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The role of immunophilins in mutant superoxide dismutase-1-linked familial amyotrophic lateral sclerosis

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ABSTRACT **It has been reported that expression of familial amyotrophic lateral sclerosis (FALS)-associated mutant Cu/Zn superoxide dismutase-1 (SOD) induces apoptosis of neuronal cells in culture associated with an increase in reactive oxygen species. SOD recently has been shown to prevent calcineurin inactivation, initiating the present investigations examining the role of calcineurin in mutant SOD-induced cell death. Wild-type or mutant SOD was expressed in neuronal cells by infection with replication-deficient adenoviruses. PC12 cells overexpressing human wild-type SOD exhibited higher calcineurin activity than cells expressing FALS-related mutant SOD (SODV148G); however, cells expressing SODV148G had calcineurin activity equal to mock-infected cells, suggesting that cell death induced by mutant SOD was not related to a decrease in calcineurin activity. Calcineurin antagonists such as cyclosporin A and FK506, as well as nonimmunosuppressant analogs of cyclosporin A, significantly enhanced SODV148G- and SODA4V-induced cell death. Because both groups of drugs inhibit the rotamase activity of cyclophilins (CyP), but only the immunosuppressant analogs inhibit calcineurin activity, these data suggest that rotamase inhibition underlies the enhanced cell death after SODV148G expression. The importance of rotamase activity in mutant SOD-mediated apoptosis was supported by experiments showing that overexpressed wild-type cyclophilin A (CyPA), but not CyPA with a rotamase active site point mutation, protected cells from death after SODV148G expression. These data suggest that mutant SOD produces a greater need for rotamase and, also, highlights possible new therapeutic strategies in FALS.**

Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disease that targets motor neurons of the cortex, brainstem, and spinal cord. Approximately 10–15% of ALS cases are familial and manifest an autosomal dominant inheritance pattern. In 1993, mutations in Cu/Zn superoxide dismutase-1 (SOD) were found to be associated with 20% of cases of familial ALS (FALS) (1). This finding led to intense investigations into the molecular mechanisms underlying the pathogenesis of mutant SOD-induced FALS.

SOD is a ubiquitously expressed homodimeric cytosolic enzyme that dismutates superoxide radical (O_2^-) into H_2O_2 and O_2 (2). Mutant SOD has been hypothesized to cause FALS not through a loss in dismutase activity but from a toxic gain of function or an augmentation of a normally present, nondismutase activity of SOD. This hypothesis has gained support from studies with non-neuronal cells (3) and transgenic mice (4, 5) that suggest cells expressing mutant SOD do not necessarily have decreased dismutase function. In addition, a SOD knockout mouse has normal motor neuron development (6), demonstrating that a loss in dismutase activity does not cause motor neuron disease. The toxicity of mutant SOD has been proposed to involve one or more of the following: an increase in peroxynitrite formation (7), an increase in peroxidase activity (8, 9), a loss of shielding of the metal ions (10), and aggregation of the enzyme (11).

It recently has been reported that the expression of FALS-associated mutant SOD in nerve growth factor (NGF)-differentiated PC12 cells, primary sympathetic neurons, and hippocampal neurons leads to altered O_2^- content and an apoptotic cell death (12). The present study explores the relationship of mutant SOD-induced death to calcineurin, a calcium/calmodulin-dependent phosphatase, and characterizes the effect of inhibitors of calcineurin, such as the immunosuppressant compounds cyclosporin A (CsA), CsG, and FK506. Immunosuppressants bind immunophilins [the drug receptor: e.g., cyclophilins (CyP) or FK binding protein], and the drug-immunophilin complex inhibits calcineurin activity as well as the rotamase (peptidyl-prolyl *cis–trans* isomerase) activity of the immunophilins (13, 14). Rotamase facilitates *cis–trans* isomerization of the peptide bond on the N-terminal side of proline residues, aids normal folding and assembly of proteins, including SOD, and has other cellular functions (15).

A recent report demonstrated that SOD normally protects calcineurin from inactivation (16). We asked whether the increase in O_2^- in mutant SOD-expressing cells leads to calcineurin inactivation and contributes to FALS-related cell death. The present investigation demonstrates that the proapoptotic activity of mutant SOD is not related to calcineurin inhibition. Most importantly, we show that immunosuppressant drugs (CsA, CsG, and FK506) enhance cell death induced by mutant SOD and that this enhancement depends on rotamase inhibition. These findings suggest that mutant SOD may lead to increased protein damage or turnover and a greater reliance on rotamase activity.

MATERIALS AND METHODS

Cells and Viruses. Growth and differentiation of rat pheochromocytoma PC12 cells, isolation and growth of hippocampal pyramidal neurons, and the preparation of adenoviruses (AdVs) expressing wild type or mutant SOD have been described previously (12). Differentiated PC12 cells and hippocampal pyramidal neurons were infected with AdVs at multiplicity of infection of 100–200 following the reported

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: ALS, amyotrophic lateral sclerosis; SOD, Cu/Zn superoxide dismutase-1; FALS, familial ALS; CyP, cyclophilin; WT, wild-type; NGF, nerve growth factor; AdV, adenovirus; CsA, cyclosporin A; NMDA, *N*-methyl-D-aspartate; MPTP, mitochondrial permeability transition pore.

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method (12). Infection procedure generally transduced >60% of the cells.

Western Blot Analysis. Expression of SOD and calcineurin A was analyzed by Western blot analysis as described (12) using horseradish peroxidase-conjugated anti-SOD polyclonal antibody (The Binding Site, San Diego; 1:300 dilution) or mouse monoclonal anti-calcineurin A antibody (Transduction Laboratories, Lexington, KY; 1:500 dilution).

Immunocytochemical Analysis. A previously described method was followed for immunohistochemical detection of SOD (12). CyPA (histidine-tagged) (17) expression was detected by immunostaining with His-probe (H-15) rabbit polyclonal antiserum (Santa Cruz Biotechnology) followed by anti-rabbit IgG -alkaline phosphatase and X-phosphate.

Cell Viability. The effect of wild-type (WT) or FALS-linked mutant SOD gene expression on viability of cells was determined as reported (12). Five random microscopic fields were counted for each coverslip, and a total of fifteen fields were examined for each drug treatment. The percentage of cells dying at the time indicated in the text was calculated from at least three different experiments as the percentage of cells that died after a particular treatment compared with the number of viable cells without treatment (12). In excitotoxicity experiments, cultured hippocampal neurons were exposed to *N*-methyl-D-aspartate (NMDA) (100 μM) for 30 min, and cell survival was evaluated 24 hr later by fluorescence assay (see above). This procedure induced ≈40% cell death.

For some experiments involving CyPA, we used a wild-type CyPA (CyPAWT) cDNA or isomerase-deficient mutant CyPA(R55A) cDNA isolated from rat brain and cloned into pcDNA1/AMP vector (17) (a gift from James Patrick, Baylor College of Medicine). Mock-, AdV148GSOD-, or AdWT-SOD-infected PC12 cells were transfected with either CyPAWT cDNA or CyPA(R55A) cDNA by using polyethylenimine as described (18, 19). As a control for these experiments, some cells were transfected with CD8 cDNA (a gift from Jeff Bluestone, University of Chicago).

Drugs were added and replenished in fresh media every 72 hr. Drugs included NMDA; the immunosuppressants CsA (1 μM), CsG (Nva-2-Cs, 1 μM), and FK506 (1 μM, Fujisawa); the nonimmunosuppressants PKF 211–811 (Me-Ile-4-Cs, 1 μM), PSC 833 (3′-keto-Bmt-1-Val-2-Cs, 1 μM); CsH (D-Me-Val-11-Cs, 1 μM), which does not bind cyclophilins; and D-lys-(dansyl)-8-Cs (1 μM), which has poor binding to cytoplasmic cyclophilins. All Cs drugs were from Novartis Pharma AG (Basel, Switzerland).

Calcineurin Activity Assay. Activity was assayed following Blumenthal *et al.* (20). In brief, R_{II} peptide (DLD-VPIPGRFDRRVSVAAE; 0.25 μM) containing the phosphorylation site of type II cAMP-dependent protein kinase (LC Services, Woburn, MA) was phosphorylated by bovine brain protein kinase A catalytic subunit (30 μg/ml) plus [γ-^{32}P]ATP (0.5 mM). The phosphopeptide then was incubated with 30 μg of cytosolic protein extracts from PC12 cells. Extracts were prepared by sonicating pelleted cells (20 sec) in ice-cold buffer (25 mM Tris, pH 7.4/1 mM EDTA/0.5 mM EGTA/100 μM/phenylmethylsulfonyl flouride/5 mM 2-mercaptoethanol) and 30 min of centrifugation at $10^4 \times g$. Dephosphorylation of ^{32}P-labeled R_{II} peptide was performed at 30°C for 5 min in a total volume of 100 μl containing 30 μg of cytosolic protein, 5 μM calmodulin, and 25 nM calyculin A and either 0.5 mM $CaCl_2$ or 1 mM EGTA without $CaCl_2$. Released ^{32}P was isolated by cation exchange chromatography and quantitated by Cerenkov counting. Ca^{2+}-dependent phosphatase activity was measured by subtracting Ca^{2+}/calmodulin-independent activity from total phosphatase activity. Calcineurin phosphatase activity in cells expressing SODV148G or SODWT or after NGF withdrawal was normalized to activity of mock-infected cells.

Rotamase Activity Assay. A previously published (21) chymotrypsin-coupled assay was used to measure rotamase activity. Cells were treated with 1 μM CsA 48 hr after infection and were harvested 24 hr later; the cytosol was prepared as described for the calcineurin assay. Succinyl-Ala-Leu-Phe-Pro-Phe-4-nitroanilide was used as the assay peptide.

SOD Activity Assay. Cells were treated with CsA and were harvested as described above for the rotamase assay. SOD activity was determined in triplicate by using a colorimetric assay kit (Calbiochem).

RESULTS

Overexpression of WT- and FALS-Associated Mutant SOD with Replication-Deficient Adenoviruses. SOD expression in PC12 cells was determined by Western blot analysis and staining with anti-SOD polyclonal antibody. Endogenous rodent SOD was present in both the mock-infected (Fig. 1*A*, lane 1) as well as the AdV-infected cells (Fig. 1*A*, lanes 2–4). An additional immunostained band of slower electrophoretic mobility consistent with that expected for human SOD was present in extracts from cells infected with AdSODWT, AdSODV148G, and AdSODA4V viruses (Fig. 1*A*, lanes 3–5, respectively) but not mock-infected cells or cells infected with a control AdLacZ (Fig. 1*A*, lanes 1 and 2, respectively). The human SOD level in cells expressing SODWT, SODV148G, and SODA4V was similar to endogenous SOD seen in mock cells (Fig. 1*A*). Under our experimental conditions, infection with AdSODWT induced expression in ≈61 ± 2% (n = 4) of the cells, as demonstrated by human-specific immunohistochemical staining. Comparable results were obtained after AdSODV148G infection.

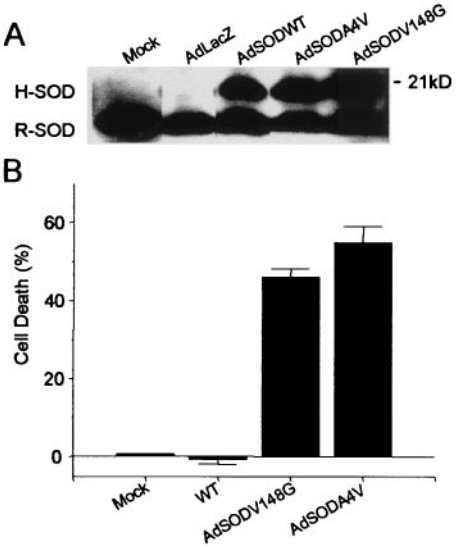

FIG. 1. (*A*) Western blot analysis of SOD from PC12 cells 3 days after mock infection (lane 1) or infection with a control AdLacZ (lane 2), AdSODWT (lane 3), AdSODA4V (lane 4), or AdSODV148G (lane 5). Cell lysates were subjected to SDS/PAGE, were blotted onto nitrocellulose, and then were incubated with an anti-SOD polyclonal antibody. Note the immunoreactivity in all lanes of a protein corresponding to the electrophoretic mobility of the rodent form of SOD (R-SOD) at ≈19 kDa (lower band). In the case of infection with AdSODWT, AdSODA4V, and AdSODV148G (lanes 3–5, respectively), an immunostained protein at ≈21 kDa (upper band) is seen, corresponding to the electrophoretic mobility of human SOD (H-SOD). (*B*) Cell death of differentiated PC12 cells 5 days after infection with mock, AdSODWT AdSODV148G, or AdSODA4V. Expression of SODV148G and SODA4V caused cell death whereas there was no significant alteration in the viability of control cells or SODWT-expressing cells.

Effect of Overexpression of Mutant SOD on Neural Cell Viability. We determined the effect of expression of WT and mutant SOD on differentiated PC12 cells, a model system for postmitotic neurons. We compared PC12 cell viability after infection with AdSODV148G with that seen after SODWT expression and mock infection. Compared with mock-infected controls, roughly half of the PC12 cells died 5 days after infection with AdSODV148G and AdSODA4V (Fig. 1*B*). This number is even more significant because the infection efficiency is ≈65–70%. In contrast, there was no decline in survival of cells after infection with AdSODWT (Fig. 1*B*). It previously was demonstrated that the cells that died were the ones expressing mutant SOD and that these cells died by apoptosis (12).

Effect of Overexpression of Mutant SOD on Calcineurin Activity. We examined the effects of WT and mutant SOD overexpression on calcineurin activity in differentiated PC12 cells. Cells expressing SODWT exhibited higher calcineurin activity than mock-infected cells or cells expressing SODV148G (Fig. 2*A*). Cells expressing SODV148G showed a similar level of calcineurin activity to that of mock-infected cells (Fig. 2*A*), suggesting that a decrease in calcineurin activity is unrelated to cell death. Similarly, there was no decline in calcineurin activity of PC12 cells after NGF-removal, a procedure that also leads to apoptotic cell death (Fig. 2*A*). As expected, treatment with CsA, a calcineurin inhibitor, dramatically decreased calcineurin in all cases by ≈60–70% (Fig. 2*B*), again indicating lack of correlation between calcineurin activity and cell death (see below).

We next tested whether the effects of SOD overexpression on calcineurin activity were related to a change in calcineurin expression. Immunoblots of PC12 cell lysates demonstrated similar levels of a protein corresponding in electrophoretic mobility to calcineurin A (≈60 kDa) after mock infection (Fig. 2*C*, lane 1), after NGF removal (Fig. 2*C*, lane 2), or 72 hr after infection with AdSODV148G and AdSODWT (Fig. 2*C*, lanes 3 and 4, respectively). These results indicate that the difference in calcineurin activity between SODWT-expressing and SODV148G-expressing cells was not attributable to differences in the amount of calcineurin expression in these cells.

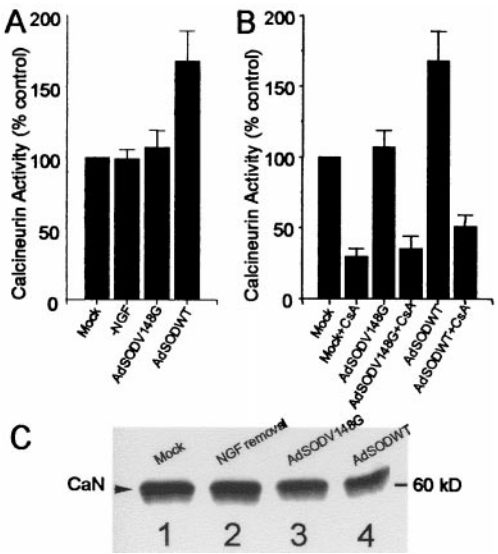

FIG. 2. Assessment of CsA influence on calcineurin activity and immunoblot analysis of calcineurin at a time before evidence of apoptotic cell loss. (*A*) Calcineurin activity was assayed 3 days after AdV infection or 1 day after NGF withdrawal. Cells expressing SODWT exhibited higher calcineurin activity than mock-infected cells, cells expressing SODV148G, or after NGF withdrawal. (*B*) Treatment with CsA (1 μM) dramatically decreased calcineurin activity in all cases. (*C*) Similar immunostaining of calcineurin is seen in Western blot analysis after mock (lane 1), NGF removal (lane 2), SODV148G (lane 3), or SODWT expression (lane 4).

Effect of CsA and CsA Analogs on Cell Death Induced by Mutant SOD Expression or NGF Withdrawal. To assess the relationship between SOD expression, calcineurin activity, and cell death, cells were treated with various drugs after AdV infection. In preliminary studies, we treated cells expressing SODV148G with CsA and found that 1 and 3 μM concentrations significantly affected their viability whereas 10 and 100 nM concentrations had little effect and a 10 μM concentration was toxic. Fig. 3*A* shows that CsA, CsG, and FK506, immunosuppressant drugs known to inhibit calcineurin and the rotamase activity of immunophilin, potentiated cell death induced by SODV148G expression (Fig. 3*A*). The potentiation of cell death by CsA did not reach 100% because the viral infection efficiency was ≈70%. Similar results were found with SODA4V (Fig. 3*A*). In contrast, mock-infected cells or cells expressing SODWT (data not shown) were not significantly affected. Although cell death was enhanced after treatment of mutant SOD-expressing cells (Fig. 3*A*), CsA dramatically decreased calcineurin activity in all cases (Fig. 2*B*). As previously noted, these results indicate that mutant SOD-induced cell death is not related to calcineurin inhibition.

We also examined the effect of PKF 211–811 and PSC 833, nonimmunosuppressant CsA analogs that inhibit cyclophilin rotamase activity but not calcineurin (22, 23). These drugs potentiated cell death induced by SODV148G expression to the same extent as immunosuppressants (Fig. 3*A*) but had no

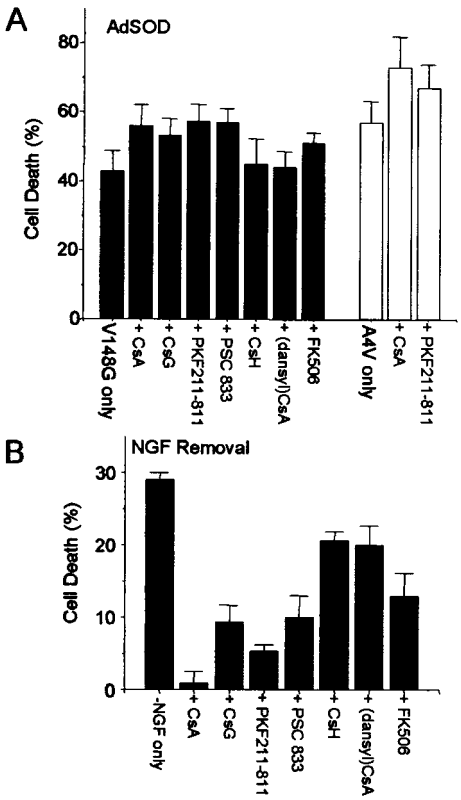

FIG. 3. CsA and its immunosuppressant and nonimmunosuppressant analogs potentiate cell death induced by SODV148G (black bars) and SODA4V expression (open bars) in PC12 cells (*A*) and show a protective effect after NGF removal (*B*). Drugs were added immediately after virus infection and were replenished in fresh media every 3 days. CsA, CsG, and FK506 are immunosuppressive drugs that inhibit calcineurin. PKF 211–811 and PSC833 are nonimmunosuppressants that do not inhibit calcineurin but do inhibit rotamase activity. The cell death induced by FALS-related SODV148G was potentiated by both drug classes (as was SODA4V) but not by CsH and D-lys(dansyl)-8-Cs. Values are expressed as means ± SEM ($n = 3$).

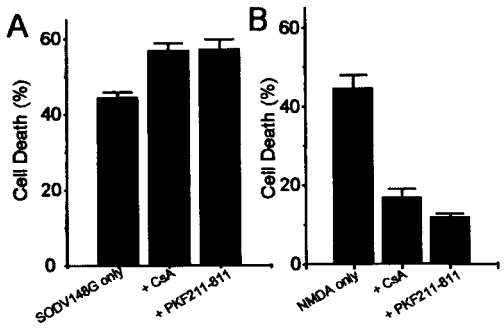

FIG. 4. The effect of the immunosuppressant CsA or its nonimmunosuppressive analog PKF211–811 on cell survival of hippocampal neurons 6 days after infection with AdSODV148G (*A*) and after treatment with NMDA (100 μM) (*B*). Treatment with these drugs did not significantly change the viability of mock-infected cells or cells expressing SODWT (data not shown). Values are expressed as means ± SEM (n = 3).

effect in mock or SODWT-expressing cells. The results suggest that CsA enhances SODV148G-induced cell death by inhibiting cyclophilin rotamase activity and not because of calcineurin inhibition. Treatment with CsH, an analogue that does not bind to cyclophilin, and treatment with dansylated CsA, which has relatively poor binding to cyclophilins at the 1 μM dosage used (24, 25), did not significantly affect the viability of cells expressing SODV148G (Fig. 3*A*).

In contrast to the above results, both immunosuppressants (CsA, CsG, and FK506) and nonimmunosuppressants (PKF 211–811 and PSC 833) protected PC12 cells from death after NGF withdrawal (Fig. 3*B*). As expected, CsH and dansylated CsA had relatively little effect on cell death after NGF withdrawal (Fig. 3*B*).

Results obtained with cultured hippocampal neurons were similar to those with PC12 cells. Six days after mutant AdSODV148G infection, there was ≈40% hippocampal neuronal cell death (Fig. 4*A*). Both CsA and PKF 211–811 significantly enhanced the mutant SOD-induced death of hippocampal neurons (Fig. 4*A*). In contrast, both CsA and PKF211–811 protected hippocampal neurons from NMDA-induced death (Fig. 4*B*).

Effect of CsA on Rotamase Activity Levels. The above studies suggested that CsA and its immunosuppressive analogs might enhance SOD mutant-induced cell death through its interference with rotamase activity. It also raised the question as to whether there is a preexisting difference in the level of rotamase activity between SODV148G-expressing and mock cells, making the former cells more sensitive to CsA. Therefore, we measured rotamase activity in mock, WT, and mutant SOD-expressing cells before and after CsA treatment. The data show that all cells had a similar amount of rotamase activity before CsA treatment and that CsA decreased rotamase activity to a similar level in all three groups (Table 1), with no significant differences in this inhibition in the SODV148G-expressing cells vs. other CsA-treated groups.

These results suggested that mutant SOD-expressing cells might be more sensitive to effects of decreased rotamase activity. Table 1 shows that the baseline SOD activity was similar in mock, SODWT-, and SODV148G-expressing cells; CsA caused a decrease in SOD activity that was significantly (albeit slightly) more in SODWT-expressing cells than in mock uninfected cells; and the mutant SOD-expressing cells had a slightly greater decrease in SOD activity than SODWT-expressing cells. Our interpretation of these studies is that human SOD/rodent SOD heterodimers that are formed in the transiently expressing cells may be more sensitive to conformational changes resulting from rotamase inhibition than the homogeneous rodent homodimers present in controls. In addition, the mutant human SOD/rodent SOD heterodimers may be more sensitive to changes in conformation than the WT human SOD/rodent SOD heterodimers.

Effect of Overexpression of Cyclophilin A in SOD Mutant-Induced Cell Death. To further examine the role of cyclophilin rotamase activity on mutant SOD-induced cell death, we transfected NGF-differentiated PC12 cells with wild-type CyPA (CyPAWT) cDNA or CyPA(R55A) cDNA that contains a point mutation in the putative rotamase active site. CyPA immunohistochemical staining showed transfection efficiencies of 68 ± 2% (n = 3) and 61 ± 4% (n = 3), respectively. PC12 cells that overexpressed CyPAWT, but not PC12 cells overexpressing CyPA(R55A), exhibited reduced cell death after SODV148G expression (Fig. 5*A*). The data show that transfection of CyPAWT produced >50% protection from cell death after mutant SOD-expression compared with that seen with transfection of a control cDNA (CD8); transfection of CyPA(R55A) was not protective. These results suggest that the rotamase function of CyPA protects cells from death induced by SODV148G. In contrast, overexpression of CyPAWT or CyPA(R55A) in cells infected with AdSODWT or in mock-infected cells had no effect on cell viability (data not shown). Furthermore, overexpression of either CyPAWT or CyPA(R55A) did not significantly protect cells from death induced by NGF withdrawal (Fig. 5*B*), indicating that the apoptotic pathway induced by mutant SOD differs from that after growth factor removal.

To further investigate these issues, we tested whether CyPA rescue of mutant SOD-induced cell death would be decreased with CsA treatment. Table 2 shows that overexpression of CyPA (but not CD8, "control" overexpression) significantly reduced SODV148G-induced cell death (from 49.1 to 21.9%) and that the CyPA rescue was decreased by CsA (changing cell death from 21.9% back to 54.6%). These results suggest that modulating the rotamase activity is vital for the survival of mutant SOD-expressing cells.

DISCUSSION

FALS-associated mutant SOD is believed to kill neurons through a gain of adverse function or enhancement of a

Table 1. Mean rotamase and SOD activity [percent of control (mock-infected) PC12 Cells]

Enzyme activity	Treatment	Baseline, mean ± SEM (n)	+CsA, mean ± SEM (n)	Differences*
Rotamase	Mock	100 ± 10.54 (4)	74.47 ± 5.95 (5)	−,+
	SODWT	98.06 ± 7.76 (5)	73.88 ± 4.53 (4)	−,+
	SODV148G	100.84 ± 8.06 (5)	68.17 ± 3.75 (5)	−,+
SOD	Mock	100 ± 1.22 (4)	98.1 ± 3.14 (4)	−,−
	SODWT	102.38 ± 1 (5)	96.4 ± 1.89 (4)	−,+
	SODV148G	102.25 ± 2.19 (5)	90.78 ± 4.58 (4)	−,+

*+ indicates significant difference ($P \leq 0.05$; Tukey post hoc analysis); the first symbol reports comparisons of baseline enzyme activity versus mock-infected groups, and the second symbol reports comparisons of enzyme activity with and without CsA per treatment (within row comparison).

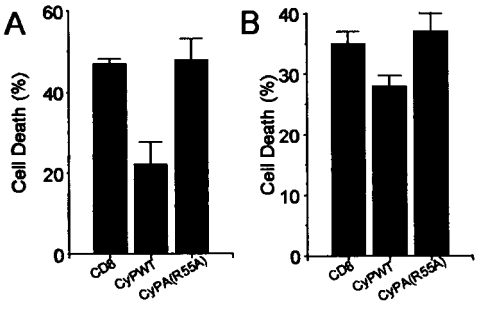

FIG. 5. Effect of overexpression of WTCyPA or an isomerase-deficient CyPA(R55A) on PC12 cell death induced by SODV148G expression (*A*) or after NGF withdrawal (*B*). As a control, cells were transfected with CD8 cDNA. Values are expressed as means ± SEM ($n = 3$).

normally present nondismutase function (7, 8, 26, 27). We recently demonstrated that overexpression of mutant SOD leads to altered superoxide content and induces the death of differentiated PC12 cells, primary sympathetic neurons, and primary hippocampal neurons (12). Our present studies were prompted by the report of Wang *et al.* (16) that WT SOD prevents calcineurin from inactivation. This finding suggested to us that mutant SOD might induce cell death because it fails to protect calcineurin from inactivation. We therefore used adenovirus as a vector to express SODWT or SODV148G in NGF-differentiated PC12 cells and hippocampal neurons to test whether calcineurin was involved in this death and to elucidate the mechanism underlying mutant SOD-induced cell death. We found that SODWT increased calcineurin activity; however, cells expressing SODV148G showed a level of calcineurin activity similar to that of mock-infected cells. For the latter reason, mutant SOD-induced cell death appeared unrelated to an effect on calcineurin activity.

To further investigate a role for calcineurin inactivation in mutant SOD-induced cell death, we tested the effect of CsA, a calcineurin inhibitor, on cell viability. CsA enhanced death of cells expressing SODV148G but not mock-infected cells, even though CsA treatment caused a greater decrease in calcineurin activity in mock-infected cells than in cells expressing SODV148G. These data again demonstrate a lack of correlation between calcineurin activity and mutant SOD-induced cell death.

We questioned whether CsA also might influence cell death by its ability to block the mitochondrial permeability transition pore (MPTP). High conductance openings of this channel are favored by oxidizing species and may lead to apoptotic cell death (28). Both NGF-withdrawal and expression of SOD mutations have been associated with the generation of O_2^- and other oxidizing radicals (8, 29–32). For NGF withdrawal and NMDA toxicity, the ability of CsA and its analogs to block the MPTP and their ability to rescue neuronal death are well correlated, suggesting that their effect on MPTP may be relevant to their protective action. On the other hand, CsA and its analogs enhanced death induced by mutant SOD. It seems unlikely that inhibition of MPTP underlies this toxicity enhancement because one would expect that MPTP inhibition would rescue cells rather than enhance cell death.

Table 2. CyPA overexpression rescues PC12 cells from SODV148G-induced death as well as from the enhanced death seen in combination with CsA

Treatment	Cell death, percent of control	SEM ($n = 3$)	Differences*
Mock + CD8	0	1.6	A
Mock + CD8 + CsA	−0.5	2.7	A
SODV148G + CD8	49.1	1.2	B
SODV148G + CD8 + CsA	69.6	1.7	C
SODV148G + CyPA	21.9	3.8	D
SODV148G + CyPA + CsA	54.6	1.3	E

*Rows with the same letter are not significantly different (Tukey post hoc analysis; $P \leq 0.05$.

In addition to inhibiting calcineurin and blocking the MPTP, CsA blocks peptidyl prolyl isomerase (rotamase) activity of cyclophilins. Immunophilin rotamase activity is believed to have a variety of actions in neurons (15). To test whether CsA enhanced SODV148G toxicity through rotamase inhibition, we examined the effect of immunosuppressants (CsA CsG, and FK506) as well as nonimmunosuppressants (PKF 211–811 or PSC 833). Nonimmunosuppressants do not influence calcineurin activity but do inhibit rotamase activity. We found that both drug classes enhanced apoptosis induced by mutant SOD, indicating that the action of CsA may be related to rotamase inhibition and not to calcineurin inhibition.

A role for rotamase in mutant SOD toxicity was supported by experiments comparing mutant SOD-induced cell death after expression of CyPAWT vs. an isomerase activity-deficient mutant CyPA(R55A). The WT, but not the mutant, CyPA protected cells from death induced by mutant SOD expression. Although a recent report concluded that CyPA(R55A) may have more rotamase activity than previously suspected (33, 34), it appears that this residual activity is insufficient to permit folding of fully functional proteins (17). Our data highlight the importance of rotamase activity in modifying the effect of mutant SOD. The findings that rotamase activity protects cells from mutant SOD-induced cell death and that there is roughly similar rotamase activity after CsA treatment in WT- and mutant SOD-expressing cells suggests that cells expressing mutant SOD have a greater reliance on rotamase activity and has implications on our understanding of the mechanism of this toxicity. We have sampled only a limited number of FALS-associated mutants in this study but suspect that a similar mechanism may underlie many (or possibly all) of the others.

Mutant SOD may increase the oxidative modification of proteins (30) and their cellular turnover as a direct result of an altered cellular free radical balance. The increased protein turnover may enhance the need for rotamase function. Rotamase also may be needed to refold proteins that are partly denatured by oxidative damage. Motor neurons may be especially sensitive to the free radical damage induced by mutant SOD because of their high oxidative activity, at least partly resulting from their large surface area and dependence on intracellular transport of proteins (35) and muscle-derived trophic factor signaling. Rotamase may be required to maintain proteins in a correct conformation during their transport in motor axons. In fact, CyPA has been shown to be transported by slow axonal transport (36), suggesting that it maintains proteins in a native state.

Rotamase also may have a role in the normal folding of SOD itself to help stabilize the enzyme or its dimers. This activity may be especially critical for abnormal FALS-associated mutant SOD function because SOD mutations may destabilize SOD or the dimer (3, 37) and may lead to misfolding. Misfolding and perturbed dimer formation could lead to aggregation of the protein (11) with a subsequent gain-of-function toxicity. We found that SOD activity decreased slightly more after mutant SOD expression than after SODWT expression, suggesting that the mutant enzyme was more sensitive than WT to the effects of CsA, perhaps because the mutant SOD was more sensitive to a decline in rotamase activity and the consequences of misfolding. It also may be that other proteins, perhaps ones that interact with SOD, are more sensitive to decreased rotamase activity.

Our observations have implications regarding possible therapy of FALS, suggesting the potential use of chaperone

proteins or of agents that modulate rotamase activity to correct the deficits. In addition, our findings raise caution concerning the use of immunosuppressants in motor neuron disease. Although these drugs may prevent certain forms of cell death (38–40) and may be therapeutic in several neuropathological states, there is other literature that indicates that these drugs may be proapoptotic for T lymphocytes and other cells (41, 42). We do not believe that the enhancement of death seen after CsA treatment of PC12 cells expressing mutant SOD is related to its proapoptotic effect on these cells because no enhancement of cell death was seen in PC12 cells expressing SODWT or mock infected cells. In addition, CsA actually decreased cell death in PC12 cells after NGF withdrawal. Rather, our studies suggest that these drugs worsen the FALS-mediated cell death because of rotamase inhibition.

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