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Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice*

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A growing body of evidence suggests that impaired mitochondrial energy production and increased oxidative radical damage to the mitochondria could be causally involved in motor neuron death in amyotrophic lateral sclerosis (ALS) and in familial ALS associated with mutations of Cu,Zn superoxide dismutase (SOD1). For example, morphologically abnormal mitochondria and impaired mitochondrial histoenzymatic respiratory chain activities have been described in motor neurons of patients with sporadic ALS. To investigate further the role of mitochondrial alterations in the pathogenesis of ALS, we studied mitochondria from transgenic mice expressing wild type and G93A mutated hSOD1. We found that a significant proportion of enzymatically active SOD1 was localized in the intermembrane space of mitochondria. Mitochondrial respiration, electron transfer chain, and ATP synthesis were severely defective in G93A mice at the time of onset of the disease. We also found evidence of oxidative damage to mitochondrial proteins and lipids. On the other hand, presymptomatic G93A transgenic mice and mice expressing the wild type form of hSOD1 did not show significant mitochondrial abnormalities. Our findings suggest that G93A-mutated hSOD1 in mitochondria may cause mitochondrial defects, which contribute to precipitating the neurodegenerative process in motor neurons.

Amyotrophic lateral sclerosis (ALS)[1] is a devastating neurodegenerative disease affecting spinal cord and cortical motor neurons. The onset of the disease is generally in the 4th and 5th decade, and it progresses over an average of 5 years leading to progressive paralysis and premature death (1). Although the majority of the cases are sporadic and due to unknown causes, about 5–10% are familial (FALS), of which ~25% are associated with mutations in the Cu,Zn superoxide dismutase gene (*SOD1*) (2–6). The symptoms and pathology of FALS patients resemble those of patients with the sporadic form of ALS, suggesting that the mechanisms of neurodegeneration share common pathways. Since the initial report (7) of mutant SOD1, more than 90 different mutations of the *SOD1* gene have been found in FALS patients. Because these mutations do not always affect the dismutase activity (8, 9), a toxic gain of function of the mutated protein has been postulated, possibly causing enhanced reactive oxygen species generation (10).

In the motor neurons of transgenic mice expressing the G93A-mutated SOD1 (11), among other pathological features is the presence of membrane-bound vacuoles deriving from mitochondrial degeneration. In these mice, the onset of the paralysis is immediately preceded by an increase in degenerating mitochondria (12), suggesting that mitochondrial alterations might represent a triggering factor in precipitating the degeneration of motor neurons. A decrease of mitochondrial membrane potential and disturbed mitochondrial calcium homeostasis have also been reported (13) in cultured primary motor neurons from G93A mice. Reduced respiratory chain activities were found in the spinal cord of G93A mice (14). These mice also showed increased vulnerability to the mitochondrial toxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a complex I inhibitor, and 3-nitropropionic acid, a complex II inhibitor (15). Another indication of mitochondrial dysfunction in FALS mice comes from the observation that supplementation of creatine, which takes part in the mitochondrial energy buffering and transfer system, improves motor performance and extends survival in G93A SOD1 mutant mice (16).

It was demonstrated that in yeast (17, 18), in rat liver (19), and in transgenic mice expressing wild type or mutated human SOD1 (20–22), a substantial amount of SOD1 is localized in the mitochondrial intermembrane space. SOD1 might have a role in protecting mitochondria from oxidative damage as suggested by an increase of oxidative damage to mitochondrial proteins in yeast lacking SOD1 (18, 19).

In the present study we further investigated the mitochondrial localization of wild type and mutated hSOD1 in transgenic mice and its role in the development of mitochondrial oxidative phosphorylation dysfunction in mice expressing G93A-mutated hSOD1.

EXPERIMENTAL PROCEDURES

hSOD1 Transgenic Mice—Mice transgenic for the G93A-mutated hSOD1 (11) and wild type hSOD1 (N1029 (11)) were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred at the Weill Medical College of Cornell University animal facility. Transgenic mice were identified by PCR of tail DNA as described (11). G93A mice started to show signs of paralysis at age 14–16 weeks. The average age of death for G93A hSOD1 transgenic mice was 19 ± 1 weeks. N1029 mice were phenotypically normal even at 1 year of life.

Mitochondrial Isolation and Purification—Mice were sacrificed, and

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[1] The abbreviations used are: ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; hSOD1, human superoxide dismutase 1; LDH, lactate dehydrogenase; MAO-B, monoamine oxidase-B; CS, citrate synthase; RCR, respiratory control ratio; COX, cytochrome *c* oxidase; SDH, succinate dehydrogenase; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

brain, liver, and spinal cord were promptly removed and mitochondria freshly isolated. For respiration, ATP synthesis assays, the whole liver was homogenized with 8 ml of buffer H, containing 0.22 M D-mannitol, 0.07 M sucrose, 20 mM HEPES, 1 mM EGTA, and 1% bovine serum albumin, pH 7.2, in a glass-Teflon pestle. The homogenate was centrifuged at 1,500 × *g* for 5 min. The supernatant was centrifuged at 10,000 × *g* for 10 min. The mitochondrial pellet was resuspended in a small volume (250 μl) of buffer H and kept on ice. The final protein concentration was 40–60 mg/ml. Mitochondria from a whole mouse brain were extracted by homogenization in 2 ml of buffer H. The homogenate was centrifuged at 1,500 × *g* for 5 min. The supernatant was kept on ice, and the resulting pellet was resuspended in 1 ml of buffer H and subjected to a second centrifugation at 1,500 × *g*. The supernatants were combined and centrifuged at 13,500 × *g* for 10 min. The mitochondrial pellet was resuspended in 50–100 μl of buffer H and kept on ice. The final protein concentration was 20–40 mg/ml. All centrifugation steps were performed at 4 °C. Mitochondria from a whole spinal cord were extracted by homogenization in 1 ml of buffer H as described above, and the mitochondrial pellet was resuspended in 50 μl of the same buffer.

For Western blot and activity gel analyses, mitochondria were purified in a 1–1.7 M sucrose gradient, and mitoplasts were obtained by digitonin treatment as described (23). Aliquots of purified mitochondria and mitoplasts were treated with 250 μg/ml proteinase K for 30 min on ice, after which 1 mM phenylmethylsulfonyl fluoride was added. To estimate the degree of purity of the post-mitochondrial supernatant, mitochondria, and mitoplasts, the residual activities of the cytosolic enzyme lactate dehydrogenase (LDH), the mitochondrial outer membrane monoamine oxidase B (MAO-B), and the mitochondrial matrix citrate synthase (CS) were measured as described (24). For biochemical assays of respiratory chain enzyme activities, mitochondria and synaptosomes from brain and spinal cord were purified in a Ficoll gradient as described (25).

Immunoblotting and Activity Gel Analyses—For SDS-PAGE and immunoblotting, post-mitochondrial supernatants, mitochondria, and mitoplasts were electrophoresed through a 15% polyacrylamide gel and electrotransfered to a polyvinylidene fluoride membrane (Bio-Rad). The membrane was immunostained with sheep anti-human SOD1 antibodies (that recognize both human and mouse SOD1) and then with donkey anti-sheep IgG HRP-conjugated secondary antibodies. Proteins were detected using a chemiluminescence system (Amersham Biosciences). Quantification of immunostained fragments was performed by densitometry using a Fluor-S MultiImager System (Bio-Rad).

SOD1 activity was detected on post-mitochondrial fractions, and isolated purified mitochondria were treated with proteinase K by activity assays in polyacrylamide gels (9) as described (8, 26). SOD1 activity was also determined by spectrophotometry as the ability to inhibit the reduction of tetrazolium salt induced by xanthine-xanthine oxidase as described (26).

Native gel electrophoresis and immunoblotting with anti-human SOD1 antibodies were also performed on post-mitochondrial supernatants and isolated purified mitochondria treated with proteinase K. Mitochondria were solubilized for 15–30 min on ice in 750 mM 6-aminocaproic acid, 50 mM Bistris, pH 7.0, with 5% lauryl maltoside for brain and spinal cord and 3% for liver plus protease inhibitors. Solubilized fractions were centrifuged at 20,000 × *g* for 30 min, and supernatants were used for non-denaturing (*i.e.* without β-mercaptoethanol and SDS) native gel electrophoresis in 4–20% gradient polyacrylamide gels and immunoblotting. Bands were detected as described above.

Measurement of Mitochondrial Respiration and ATP Synthesis—For measurements of mitochondrial oxygen consumption, ~600 μg of liver, 400 μg of brain, and 250 μg of spinal cord mitochondrial proteins were resuspended in 0.3 ml of buffer R containing 0.25 M sucrose, 50 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM KH_2PO_4, pH 7.4. Oxygen consumption was measured in a Clark-type electrode oxygraph (Hansatech Inc., UK) with either 20 mM succinate or 30 mM glutamate plus 30 mM malate in the absence of exogenous ADP (state 2 respiration) and after addition of 300 mM ADP (state 3 respiration). Respiratory control ratios (RCR) were calculated as the ratios between state 3 and state 2 respiration. The ATPase inhibitor oligomycin (100 μg/ml) was then added to inhibit mitochondrial respiration. In normally coupled mitochondria the addition of oligomycin slows respiration to a rate similar to that of state 2, whereas in uncoupled mitochondria oligomycin inhibition is reduced.

Thirty μg of liver, 100 μg of brain, and 50 μg of spinal cord mitochondrial proteins were utilized to measure ATP synthesis with a luciferase/luciferin-based system as described elsewhere (27). The digitonin treatment step in the described procedure was omitted, because purified mitochondria do not require permeabilization for substrates uptake.

Measurements of Protein and Lipid Oxidative Damage—Mitochondrial carbonylated proteins were detected using an OxyBlot Kit (Intergen, Portland, OR). Twenty μg of post-mitochondrial supernatants and proteinase K-treated purified mitochondria were reacted with 2,4-dinitrophenylhydrazine according to the manufacturer's protocol. Proteins were electrophoresed through a 15% SDS-PAGE, electrotransfered to a polyvinylidene fluoride membrane, and immunodetected with anti-2,4-dinitrophenyl antibodies.

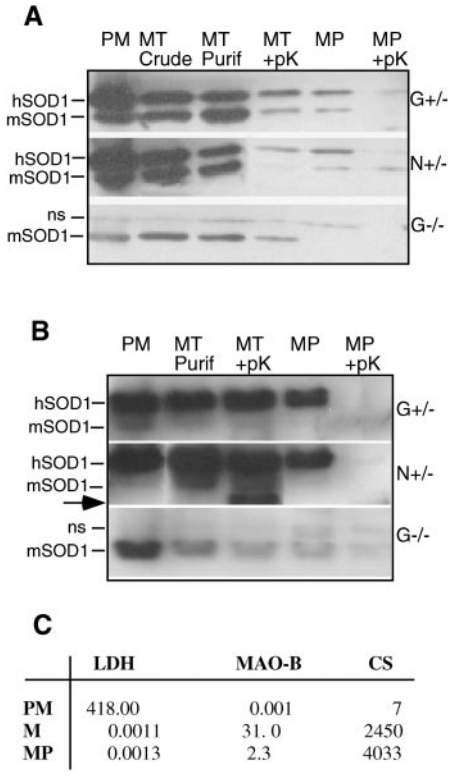
For lipid peroxidation measurements, isolated brain mitochondria (0.8–1.7 mg of mitochondrial proteins) were resuspended in 90 μl of phosphate-buffered saline and pretreated with a combination of respiratory chain inhibitors (1.3 μM rotenone, 1.8 μM antimycin, 2 mM KCN, 10 mM malonate). Lipid hydroperoxides were measured using an LPO-560 Kit (OxisResearch, Portland, OR) according to the manufacturer's protocol.

Respiratory Chain Enzyme Activities and Histoenzymatic Staining—Respiratory chain activity assays were performed on purified mitochondria and synaptosomes from brain and spinal cord. Complexes I + III (NADH-cytochrome *c* oxidoreductase), II + III (succinate-cytochrome *c* oxidoreductase), IV (cytochrome *c* oxidase, COX), and CS activities were measured as described elsewhere (28).

For histoenzymatic staining, spinal cords were dissected and immediately frozen in dry ice-cooled isopentane. Ten-μm-thick cryosections were obtained from the lumbar portion of the spinal cord. Sections were histochemically stained for COX and SDH as described (29).

RESULTS

SOD1 Is Localized in the Intermembrane Space of Mitochondria—Western blot analyses for SOD1 were performed on mitochondrial fractions isolated from liver and brain and on post-mitochondrial supernatants (*i.e.* cytosolic fractions) of 13-week-old heterozygote G93A, age-matched non-transgenic controls, and 1-year-old heterozygote N1029 mice expressing wild type hSOD1. Mitoplasts were obtained by removal of the outer mitochondrial membrane. The purity of mitochondrial and mitoplast fractions was assessed by measuring residual activities of LDH (a cytosolic enzyme), MAO-B (an enzyme of the outer membrane of mitochondria), and CS (a soluble mitochondrial matrix enzyme). The purified mitochondrial fraction was virtually devoid of LDH but rich in MAO-B activity, whereas MAO-B was reduced to ~7% in mitoplast fractions. CS was preserved in both mitochondrial and mitoplast fractions, indicating that the integrity of the inner mitochondrial membrane was preserved (Fig. 1*C*). Aliquots of both mitochondria and mitoplasts were treated with proteinase K to digest all proteins localized on the outside of the outer and inner mitochondrial membranes, respectively. Although the antibody used for the Western blots recognized both human and mouse SOD1, due to different gel mobility, mouse SOD1 migrated faster in the gel than the human form and was recognizable on the membrane as a lower band. In liver (Fig. 1*A*) and to a larger extent in brain (Fig. 1*B*), a considerably large proportion of SOD1 appeared to be localized into mitochondria. Based on the amounts of mitochondrial proteins loaded in the gel and because in our preparation the mitochondrial fraction amounted to about 1% of total liver proteins, we estimated that ~0.5% of total liver hSOD1 actually localized to mitochondria. After proteinase K treatment of purified mitochondria, the absolute amount of SOD1 was decreased severalfold, suggesting that a large proportion of the protein was localized on the external side of the outer membrane. In proteinase K-treated mitochondria, the proportion of hSOD1 was almost 3-fold higher than mouse SOD1. This proportion of mitochondrial SOD1 was mostly preserved in mitoplasts, but it almost totally disappeared after mitoplasts were treated with proteinase K (Fig. 1*A*), suggesting that SOD1 localized in mitochondria actually resided in the intermembrane space and was therefore sensitive to proteinase K digestion of the mitoplast fraction. The fact that SOD1 was still present in the mitoplasts after mild washes and that protein-



C

	LDH	MAO-B	CS
PM	418.00	0.001	7
M	0.0011	31. 0	2450
MP	0.0013	2.3	4033

FIG. 1. **SOD1 Western blot analysis of liver (*A*) and brain (*B*) from 13-week-old G93A transgenic mice (G+/−), age-matched non transgenic mice (G−/−), and 1-year-old N1029 wild type hSOD1 transgenic mice (N+/−).** The anti-SOD1 antibody used for immunodetection recognizes both the human (*hSOD1*) and the mouse (*mSOD1*) forms. A faint nonspecific band (*ns*) that co-migrates with hSOD1 is also recognized by the anti-SOD1 antibody. *PM,* post-mitochondrial fraction; *MT crude*, unpurified isolated mitochondria; *MT purif*, gradient purified mitochondria; *MT + pK*, purified mitochondria treated with proteinase K; *MP*, mitoplasts; *MP + pK*, mitoplasts + proteinase K. The *arrow* in *B* indicates a band presumably deriving from proteinase K digestion of SOD1. *C*, residual activities of the cytosolic enzyme LDH, of the mitochondrial intermembrane space MAO-B, and of the mitochondrial matrix CS. Activities are expressed as nmol/min/mg protein.

ase K treatment was necessary to entirely degrade it indicates that SOD1 is physically associated with components of the inner mitochondrial membrane.

In brain mitochondria, the proportion of hSOD1 relative to the endogenous mouse SOD1 was far greater than in liver. The amount of SOD1 in mitochondria was estimated to represent 2–5% of total brain hSOD1. Also in brain most of the mitochondrial hSOD1 was contained in the intermembrane space, as demonstrated by resistance to proteinase K treatment of purified mitochondria. Again, hSOD1 in brain largely persisted after mitoplast preparation but totally disappeared when mitoplasts were treated with proteinase K (Fig. 1*B*).

Native gel electrophoresis and immunoblotting with anti-SOD1 antibodies showed a band of immunoreactive material of ~40–50 kDa, presumably corresponding to SOD1 dimers, in brain post-mitochondrial fraction, mitochondria, and mitochondria treated with proteinase K (Fig. 2). Although equal amounts of proteins were loaded in the gel, the intensity of these bands was noticeably higher in the G93A sample. Moreover, in post-mitochondrial and mitochondrial fractions of G93A mice there were high molecular mass bands of ~140 kDa, presumably corresponding to aggregated forms of mutated hSOD1 (Fig. 2). Aggregated forms of high molecular weight were not detected in N1029 and in non-transgenic mice, probably because wild type hSOD1 and endogenous mouse SOD1

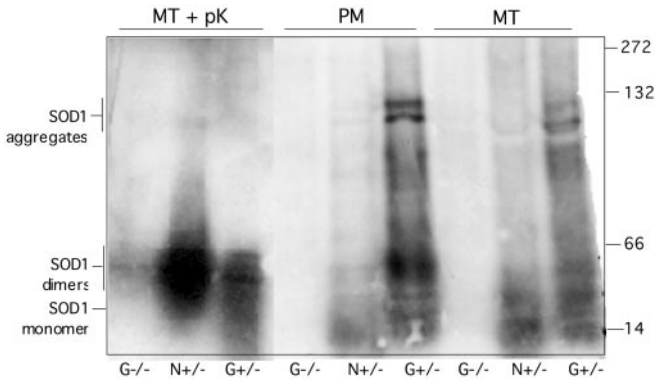


FIG. 2. **SOD1 native Western blot analyses of brain from 13-week-old G93A transgenic mice (G+/−), age-matched non transgenic mice (G−/−), and 1-year-old N1029 wild type hSOD1 transgenic mice (N+/−).** Lane notations are same as in Fig. 1. Positions of the bands presumably corresponding to dimers and aggregated forms of SOD1 are indicated at *left.* Positions of molecular weight markers are indicated at *right.*

have a lower tendency to aggregate. Proteinase K-treated mitochondria showed virtually no aggregated SOD1, suggesting that aggregation might occur predominantly in the cytosol and that the high molecular forms may not be able to penetrate the outer mitochondrial membrane. A similar pattern of aggregation was found in liver post-mitochondrial fractions and isolated mitochondria; however, no aggregated forms were detected in the mitochondrial fraction after proteinase K treatment (not shown). In liver, the intensities of the bands presumably corresponding to SOD1 were rather faint compared with those observed in brain. These findings were in agreement with the results obtained by denaturing Western blot showing higher SOD1 content in brain mitochondria (Fig. 1).

Mitochondrial hSOD1 Is Enzymatically Active—The enzymatic activity of SOD1 was tested by gel assays of liver, brain, and spinal cord in post-mitochondrial and mitochondrial fractions treated with proteinase K. In the latter only the portion of SOD1 contained in the intermembrane space was left after proteinase K digestion. Dismutase activity was found in the post-mitochondrial fractions of liver, brain, and spinal cord of 17-week-old heterozygote G93A, age-matched non-transgenic littermate controls, and 1-year-old homozygote N1029 mice (Fig. 3, *A* and *C*). As expected, in all tissues tested SOD1 activities were higher in transgenic mice as compared with non-transgenic ones. In agreement with the results obtained by denaturing and native Western blots (Figs. 1 and 2), proteinase K-treated brain and spinal cord mitochondria showed high levels of SOD1 activity, whereas SOD1 activity was low in liver mitochondria (Fig. 3, *B* and *C*). These data confirmed that enzymatically active SOD1 was present in mitochondria and that the levels of active mitochondrial SOD1 were higher in the central nervous system. Moreover, despite the fact that the expression levels of SOD1 as determined by Western blot were similar, the activity of wild type hSOD1 in N1029 mice was clearly higher than in G93A mice both in post-mitochondrial supernatant and in mitochondria. This suggested that G93A-mutated hSOD1 has decreased dismutase activity. In the activity gel the high molecular weight forms of SOD1 observed in brain by native electrophoresis and immunoblotting experiments (Fig. 2) were not detected (Fig. 3*A*) and therefore did not appear to be enzymatically active. To confirm that G93A SOD1 had lower dismutase activity, SOD1 activity was also measured by spectrophotometry in brain post-mitochondrial fractions and in mitochondria treated with proteinase K. In G9A mice, SOD1 activity was reduced by ~40% in post-mitochondrial

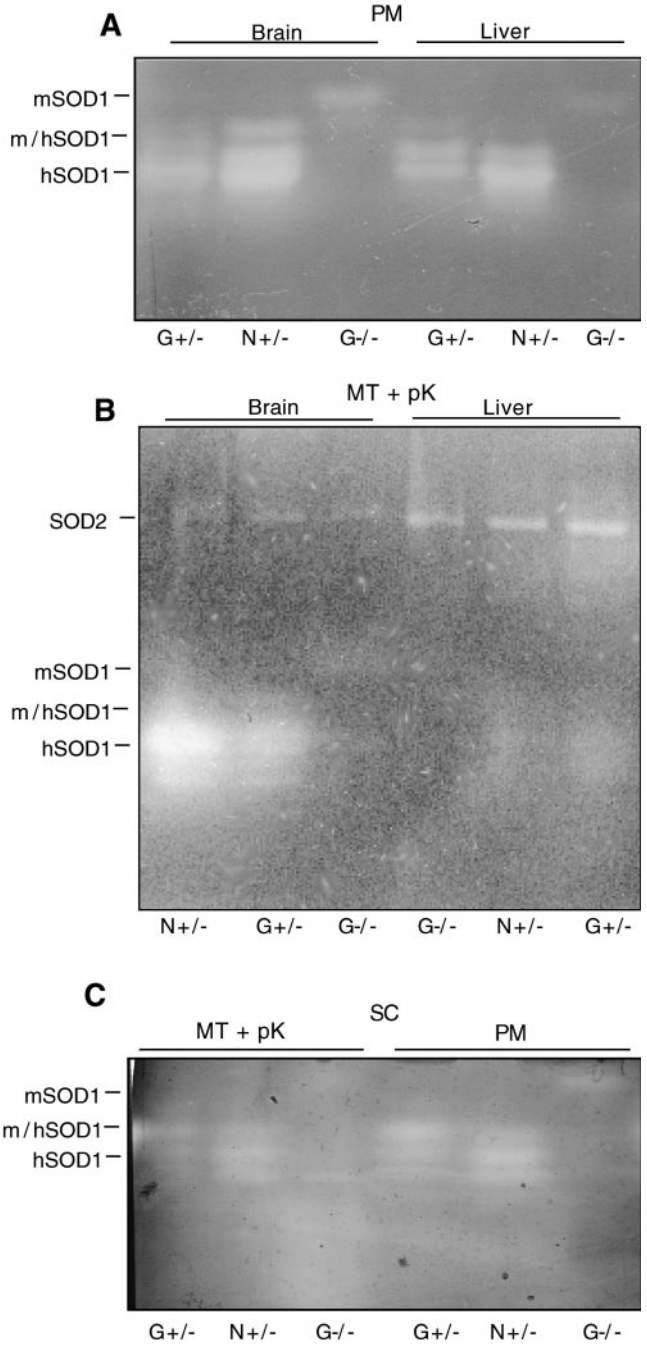


FIG. 3. **SOD activity gel assays.** *A*, brain and liver post-mitochondrial fractions (*PM*) from 13-week-old G93A transgenic mice (G+/−), age-matched non transgenic mice (G−/−), and 1-year-old N1029 wild type hSOD1 transgenic mice (N+/−). *B*, proteinase K-treated (*MT + pK*) purified mitochondria from liver and brain. *C*, proteinase K-treated mitochondria (*MT + pK*) from spinal cord. The position of human SOD1 dimers (*hSOD1*) was determined based on the migration of recombinant hSOD1 (Sigma, not shown). Positions of the bands presumably corresponding to mouse SOD1 dimers (*mSOD1*) and mouse/human SOD1 hybrid dimers (*m/hSOD1*) are also indicated.

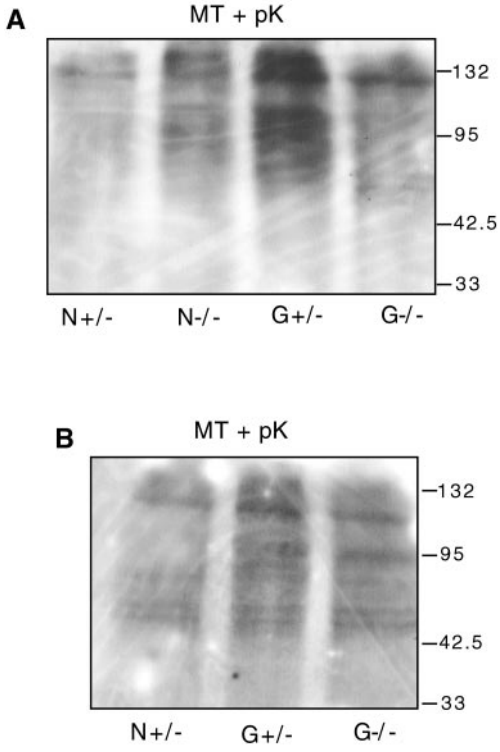


FIG. 4. **Protein carbonylation assays.** Western blot with anti-2,4-dinitrophenyl antibodies to detect protein carbonyl groups in brain (*A*) and spinal cord (*B*) proteinase K-treated purified mitochondria (*MT + pK*) from 17-week-old G93A (G+/−), 1-year-old N1029 (N+/−), and age-matched non-transgenic mice (G−/− and N−/−, respectively). Positions of molecular weight markers are indicated at *right*.

fractions as compared with N1029 mice (188.2 ± 53.7 and 112.6 ± 31.6 units/mg, respectively; $n = 8$, $p < 0.006$) and by 34% in mitochondrial fractions (14.2 ± 5.5 and 9.5 ± 5.5 units/mg; $n = 10$, $p < 0.06$).

hSOD1 Increases Mitochondrial Protein and Lipid Oxidative Damage—Protein carbonylation, a marker of protein oxidative damage, was measured on mitochondria treated with proteinase K from brain and spinal cords of G93A, N1029, and non-transgenic littermate controls. Protein carbonyls of the mitochondrial fractions were derivatized with 2,4-dinitrophenylhydrazine, and proteins were subjected to gel electrophoresis and immunoblotting. Bands corresponding to carbonylated proteins were detected with anti-2,4-dinitrophenyl antibodies. In brain and to a lesser extent in spinal cord from 17-week-old G93A mice proteins showed higher levels of oxidative damage as compared with age-matched non-transgenic controls and 1-year-old N1029 mice (Fig. 4, *A* and *B*). Interestingly, 1-year-old N1029 mice showed less protein oxidative damage both in brain and spinal cord mitochondria, not only compared with age-matched non-transgenic controls but also compared with 17-week-old non-transgenic animals (Fig. 4, *A* and *B*). These results were replicated on three different sets of animals and strongly suggested that in the central nervous system of this strain of mice expression of G93A-mutated hSOD1 enhanced oxidative damage of mitochondrial proteins, whereas expression of wild type hSOD1 seemed to have a protective effect.

Mice expressing G93A-mutated hSOD1 also exhibited a marked increase in the content of brain mitochondrial lipid hydroperoxides, a product of lipid oxidative damage. Lipid hydroperoxides in 13-week-old G93A mice were 1.37 ± 0.4 nmol/mg mitochondrial proteins (mean ± S.D.), whereas in non-transgenic controls they were 0.66 ± 0.28 nmol/mg mitochondrial proteins ($n = 6$; $p < 0.006$). The increase in mitochondrial lipid peroxidation was even more pronounced at 17 weeks of age, when lipid hydroperoxides were 1.99 ± 0.82 and 0.71 ± 0.31 nmol/mg mitochondrial proteins in G93A and control brains, respectively ($n = 10$; $p < 0.0006$).

Mitochondrial Respiration and ATP Synthesis Are Impaired in Mice Expressing G93A hSOD1—Mitochondrial respiration was assayed by oxygen consumption on freshly isolated intact mitochondria from liver, brain, and spinal cord. Oxygen consumption was measured in a Clark-type electrode oxygraph

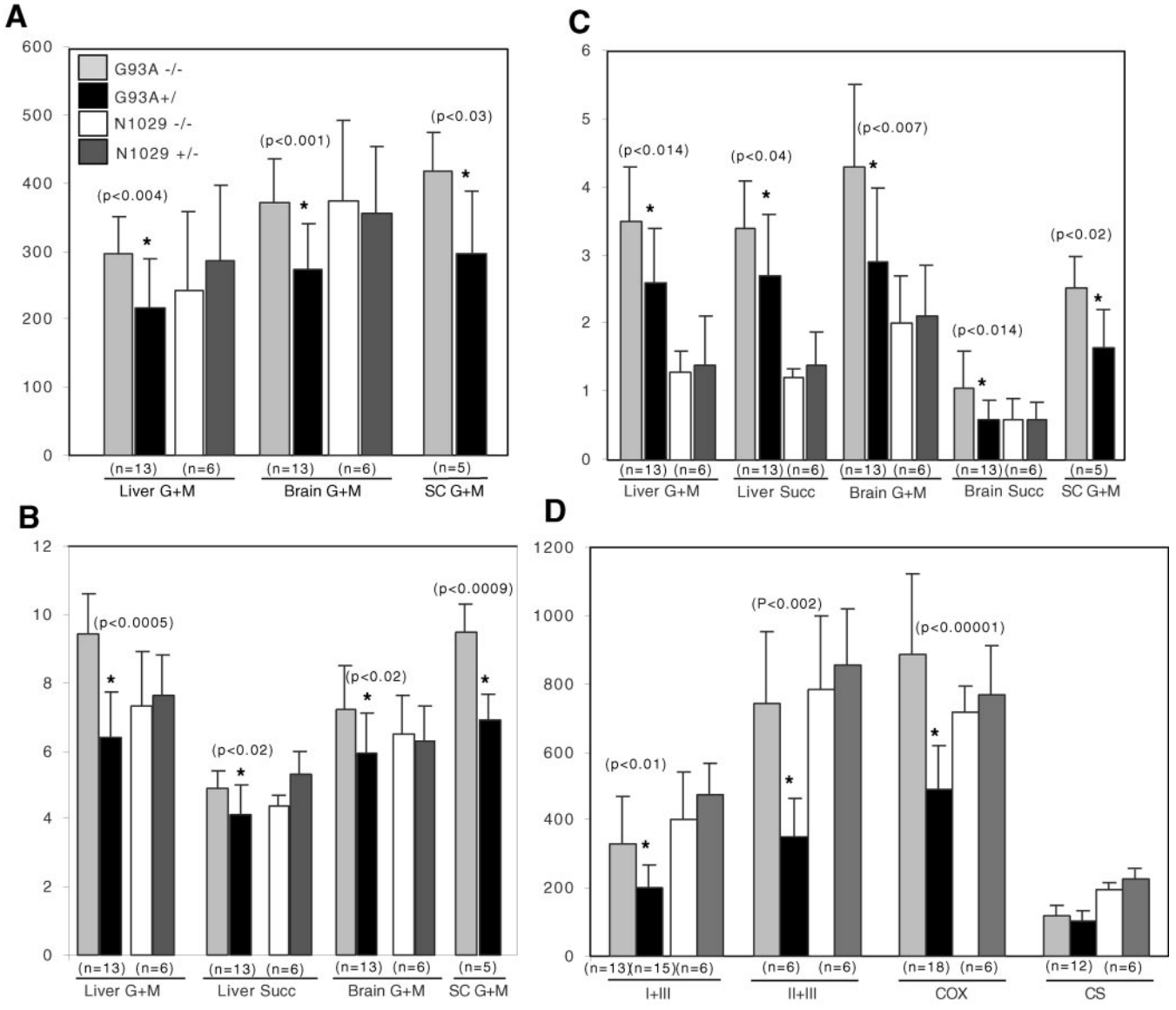


FIG. 5. **Biochemical assays on isolated mitochondria.** *G + M*, glutamate plus malate; *Succ*, succinate. *G93A +/−*, G93A SOD1 transgenic mice, age 17 weeks; *G93A −/−*, G93A non-transgenic mice, age 17 weeks; *N1029 +/−*, N1029 wild type hSOD1 transgenic mice, age 1 year; *N1029 −/−*, N1029 non-transgenic mice, age 1 year. Number of samples tested is indicated at *bottom* for each group. *Error bars* represent ± S.D. Significantly decreased values in transgenic animals compared with their non-transgenic age-matched controls are indicated by *asterisks*; *p* values (unpaired Student's *t* test) are shown in *parentheses*. *A*, state 3 respiration expressed as nmol of O_2/min/mg mitochondrial protein. *B*, respiratory control ratio (*RCR*) expressed as the ratio of state 3 over state 2 respiration. *Bar* denotations as in *A*. *C*, ATP synthesis expressed as RLU/min/mg mitochondrial protein. *Bar* denotations as in *A*. *D*, respiratory chain enzyme activities: complex *I + III,* NADH-cytochrome *c* oxidoreductase; *II + III*, succinate-cytochrome *c* oxidoreductase; COX, cytochrome *c* oxidase; *CS*, citrate synthase. Enzyme activities are expressed as nmol of substrate/min/mg mitochondrial protein (except for COX and CS, 10^{-2} μmol substrate/min/mg protein). *Bar* denotations as in *A*.

using as substrates either succinate or glutamate plus malate in the absence of exogenous ADP (state 2 respiration) and after addition of ADP (state 3 respiration). We found no significant difference in state 2 respiration between G93A and non-transgenic age-matched control mice (not shown). However, in all three tissues, state 3 respiration, indicating the maximal rate at which oxygen can be utilized by the respiratory chain in coupled mitochondria, was significantly reduced in 17-week-old G93A mice as compared with non-transgenic age-matched controls (Fig. 5*A*). The RCR, defined as the ratio between state 3 and state 2 respiration, was also significantly reduced in G93A mice (Fig. 5*B*). Upon addition of the ATPase inhibitor oligomycin, respiratory rates were decreased to levels similar to those of state 2 respiration (not shown), suggesting that respiration and ADP phosphorylation in mitochondria of G93A animals were still coupled. Therefore, we concluded that the decrease in RCR was to be attributed mainly to lower respiratory capacity rather than to uncoupling of mitochondria. ATP synthesis rate in 17-week-old G93A mice mitochondria was also significantly reduced in liver and brain using both succinate and glutamate plus malate and in spinal cord using glutamate plus malate as substrates (Fig. 5*C*). In 1-year-old N1029 mice expressing wild type hSOD1 state 3 respiration and RCR were unchanged as compared with their age-matched controls and to 17-week-old non-transgenic animals (Fig. 5, *A* and *B*). Despite normal respiration, ATP synthesis was reduced in liver, brain, and spinal cord mitochondria from 1-year-old N1029 animals compared with younger non-transgenic controls. However, there was no difference in ATP synthesis between transgenic N1029 and age-matched non-transgenic controls (Fig. 5*C*). Therefore, we concluded that the reduction in mitochondrial ATP synthesis in these mice was related to aging and not to the expression of

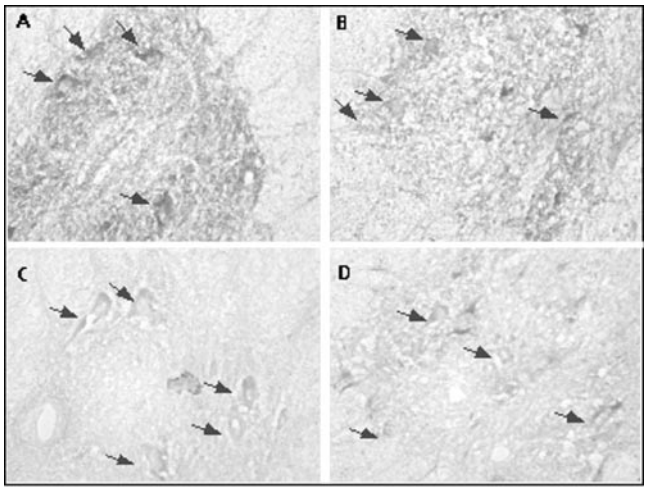


FIG. 6. **COX (*A* and *B*) and SDH (*C* and *D*) histoenzymatic staining on 10-μm-thick sections of spinal cord from 17-week-old G93A (*A* and *C*) and age-matched non transgenic mice (*B* and *D*).** Motor neurons are indicated by *arrows.* Magnification was ×40.

wild type hSOD1. We found no significant change in state 3 respiration, RCR, and ATP synthesis in brain, spinal cord, and liver of 13-week-old mice, as compared with age-matched non-transgenic controls (not shown). These results demonstrated that the expression of G93A mutated hSOD1, but not wild type hSOD1, caused impairment of mitochondrial oxidative phosphorylation and that this impairment became detectable by functional assays on isolated mitochondria only in the advanced stages of the disease (*i.e.* when the animals start becoming symptomatic).

Mitochondrial Respiratory Chain Activities Are Defective in the Spinal Cord of Mice Expressing G93A hSOD1—Enzymatic activities of mitochondrial respiratory chain complexes I + III, II + III, COX, and of the mitochondrial matrix enzyme CS were measured spectrophotometrically on purified mitochondrial fractions from spinal cord and brain as well as on purified brain synaptosomes (*i.e.* synaptic buttons). We found a statistically significant reduction in the activities of complexes I + III, II + III, and IV in spinal cord mitochondria from 17-week-old G93A mice as compared with non-transgenic animals (Fig. 5*D*). On the other hand, CS activity was unchanged suggesting that the decrease in respiratory chain activities was not due to loss of mitochondrial mass or to increased fragility of mitochondria, which would have caused membrane disruption and leakage of the soluble CS during the mitochondria isolation process. However, G93A mice brain mitochondria and synaptosomes at 9 and 17 weeks of age and spinal cord mitochondria at 13 weeks of age had normal respiratory chain activities (not shown). Also in 1-year-old N1029 mice, spinal cord mitochondria respiratory chain activities were unchanged as compared with age-matched non-transgenic controls. These data again suggested that only the expression of G93A mutated hSOD1 was able to cause mitochondrial respiratory chain dysfunction, which developed in the advanced stages of the disease, affecting more severely mitochondria in the spinal cord.

Histoenzymatic staining for COX and SDH were performed on cryosections of the lumbar portion of the spinal cord of 17-week-old G93A transgenic mice and age-matched non-transgenic controls to establish whether the respiratory chain defect was predominantly localized to the motoneurons. We found that in the anterior horns of the spinal cord not only the majority of motoneurons stained less intensely for COX in the G93A animals than in controls but also the neuropil showed reduced COX staining (Fig. 6, *A* and *B*). On the other hand, there was no detectable difference in the staining for SDH (Fig. 6, *C* and *D*). These findings were replicated in a series of three sets of transgenic animals and non-transgenic controls.

DISCUSSION

The existence of a mitochondrial SOD1, originally postulated by Weisiger and Freidovich (17), has been clearly demonstrated in the yeast *Saccharomyces cerevisiae* (18), in rat liver (19), and in transgenic mice (22). These authors have shown that ~1–5% of total SOD1 is contained in the intermembrane space of mitochondria where it presumably plays an important role in protecting mitochondrial components from oxidative damage. The mitochondrial respiratory chain is indeed the leading source of superoxide in the cells (30), and it is conceivable that the presence of SOD1 in mitochondria might serve to provide an additional line of defense against the oxygen-reactive species originated in mitochondria. The presence of hSOD1 in vacuolated mitochondria in the central nervous system of G93A transgenic mice has been also demonstrated by electron microscopy (21). Our immunochemical findings confirm that in isolated mammalian mitochondria there is a substantial amount of SOD1 localized in the intermembrane space.

In yeast, the presence of SOD1 in mitochondria seemed to be highly dependent on that of its copper chaperon, CCS (18). Unfortunately, we could not verify that this was the case in mouse mitochondria because anti-CCS antibodies were not available to us. It might be hypothesized that SOD1 enters mitochondria in transgenic animals because they express the protein at concentrations severalfold above normal levels. However, we found detectable amounts of enzymatically active SOD1 also in mitochondria from brain and liver of non-transgenic animals, suggesting that SOD1 is a natural protein component of the intermembrane space. Based on Western blot hSOD1 band intensities and on the dilution factor for post-mitochondrial supernatants and purified mitochondria, we estimated that the proportion of mitochondrial hSOD1 in transgenic animals was ~0.5–2% of total cellular hSOD1 in both liver and brain. These proportions were similar to those found in yeast (18) and rat liver (19). However, when mitochondria were treated with proteinase K, the amount of residual hSOD1 in liver was reduced ~10-fold (*i.e.* less than 0.1% of total hSOD1), whereas in brain there was no change in the intensity of the immunoreactive band. This suggested that in brain a considerably larger proportion of SOD1 in mitochondria is proteinase K-resistant and therefore presumably located in a protected compartment within the intermembrane space. These data are consistent with the finding that hSOD1 seems to accumulate with age in the central nervous system of transgenic animals but not in non-neural tissues (21). Although we don't know the reason for the difference in importation efficiency of hSOD1 into mitochondria between liver and brain, it is tempting to speculate that the finding of higher amounts of G93A-mutated mitochondrial SOD1 in the central nervous system is correlated with the neuronal phenotype of the disease.

Several mitochondrial functions were abnormal in mice expressing G93A mutated hSOD1. In liver, brain, and spinal cord mitochondrial state 3 respiration was reduced causing lower RCR and lower mitochondrial ATP synthesis. This respiratory defect presumably resulted in impairment of energy stores. These findings might contribute to explain the mechanisms of the neuroprotective effect of mitochondrial energy-buffering compounds such as creatine in G93A mice (16).

Respiratory chain enzyme activities were significantly reduced in spinal cord but not in brain mitochondria despite the aforementioned respiratory defect. It is interesting that in G93A mice the first signs of a reduction in respiratory chain enzyme activities was in the spinal cord, where neurodegeneration occurs predominantly, suggesting that spinal cord mi-

tochondria might be particularly sensitive to the mitochondrial damage caused by G93A-mutated hSOD1. COX histochemical staining of spinal cord sections showed reduced activity both in motor neurons and in the neuropil of G93A mice. This result was not unexpected, because it would be difficult to imagine that an enzymatic defect purely confined to the motor neurons could be detectable by spectrophotometric assays on mitochondria isolated from the whole spinal cords, where motor neurons, albeit being large cells, only represent a small fraction of all cells.

Abnormally vacuolated and swollen mitochondria have been observed in G93A mice prior to the onset of muscle weakness and motor neuron death (12, 21, 31). However, in one report, COX histochemistry on spinal cord sections of G93A mice failed to show any reduction in activity in the residual motor neurons, even at the terminal stages of the disease (31). In our strain of G93A mice we could not detect any mitochondrial function that was significantly impaired at 13 weeks of age or earlier. Although we observed a trend for reduction of respiration, ATP synthesis, and some enzymatic activities at age 13 weeks (Fig. 5), these only reached statistically significant levels at 17 weeks of age (average age of death was 19 $\pm$ 1 weeks).

Heterozygote mice expressing wild type hSOD1 tested at age 1 year did not show defects in mitochondrial respiratory functions compared with age-matched non-transgenic controls. Although in a previous report (20) mitochondrial swelling and vacuolization were observed in a similar strain of mice as early as at 30–40 weeks of age, in those animals the vacuoles did not appear to contain SOD1, and COX histochemistry in neurons was normal. Although we did not look at the mitochondrial structure by electron microscopy in our mice, from a functional standpoint, our results agree with those by Jaarsma *et al*. (20), confirming that expression of wild type hSOD1 is not sufficient to cause mitochondrial dysfunction *per se*.

How G93A-mutated hSOD1 causes dysfunction in mitochondria is not certain at this point. However, a number of mechanisms might be hypothesized. First, our finding of increased oxidative lipid and protein damage in mitochondria seems to support the concept that mutated SOD1 might gain an aberrant catalytic function leading to excessive production of free radical species (10, 32, 33). Evidence of oxidative damage of proteins (34), lipid membranes (35, 36), and nucleic acids (37–39) has been found in ALS tissues. Furthermore, levels of oxidative damage to DNA are increased in urine, plasma, and cerebrospinal fluid of ALS patients; they increase over time and correlate with the severity of the disease (40). Thus, free radical damage might become prominent in mitochondria where the majority of cellular superoxide is produced and target mitochondrial proteins, lipids, as well as mitochondrial DNA. We found a highly significant increase of lipid peroxidation in G93A mice at 13 weeks of age, which became more pronounced at 17 weeks. Oxidative damage to the lipid milieu of mitochondria might very well explain the loss of respiratory function and ATP synthesis, even before individual respiratory chain enzymes become defective, as observed in brain from G93A mice. We also found evidence that mitochondria of the central nervous system of these mice contained high molecular weight aggregates of SOD1, which were not observed in mitochondria from control mice nor from mice expressing wild type hSOD1. Aggregated hSOD1 was more abundant on the external side of the outer mitochondrial membrane, where it might interfere, for example, with exchange of substrates and ions between mitochondria and other cell compartments and import of proteins from the cytosol.

In conclusion, in this work we provide a molecular and biochemical characterization of mitochondrial dysfunction in an animal model of FALS. We suggest that mutated hSOD1 in mitochondria may cause such mitochondrial defects which, in turn, may contribute to precipitating the neurodegenerative process in these animals. Because of the time frame of their development, it is likely that mitochondrial abnormalities and the ensuing energy metabolism defects contribute to the demise of motor neurons but do not necessarily initiate the neurodegeneration. Although the precise pathogenic role of mutated hSOD1 remains to be fully clarified, we believe that a better comprehension of the molecular basis of mitochondrial dysfunction in FALS might eventually help us to identify more effective therapeutic strategies.

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