Phenotypes of Mice Lacking Extracellular Superoxide Dismutase and Copper- and Zinc-containing Superoxide Dismutase*

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Mice lacking the secreted extracellular superoxide dismutase (EC-SOD) or the cytosolic copper- and zinc-containing SOD (CuZn-SOD) show relatively mild phenotypes. To explore the possibility that the isoenzymes have partly overlapping functions, single and double knockout mice were examined. The absence of EC-SOD was found to be without effect on the lifespan of mice, and the reduced lifespan of CuZn-SOD knockouts was not further shortened by EC-SOD deficiency. The urinary excretion of isoprostanes was increased in CuZn-SOD knockout mice, and plasma thiobarbituric acid-reactive substances levels were elevated in EC-SOD knockout mice. These oxidant stress markers showed potentiated increases in the absence of both isoenzymes. Other alterations were mainly found in CuZn-SOD knockout mice, such as halfed glutathione peroxidase activity in the tissues examined and increased glutathione and iron in the liver. There were no changes in tissue content of the alternative superoxide scavenger ascorbate, but there was a 25% reduction in ascorbate in blood plasma in mice lacking CuZn-SOD. No increase was found in the urinary excretion of the terminal metabolites of NO, nitrite, and nitrate in any of the genotypes. In conclusion, apart from the increases in the global urinary terminal metabolites of NO, nitrite, and nitrate in any of the genotypes, knockouts can be explained by partial functional overlap of the isoenzymes. To investigate this possibility, we examined biochemical phenotypes of double and single EC-SOD and CuZn-SOD knockouts. Furthermore, because oxygen free radicals have been implicated in aging in many studies in a variety of species (25), the lifespans associated with the various SOD mutant genotypes were studied.

EXPERIMENTAL PROCEDURES

Mice—EC-SOD null mutant mice (female, background: five times backcrossed with C57BL/6), (21) were bred with male CuZn-SOD null mutant mice (background 129/CD1) (23). The double heterozygous offspring were then bred to each other to generate homozygous double mutant mice, single null mutants, and wild-type littermates. Various other combinations from these litters were also thereafter used in breeding to generate the desired genotypes (see Table 1). The EC-SOD geneotype was determined by PCR with primers specific for the gene and for the neomycin gene disruption insert (26, 21). The CuZn-SOD genotpe was determined by measuring CuZn-SOD activity in whole blood (27, 28). The pups were weaned at 3 weeks of age and then maintained on a 12-h day and night cycle with food and water provided ad libitum. The study was approved by the Animal Research Ethics Board.
(National Institutes of Health publication 86-23, revised 1985) and was approved by the local animal ethics committee. The samples for the biochemical analyses were generally collected at 3 months of age. Another group of mice was kept and monitored during aging.

NO Turnover Study—The mice were kept on a fluid nutrient lacking nitrite and nitrate (50 g/liter glucose, 40 mM sodium, and 20 mM potassium) for 6 days. The mice were put in clean cages each day to limit the complication of feces being an external source of nitrate. Urine samples were collected on days 5 and 6 by holding the mice over a clean surface and applying a gentle pressure on the stomach to induce micturition. The urine was immediately collected and stored at −80 °C until assayed.

Chemical Analysis—Serum iron and total iron binding capacity were determined with a reagent kit from Roche Applied Science. The iron and copper content of mouse liver was analyzed with inductively coupled plasma atomic emission spectrometry. Urine creatinine was determined in a Vitros 950 apparatus (Ortho-Clinical Diagnostics). Thiobarbituric acid-reactive substances (TBARS) in EDTA plasma were measured by fluorimetry (29, 30). Unextracted urine (50 μl) was analyzed for 8-iso-prostaglandin F_2α by radioimmunoassay (31). Nitrite plus nitrate in urine were analyzed using a nitric oxide assay kit from R & D Systems Inc. (Minneapolis, MN).

Glutathione and Ascorbate Analysis—Tissues and plasma were homogenized in 50 g/liter metaphosphorous acid, at a 1:10 (w/v) ratio. After centrifugation for 15 min at 15,000 g, the supernatant was stored at −80 °C until analysis. The reduced glutathione GSH content was determined with a kit, GSH 400, from Oxis Research (Portland OR). Ascorbate was analyzed by high pressure liquid chromatography using an electrochemical detector (32).

Analysis of Enzymes—Tissues were homogenized with an Ultraturrax in 10 volumes of 50 mM sodium phosphate (pH 7.4) with 0.3 M KBr containing the Complete protease inhibitor mixture (Roche Applied Science) and centrifuged at 20,000 g for 15 min. The SOD activity of the supernatants was determined by the direct spectrophotometric method using KO_2 (27, 28). To distinguish between the resistant isoenzyme Mn-SOD and the sensitive isoenzymes CuZn-SOD and EC-SOD, 3 mM cyanide was used. The glycoprotein EC-SOD was separated by its binding to a concanavalin A-Sepharose column (21, 33). The CuZn-SOD activity is calculated as the total cyanide-sensitive activity minus the EC-SOD activity. The detection limits in the procedure are for CuZn-SOD, EC-SOD, and Mn-SOD 5 (20 ng), 50 (430 ng), and 5 (325 ng) units/g of wet weight, respectively. Glutathione peroxidase (GSHPX) was determined by a coupled spectrophotometric method (34). Catalase was determined with a kit, GSH 400, from Oxis Research (Portland OR).

Statistics—Differences between the various groups of mice were evaluated using the nonparametric Mann-Whitney U test. Survival curves were made from Kaplan-Meier plots, and the survival statistics were calculated with Cox regression, Forward: Wald. Unless otherwise stated, the values (number of animals) in the tables are presented as the means ± S.D.

RESULTS

Breeding, Body Weight, and Lifespan of Mice—Several genotype combinations were used to produce the single and double EC-SOD and CuZn-SOD knockouts, all resulting in similar litter sizes (Table 1). All of the genotypes produced were viable and developed normally to fertile age. Attempts to breed with CuZn-SOD null females failed as previously reported (22).

The absence of EC-SOD did not influence the lifespan of the mice (Fig. 1). As previously found (24), the lifespan of the CuZn-SOD mice was shortened, but there was no further shortening in the double knockouts. Throughout life, the CuZn-SOD knockout mice showed reduced body weight compared with controls (Fig. 2). Body weights tended to be higher in mice lacking EC-SOD, but because of a large degree of spread, the effect was not statistically significant.

<table>
<thead>
<tr>
<th>Genotype male</th>
<th>Genotype female</th>
<th>Number of litters</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEe/Cc</td>
<td>Ee/Cc</td>
<td>36</td>
<td>7.8 ± 3.8</td>
</tr>
<tr>
<td>ee/Cc</td>
<td>ee/Cc</td>
<td>40</td>
<td>7.4 ± 2.7</td>
</tr>
<tr>
<td>ee/cc</td>
<td>ee /Cc</td>
<td>15</td>
<td>8.9 ± 2.9</td>
</tr>
<tr>
<td>EE/Cc</td>
<td>EE/Cc</td>
<td>10</td>
<td>8.5 ± 3.9</td>
</tr>
<tr>
<td>EE/cc</td>
<td>EE/Cc</td>
<td>2</td>
<td>9,7</td>
</tr>
</tbody>
</table>

FIGURE 1. Lifespans of mice of the different genotypes. Thick dotted line, EE/CC; thick solid line, ee/cc; thin dotted line, EE/cc; thin solid line, ee/Cc. The cumulative survival is plotted against the ages of the mice at the time of death. Because of a period of problems with infection in the mouse quarters, some early deaths of mice occurred. Only mice surviving 40 weeks were entered into the analysis. The numbers of mice excluded/initial
number of mice were 3/22, 0/60, 1/20, and 5/56 in the control, EE/cc, ee/Cc, and ee/cc groups, respectively. The significance for the differences versus wild-type mice were, for the ee/cc, EE/cc, and ee/cc groups, p = 0.001, p = 0.056, and p = 0.49, respectively. The survivals between the ee/cc mice and the ee/Cc mice were significantly different (p < 0.001), whereas the further possible comparisons lacked significance.

FIGURE 2. Weight development in female mice of the different genotypes. Only mice entering the survival analysis were included (cf. Fig. 1). Filled circles, EE/CC (n = 12); open triangles, ee/Cc (n = 18); filled triangles, EE/cc (n = 6); open circles, ee/cc (n = 16). *p < 0.05 relative to control mice.

Phenotypes of Superoxide Dismutase Double Knockouts

**TABLE 1**

Fertilites of breeding combinations of EC-SOD and CuZn-SOD null mutant mice

The values are the means ± S.D. E/e, EC-SOD replete/null mice; C/c, CuZn-SOD replete/null mice.
Glutathione and Ascorbate—Whereas no changes in GSH were noted in EC-SOD null mice, the levels were elevated by about 35% in the livers of CuZn-SOD null mice (Table 2). There was no further increase in GSH levels in the livers of double knockout mice, but there was a rise in GSH levels in the kidney.

In the absence of SOD, ascorbate should be the major scavenger of superoxide radicals (37). The EC-SOD null mice showed a slightly reduced level of ascorbate in the lung relative to wild-type mice (1520 ± 390 (n = 25) and 1610 ± 740 (n = 20) μmol/kg of wet weight, respectively; p < 0.05). No other changes in ascorbate in liver, kidney, heart, lung, and skeletal muscle were found in the mice of the different genotypes (data not shown). There was no change in blood plasma ascorbate in the EC-SOD null mice, but there was a 25% reduction in the CuZn-SOD null mice and in the double knockout mice (Table 3).

Oxidative Stress Markers—The urinary excretion of the isoprostane 8-iso-prostaglandin F₂α is a specific marker for the extent of systemic lipid peroxidation (30). Although there was no change in EC-SOD null mice, there was a significant increase in excretion of this marker in the CuZn-SOD null mice. This increase was enhanced by the additional absence of EC-SOD (Table 3). Overall, the females of all genotypes had higher excretion rates than the males. TBARS are nonspecific markers of systemic lipid peroxidation (30, 29). The plasma TBARS levels were slightly elevated in EC-SOD null mice, but not in the CuZn-SOD knockouts. There was a marked potentiation in the double knockout mice, however.

Nitric Oxide Turnover—Nitric oxide reacts rapidly with superoxide to form peroxynitrite (38). The absence of SOD isoenzymes should increase the consumption of NO, which could conceivably be produced in increased amounts through autoregulation. The production of NO in the body can be estimated by analysis of the stable metabolites nitrite and nitrate in urine. There was no difference, however, in nitrate plus nitrite excretion in the urine between any of the genotypes in the study (Table 3).

Iron and Copper Analysis—The superoxide radical can influence iron and iron turnover in several different ways (2, 3, 39). The iron regulatory protein-1, also known as cytosolic aconitase, is inactivated by superoxide radicals (38). The absence of SOD isoenzymes should increase the consumption of NO, which could conceivably be produced in increased amounts through autoregulation. The production of NO in the body can be estimated by analysis of the stable metabolites nitrite and nitrate in urine. There was no difference, however, in nitrate plus nitrite excretion in the urine between any of the genotypes in the study (Table 3).
Phenotypes of Superoxide Dismutase Double Knockouts

The values are the means ± S.D. (number of animals). E/e, EC-SOD replete/null mice; C/c, CuZn-SOD replete/null mice. ND, not detected (value below detection level of the assay); NA, not analyzed.

**TABLE 4**
Iron and copper in 3-month-old mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Serum iron</th>
<th>Iron in liver</th>
<th>Copper in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µM</td>
<td>mg/kg of wet weight</td>
<td>mg/kg of wet weight</td>
</tr>
<tr>
<td>EE/CC</td>
<td>F</td>
<td>31.6 ± 4.9 (10)</td>
<td>90.5 ± 33.4 (7)</td>
<td>4.57 ± 0.29 (5)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>33.6 ± 7.5 (11)</td>
<td>5.97 ± 0.52 (5)</td>
<td>5.27 ± 0.84 (10)</td>
</tr>
<tr>
<td>ee/CC</td>
<td>F</td>
<td>32.5 ± 3.2 (6)</td>
<td>99.4 ± 36.6 (10)</td>
<td></td>
</tr>
<tr>
<td>ee/CC</td>
<td>M</td>
<td>34.7 ± 5.0 (10)</td>
<td>99.4 ± 36.6 (10)</td>
<td></td>
</tr>
<tr>
<td>EE/cc</td>
<td>F</td>
<td>38.4 ± 11.6(10)</td>
<td>138.3 ± 38.7 (9)</td>
<td>2.57 ± 0.21 (5)</td>
</tr>
<tr>
<td>EE/cc</td>
<td>M</td>
<td>40.0 ± 5.7 (10)p</td>
<td>138.3 ± 38.7 (9)</td>
<td>2.57 ± 0.21 (5)</td>
</tr>
<tr>
<td>ee/cc</td>
<td>F</td>
<td>36.6 ± 7.5 (8)</td>
<td>142.5 ± 29.2 (10)</td>
<td>2.77 ± 0.29 (10)</td>
</tr>
<tr>
<td>ee/cc</td>
<td>M</td>
<td>39.4 ± 5.6 (2)</td>
<td>142.5 ± 29.2 (10)</td>
<td>2.77 ± 0.29 (10)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>37.1 ± 7.0 (10)</td>
<td>142.5 ± 29.2 (10)</td>
<td>2.77 ± 0.29 (10)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In this study, we searched for changes associated with single and double knockout of the genes encoding EC-SOD and CuZn-SOD. The copper levels in livers of CuZn-SOD knockouts were nearly halved (Table 4).

**Tissue Antioxidant Enzymes**—To search for compensatory changes, tissue antioxidant enzyme levels were examined in liver, kidney, and lung (Table 5). No change in CuZn-SOD was noted in any of the organs as a result of the absence of EC-SOD. Absence of CuZn-SOD did not significantly affect the EC-SOD activity. In the CuZn-SOD null mice, the Mn-SOD activity was about 30% increased in lung and liver, but not in the kidney. The Mn-SOD activity in the liver was slightly reduced in EC-SOD knockouts, but not in the other organs. Tissue catalase levels were generally not affected in the knockouts.

The most marked change detected was in GSHPX, which was reduced by 55–60% in all three organs examined in CuZn-SOD knockouts. Except for a minor reduction in the liver, the absence of EC-SOD had no influence on GSHPX activity.
Phenotypes of Superoxide Dismutase Double Knockouts

main question was whether there was any evidence for overlapping functions that could explain the relatively mild phenotypes previously noted in single knockouts. Absence of EC-SOD is here shown to be without effect on the lifespan of mice, and the reduced lifespan of CuZn-SOD knockouts was not further shortened by the absence of EC-SOD (Fig. 1). It should, however, be noted that the genetic backgrounds of the mice are complex, which may have influenced the comparison of lifespans of the different genotypes. Knockout of EC-SOD, however, tended to increase the body weight of the mice. It is possible that extra-cellular superoxide radicals at some critical locations influence appetite or energy turnover. Loss of EC-SOD in tanytelys and neurons in discrete areas of the thalamus and hypothalamus may be involved in this effect (14).

The only evidence for any overlapping functions of the SOD iso-enzymes was the potentiated increase in urinary excretion of 8-iso-prostaglandin F_2alpha and in plasma levels of TBARS found in the double knockouts (Table 3). These markers indicate different types of stress, because the former was primarily associated with the absence of CuZn-SOD and the latter with the absence of EC-SOD. A variety of aldehydes can show TBARS reactivity, and increased levels are caused by, for example, prostanoid synthesis (30).

The other alterations found were almost exclusively related to the absence of CuZn-SOD. Changes were most commonly seen in the liver, which is the organ of the body that contains by far most CuZn-SOD (41) (Table 5). The versatile antioxidant GSH can be induced by oxidant stress (42), and here it was found to be up-regulated in the liver, but not in the other organs analyzed. In the absence of SOD, ascorbate should be the major scavenger of superoxide radicals (37). There were, however, no changes in ascorbate content in the organs analyzed. Mice can synthesize ascorbate in the liver, and dehydroascorbate is easily reduced to ascorbate in the tissues, which may explain the absence of alterations. There was a reduction in plasma ascorbate, but surprisingly, this was only associated with the absence of the intracellular CuZn-SOD. Perhaps there was an increased uptake of ascorbate from plasma by non-synthesizing organs, because of intracellular degradation of the labile dehydroascorbate.

The superoxide radical might interfere with iron turnover by several different mechanisms. Ferric iron can be reduced to ferrous iron (3), and in the extracellular space, the ferric form is necessary for transport from intestinal mucosal cells (43) and for part of the cellular uptake and turnover (44). In the cytosol, reduction by superoxide may lead to iron release from ferritin (39). In addition, the superoxide radicals may cause disruption of the FeS cluster of iron regulatory protein-1, which may increase the uptake of iron into tissues (2). The activity of cytosolic aconitate was greatly reduced in the liver of CuZn-SOD knockouts, as has been reported previously (24, 45). Both the liver and serum iron levels were significantly increased, suggesting a perturbed iron homeostasis (30).

Minor additional effects of the enzyme on the homeostasis of Cu in liver cannot be ruled out.

The superoxide radical reacts extremely rapidly with NO (38). The absence of SODs should lead to increased concentrations of superoxide and consequently increased rates of NO degradation. The product of the reaction, peroxynitrite, is strongly oxidizing and may also nitrate tyrosines in proteins (6, 48). NO, on the other hand, may exert antioxid-ant effects (48). Because the endothelial and neuronal nitric-oxide syntheses are regulated, increased consumption may conceivably lead to increased NO synthesis. However, no significant increases in the urinary excretion of the stable metabolites of NO, nitrite and nitrate, were found in the SOD knockouts.

CuZn-SOD is generally regarded as being constitutively expressed, and it was not influenced by absence of EC-SOD, nor was EC-SOD activity influenced by the absence of CuZn-SOD (Table 5). Mn-SOD was elevated in the liver, as has been found previously (24), and in the lungs of mice lacking CuZn-SOD. There was a marked reduction in GSHPx in all organs lacking CuZn-SOD. Previous studies on CuZn-SOD null mice have reported reduced levels in liver (46, 24) but increases in the lung (46). Possible causes of the reductions in GSHPx are inactivation by superoxide (49) and peroxynitrite (50). Changes in hydrogen peroxide levels might also be involved, because hydrogen peroxide may induce GSHPx synthesis (51). The effects of the loss of CuZn-SOD on hydrogen peroxide levels are, however, difficult to predict. Spontaneous superoxide dismutation would lead to formation of equal amounts of hydrogen peroxide, whereas reaction with ascorbate and nitric oxide would lead to doubled amounts and no formation of hydrogen peroxide, respectively.

In conclusion, the present study has failed to reveal any evidence for major redundancy in the biological roles of EC-SOD and CuZn-SOD. The findings further strengthen the notion that mitochondria are both the major sources and targets of the superoxide radical in mammals.

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REFERENCES