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Pyrrolidine Dithiocarbamate Inhibits Induction of Immunoproteasome and Decreases Survival in a Rat Model of Amyotrophic Lateral Sclerosis

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ABSTRACT

Pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear transcription factor κ-B (NF-κB) and an antioxidant, has beneficial effects in animal models of various diseases, including arthritis, brain ischemia, spinal cord injury, Alzheimer's disease, and Duchenne muscular dystrophy. Because inflammation and oxidative damage are also hallmarks of amyotrophic lateral sclerosis (ALS), we studied the effect of oral PDTC treatment on G93A-superoxide dismutase 1 (SOD1) transgenic (TG) rat model of human ALS and observed that PDTC treatment significantly decreases the survival. PDTC treatment evoked the end stage of the disease at 121 ± 21 days, whereas untreated TG animals reached the end stage at 141 ± 13 days ($p < 0.01$). The DNA binding activity of NF-κB was not altered in G93A-SOD1 TG rats by PDTC treatment. The copper concentration in the spinal cord was increased after PDTC treatment both in G93A-SOD1 TG and wild-type rats, suggesting that increased copper may enhance the neurotoxicity of mutant SOD1. The amount of ubiquitinated proteins were significantly higher and proteasomal activity was decreased in the spinal cords of PDTC-treated TG rats compared with other groups, suggesting that PDTC treatment decreases proteasome function. Immunoblotting and immunocytochemistry showed that the level of immunoproteasome but not constitutive proteasome was increased in glia of G93A-SOD1 TG rats along with disease development. PDTC treatment completely blocked the induction of immunoproteasome expression without affecting constitutive proteasome. These results suggest that PDTC acts as an immunoproteasome inhibitor in mutant SOD1 rats and that immunoproteasome may help the nervous system to cope with deleterious effects of SOD1-G93A mutation.

Pyrrolidine dithiocarbamate (PDTC) belongs to a class of dithiocarbamates, which have been used previously in the treatment of bacterial and fungal infections and have been considered for use in the treatment of AIDS (Reisinger et al., 1990). PDTC is also known as an inhibitor of nuclear transcription factor κ-B (NF-κB) that regulates the expression of several proinflammatory genes and some genes related to apoptosis (Schreck et al., 1992; Liu et al., 1999; Hayakawa et al., 2003). In addition, PDTC has been shown to be a potent antioxidant both in vitro and in vivo (Schreck et al., 1992; Liu et al., 1999). Because inflammation, apoptosis, and oxidative stress are implicated in a large range of diseases, it is not surprising that PDTC has been reported to have beneficial effects in models of diseases such as arthritis, pleurisy, liver and brain ischemia, spinal cord injury, Alzheimer's disease, Duchenne muscular dystrophy, and neonatal asphyxia (Cuzzocrea et al., 2002; La Rosa et al., 2004; Nurmi et al., 2004a, 2006; Matsui et al., 2005; Cheng et al., 2006; Messina et al., 2006). There is evidence that mechanisms of PDTC's beneficial effects in these models are indeed related to its ability to act as an antioxidant and to inhibit the expression of proinflammatory genes, including cyclooxygenase-2, tumor necrosis factor-α, and interleukin-1β (Nurmi et al., 2004a,b). In addition, PDTC may also provide protection by activating Akt-GSK3β signaling (Nurmi et al., 2006).

Amyotrophic lateral sclerosis (ALS) is a late-onset motor neuron degenerative disease of the spinal cord, brainstem, and cortex occurring both sporadically and as a familial disorder, the latter accounting for approximately 10% of the cases. The patients with ALS typically become progressively

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ABBREVIATIONS: PDTC, pyrrolidine dithiocarbamate; ALS, amyotrophic lateral sclerosis; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; SOD, superoxide dismutase; WT, wild type; TG, transgenic; GLT, glutamate transporter.

paralyzed, and respiratory failure eventually leads to death within 3 to 5 years (Mulder et al., 1986). The exact mechanism responsible for the motor degeneration is largely unknown, and there is no known effective treatment for this fatal disease. However, mutations in the ubiquitously expressed protein Cu,Zn-superoxide dismutase (SOD1) are associated with approximately 20% of familial ALS cases (Rosen et al., 1993). Because the pathology and clinical symptoms of familial and sporadic ALS cannot be distinguished, TG animal models overexpressing mutant SOD1 offer a valuable tool for understanding the pathogenic mechanisms shared by both sporadic and familial forms of ALS. It is noteworthy that several pathogenic SOD1 mutations do not affect SOD1 activity significantly, and "a toxic gain of function" of the mutated protein rather than a lack of its antioxidant function has been postulated. The nature of this gained toxic function is not known, even though several putative mechanisms have been proposed, including the formation of protein aggregates, mislocalization and aggregation of neurofilaments, increased free radical generation, mitochondrial dysfunction, and proapoptotic alterations (Bruijn et al., 2004). In addition, inflammation is strongly implicated in the pathogenesis of ALS, because microgliosis and astrogliosis with activation of stress-induced kinases and transcription factor NF-κB accompany motor neuron degeneration either in ALS or SOD1 mutant mice (Migheli et al., 1997; Tortarolo et al., 2003). Moreover, expression of several proinflammatory mediators is increased both in ALS and SOD1 mutant mice (Alexianu et al., 2001; Elliott, 2001; Nguyen et al., 2001), and numerous anti-inflammatory compounds prolong survival of TG SOD1 mutant mice (Drachman et al., 2002; Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002; Kiaei et al., 2006). In addition, animal models that express mutant SOD1 exclusively either in motor neurons (Pramatarova et al., 2001; Lino et al., 2002) or in astrocytes (Gong et al., 2000) do not develop the disease or pathologic phenotype, suggesting that interplay between glia and motor neurons is required for the ALS pathogenesis.

Because oxidative damage, inflammation, and even apoptosis play major roles in the pathology of ALS, we decided to test the effect of PDTC treatment on G93A-SOD1 TG rats, a disease model for human amyotrophic lateral sclerosis (Howland et al., 2002). We show that daily oral treatment with PDTC significantly decreases the survival of G93A-SOD1 TG rats. Our data indicate that this unexpected effect may be mediated by the prevention of induction of immunoproteasome, suggesting that immunoproteasome is a beneficial response in ALS to cope with accumulating protein aggregates. Because we found that the immunoproteasome was induced exclusively in astrocytes and microglia, our results support the idea of the important role of the non-neuronal cells adjacent of dying motor neurons.

Materials and Methods

PDTC Treatment of ALS Rats. All animal studies were carried out under permission of The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government according to the National Institutes of Health guidelines for the care and use of laboratory animals. Transgenic rats [Tac:N:(SD)-TgN(SOD1G93A)L26H, Emerging Models Program sponsored by Amyotrophic Lateral Sclerosis Association, Taconic] expressing human mutant G93A-SOD1 were treated with PDTC on a dose of 50 mg/kg/day starting at 70 days of age (n = 19). Fresh PDTC (Sigma, St. Louis, MO) was administered into drinking water every other day. The control transgenic rats (n = 12) received plain water. In addition, wild-type (WT) litter mates of corresponding age received either PDTC (n = 6) or plain water (n = 7). Neurological signs of disease, such as overall locomotor activity, ataxia, extension reflex of hind limps, limp gait, paralysis of hind limp, or paralysis of fore limp and weight gain of the animals were followed daily, and appearance of limp gait was determined as the onset of the disease. New dose of PDTC was calculated weekly according to animal weight and water consumption, and the treatment was continued till the end stage of the disease when the animals were killed. The end stage of the disease was determined by righting reflex test where animal was placed on its side, and if it was not able to right itself in 30 s, it was scored as death. Sacrificed animals were perfused transcardially with saline, and extracted tissues were either frozen in liquid nitrogen and stored at −80°C or postfixed with 4% paraformaldehyde for 24 h and cryoprotected with 30% sucrose for 3 days and then frozen in liquid nitrogen and stored at −80°C.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays (EMSA) for NF-κB-DNA binding were carried out as described earlier in detail (Helenius et al., 1996) with 5 μg of nuclear protein of the spinal cord tissues at the presymptomatic age (100 d) and endstage. Double-stranded oligonucleotides for NF-κB binding sites were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). The probe was labeled with T4 polynucleotide kinase (Promega, Madison, WI). Nonspecific binding was blocked with 2 μg of poly(dI-dC)/poly(dI-dC) (Roche Applied Science, Basel, Switzerland) in a 20-μl assay volume. Bound and free probes were separated in a native 4% polyacrylamide gel. Radioactive bands were visualized with a Storm 860 Imager (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and pixel volumes of specific bands were calculated with ImageQuant software (GE Healthcare).

Atomic Absorption Spectrophotometry. Copper concentrations in the spinal cord, cortex, and liver tissues from each treatment group were measured by atomic absorption spectrophotometry at the City of Kuopio Environmental Health Laboratory by Hitachi Z-8100 Polarized Zeeman (Hitachi, Tokyo, Japan) graphite furnace atomic absorption spectrophotometry from pyrolyzed samples. Copper concentrations shown as microgram of copper per gram of tissue wet weight.

Proteasomal Activity. Proteasomal activity was measured from cytosolic fractions of the spinal cord samples as chymotrypsin-like activity by cleavage of *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Sigma). The activity was measured as increasing fluorescence of cleaved 7-amino-4-methylcoumarin-peptides. Tissues were homogenized in ice-cold buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 0.25 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM ATP) and centrifuged 3500 rpm at 4°C for 7 min. Protein concentration of the supernatant was determined using Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Proteasomal activity was measured in aliquots of 10 μg of protein in 50-μl volume of assay buffer (20 mM Tris-HCl pH 7.5, 1 mM ATP, 2 mM $MgCl_2$, and 0.1% bovine serum albumin containing 100 μM *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin). Fluorescence of cleaved 7-amino-4-methylcoumarin-peptides was monitored every 10 min at 37°C at 355 nm excitation and 460 nm emission using a Wallac 1420 multilabel counter (PerkinElmer Wallac, Gaithersburg, MD).

Western Blotting. Cytosolic proteins were separated by 10% or 12% SDS-polyacrylamide gel electrophoresis on Mini-Protean III electrophoresis device (Bio-Rad). After electrophoresis proteins were transferred to polyvinylidene difluoride membrane (GE Healthcare, Uppsala, Sweden) with a Mini-Protean II blotting cell (Bio-Rad) according to manufacturer's instructions and immunostained using rabbit polyclonal anti-proteasome 20S LMP7 (dilution 1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-proteasome 20S X (dilution 1:1000; Abcam), rabbit polyclonal anti-GLT-1 (dilution 1:1000;

Calbiochem, La Jolla, CA), monoclonal anti-β-actin (dilution 1:4000, Sigma) or rabbit polyclonal anti-ubiquitin antibodies (dilution 1:1000; DakoCytomation Denmark A/S, Glostrup, Denmark) and horseradish peroxidase-labeled anti-mouse IgG (dilution 1:4000; GE Healthcare) or horseradish peroxidase-labeled anti-rabbit IgG (dilution 1:3000; GE Healthcare) secondary antibodies, followed by enhanced chemiluminescence detection (GE Healthcare). Quantifications were done on a STORM Imager (GE Healthcare) with ImageQuant software (GE Healthcare).

Immunohistochemistry. Three coronal 20-μm cryosections from two animals per group of the lumbar spinal cord were used for immunohistochemistry with rabbit polyclonal anti-proteasome 20S LMP7 (dilution 1:500; Abcam) or rabbit polyclonal anti-proteasome 20S X antibodies (dilution 1:500; Abcam) and with cell markers monoclonal glial fibrillary acidic protein (dilution 1:500; Chemicon, Temecula, CA), monoclonal NeuN (dilution 1:500; Chemicon) or monoclonal CD68 (dilution 1:500; Serotec, Oxford, UK) antibodies. For the detection of proteasome, Alexa Fluor 488 conjugated anti-rabbit IgG antibody (Invitrogen) was used and for detection of cell markers, Alexa Fluor 568 conjugated anti-mouse IgG antibody (Invitrogen) was used. Fluorescence stains were visualized using a confocal microscope (Bio-Rad Radiance Laser Scanning Systems 2100; Bio-Rad Microscience Ltd., Hertfordshire, UK) with a Laser-Sharp 2000 software (Bio-Rad Microscience Ltd.). For the visualization of proteasome immunoreactivity in light microscopy (Olympus AX70, Tokyo, Japan), incubation with biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) antibody was followed by avidin-biotin complex (Vectastain Elite Kit; Vector Laboratories) and the immunoreaction was visualized using nickel-enhanced diaminobenzidine (Sigma) as a substrate.

Statistical Analyses. The results are presented as mean ± S.D. Differences between groups (*, $p < 0.05$ was considered statistically significant) were determined by Student's t test (two-group comparisons) or with analysis of variance combined with Bonferroni post-hoc test on SPSS software version 11.5 (SPSS Inc., Chicago, IL).

Results

PDTC Treatment Decreased Survival of G93A-SOD1 Transgenic Rats. PDTC treatment decreased the survival of G93A-SOD1 TG rats by 15% (140 ± 13 days in untreated group and 122 ± 21 days in PDTC group, $p < 0.01$) (Fig. 1A). Compared with untreated group of G93A-SOD1 TG rats, the onset of paralysis (Fig. 1B) occurred significantly earlier in PDTC-treated animals (109 ± 22 and 120 ± 14 days for PDTC TG and untreated TG, respectively; $p < 0.01$), whereas there was no difference in the duration of the disease from the onset to the end stage between PDTC- (12 ± 4 days) and vehicle-treated (11 ± 3 days) ALS rats (Fig. 1, C and D). Weight gain measured at or after the onset of the disease showed no significant differences between PDTC and untreated TG or WT rats (Fig. 1, E and F). The mean weight decreased earlier (weeks 13–14) in PDTC-treated TG mice because of the earlier onset of the disease in PDTC-treated group. We did not observe any significant signs of toxicity of the PDTC treatment, as judged by weight gain, consumption of drinking water, development of diarrhea, copper concentration of the liver, ataxia, overall locomotor activity, or immunohistochemical signs of increased gliosis or myelin loss in the ventral spinal cord.

The Effect of PDTC Was Not Mediated through NF-κB Inhibition. NF-κB activation may promote the expression of the genes that mediate inflammation or apoptosis and some genes that support survival. EMSA analysis of the spinal cord samples showed no differences in DNA binding activity of NF-κB between PDTC and untreated groups (Fig. 2). However, although there was no statistically significant difference between any of groups, there was a trend toward increased DNA binding activity of NF-κB in G93A-SOD1 TG rats.

PDTC Increased Copper Concentration in the Spinal Cord. Because SOD1 is a major contributor to the cellular Cu^{2+} concentration, copper concentrations of the spinal cord were significantly higher in both presymptomatic (3.3 ± 0.2 μg/g, $n = 5$, $p < 0.001$) and end-stage (3.1 ± 0.5 μg/g, $n = 7$, $p < 0.001$) G93A-SOD1 TG rats than in WT rats (1.7 ± 0.1 μg/g, $n = 4$). PDTC treatment increased the copper levels of the spinal cord tissue in G93A-SOD1 TG rats by 36% (4.2 ± 0.8 μg/g, $n = 7$, $p < 0.05$) and in WT rats by 200% (5.1 ± 1.9 μg/g, $n = 4$, $p < 0.001$). There was no statistically significant difference in the spinal cord copper concentration between G93A-SOD1 TG and WT rats after PDTC treatment. PDTC treatment did not cause significant increases in copper concentration in the cortex or liver of G93A-SOD1 rats.

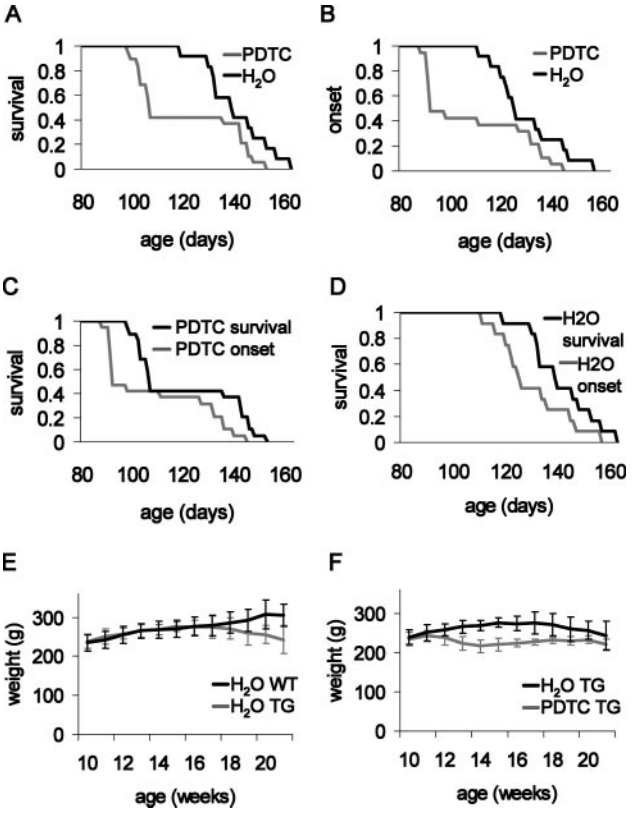

Fig. 1. PDTC treatment of ALS rats resulted in decreased survival. A, for all PDTC-treated TG animals, survival was 121 ± 21 days (gray line, $n = 19$) and 141 ± 12 days for TG animals that received plain H_2O (black line, $n = 12$); **, $p < 0.01$. B, similarly, the onset of paralysis occurred earlier in PDTC-treated rats. Onset of paralysis occurred on average at 109 ± 22 days for TG PDTC (gray line) and 120 ± 14 days for untreated TG (black line) rats; **, $p < 0.01$. The disease duration from onset to the end stage was 12 ± 4 days for TG PDTC rats (C) and 11 ± 3 days for untreated TG rats (D). Weight gain for untreated TG animals compared with WT untreated (E) and PDTC-treated TG animals compared with untreated TG rats (F) did not show significant differences, but a decrease in weight was observed after the onset of disease, which occurred earlier in PDTC-treated rats compared with untreated animals.

PDTC Treatment Increased the Levels of Ubiquitinated Proteins, Decreased Proteasome Activity, and Prevented the Immunoproteasome Induction in G93A-SOD1 TG Rats. As Cu-PDTC complexes may interfere with proteasomes, we investigated whether PDTC alters the amount of ubiquitinated proteins in the spinal cord. Immunoblotting revealed that the levels of ubiquitinated proteins were increased by 33% in the cytosolic fraction of the spinal cord of PDTC-treated TG rats at the end stage of the disease compared with untreated endstage animals (1507 ± 167 and 1136 ± 156, respectively; $p < 0.05$) (Fig. 3).

To find out whether proteasomal activity is changed in G93A-SOD1 TG rats or by PDTC treatment, we measured chymotrypsin-like activity in the spinal cord tissues. Chymotrypsin-like activity was approximately on the same level in untreated WT group (902 ± 85, $n = 5$) and untreated G93A-SOD1 TG group at the presymptomatic stage (1015 ± 167, $n = 5$). In the end stage, the G93A-SOD1 TG rats had significantly increased proteasomal activity (1427 ± 184, $n = 5$, $p < 0.05$, 41% increase), and this increase was completely prevented (990 ± 255, $n = 5$) by PDTC treatment. PDTC treatment had no effect on proteasome activity in WT animals (824 ± 87 and 902 ± 85 for PDTC and untreated WT rats, respectively; $n = 5$).

To investigate in detail whether PDTC alters the proteasome expression in the spinal cord, we used immunoblotting for 20S X and 20S LMP7, markers of constitutive and inducible proteasome, respectively. The level of 20S X was not changed in G93A-SOD1 TG rats at any time point of the disease progression (Fig. 4, A and C). Instead, the expression of immunoproteasome measured by immunoblotting for 20S LMP7, an inducible β subunit, was strongly increased in the spinal cord but not in the cortex along with the disease progression of G93A-SOD1 TG rats (Fig. 4A). The amount of LMP7 protein was increased 6-fold between 8 and 16 weeks of age (334 ± 236 and 1905 ± 174, $p < 0.01$, when 8 weeks and 16 weeks were compared, respectively) and reached 9-fold increase at the end stage (3101 ± 603, $p < 0.001$) (Fig. 4B). PDTC treat-

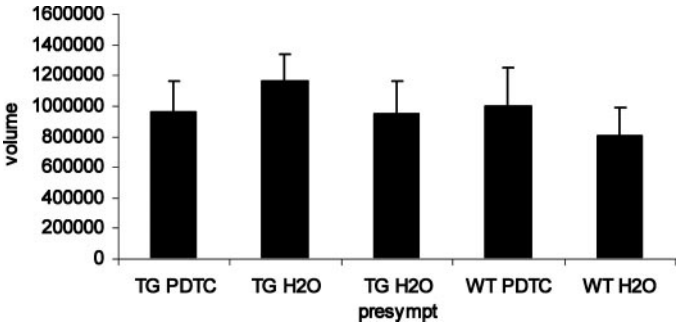

Fig. 2. EMSA analysis of spinal cord samples showed no statistically significant differences in DNA binding activity of NF-κB between PDTC and untreated groups; $n = 5$, results shown are mean ± S.D.

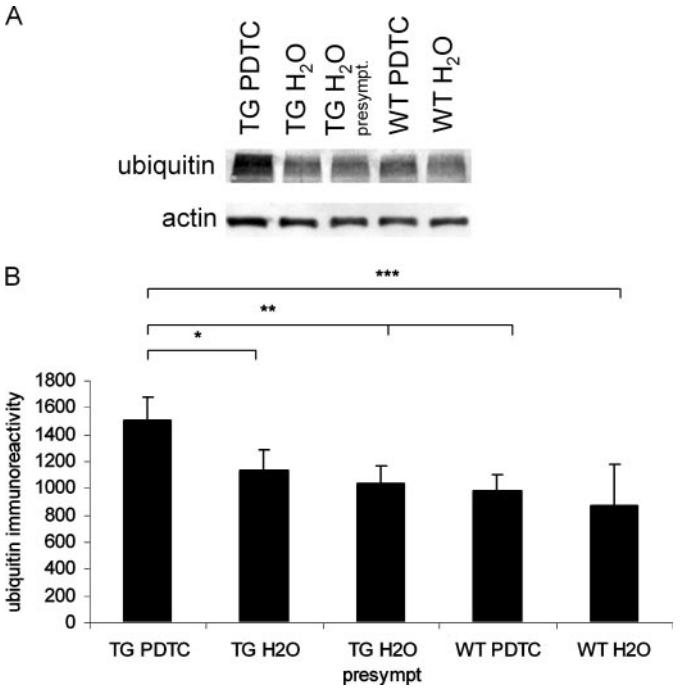

Fig. 3. Levels of ubiquitinated proteins increased significantly in PDTC-treated TG rats at the end stage of the disease (A) compared with untreated end-stage animals (*, $p < 0.05$), presymptomatics (**, $p < 0.01$), or WT rats (***, $p < 0.001$) (B); $n = 5$, results shown are mean ± S.D.

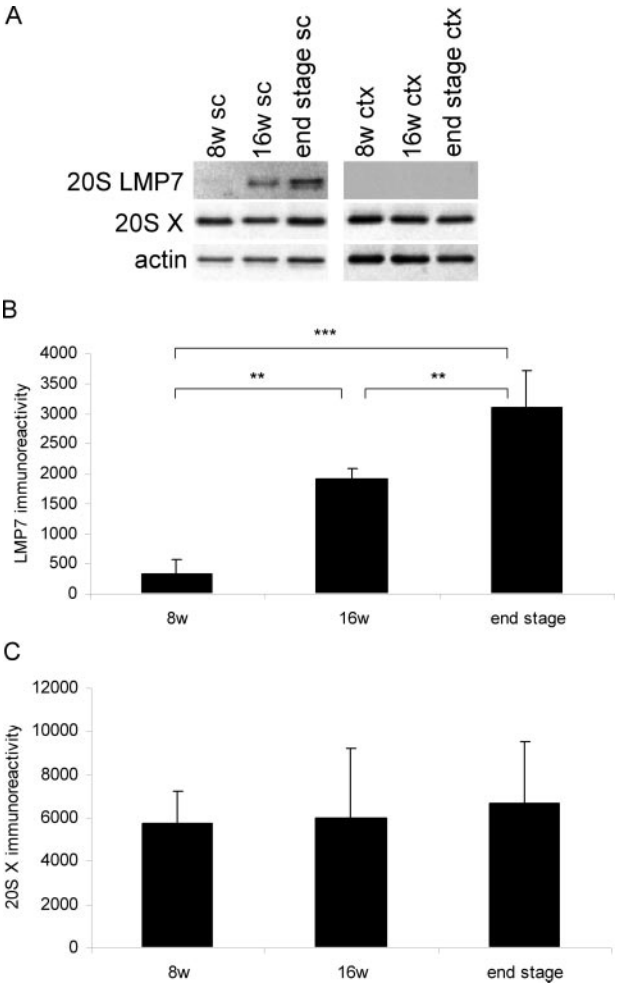

Fig. 4. The levels of immunoproteasome (LMP7) increased along with the disease progression in the spinal cord of G93A-SOD1 TG rats but not in the cortex. In G93A-SOD1 TG rats the levels of 20S LMP7 protein in the spinal cord was increased 6-fold between 8 and 16 weeks (**, $p < 0.01$) of age and reached 9-fold increase (***, $p < 0.001$) at the end stage (A and B). Levels of constitutive proteasome 20S X remained unchanged through the disease progression in the spinal cord (A and C); $n = 4$, results shown are mean ± S.D.

ment completely prevented the induction of 20S LMP7 at the end stage of G93A-SOD1 TG rats (1476 ± 479 and 2978 ± 1296, for PDTC treated and untreated, respectively; $p < 0.05$; Fig. 5, A and B), whereas no effect of PDTC on the constitutive proteasome subunit was detected. It is noteworthy that in WT animals, 20S LMP7 was barely detectable or undetectable in the cytosolic fraction (Fig. 5A), and PDTC had no effect on expression of this protein or 20S X in WT animals (Fig. 5A).

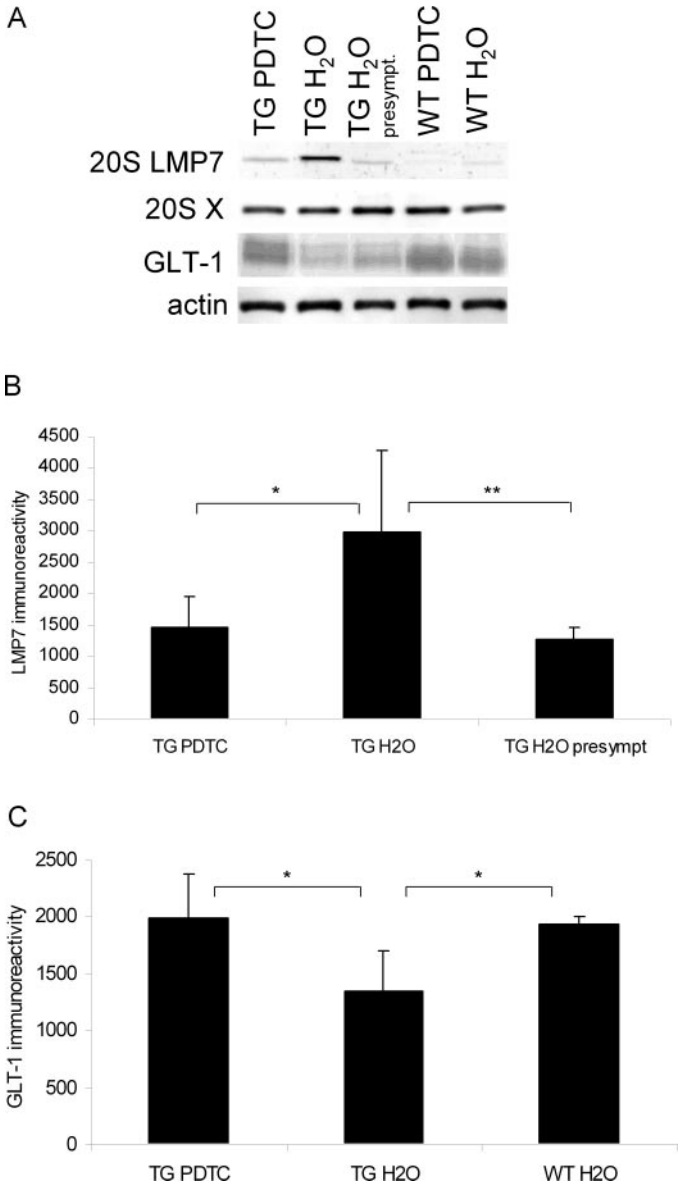

Fig. 5. PDTC led to decreased immunoproteasome levels and increased GLT-1 levels in TG rats at the end stage, whereas PDTC had no effect on the levels of constitutive proteasome, immunoproteasome, or GLT-1 in WT rats. PDTC treatment completely prevented the induction of 20S LMP7 at the end stage of G93A-SOD1 TG rats (A and B), whereas no effect of PDTC on the constitutive proteasome subunit was detected. A, it is noteworthy that in WT animals, 20S LMP7 was barely detectable or undetectable in the cytosolic fraction, and PDTC had no effect on the expression of this protein or 20S X in WT animals. PDTC also had an effect on the levels of astrocyte specific glutamate transporter (GLT-1). A and C, in the spinal cords of untreated TG rats, the levels of GLT-1 were decreased, whereas in PDTC-treated TG rats, the levels of GLT-1 were at the same levels as in WT rats. *, $p < 0.05$, $n = 5$, results shown are mean ± S.D.

The changes in immunoproteasome levels after PDTC treatment were not due to common reduction of astroglial functions, because PDTC also increased the levels of astrocyte-specific glutamate transporter (GLT-1). In the spinal cords of untreated TG rats, the levels of GLT-1 were decreased, whereas in PDTC-treated TG rats, the levels of GLT-1 were at the same levels as in WT rats (Fig. 5, A and C).

Immunoproteasome Induction in G93A-SOD1 TG Was Localized in Glia. Constitutive proteasome was expressed in cells throughout the gray matter in the ventral horn of the lumbar spinal cord in both PDTC-treated TG and WT rats and in untreated TG and WT rats (Fig. 6A). However, immunoproteasome was expressed only in untreated TG rats at the end stage (Fig. 6B). From the appearance, the cells showing immunoproteasome staining looked non-neuronal. Double-labeling immunohistochemistry with confocal imaging showed that the immunoproteasome 20S LMP7 was expressed in astrocytes and microglia (Fig. 7B), whereas proteasome 20S X was also expressed in neurons (Fig. 7A).

Discussion

Previous studies have shown that PDTC provides protection in a number of central nervous system and peripheral disease models by inhibiting the activation of transcription factor NF-κB, serving as a strong antioxidant, or by activating Akt-GSK3β pathway (Cuzzocrea et al., 2002; La Rosa et al., 2004; Nurmi et al., 2004a, 2006; Matsui et al., 2005; Cheng et al., 2006; Messina et al., 2006). Even though several antioxidants (Gurney et al., 1996; Wu et al., 2003) and inhibitors of NF-κB-driven genes, such as cyclooxygenase-2, tumor necrosis factor-α, and interleukin-1β (Drachman et al., 2002; Kiaei et al., 2006), prolong the survival of TG ALS mice, and activation of Akt-GSK3β pathway reduced mutant SOD1-mediated motor neuron cell death in vitro (Koh et al., 2005), we found that PDTC treatment does not provide protection but instead significantly decreases the survival of G93A-SOD1 TG rats. The fact that PDTC treatment prevented the reduction of the glutamate transporter GLT-1, a potential therapeutic target verified in numerous animal studies of ALS (Gurney et al., 1996; Howland et al., 2002) and that PDTC treatment did not induce toxic side effects in the TG or WT rats, the negative finding is of interest and implies that PDTC treatment caused some harmful alterations in cellular functions, which were able to override the potentially beneficial effects of the drug.

In agreement with the previous studies on mouse ALS models (Cheroni et al., 2005; Puttaparthi and Elliott, 2005), we observed that both increase in proteasome activity and induction of immunoproteasome selectively occur in the affected spinal cord tissue of a G93A-SOD1 TG rat model. Three catalytic subunits of the proteasome, namely β1, β2, and β5, have close homologs, LMP2, MECL-1, and LMP7, that are selectively induced under certain conditions, such as the treatment of cells with γ-interferon. This chance in subunit composition is believed to be involved in antigen processing/presentation and may influence catalytic specificity, at which different catalytic sites hydrolyze peptide bonds which may be relevant for immune response (DeMartino and Slaughter, 1999). It is noteworthy that we

found that PDTC treatment strongly inhibited these ALS model-specific changes in proteasome. On the other hand, in line with previous reports showing that PDTC is a metal chelator and transports Cu^{2+} from the extracellular medium into the cell, we found that PDTC treatment increased copper concentration in the spinal cord of both G93A-SOD1 TG and WT rats. Considering that PDTC is also known as a proteasome inhibitor (Kim et al., 2004) and that increased Cu^{2+} concentration may be needed for the proteasome inhibitory activity of PDTC, it is likely that the detrimental effect of PDTC on G93A-SOD1 TG rats is mediated by the inhibition of immunoproteasome. This inhibition of immunoproteasome was also evidenced as increased levels of ubiquitinated proteins in PDTC-treated G93A-SOD1 TG rats. Because 20S X, a marker of constitutive proteasome, was not affected in G93A-SOD1 TG rats or by PDTC treatment, whereas 20S LMP7, an inducible β-subunit of immunoproteasome, was induced selectively in astrocytes and microglia, our results suggest that immunoproteasome in non-neuronal cells plays a protective role in G93A-SOD1 TG rats.

In addition to its effect on proteasome activity, increased copper concentration may well trigger other cellular processes, which in animals overexpressing G93A-SOD1 could accelerate neurodegeneration. An oral, 15-day PDTC treatment at millimolar concentrations has been reported to increase Cu^{2+} concentration and levels of lipid peroxidation products in rat peripheral nerves (Calviello et al., 2005), implicating that at least in some rodent tissues, a long-term PDTC treatment may cause oxidative stress associated with increased Cu^{2+} concentrations. On the other hand, a 7-month PDTC treatment with the same protocol as in the present study significantly increases cortical copper concentrations in a TG mouse model of Alzheimer's disease and prevents the decrease in cognition without increasing oxidative stress in the brain (Malm et al., submitted for publication). Even though the induction of immunoproteasome was observed selectively in G93A-SOD1 TG rat spinal cords and PDTC prevented this induction without causing changes in other known targets of PDTC, we cannot exclude the possibility that the increased copper concentration enhances motor neuron degeneration in G93A-SOD1 TG rats also by other mechanisms in parallel with immunoproteasome inhibition. However, it should be noted that β-lactams, such as ceftriaxone, which have been reported to be neuroprotective in models of ALS by increasing expression of glutamate transporter GLT-1 (Rothstein et al., 2005), are also metal chelators. PDTC resembles β-lactams because it significantly increases the expression of GLT-1 and Cu^{2+} concentration in the spinal cord. We hypothesize that even though β-lactams and PDTC might both be able to modulate GLT-1 and Cu^{2+} concentration, only PDTC but not β-lactams inhibits immunoproteasome. Because induction of immunoproteasome may be a rather selective characteristic for ALS (models) compared with models of ischemia, trauma, and amyloid-accumulating diseases, inhibition of immunoproteasome alone in ALS models could increase the accumulation of ubiquitinated proteins, including ubiquitinated SOD1, which has been suggested to gain neurotoxic functions such as increased peroxidase activity.

PDTC is an established inhibitor of NF-κB. Even though inhibition of NF-κB has frequently been associated with tissue and cellular protection, inhibition of NF-κB may also accelerate neurodegeneration because of the survival-supporting role of some NF-κB-regulated genes, such as

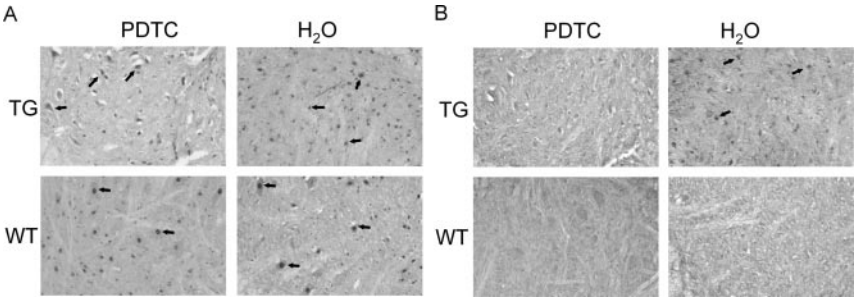

Fig. 6. 20S X and 20S LMP7 expression in lumbar spinal cord. A, 20S X, a marker for constitutive proteasome, was expressed throughout the gray matter in the ventral horn in both PDTC-treated TG and WT rats and in untreated TG and WT rats. B, 20S LMP7, an inducible β-subunit of immunoproteasome, was expressed only in the spinal cords of the untreated TG rats in the end stage but not in the PDTC-treated or WT rats. Magnification, 100×.

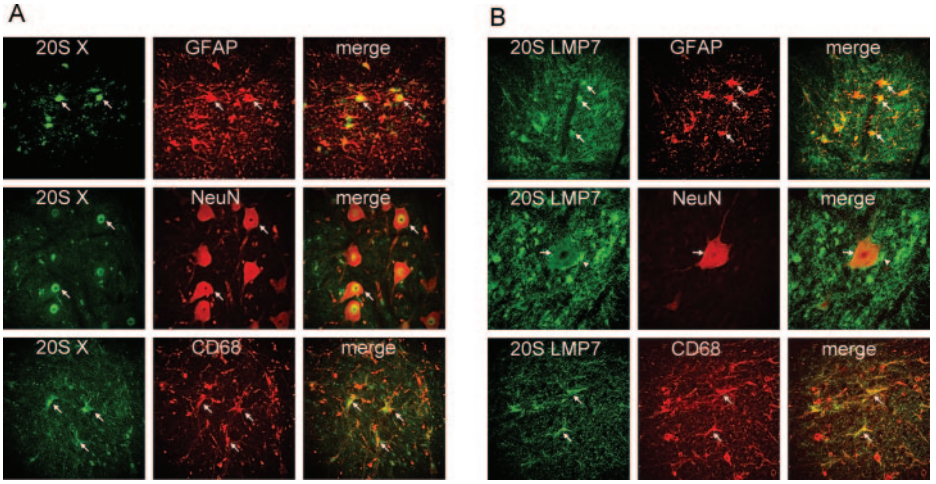

Fig. 7. Expression of constitutive proteasome was localized in neurons and glia, whereas immunoproteasome was expressed only in glia. A, constitutive proteasome 20S X was expressed in astrocytes (stained with glial fibrillary acidic protein), neurons (stained with NeuN), and microglia (stained with CD68). B, immunoproteasome 20S LMP7 was expressed mainly in astrocytes and microglia. Arrows point to cells that have colocalized cell marker and proteasome staining. Arrowheads point to non-neuronal, presumably microglial cell. Double-staining with confocal imaging. Magnification, 400×.

manganese SOD and Bcl-2 (Mattson and Camandola, 2001). Even though we detected a trend for increased NF-κB binding activity in the spinal cords of G93A-SOD1 TG rats, no statistically significant differences between any of the untreated/PDTC-treated TG and WT rat groups were observed. It is possible that in a long-term disease such as ALS, NF-κB activity, even though being induced, is not maintained at so high levels, and on the other hand, oral PDTC administration may not allow achieving tissue concentrations as high as intraperitoneal administration does, and thereby does not result in efficient inhibition of NF-κB. In addition, we cannot exclude the possibility that NF-κB binding to DNA is increased at time points other than the presymptomatic (100 days) and end-stage time points studied here. Nevertheless, our experiments do not provide evidence for a central role of NF-κB in ALS. In a previous study, NF-κB immunoreactivity was found to be increased in astrocytes surrounding degenerating motor neurons (Migheli et al., 1997). Therefore, the possible antiapoptotic effects of NF-κB may not be, at least, directly involved in neuronal survival in TG ALS models.

In summary, we report that PDTC, a multipotent drug providing protection in various animal models by inhibiting NF-κB, acting as an antioxidant and activating Akt-GSK3β pathway, also induces GLT-1, a potential drug target in brain diseases. Regardless of these beneficial effects, oral daily treatment with PDTC at the dose of 50 mg/kg reduces the survival of G93A-SOD1 TG rats, possibly by preventing immunoproteasome activity in non-neuronal cells and thereby accelerating the formation of toxic SOD1-containing protein aggregates. The reduced survival of PDTC-treated G93A-SOD1 TG rats is likely not due to PDTC toxicity, because the onset of specific disease symptoms also occurred earlier, and we did not observe any side effects in PDTC-treated rats. Moreover, a similar treatment protocol is beneficial in a rodent model of Alzheimer's disease (Malm et al., submitted for publication), whereas PDTC treatment even at substantially lower doses (10 mg/kg) is not beneficial in a TG model of ALS (L. van den Bosch, T. Ahtoniemi, J. Kolstinaho, unpublished data). Immunoproteasome may be important for coping with the toxic consequences of mutant SOD1 in tissues affected by ALS.

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