Extracellular superoxide dismutase, nitric oxide, and central nervous system O$_2$ toxicity

(transgenic)

TIM D. OURY*†, YE-SHIH HO‡, CLAUDE A. PIANTADOSI‡, AND JAMES D. CRAPO*‡

*Department of Pathology, Duke University Medical Center, P.O. Box 3177, Durham, NC 27710; and ‡Department of Medicine, Duke University Medical Center, Durham, NC 27710

ABSTRACT   Although reactive O$_2$ species appear to participate in central nervous system (CNS) O$_2$ toxicity, the exact roles of different reactive O$_2$ species are undetermined. To study the contribution of extracellular superoxide anion (O$_2^-$) to CNS O$_2$ toxicity we constructed transgenic mice overexpressing human extracellular superoxide dismutase (ECSOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) in the brain. Remarkably, when exposed to 6 atm (1 atm = 101.3 kPA) of hyperbaric oxygen for 25 min, transgenic mice demonstrated higher mortality (83%) than nontransgenic littermates (33%; $P < 0.017$). Pretreatment with diethyldithiocarbamate, which inhibits both ECSOD and Cu/Zn superoxide dismutase (Cu/Zn SOD) activity, increased resistance to CNS O$_2$ toxicity, in terms of both survival (100% in transgenics and 93% in nontransgenics) and resistance to seizures (4-fold increase in seizure latency in both transgenic and nontransgenic mice; $P < 0.05$). Thus, O$_2^-$ apparently protects against CNS O$_2$ toxicity. We hypothesized that O$_2^-$ decreased toxicity by inactivating nitric oxide (NO·). To test this, we inhibited NO· synthase (EC 1.14.23) with N^ω-nitro-L-arginine to determine whether NO· contributes to enhanced CNS O$_2$ toxicity in transgenic mice. N^ω-nitro-L-arginine protected both transgenic and nontransgenic mice against CNS O$_2$ toxicity (100% survival and a 4-fold delay in time to first seizure; $P < 0.05$), as well as abolishing the difference in sensitivity to CNS O$_2$ toxicity between transgenic and nontransgenic mice. These results implicate NO· as an important mediator in CNS O$_2$ toxicity and suggest that ECSOD increases CNS O$_2$ toxicity by inhibiting O$_2^-$-mediated inactivation of NO·.

Several important antioxidant enzymes, such as catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6), glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9), and the superoxide dismutases (SOD; superoxide:superoxide oxidoreductase EC 1.15.1.1) are known to exist within cells. However, extracellular fluids and the extracellular matrix contain only small amounts of these enzymes. It has been argued that the major extracellular antioxidants are proteins that sequester transition metals so that they cannot act as catalysts to form the highly reactive hydroxyl radical (OH·) (1). Other extracellular antioxidants include radical scavengers and inhibitors of lipid peroxidation, such as ascorbic acid, uric acid, and α-tocopherol (1). The relative lack of extracellular antioxidant enzymes may reflect the possible function of extracellular reactive oxygen species as bioeffector molecules (1); however, this may also lead to greater susceptibility to extracellular oxidant stresses.

The enzyme extracellular superoxide dismutase (ECSOD) exists only at low concentrations in extracellular fluids and is not thought to function as a bulk scavenger of O$_2^-$ (1). The physiologic role of ECSOD in vivo remains a mystery.

ECSOD is a tetrameric Cu/Zn-containing glycoprotein with a subunit molecular weight of 30,000 (2, 3). Biochemical data suggest that ECSOD binds to heparan sulfate proteoglycans on endothelial cells, where it has been speculated to serve as a "protective coat" (4, 5). Endothelial cells secrete both O$_2^-$ (reviewed in ref. 6) and endothelium-derived relaxing factor, putatively identified as nitric oxide (NO·) (7). In addition to vasoregulation, NO· functions to regulate neurotransmission (8), although it can be toxic to neurons in some situations (9). Because O$_2^-$ is known to inactivate NO·-induced vasorelaxation (10–14), one possible function for ECSOD may be to protect NO· released from cells from O$_2^-$-mediated inactivation. In support of the potential importance of ECSOD acting as a "protector" of NO·, a recent report has shown that blood pressure in spontaneously hypertensive rats could be lowered by giving them an intravenous injection of a SOD construct containing a high affinity to heparan sulfate on endothelial cells (15).

In addition to inactivating NO·-induced vasorelaxation, the reaction of O$_2^-$ with NO· also produces a potentially toxic intermediate in the form of the peroxynitrite anion (ONOO$^-$) (16–19). While ONOO$^-$ has recently been shown to be capable of stimulating cGMP formation, it is a much less potent stimulator than NO· itself (20).

Hyperbaric oxygen exposures equal to or greater than 2 atm (1 atm = 101.3 kPa) are well known to be toxic to the central nervous system (CNS), leading to generalized convulsions and eventually to death (21). The mechanisms of this toxicity have been proposed to involve increased production of intracellular reactive oxygen species like O$_2^-$ and H$_2$O$_2$. The role of extracellular O$_2^-$ and NO· have not yet been studied. To begin addressing the roles of extracellular O$_2^-$, ECSOD, and NO· in CNS O$_2$ toxicity, we constructed transgenic mice that express elevated levels of ECSOD in the brain. These mice were then exposed to hyperbaric oxygen to see what effect this had on resistance to CNS O$_2$ toxicity.

MATERIALS AND METHODS

Materials. Concanavalin A-Sepharose, N^ω-nitro-L-arginine, equine cytochrome c, and diethyldithiocarbamate were purchased from Sigma. The plasmid pMSG was purchased from Pharmacia LKB. pKS was purchased from Stratagene. The human β-actin promoter was provided by Larry Kedes (University of Southern California, Los Angeles). Restriction endonucleases were purchased from New England Biolabs.

Animals. (C57BL/6 × C3H)F$_1$ and CD1 mice were purchased from Charles River Breeding Laboratories.

Abbreviations: SOD, superoxide dismutase; CNS, central nervous system; ECSOD, extracellular SOD.
†To whom reprint requests should be addressed.

Construction of Human ECSOD Expression Vector. The ECSOD expression vector (Fig. 1) was constructed as follows: The entire human ECSOD cDNA fragment (22, 23) flanked by *Eco*RI restriction sites was converted and treated with mung bean nuclease to form blunt ends, ligated to *Sal* I linkers, digested with *Sal* I, and then inserted into the *Sal* I site of the human β-actin expression vector pHβAPr-1. The *Eco*RI/*Hin*dIII fragment of the resultant plasmid containing the human β-actin promoter, intron, and ECSOD cDNA was isolated. In addition, the *Hpa* I site of simian virus 40 (SV40) at position 2666 in plasmid pMSG was converted to a *Hin*dIII site by linker ligation and the *Hin*dIII/*Pst* I fragment containing the polyadenylylation site of the SV40 early region was isolated. These two DNA fragments were then ligated to an *Eco*RI plus *Pst* I-digested pKS vector. The *Eco*RI/*Xba* I fragment containing the entire expression construct free of plasmid sequences was isolated and used to establish transgenic mice. All of the recombinant DNA procedures were done according to established methods (24).

Development of Transgenic Mice. Purified DNA at 2.5 μg/ml in 5 mM Tris·HCl, pH 7.4/0.1 mM EDTA was injected into the pronuclei of fertilized eggs isolated from mice [(C57BL/6 × C3H)F₁ × (C57BL/6 × C3H)F₁]. Mouse eggs surviving microinjection were then implanted into the oviducts of pseudopregnant foster mothers (CD1) following procedures described by Hogan *et al.* (25). Mice carrying the transgene were identified by Southern blot analysis of tail DNA probed with the entire human ECSOD cDNA. Transgenic founders were bred with (C57BL/6 × C3H)F₁ to produce offspring for further studies.

Northern Blot Analysis. Transgenic mice and nontransgenic littermates were killed with an overdose of pentobarbital. Tissues were quickly excised and frozen in liquid nitrogen. Total RNA was then isolated by the CsCl procedure as described (24). Twenty micrograms of total RNA from the tissues of transgenic mice and nontransgenic littermates and an RNA ladder was then denatured with glyoxal, electrophoresed through a 1.2% agarose gel, and blotted to nitrocellulose as described (24). The blots were then probed with the entire human ECSOD cDNA.

Separation of SOD Isoenzymes by Concanavalin A-Sepharose Chromatography. Tissues from three mice were weighed, combined, and homogenized in 10 vol of ice-cold 50 mM potassium phosphate (pH 7.4) with 0.3 M KBr/3 mM diethylenetriaminepentaacetic acid/0.5 mM phenylmethylsulfonyl fluoride. Separation of ECSOD from Cu/Zn SOD and Mn SOD was accomplished by passing tissue homogenates over a concanavalin A-Sepharose column as described (26).

SOD Activity. ECSOD activity and total SOD activity (Cu/Zn SOD and Mn SOD) remaining after ECSOD extraction were measured by inhibition of cytochrome *c* reduction at pH 10 as described (27). Total protein was determined by the BCA protein assay (Pierce).

Oxygen Exposures. Mice (7–8 weeks old) were exposed to hyperbaric oxygen five at a time in a small-animal chamber (Bethlehem, PA). After flushing the chamber with pure O_2, compression to 50 m (6 atm) was performed within 5 min. The O_2 concentration in the chamber was monitored continuously with a Servomex O_2 analyzer (model 572; Sybron, Norwood,

MA) and maintained at ≥99%. The CO_2 concentration was analyzed from intermittent samples of chamber gas with an IR detector (IR Industries, Santa Barbara, CA) and not allowed to rise above 0.1%. Compression of O_2 in the chamber raised the internal temperature to 30°C–32°C transiently, but an environmental control system returned the chamber temperature to 25°C–26°C within 3 min. The exposures lasted 25–75 min and were followed by decompression for 5 min. The mice were observed continuously for signs of O_2 toxicity. The time to the first generalized convulsion (seizure latency) and the time to death were recorded. These exposure conditions are designed to cause CNS O_2 toxicity without appreciable evidence of pulmonary O_2 toxicity.

Treatment with Diethyldithiocarbamate. One hour before exposure to 6 atm of O_2, mice were given either i.p. injections of saline (0.008 ml/g) or diethyldithiocarbamate (400 mg per kg of body weight) dissolved in normal saline (0.008 ml/g). The mice were then exposed to 6 atm of O_2 for 25 min as described above.

To determine the extent of ECSOD and Cu/Zn SOD inhibition by diethyldithiocarbamate, mice were given diethyldithiocarbamate and sacrificed 1 hr later. The brains were removed and assayed for ECSOD and Cu/Zn SOD activity as described above.

Treatment with 2-Mercaptoethanol. One hour before exposure to 6 atm of O_2, mice were given either i.p. injections of saline (0.008 ml/g) or 2-mercaptoethanol (40 mg per kg of body weight) dissolved in normal saline (0.008 ml/g). This dose of 2-mercaptoethanol was selected because it contains an equal number of reducing thiols as the dose of diethyldithiocarbamate. The mice were then exposed to 6 atm of O_2 for 30 min as described above.

Treatment with N^ω-Nitro-L-Arginine, an Inhibitor of Nitric Oxide Synthase (EC 1.14.23). Ten minutes before compression, saline (0.008 ml/g) or N^ω-nitro-L-arginine (20 mg per kg of body weight) dissolved in saline (0.008 ml/g) was given i.p. to the transgenic and nontransgenic mice. Mice were then exposed at 6 atm of O_2 for 25 or 75 min as described above.

Statistics. A paired Student's *t* test was used to compare enzyme activities in transgenic and nontransgenic mice. A χ^2 test with Bonferroni correction was used to assess significance in survival differences to hyperbaric exposures. Analysis of variance with a Scheffe *F* test was used to compare differences in seizure latency in the different groups of mice.

RESULTS

Characterization of Transgenic Mice. Mice carrying the human ECSOD transgene were detected by Southern blot analysis. Northern blot analysis of various tissues from the F₁ of one mouse found to carry the transgene is shown in Fig. 2. High levels of message for human ECSOD were detected in the heart, skeletal muscle, and brain of transgenic mice, with little or no message observed in the lung, liver, and spleen. No message was detectable in nontransgenic littermates (results not shown).

ECSOD activities in the tissues of transgenic and nontransgenic mice were measured and compared (Table 1). Transgenic mice displayed a 5-fold increase in ECSOD activity in the brain and a 2- to 3-fold increase in heart and skeletal muscle. A study of three organs each of lung, liver, and spleen from transgenic mice were pooled for a single analysis of ECSOD activity. The results suggested that there were no major differences in ECSOD activity in these tissues compared to nontransgenic mice. Thus, the tissues that contained ECSOD message also displayed higher levels of activity. The total activity of the other SODs (Cu/Zn SOD and Mn SOD) was also assayed in brain, heart, muscle, liver, spleen, and lung, and no differences were found between transgenic and nontransgenic mice in any of these tissues.
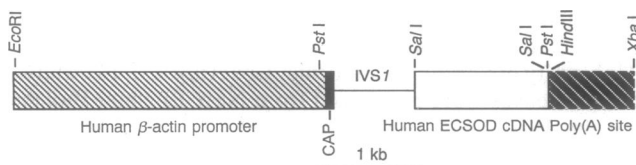

FIG. 1. Diagram showing structure of human ECSOD transgene. Transgenic mice were generated with the *Eco*RI/*Xba* I fragment. IVS1, intervening sequence 1 from human β-actin gene; kb, kilobase.
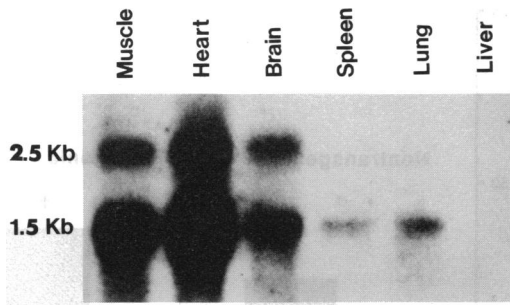
FIG. 2. Northern blot analysis of tissues from transgenic mice. Twenty micrograms of total RNA from tissues of transgenic mice was denatured with glyoxal and electrophoresed through a 1.2% agarose gel and blotted onto nitrocellulose. The filter was probed with the entire human ECSOD cDNA. The 2.5-kilobase (kb) band corresponds to mRNA of the human ECSOD transgene containing the 1-kb intervening sequence (see Fig. 1). The 1.5-kb band corresponds to the fully processed mRNA of the human ECSOD transgene.

Hyperbaric Oxygen Exposures. To test the effects of increased ECSOD levels in the brain on CNS O_2 toxicity, we exposed both transgenic and nontransgenic mice to 6 atm of O_2 for 25 min. Remarkably, transgenic mice were more susceptible (83% mortality) to CNS O_2 toxicity than nontransgenic mice (33% mortality) (Fig. 3).

Transgenic and nontransgenic mice were treated with an inhibitor of Cu/Zn SOD, diethyldithiocarbamate, to confirm that the increased sensitivity of transgenic mice to CNS O_2 toxicity was the result of increased SOD activity. In both transgenic and nontransgenic mice, treatment with diethyldithiocarbamate (400 mg/kg) resulted in 80% inhibition of ECSOD and 60% inhibition of Cu/Zn SOD in the brain. This is consistent with previous findings (28, 29). Treatment with diethyldithiocarbamate conferred increased resistance to CNS O_2 toxicity for both transgenic and nontransgenic mice. Survival increased to 100% in transgenic mice and to 93% in nontransgenic mice (Fig. 3). The onset of seizures was also delayed 4-fold in mice treated with diethyldithiocarbamate (Fig. 4).

To evaluate whether or not diethyldithiocarbamate protects against CNS O_2 toxicity by acting as a reducing agent rather than as an inhibitor of SOD activity, mice were treated with an equimolar amount of reducing thiols in the form of 2-mercaptoethanol and reexposed to hyperbaric oxygen. Fig. 5 shows that 2-mercaptoethanol did not protect against CNS O_2 toxicity.

Treatment with the NO· synthase inhibitor, N^ω-nitro-L-arginine, dramatically reduced CNS O_2 toxicity in both transgenic and nontransgenic mice. Survival after a 25-min exposure to 6 atm of O_2 increased to 100% in both groups (Fig. 3). Seizure latency was also significantly delayed (Fig. 4). We then increased the exposure time to 75 min to
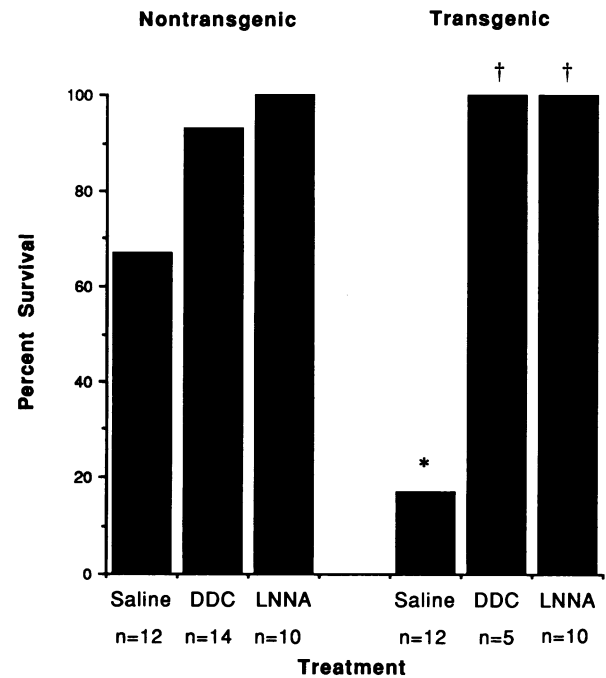

FIG. 3. Percentage survival in transgenic and nontransgenic mice exposed to 6 atm of O_2 for 25 min. Mice were injected with saline or given N^ω-nitro-L-arginine (LNNA) (20 mg/kg) i.p. 10 min before compression. Diethyldithiocarbamate (DDC) (400 mg/kg) in saline was injected i.p. 55 min before compression. *, $P < 0.017$ by χ^2 test compared to nontransgenic saline-treated mice with Bonferroni correction; †, $P < 0.017$ by χ^2 test compared to transgenic saline-treated mice with Bonferroni correction.

investigate whether transgenic mice were still more sensitive than nontransgenic mice to hyperbaric oxygen. The results in Fig. 6 indicate that treatment with N^ω-nitro-L-arginine abolished the difference in sensitivity between transgenic and nontransgenic mice that was observed in the 25-min exposure shown in Fig. 3 (saline treatment).

DISCUSSION

We successfully established a line of transgenic mice expressing 5-fold higher levels of ECSOD in the brain compared to nontransgenic littermates. The levels of intracellular SODs were unaffected. These mice were exposed to 6 atm of O_2 and displayed increased sensitivity to CNS O_2 toxicity compared to nontransgenic littermates (Fig. 3). Decreasing the activity of ECSOD and Cu/Zn SOD with diethyldithiocarbamate (28, 29, 31) resulted in increased resistance to CNS O_2 toxicity (Figs. 3 and 4).

In addition to its ability to inhibit ECSOD and Cu/Zn SOD, diethyldithiocarbamate can also inhibit dopamine β-hydrox-

Table 1. ECSOD activity in tissues of nontransgenic and transgenic mice

Tissue	Units per mg of total protein		Units per g of tissue	
	Nontransgenic	Transgenic	Nontransgenic	Transgenic
Brain	0.18 ± 0.01	$0.86 \pm 0.01*$	7.0 ± 0.3	$33.9 \pm 0.3*$
Heart	0.51 ± 0.03	$1.53 \pm 0.06*$	22.5 ± 1.3	$71.1 \pm 2.8*$
Muscle	0.16 ± 0.01	$0.36 \pm 0.01*$	12.7 ± 0.9	$29.2 \pm 0.4*$
Liver	0.40	0.37	40.2	51.5
Spleen	0.34	0.27	31.8	24.9
Lung	7.64	8.57	469	497

Results are means $\pm$ SD. Tissues from three mice were combined for each measurement and triplicate measurements were made for brain, heart, and muscle (total of seven to nine mice for each value). Single measurements were made for liver, spleen, and lung (total of three mice for each value).
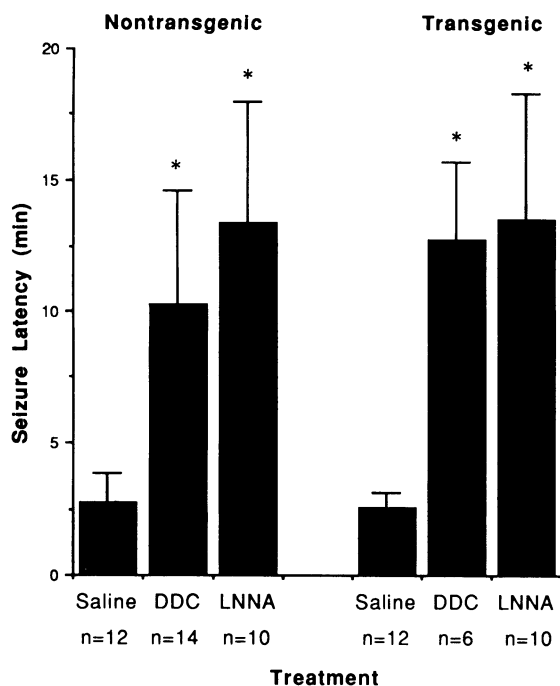$*P < 0.05$ compared to nontransgenic group tested by paired Student's t test.

FIG. 4. Time to onset of first seizure in transgenic and nontransgenic mice exposed to 6 atm of O_2. Mice were injected with saline or given N^ω-nitro-L-arginine (LNNA) (20 mg/kg) i.p. 10 min before compression. Diethyldithiocarbamate (DDC) (400 mg/kg) was injected i.p. 55 min before compression. Results are expressed as means ± SD of time to first seizure with zero time taken once chamber reached 6 atm. *, $P < 0.05$ by analysis of variance with the Scheffe F test compared to nontransgenic saline-treated mice.

ylase [dopamine β-monooxygenase; 3,4-dihydroxyphenethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1]. Previous studies have shown, however, that inhibition of dopamine β-hydroxylase does not protect
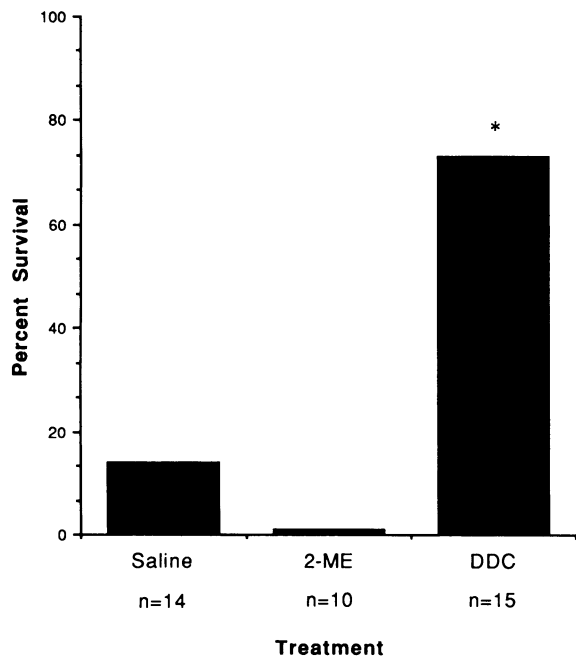

FIG. 5. Percentage survival in (C57BL/6 × C3H)F₁ mice exposed to 6 atm of O_2 for 30 min. Mice were injected i.p. with saline, 2-mercaptoethanol (2-ME) (180 mg/kg), or diethyldithiocarbamate (DDC) (400 mg/kg) in saline 55 min before compression. *, $P < 0.025$ by x^2 test with Bonferroni correction compared to saline-treated mice.
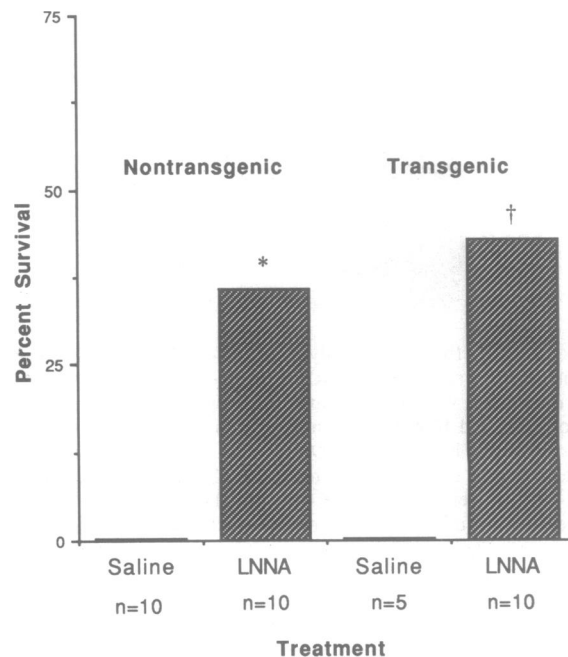

FIG. 6. Percentage survival in transgenic and nontransgenic mice exposed to 6 atm of O_2 for 75 min. Mice were injected with saline or given N^ω-nitro-L-arginine (LNNA) (20 mg/kg) i.p. 10 min before compression. *, $P < 0.05$ by x^2 test compared to nontransgenic saline-treated mice. †, $P < 0.05$ by x^2 test compared to transgenic saline-treated mice.

against CNS O_2 toxicity (32). Another potential explanation for the observed effect of diethyldithiocarbamate may involve its reducing potential. We explored this possibility by treating mice with a similar reducing agent, 2-mercaptoethanol, to determine whether reducing properties alone could protect against CNS O_2 toxicity under these conditions. Fig. 5 shows that 2-mercaptoethanol did not protect against CNS O_2 toxicity. These results suggest that diethyldithiocarbamate protected against CNS O_2 toxicity by inhibiting ECSOD and/or Cu/Zn SOD.

These hyperbaric O_2 studies imply that increased scavenging of extracellular O_2^- enhances sensitivity to CNS O_2 toxicity, while inhibition of these scavengers affords protection. O_2^- and other reactive oxygen species are thought to mediate damage in most models of oxygen toxicity, and scavengers of reactive oxygen species generally provide protection in these other systems. The results we obtained for CNS O_2 toxicity using transgenic mice overexpressing human ECSOD in the brain conflict with the prediction that antioxidants would confer protection. This led us to consider the possibility that O_2^- may protect against CNS O_2 toxicity by inactivating another reactive species.

The fact that our transgenic mice possessed altered levels of only ECSOD as well as the fact that O_2^- has a relatively short diffusion distance suggested involvement of a species in the extracellular space, specifically in the regions where ECSOD is located. Since ECSOD is capable of binding to cell surfaces via heparan sulfate proteoglycans (4, 5), we proposed that a species released from cells, which is susceptible to inactivation by O_2^-, may be responsible for the increased sensitivity to CNS O_2 toxicity observed in ECSOD transgenic mice. NO˙ is secreted from cells and can be inactivated by O_2^-, which can also be released into the extracellular space. Because NO˙ is lipid soluble and has a half-life between 6 and 30 sec, it can diffuse freely into surrounding cells where it has the potential to exacerbate CNS O_2 toxicity through a number of mechanisms (see below). These properties led us to

hypothesize that $NO^\bullet$ is an important mediator in CNS O_2 toxicity, and that O_2^- may modulate these effects of $NO^\bullet$.

We tested this hypothesis by giving both transgenic and nontransgenic mice the nitric oxide synthase inhibitor N^ω-nitro-L-arginine and exposing them to 25 min of hyperbaric O_2. Treatment with N^ω-nitro-L-arginine dramatically reduced CNS O_2 toxicity in both transgenic and nontransgenic mice (Figs. 3 and 4). We then increased the exposure time to 75 min to determine whether the increased sensitivity of the transgenic mice relative to nontransgenic controls was truly abolished by the $NO^\bullet$ synthase inhibitor. Indeed, Fig. 6 shows that, in the presence of an inhibitor of $NO^\bullet$ synthase, mice expressing excess ECSOD no longer displayed any increased sensitivity to CNS O_2 toxicity compared to nontransgenic mice.

There are several mechanisms by which $NO^\bullet$ may contribute to CNS O_2 toxicity. One potential mechanism involves the ability of $NO^\bullet$ to function as the vasodilator endothelium-derived relaxing factor. Hyperoxia-induced vasoconstriction helps reduce O_2 delivery to the brain, partially protecting the brain from excess tissue oxygenation (21). By acting as a local vasodilator, $NO^\bullet$ could antagonize the vasoconstrictor effect of oxygen and increase brain oxygenation, thereby resulting in increased damage during CNS O_2 toxicity. $NO^\bullet$ could also contribute to CNS O_2 toxicity through a neural excitatory mechanism. Recently, $NO^\bullet$ has been shown to be the mediator of glutamate toxicity in rat cortical cultures (9). In support of this mechanism, inhibition of excitatory amino acid neurotransmitters has been shown to protect against CNS O_2 toxicity (33, 34). A third mechanism by which $NO^\bullet$ may mediate CNS O_2 toxicity is by impeding mitochondrial respiration. $NO^\bullet$ can inhibit aconitase and complexes I and II in the electron transport chain (35–40). This inhibition could lead to ATP depletion and eventually to cell death. Metabolites that supplement ATP production have been shown to protect against CNS O_2 toxicity (41), supporting this as a mechanism by which $NO^\bullet$ could be acting in CNS O_2 toxicity. Finally, H_2O_2 has been shown to be an important mediator in CNS O_2 toxicity (30). An iron-independent reaction between $NO^\bullet$ and H_2O_2 during CNS hyperoxia could produce the highly reactive $OH^\bullet$ (42).

An additional mechanism in which $NO^\bullet$ could contribute to CNS O_2 toxicity is by reacting with O_2^- to form $ONOO^-$ (16–19). However, if $ONOO^-$ formation were truly important in the pathogenesis of CNS O_2 toxicity, increased scavenging of O_2^- in transgenic mice should have protected mice by inhibiting $ONOO^-$ formation, while inhibition of O_2^- scavenging with diethyldithiocarbamate should have increased toxicity by enhancing $ONOO^-$ formation. The exact opposite was observed, indicating that the direct effects of $NO^\bullet$ discussed above are the most likely pathways whereby $NO^\bullet$ contributes to injury in CNS O_2 toxicity.

In conclusion, we have shown that enhanced extracellular scavenging of O_2^- can potentiate CNS O_2 toxicity, while decreased scavenging of O_2^- reduces toxicity. These results indicate that extracellular O_2^- is able to protect the brain against hyperoxia. We have also demonstrated that $NO^\bullet$ is an important mediator in CNS O_2 toxicity in normal mice as well as in those superexpressing ECSOD. Based on these results, we propose that increased levels of ECSOD enhance CNS O_2 toxicity by inhibiting O_2^--mediated inactivation of $NO^\bullet$.

1. Halliwell, B. & Gutteridge, J. M. C. (1990) *Arch. Biochem. Biophys.* **280**, 1–8.
2. Marklund, S. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7634–7638.
3. Tibell, L., Hjalmarsson, K., Edlund, T., Skogman, G., Engström, Å. & Marklund, S. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6634–6638.
4. Marklund, S. L. (1984) *J. Clin. Invest.* **74**, 1398–1403.
5. Karlsson, K. & Marklund, S. L. (1988) *Biochem. J.* **255**, 223–228.
6. Halliwell, B. (1989) *Free Radical Res. Commun.* **5**, 315–318.
7. Noack, E. & Murphy, M. (1991) in *Oxidative Stress Oxidants and Antioxidants*, ed. Sies, H. (Academic, San Diego), pp. 445–489.
8. Schuman, E. M. & Madison, D. V. (1991) *Science* **254**, 1503–1506.
9. Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6368–6371.
10. Gryglewski, R. J., Palmer, R. M. J. & Moncada, S. (1986) *Nature (London)* **320**, 454–456.
11. Rubanyi, G. M. & Vanhoutte, P. M. (1986) *Am. J. Physiol.* **250**, H822–H827.
12. Rubanyi, G. M. & Vanhoutte, P. M. (1986) *Am. J. Physiol.* **250**, H815–H821.
13. Bult, H., Fret, H. R. L., Bossche, R. M. V. d. & Herman, A. G. (1988) *Br. J. Pharmacol.* **95**, 1308–1314.
14. Nucci, G. D., Gryglewski, R. J., Warner, T. D. & Vane, J. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2334–2338.
15. Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T. & Inoue, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10045–10048.
16. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624.
17. Mulligan, M. S., Hevel, J. M., Marletta, M. A. & Ward, P. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6338–6342.
18. Hogg, N., Darley-Usmar, V. M., Wilson, M. T. & Moncada, S. (1992) *Biochem. J.* **281**, 419–424.
19. Matheis, G., Sherman, M. P., Buckberg, G. D., Haybron, D. M., Young, H. H. & Ignarro, L. J. (1992) *Am. J. Physiol.* **262**, H616–H620.
20. Tarpy, M. M., Ischiropoulos, H., Gore, J. Z., Beckman, J. S. & Brock, T. A. (1992) *Am. Rev. Respir. Dis.* **145**, A714 (abstr.).
21. Balentine, J. D. (1982) *Pathology of Oxygen Toxicity* (Academic, New York), pp. 172–213.
22. Hjalmarrson, K., Marklund, S. L., Engstrom, A. & Edlund, T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6340–6344.
23. Hendrickson, D. J., Fisher, J. H., Jones, C. & Ho, Y.-S. (1990) *Genomics* **8**, 736–738.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
25. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
26. Marklund, S. L., Holme, E. & Hellner, L. (1982) *Clin. Chim. Acta* **126**, 41–51.
27. Crapo, J. D., McCord, J. M. & Fridovich, I. (1978) *Methods Enzymol.* **53**, 382–393.
28. Heikkila, R. E., Cabbat, F. S. & Cohen, G. (1976) *J. Biol. Chem.* **251**, 2182–2185.
29. Frank, L., Wood, D. L. & Roberts, R. J. (1978) *Biochem. Pharmacol.* **27**, 251–254.
30. Zhang, J. & Piantadosi, C. A. (1991) *J. Appl. Physiol.* **71**, 1057–1061.
31. Forman, H. J., York, J. L. & Fisher, A. B. (1979) *J. Pharmacol. Exp. Ther.* **212**, 452–455.
32. Faiman, M. D., Mehl, R. G. & Oehme, F. W. (1971) *Biochem. Pharmacol.* **20**, 3059–3067.
33. Clark, J. M. (1982) in *The Physiology and Medicine of Diving*, eds. Bennett, P. B. & Elliot, D. H. (Best Publ., San Pedro, CA), pp. 200–238.
34. Colton, C. A. & Colton, J. S. (1984) *Can. J. Physiol. Pharmacol.* **63**, 519–521.
35. Granger, D. L., Taintor, R. R., Cood, J. L. & Hibbs, J. B., Jr. (1980) *J. Clin. Invest.* **65**, 357–370.
36. Granger, D. L. & Lehninger, A. L. (1982) *J. Cell Biol.* **95**, 527–535.
37. Drapier, J.-C. & Hibbs, J. B., Jr. (1986) *J. Clin. Invest.* **78**, 790–797.
38. Drapier, J.-C. & Hibbs, J. B., Jr. (1988) *J. Immunol.* **140**, 2829–2838.
39. Wharton, M., Granger, D. L. & Durack, D. T. (1988) *J. Immunol.* **141**, 1311–1317.
40. Stradler, J., Billiar, T. R., Curran, R. D., Stuehr, D. J., Ochoa, J. B. & Simmons, R. L. (1991) *Am. J. Physiol.* **260**, C910–C916.
41. Beubler, V. E., Lembeck, F. & Stolze, A. (1977) *Weiner Klenische Wochenschrift* **89**, 260–265.
42. Halliwell, B. & Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine* (Oxford Univ. Press, New York).