Regulation of the Brain's Vascular Responses to Oxygen

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Abstract—The mechanism of oxygen-induced cerebral vasoconstriction has been sought for more than a century. Using genetically altered mice to enhance or disrupt extracellular superoxide dismutase (EC-SOD, SOD3), we tested the hypothesis that this enzyme plays a critical role in the physiological response to oxygen in the brain by regulating nitric oxide (NO·) availability. Cerebral blood flow responses in these genetically altered mice to changes in P_{O_2} demonstrate that SOD3 regulates equilibrium between superoxide ($\cdot O_2^-$) and NO·, thereby controlling vascular tone and reactivity in the brain. That SOD3 opposes inactivation of NO· is shown by absence of vasoconstriction in response to P_{O_2} in the hyperbaric range in $SOD3^{+/+}$ mice, whereas NO-dependent relaxation is attenuated in $SOD3^{-/-}$ mutants. Thus, EC-SOD promotes NO· vasodilation by scavenging $\cdot O_2^-$ while hyperoxia opposes NO· and promotes constriction by enhancing endogenous $\cdot O_2^-$ generation and decreasing basal vasodilator effects of NO·. **(*Circ Res*. 2002;91:●●●-●●●.)**

Key Words: cerebral blood flow ■ hyperoxia ■ superoxide dismutase ■ nitric oxide ■ peroxynitrite

A physiological role for extracellular superoxide dismutase (EC-SOD, SOD3), which catalyzes superoxide ($\cdot O_2^-$) dismutation to hydrogen peroxide and oxygen (O_2) extracellularly, has never been demonstrated experimentally.[1–5] Because of relatively low content in most, SOD3 was initially presumed not to be an efficient antioxidant enzyme.[6] Actually, SOD3 is highly expressed in blood vessels where it constitutes up to 70% of aortic SOD activity.[7] SOD3 activity in arterial wall is approximately 10-fold higher than in other tissues and present at roughly the same activity as SOD1.[8,9] The SOD3 tetramer contains Cu, Zn, and a positively charged heparin domain linking it to extracellular matrix and cell surfaces.[4,8,10] It is located strategically between endothelium and smooth muscle where $\cdot O_2^-$ is generated[11,12] and dilatory NO· is present.

Endothelial cells release $\cdot O_2^-$,[13] which is converted to H_2O_2 or reacts with NO· to generate the strong oxidant, peroxynitrite ($ONOO^-$).[14] By reacting rapidly with NO·, extracellular $\cdot O_2^-$ should decrease biologically available NO·, which diffuses from endothelium, erythrocytes, and vascular nerves to smooth muscle. Catalytic removal of $\cdot O_2^-$ from this domain should prevent inactivation of NO· by $\cdot O_2^-$ and increase its availability for vasodilation. Hence, we have proposed a primary function for EC-SOD in blood vessels to minimize microenvironment concentrations of $\cdot O_2^-$ and prevent inactivation of NO·.[15] However, the validity of this hypothesis and the actual importance of SOD3 to NO·-mediated control of vascular tone are not known. In vitro studies have reported antagonism of NO·-dependent vasorelaxation after exposure to exogenous $\cdot O_2^-$ or chemical inhibition of CuZn-SOD,[16–20] but no physiological studies have yet linked SOD3 function to NO·-regulated blood flow in vivo. Genetically altered animals with increased or absent expression of SOD have made it possible to investigate this hypothesis in the brain.

Using mice that overexpress (+/+) or lack (−/−) SOD3, we performed experiments to determine whether the enzyme regulates the balance between $\cdot O_2^-$ and NO· in the brain, and how the two radicals affect basal tone and vasoconstrictor responses to increasing O_2 concentrations. $SOD3^{+/+}$ and $SOD3^{-/-}$ mice were compared for blood flow changes in specific brain regions (rCBF) in response to P_{O_2} changes. rCBF was correlated by cerebral microdialysis with NO· release (NO_x), and a biochemical marker, 3-nitrotyrosine, to assess $ONOO^-$ as a product of SOD3-controlled reactions between $\cdot O_2^-$ and NO.

Materials and Methods

Mice Strains

The SOD3 mutant strains have been described and characterized.[15,21] $SOD3^{+/+}$ mice were generated by microinjecting nuclei of fertilized oocytes from (C57BL/6) (C3H) F1 female mice with cDNA of human SOD3 containing a β-actin promoter. Mice carrying the transgene were identified by polymerase chain reaction (PCR) amplification of the human SOD3 gene and backcrossed into (C57BL/6×C3H) F1 mice. SOD3 transgenic mice were out-bred to improve fertility. Thus, the background of the $SOD3^{+/+}$ was not identical to the BL/6 WT strain for null mice (see blood flow methods). Overexpression of EC-SOD in the brains and aortas of the transgenic mice was verified by activity analysis using xanthine/xanthine oxidase and cytochrome *c*. $SOD3^{+/+}$ mice had a 5-fold increase in brain and 2-fold in aortic EC-SOD activity. Other antioxidant enzyme levels were not changed. Activity measurements were not done on isolated mouse brain vessels because tissue was insufficient for assay.

The EC-SOD gene was disrupted in Neo^r-generated SOD3-deficient mice ($SOD3^{-/-}$). Null mice were bred into WT C57BL/6 mice to yield heterozygotes, which were crossed to produce homozy-

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TABLE 1. Blood Pressure and rCBF After L-NAME

		Before L-NAME rCBF					After L-NAME rCBF			
Group	MABP	PC	HC	CP	SN	MABP	PC	HC	CP	SN
WT	91±8	78±7	73±6	79±8	91±9	109±10	60±5	57±6	59±6	69±6
$SOD3^{-/-}$	93±9	77±6	68±6*	74±7*	87±9	113±10	61±5	55±7	60±5	68±7
$SOD3^{+/+}$	89±8	84±8	81±7	88±9	96±10	115±11	64±6	63±8	61±6	74±8

MABP indicates mean arterial BP (mm Hg) before and 30 minutes after L-NAME; rCBF (mL/100g per min) in parietal cortex (PC), hippocampus (HC), caudate putamen (CP), and substantia nigra (SN) before and 30 minutes after L-NAME. WT is averaged data set for two strains (n=14).

Values are mean±SE; *$P<0.05$ vs $SOD3^{+/+}$. All MABP and rCBF values after L-NAME are significantly different than before L-NAME (no symbols).

gous knockout and WT breeding pairs. Control mice were derived from WT pairs, whereas SOD3 deficient mice were derived from knockout breeding pairs. The genetic identity of breeders was identified by PCR using unique primers for SOD3 and Neo^r. Homozygote SOD3 mice have no brain SOD3 but normal activities of other SOD isozymes. $SOD3^{-/-}$ mice have a normal phenotype.

Animal Studies

The Institutional Animal Care and Use Committee approved the protocol. At 10 to 12 weeks of age, mice were anesthetized with urethane (800 mg/kg, IP) followed by continuous infusion of 1/4 initial dose/hour by IP pump. Anesthetized mice underwent tracheotomy and ventilation (tidal volume=0.4 mL, rate 110/min) with 30% O_2 (balance N_2) and paralysis (pancuronium 0.1 mg/kg, IV). The head was fixed in a stereotaxic frame, and hydrogen/oxygen-sensitive microelectrodes inserted through a burr hole into substantia nigra (SN), caudate putamen (CP), hippocampus (HC), and/or parietal cortex (PC). Electrodes were made of platinum wire of 100-μm diameter insulated with epoxy except 1.0 mm at the pointed tip (15 to 20 μm) covered by Nafion to prevent fouling. In some mice, a microdialysis probe (CMA/10: 2-mm membrane, 0.24-mm OD, CMA/AB, Sweden) was placed into CP and perfused with artificial CSF at 2 μL/min using a microinjection pump. For EEG recording, stainless steel screws were inserted into the skull symmetrically over parietal cortex.

Regional Cerebral Blood Flow and Brain Po_2

Regional cerebral blood flow (rCBF) was measured by a H_2 clearance method.[22] Platinum electrodes were inserted into brain and reference Ag-AgCl electrodes attached to the base of the tail. H_2 (2.5%) was added via the respirator for 60 seconds then switched to the base breathing gas and the H_2-washout curve recorded. Absolute values of CBF ($mL \cdot g^{-1} \cdot min^{-1}$) were calculated by initial slope using a WINDAQ data system (DATAQ Inst) and Mathematica 3.0 software (Wolfrom Research, Inc) with minor modification.[22] CBF data from the two WT strains, C57/BL6 (n=6) and C57BL6/C3H F1 (n=9), were compared for baseline flow and responses to HBO_2, CO_2, or L-NAME. There were no significant differences in the mean values; therefore, nontransgenic C57BL6/C3H F1 littermates were used as WT unless indicated otherwise.

Brain Po_2 during HBO_2 exposures was measured continuously in CP of rats using the same microelectrodes as for rCBF measurements. Po_2 electrode calibration was performed with 100% N_2 and 20% O_2 at 1 ATA and 100% O_2 at 1 ATA and 5 ATA. Absolute values for brain Po_2 were calculated from a calibration curve for each electrode.

Measurement of Interstitial $NO^{\cdot}$ and Peroxynitrite

Extracellular $NO^{\cdot}$ was estimated from the microdialysis yield of nitrite and nitrate (NO_x).[23] After system equilibration, dialysate was collected every 15 minutes before and during HBO_2. Samples were assayed for NO_x after catalytic reduction to NO gas and chemiluminescence detection. Nitrite and nitrate signals, quantitatively linear between 25 and 500 pmol/25 μL, were used for calibrations. Recovery efficiency for the dialysis probes were 31±6% and 29±8% for NO_2 and NO_3, respectively. The data were expressed as [μmol/L] nitrite and nitrate (NO_x). The amount of 3-nitrotyrosine (3-NTyr) in dialysate was measured relative to tyrosine by HPLC and electrochemical detection in timed samples and retention time compared with authentic standards. 3-NTyr values were expressed as 3-NTyr/Tyr ×100.

Hyperbaric O_2 Exposures

Hyperbaric O_2 (HBO_2) exposures was used to maximize O_2-induced vasoconstriction in the brain, which in mice occurs at 4 to 5 atmospheres absolute (ATA). Mice were instrumented and placed in a chamber containing the respirator, EEG amplifier, pressure transducer and amplifier, heating pad, and microdialysis system. After placing electrodes in the brain, the animals were recovered for 60 minutes. For microdialysis studies, the recovery period was 2 hours. Once the mice were stable and blood gases and temperature in the physiological range, three control H_2 clearance curves were recorded and three dialysate samples collected. The respirator was connected to 100% O_2 and the chamber compressed to a maximum pressure of 5 ATA at 0.6 ATA per min. During HBO_2, rCBF was measured every 15 minutes using a remotely operated pneumatic system for H_2 delivery. Brain Po_2, EEG and blood pressure were monitored continuously. Body temperature was maintained between 36° and 37°C. Dialysate was collected every 15 minutes and NO_x and 3-NTyr measured later. Arterial blood was taken at 1 ATA to determine Pao_2, $Paco_2$, and pH while the mice breathed 100% O_2.

Experimental Design

The hypothesis was tested first by evaluating differences in basal rCBF and responses to pharmacological agents known to alter brain $NO^{\cdot}$ and $\cdot O_2^-$ availability. These studies were conducted in mice breathing 30% O_2 (balance N_2) at sea level. Then, time-dependent measurements of rCBF, EEG, and physiological variables were made in mice exposed to HBO_2 at 3 and 5 ATA. Timed accumulation of NOx and 3-NTyr was correlated with rCBF responses and EEG activity.

Statistical Analysis

Group values are given as mean±SE. Group sizes for mutant strains were 6 to 9 animals each. Changes in rCBF and physiological variables were compared using ANOVA followed by post hoc comparison (Fisher's exact test) or unpaired *t* test using computer software. A value of $P<0.05$ was accepted as significant.

Results

Modulation of Basal CBF by EC-SOD

Using hydrogen clearance, rCBF was measured in WT, $SOD3^{-/-}$, or $SOD3^{+/+}$ mice before and after interventions designed to unbalance the equilibrium between $\cdot O_2^-$ and $NO^{\cdot}$. Resting rCBF in WT and mutant strains were similar in the same brain regions, but lower absolute values were observed in $SOD3^{-/-}$ than $SOD3^{+/+}$ mice in CP and HC (Table 1). CBF responses were statistically identical in the inbred and out-

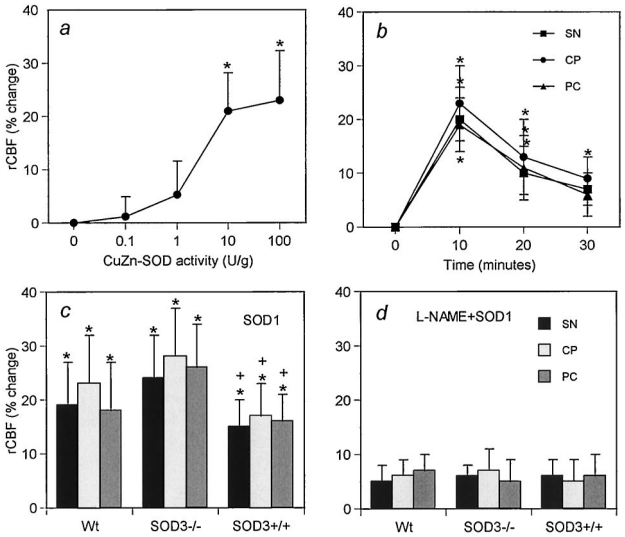

Figure 1. CBF in CuZn-SOD in WT and SOD3 mutant mice. a, Dose-response curve of rCBF in caudate of WT mice after CuZn-SOD injection (0.1 to 100 U/g body weight IV). *$P<0.05$ vs pretreatment. b, Time-course of rCBF in substantia nigra (SN), caudate (CP), and parietal cortex (PC) of WT mice after CuZn-SOD (10 U/g, IV). *$P<0.05$ vs pretreatment. c and d, CuZn-SOD and rCBF in SN, CP, and PC in pooled WT, $SOD3^{-/-}$, and $SOD3^{+/+}$ mice. c, CBF responses to CuZn SOD are greater in $SOD3^{-/-}$ mice than $SOD3^{+/+}$ mice. *$P<0.05$ vs pretreatment; +$P<0.05$ vs $SOD3^{-/-}$. d, rCBF responses to CuZn-SOD after NOS inhibition with L-NAME (30 mg/kg, IP) were attenuated equally in both mutants and WT mice.

bred WT mice (data not shown); therefore, these values were pooled into one control group of 14 animals. To determine whether SOD3 modulates NO-mediated basal tone, we measured rCBF after treating mice with NOS inhibitor, L-NAME to block endothelial and neuronal NO synthesis. rCBF was reduced significantly in an L-arginine–dependent manner in all groups of L-NAME–treated mice, but $SOD3^{+/+}$ mice showed larger decreases in rCBF in selected brain regions (Table 1). These data indicate that basal tone in brain vessels depends on both NO availability and SOD3 activity.

To confirm this assertion we measured changes in rCBF after infusing mice intravenously with a high concentration of SOD1 (10 000 U/kg body weight) to reduce [$\cdot O_2^-$] at the endothelial surface. Preliminary studies showed that active but not inactive SOD1 produced dose- and time-dependent increases in rCBF in WT mice: rCBF responses were reproducible and statistically significant after SOD1 infusion and reached maximal amplitude within 10 minutes (Figures 1a and 1b). This SOD1 dose and time was used for rCBF measurement in mutant mice. In mice treated with SOD1, rCBF in SN, CP, and PC increased significantly in WT, $SOD3^{-/-}$, and $SOD3^{+/+}$ mice, but significantly less in $SOD3^{+/+}$ than $SOD3^{-/-}$ mice (Figure 1c). Because these increases in rCBF after SOD1 treatment could reflect either an increase in $NO^{\cdot}$ availability in vessels due to $\cdot O_2^-$ removal or a dilator effect of the enzyme, we tested these possibilities by treating the mice with L-NAME to inhibit endothelial and neuronal NOS. Infusion of SOD1 into WT and mutant mice pretreated with L-NAME had no effect on rCBF (Figure 1d), suggesting SOD1 altered rCBF by preventing $\cdot O_2^-$-mediated inactivation of $NO^{\cdot}$. $SOD3^{-/-}$ mice have higher extracellular levels of $\cdot O_2^-$ compared with $SOD3^{+/+}$ mice, thus SOD1 infusion has less influence on the equilibrium between $\cdot O_2^-$ and $NO^{\cdot}$ in $SOD3^{+/+}$ animals.

Functional Implications of EC-SOD in Endothelium-Derived Relaxation

Endothelium-derived relaxation is a physiological response of vessels to stimuli that activate $NO^{\cdot}$ signals.[24,25] If $\cdot O_2^-$ opposes endothelium-derived relaxation, then NO-mediated cerebrovascular responses will depend critically on vascular SOD3 activity. To determine the extent to which SOD3 contributes to endothelium-derived vasodilation, we compared rCBF responses in $SOD3^{+/+}$ and $SOD3^{-/-}$ mice to an NO donor and to other stimuli that operate via NO-dependent mechanisms. Representative data are shown in Figure 2.

The NO donor PAPA NONOate (at ≥0.1 mg/kg) increased rCBF in WT and both genetic strains of mice, but rCBF responses in $SOD3^{+/+}$ mutant were more pronounced and significantly greater than that of $SOD3^{-/-}$ mice (representative data in Figure 2a). At higher NONOate concentrations, $SOD^{-/-}$ mice also were found to have attenuated CBF responses relative to the WT, but these infusions caused hypotension in $SOD^{+/+}$ mice (data not shown).

The role of EC-SOD in vascular CO_2 reactivity was examined by measuring the rCBF responses in WT and mutant mice to inhaled CO_2. The use of 5% CO_2 produced rCBF increases on the flat part of the CO_2 response curve and relatively rapid reversal of the CBF effect allowed multiple interventions per animal (Figure 2b). WT and both genetically altered mouse strains showed rCBF increases of 17% to 87% to 5% CO_2 relative to baseline, but the greatest increases (in striatum) were observed in $SOD3^{+/+}$ mice. This response was greater than that of $SOD3^{-/-}$ mutants (Figure 2b). Increases in rCBF to inhaled CO_2 were attenuated significantly and to approximately the same extent in WT and both mutant strains pretreated with L-NAME (data not shown). In mice given acetylcholine, arterial blood pressure decreased slightly and transiently for ≈5 minutes followed by a peak rCBF response within 10 minutes. The peak response was

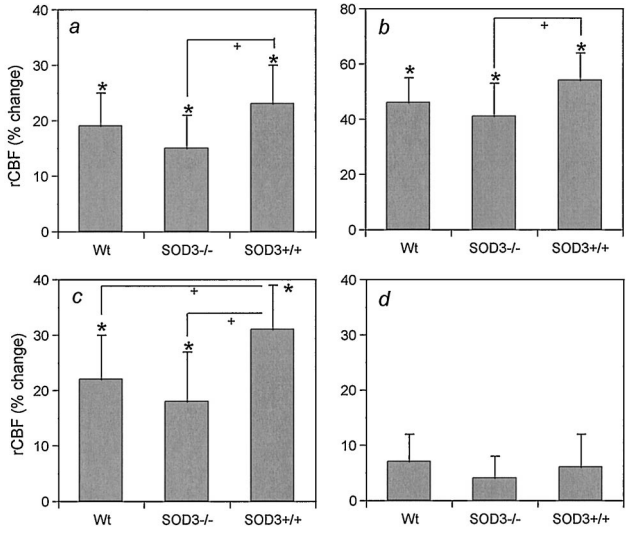

Figure 2. Integrity of NO-dependent cerebrovascular responses in SOD3 mutant mice. a, Representative data of effect of NO-donor PAPA NONOate in caudate showing greater rCBF response in $SOD3^{+/+}$ than $SOD3^{-/-}$ mice. *$P<0.05$ vs pretreatment; +$P<0.05$ between groups. b, rCBF responses in striatum to 5% CO_2 (in air) were greater in $SOD^{+/+}$ mice compared with $SOD3^{-/-}$ mutants. *$P<0.05$ vs pretreatment; +$P<0.05$ between the two mutant groups. c, Acetylcholine injection produces greater CBF responses in caudate (CP) of $SOD^{+/+}$ mice compared with WT. *$P<0.05$ vs pretreatment; +$P<0.05$ between groups. d, After L-NAME (30 mg/kg), CBF responses to acetylcholine were abolished in WT and SOD3-mutant strains. Acetylcholine responses at 0.05 mg/kg IV are shown.

augmented significantly when a constant dose of acetylcholine (0.05 mg/kg) was administered to $SOD3^{+/+}$ mice, whereas it was attenuated in $SOD3^{-/-}$ mice (Figure 2c). WT controls had similar responses to acetylcholine (not shown). NOS inhibitor, L-NAME completely blocked the rCBF responses to acetylcholine (Figure 2d). The data of Figure 2 collectively support the interpretation that EC-SOD in mouse brain modulates NO-dependent vasodilation via $\cdot O_2^-$-dependent regulation of NO availability.

Role of EC-SOD in regulating cerebrovascular responses to hyperoxia. We assessed regulation of the vasoconstrictor response to O_2 by SOD3 by exposing mice to O_2 during HBO_2 to increase brain $\cdot O_2^-$ production.[26] We report here rCBF in WT and mutant mice exposed to HBO_2 that showed stable, normal arterial BP, P_{CO_2}, and pH values for 60 minutes at 3 and 5 atmospheres absolute (ATA) (Tables 1 and 2).

HBO_2 altered rCBF in an O_2 dose- and time-dependent manner unrelated to the change in hydrostatic pressure per se (data not shown). Mean blood pressure in mice at 3 or 5 ATA rose during the first few minutes of HBO_2 exposure and remained elevated until decompression. At 3 ATA, EEG discharges of HBO_2 toxicity were not apparent. At 5 ATA, HBO_2 produced marked differences in EEG pattern in all strains consisting of paroxysmal EEG discharges of single or multiple spiking activity of amplitude >100 μV. These changes were observed after HBO_2 exposures of 40 ± 4 minutes in $SOD3^{+/+}$ mice, 49 ± 5 minutes in $SOD3^{-/-}$ mice, and 56 ± 6 minutes in WT mice consistent with the enhanced susceptibility reported for both mutant strains (Figure 3a).

At 3 ATA, the rCBF flow in cortex and deep brain structures decreased significantly over 60 minutes of HBO_2 in WT and $SOD3^{-/-}$ mice. At 30 to 45 minutes, vasoconstriction reached maximum where it was maintained until decompression (Figure 3b). In contrast, rCBF responses in $SOD3^{+/+}$ mice to HBO_2 were minimal and not statistically different from preexposure levels (Figure 3b). At 5 ATA, HBO_2 decreased rCBF in WT and $SOD3^{-/-}$ mice over the initial 30 minutes, but then rCBF gradually rose. By 45 minutes, rCBF had returned to preexposure levels and over the next 15 minutes increased above baseline (Figure 3c). This secondary increase in rCBF always preceded abnormal EEG discharges. $SOD3^{+/+}$ mice did not show significant cerebral vasoconstriction at 5 ATA of HBO_2, and within 30 minutes rCBF had increased significantly. After 60 minutes of HBO_2, rCBF had increased 57% above control values (Figure 3c).

TABLE 2. Arterial Blood Gases After HBO_2 at 3 and 5 ATA

	3 ATA (Before/After)			5 ATA (Before/After)		
Group	Pa_{O_2}	Pa_{CO_2}	pH	Pa_{O_2}	Pa_{CO_2}	pH
WT	112±11/412±21	38±2/42±2	7.43/7.29	107±9/398±19	36±2/40±2	7.42/7.39
$SOD3^{-/-}$	109±8/397±17	36±1/40±2	7.39/7.36	105±8/383±18	37±1/41±2	7.41/7.29
$SOD3^{+/+}$	106±10/421±22	38±2/41±2	7.42/7.31	111±11/411±24	37±2/39±2	7.35/7.25

Pa_{O_2} (mm Hg), Pa_{CO_2} (mm Hg), and pH measured before and immediately after HBO_2 exposure at 3 or 5 ATA. Mice breathed 30% O_2 before and 100% O_2 after HBO_2.

Blood gases are mean±SE; pH is mean (SE<0.02 units).

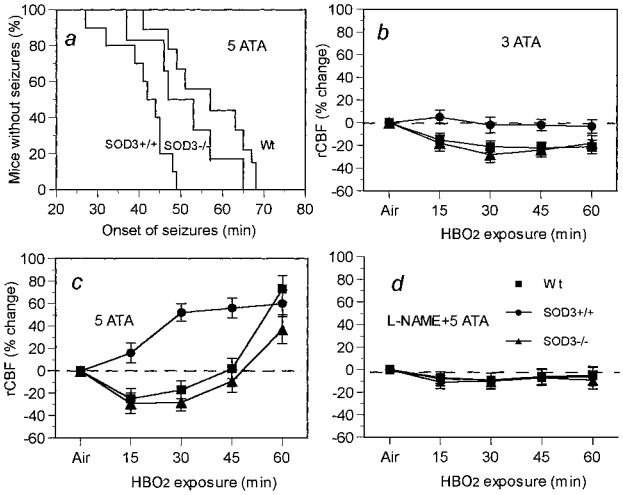

Figure 3. Cerebral responses of WT and SOD3-mutant mice during hyperbaric oxygen (HBO_2). a, Onset of oxygen EEG spikes in WT and SOD3 mutants exposed to oxygen at 5 ATA. No. of mice: WT=9; $SOD3^{+/+}$=9; $SOD3^{-/-}$=7. b, rCBF changes during HBO_2 at 3 ATA. CBF in striatum was measured every 15 minutes and compared with rCBF measured in the same mice breathing 30% O_2 (balance N_2) before HBO_2. CBF responses to 3 ATA are significant in WT and $SOD3^{-/-}$ mice. Hyperoxic vasoconstriction is absent in $SOD3^{+/+}$ mice. c, Representative rCBF changes in WT and SOD3-mutant mice at 5 ATA showing no vasoconstriction in $SOD3^{+/+}$ mice and escape of vasoconstriction in WT and $SOD3^{-/-}$ mice. d, CBF in WT and SOD3 mutants treated with L-NAME (30 mg/kg) and exposed at 5 ATA. CBF in striatum was measured every 15 minutes and compared with rCBF in the same mice breathing 30% O_2 (balance N_2) in 30 minutes after L-NAME injection before HBO_2.

P_{O_2} values in the striatum of WT mice and SOD3 mutant strains breathing 30% O_2 at 1 ATA ranged from 24 to 45 mm Hg. During compression, P_{O_2} progressively rose and after one minute at 5 ATA ranged from 497 to 738 mm Hg. During HBO_2 exposure, P_{O_2} increased gradually and immediately before EEG spikes reached values of 1276 to 2197 mm Hg. No significant differences in P_{O_2} responses to HBO_2 were observed among strains of mice.

The rCBF responses to HBO_2 in these mice collectively are consistent with the SOD3 mechanism of $\cdot O_2^-$-dependent regulation of NO availability. This was confirmed by measuring the effects of HBO_2 on CBF after treating mice with L-NAME. L-NAME decreased rCBF equally in all strains of mice breathing air (data not shown). During exposure to HBO_2 at 5 ATA, rCBF remained at or near the control level for air-breathing mice given L-NAME (Figure 3d) throughout 60 minutes exposures. Abnormal EEG discharges were not observed in L-NAME–treated mice exposed to HBO_2 at 5 ATA for 60 minutes.

EC-SOD Regulates Interstitial NO and O_2^- Equilibrium in the Brain

Intracerebral microdialysis experiments revealed dynamic changes in equilibrium between $NO^{\cdot}$ and $\cdot O_2^-$ in brain extracellular fluid as a function of EC-SOD expression. We measured interstitial NO_x to assess $NO^{\cdot}$ release and 3-NTyr to assess $ONOO^-$ formation in mice at 5 ATA. Basal interstitial NO_x in the striatum of WT mice was stable at 3.9±0.4 μmol/L, in $SOD3^{-/-}$ and $SOD^{+/+}$ mice these values were 3.8±0.5 μmol/L and 4.3±0.6 μmol/L, respectively. Within 30 minutes at 5 ATA, brain interstitial NO_x decreased significantly. Thereafter, NO_x gradually increased to above control levels (Figure 4a). The recovery of NO_x in brain interstitial fluid at 5 ATA was remarkably different in $SOD3^{+/+}$ and $SOD3^{-/-}$ mice. In $SOD^{+/+}$ mice, NO_x levels gradually rose during 60 minutes of HBO_2 (Figure 4a). In $SOD3^{-/-}$ mice, NO_x decreased significantly over 30 minutes at 5 ATA and then stabilized (Figure 4a) indicating HBO_2-related differences in NO varied with EC-SOD expression in the different strains.

Brain interstitial 3-NTyr content, unlike NO_x, accumulated steadily at different rates during HBO_2 in WT and SOD3 mutant mice exposed to 5 ATA (Figure 4b). Marked increases in 3-NTyr were detected in WT and $SOD3^{-/-}$ mice at 5 ATA consistent with an $NO\text{-}\cdot O_2^-$-dependent mechanism of $ONOO^-$ formation. This was confirmed by less accumulation of 3-NTyr in striatum of mice pretreated before HBO_2 with L-NAME. Thus, HBO_2-mediated increases in $ONOO^-$ production at 5 ATA were blocked by NOS inhibition to a similar degree in both mutants (Figure 4c).

Discussion

These physiological measurements in genetically altered mice provide definite evidence that SOD3 regulates rCBF in vivo by controlling the equilibrium between NO and $\cdot O_2^-$ in the extracellular brain space. Furthermore, SOD3 regulates both basal tone and NO-mediated rCBF responses during increases in P_{O_2}. The different rCBF responses to raising the P_{O_2} of mice that overexpress or lack SOD3 supports the central hypothesis of the study: oxygen alters the equilibrium between $\cdot O_2^-$ and $NO^{\cdot}$, which regulates vessel tone and blood flow. Enhanced $\cdot O_2^-$ scavenging by SOD3 augments the relaxing effect of $NO^{\cdot}$ on vascular smooth muscle. Because $NO^{\cdot}$ is a central regulator of vascular tone, extracellular $\cdot O_2^-$ production functionally reduces the influence of eNOS on guanylate cyclase in smooth muscle. As a major vascular sink for extracellular $\cdot O_2^-$, SOD3 activity regulates the vasodilator effect of NO on cerebral vessels. Evidence for this premise

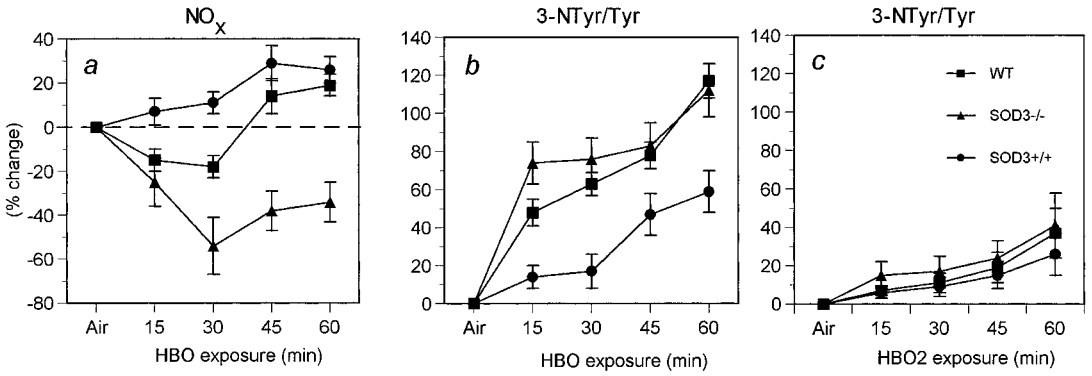

Figure 4. Effects of HBO_2 on brain NO_x and 3-NTyr in WT and SOD3-mutant mice. a, Time-course of NO_x in WT and SOD3-mutant mice exposed to HBO_2 at 5 ATA. For NO_x (NO_2+NO_3) measurement, dialysate was collected from striatum every 15 minutes. NO_x values at 5ATA were compared with NO_x measured in the same mice breathing 30% O_2 at 1 ATA. Accumulation of NO_x was less rapid in $SOD3^{-/-}$ mice ($P<0.05$). b and c, Time-course of 3-NTyr in WT and SOD3-mutant mice at 5 ATA (b) and in mice treated with L-NAME (30 mg/kg, IP) and exposed to 5 ATA (c). For measurement of 3-NTyr, dialysate was collected from striatum every 15 minutes and 3-NTyr expressed as 3-NTyr/Tyr ×100. $SOD3^{+/+}$ mice accumulate less 3-NTyr than $SOD3^{-/-}$ mice ($P<0.05$).

derives from differences in basal rCBF lacking in WT, $SOD3^{+/+}$, and $SOD^{-/-}$ mice brought forth by interventions to alter the equilibrium between $NO^{\cdot}$ and $\cdot O_2^-$. These interventions yield the predicted differences in rCBF response in the mutants. $SOD3^{+/+}$ mice treated with an NO donor show more pronounced rCBF responses than in $SOD3^{-/-}$ mutants, which is consistent with less $\cdot O_2^-$ bioactivity in cerebral vessels of $SOD3^{+/+}$ and a lower tendency for $NO^{\cdot}$ inactivation. Thus, we surmise that $SOD3^{-/-}$ mice have higher steady-state vascular $\cdot O_2^-$ levels, which allow a more substantial increase in the $NO^{\cdot}$ effect when SOD1 is administered.

One difference between this study and several earlier ones is that basal rCBF in these mice responded to active and not inactivated SOD1. This response could be related in part to blood brain barrier (BBB) damage by electrode insertion, which allowed SOD1 entry into parenchyma. However, $\cdot O_2^-$ and $NO^{\cdot}$ flux are regulated on both sides of a thin endothelium, and a large sink for $\cdot O_2^-$ within vessels might well increase $NO^{\cdot}$ flux on the other side of the barrier. NO released by RBCs also might have a greater dilator effect if $\cdot O_2^-$ scavenging were increased. Notably, rCBF responses to SOD1 were abolished completely in mice pretreated with L-NAME, suggesting the vasoactivity of SOD1 was NO-dependent and did not require BBB damage. In any event, rCBF response to SOD1 infusion in $SOD3^{-/-}$ mice was significantly greater than in $SOD3^{+/+}$ animals.

A consistent finding in mice exposed to HBO_2 is that seizure onset is accelerated in $SOD3^{+/+}$ mutants,[15] indicated here by abnormal EEG activity. Although this conflicts with predictions that antioxidant enzymes protect against O_2 toxicity, $SOD3^{-/-}$ mice are also more sensitive to CNS O_2 toxicity than WT animals. When inactivation of NO by $\cdot O_2^-$ is circumvented in $SOD3^{+/+}$ mice, rCBF is higher at high P_{O_2}, which appears to contribute to sensitivity to O_2 toxicity. HBO_2 also alters recovery of NO_x from the brain in accordance with "escape" of rCBF from vasoconstriction before the EEG discharges. This escape is prevented by NOS inhibition in the different mice, as well as in rats in an L-arginine–dependent manner.[22] The genetic changes in EC-SOD expression in the mice provide evidence that O_2 initially alters brain NO in accordance with this concept as opposed to stimulating its production by providing substrate. Indeed, WT and $SOD3^{-/-}$ mice first show less interstitial NO_x in HBO_2 at 5 ATA followed by an increase in NO_x to above control. By comparison, $SOD3^{+/+}$ mice consistently show elevated brain NO_x levels in HBO_2 at 5 ATA. The directional changes in NO metabolites are appropriate to the O_2-dependent rCBF responses, and these measurements are the first in vivo evidence that O_2 causes cerebral vasoconstriction by interfering with $NO^{\cdot}$.

Oxygen may inactivate $NO^{\cdot}$ because, in addition to heme and thiol interactions, NO residence time shows dependence on P_{O_2} and rate of $\cdot O_2^-$ generation.[27–29] At the onset of HBO_2, hemoglobin and thiol pools are stable while brain P_{O_2} and $\cdot O_2^-$ production rate increase in proportion to P_{O_2}.[26] If $[O_2]$ greatly exceeds $[NO^{\cdot}]$, NO_2 and NO_3 are produced by autoxidation of $NO^{\cdot}$.[30] If autoxidation had been a major factor in NO_x formation, high instead of low NO_x would have been expected. On the other hand, the transient decrease in NO_x is most readily explained by a decrease in extracellular $NO^{\cdot}$ from rapid inactivation by $\cdot O_2^-$. This effect is also qualitatively independent of any effect of CBF on recovery of the compounds by dialysis because decreases in CBF tend to increase NO_x recovery and vice versa.

$NO^{\cdot}$ and $\cdot O_2^-$ also produce $ONOO^-$ in a nearly diffusion limited reaction, and at steady $\cdot O_2^-$ flux, $ONOO^-$ is formed almost as rapidly as $NO^{\cdot}$ is released.[31] This chemistry predicts NO activity is regulated directly by $\cdot O_2^-$ rather than by independent and opposite effects of NO and $\cdot O_2^-$ on tone. The lack of difference in O_2-dependent vasoconstriction between WT and $EC\text{-}SOD^{-/-}$ mice and the lack of a greater fall in rCBF in HBO_2 after L-NAME in $EC\text{-}SOD^{-/-}$ mice support this concept because HBO_2 generates $\cdot O_2^-$. It is not possible to assess the reaction of $NO^{\cdot}$ and $\cdot O_2^-$ directly in brain, but the initial decline in NO_x with HBO_2 suggests $NO^{\cdot}$ inactivation by increased $\cdot O_2^-$ production.[26] HBO_2-mediated decreases in rCBF were not observed in $SOD3^{+/+}$ mice, which together with increased NO_x and decreased 3-NT are compatible with SOD3 opposition of $NO^{\cdot}$ inactivation.

In WT mice at 5 ATA, the initial decrease in interstitial NO_x was followed by an increase to above control in concert with increases in rCBF. This observation suggests that prolonged HBO_2 exposure promotes $NO^{\cdot}$ production, which augments CBF.[22] Because NOS requires O_2 it is possible that $[O_2]$ is limiting,[32] and HBO_2 stimulates NOS activity by increasing cerebral P_{O_2}. HBO_2 also elevates brain L-arginine, the substrate for NOS.[33] L-arginine synthesis is stimulated by O_2-dependent catecholamine deamination, which produces H_2O_2 and ammonia. Ammonia is detoxified by transamination of glutamate to glutamine, which generates L-arginine.[33] Finally, high P_{O_2} increases sympathetic and metabolic activity in the preconvulsive period[34] and calcium/calmodulin-dependent neuronal NOS is stimulated by postsynaptic receptor activation. These data, however, do not discriminate among these possibilities.

In summary, O_2 vasoconstriction defines a physiological role for SOD3 in regulating CBF in vivo. SOD3 is present in high concentration in vessels where $NO^{\cdot}$ is important for vascular relaxation. Inactivation by $\cdot O_2^-$ interferes with NO-dependent basal tone and vasorelaxation. Scavenging of $\cdot O_2^-$ by EC-SOD plays a critical role in $NO^{\cdot}$ CBF function as shown by predictable differences in rCBF response to high P_{O_2} in mice with varying enzyme expression. These findings have implications in vascular diseases where extracellular $\cdot O_2^-$ production exceeds SOD3 function, diminishing responsiveness to $NO^{\cdot}$, and allowing production of oxidative/nitrosative stress. The strategic location of vascular SOD3 in the brain between endothelium and smooth muscle preserves and regulates the endogenous dilator function of $NO^{\cdot}$.

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