Background
Oxygen under pressure (hyperbaric oxygen or HBO2) induces the reactive nitrogen species (RNS) formation in the brain involved in central nervous system (CNS) O2 toxicity. In particular, nitric oxide (NO) has been implicated in the pathogenesis of CNS O2 toxicity because inhibition of nitric oxide synthase (NOS) isoforms with N,N-nitro-L-arginine methyl ester (L-NAME) or N-nitro-L-arginine (L-NNA) protects rats and mice against O2 convulsions [3-6,15,17]. HBO2 exposure at 5 ATA of sufficient duration to trigger EEG spikes is accompanied by biphasic changes in rCBF, wherein initial vasoconstriction is followed by increase in blood flow before the onset of seizure activity [1,6,7,16]. These changes in rCBF correlate with extracellular NO availability: increases in rCBF during HBO2 exposure are associated with large increases in NO preceding EEG spikes [8,9]. Thus NO production during prolonged HBO2 exposure may be responsible for the development of CNS O2 toxicity because hyperoxic hyperemia, O2 toxicity-related EEG spikes and NO overproduction were prevented by NOS inhibition with L-NAME, which is counteracted by treatment with L-arginine [3,5,7,15]. The origins of HBO2-induced NO augmentation are uncertain since there are multiple sources of NO production in the brain: vessels endothelium, pre-vascular nerves and neuronal/glial cells [12,14]. However it has been difficult to evaluate specific contributions of endothelial or neuronal NO in CNS O2 toxicity. Knockout mice that lack eNOS or nNOS isoforms provide an opportunity to address this problem [10,11]. In the present study we measured the temporal profile of interstitial NO availability and peroxynitrite formation during HBO2 exposure and to correlate them with time dependent changes in rCBF and EEG discharges as an early manifestation of CNS O2 toxicity. We have used mutant mice lacking eNOS or nNOS (-/-) to assess relative contribution of neuronal or endothelial NO in CNS O2 toxicity.

Materials and Methods
Wild-type (C57BL/6) and eNOS-/- or nNOS-/- mutant mice (8-10 weeks of age) anesthetized with urethane (800 mg/kg, i.p.) were intubated, and the femoral artery and vein catheterized to monitor blood pressure, withdraw blood samples and administer pharmacological agents. The animals were placed in a stereotaxic frame and hydrogen/oxygen sensitive microelectrodes of platinum wire (100 µm diameter) inserted through a burr hole into striatum (ST) and parietal cortex (PC) to measure rCBF and brain PO2. A microdialysis probe (CMA/10: 2 mm membrane length, 0.24 mm O.D., CMA/ AB, Sweden) was placed into ST and perfused continuously with artificial CSF using a microinjection pump. Samples of dialysate were collected automatically every 15 minutes and used to measure NOx as an index of NO availability and 3-nitrotyrosine as index of peroxynitrite (ONOO-) formation [2,13]. Stainless steel screws were inserted into the skull over the left and right parietal cortex for EEG recording. The mice were paralyzed with pancuronium bromide (0.5 mg/kg, i.p.) and ventilated with 30% oxygen.

These animals were placed in the hyperbaric chamber contained the respirator, EEG amplifier, blood pressure transducer and amplifier, heating pad, infusion pump and the cerebral microdialysis set-up. To maintain constant anesthesia, urethane (0.2 g/kg/hr) and pancuronium bromide (0.1 mg/kg per hr) were infused continuously by pump. Once the mice were stable and arterial blood gases and body temperature in the physiological range, three H2 clearance curves were recorded to measure resting rCBF and brain PO2. A microdialysis probe (CMA/10: 2 mm membrane length, 0.24 mm O.D., CMA/ AB, Sweden) was placed into ST and perfused continuously with artificial CSF using a microinjection pump. Samples of dialysate were collected automatically every 15 minutes and used to measure NOx as an index of NO availability and 3-nitrotyrosine as index of peroxynitrite (ONOO-) formation [2,13]. Stainless steel screws were inserted into the skull over the left and right parietal cortex for EEG recording. The mice were paralyzed with pancuronium bromide (0.5 mg/kg, i.p.) and ventilated with 30% oxygen.
Cerebral blood flow was calculated (in ml/g/min) from H2-clearance curves using a WINDAQ data acquisition and analysis system (D-1200 AC, DATAQ Inst., OH). For brain PO2 measurements, electrodes were calibrated with 100% N2 and 20% O2 at 1 ATA and 100% O2 at 1 ATA and 5 ATA. Absolute values for brain tissue PO2 (mm Hg) were calculated from the calibration curve for each electrode. Statistical analyses were performed by Student’s t-test or analysis of variance followed by a post hoc comparison (Fisher’s exact test). Values are provided as mean ± SEM.

Results
Resting rCBF, brain PO2 and physiological variables did not significantly differ between in WT, nNOS— and eNOS— animals. Because endothelium-derived vascular relaxation is a physiological response to stimuli that activate NO signaling, we determined the extent to which eNOS or nNOS contribute to cerebrovascular reactivity in response to NOS inhibition, NO donor, acetylcholine and superoxide dismutase (SOD) administration. L-NAME treatment (30 mg/kg, i.p.) significantly reduced rCBF in WT and nNOS— mice, but eNOS— mutants showed smaller decreases in rCBF. The NO donor, PAPA NONOate (0.05 mg/kg), increased rCBF in all three groups of animals, but rCBF responses were significantly less in eNOS— mutants than in WT and nNOS— mice. In mice given acetylcholine (Ach), a peak rCBF response occurred within 10 min and was augmented significantly when a constant dose of Ach (0.05 mg/kg) was administered to WT and nNOS— mice, but not to eNOS— mice. After treatment with SOD1 (25 U/g body weight), rCBF increased in all groups of mice, but significantly less in the eNOS— strain compared to nNOS—. L-NAME completely blocked the rCBF responses to Ach and SOD1 in all groups of animals.

We assessed cerebrovascular responses to extreme hyperoxia exposing WT and NOS— mutant mice to O2 at 5 ATA at physiological arterial PCO2 and pH values. In WT mice the hyperbaric oxygen exposure reduced rCBF over 30 min by 19±7% (P<0.05) in cortex and by 25±8% (P<0.01) in striatum but by 45 min rCBF had returned to pre-exposure levels and then over the next 15 min increased above control baseline by 48±9% and 108±24% in cortex and striatum, respectively. In nNOS— mutants, rCBF changes during HBO2 exposure were similar to WT animals. Mice lacking nNOS also showed significant cerebral vasocostriction the first 30 min followed by hyperemia. In contrast, eNOS— mice did not show cerebral vasocostriction at 5 ATA and after 30 min HBO2 exposure rCBF increased only by 15±6% and 17±7% in the two brain regions.

At 5 ATA, HBO2 produced marked differences in EEG pattern in all three strains of mice. Paroxysmal EEG discharges of single or multiple spiking activity of amplitude more than 100 µV were observed after exposure to HBO2 for 41±4 min in WT mice, 49±7 min for nNOS— mice and 54±1 min for the eNOS— animals. The different rCBF responses to HBO2 in the three strains of mice support NO-mediated involvement in CNS O2 toxicity. This concept was also supported by the effects of HBO2 on rCBF in the three strains of mice after L-NAME treatment. During exposure to HBO2 at 5 ATA, rCBF did not increase and abnormal EEG spikes were not observed. Intracerebral microdialysis data revealed dynamic changes in interstitial NOx and 3-nitrotyrosine during HBO2 at 5 ATA. In WT mice, brain interstitial NOx decreased significantly within 30 min at 5 ATA, but after 45 min NOx rose to above control levels. In eNOS— mice basal NOx decreased during the first 30 min of HBO2 but less so than in WT mice. In the nNOS— mice, striatal NOx did not increase significantly over 60 min at 5 ATA indicating HBO2-related differences in NO availability varied with NOS activity in the different strains. Brain interstitial 3-nitrotyrosine content, unlike NOx, accumulated steadily but at different rates during HBO2 at 5 ATA in WT and the NOS— mutant mice. Marked increases in 3-nitrotyrosine were detected in WT and eNOS— mice consistent with ONOO— formation. The accumulation of 3-nitrotyrosine was much less in striatum of nNOS— mice.

Conclusions
Mice lacking endothelial or neuronal NOS exhibit different cerebrovascular responses and tolerance to extreme hyperoxia. These result suggest that eNOS-dependent constitutive NO production plays a key role in the escape from HBO2-mediated vasoconstriction and the hyperemia preceding oxygen seizures. Meanwhile, nNOS may be involved in CNS O2 toxicity by contributing to nitrosative stress, e.g. ONOO— formation.
References


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