Reduced Fertility in Female Mice Lacking Copper-Zinc Superoxide Dismutase*

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Copper-zinc superoxide dismutase (CuZn-SOD) is believed to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals to form hydrogen peroxide and molecular oxygen. Recent studies have shown that missense mutations in this gene contribute, evidently through a gain-of-function mechanism, to about 20% of familial amyotrophic lateral sclerosis. To define further the physiologic role of this enzyme, a model of mice deficient in this enzyme was generated using gene targeting technology. Mice lacking this enzyme were apparently healthy and displayed no increased sensitivity to hyperoxia. However, they exhibited a pronounced susceptibility to paraquat toxicity. Most surprisingly, female homozygous knock-out mice showed a markedly reduced fertility compared with that of wild-type and heterozygous knock-out mice. Further studies revealed that although these mice ovulated and conceived normally, they exhibited a marked increase in embryonic lethality. These data, for the first time, suggest a role of oxygen free radicals in causing abnormality of female reproduction in mammals.

Reactive oxygen species (ROS),¹ which are produced as by-products of normal metabolism, are capable of causing cellular damage, leading to cell death and tissue injury (for review, see Ref. 1). Mammalian cells are equipped with both enzymatic and nonenzymatic antioxidant defense mechanisms to minimize the cellular damage resulting from interaction between cellular constituents and ROS (for review, see Ref. 2). Despite the presence of these delicate cellular antioxidant systems, an overproduction of ROS in both intracellular and extracellular spaces often occurs upon exposure of cells or individuals to radiation, hyperoxia, and certain chemicals. An unbalanced production of ROS has been postulated to play a role in the pathogenesis of a number of clinical disorders such as acute respiratory distress syndrome, ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, and cancer (for review, see Ref. 3). This understanding illustrates the importance of the antioxidant defense system in maintaining normal cellular physiology. However, due to the overlapping activity among some of the antioxidant enzymes, it is generally difficult to define the role of each individual antioxidant enzyme. We are interested in understanding the physiologic relevance of copper-zinc superoxide dismutase (CuZn-SOD) under normal physiologic conditions and in defending cells and animals against the pathogenesis of ROS-mediated diseases. The results from previous studies for defining the protective function of this enzyme using cells and animals with augmented enzyme expression have been controversial (4–9), since some of them develop an increased susceptibility to certain oxidants relative to that of parental cells and control animals. It is not clear whether the detrimental effect of CuZn-SOD overexpression is a result of the associated free radical generating activity of this enzyme or its capability in enhancing nitration of tyrosine by peroxynitrite (10–15). Therefore, overexpression of this enzyme may not provide a suitable model to address the nature of this enzyme in cellular antioxidant mechanisms. To define further the role of CuZn-SOD in cellular antioxidant defense mechanisms, we generated, by gene targeting technology, mice lacking this enzyme.

MATERIALS AND METHODS

Targeted Disruption of the Mouse Sod1 Gene—Eleven mouse Sod1 genomic clones were isolated from a 129/SvJ genomic library purchased from Stratagene (La Jolla, CA) by screening with a rat Sod1 cDNA probe (16). An approximately 7.2-kb SacI genomic fragment from clone 30 was found to contain the entire mouse Sod1 gene with a sequence very similar to that published by Benedetto et al. (17). To inactivate the mouse Sod1 gene, the Smal and HindIII restriction sites flanking the Smal-HindIII fragment, which contains sequences from intron 1 to intron 4, were converted into XhoI sites by linker ligation and then inserted into the XhoI site in plasmid vector pPNT (see Ref. 18, Fig. 1a). Similarly, linker ligation was also used to clone the EcoRI-Sod1 fragment containing the 3'-flanking sequence of the gene into the BamHI site in the pPNT vector.

The Sod1 targeting vector, in which exon 5 was deleted, was linearized by HindIII digestion and transfected into R1 embryonic stem cells (19). Clones resistant to G418 and ganciclovir were screened by Southern blot analysis using a probe 5' external to the genomic sequence present in the targeting vector. Fifty-two clones were identified from 666 clones screened to contain the expected targeted Sod1 allele. Targeted clones were microinjected into C57BL/6 blastocysts following the standard procedure (20). Thirty chimeric mice with near 100% chimerism were generated using Sod1 knock-out clones 5 and 8. Chimeric mice derived from either clone showed 100% transmission of the chimeric alleles to the offspring. The heterozygous CuZn-SOD knock-out (Sod1¹⁻) mice were initially generated from breeding between the chimeric mice and C57BL/6 mice. They, therefore, are F1 hybrid between the 129SvJ and C57BL/6 inbred genetic backgrounds. These Sod1¹⁻ mice were interbred to generate mice with three Sod1 geno-
types (Sod1\(^{+/+}\), Sod1\(^{+-}\), and Sod1\(^{-/-}\)). These F2 littermates were used in expression studies including RNA and protein analysis. Since the female Sod1\(^{2/-}\) mice are not very fertile, breeding was performed between F2 male Sod1\(^{2/-}\) and female Sod1\(^{1/-}\) littermates as well as between F2 Sod1\(^{1/-}\) male and female littermates to generate a large number of wild-type and knock-out mice for various pathologic and physiologic studies described in this report.

**RNA Blot Analysis**—Total RNA was isolated from tissues by the treatments and analyzed by Northern blot hybridization using a cDNA probe derived from the CuZn-SOD gene. Blots were hybridized with the probe and then probed with a GAPDH cDNA to check for potential variation in sample loading. The results show the expression levels of CuZn-SOD in various tissues, with higher expression in the brain compared to other organs.

**Fig. 1. Generation and characterization of CuZn-SOD-deficient mice.**

**a.** Schematic diagram showing the genomic and partial restriction map of the mouse Sod1 locus (top), the targeting vector (middle), and the predicted structure of the targeted locus (bottom). Numbered black boxes represent exons. Striped box represents the 5' external sequence used as a hybridization probe. neo, neomycin resistance gene cassette; TK, herpes thymidine kinase gene cassette. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SacI; Sa, Sall; Sm, SmaI. The approximate sizes of hybridizing PstI genomic fragments of the wild-type allele and the targeted allele are indicated at the top and bottom of the figure, respectively. kb, kilobase pairs.

**b.** DNA blot analysis of mouse offspring. Mouse tail DNA was digested with PstI and probed with the 5' external probe shown in a. 1/1, 1/2, and 2/2 represent wild-type, heterozygous, and homozygous knock-out mice, respectively.

**c.** RNA blot analysis of total cellular RNA isolated from tissues of Sod1\(^{+/+}\), Sod1\(^{+-}\), and Sod1\(^{-/-}\) mice. Twenty-five micrograms of total RNA from each tissue were separated on agarose gel for blot analysis. The RNA blot was initially hybridized with a rat CuZn-SOD cDNA probe (top panel), and then re-hybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, bottom panel) cDNA to check the potential variation in sample loading.

**d.** A native polyacrylamide gel showing activity staining for SOD in tissues of Sod1\(^{+/+}\), Sod1\(^{+-}\), and Sod1\(^{-/-}\) mice (24). The types of tissues and Sod1 genotypes are shown at the top of the gel. The positions of CuZn-SOD and Mn-SOD migration are indicated to the left.
CuZn-SOD activities were detected.

With plugs were then housed individually and the birth date and numerical plugs were checked at around 9 a.m. each morning. Female mice were counted three times each day.

12-h off light cycle at all times. The numbers of surviving animals were determined using a moisturized cotton tip and examined under a light microscope.

Measurement of Estrous Cyclicity (27)—Vaginal smears were taken daily using a moisturized cotton tip and examined under a light microscope. Entry into the estrous cycle is indicated by an increase in the concentration of progesterone.

Measurement of Fecundity Index Determination—Total number of surviving animals was recorded over a 12-h period at 10 a.m., and the animals were kept in a 12-h on, 12-h off light cycle at all times.

Histological Analysis—Wild-type and age-matched homozous knock-out mice were fixed by systemic perfusion with Bouin’s fixative through the left ventricle. The tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were examined under a light microscope.

As shown in Fig. 1a, exon 5 of the mouse Sod1 gene (which encodes the C-terminal of the protein from amino acid residues 120–154 that constitute both the structure and function of the active site channel (28)) and some of the flanking intron sequences were replaced by a neomycin resistance cassette (neo). Insertion of the neo in the mouse Sod1 gene creates a new PstI restriction site, resulting in a shorter PstI genomic fragment from the targeted allele (−12.5 kb) than that from the wild-type allele (−16.5 kb). Mice heterozygous (Sod1+/−) for the targeted allele were interbred to generate homozous knock-out (Sod1−/−) mice. An example of DNA blot analysis of mouse DNA is shown in Fig. 1b. In addition to the −16.5-kb wild-type and the −12.5-kb targeted genomic fragments, the 5′ external probe containing exon 1 sequence also hybridized with a PstI fragment of 6.5 kb. This is believed to result from cross-hybridization between the probe and the mouse Sod1 pseudogene(s) (29).

Statistical Analysis—One-way analysis of variance was used to examine differences in each measurement performed on wild-type, heterozygous, and homozous knock-out mice. If a significant difference was observed (p < 0.05), then pairwise comparisons among mice were made using Duncan’s test. Fecundity indices of mice were analyzed by one-sided Fisher’s exact test. Survival of wild-type and knock-out mice exposed to >99% oxygen or following intraperitoneal administration of paraquat at 10 mg/kg body weight was analyzed using the Kaplan-Meier method.

RESULTS AND DISCUSSION

Generation and Characterization of Sod1 Knock-out Mice—As shown in Fig. 1a, exon 5 of the mouse Sod1 gene (which encodes the C-terminal of the protein from amino acid residues 120–154 that constitute both the structure and function of the active site channel (28)) and some of the flanking intron sequences were replaced by a neomycin resistance cassette (neo). Insertion of the neo in the mouse Sod1 gene creates a new PstI restriction site, resulting in a shorter PstI genomic fragment from the targeted allele (−12.5 kb) than that from the wild-type allele (−16.5 kb). Mice heterozygous (Sod1+/−) for the targeted allele were interbred to generate homozous knock-out (Sod1−/−) mice. An example of DNA blot analysis of mouse DNA is shown in Fig. 1b. In addition to the −16.5-kb wild-type and the −12.5-kb targeted genomic fragments, the 5′ external probe containing exon 1 sequence also hybridized with a PstI fragment of 6.5 kb. This is believed to result from cross-hybridization between the probe and the mouse Sod1 pseudogene(s) (29).

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Inactivation of the functional mouse Sod1 gene by gene targeting was then demonstrated by expression study. RNA blot analysis revealed an approximate 40–60% reduction of Sod1 mRNA in tissues of Sod1−/− mice compared with that of wild-type (Sod1+/+ or Sod1+/−) mice (Fig. 1c). Furthermore, no CuZn-SOD mRNA could be found in the tissues from Sod1−/− mice, indicating that the truncated CuZn-SOD or CuZn-SOD-neo fusion mRNA was degraded rapidly in these tissues. Reduction of CuZn-SOD activity in tissues of Sod1−/− and Sod1+/− mice was also confirmed by activity staining on a native polyacrylamide gel (Fig. 1d) and the enzyme assay (Table I). It should be noted that whereas no CuZn-SOD activities were found in brain and liver of Sod1−/− mice, a very low level of CuZn-SOD activity was present in the lung samples. This activity presumably represents the activity of extracellular superoxide dismutase, as expression of this SOD isoform is relatively high in the lungs compared with other tissues (30). A decrease in CuZn-SOD activity apparently had no effect on the activity of other cellular antioxidant enzymes such as manganese superoxide dismutase (Mn-SOD) (Fig. 1d and Table I), catalase, and glutathione peroxidase and the enzymes that participate in the recycling of oxidized glutathione including glutathione reductase and glucose-6-phosphate dehydrogenase in these tissues.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sod1 genotype</th>
<th>Brain</th>
<th>Liver</th>
<th>Lung</th>
</tr>
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<tbody>
<tr>
<td>CuZn-SOD</td>
<td>+/+</td>
<td>23.0 ± 5.4</td>
<td>61.2 ± 15.9</td>
<td>21.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>−/+</td>
<td>10.7 ± 1.2</td>
<td>28.5 ± 4.5</td>
<td>11.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>+/+</td>
<td>11.3 ± 3.1</td>
<td>13.9 ± 2.7</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>−/+</td>
<td>11.1 ± 2.2</td>
<td>12.9 ± 3.1</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>11.4 ± 1.4</td>
<td>11.5 ± 1.3</td>
<td>8.3 ± 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.01 when comparing +/+ and −/− mice.

<sup>b</sup> p < 0.001 when comparing +/+ and −/+ mice.

<sup>c</sup> p < 0.0001 when comparing +/+ and −/− mice.

<sup>d</sup> p < 0.0001 when comparing +/+ and −/− mice, or +/−, and −/− mice.

**Fig. 2. Increased susceptibility in Sod1−/− mice to paraquat but not hyperoxia.** a, survival analysis of Sod1+/+ and Sod1−/− mice under hyperoxia. The survival times of age-matched Sod1+/+ and Sod1−/− mice of both genders under >99% oxygen were measured. b, survival curves of age-matched male Sod1+/+, Sod1−/−, and Sod1−/− mice following intraperitoneal administration of paraquat at a dose of 10 mg/kg body weight.
with either Sod1 on five age. Histologic survey at the light microscopic level performed Sod1 kocyte counts including lymphocytes, monocytes, neutrophils, in the numbers of red cells, reticulocytes, and differential leu- ciency on homeostasis of these cells. No differences were found (data not shown). Since CuZn-SOD is highly expressed in erythrocytes shown). Since CuZn-SOD is highly expressed in erythrocytes (31) and is believed to play a protective role against the super- oxide-mediated damage in these cells, a survey of hematologic profile was performed to assess the effect of CuZn-SOD defi- ciency on homeostasis of these cells. No differences were found in the numbers of red cells, reticulocytes, and differential leu- kocyte counts including lymphocytes, monocytes, neutrophils, eosinophils, and platelets of Sod1+/+, Sod1−/−, or Sod1−/− mice (data not shown).

from Sod1+/+, Sod1+/−, and Sod1−/− mice (data not shown).

Mice Lacking CuZn-SOD Are Phenotypically Normal and Show No Increased Sensitivity to Hypoxia, but Exhibit a Pronounced Susceptibility to Paraquart—The ratio of the three Sod1 genotypes of mouse progeny obtained from interbreeding of Sod1+/− mice was in agreement with Mendelian inheritance, indicating that there was no lethality in development of Sod1+/− mice. Male and female Sod1−/− mice grew normally and were apparently healthy upon observation to 16 months of age. Histologic survey at the light microscopic level performed on five Sod1−/− mice at 4.5 months of age showed no evidence for abnormalities in various tissues including the brain, heart, intestine, kidney, liver, lung, testis, uterus, and ovary (data not shown). Since CuZn-SOD is highly expressed in erythrocytes (31) and is believed to play a protective role against the superoxide-mediated damage in these cells, a survey of hematologic profile was performed to assess the effect of CuZn-SOD deficiency on homeostasis of these cells. No differences were found in the numbers of red cells, reticulocytes, and differential leukocyte counts including lymphocytes, monocytes, neutrophils, eosinophils, and platelets of Sod1+/+, Sod1−/−, or Sod1−/− mice (data not shown).

Since the protective role of CuZn-SOD against hypoxia has been implicated in earlier studies on rats and CuZn-SOD transgenic mice (32, 33), we next determined whether a deficiency in pulmonary CuZn-SOD activity would render animals more sus- ceptible to hypoxic exposure. As shown in Fig. 2a, a virtually identical survival curve with a median survival time of 3.4 ± 0.1 (± S.E.) days was found in both Sod1+/+ and Sod1−/− mice, indicating that the function of this enzyme in lung defense against the damage from increased production of superoxide anion radicals during hypoxia is very limited. However, the Sod1−/− mice were extremely sensitive to paraquat at a dose of 10 mg/kg body weight with a median survival time of 1.4 ± 0.3 (S.E.) days (Fig. 2b, p < 0.0001 compared with Sod1+/+ or Sod1−/− mice). Remarkably, the Sod1−/− mice became listless at about 30 min after intraperitoneal administration of paraquat, while Sod1+/+ and Sod1−/− mice were phenotypically normal even at the end of 7 days of observation.

Female Mice Lacking CuZn-SOD Exhibit a Marked Increase in Post-implantation Embryo Death—During the study, we intended to generate a large number of Sod1−/− mice for various pathologic and physiologic studies by interbreeding between Sod1−/− mice. To our surprise, the reproductive performance of female Sod1−/− mice was inferior to that of female Sod1+/− mice. As shown in Table II, while 16 female Sod1+/− mice gave birth to 26 litters (mean litter size 7.5 ± 2.5) in a period of 3 months, only 16 litters (mean litter size 1.6 ± 1.0) were yielded from an equal number of Sod1−/− female mice. Of these 16 litters, 6 litters contained only 1 pup, 3 litters contained 2 pups, and one litter contained 4 pups. It should be noted that the drastic reduction in reproduction of Sod1−/− females is not a result of a defect in the development of Sod1−/− fetuses, since of the 194 pups derived from the breeding between Sod1−/− female and Sod1−/− male mice, 52% were heterozygous and 48% homozygous for the targeted Sod1 allele.

To understand further this unexpected observation, the re- productive performance of the female mice with three Sod1 genotypes was closely followed. As shown in Table III, male Sod1−/− mice were as fertile as Sod1+/− males, and female Sod1+/− and Sod1−/− mice were similarly fertile when bred with either Sod1+/− or Sod1−/− males. However, the fecu- dity index (number of litters/number of copulations) and size of the litters of Sod1−/− females were much less than those of Sod1+/+ and Sod1+/− females.

The mechanism underlying the poor reproductive performance of Sod1−/− females was further investigated. Examination of vaginal smears indicated that all types of mice had similar estrous cycles, with an average length of 4 to 5 days. The frequency of female mice that became receptive to males was also measured. For 6 weeks, eight female Sod1+/−, Sod1−/−, and Sod1+/− female mice mated 17, 20, and 18 times with vasecto- mized males, respectively. Apparently, the reduced fertility in Sod1−/− mice was not a result of altered estrous cycles.

The numbers of ova ovulated by these three types of females at each estrous cycle were also found to be equivalent (Table IV). In addition, female Sod1−/− mice exhibited a normal ovarian histol- ogy including the number, size, or morphology of antral follicles and corpora lutea compared with that of Sod1+/+ and Sod1+/− mice (data not shown). These results suggested that the reduced fertility in Sod1−/− female mice might result from

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of pups from each copulation</th>
<th>Fecundity index</th>
<th>Litter size</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>11, 9, 9, 9, 8, 7, 6, 5, 4</td>
<td>100</td>
</tr>
<tr>
<td>+/+</td>
<td>−/+</td>
<td>9, 9, 7, 7, 6, 5, 5, 5, 0</td>
<td>90</td>
</tr>
<tr>
<td>+/+</td>
<td>−/−</td>
<td>5, 5, 3, 1, 0, 0, 0, 0, 0</td>
<td>36</td>
</tr>
<tr>
<td>−/−</td>
<td>+/+</td>
<td>11, 10, 9, 8, 7, 7, 0</td>
<td>88</td>
</tr>
<tr>
<td>−/−</td>
<td>−/+</td>
<td>14, 11, 11, 9, 9, 8, 7, 5, 2</td>
<td>100</td>
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<tr>
<td>−/−</td>
<td>−/−</td>
<td>3, 1, 1, 1, 0, 0, 0, 0, 0</td>
<td>36</td>
</tr>
</tbody>
</table>

* Fecundity index = number of litters/number of copulations × 100.
* Numbers are means ± S.D.
* p < 0.05 when comparing +/+ females to +/+ or −/+ females bred with either +/+ or −/+ males.
* p < 0.01 when comparing −/+ females to +/+ or −/+ females bred with either +/+ or −/+ males.

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of ova produced by female Sod1+/+, Sod1+/−, and Sod1−/− mice at each estrus cycle</th>
<th>Average number of ova</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>9, 6, 10, 7, 8, 8</td>
<td>8.0 ± 1.4</td>
</tr>
<tr>
<td>+/+</td>
<td>8, 5, 6, 7, 7, 9</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td>−/−</td>
<td>5, 4, 8, 10, 7, 9, 9, 9</td>
<td>7.6 ± 2.1</td>
</tr>
</tbody>
</table>

* Numbers are means ± S.D.

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of ova produced by female Sod1+/+, Sod1+/−, and Sod1−/− mice</th>
<th>Average number of ova</th>
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<tbody>
<tr>
<td>+/+</td>
<td>14/15</td>
<td>122</td>
</tr>
<tr>
<td>−/−</td>
<td>13/15</td>
<td>91</td>
</tr>
</tbody>
</table>

* Numbers are means ± S.D.
* p < 0.0001 when comparing to −/+ female mice.

TABLE II

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of progeny produced from breeding of female Sod1 knock-out mice</th>
<th>Total number of pups</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/−</td>
<td>+/+</td>
<td>26</td>
<td>194</td>
</tr>
<tr>
<td>−/−</td>
<td>−/+</td>
<td>16</td>
<td>10</td>
</tr>
</tbody>
</table>

* Numbers are means ± S.D.

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of ova per cycle</th>
<th>Average number of ova</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>9, 6, 10, 7, 8, 8</td>
<td>8.0 ± 1.4</td>
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<tr>
<td>+/+</td>
<td>8, 5, 6, 7, 7, 9</td>
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<tr>
<td>−/−</td>
<td>5, 4, 8, 10, 7, 9, 9, 9, 9</td>
<td>7.6 ± 2.1</td>
</tr>
</tbody>
</table>

* Numbers are means ± S.D.

TABLE IV

Post-implantation embryonic lethality in female Sod1−/− and Sod1−/− mice in breeding with male Sod1−/− mice

Pregnant female mice were sacrificed between 10.5 and 14.5 days postcoitum, and the number and viability of embryos were determined.

<table>
<thead>
<tr>
<th>Sod1 genotype</th>
<th>Number of pregnancies/number of copulations</th>
<th>Total number of implanted embryos</th>
<th>Average number of implanted embryos</th>
<th>Total number of dead embryos</th>
<th>Frequency of embryonic death</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>14/15</td>
<td>122</td>
<td>8.2 ± 3.0</td>
<td>9</td>
<td>7.0 ± 7.0</td>
</tr>
<tr>
<td>−/−</td>
<td>13/15</td>
<td>91</td>
<td>6.3 ± 4.4</td>
<td>75</td>
<td>83 ± 23*</td>
</tr>
</tbody>
</table>

* Numbers are means ± S.D.
* p < 0.0001 when comparing to −/+ female mice.
a defect in implantation of embryos to the wall of the uterine horns or premature death of the fetuses.

To evaluate further these two possibilities, we then examined the number and viability of fetuses in Sod1−/− female mice bred with Sod1+/− male mice between 10.5 and 14.5 days postcoitum. As shown in Table V, of 15 mice examined, only 2 of them were not pregnant. However, 75 of 91 implanted embryos in a variety of sizes were found dead and in the process of being resorbed. Compared with the normal 12.5-day fetuses, most of those dead and resorbed embryos were loosely attached to the wall of the uterine horns without a developed placenta or yolk sac (data not shown). Judging from the sizes of these embryos, embryonic death might have occurred at various times before 10 days of pregnancy. In control experiments, 14 of 15 female Sod1−/− mice were pregnant from breeding with Sod1+/− males. A total of 122 implanted embryos was found, and of them only 9 were dead. Although the average number of successfully implanted embryos in Sod1−/− females (6.3 ± 4.4), including both the live and dead ones, was less than that in Sod1+/− females (8.2 ± 3.0), the difference was not statistically significant (p = 0.2). These results indicated that the efficiency of embryo implantation in the former mice was equivalent to that in the latter mice. However, the rate of post-implantation embryonic death that occurred in Sod1−/− females was significantly higher than that in Sod1+/− females (83 ± 23% versus 7.0 ± 7.0%, respectively; p < 0.0001).

During the course of this study, generation and characterization of a line of mice lacking the entire Sod1 locus have also been reported (34). Although we inactivated the Sod1 gene by a different approach, our results on the apparently normal phenotype of the Sod1−/− mice are in agreement with those reported previously. However, our study has focused on some other antioxidant functions of this enzyme that were not described previously (34). Our data reveal that although the role of CuZn-SOD in defending against lethal exposure of hyperoxia is negligible, it is essential in protecting animals against paraquat toxicity. These data indicate that the protective role of CuZn-SOD is dependent on the cellular site of oxygen radical generation. The mitochondrion is known to be a major subcellular site producing oxygen radicals under normal physiologic conditions (for review, see Ref. 1), and the rate of radical production is further enhanced in mitochondria of hyperoxic lungs (35–37). This understanding and our results suggest that Mn-SOD may play a more critical role than does CuZn-SOD in antioxidant defense mechanism(s) under normal physiologic conditions, and in defending against lung injury resulting from hyperoxic insults. This notion is supported by the recent findings that mice lacking Mn-SOD die at very young ages (38–39). However, CuZn-SOD is apparently critical for animals to survive under a lethal exposure to paraquat, a bipyrilid herbicide capable of generating oxygen radicals through the redox cycling mechanism. This reaction is believed to be catalyzed by the enzyme NADPH-dependent cytochrome P-450 reductase, primarily located in the endoplasmic reticulum. Our data suggest that both the cytosolic and microsomal enzymes may be the primary targets of superoxide radicals generated during paraquat toxicity. This conclusion is in agreement with an earlier study reported by Phillips and colleagues (40) that Drosophila deficient in CuZn-SOD is hypersensitive to paraquat.

The most intriguing observation made in this study is the reduced fertility of female mice lacking this enzyme. Apparently, this defect is associated with CuZn-SOD deficiency in the female mice and unrelated to the Sod1 genotypes of the fetuses. Since the female Sod1−/− mice exhibited a normal estrous cycle and generated comparable numbers of ova compared with those of Sod1+/− and Sod1+/− females, the reduced fertility might not have been a result of a gross defect in the hypothalamic-pituitary axis in these mice. However, post-implantation embryonic loss did occur, and this could certainly be endocrine-related. Interestingly, male CuZn-SOD-deficient fruit flies are sterile, and females show a markedly reduced fertility (40). These and our results suggest that CuZn-SOD plays a critical role in female reproduction. The exact mechanism(s) underlying the observed reduced fertility in female CuZn-SOD-deficient mice as well as its implication in human reproductive dysfunction remain to be defined.

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