in the Rat Brain: A Possible Neuroprotective Mechanism

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Abstract A decrease in nitric oxide availability in the brain tissue due to the inhibition of nitric oxide synthase (NOS) activity during the early phases of hyperbaric oxygen (HBO) exposure was found to be involved in hyperoxic vasoconstriction leading to reduced regional cerebral blood flow. We hypothesized that the concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), may be an important factor during this hyperoxic vasoconstriction state. Rats were exposed to 1, 2 and 3 atmospheres pure oxygen for two hours. A fourth group of animals served as control. Asymmetric dimethylarginine, L-Arginine and nitrite/nitrate (NO_x) concentrations were measured from deproteinized rat brain cytosols. In rat brains exposed to 3 atmospheres O_2, ADMA and L-Arginine levels were found to be significantly higher and NO_x significantly lower than control levels. Additionally, statistically significant correlations between ADMA and L-Arginine, and ADMA and NO_x concentrations were detected. In conclusion, this is the first study indicating increased ADMA levels in rat brains exposed to HBO. The simultaneously decreased NO_x values suggest that ADMA elevation resulted in NOS inhibition and therefore may be responsible for the early phase hyperoxic vasoconstriction.

Keywords ADMA · Nitric oxide · Hyperbaric oxygen · L-Arginine

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Introduction

Hyperbaric oxygen (HBO) therapy, i.e. breathing of 100% oxygen while inside a pressurized treatment chamber, is used in the management of different diseases and conditions. The efficacy of the treatment depends on either increased dissolved oxygen in the blood (e.g. ischemia-reperfusion damage, soft tissue infections, radiation necrosis, impaired wound healing) or the high exposure pressure (e.g. decompression sickness, air embolism). Edema reduction, impairment of leukocyte adhesion, enhancement of antibacterial mechanisms and stimulation of fibroblast proliferation and neovascularization constitute the majority of the beneficial pharmacological effects of HBO therapy but the basic mechanisms are not well established [1]. However, oxygen under pressure behaves as a drug with both beneficial and toxic effects. The central nervous system (CNS) is a major site of oxygen toxicity at high inspired partial oxygen pressure as manifested by neurological impairment and convulsions. The toxic effect of increased oxygen in cells has been attributed to a proportional increase in the rate of formation of reactive

oxygen species (ROS) such as superoxide anion, singlet oxygen, and hydroxyl radical [2, 3]. Reactive oxygen species may alter functions of biological membranes and enzymes; however, the prime site of ROS attack and its relation to HBO toxicity remain unknown.

Nitric oxide (NO), a multifunctional small radical molecule, is involved in a number of regulatory mechanisms, including vasodilatation, neurotransmission and neuromodulation, inhibition of platelet aggregation, and modulation of leukocyte adhesion [4, 5]. In addition to the role of ROS, recent studies have implicated NO as a mediator of CNS HBO toxicity. One mechanism by which NO may contribute to HBO-induced brain toxicity involves the ability of NO to act as a vasodilator of cerebral vessels and antagonize the vasoconstrictive effects of oxygen. Another mechanism involves the neurotoxic, pro-oxidative action of NO. Nitric oxide is a highly diffusible, relatively nonreactive free radical, that combines very rapidly with oxygen radicals (i.e., superoxide anion) to form the potent oxidant peroxynitrite [6–8].

Nitric oxide has a critical role in neuronal function; however, high levels lead to cellular injury. It is a ubiquitous intracellular messenger synthesized from the guanidino group of L-arginine in a reaction catalyzed by nitric oxide synthase (NOS) [9, 10]. The endogenous methylarginines (MA) asymmetric dimethylarginine (ADMA) and *NG*-monomethyl-L-arginine (L-NMMA) regulate NO production from NOS. Asymmetric dimethylarginine and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferases. Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS is inhibited [11, 12]. The biological significance of MA derivatives has been known since the inhibitory actions of L-NMMA on macrophage-induced cytotoxicity were first demonstrated. It was subsequently determined that these effects were mediated through inhibition of NO formation [13]. This naturally occurring arginine analogue, together with its structural congener ADMA, forms a pair of L-arginine derivatives with the ability to regulate the L-arginine–NO pathway. In fact, these two compounds have been shown to be potent inhibitors of NOS activity [14].

Despite the known role of NO in hyperoxic neurotoxicity, there is still a lack of knowledge regarding the intracellular concentration of methylarginines in the neurons during HBO exposure. Considering the importance of NO in neuronal signaling and, when present in excess, as a mediator of brain injury, it is of great importance to determine the levels of methylarginine in neuronal tissue exposed to HBO. In this study, we aimed to determine ADMA, L-arginine and NO concentrations of rat brains exposed to HBO in a therapeutic range and to elucidate a possible relationship of this NOS inhibitor with the hyperoxic state.

Experimental procedure

Animals and groups

Forty male Sprague-Dawley rats, weighing 200–250 g and obtained at three months of age, were used in the study. The animals were housed in individual cages in a temperature-controlled (22–24°C) room with a 12 h light/dark cycle. All animals were fed a commercial diet during the experiment. They were randomly distributed into four equal groups (n = 10 for each group):

Group 1; control group (without any exposure)
Group 2; normobaric oxygen group (exposed to 1 ATA 100% O_2; ATA = atmospheres absolute)
Group 3; intermediate hyperbaric oxygen group (exposed to 2 ATA 100% O_2)
Group 4; maximal hyperbaric oxygen group (exposed to 3 ATA 100% O_2)

All experimental procedures were carried out in accordance with the regulations of the Gülhane Military Medical Academy Committee on Ethics in the Care and Use of Laboratory Animals.

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and all organic solvents from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade and were prepared each day (except the phosphate buffer) and stored in the refrigerator at +4°C. The reagents were equilibrated at room temperature for 0.5 h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4°C for 1 month.

HBO exposure

An animal hyperbaric chamber (made in Etimesgut Military Equipment Factory, Ankara, Turkey) was used. After the animals were placed into the chamber it was first flushed with 100% O_2 for 1.5 min to raise the O_2 level to >99%. The chamber was then compressed slowly at a rate of 1 ATA/min to the exposure pressure of each group. The ventilation rate during exposure was set as 3–4 l/min. After two hours of exposure, decompression was performed slowly at the same rate. All administrations were initiated

at the same hour in the morning (8 AM) to prevent biological rhythm variability.

Tissue preparation

At the end of HBO exposure the animals were decapitated. The brains were removed immediately and homogenized in nine volumes of 10 mM Tris-HCl buffer (pH 7.5). The homogenates were then centrifuged for 75 min at 90000 g and the supernatant removed and stored at –70°C until use.

Biochemical analysis

Measurement of L-arginine and ADMA was accomplished by HPLC, using the method described by Chen et al [15]. In brief, 20 mg of 5-sulfosalisilic acid (5-SSA) was added to 1 ml brain cytosol and the mixture was left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 2000 g for 10 min. Ten micro liters of the supernatant filtered through a 0.2 μm filter was mixed with 100 μl of derivatization reagent (prepared by dissolving 10 mg o-phthaldialdehyde in 0.5 ml of methanol, and the addition of 2 ml of 0.4 M borate buffer (pH 10.0) and 30 μl of 2-mercaptoethanol) and then injected into the chromatographic system. Separation of arginine and ADMA was achieved with a 150 × 4 mm I.D. Nova-pak C_{18} column had a particle size of 5 μm (Waters Millipore, Milford, MA, USA) using 50 mM sodium acetate (pH 6.8), methanol and tetrahydrofurane as mobile phase (A, 82:17:1; B, 22:77:1) at a flow-rate of 1.0 ml/min. The areas of peaks detected by the fluorescent detector (Ex: 338 nm; Em: 425 nm) were used for quantification. L-Arginine and ADMA levels in brain tissues were expressed as μmol/g-protein. The amount of protein was determined using the method of Lowry et al [16].

Nitrite and nitrate (NO_x) levels of brain homogenates were measured in triplicate after conversion of nitrate to nitrite by nitrate reductase, and nitrite was measured by using the Griess reaction as described by Moshage et al [17]. The intraessay and interassay coefficients of variation were 3% and 7%, respectively. Recoveries of both nitrites and nitrates in our samples were greater than 95%. The final calculated levels were presented as ng/g-protein.

Statistical analysis

Changes in brain concentrations of measured laboratory parameters at different pressure levels of HBO were compared using the Mann Whitney-U test. In addition, Pearson correlation coefficients were calculated for evaluating the relationship between ADMA and L-arginine levels. Values from each group of animals were expressed as mean ± standard deviation. The statistical significance level was chosen as $P < 0.05$.

Results

ADMA and L-arginine levels

Asymmetric dimethylarginine levels were found to be significantly higher than control animals ($P < 0.05$) in rat brains of group 4 exposed to 3 ATA HBO for 2 h, which is the maximal pressure-duration range clinically used [1]. However HBO did not significantly affect ADMA levels ($P > 0.05$) in exposure pressures lower than 3 ATA (group 2 and 3). Similarly, the highest L-arginine concentrations were found in 3 ATA group ($P < 0.05$ for group 4 vs. group 1). Asymmetric dimethylarginine and L-Arginine levels of group 4 were also found to be significantly higher than the normobaric oxygen-exposed group 2 ($P < 0.05$ for both).

Nitrite–nitrate levels

NO_x levels were found to be lowest in 3 ATA group; these levels were significantly lower than control and normobaric oxygen-exposed animals ($P < 0.05$ when compared with both group 1 and group 2).

The detailed ADMA, L-Arginine and NO_x level figures are presented in Table 1.

Correlation analysis

There was a strong positive correlation between ADMA and L-arginine($r = 0.622$, $P < 0.001$; Fig. 1) and negative correlation between ADMA and NO_x concentrations ($r = 0.554$, $P < 0.01$). This relationship is in agreement with the inhibitory effect of ADMA on NOS activity that utilizes L-arginine as its substrate.

Additionally, we calculated the logarithmic (log) L-arginine to ADMA concentration ratio that was previously reported to be positively correlated with the NO production rate [18]. Although no statistical significance

Table 1 ADMA, L-Arginine and NOx levels in brain tissue (Mean ± SD)

Groups	ADMA (μmol/g-prot)	L-Arginine (μmol/g-prot)	NO_x (ng/g-prot)
Group 1 (Control)	0.90 ± 0.08	59.3 ± 6.1	70.8 ± 5.1
Group 2 (1 ATA)	0.91 ± 0.20	56.7 ± 5.2	70.2 ± 3.9
Group 3 (2 ATA)	0.98 ± 0.20	54.9 ± 7.6	64.5 ± 4.7
Group 4 (3 ATA)	1.14 ± 0.08[a, b]	72.8 ± 10.8[a, b]	58.4 ± 5.6[a, b]

[a] $P < 0.05$ vs. group 1 (control), [b]$P < 0.05$ vs. group 2 (1 ATA; normobaric hyperoxia)

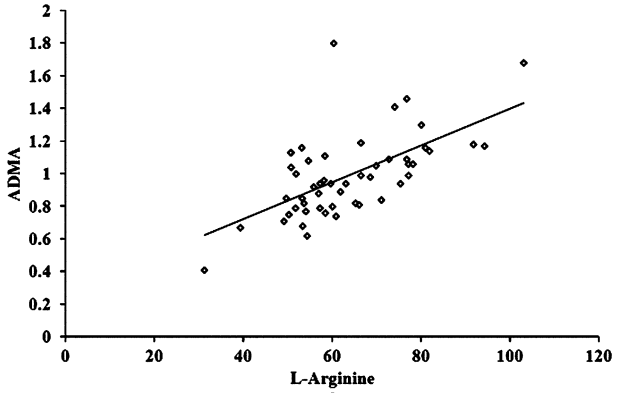

Fig. 1 Correlation of brain ADMA levels with L-arginine concentrations. The estimated Pearson's correlation coefficient of 0.622 was highly significant ($P < 0.001$)

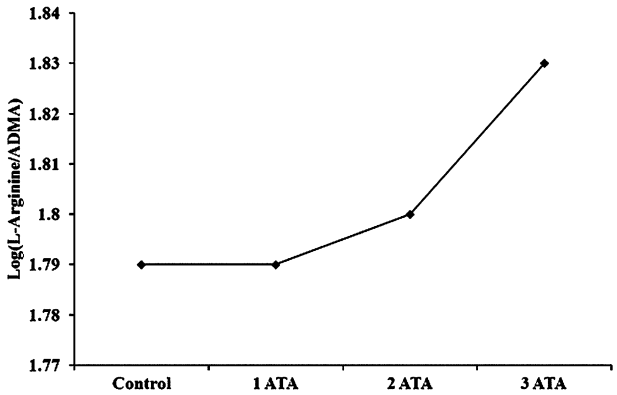

Fig. 2 Logarithmic L-arginine to ADMA concentration ratio. Note that the slight increase with incremental pressure from 1.79 to 1.83 is not significant

was found between study groups, there was a slight increase with pressure elevations (Fig. 2).

Discussion

Despite the wide range of HBO therapy indications, the mechanisms of its therapeutic as well as adverse effects are not fully understood. Most of the investigations on this matter were performed with excessive exposure ranges of HBO longer than 2 h and higher than 2 ATA. The effects of therapeutically used doses of HBO have been less well studied. The Undersea & Hyperbaric Medical Society has defined and limited the use of HBO with a maximal exposure pressure of 3 ATA and duration of 2 h, as used in our study [1].

Breathing oxygen at high partial pressures produces marked hemodynamic and neurological effects. Among the most significant are cerebral vasoconstriction and hyperoxia-induced seizures [5, 19]. At a certain point in time, the initial oxygen-induced vasoconstriction is reported to be impaired and an increase in cerebral blood flow (CBF) is observed [20]. It has been suggested that this increase in cerebral blood flow might be correlated with the development of hyperoxia-induced seizures. Additionally, it is well accepted that reactive oxygen species, which are produced in excess on exposure to HBO, mediate the hyperoxic insult [2]. The exact mechanisms responsible for the hyperoxia-induced hemodynamic changes and neurological manifestations as well as the specific biological messengers responsible for the various expressions of the hyperoxic insult still remain unknown.

NO from endothelial or neuronal NO synthases (eNOS or nNOS) may contribute both to the cerebrovascular responses to oxygen and potentially to the peroxynitrite-mediated toxic effects of HBO on the central nervous system [21]. A variety of direct and indirect cytotoxic and cytoprotective effects of NO have been reported in different models of cellular injuries [22]. Specific redox-based neuroprotective and neurodestructive effects of NO were reported by Lipton et al [18]. The balance between the cytotoxic and cytoprotective effects of NO, and the net outcome of these effects in any given pathophysiological condition, seem to depend on myriad factors and mechanisms. The implication of NO as a mediator of CNS HBO toxicity is supported by two lines of evidence: (i) nitric oxide synthase (NOS) inhibitors, which block NO production and protect against hyperoxic seizures [6, 7], (ii) increased NO concentrations in the brain during exposure to HBO [23, 24]. It is still not clear whether NOS inhibitors protect against hyperoxic brain toxicity by decreasing NO concentration and reducing peroxynitrite formation or by simply lowering oxygen delivery to tissues as a result of the reduced cerebral blood flow [25]. In the present study, we found that ADMA as well as L-arginine levels increased significantly with 3 ATA HBO exposure accompanied by an decrease in NO_x levels. No hyperoxic seizures were observed in the animals during exposure and although CBF was not measured, one can say that the end-point of the session coincided with the early phase state of HBO exposure where vasoconstriction was reported by previous investigators [26–28]. Those studies suggested that the relative lack of NO activity contributes to decreased CBF during hyperoxia and they implicate NO metabolism as a factor in hyperoxic vasoconstriction.

The endogenous MA compounds including ADMA and L-NMMA are potent competitive inhibitors of NOS and are released upon protein degradation. It has been demonstrated that the methylarginine levels in isolated neurons and in the intact brain are sufficient to regulate NO production from nNOS [14]. In our study we showed the increased ADMA concentrations that may be one of the reasons of hyperoxic vasoconstriction via decreased NOS

activity in the rat brains after exposure to HBO. Using the same HBO procedure (3 ATA for 2 h), Ito et al reported elevated L-arginine levels in rat brains. They measured arginase and arginine:glycine amidino transferase activities in order to reveal these elevated arginine levels and found a decreased arginase activity that may cause higher arginine concentrations in the rat cerebral cortex. However they did not determine NOS inhibition which may also contribute to the elevated arginine levels [29]. Demchenko et al showed reduced CBF percentages of different rat brain sites during 75 min of 4 ATA HBO exposure. In the same group, the NO_x levels showing NOS activity indirectly were found to be lower than the control group during the first hour [20]. The increased ADMA level in our study is also compatible with these findings. Elevated ADMA levels may provide a critical feedback mechanism to protect the brain against a vicious cycle whereby excess NOS activation and NO production from injured neurons would otherwise lead to uncontrolled expanded injury of neighboring neurons. Nevertheless, we have to concede that the lack of NOS measurement in the present study restricts further detailed interpretation.

Oxidative stress has been shown to increase the activity of arginine methylating and ADMA degrading enzymes leading to increased ADMA concentrations [30]. Our previous studies showed that exposing rats to HBO at 3 ATA for 2 h results in a significant increase in lipid peroxidation in the rat brain that can be effectively prevented by antioxidant administration [31, 32]. This finding provides a simple explanation for the increased ADMA levels of the present study. Nevertheless, the mechanisms which lead to increased NO concentrations and CBF in the prolonged state of HBO exposure are still unexplained. Further studies focused on the arginine methylation pathway may be able to elucidate this subject.

The slight elevation of log (L-arginine/ADMA) with pressure increase is another interesting finding of the study (Fig. 2). One can alternatively think that unchanged ratios between our study groups show indirectly unchanged NO production rates as well. This means that NO production is held within normal ranges with short exposure to HBO probably due to the elevated ADMA levels. However, we believe that additional studies with longer HBO exposure durations should be performed for a better interpretation of this finding.

In conclusion, the present study provides the first direct evidence regarding the elevated levels of ADMA in rat brains exposed to therapeutic HBO at maximal limits. The elevated ADMA concentration seems to be responsible for the hyperoxic vasoconstriction in the early phase of HBO exposure. Due to the basis of the double-edged nature of NO, this inhibition may cause some harmful as well as protective effects in the central nervous system which need to be elucidated with additional studies. Further investigations should be performed to address how the ADMA regulatory enzymes, protein-arginine methyltransferase (PRMT) and dimethylarginine dimethylaminohydrolase (DDAH), function to regulate intrinsic ADMA levels under both normal and hyperoxic conditions. Additional NOS measurement involving studies focused on exposure duration related effects of HBO should complement the present results.

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