Journal of Neurochemistry, 2007, **102**, 609–618 doi:10.1111/j.1471-4159.2007.04502.x

Mutant SOD1-induced neuronal toxicity is mediated by increased mitochondrial superoxide levels

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Abstract

Amyotrophic lateral sclerosis (ALS), the most common motor neuron disease in adults, is characterized by the selective degeneration and death of motor neurons leading to progressive paralysis and eventually death. Approximately 20% of familial ALS cases are associated with mutations in SOD1, the gene encoding Cu/Zn-superoxide dismutase (CuZnSOD). Previously, we reported that overexpression of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD or SOD2) attenuates cytotoxicity induced by expression of the G37R-SOD1 mutant in a human neuroblastoma cell culture model of ALS. In the present study, we extended these earlier findings using several different SOD1 mutants (G93C, G85R, and I113T). Additionally, we tested the hypothesis that mutant SOD1 increases mitochondrial-produced superoxide ($O_2^{\bullet-}$) levels and that SOD2 overexpression protects neurons from mutant SOD1-induced toxicity by reducing $O_2^{\bullet-}$ levels in mitochondria. In the present study, we demonstrate that SOD2 overexpression markedly attenuates the neuronal toxicity induced by adenovirus-mediated expression of all four SOD1 mutants (G37R, G93C, G85R, or I113T) tested. Utilizing the mitochondrial-targeted $O_2^{\bullet-}$-sensitive fluorogenic probe MitoSOX Red^{TM}, we observed a significant increase in mitochondrial $O_2^{\bullet-}$ levels in neural cells expressing mutant SOD1. These elevated $O_2^{\bullet-}$ levels in mitochondria were significantly diminished by the overexpression of SOD2. These data suggest that mitochondrial-produced $O_2^{\bullet-}$ radicals play a critical role in mutant SOD1-mediated neuronal toxicity and implicate mitochondrial-produced free radicals as potential therapeutic targets in ALS.

Keywords: familial amyotrophic lateral sclerosis, free radicals, manganese superoxide dismutase, neuroblastoma, neuronal toxicity.

J. Neurochem. (2007) **102**, 609–618.

Identification of mutations in the gene encoding copper/zinc-superoxide dismutase (CuZnSOD), SOD1, in a subset of familial amyotrophic lateral sclerosis (FALS) patients has been one of the most significant findings to date in ALS research. Currently, more than 100 different mutations in SOD1 have been reported in 15–20% of FALS cases (Valentine *et al.* 1999). As SOD1 is a key enzyme in protection against oxidative-induced cellular damage, it was originally hypothesized that mutations in SOD1 resulted in a decrease in enzymatic activity and a subsequent increase in destructive reactive oxygen species (ROS) (Rowland 1995). However, it is now believed that mutations in SOD1 may lead to ALS through the gain of a novel adverse function. Support for the gain of function hypothesis comes from studies in transgenic mice in which expression of a human SOD1 mutation was associated with an ALS-phenotype, despite the fact no differences were observed in SOD1 enzymatic activity (Gurney *et al.* 1994). Additionally, it has been shown that many of the ALS-associated SOD1 mutants retain normal SOD activity (Borchelt *et al.* 1994). Further evidence for a toxic gain of function comes from the observations that SOD1 null mice do not develop an ALS phenotype (Reaume *et al.* 1996). As such, it is generally well accepted that mutations in SOD1 lead to a toxic gain of function; however, that so-called function remains in contention. To this end, numerous hypotheses involving mitochondrial dysfunction, increased oxidative stress, and abnormal protein aggregation are currently being tested. In

Received July 11, 2006; revised manuscript received December 15, 2006; accepted December 18, 2006.

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Abbreviations used: ALS, amyotrophic lateral sclerosis; CuZnSOD or SOD1, copper/zinc-superoxide dismutase; ETC, electron transport chain; FALS, familial amyotrophic lateral sclerosis; LAMP2, lysosome-associated membrane protein 2; LDH, lactate dehydrogenase; MnSOD or SOD2, manganese superoxide dismutase; MOI, multiplicity of infection; $O_2^{\bullet-}$, superoxide; ROS, reactive oxygen species.

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this regard, it has become evident that the development and progression of ALS is multi-factorial and there may be considerable interplay between numerous mechanisms.

Recently, motor neuron mitochondria have become increasingly more suspicious as key players in the pathogenesis of ALS. Interestingly, mutant SOD1 has been shown to accumulate in spinal cord mitochondria from ALS transgenic mice expressing mutant human SOD1 (Higgins *et al.* 2002; Jaarsma *et al.* 2001). In addition, it appears that mutant SOD1 is more abundant in mitochondria from nervous tissue compared with non-affected tissue, including heart and liver, in ALS transgenic mice (Liu *et al.* 2004; Mattiazzi *et al.* 2002; Vijayvergiya *et al.* 2005). Support for the involvement of mitochondrial dysfunction in the development of ALS comes from the observation that morphological abnormalities of motor neuron mitochondria appear prior to neuronal degeneration in ALS transgenic mice (Dal Canto and Gurney 1994; Kong and Xu 1998; Wong *et al.* 1995). Additionally, mitochondrial electron transport chain (ETC) dysfunction (Menzies *et al.* 2002; Jung *et al.* 2002; Manfredi and Xu 2005; Gunther *et al.* 2004), mitochondrial Ca^{2+} loading capacity impairment (Damiano *et al.* 2006), and the initiation of the mitochondrial apoptosis pathway (Guegan *et al.* 2001; Takeuchi *et al.* 2002) have all been reported to play a role in mutant SOD1-associated motor neuron degeneration.

In cells, mitochondria are the major sites for generation of ROS, including superoxide ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2). Increased levels of ROS within mitochondria may, at least in part, lead to mitochondrial dysfunction or, alternatively, may be a result of mitochondrial dysfunction (Adam-Vizi 2005). A growing amount of evidence suggests that oxidative damage as a result of increased ROS levels may play a primary role in several neurodegenerative diseases, including both familial and sporadic cases of ALS. Biomarkers of oxidative damage, including protein carbonyls, lipid peroxidation, and DNA damage have been observed in both mutant SOD1 transgenic mice (Warita *et al.* 2001; Liu *et al.* 1999; Hall *et al.* 1998) and neural tissue from ALS patients (Ferrante *et al.* 1997). Although these studies have examined cellular oxidative damage, only a few reports have investigated the contribution of mitochondrial-produced ROS in the pathogenesis of ALS. Most recently, it was reported that cells expressing mutant SOD1 exhibited a decrease in peroxiredoxin 3, a mitochondrial-targeted antioxidant (Wood-Allum *et al.* 2006), and an increase in the oxidative environment of mitochondria (Ferri *et al.* 2006). Earlier, Andreassen *et al.* reported that ALS transgenic mice with a partial depletion of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD or SOD2) exhibit an exacerbation of motor dysfunction and a significant decrease in survival (Andreassen *et al.* 2000). In addition, we have previously demonstrated in a novel cell culture model of mutant SOD1-associated ALS that overexpression of SOD2 attenuates cell death induced by the SOD1 mutant, G37R (Flanagan *et al.* 2002). Together, these studies suggest a role for mitochondrial-produced ROS, particularly $O_2^{\bullet -}$, in the development of mutant SOD1-associated ALS; however, the precise mechanisms of SOD2 protection and mitochondrial $O_2^{\bullet -}$ levels in neural cells expressing mutant SOD1 remains unclear.

In the present study, we sought to extend our previous findings that SOD2 protects against mutant G37R-SOD1-mediated toxicity in our cell culture model of ALS by using several different SOD1 mutants (G93C, G85R, and I113T) with varying levels of SOD activity. In addition, we tested the hypothesis that mutant SOD1 expression increases $O_2^{\bullet -}$ levels in mitochondria and that overexpression of SOD2 protects neural cells from mutant SOD1-induced toxicity by scavenging mitochondrial-produced $O_2^{\bullet -}$. In the present study, we report that overexpression of SOD2 protects against neuronal toxicity induced by numerous SOD1 mutants regardless of the level of SOD activity exhibited by the mutant. Furthermore, expression of mutant SOD1 in neuronal cells leads to an increase in mitochondrial-produced $O_2^{\bullet -}$ levels, which are diminished by overexpression of SOD2.

Materials and methods

Construction of recombinant adenovirus

The cDNA for the human SOD1 mutants (G37R, G93C, G85R, and I113T) were kindly provided by David Borchelt (University of Florida, FL, USA). Replication-deficient mutant SOD1 adenovirus (AdG37R, AdG93C, AdG85R, and AdI113T) was generated by direct ligation of a plasmid fragment containing the human cDNA to a viral backbone derived from the parent plasmid pAd-CMVKpn-Not1 using current methods of homologous recombination. After ligation, plasmids containing insert DNA were purified by phenol–chloroform extraction and ethanol precipitation and transfected into 293 cells with near full-length adenoviral DNA previously restricted to remove a portion of the E1 region by Viraquest Inc. (North Liberty, IA, USA). Following observation of a cytopathic effect, cells were harvested, lysed, and functional *in vitro* analyzes were perfromed to score for recombinants. Functional adenovirus was characterized by western blotting, after which, recombinant adenovirus underwent two additional rounds of plaque purification. Replication-deficient recombinant adenovirus with the E1 region substituted with human wild-type SOD1 (AdwtSOD1) or SOD2 cDNA (AdSOD2) was provided by Viraquest Inc. Control cells were exposed to adenovirus containing cytomegalovirus promoter only (AdEmpty) also provided by Viraquest Inc. All purified stocks of adenovirus were checked for the presence of contaminating replication competent/E1 positive virus using a PCR assay for E1 sequence and plaque assay on A549 cells. Adenoviral stocks were stored in 3% sucrose at $-80°$ C. Adenoviral titers were typically $1–2 \times 10^{12}$ DNA particles/mL. Multiplicity of Infection (MOI) is given throughout the text.

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Cell culture

The human neuroblastoma cell line CHP212 (ATCC) was cultivated in Ham's F12 media containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics. Cells were transduced with adenoviral vectors in the absence of serum for 24 h, after which media was removed and replaced with full media. It should be noted that this protocol results in a 90–95% transduction efficiency of CHP212 cells.

Cell survival assay

Two different assays were used to assess cell viability: (i) Direct cell counts using a Coulter counter (Fullerton, CA, USA) and (ii) Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA), a WST-8 based assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt used according to the manufacturer's directions.

Western blot analysis

For protein analysis, cells lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis electrophoresis and electrotransferred to a nitrocellulose membrane. The membrane was incubated with human anti-SOD1 or human anti-SOD2 primary antibody (1 : 2000) developed in our laboratory and human anti-actin (1 : 2000; Sigma-Aldrich, St Louis, MO, USA) followed by incubation with secondary IgG peroxidase-labeled antibody (1 : 10 000; Chemicon International, Temecula, CA, USA). Protein expression was detected using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA) as described by the manufacturer. Protein levels were semi-quantitatively measured using densitometry (Image J analysis software version 1.31; NIH, Bethesda, MD, USA).

Isolation of mitochondrial fractions

To determine mitochondrial localization of mutant SOD1 following adenovirus transduction, mitochondrial fractions were isolated as described (Dobson *et al.* 2000). Briefly, 12×10^6 cells were plated in 150 mm plates and exposed to AdwtSOD1, AdG37R, AdG85R, AdG93C, or AdII3T for 24 h. Three days following removal of adenovirus, cells were washed with ice-cold phosphate-buffered saline, harvested by trypsinization, and treated with ice-cold digitonin (325 mmol/L digitonin, 2.5 mmol/L EDTA, 250 mmol/L mannitol, and 17 mmol/L 4-morpholinepropanesulfonic acid, pH 7.4) for 80 s. The lysed cells were then added to 2x mannitol–sucrose buffer (525 mmol/L mannitol, 175 mmol/L sucrose, 12.5 mmol/L Tris, and 12.5 mmol/L EDTA, pH 7.4) and centrifuged at 800 *g* for 10 min at 4°C to pellet nuclei. The supernatant was saved and the pellet material was resuspended in 1x mannitol–sucrose buffer (210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L Tris, and 5 mmol/L EDTA, pH 7.4) and centrifuged at 1100 *g*. This was repeated three more times. The combined supernatants were then centrifuged at 1100 *g* to remove any remaining nuclei, and the resulting supernatant centrifuged at 10 000 *g* to pellet mitochondria. The mitochondrial pellet was resuspended in isolation medium (0.25 mol/L sucrose, 5 mmol/L HEPES, and 0.1 mmol/L EDTA), briefly sonicated on ice, and then subjected to western blot analysis as described above. This mitochondrial isolation protocol results in 11-fold enrichment of mitochondria as determined by SOD2 protein levels in the mitochondrial fraction compared with whole cell lysates. The content and purity of the mitochondrial fraction was monitored using an antibody directed against Bcl-xL (1 : 500; Abcam, Cambridge, MA, USA), a commonly used mitochondrial marker. Additionally, contamination of the mitochondrial fraction by other cellular components was analyzed using antibodies directed against lactate dehydrogenase (LDH, 1 : 1000; Abcam), a cytosolic marker, and lysosome-associated membrane protein 2 (LAMP2, 1 : 1000; Abcam), a lysosomal marker.

Detection of mitochondrial $O_2^{\bullet -}$

Five days following adenovirus transduction, CHP212 cells were loaded with the fluorogenic probe MitoSOX Red^{TM} (3 μmol/L; Molecular Probes, Inc., Eugene, OR, USA) for 20 min. After removing MitoSOX Red^{TM} and washing cells with Hanks' balanced salt solution, fluorescent images were captured by confocal microscopy using a Zeiss 510 inverted confocal laser-scanning microscope equipped with lasers and bright-field optics. MitoSOX Red^{TM} is a fluorescent dye specific for the detection of $O_2^{\bullet -}$ in the mitochondria of live cells (Robinson *et al.* 2006). To confirm the localization of MitoSOX Red^{TM} to mitochondria, cells were also incubated with MitoTracker Green (20 nmol/L; Molecular Probes, Inc.) for 20 min. To confirm the specificity of MitoSOX for $O_2^{\bullet -}$, additional cultures were treated with the mitochondrial Complex I inhibitor rotenone (50 μmol/L, 60 min), which increases mitochondrial-produced $O_2^{\bullet -}$ (Adam-Vizi 2005; Kowaltowski *et al.* 2001). MitoSOX Red^{TM} fluorescence per cell was quantified using Image J analysis software.

Antioxidant native activity gel

Copper/zinc-superoxide dismutase and SOD2 activity was measured using a native-gel activity stain (Beauchamp and Fridovich 1971). After separating cell lysates on a native gel using electrophoresis, enzyme activity bands were visualized by saturating the gel with 2.6 μmol/L nitroblue tetrazolium, 28 μmol/L riboflavin, and 28 mmol/L tetramethylethylenediamine for 20 min in the dark and then illuminated under a fluorescent light until achromatic bands appeared. The intensities of the achromatic bands were analyzed using Image J analysis software to semi-quantitatively measure SOD1 and SOD2 activity.

Statistics

Data were analyzed by Student's *t*-test when comparing only two groups and by one-way analysis of variance (ANOVA) followed by Newman–Keuls correction for multiple comparisons when comparing more than two groups. Data are expressed as mean ± SEM and differences were considered significant at $p < 0.05$.

Results

Expression of mutant SOD1 results in cell death

We have previously developed a novel adenovirus-based gene delivery system in human neuroblastoma cells to examine the pathogenesis of the ALS-associated mutant SOD1, G37R (Flanagan *et al.* 2002). In order to determine the effect of other SOD1 mutants with varying levels of SOD

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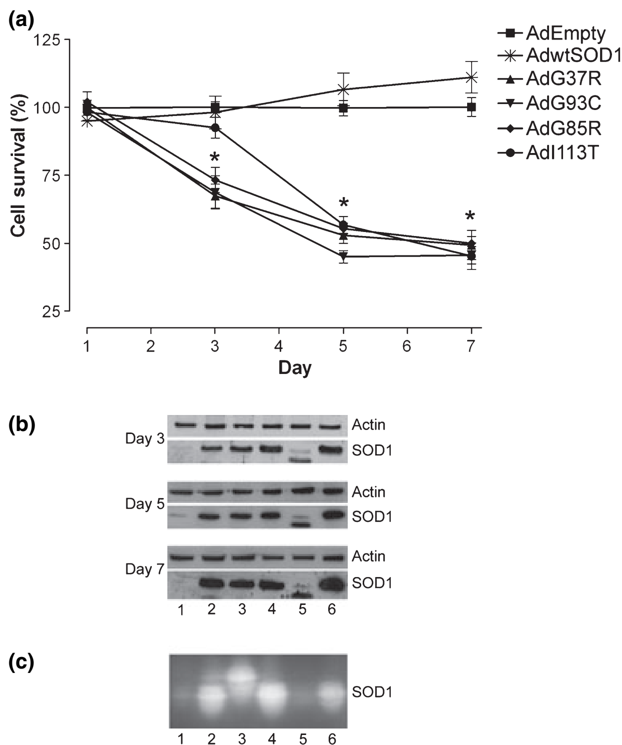

Fig. 1 Expression of mutant copper/zinc-superoxide dismutase (SOD1) decreases cell survival. (a) Summary data from three to four separate experiments performed in triplicate showing a time-dependent decrease in cell survival of neuroblastoma cells (CHP212) infected with AdEmpty, AdwtSOD1, AdG37R, AdG93C, AdG85R, or AdI113T (50 multiplicity of infection). *$p < 0.05$ versus AdEmpty. (b and c) Representative western blots (b) showing time-dependent expression of SOD1 3, 5, or 7 days after adenovirus infection of CHP212 cells and representative SOD1 activity assay (c) 5 days after adenovirus infection. AdEmpty (lane 1), AdwtSOD1 (lane 2), AdG37R (lane 3), AdG93C (lane 4), AdG85R (lane 5), or AdI113T (lane 6).

activity on neuronal survival, CHP212 human neuroblastoma cells were transduced with replication-deficient adenovirus expressing the human FALS-associated SOD1 mutations G37R, G93C, G85R, or I113T. After 1, 3, 5, or 7 days, cell survival was determined using WST-8 reduction. Cells transduced with AdG37R, AdG93C, AdG85R, or AdI113t exhibited a time-dependent decrease in cell survival compared with cells infected with the control vector AdEmpty (Fig. 1a). Adenovirus-mediated expression of mutant SOD1 was undetectable after 24 h of infection (data not shown); however, at 3, 5, and 7 days after infection mutant SOD1 protein was increased 10 to 15-fold on each respective day (Fig. 1b, lanes 3–6) compared with the endogenous levels in control cells infected with AdEmpty (Fig. 1b, lane 1). Furthermore, SOD1 activity was elevated approximately fivefold in AdG37R, AdG93C, and AdI113T cell lysates (Fig. 1c, lanes 3, 4, and 6) compared with AdEmpty-treated cell lysates (Fig. 1c, lane 1). In contrast, but as previously reported (Borchelt *et al.* 1994), the G85R mutant showed no detectable SOD activity (Fig. 1c, lane 5). It should be noted that the differential migration of G85R and G37R observed in the western blot analysis (Fig. 1b) and the SOD activity assay (Fig. 1c), respectively, was similar to previous reports (Borchelt *et al.* 1994; Hayward *et al.* 2002). To control for the increase in SOD1 expression and activity observed in AdG37R-, AdG93C-, and AdI113T-treated cells, additional CHP212 cells were infected with adenovirus encoding wild-type SOD1 (AdwtSOD1). Although AdwtSOD1 resulted in an increase in SOD1 protein and activity (Figs 1b and c, lane 2), no effect on cell survival was observed (Fig. 1a). The effects of mutant SOD1 expression on cell survival, as measured by the WST-8 reduction assay, was confirmed by directly counting cells using a Coulter cell counter (data not shown). These data demonstrate that adenovirus-mediated expression of different SOD1 mutants with varying levels of SOD activity in CHP212 neuroblastoma cells results in cytotoxicity. It should be noted that unlike other cell culture models of mutant SOD1-associated ALS, the toxicity observed in our model does not require an additional stress. That is, expression of the SOD1 mutants themselves is sufficient for toxicity.

SOD2 overexpression attenuates mutant sod1 toxicity

We have previously shown that overexpression of the mitochondrial $O_2^{\bullet-}$ scavenger SOD2, but not the cytosolic-localized wild-type SOD1, significantly increases survival of neuronal cells expressing the G37R-SOD1 mutant (Flanagan *et al.* 2002). In order to determine the ability of SOD2 to attenuate cell death following expression of other SOD1 mutants with different biochemical properties, CHP212 cells were exposed to 10 MOI of AdSOD2 or to control for total viral MOI, AdEmpty at the same time as mutant SOD1 adenovirus (50 MOI). Overexpression of SOD2, which resulted in a fivefold increase in SOD2 activity (Fig. 2b) significantly increased survival of neural cells expressing G37R, G93C, G85R, or I113T mutant SOD1 (Fig. 2a, black bars) compared with cells co-transduced with Ad-mutant SOD1 plus AdEmpty (Fig. 2a, white bars). In contrast, overexpression of wild-type SOD1 had no effect on mutant SOD1-induced toxicity (Fig. 2a, gray bars). Importantly, the SOD1 activity demonstrated by the mutants was not altered by overexpression of SOD2 (Fig. 2b). Taken together, these data suggest that mitochondrial-derived $O_2^{\bullet-}$ plays a critical role in mutant SOD1-induced cytotoxicity.

Mutant SOD1 localizes to the mitochondria

Overexpression of SOD2, an antioxidant found in the mitochondrial matrix, significantly attenuates mutant SOD1-induced neuronal death (Fig. 2a). As recent studies suggest the presence of a small fraction of SOD1 in the intermembrane space of the mitochondria, we sought to

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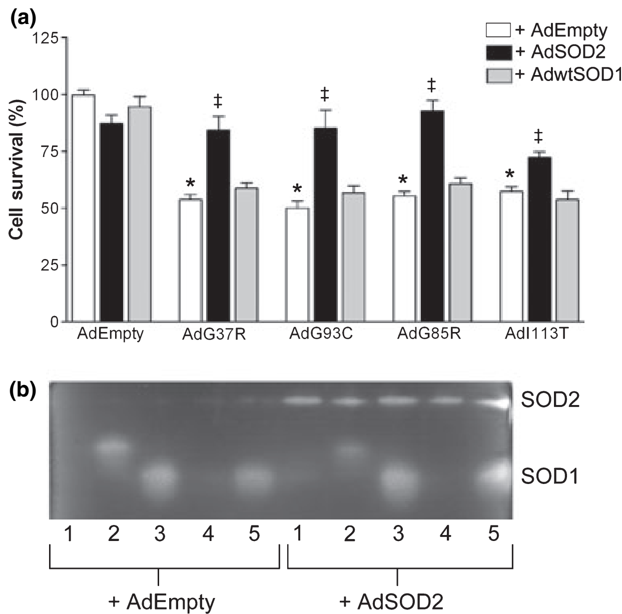

Fig. 2 Overexpression of manganese superoxide dismutase (SOD2) inhibits mutant copper/zinc-superoxide dismutase (SOD1) -mediated neuronal toxicity. (a) Summary data from three to four separate experiments performed in triplicate showing CHP212 cell survival 5 days following adenoviral co-infection with 50 multiplicity of infection (MOI) of AdEmpty, AdG37R, AdG93C, AdG85R, or Ad113T plus 10 MOI of either AdSOD2 (black bars), AdwtSOD1 (gray bars), or, to control for total viral MOI, AdEmpty (white bars). $^{*}p < 0.05$ versus AdEmpty + AdEmpty, $\ddagger p < 0.05$ versus the respective mutant SOD1 + AdEmpty. (b) Representative SOD activity assay performed on CHP212 cell lysates 5 days after co-infecting cells with AdEmpty (lane 1), AdG37R (lane 2), AdG93C (lane 3), AdG85R (lane 4), or Ad113T (lane 5) plus AdSOD2 or AdEmpty.

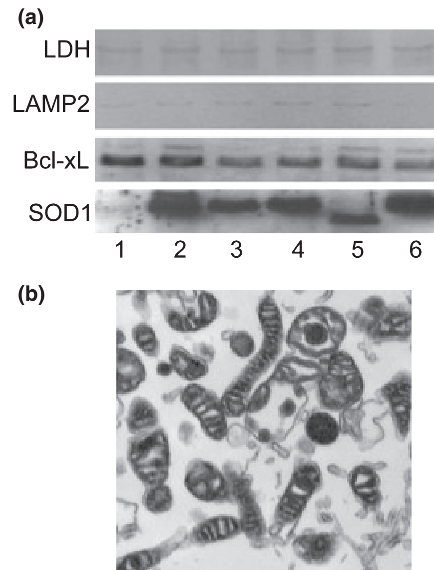

Fig. 3 Mutant copper/zinc-superoxide dismutase (SOD1) accumulates in mitochondria. (a) Representative western blot analysis showing SOD1 expression in mitochondria isolated from CHP212 cells exposed to 50 multiplicity of infection of AdEmpty (lane 1), AdwtSOD1 (lane 2), AdG37R (lane3), AdG93C (lane 4), AdG85R (lane 5), or AdI113T (lane 6). Mitochondrial content and purity was assessed using an antibody against the mitochondrial marker Bcl-xL, while contamination of other cellular compartments was analyzed using antibodies against cytosolic [lactate dehydrogenase (LDH)] and lysosomal markers [lysosome-associated membrane protein 2 (LAMP2)]. (b) Representative electron micrograph of isolated mitochondria showing a relatively pure population of intact mitochondria.

determine if mutant SOD1 localizes to the mitochondria in our *in vitro* model. Three days after transducing CHP212 cells with 50 MOI of AdEmpty, AdwtSOD1, AdG37R, AdG93C, AdG85R, or AdI113T, mitochondrial fractions were subjected to western blot analysis. Similar to previous studies using mitochondria isolated from spinal cord of ALS transgenic mice (Liu *et al.* 2004; Higgins *et al.* 2002; Mattiazzi *et al.* 2002; Jaarsma *et al.* 2001), mutant SOD1 was localized in mitochondria isolated from our cell culture model of ALS (Fig. 3a). To determine the purity of the mitochondrial isolation, mitochondrial fractions were examined for expression of Bcl-xL, which is highly associated with the mitochondrial membrane. In addition, contamination of the mitochondrial fraction was assessed using antibodies directed against cytosolic (LDH) and lysosomal markers (LAMP2). The high expression of Bcl-xL in the mitochondrial fraction (Fig. 3a) and low expression of LDH and LAMP2 indicates that relatively pure mitochondrial fractions were obtained. In addition, an electron micrograph taken of the mitochondrial pellet (Fig. 3b) provides further evidence that mitochondrial fractions consist mostly of intact mitochondria. The presence of mutant SOD1 in mitochondria support the use of our cell culture model to investigate the mechanism(s) of mutant SOD1-associated ALS, as our model appears to recapitulate the characteristics of the *in vivo* model.

Mutant SOD1 increases mitochondrial $O_2^{\bullet-}$ levels

Due to the localization of mutant SOD1 in mitochondria (Fig. 3) and the protective effect of the mitochondrial-targeted $O_2^{\bullet-}$ scavenger SOD2 on mutant SOD1-induced toxicity (Fig. 2), we hypothesized that mutant SOD1 increases mitochondrial-produced $O_2^{\bullet-}$ levels. To test this hypothesis, CHP212 cells were transduced with 50 MOI of AdEmpty, AdwtSOD1, AdG37R, AdG93C, AdG85R, or AdI113T, and 5 days later mitochondrial $O_2^{\bullet-}$ levels were measured using MitoSOX Red^{TM} fluorescence. As shown in the representative confocal microscopy images (Fig. 4), expression of all SOD1 mutants examined, but not wild-type SOD1, resulted in an increase of MitoSOX Red^{TM} fluorescence compared with control cells transduced with

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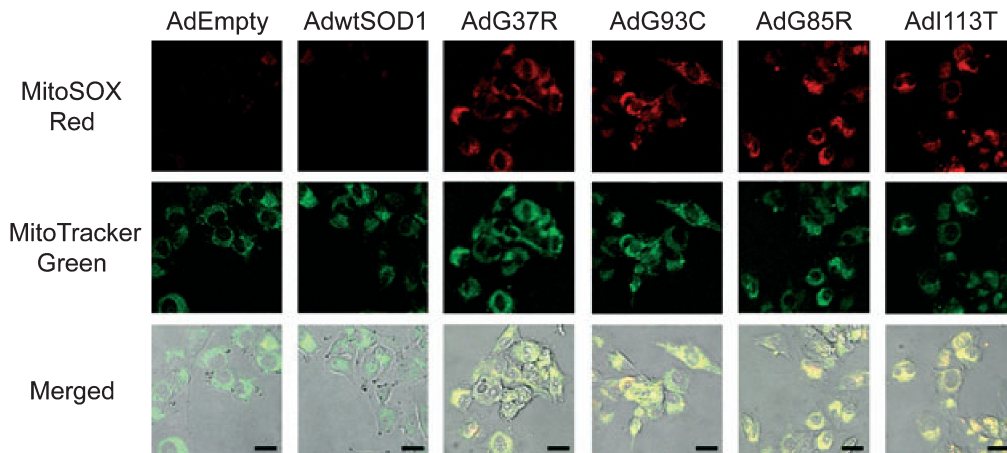

Fig. 4 Mutant copper/zinc-superoxide dismutase (SOD1) increases mitochondrial-produced superoxide levels. Representative confocal images showing co-localization (yellow fluorescence in merged image) of MitoSOX Red™ fluorescence and MitoTracker Green fluorescence in CHP212 cells 5 days after exposure to 50 multiplicity of infection of AdEmpty, AdwtSOD1, AdG37R, AdG93C, AdG85R, or AdI113T. Magnification bar equals 20 μm.

AdEmpty. Importantly, MitoSOX Red™ fluorescence co-localized (yellow fluorescence in merged image) with the green fluorescence of MitoTracker Green (Fig. 4), thus indicating an increase in mitochondrial-produced $O_2^{\bullet-}$.

SOD2 decreases mitochondrial $O_2^{\bullet-}$ levels in neural cells expressing mutant SOD1

In order to determine if attenuation of mutant SOD1 cytotoxicity following SOD2 overexpression (Fig. 2) is associated with decreases in mitochondrial $O_2^{\bullet-}$ levels, confocal microscopy was used to capture MitoSOX Red™ fluorescence in cells expressing mutant SOD1 plus SOD2 (Fig. 5). As shown in the representative confocal microscopy images, the increase in MitoSOX Red™ fluorescence in CHP212 cells expressing mutant SOD1 was markedly attenuated by overexpression of SOD2 (Fig. 5a). Similar to Fig. 4, MitoSOX fluorescence co-localized with MitoTracker Green fluorescence (Molecular Probes, Inc.). In addition, the high magnification images in Fig. 5 show punctate staining characteristic of mitochondrial localization, thus providing additional support for an increase in *mitochondrial*-produced $O_2^{\bullet-}$. Figure 5b shows the summary data of MitoSOX fluorescence from cells transduced with AdG37R, AdG93C, AdG85R, or AdI113T plus AdSOD2, AdwtSOD1, or, to control for total viral MOI, AdEmpty. As suggested by the confocal images, all SOD1 mutants tested increased mitochondrial-produced $O_2^{\bullet-}$, which was inhibited by overexpression of SOD2 (Fig. 5b, black bars). However, overexpression of wild-type SOD1 had no effect on the increase in MitoSOX fluorescence induced by mutant SOD1 (Fig. 5b, gray bars). To determine if the increase in mitochondrial $O_2^{\bullet-}$ was specific to mutant SOD1, an additional group of cells were transduced with AdwtSOD1 alone (Fig. 5b, hatched bar). In contrast to mutant SOD1, wild-type SOD1 did not change mitochondrial $O_2^{\bullet-}$ levels compared with control cells infected with AdEmpty. To confirm the specificity of the assay, an additional group of cells were incubated with the mitochondrial Complex I inhibitor, rotenone (Fig. 5b). Rotenone exposure increased mitochondrial-produced $O_2^{\bullet-}$, which was inhibited by SOD2 but not wild-type SOD1. These data demonstrate that expression of mutant SOD1 in neural cells leads to an increase in mitochondrial-produced $O_2^{\bullet-}$, which is attenuated by overexpression of SOD2.

Discussion

We have previously demonstrated that overexpression of SOD2 protects neuronal cells against toxicity induced by the G37R-SOD1 mutant (Flanagan *et al.* 2002). The goal of the present work was to examine the protective effect of SOD2 in neural cells expressing various SOD1 mutants with different levels of SOD activity and to define the mechanisms by which SOD2 attenuates mutant SOD1-induced cell death. In the present study, we report, for the first time, that the expression of SOD1 mutants with different biochemical properties in neuronal cells leads to an increase in mitochondrial-produced $O_2^{\bullet-}$. Furthermore, overexpression of the mitochondrial-targeted $O_2^{\bullet-}$ scavenger SOD2 decreases the elevated $O_2^{\bullet-}$ levels in mitochondria and protects against neuronal toxicity induced by mutant SOD1.

Although the present study is the first to specifically investigate the levels of $O_2^{\bullet-}$ in mitochondria in a mutant SOD1-associated ALS model, previous reports have suggested that mitochondrial $O_2^{\bullet-}$ and/or mitochondrial oxidative damage play a key role in the pathogenesis of the disease. For example, an increase in mitochondrial oxidative stress, as measured by dihydrorhodamine-123, has been observed in neuroblastoma cells expressing the G93A-SOD1 mutant

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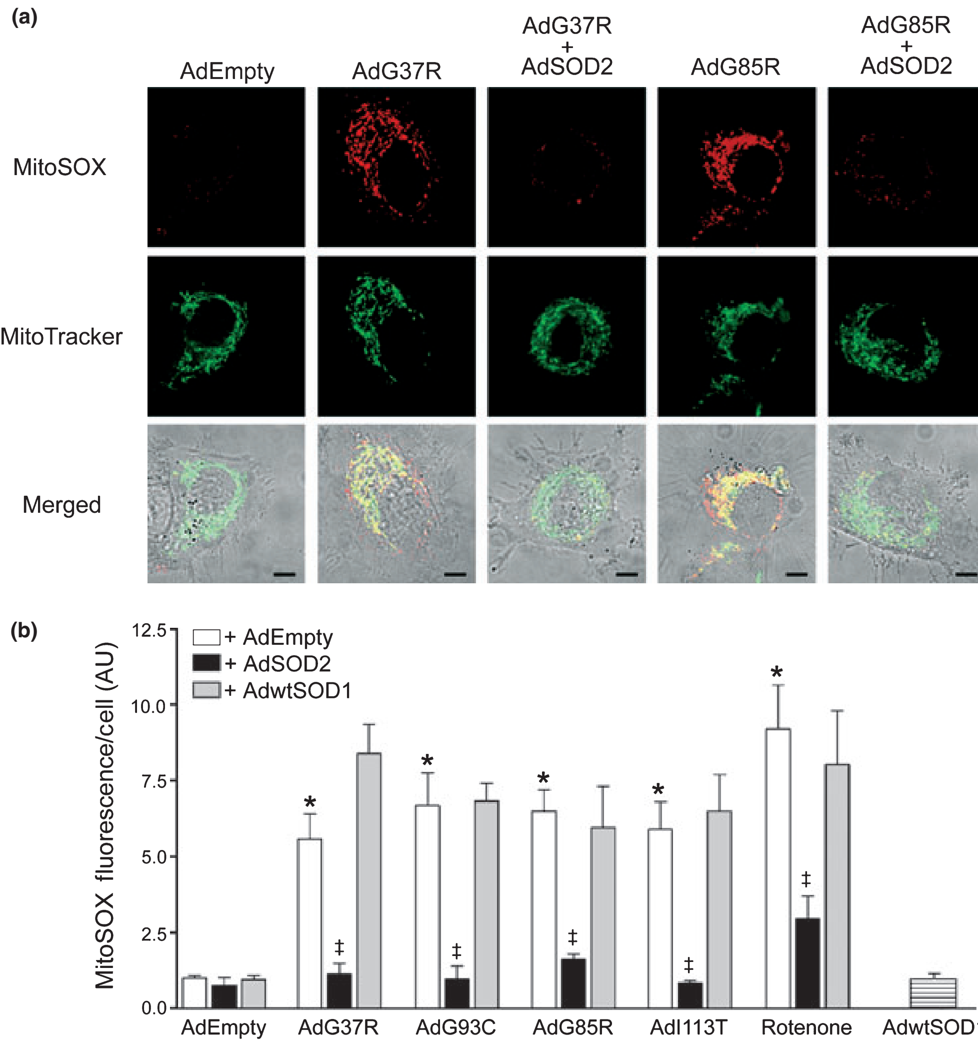

Fig. 5 Manganese superoxide dismutase (SOD2) decreases mutant copper/zinc-superoxide dismutase (SOD1) -induced mitochondrial-produced superoxide levels. (a) Representative confocal images showing co-localization (yellow fluorescence in merged image) of superoxide (MitoSOX Red™ fluorescence) in mitochondria (MitoTracker Green fluorescence). CHP212 cells were transduced with AdEmpty, AdG37R, or AdG85R [50 multiplicity of infection (MOI)] with and without AdSOD2 (10 MOI) for 5 days. Magnification bar equals 5 μm. (b) Summary data of MitoSOX fluorescence for all four mutants in CHP212 cells 5 days following adenovirus infection. Cells were co-transduced with 50 MOI of AdEmpty, AdG37R, AdG93C, AdG85R, or AdI113T plus 10 MOI of AdSOD2 (black bars), AdwtSOD1 (gray bars), or, to control for total viral MOI, AdEmpty (white bars). An additional group of cells were transduced with AdwtSOD1 (hatched bar). As a positive control, cells were treated with rotenone (50 μmol/L, 1 h). MitoSOX fluorescence per cell (n = 250–400 cells) from at least three separate experiments was quantified using ImageJ analysis software. *$p < 0.05$ versus AdEmpty + AdEmpty, ‡$p < 0.05$ versus the respective treatment + AdEmpty. AU = arbitrary units.

(Beretta *et al.* 2003) and in cells isolated from the spinal cord of G93A transgenic mice (Kruman *et al.* 1999). Overexpression of SOD2 plus phospholipid glutathione peroxidase, a mitochondrial-expressed enzyme that protects membranes against oxidative damage, decreases neuronal cell death mediated by the G93A-SOD1 mutant (Liu *et al.* 2002). Similarly, ebselen, an antioxidant effective in reducing hydroperoxides, protects motor neuron-like cells (NSC34) against mutant SOD1-mediated toxicity (Wood-Allum *et al.* 2006). Further evidence for increased levels of oxidants in motor neuron mitochondria comes from the recent observation that mutant SOD1 associated with mitochondria results in a shift in the mitochondria redox potential to a more oxidizing state (Ferri *et al.* 2006). In addition, levels of protein carbonylation and lipid hydroperoxides, markers of oxidant-induced damaged proteins and lipids, are elevated in spinal cord mitochondria isolated from G93A mice (Kirkinezos *et al.* 2005; Mattiazzi *et al.* 2002). More specifically, brain mitochondria from G93A mice exhibit increased oxidation of cardiolipin, an inner mitochondrial membrane

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lipid associated with cytochrome *c* (Kirkinezos *et al.* 2005). Evidence for an *in vivo* therapeutic effect of increasing mitochondrial antioxidant levels comes from a preliminary study showing that the systemic administration of a mitochondrial-targeted antioxidant nitroxide delays disease progression, improves motor function, and increases survival of G93A mice (Joseph *et al.* 2006). In contrast, decreasing mitochondrial antioxidant levels via a partial depletion of SOD2 in ALS transgenic mice expressing the G93A SOD1 mutation exacerbates motor neuron degeneration and motor dysfunction and significantly decreases animal survival compared with G93A mice expressing normal levels of SOD2 (Andreassen *et al.* 2000). Taken together, these studies provide compelling evidence for the involvement of mitochondrial-produced oxidants and mitochondrial oxidative damage in mutant SOD1-associated ALS. The present study supports this hypothesis and, for the first time, demonstrates that decreasing mitochondrial $O_2^{\bullet -}$ levels protects neural cells from mutant SOD1-induced toxicity.

Accumulating evidence suggests that mitochondrial dysfunction is involved in the pathogenesis of mutant SOD1-associated ALS. For example, morphologically abnormal motor neuron mitochondria are one of earliest pathological signs of degenerating motor neurons in ALS transgenic mice (Dal Canto and Gurney 1994). Although the causality of morphologically abnormal motor neuron mitochondria in the development of ALS remains to be fully elucidated, numerous studies have identified significant mitochondrial abnormalities in mutant SOD1-associated ALS. Of particular interest is the observation that mutant SOD1 accumulates in the intermembrane space of motor neuron mitochondria of ALS transgenic mice expressing mutant SOD1 (Liu *et al.* 2004; Higgins *et al.* 2002; Mattiazzi *et al.* 2002; Jaarsma *et al.* 2001). It has also been reported that in brain mitochondria from G93A mice a significant portion of mutant SOD1 is localized in the mitochondrial matrix (Vijayvergiya *et al.* 2005). Further, the levels of mutant SOD1 in mitochondria appears greater in nervous tissue of ALS mice compared with non-affected tissues (Liu *et al.* 2004; Mattiazzi *et al.* 2002; Vijayvergiya *et al.* 2005), thus suggesting that the localization of mutant SOD1 in mitochondria plays a specific role in the selective degeneration of motor neurons in ALS. Importantly, we observed mutant SOD1 expression in mitochondria isolated from our neuroblastoma cell culture model of ALS, thus supporting the use of this *in vitro* model to examine the intra-neuronal mechanisms involved in mutant SOD1-associated ALS.

Additional evidence for mutant SOD1-induced mitochondrial dysfunction comes from reports that mutant SOD1 modulates the mitochondrial ETC. In a yeast model of ALS, expression of the A4V-SOD1 mutant results in a decrease in the activities of the mitochondrial ETC complexes II, III, and IV (Gunther *et al.* 2004). In ALS transgenic mice expressing the human G93A-SOD1 mutant, the activities of ETC complexes are reduced in brain and spinal cord mitochondria compared with mice expressing wild-type human SOD1 (Jung *et al.* 2002; Mattiazzi *et al.* 2002). Using motor neuron-like cells (NSC34), Menzies *et al.* demonstrated that the expression of G93A- or G37R-SOD1 mutant decreases the activity of the mitochondrial ETC complexes II and IV (Menzies *et al.* 2002). Further, these authors show that the activities of complex II and IV are attenuated in NSC34 cells under an oxidative environment induced by serum withdrawal regardless of mutant SOD1 expression (Menzies *et al.* 2002). Thus, as the authors speculate, increased levels of ROS may, at least in part, cause the mitochondrial dysfunction observed in mutant SOD1-associated ALS.

Alternatively, mitochondrial dysfunction and modulation of ETC activity may lead to an increase in mitochondrial-produced ROS. Inhibiting ETC complexes slows the flow of electrons through the respiratory chain, thus favoring the escape of electrons from the chain and the reduction of molecular oxygen to $O_2^{\bullet -}$ and other ROS (Adam-Vizi 2005). For example, inhibition of complex I by rotenone causes electrons to 'leak' from the complex, react with oxygen, and increase the levels of $O_2^{\bullet -}$ within the mitochondrial matrix (Adam-Vizi 2005). Additionally, increased mitochondrial levels of H_2O_2 have been reported in brain mitochondria following the inhibition of complex I by rotenone or the inhibition of complex III by antimycin (Votyakova and Reynolds 2001; Zoccarato *et al.* 1988). Although a direct link between mitochondrial ETC inhibition and the production of mitochondrial ROS in the pathogenesis of ALS remains unclear, this phenomenon has been associated with other neurodegenerative diseases. In particular, deficiency of complex I and an increase in mitochondrial-produced ROS has been linked to the pathogenesis of Parkinson's disease (Seaton *et al.* 1997; Mizuno *et al.* 1998; Schapira *et al.* 1990). The cause and effect of increased mitochondrial-produced ROS, particularly $O_2^{\bullet -}$, in mutant SOD1-induced mitochondrial dysfunction requires further investigation. However, the data presented herein showing that overexpression of SOD2 and the subsequent reduction of mitochondrial $O_2^{\bullet -}$ levels protects neuronal cells against mutant SOD1-induced toxicity strongly suggests that mitochondrial-produced $O_2^{\bullet -}$ plays a key role in the pathogenesis of mutant SOD1-associated ALS.

It is assumed that the SOD2-mediated protection is due to reductions in mitochondrial $O_2^{\bullet -}$ levels; however, in the dismutation of $O_2^{\bullet -}$, SOD2 produces H_2O_2. It is possible that an increase in H_2O_2 within mitochondria provides protection to neural cells expressing mutant SOD1. Interestingly, it has recently been observed that oxidative modification of normal SOD1 by H_2O_2 is necessary for activation of the enzyme in the mitochondrial intermembrane space (Inarrea *et al.* 2005). Thus, it is tempting to speculate that overexpression of SOD2 protects against mutant SOD1 toxicity because it increases mitochondrial H_2O_2 levels, which in turn modulates the

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activity of both mutant and wild-type CuZnSOD within the mitochondrial intermembrane space. Experiments to measure the levels of mitochondrial H_2O_2 levels following SOD2 overexpression in neuronal cells expressing mutant SOD1 are currently underway in our laboratory. In addition, the role of mitochondrial H_2O_2 in mutant SOD1-induced toxicity using H_2O_2 scavenging enzymes targeted to mitochondria is being investigated.

In summary, utilizing a neuroblastoma cell culture model of ALS in which expression of different SOD1 mutants results in a time-dependent decrease in cell survival, the present study provides the first known evidence that mutant SOD1 increases mitochondrial $O_2^{\bullet-}$ levels. Furthermore, overexpression of the mitochondrial-targeted $O_2^{\bullet-}$ scavenger SOD2 attenuates the increase in mitochondrial $O_2^{\bullet-}$ levels and protects neuronal cells against mutant SOD1-induced toxicity. Although these data suggest that mitochondrial-produced $O_2^{\bullet-}$ is involved in the pathogenesis of mutant SOD1-associated ALS, further work using both *in vitro* and *in vivo* models of ALS is necessary to determine the precise source of $O_2^{\bullet-}$ within mitochondria and to determine the mechanisms involved in mitochondrial $O_2^{\bullet-}$-induced toxicity. Nevertheless, the protective effect of SOD2 in this *in vitro* model of ALS implicates mitochondrial-produced oxidants as important new therapeutic targets in ALS.

Acknowledgements

This study was funded by grants from the Muscular Dystrophy Association (SWF) and from the National Institutes of Health (CA66081, LWO; CA78586, MCZ and LWO).

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