Extracellular superoxide dismutase, nitric oxide, and central nervous system O2 toxicity (transgenic)

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ABSTRACT Although reactive O2 species appear to participate in central nervous system (CNS) O2 toxicity, the exact roles of different reactive O2 species are undetermined. To study the contribution of extracellular superoxide anion (O2-) to CNS O2 toxicity we constructed transgenic mice overexpressing human extracellular superoxide dismutase (EC2SOD; 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 EC2SOD and Cu/Zn superoxide dismutase (Cu/Zn SOD) activity, increased resistance to CNS O2 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, O2- apparently protects against CNS O2 toxicity. We hypothesized that O2- decreased toxicity by inactivating nitric oxide (NO). To test this, we inhibited NO synthase (EC 1.14.23) with Nω-nitro-l-arginine to determine whether NO protects against CNS O2 toxicity in transgenic mice. Nω-nitro-l-arginine protected both transgenic and nontransgenic mice against CNS O2 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 O2 toxicity between transgenic and nontransgenic mice. These results implicate NO as an important mediator in CNS O2 toxicity and suggest that EC2SOD increases CNS O2 toxicity by inhibiting O2- 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 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 (EC2SOD) exists only at low concentrations in extracellular fluids and is not thought to function as a bulk scavenger of O2- (1). The physiologic role of EC2SOD in vivo remains a mystery.

EC2SOD is a tetrameric Cu/Zn-containing glycoprotein with a subunit molecular weight of 30,000 (2, 3). Biochemical data suggest that EC2SOD 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 O2- (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 O2- is known to inactivate NO-induced vasorelaxation (10–14), one possible function for EC2SOD may be to protect NO released from cells by O2- mediated inactivation. In support of the potential importance of EC2SOD 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 O2- 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 O2- and H2O2. The role of extracellular O2- and NO have not yet been studied. To begin addressing the roles of extracellular O2-, EC2SOD, and NO in CNS O2 toxicity, we constructed transgenic mice that express elevated levels of EC2SOD in the brain. These mice were then exposed to hyperbaric oxygen to see what effect this had on resistance to CNS O2 toxicity.

MATERIALS AND METHODS

Materials. Concanaavalin A-Sepharose, Nω-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)F1 and CD1 mice were purchased from Charles River Breeding Laboratories.

Abbreviations: SOD, superoxide dismutase; CNS, central nervous system; EC2SOD, extracellular SOD.
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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 EcoRI 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 EcoRI/HindIII 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 HindIII site by linker ligation and the HindIII/Pst I fragment containing the polyadenylation site of the SV40 early region was isolated. These two DNA fragments were then ligated to an EcoRI plus Pst I-digested pKS vector. The EcoRI/Xba I fragment containing the entire expression construct free of plasmid sequences was isolated and used to establish transgenic mice. All of the recombiant 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 diethyltetraminepentaacetic 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 hyperoxic oxygen five at a time in a small-animal chamber (Bethlehem, PA). After flushing the chamber with pure O2, compression to 50 m (6 atm) was performed within 5 min. The O2 concentration in the chamber was monitored continuously with a Servomex O2 analyzer (model 572; Sybron, Norwood, MA) and maintained at >99%. The CO2 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 O2 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 O2 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 O2 toxicity without appreciable evidence of pulmonary O2 toxicity.

Treatment with Diethyldithiocarbamate. One hour before exposure to 6 atm of O2, 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 O2 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-Mercaptocoethanol. One hour before exposure to 6 atm of O2, 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 O2 for 30 min as described above.

Treatment with Nω-Nitro-L-Arginine, an Inhibitor of Nitric Oxide Synthase (EC 1.14.23). Ten minutes before compression, saline (0.008 ml/g) or Nω-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 O2 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 χ2 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 same 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 F2 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. 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 O₂ toxicity, we exposed both transgenic and nontransgenic mice to 6 atm of O₂ for 25 min. Remarkably, transgenic mice were more susceptible (83% mortality) to CNS O₂ 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 O₂ 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 O₂ 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 O₂ toxicity by acting as a reducing agent rather than as an inhibitor of SOD activity, mice were treated with an equimolar amount of reducing thiol in the form of 2-mercaptoethanol and reexposed to hyperbaric oxygen. Fig. 5 shows that 2-mercaptoethanol did not protect against CNS O₂ toxicity.

Treatment with the NO⁺ synthase inhibitor, N⁶-nitro-1-arginine, dramatically reduced CNS O₂ toxicity in both transgenic and nontransgenic mice. Survival after a 25-min exposure to 6 atm of O₂ 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 investigate whether transgenic mice were still more sensitive than nontransgenic mice to hyperbaric oxygen. The results in Fig. 6 indicate that treatment with N⁶-nitro-1-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 O₂ and displayed increased sensitivity to CNS O₂ 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 O₂ toxicity (Figs. 3 and 4).

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

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Units per mg of total protein</th>
<th>Units per g of tissue</th>
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<tbody>
<tr>
<td></td>
<td>Nontransgenic</td>
<td>Transgenic</td>
</tr>
<tr>
<td>Brain</td>
<td>0.18 ± 0.01</td>
<td>0.86 ± 0.01*</td>
</tr>
<tr>
<td>Heart</td>
<td>0.51 ± 0.03</td>
<td>1.53 ± 0.06*</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.16 ± 0.01</td>
<td>0.36 ± 0.01*</td>
</tr>
<tr>
<td>Liver</td>
<td>0.40</td>
<td>0.37</td>
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<tr>
<td>Spleen</td>
<td>0.34</td>
<td>0.27</td>
</tr>
<tr>
<td>Lung</td>
<td>7.64</td>
<td>8.57</td>
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Results are means ± 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 O₂. 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.

FIG. 5. Percentage survival in (C57BL/6 × C3H)F₁ mice exposed to 6 atm of O₂ 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 χ² test with Bonferroni correction compared to saline-treated mice.

FIG. 6. Percentage survival in transgenic and nontransgenic mice exposed to 6 atm of O₂ 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 χ² test compared to nontransgenic saline-treated mice. †, P < 0.05 by χ² test compared to transgenic saline-treated mice.

against CNS O₂ 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 O₂ toxicity under these conditions. Fig. 5 shows that 2-mercaptoethanol did not protect against CNS O₂ toxicity. These results suggest that diethyldithiocarbamate protected against CNS O₂ toxicity by inhibiting ECSOD and/or Cu/Zn SOD.

These hyperbaric O₂ studies imply that increased scavenging of extracellular O₂ enhances sensitivity to CNS O₂ toxicity, while inhibition of these scavengers affords protection. O₂ 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 O₂ 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 O₂ may protect against CNS O₂ toxicity by inactivating another reactive species.

The fact that our transgenic mice possessed altered levels of only ECSOD as well as the fact that O₂ 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 O₂, may be responsible for the increased sensitivity to CNS O₂ toxicity observed in ECSOD transgenic mice. NO' is secreted from cells and can be inactivated by O₂, 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 O₂ toxicity through a number of mechanisms (see below). These properties led us to
hypothesize that NO\(^\cdot\) is an important mediator in CNS O\(_2\) toxicity, and that O\(_2\) may modulate these effects of NO\(^\cdot\).

We tested this hypothesis by giving both transgenic and nontransgenic mice the nitric oxide synthase inhibitor \(L-NAME\) and exposing them to 25 min of hyperbaric O\(_2\). Treatment with \(L-NAME\) 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\(^\cdot\) synthase inhibitor. Indeed, Fig. 6 shows that, in the presence of an inhibitor of NO\(^\cdot\) 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\(^\cdot\) may contribute to CNS O\(_2\) toxicity. One potential mechanism involves the ability of NO\(^\cdot\) 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\(^\cdot\) could antagonize the vasconstrictor effect of oxygen and increase brain oxygenation, thereby resulting in increased damage during CNS O\(_2\) toxicity. NO\(^\cdot\) could also contribute to CNS O\(_2\) toxicity through a neural excitatory mechanism. Recently, NO\(^\cdot\) 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\(^\cdot\) may mediate CNS O\(_2\) toxicity is by impeding mitochondrial respiration. NO\(^\cdot\) can inhibit aconitase and complex 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\(^\cdot\) could be acting in CNS O\(_2\) toxicity.

Finally, H\(_2\)O\(_2\) has been shown to be an important mediator in CNS O\(_2\) toxicity (30). An iron-independent reaction between NO\(^\cdot\) and H\(_2\)O\(_2\) during CNS hyperoxia could produce the highly reactive OH\(^\cdot\) (42).

An additional mechanism in which NO\(^\cdot\) 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 \(O_2^\cdot\) 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\(^\cdot\) discussed above are the most likely pathways whereby NO\(^\cdot\) 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\(^\cdot\) 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\(^\cdot\).

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