

## Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice

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**ABSTRACT** Manganese superoxide dismutase (SOD2) converts superoxide to oxygen plus hydrogen peroxide and serves as the primary defense against mitochondrial superoxide. Impaired SOD2 activity in humans has been associated with several chronic diseases, including ovarian cancer and type I diabetes, and SOD2 overexpression appears to suppress malignancy in cultured cells. We have produced a line of SOD2 knockout mice (*SOD2*<sup>m1BCM</sup>/*SOD2*<sup>m1BCM</sup>) that survive up to 3 weeks of age and exhibit several novel pathologic phenotypes including severe anemia, degeneration of neurons in the basal ganglia and brainstem, and progressive motor disturbances characterized by weakness, rapid fatigue, and circling behavior. In addition, *SOD2*<sup>m1BCM</sup>/*SOD2*<sup>m1BCM</sup> mice older than 7 days exhibit extensive mitochondrial injury within degenerating neurons and cardiac myocytes. Approximately 10% of *SOD2*<sup>m1BCM</sup>/*SOD2*<sup>m1BCM</sup> mice exhibit markedly enlarged and dilated hearts. These observations indicate that SOD2 deficiency causes increased susceptibility to oxidative mitochondrial injury in central nervous system neurons, cardiac myocytes, and other metabolically active tissues after postnatal exposure to ambient oxygen concentrations. Our SOD2-deficient mice differ from a recently described model in which homozygotes die within the first 5 days of life with severe cardiomyopathy and do not exhibit motor disturbances, central nervous system injury, or ultrastructural evidence of mitochondrial injury.

Superoxide radicals produced as by-products of metabolic oxidation can cause extensive cellular injury, and several different superoxide dismutases (SODs) have evolved to inactivate both intracellular and extracellular superoxide (1–7). Two closely related SODs containing either manganese or iron as cofactors are produced in most bacterial species, whereas most eukaryotic species contain at least two different intracellular SODs: (i) manganese superoxide (Mn SOD/SOD2) localized within the mitochondrial matrix and (ii) copper- and zinc-containing SOD1 (Cu/Zn SOD1/SOD1) localized predominantly in cytoplasmic and nuclear compartments (8). Another copper- and zinc-containing SOD found predominantly in extracellular compartments (EC SOD/SOD3) has recently been described (9). Although Mn SOD is located within the mitochondrial matrix, the *SOD2* gene encoding Mn SOD is located within nuclear chromosomal DNA (10).

Yeast and bacterial mutants devoid of all SOD activities exhibit hypersensitivity to oxygen and redox compounds such as paraquat, and they survive ambient oxygen by using predominantly anaerobic metabolic pathways to minimize superoxide production (2, 11–13). SOD1-deficient mutants of *Drosophila melanogaster* are viable, but exhibit oxygen and paraquat sensitivity, decreased lifespan, and female infertility (14). Although other potent antioxidants such as glutathione, ascor-

bate, and tocopherols are present to varying degrees within eukaryotic and prokaryotic cells, none of these inactivates superoxide as rapidly or effectively as SODs.

Several recent studies have suggested that decreased Mn SOD activity may be associated with one or more chronic diseases, including ovarian cancer (15, 16) and type I diabetes (17–19). Mn SOD expression has also been shown to decrease metastatic potential and tumorigenicity in cultured cells (20–24) and to protect against neoplastic transformation by ionizing radiation (25). The putative role of Mn SOD as a tumor-suppressor protein plus recent findings that mutations in the *SOD1* gene are linked to amyotrophic lateral sclerosis (26–30) have led us to study the effects of a null *SOD2* mutation on growth, development, and maturation in mice using homologous recombination. A line of SOD2-deficient mice has recently been shown to die within a few days after birth with cardiomyopathy and fatty liver, but without morphologic evidence of mitochondrial damage (31). We have independently produced a line of SOD2 knockout mice (*SOD2*<sup>m1BCM</sup>/*SOD2*<sup>m1BCM</sup>) that survive for up to 3 weeks of age and exhibit several novel pathologic phenotypes including severe anemia, degeneration of neurons in the basal ganglia and brainstem, and progressive motor disturbances characterized by weakness, rapid fatigue, and circling behavior.

### MATERIALS AND METHODS

#### Targeting Constructs and Production of *SOD2*<sup>m1BCM</sup> Mice.

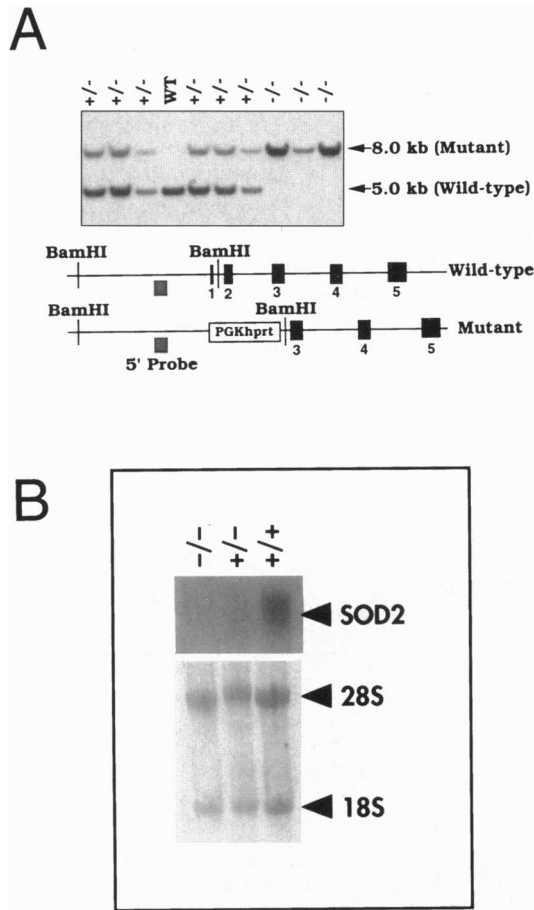
A 1.5-kb *Hind*III fragment containing exons 1 and 2 of the *SOD2* gene was replaced with a human hypoxanthine phosphoribosyltransferase (HPRT) minigene driven by the phosphoglycerate kinase (PGK) promoter (PGK-HPRT) (32, 33). Constructs were transfected into murine ES cells by electroporation, and recombinants were selected in hypoxanthine/aminopterin/thymidine (HAT) medium containing gancyclovir. DNA from selected embryonic stem cell clones and knockout mice was digested with either *Bam*HI or *Eco*RI/*Hind*III, followed by Southern blot analysis using an external probe from the 5' or 3' end of the *SOD2* gene, respectively. For Northern blot analysis, 10 µg per lane of total brain RNA from postnatal day (P) 10 wild-type, *SOD2*<sup>m1BCM</sup>/*SOD2*<sup>m1BCM</sup>, and *SOD2*<sup>m1BCM</sup>/+ mice were run on formaldehyde gels, blotted to nylon (Hybond-N, Amersham), and hybridized to <sup>32</sup>P-labeled mouse *SOD2* cDNA. The filters used for Northern blots were stained with methylene blue dye before hybridization to demonstrate the relative abundance of 28S and 18S ribosomal RNA in each lane.

**Direct SOD Assay.** Measurements of Mn- and Cu/Zn-containing SOD activities were performed as described by

Abbreviations: SOD, superoxide dismutase; CNS, central nervous system; *SOD2*<sup>m1BCM</sup>, *SOD2* mutation resulting in deletion of exons 1 and 2; P, postnatal day.

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**FIG. 1.** Targeting strategy and SOD2 mRNA expression in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice. (A) Germline transmission of the targeted SOD2 gene. Southern blot analysis of genomic DNA from 10 offspring in a single litter immediately after birth shows the presence of wild-type mice (WT), as well as both *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* (-/-) and *SOD2<sup>m1BCM</sup>/+* (+/-) mice. Filters were probed using an external fragment from the 5' end of the SOD2 gene. (B) Expression of SOD2 mRNA in SOD2-knockout mice. 10  $\mu$ g per lane of total brain RNA from P10 wild-type, *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* (-/-), and *SOD2<sup>m1BCM</sup>/+* (+/-) mice was run in each lane and probed with SOD2 cDNA (Upper). (Lower) The methylene blue-stained filter, demonstrating the relative abundance of 28S and 18S ribosomal RNA in each lane, is shown.

Marklund (34) with only minor modifications. One unit of SOD activity in this assay corresponds to  $3 \times 10^{-9}$  g of purified SOD1 (Sigma), and a minimum of 0.1 units could be reliably detected per reaction. Frozen tissues ( $-80^{\circ}\text{C}$ ) were thawed and homogenized in 10 vol of buffer, and SOD activity was extracted for 30 min at  $4^{\circ}\text{C}$  before centrifugation at  $20,000 \times g$  (9). Potassium superoxide (Sigma) was added to each assay as described, and  $t_{1/2}$  of superoxide was measured directly by  $A_{250}$  (34).

**Light and Electron Microscopy.** Vertebral columns from *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* and control mice were fixed in formalin, decalcified in 10% formic acid, embedded in paraffin, and stained with hematoxylin/eosin. Hepatic glycogen stores were evaluated after periodic acid/Schiff base staining of paraffin-embedded, formalin-fixed tissue sections. The presence of glycogen was confirmed by predigestion with diastase, which removes glycogen but not glycoprotein-associated carbohydrate (35). Hepatic stores of neutral lipid were evaluated by staining formalin-fixed frozen sections with Oil red O stain (35).

For electron microscopy, anesthetized mice were perfusion-fixed with 0.25% glutaraldehyde, 2% paraformaldehyde,

**Table 1.** SOD levels in wild-type and -/- heart tissue

SOD type	Genotype	Average units/mg protein $\pm$ SD	P value
Cu/Zn SOD	WT	309.9 $\pm$ 39.5	0.0163
	-/-	391.1 $\pm$ 58.3	
Mn SOD	WT	30.7 $\pm$ 24.7	0.0446
	-/-	0.0 $\pm$ 0.0*	

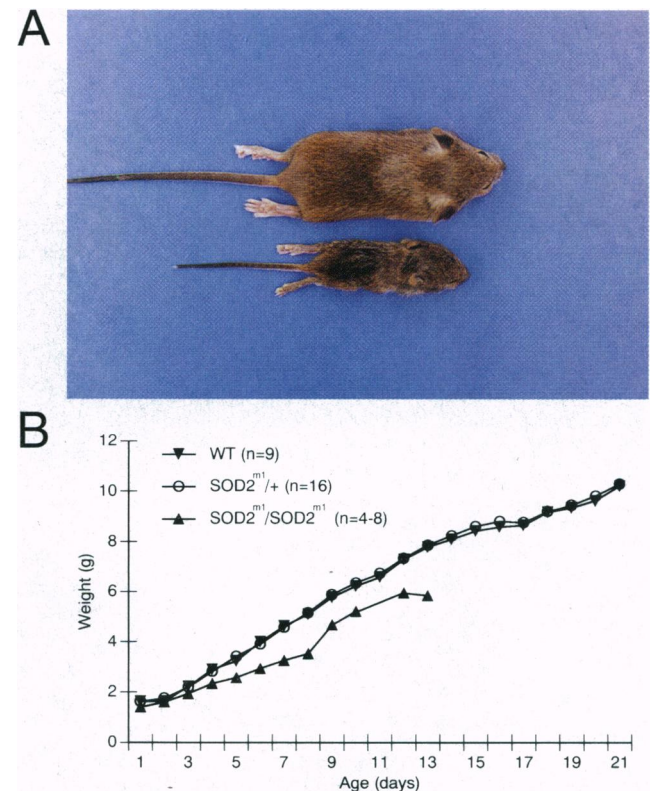
Four 8 to 14 day-old homozygous mutant mice, plus four wild-type littermate controls, were used for this study. Data are represented in SOD units as determined by the direct SOD assay. Paired two-sample Student's *t* tests were conducted to obtain *P* values. A one-tailed and two-tailed test were applied to the Cu/Zn SOD and the Mn SOD data, respectively. WT, wild type.

\*All -/- SOD2 measurements resulted in activities of less than 0.1 unit, the detection limit for this assay. These were recorded as zero.

0.25% CaCl<sub>2</sub>, 0.1 M sodium cacodylate buffer (pH 7.4), and specific regions of central nervous system (CNS), including cortex, basal ganglia and adjacent subcortical gray matter, brainstem, and spinal cord were individually dissected. Tissues were fixed overnight, dehydrated, and processed for electron microscopy as described (36).

**RESULTS**

The *SOD2* gene was inactivated by deletion of exons 1 and 2, plus  $\approx 500$  bp immediately 5' of exon 1 (Fig. 1A). This deletion (*SOD2<sup>m1BCM</sup>*) eliminates the transcription and translation start



**FIG. 2.** Growth abnormalities in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice. (A) The *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mouse at day P10 (bottom mouse) is markedly smaller than its wild-type littermate (top mouse). Although growth abnormalities in these mice effect length as well as weight, no skeletal abnormalities have been detected in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice. (B) Growth rates of *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice are significantly reduced compared with *SOD2<sup>m1BCM</sup>/+* or wild-type littermates. The growth rates of *SOD<sup>m1BCM</sup>* heterozygous and wild-type mice are indistinguishable. The cohort of *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice used in this study ( $n = 8$ ) was reduced by the death of four mice during the first postnatal week.



sites, the mitochondrial targeting sequence, and one of three histidines that bind directly to the manganese cofactor (1, 3, 37–39). Northern blots comparing steady-state SOD2 mRNA expression in mutant and wild-type mice indicate that  $SOD2^{m1BCM/+}$  heterozygotes contain  $\approx 40\%$  of wild-type SOD2 mRNA levels, and  $SOD2^{m1BCM/SOD2^{m1BCM}}$  mice do not contain detectable SOD2 mRNA (Fig. 1B). In addition, assays of SOD activity indicate that any residual SOD2 activity in homozygotes is below limits of detection, whereas cyanide-sensitive SOD levels are increased by  $\approx 25\%$  in  $SOD2^{m1BCM}$  relative to wild-type mice ( $P = 0.016$ ) (Table 1).

To assess whether SOD2 deficiency increases the frequency of embryonic lethality, we evaluated the genotypes of embry-

onic day 18.5 embryos from  $SOD2^{m1BCM/+}$  matings. We observed 26  $SOD2^{m1BCM/SOD2^{m1BCM}}$  (24.3%), 53  $SOD2^{m1BCM/+}$  (49.5%), and 28 wild-type (26.2%) mice, which is close to the expected Mendelian ratio of 1:2:1 and indicates that absence of SOD2 does not cause embryonic lethality. Moreover, embryonic day 18.5  $SOD2^{m1BCM/SOD2^{m1BCM}}$  embryos are indistinguishable from  $SOD2^{m1BCM/+}$  and wild-type embryos with respect to size, gross morphology, and histology (data not shown).

Although  $SOD2^{m1BCM/SOD2^{m1BCM}}$  mice cannot be easily distinguished from their littermates at birth, their diminished growth rate (Fig. 2A) becomes readily apparent between days P2 and P7 and progresses until death by P18 (Fig. 2B). All

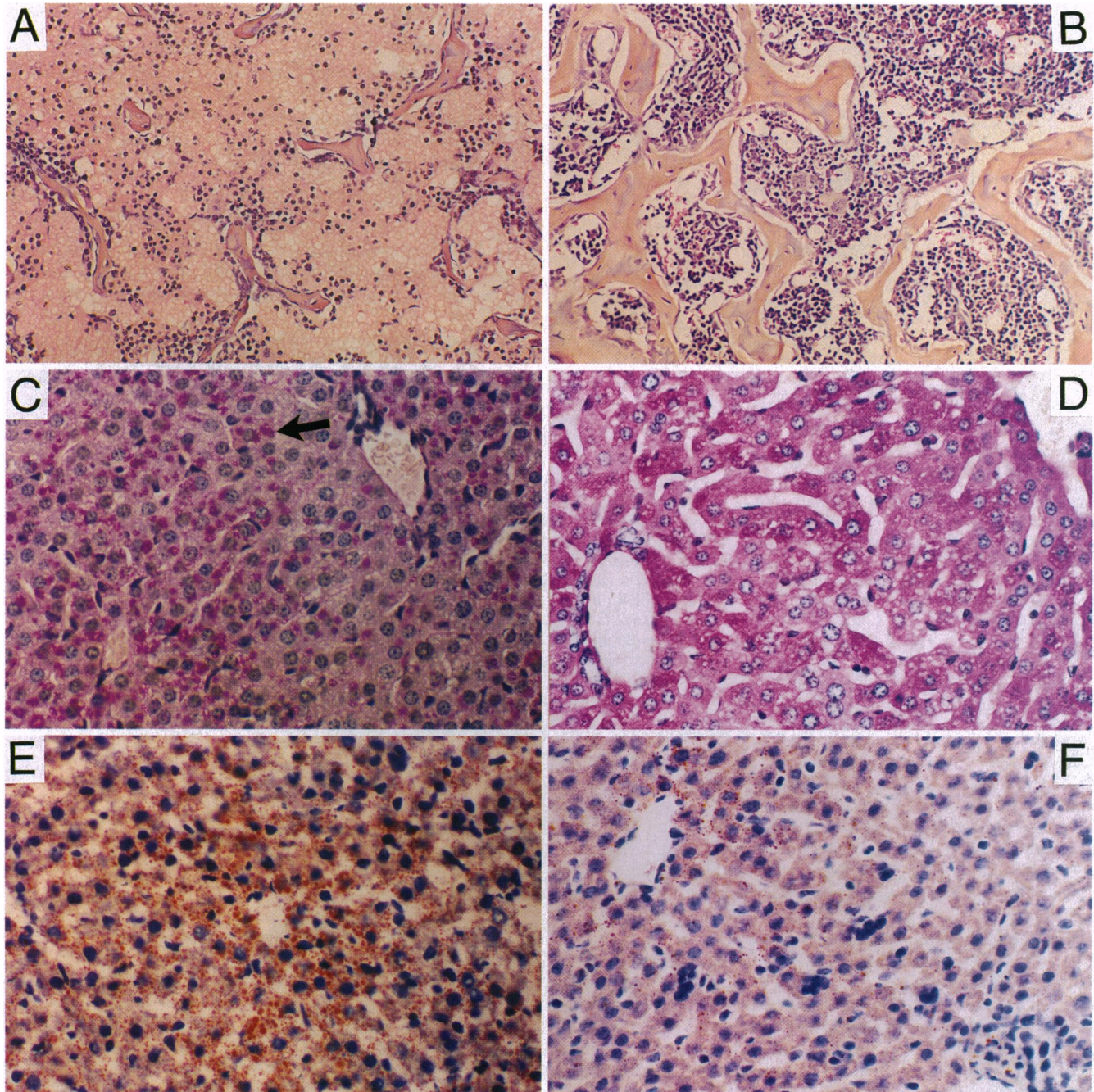


FIG. 3. Histological abnormalities in SOD2-deficient mice. (A and B) Vertebral bone marrow, hematoxylin/eosin stained. Marrow from a P17  $SOD2^{m1BCM/SOD2^{m1BCM}}$  mouse (A) is depleted of hematopoietic cells compared with marrow from a control mouse (B). Periodic acid/Schiff base stained hepatic glycogen from  $SOD2^{m1BCM/SOD2^{m1BCM}}$  mice (C) is coarsely granular (arrow) with a tendency toward centrilobular accumulation. In contrast, hepatic glycogen from control mice (D) is finely and evenly distributed within the hepatic lobule. Oil red O-stained neutral lipid within hepatocytes is increased substantially in  $SOD2^{m1BCM/SOD2^{m1BCM}}$  mice (E) as compared with controls (F). (A and B,  $\times 60$ ; C–F,  $\times 120$ .)



$SOD2^{m1BCM}/SOD2^{m1BCM}$  mice exhibit reduced growth rates compared with control mice, but the observed growth rates vary among  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice. Mutants with the slowest growth rates tend to die within the first postnatal week, whereas those with intermediate growth rates die within the second or third week. Besides their small size, the  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice appear slightly pale. No skeletal abnormalities have been observed other than an overall reduction in size, and  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice exhibit a striking reduction in both adipose tissue and skeletal muscle mass (Fig. 2A). The small size and failure to thrive are not related to nursing difficulties, since milk is always noted in the stomachs of these mutants. In contrast to  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice,  $SOD2^{m1BCM}/+$  mice are indistinguishable from wild-type mice with respect to size and growth rate (Fig. 2B).

Histological analysis of tissues from  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice reveals hypocellular bone marrow (Fig. 3A and B), atypical patterns of glycogen deposition in hepatocytes (Fig. 3C and D), and an abundance of intracellular lipid vacuoles in hepatocytes (Fig. 3E and F). Within the bone marrow of  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice, all hematopoietic lineages appear to be reduced (Fig. 3A), resulting in marked anemia at the time of death. The extent of marrow hypocellularity progresses with increasing age.

Analysis of the brain and spinal cord in  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice by electron microscopy reveals degenerative injury to large CNS neurons, particularly in the basal ganglia and brainstem. This injury is characterized by extensive mitochondrial damage, loss of polysomes, and clearing of the cytoplasm (Fig. 4A). We have also observed a relative absence of rough endoplasmic reticulum, focal dilatation of smooth

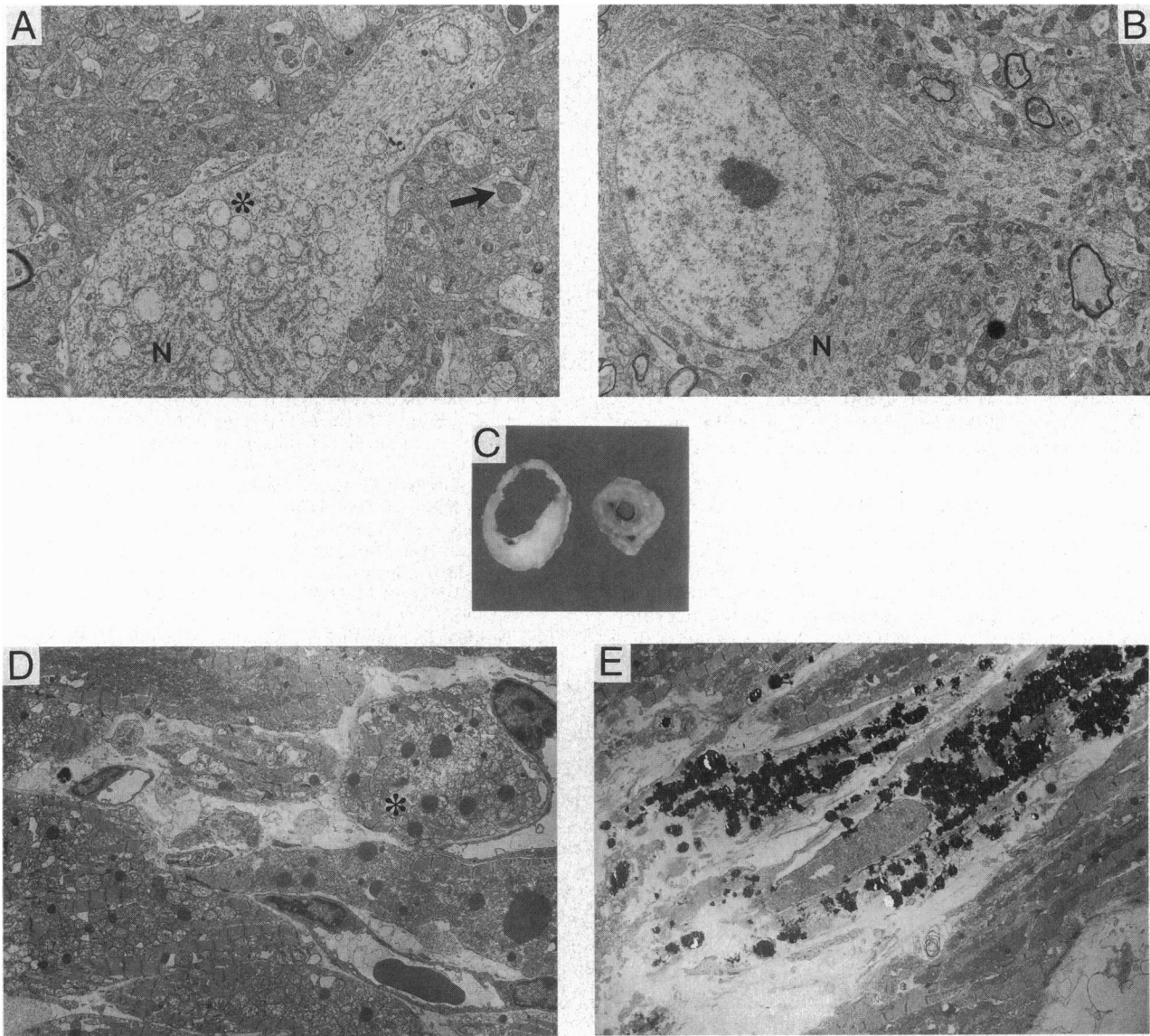


FIG. 4. CNS and cardiac abnormalities in  $SOD2$ -deficient mice. (A) Sections of basal ganglia and adjacent subcortical gray matter from a P10  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mouse demonstrate features of early cellular degeneration, including dispersion of cytoplasm, shedding of ribosomes, and ballooning of mitochondria (arrow). In the brain regions surrounding the degenerating neurons, occasional swollen neurites are present (\*), but most structures are normal and mitochondria are intact. Similar degenerative neuronal changes were also observed repeatedly in brainstem from  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice, but very rarely in cortex or spinal cord. (B) Basal ganglia and other CNS regions from control mice were sampled repeatedly without success for similar degenerative neuronal changes. (C) Sections (3-mm thick) of heart from P8  $SOD2^{m1BCM}/SOD2^{m1BCM}$  (Left) and control (Right) mice demonstrate the marked cardiac dilatation observed in approximately 10% of  $SOD2^{m1BCM}/SOD2^{m1BCM}$  mice. (D) Electron micrographs of myocardium from the dilated heart in C exhibit focal cell death (\*) with swollen, damaged mitochondria and abundant lipid-filled vacuoles. (E) More extensively damaged regions of the myocardium show degenerative mitochondrial changes characterized by diffuse, electron-dense deposits. (A and B,  $\times 1800$ ; D and E,  $\times 2100$ .)

endoplasmic reticulum, and ruffling of nuclear membranes in these cells. Cells surrounding the injured neurons are usually normal in appearance (Fig. 4A). Similar degenerating neurons are lacking in littermate controls, despite extensive searches.

Although dilated cardiomyopathy in SOD2-deficient mice has previously been demonstrated (31), only 10% of our *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice exhibit extreme balloon-like cardiac dilatation with thinning of the ventricular wall (Fig. 4C). Electron microscopy of myocardium from these mice demonstrates widespread cellular injury and death characterized by swelling and fragmentation of mitochondria (Fig. 4D) and, in the most severely affected regions, by deposition of electron-dense material within mitochondria (Fig. 4E). Analysis of the electron-dense mitochondria by energy dispersive x-ray elemental analysis (40) indicates that the mitochondrial densities in Fig. 4E do not contain calcium (data not shown), and we infer from these findings that the observed injury probably represents lipid peroxidation of mitochondrial membranes. Myocytes from *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice without extreme cardiac dilation exhibit increased accumulation of neutral lipid, but do not exhibit mitochondrial injury at the ultrastructural level (data not shown).

In addition to defects in growth and maturation, *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice exhibit striking and progressive motor and behavioral abnormalities including limb weakness, early onset of fatigue, and circling behavior. Limb strength was assessed in mice over 10 days of age by the ability to hold onto a bar suspended above the cage for at least 5 sec without falling, as well as by the ability to scale a 60° incline. *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice perform poorly at both of these tasks relative to littermates. Fatigue was evaluated by comparing the endurance of homozygous, heterozygous, and wild-type mice after immersion into a small room-temperature water bath for a series of 1-min cycles. *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice exhibited fatigue after 2–3 cycles, whereas control mice continued to swim actively after 10 cycles.

## DISCUSSION

SOD2 deficiency causes selective mitochondrial injury in cells with obligatory requirements for high levels of oxidative metabolism, including cardiac myocytes, neurons, hepatocytes, and hematopoietic cells. The apparently normal embryonic development of *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice suggests that embryos are protected by a combination of low oxygen tension and relatively low levels of oxidative metabolism. This hypothesis is supported by observations indicating that fetal levels of Mn SOD in rodents and primates remain very low until relatively late in gestation (41, 42). Our results also indicate that Cu/Zn SOD activity is elevated by approximately 25% in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice, perhaps as part of a compensatory response. It is not clear at present whether this increase is due to SOD1, SOD3, or both.

Histological and ultrastructural evidence of mitochondrial injury in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice has been detected predominantly in older mice (P8–P18), while inactivation of mitochondrial enzymes including aconitase and succinate dehydrogenase can be detected during the first week (31). These findings suggest a model for mitochondrial oxidative damage in which superoxide-induced injury to soluble mitochondrial proteins compromises mitochondrial function and accelerates damage to mitochondrial membranes. Alternatively, the apparent hypersensitivity of soluble mitochondrial enzymes to superoxide may reflect the fact that ultrastructural injury to mitochondrial membranes represents a relatively insensitive assay.

*SOD2<sup>m1BCM</sup>* mutants, missing exons 1 and 2, survive for up to 18 days, and during the second and third weeks of life they develop CNS injury, motor abnormalities, and ultrastructural evidence of mitochondrial damage. These changes have not

been seen in another SOD2-deficient model in which exon 3 has been deleted (*SOD2<sup>m1UCSF</sup>*) and the mice live only for 5 days (31). It is not immediately clear why some *SOD2<sup>m1BCM</sup>* mice outlive *SOD2<sup>m1UCSF</sup>* mice. The possibility of “leaky” expression of SOD2 in our model is highly unlikely considering the nature of our deletion construct. Moreover, Northern blots and direct SOD2 assays demonstrate that neither SOD2 mRNA nor activity can be detected in *SOD2<sup>m1BCM</sup>* homozygotes. The observation that cyanide-sensitive SOD levels are increased in *SOD2<sup>m1BCM</sup>/SOD2<sup>m1BCM</sup>* mice raises the additional possibility that levels of other antioxidants, such as glutathione or glutathione peroxidase, may also be increased.

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