Cold-induced Brain Edema in Mice
INVolvEMENT OF EXTRACELLULAR SUPEROXIDE DISMUTASE AND NITRIC OXIDE*

(Received for publication, January 8, 1993, and in revised form, March 22, 1993)

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The role of extracellular superoxide in the pathogenesis of vasogenic edema was studied using transgenic mice expressing a 5-fold increase in extracellular superoxide dismutase (EC-SOD) activity in their brains. Increased EC-SOD expression offered significant protection against edema development after cold-induced injury (44% less edema than nontransgenic littermates, p < 0.05). Since iron may contribute to vasogenic edema by catalyzing the production of hydroxyl radical from superoxide and hydrogen peroxide, the effects of the chelator deferoxamine were studied. Deferoxamine reduced edema formation after cold-induced injury (43% less edema than controls, p < 0.05); however, treatment with iron-saturated defereroxamine also reduced edema development in mice (32–48% less edema, p < 0.05). This suggested that the protection offered by deferoxamine was independent of its ability to chelate iron. An iron-independent mechanism by which superoxide can contribute to vasogenic edema is via reaction with nitric oxide to produce the potentially toxic peroxynitrite anion, which is also scavenged by deferoxamine. Mice treated with an inhibitor of nitric oxide synthase were protected against cold-induced edema (37% less edema, p < 0.05). EC-SOD transgenic mice received no additional protection by inhibition of nitric oxide synthesis, supporting this novel alternative mechanism of edema formation.

Evidence supporting a role for the hydroxyl radical in vasogenic edema is based on the necessity for the presence of free iron as a catalyst in the production of hydroxyl radical from superoxide as shown above. These experiments demonstrate protection against vasogenic edema when animals were pretreated with iron chelators such as deferoxamine (1, 8) and from observations that vasogenic edema is exacerbated when free iron is added to the system (9). However, because deferoxamine is also a potent scavenger of hydroxyl radical (k = 1.3 × 10^10 M^-1.s^-1 (10)) it may protect against vasogenic edema in this manner.

Recently, the production of hydroxyl radicals from superoxide in a reaction that is independent of catalytic transition has been proposed and supported experimentally (11, 12). In this reaction superoxide (O2⁻) reacts with nitric oxide (NO·) (k = 3.7 × 10^10 M^-1.s^-1 (13)) to form the potentially toxic peroxynitrite anion (ONOO⁻, Reaction 3) (14). Peroxynitrite has a pKa of 6.5 (15, 16) and once protonated, forms peroxynitrous acid which rapidly decomposes by homolytic cleavage to produce hydroxyl radical (·OH) and nitrogen dioxide (NO₂, Reaction 4).

O2⁻ + NO· → ONOO⁻ (Reaction 3)
ONOO⁻ + H⁺ → HONO + NO₂ (Reaction 4)

These reactions may also contribute to the pathogenesis of vasogenic edema but have not yet been examined experimentally.

An enzyme that is ideally situated for protecting against superoxide-mediated endothelial cell injury during vasogenic edema is extracellular superoxide dismutase (EC-SOD). EC-SOD is a tetrameric Cu/Zn containing glycoprotein with a subunit molecular mass of 30,000 daltons (17, 18). There is a cluster of positively charged amino acids near the C terminus of the protein that contributes to the ability of EC-SOD to bind heparan sulfate proteoglycans (19, 20). This heparan sulfate affinity may be important in targeting EC-SOD to cell surfaces, serving as a "protective coat" against extracellular superoxide. Endothelial cells in particular have been hypothesized to contain EC-SOD on their surface based on experiments which show increases in plasma EC-SOD activity after intravenous administration of heparin which can compete with heparan sulfate for binding with EC-SOD (21, 22).

Endothelial cells are capable of secreting both superoxide anion (reviewed in Ref. 23) and endothelium-derived relaxing factor, putatively identified as nitric oxide. Because superoxide is known to inactivate nitric oxide (24–28) via Reaction 3 (above), one possible function for EC-SOD may be protecting nitric oxide from superoxide-mediated inactivation. Recently, we have shown, using transgenic mice, that EC-SOD is capable of modulating nitric oxide activity in the brain by preventing superoxide-mediated inactivation of this impor-

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*This work was supported by National Institutes of Health Grants HL 44571 and PO1 HL 31992. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SOD, superoxide dismutase; EC-SOD, extracellular SOD.
tant intercellular messenger (29). In addition to preserving
nitric oxide activity, the scavenging of extracellular superoxide may also be important in preventing formation of the potentially toxic peroxynitrite anion.

We utilized transgenic mice which express a 5-fold increase in EC-SOD in the brain (29) to study the role of extracellular superoxide in the pathogenesis of vasogenic edema. We show that EC-SOD is capable of protecting against vasogenic edema. In addition, we have explored the mechanism by which the iron chelator and hydroxyl radical scavenger, deferoxamine,
exerts its protective effects in vasogenic edema.

MATERIALS AND METHODS

Reagents—Deferoxamine mesylate (Desferal) was purchased from CIBA-GEIGY (Summit, N.J). N-ω-nitro-L-arginine methyl ester and L-arginine were purchased from Sigma.

Animals—(C57BL/6 X C3H) F1 mice were purchased from Taconic Laboratory Animals (Germantown, NY). Transgenic mice were constructed with the EC-SOD expression vector shown in Fig. 1. The vector utilizes the human β-actin promoter and contains the entire coding sequence of human EC-SOD (30, 31). Mice carrying the human EC-SOD transgene were detected by Southern blot analysis of tail DNA probed with the entire human EC-SOD cDNA (32). EC-SOD transgenic mice expressed a 5-fold increase in EC-SOD activity in the brain compared to nontransgenic littermates as previously described (29).

Cold-induced Brain Edema—Young (6–7 week) mice were anesthetized with 80 mg/kg pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL). An incision was then made in the scalp and a metal probe, 2 mm in diameter, equilibrated in liquid nitrogen, was placed on the skull over the right cerebral hemisphere for 30 s. The skin incision was then nurtured.

Two h after the injury the mice were given an additional dose of pentobarbital. The chest cavity was opened, the lungs were excised, and the mouse was then perfused with 20 ml of saline through the left ventricle of the heart. The brain was then removed and the cerebellum was excised. The right (R) and left (L) cerebral hemispheres were separated and immediately weighed (wet weight, W). Each hemisphere was then dried at 70 °C for 2–5 days until a constant weight was achieved (dry weight, D). An index of edema (I) was then calculated as shown in Equation 1.

\[
I = \frac{(W/D)}{(W/D)} \times 100 \quad (\text{Eq. 1})
\]

This calculation allowed the left hemisphere to serve as an internal control for the injured right hemisphere in each mouse.

Chemical Treatments—Six groups of experiments were conducted to investigate the importance of extracellular superoxide, iron, and nitric oxide in cold-induced brain edema. In all groups drugs were dissolved in saline and injected at 8 ml/kg 15 min prior to cold injury. In group 1 edema formation of EC-SOD transgenic mice was compared with that of nontransgenic littermates. Group 2 compared edema formation between (C57BL/6 × C3H) F1 mice treated with saline and (C57BL/6 × C3H) F1 mice treated with 0.51 μmol/g deferoxamine. Group 3 compared (C57BL/6 × C3H) F1 mice treated with saline to (C57BL/6 × C3H) F1 mice treated with 0.51 μmol/g Fe(II)-saturated deferoxamine. Group 4 consisted of (C57BL/6 × C3H) F1 mice treated with saline and (C57BL/6 × C3H) F1 mice treated with 0.02 mg/g N-ω-nitro-L-arginine methyl ester. Group 5 consisted of (C57BL/6 × C3H) F1 mice treated with saline and (C57BL/6 × C3H) F1 mice treated with 0.02 mg/g N-ω-nitro-L-arginine methyl ester plus 0.05 mg/g L-arginine. Group 6 compared edema formation between nontransgenic mice, EC-SOD transgenic mice treated with saline, and EC-SOD transgenic mice treated with 0.02 mg/g N-ω-nitro-L-arginine methyl ester.

Because deferoxamine is known to react directly with hydroxyl radical, a final concentration of less than 1 mM has been recommended when using this compound (reviewed in Ref. 33). The dose of deferoxamine used in these experiments was chosen based on this recommendation so that the final concentration in the mice, assuming 60% water/g, would be 0.85 mM. The dose of N-ω-nitro-L-arginine methyl ester given was chosen based on a dose response of the inhibitor used in central nervous system oxygen toxicity that gave maximal protection against injury (34).

Iron-saturated deferoxamine was made by dissolving equimolar amounts of deferoxamine and then ferric chloride in saline. Satura-

tion of deferoxamine with ferric iron was determined spectrophotometrically by measuring the absorbance at 425 nm (ε = 2500 M⁻¹ cm⁻¹ for Fe(III)-deferoxamine) (35).

Evan’s Blue Treatment—One h and 50 min after cold injury 5 ml/kg of 1% Evan’s Blue in saline was injected into the femoral artery of transgenic and nontransgenic mice. The mice were sacrificed 10 min later and perfused with normal saline through the left ventricle until there was no more blue color in the effluent. The brains were then removed and photographed.

Statistical Analysis—A paired Student’s t test was used to compare significance of edema development compared to nontransgenic mice or saline-treated mice for each of the groups examined. Analysis of variance with a Fisher’s LSD test was used to compare significance in group 6. p values less than 0.05 were considered to be significant.

RESULTS

The construction and characterization of the EC-SOD transgenic mice used in this study were described previously (29). These mice were generated with the EC-SOD expression vector shown in Fig. 1. The transgenic mice were found to express messenger RNA for the human EC-SOD gene as well as increased levels of EC-SOD activity in brain, heart, and skeletal muscle. EC-SOD activity in the brains of transgenic mice was found to be increased by 5-fold compared to nontransgenic littermates.

When transgenic mice and nontransgenic littermates were subjected to cold-induced injury to the right cerebral hemisphere we found that the transgenic mice were significantly protected against edema formation compared to nontransgenic littermates (Fig. 2). Percent edema was 44% less in transgenic mice than in nontransgenic littermates, and Evan’s
Blue dye extravasation was visibly less in transgenic mice compared to nontransgenic littermates (Fig. 3). The value for the no injury bar in Fig. 2 is not statistically different from zero.

To test the contribution of iron to edema formation in this model, mice were pretreated with intraperitoneal injections of defereroxamine or saline prior to cold-induced injury. Table I shows that pretreatment with defereroxamine resulted in 43% less edema formation compared to mice only given saline. We then pretreated mice with intraperitoneal injections of iron-saturated defereroxamine or saline before cold-induced injury to see if the iron-chelating properties of this compound were truly necessary for protection against edema formation. Table I shows that even when defereroxamine was saturated with iron it was still capable of protecting against edema formation and resulted in 32-48% less edema than that found in saline-treated controls. Of note, the absolute values for the edema index varied from day to day, however, repeated experiments consistently show the same significant trends in protection against edema formation in the various treatments examined.

These results suggest that defereroxamine is capable of protecting against edema formation by a mechanism independent of its ability to scavenge iron. Because defereroxamine is capable of scavenging both the hydroxyl radical (10) as well as the peroxynitrite anion (36) even when it was presaturated with iron, we hypothesized that it is these properties of defereroxamine that enable it to protect against vasogenic edema.

To test this hypothesis we inhibited the synthesis of nitric oxide with N-ω-nitro-L-arginine methyl ester, a competitive inhibitor of the enzyme nitric oxide synthase, to determine if this would result in protection against edema formation after a cold-induced injury. Table II shows that treatment with N-ω-nitro-L-arginine methyl ester significantly protected mice against edema formation resulting in 37% less edema formation than that occurring in saline-treated controls. This protection by N-ω-nitro-L-arginine methyl ester was reversed by simultaneous administration of an excess of L-arginine to the mice (Table II).

In the final experiments, EC-SOD transgenic mice were treated with either saline or N-ω-nitro-L-arginine methyl ester to determine if there was an additive effect in preventing edema formation in mice which have both increased levels of EC-SOD as well as the inhibitor of nitric oxide synthase. Table III shows that when EC-SOD transgenic mice were given the inhibitor of nitric oxide synthase, no added protection against edema formation was detected relative to transgenic mice protected only by increased levels of EC-SOD in the brain.

**DISCUSSION**

Vasogenic edema is associated with the disruption of endothelial cells and a resultant increase in vascular permeability. The biochemical mechanisms involved in the pathogenesis of the edema are poorly understood. However, reactive oxygen species as well as arachidonic acid metabolism, which may produce reactive oxygen species, have been postulated to contribute to the injury (reviewed in Ref. 37). In this study we investigated the role of EC-SOD in protection against vasogenic edema using transgenic mice which expressed a 5-fold increase in EC-SOD activity in the brain (29). We found that overexpression of EC-SOD is indeed capable of protecting against vasogenic edema and decreasing vascular compro-

**TABLE II**

*The effect of inhibition of nitric oxide synthase on edema formation after cold-induced brain injury*

(C57BL/6 × C3H) F1 mice were treated with the competitive inhibitor of nitric oxide synthase, N-ω-nitro-L-arginine methyl ester (LNAME) to determine what effect nitric oxide had on vasogenic edema. Mice were also given N-ω-nitro-L-arginine methyl ester plus an excess of L-arginine (LNAME + L-Arg) to see if the effects seen with LNAME alone could be reversed. Values are presented as mean ± standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Edema index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>5.77 ± 0.29</td>
</tr>
<tr>
<td>LNAME</td>
<td>6</td>
<td>3.65 ± 0.51</td>
</tr>
<tr>
<td>Saline + L-Arg</td>
<td>6</td>
<td>6.56 ± 0.21</td>
</tr>
<tr>
<td>LNAME + L-Arg</td>
<td>6</td>
<td>6.03 ± 0.71</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to Edema index of respective saline treated controls using a paired Student’s t test.

**TABLE III**

*Evaluation of edema formation in transgenic mice to edema formation in transgenic mice with elevated levels of EC-SOD activity in the brain or edema formation in transgenic mice also treated with an inhibitor of nitric oxide synthase (20 mg/kg N-ω-nitro-L-arginine; Transgenic + LNAME) 15 min prior to cold-induced injury*

Values are presented as mean ± standard error and were compared using analysis of variance with a Fisher PLSD test. No significant difference was seen between transgenic and transgenic + LNAME mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Edema index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic</td>
<td>6</td>
<td>7.91 ± 0.67</td>
</tr>
<tr>
<td>Transgenic</td>
<td>6</td>
<td>4.91 ± 0.78</td>
</tr>
<tr>
<td>Transgenic + LNAME</td>
<td>6</td>
<td>4.30 ± 0.98</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to Edema index of nontransgenic mice.
mize in the brains of transgenic mice (Figs. 2 and 3). These results agree with other studies which have shown that increased scavenging of superoxide can protect against vasogenic edema (1–7).

The precise mechanisms by which reactive oxygen species, such as the superoxide anion, contribute to vasogenic brain edema are unknown. Superoxide is generally not thought to contribute directly to damage leading to edema development. Rather, superoxide was proposed to contribute to the pathogenesis of vasogenic edema by producing the hydroxyl radical in an iron-catalyzed Haber-Weiss reaction as shown in Equations 1 and 2 (1, 8, 9). To investigate the role of iron in the pathogenesis of vasogenic edema, we treated mice with either deferoxamine, a chelator of ferric-iron, or iron-saturated deferoxamine prior to cold-induced injury. Table I shows that deferoxamine and iron-saturated deferoxamine provided nearly equivalent protection against edema formation. These results suggest that deferoxamine protection against vasogenic edema is not entirely due to its iron chelating properties.

An alternative mechanism for edema formation that is independent of iron, in which both EC-SOD and deferoxamine offer protection, must therefore be considered. We have recently shown that EC-SOD is capable of modulating nitric oxide activity during central nervous system oxygen toxicity (29). Therefore, we hypothesized that EC-SOD may also be modulating nitric oxide activity during vasogenic edema, and this interaction may be responsible for the protection seen in this injury. Support for a possible toxic role for nitric oxide in vasogenic edema was provided recently when nitric oxide was found to mediate excitotoxicity produced by glutamate in the brain (38). In addition, nitric oxide has been proposed to mediate neurotoxicity via a similar mechanism during cerebral ischemia (39–41). A recent report supported this possibility by showing that nitric oxide contributed to neuronal death in focal ischemia in the mouse (42); however, conflicting evidence has been seen in cerebral ischemia in rats (43). If nitric oxide is also a mediator of vasogenic edema in the brain, then one relatively simple mechanism could explain how both EC-SOD and deferoxamine can protect against edema formation. This mechanism is illustrated in Fig. 4.

In this pathway, peroxynitrite is formed as a result of a rapid reaction ($k = 3.7 \times 10^7$) between superoxide and nitric oxide. Peroxynitrite has a relatively long half-life ($t_{1/2} = 1.9$ s) (11) and can therefore diffuse up to several cell diameters before exerting its toxic effects. Once protonated peroxynitrous acid can then spontaneously decompose to form the highly reactive hydroxyl radical and nitrogen dioxide as shown in reaction 4 above. Peroxynitrite was recently shown to induce lipid peroxidation in a reaction independent of iron (36). Both the hydroxyl radical and nitrogen dioxide are capable of initiating lipid peroxidation. The hydroxyl radical can react with fatty acids at diffusion controlled rates ($k = 10^9$ M$^{-1}$ s$^{-1}$) (44), while nitrogen dioxide, a lipophilic oxidant radical, reacts with unsaturated fatty acids in a somewhat slower reaction to initiate lipid peroxidation by hydrogen abstraction ($k = 10^6$ M$^{-1}$ s$^{-1}$) (45).

If this reaction pathway is operative during vasogenic edema formation, then EC-SOD would protect by scavenging superoxide and thus inhibiting peroxynitrite formation. Deferoxamine, as well as iron-saturated deferoxamine, could also protect either by directly scavenging peroxynitrite even when deferoxamine is only present at concentrations as low as 200 micromolar (36) or by scavenging the hydroxyl radical generated by decomposition of peroxynitrous acid (10). In addition, inhibition of nitric oxide synthesis should also protect against edema formation by inhibiting the formation of peroxynitrite.

To determine if inhibition of nitric oxide synthesis could protect against vasogenic brain edema, we treated mice with a competitive inhibitor of nitric oxide synthase and repeated the injury. As can be seen in Table II, mice treated with N-ω-nitro-L-arginine methyl ester demonstrated less edema formation compared to saline-treated controls. This protection by N-ω-nitro-L-arginine methyl ester was completely lost by addition of an excess of L-arginine.

In the proposed pathway of edema formation shown in Fig. 4, both superoxide and nitric oxide contribute to edema formation by reacting together to form peroxynitrite. To determine if an additive protective effect could occur when EC-SOD activity is increased in the brain and nitric oxide synthesis is inhibited simultaneously, EC-SOD transgenic mice were treated with either saline or a competitive inhibitor of nitric oxide synthase. Table III shows that having both increased levels of EC-SOD and decreased synthesis of nitric oxide offered no increased protection against edema formation than EC-SOD alone. These results suggest that superoxide and nitric oxide are not acting by independent pathways in edema formation as might be predicted if an additive protection was seen.

We have previously shown that EC-SOD can modulate nitric oxide functions during central nervous system oxygen toxicity (29). Interestingly, during central nervous system oxygen toxicity, protecting nitric oxide from superoxide-mediated inactivation with EC-SOD actually increased the toxicity of the hyperoxia. This increased toxicity is apparently related to the effects of nitric oxide as an intercellular messenger (i.e. vasodilator or neurotransmitter) in this form of oxygen toxicity. However, in vasogenic edema modulation of nitric oxide activity by elevated levels of EC-SOD appears to be beneficial presumably because it prevents the formation of peroxynitrite.

In conclusion, our results indicate that superoxide and nitric oxide are important mediators of injury in vasogenic brain edema. In addition we have shown that deferoxamine as well as iron-saturated deferoxamine can protect against vasogenic edema. These results indicate that the iron-chelating properties of deferoxamine are not the only mechanisms by which this agent protects against vasogenic edema. In addition, we have proposed a novel pathway by which vasogenic edema may be occurring through the production of peroxynitrite.

Acknowledgements—We thank Craig Marshall for his technical expertise in the intrarterial injections and to Charleen Chu and Dr. Brian Day for their helpful suggestions.
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