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# Research Article

# Prevention of glutamate excitotoxicity in motor neurons by 5,6-dihydrocyclopenta-1,2-dithiole-3-thione: implication to the development of neuroprotective drugs

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**Abstract.** Glutamate-induced excitotoxicity and oxidative damage are believed to play an important role in the development of a number of central nerve system disorders. Nuclear-factor erythroid 2-related factor 2 (Nrf2) is a master transcriptional regulator of many cytoprotective genes. We report herein that 5,6-dihydrocyclopenta-1,2-dithiole-3-thione (CPDT), which was previously shown to induce several Nrf2 target genes in non-nervous cells and tissues, significantly activates Nrf2 and Nrf2 target genes in rat spinal cord explants. More importantly, such activation is accompanied by complete inhibition of glutamate-induced motor neuron death in these explants. Further studies show that CPDT inhibits glutamate-induced intracellular $Ca^{2+}$ rise, loss of mitochondrial transmembrane potential and depletion of tissue glutathione. CPDT did not appear to modulate glutamate transport or to interfere with glutamate interaction with postsynaptic receptors. Taken together, our studies have identified CPDT as a promising neuroprotective agent and suggest that pharmacological activation of Nrf2 signaling is an important strategy for protection against glutamate-induced excitotoxicity.

**Keywords.** Nrf2, glutamate excitotoxicity, cytoprotection, motor neuron, 5,6-dihydrocyclopenta-1,2-dithiole-3-thione, phase 2 gene.

## Introduction

Glutamate is a major excitatory neurotransmitter, release of which from presynaptic terminals activates glutamate receptors on the postsynaptic neurons. However, the level of extracellular or synaptic cleft glutamate is tightly controlled by glutamate transporters, as excessive glutamate exposure harms neurons. Glutamate excitotoxicity, which refers to neuronal injury/death caused by over-stimulation of the postsynaptic glutamate receptors, is believed to play an important role in the development of amyotrophic lateral sclerosis (ALS) and many other central nervous system (CNS) disorders [1–3]. Glutamate excitotoxicity is thought to result mainly from excess $Ca^{2+}$ entry into neurons triggered by over-stimulation of postsynaptic glutamate receptors [4], and a large rise

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in intracellular $Ca^{2+}$ levels in these cells causes rapid mitochondrial $Ca^{2+}$ overload, resulting in mitochondrial damage and mitochondrial generation of reactive oxygen species (ROS) [5–7]. Mitochondrial damage may lead to necrosis and apoptosis, as a result of potential loss of ATP synthesis and release to cytoplasm of apoptogenic factors [7, 8]. Increased ROS levels may cause oxidative damage within motor neurons and also cause oxidative damage and disruption of glutamate transport in surrounding astrocytes, the latter resulting from the release of ROS from injured motor neurons [9].

Many cytoprotective genes, such as heme oxygenase-1 (*HO-1*) and glutamate cysteine ligase (*GCL*), share a common regulatory mechanism. One or more copies of a *cis*-acting DNA regulatory element, namely the antioxidant response element (ARE), exist in the 5'-flanking region of these genes, and activation of ARE leads to coordinate transcriptional gene up-regulation [10–12]. Nuclear-factor erythroid 2-related factor 2 (Nrf2) has been identified as the key transcriptional factor that activates ARE. Nrf2 is normally bound to its suppressor Keap1 in the cytoplasm but is freed from the latter under certain conditions, and free Nrf2 translocates to the nucleus and binds to ARE as a heterodimer with partners such as small Maf. Many chemical agents have been shown to activate this signaling pathway [11, 13], and Nrf2 and several Nrf2 activators have been shown to inhibit cancer development and development of other inflammation- and/or oxidation-related diseases in animal models [14–17]. In fact, Nrf2 was recently shown to confer neuroprotection against mitochondrial stress induced by the mitochondrial complex II inhibitor 3-nitropropionic acid [18].

In the present study, we show that a chemical agent that activates Nrf2-ARE signaling protects motor neurons against glutamate-induced excitotoxicity. Our experiments were performed using a rat spinal cord explant model of chronic glutamate excitotoxicity and 5,6-dihydrocyclopenta-1,2-dithiole-3-thione (CPDT). CPDT was previously shown to be a potent activator of ARE-mediated gene transcription in cultured murine Hepa1c1c7 hepatoma cells [19] and also to induce several Nrf2 target genes in a number of rat tissues *in vivo* [20].

## Materials and methods

**Chemicals.** CPDT was generously provided by Dr. Rex Munday (Ruakura Agricultural Research Center, Hamilton, New Zealand) [20]. Threo-hydroxyaspartate (THA), rhodamine 123, Fluo 3-AM, and an anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Antibodies recognizing neurofilament (SMI-32), HO-1 and glutamate transporter GLT1 were purchased from Sternberger Monoclonals (Lutherville, MD), Stressgen Biotechnologies (San Diego, CA) and Chemicon (Temecula, CA), respectively. Other antibodies including anti-Nrf2, anti-glutathione S-transferase mu (GSTmu) and anti-NAD(P)H:quinone oxidoreductase 1 (NQO1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Organotypic spinal cord culture.** Glutamate excitotoxicity was assessed using the spinal cord explant model originally developed by Rothstein and coworkers [21]. Briefly, lumbar spinal cords were removed from 7-day old Sprague Dawley rats (Animal Center of Hebei Medical University) under sterile conditions and sectioned transversely at 350-μm intervals using a tissue chopper (Mickle Laboratory Engineering, Surrey, UK). Sections were quickly transferred to sterile Gey's balanced salt solution containing glucose (6.4 mg/ml) and separated from one another at room temperature. The tissue slices were placed on the surface of 30-mm Millipore Millicell-CM membranes (Bedford, MA), five slices/membrane, and each membrane was then placed in a 33-mm culture well containing 1 ml medium (pH 7.2), which consisted of 50 % (v/v) minimal essential medium with 25 mM HEPES, 25 % (v/v) heat-activated horse serum (56°C for 30 min), and 25 % (v/v) Hanks' balanced salt solution supplemented with 25.6 mg/ml glucose and 2 mM glutamine. The cultures were maintained at 37°C in a humidified incubator with 5 % $CO_2$ for up to 4 weeks. Culture media along with test chemicals were changed biweekly, unless specified otherwise.

**Immunoblotting analysis.** Rat spinal cord explants were removed from the Millipore membranes at the end of an experimental treatment and processed to prepare whole tissue extracts or nuclear extracts using NE-PER nuclear and cytoplasmic extraction reagents kit from Pierce (Rockford, IL). The samples were resolved by SDS-PAGE and the resolved proteins were transferred to PVDF membranes. After probing the membranes with specific antibodies, the bands of interest were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). The usual green or red color of a band was converted to black and white colors for data presentation.

**RT-PCR.** This technique was used to measure the expression of two genes, including Nrf2 and the catalytic subunit of GCL (GCLc) along with β-actin gene as a control. The TRIzol method was used to extract total RNA from the rat spinal cord explants removed from the Millipore membranes after an experimental treatment. cDNA was synthesized using random primers and ExScriptTM RTase. PCR primers specific to each gene are listed below: Nrf2, 5'-ttcctctgctgccattagtcagtc-3' and 5'-gctcttccatttccgagtcactg-3', which amplify a 242-bp fragment; GCLc, 5'-atgaaagtggcacaggagcgag-3' and 5'-aaacacgccttccttcccattg-3', which amplify a 186-bp fragment; and β-actin, 5'-gccatgtacgtagccatcca-3' and 5'-gaaccgctcattgccgatag-3', which amplify a 375-bp fragment. PCR was carried out in a 25-μl reaction volume and run for 35 cycles, using an assay kit purchased from Takara Bio (Otsu, Shiga, Japan). The amplified products were electrophoresed in 2 % agarose gel and stained with GoldView™ (Solarbio, Beijing, China).

**Immunohistochemistry.** Immunohistochemical staining was used to visualize motor neurons in spinal cord explants. The explants after removing from the Millipore membranes were washed in Tris-buffered saline for 30 min and then treated with 10 % horse serum for 1 h at room temperature. The explants were subsequently incubated with an anti-neurofilament antibody (SMI-32) overnight at 4°C, which was followed by three-time washing with TBS-T and incubation with a biotinylated secondary antibody for 1 h. The explants were further washed and then incubated with a horseradish peroxidase-conjugated ABC staining solution (Vector Laboratories, Burlingame, CA). The explants were finally mounted on glass slides, and motor neurons in the ventral horns, which were stained dark brown, were counted under the light microscope. Each experimental condition involved 10–15 explants.

**Measurement of tissue glutathione levels and glutamate levels in culture medium.** Tissue glutathione (GSH) levels were determined using a previously published method [22]. Briefly, cultured rat spinal cord explants were weighed (30 explants that received identical treatment were pooled) and homogenized in ice-cold 50 mM Tris buffer (pH 7.4). GSH in tissue homogenates was

derivatized with monochlorobimane (Sigma) in the presence of exogenous GST (Sigma), and the derivatives (GSH-monochlorobimane) were measured using a fluorescence plate reader. Glutamate concentrations in culture media were measured using a biochemical assay kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing City, China), which is based on the conversion of glutamate to α-ketoglutarate catalyzed by glutamic dehydrogenase with concomitant conversion of $NAD^+$ to NADH [23].

**Flow cytometry.** Mitochondrial transmembrane potential ($\Delta\Psi m$) and intracellular $Ca^{2+}$ level were measured using flow cytometry. Thirty spinal cord explants that received identical treatment were pooled and processed to prepare single-cell suspensions. Briefly, the tissues were gently rubbed against a steel mesh (75-μm pore size) with ophthalmological forceps, and cells were collected by rinsing the tissues with PBS. The cell suspensions were filtered through a copper mesh (53-μm pore size) to remove cell aggregates and tissue debris. The filtrate was centrifuged at 800 *g* for 3 min and the cells were resuspended in PBS. The entire procedure was performed at room temperature. For measurement of $\Delta\Psi m$, 1 ml cell suspension ($10^6$ cells) was incubated with 26.3 μM rhodamine 123 at 37°C for 30 min. The cells were then washed twice with PBS and immediately analyzed by flow cytometry to determine fluorescence intensity. Rhodamine 123 is readily and selectively sequestered by normal mitochondria (cells showing strong fluorescence) but is removed from the latter when $\Delta\Psi m$ is lost (cells showing diminished fluorescence). At least 30 000 cells per sample were analyzed. Measurement of intracellular $Ca^{2+}$ levels essentially followed the same protocol as described above, except that the cells were incubated with 8 μM Fluo 3-AM (a cell permeable fluorescent indicator of intracellular $Ca^{2+}$) and the PBS was calcium free [24].

**Statistical analysis.** Results are expressed as means ± SD. Statistical analyses were performed using one-way ANOVA followed by Student's *t*-test, using SAS 6.12 statistical software. Differences were considered significant at $p<0.05$.

## Results

**CPDT stimulates Nrf2 and Nrf2 target genes in rat spinal cord explants.** Although CPDT (see Fig. 1 for its chemical structure) was previously shown to potently induce the expression of Nrf2 target genes in Hepa 1c1c7 cells *in vitro* [19] and in several rat tissues *in vivo* [20], its impact on these genes in nervous tissues was previously unknown. In the present study, lumbar spinal cord explants prepared from 7-day-old rats, after 1 week of culture, were treated with CPDT for 48 h and then harvested for measurement of activation of Nrf2 (nuclear translocation) and four Nrf2 target genes, including *GCLc*, *GSTmu*, *HO-1* and *NQO1* [25–28]. These Nrf2 target genes are well known to play an important role in protecting cells against oxidative stress and other insults. In the case of GSTmu, it is of note that there are multiple GSTmu isoforms in mammalian cells and the antibody used may recognize more than one isoform. As shown in Figure 1, CPDT at 15 and 30 μM caused a significant and dose-dependent increase in nuclear Nrf2 translocation and an increase in expression of all four Nrf2 target genes, which was detected either at the protein level (GSTmu, HO-1 and NQO1) or at the mRNA level (GCLc; an antibody recognizing this protein is unavailable). These results show that not only is the Nrf2-ARE signaling system responsive in spinal cord tissues but also it can be significantly activated by CPDT. Furthermore, given that Nrf2 is known to regulate a large number of cytoprotective genes, the result of the four genes described above may represent only a partial response of a much greater number of genes to CPDT.

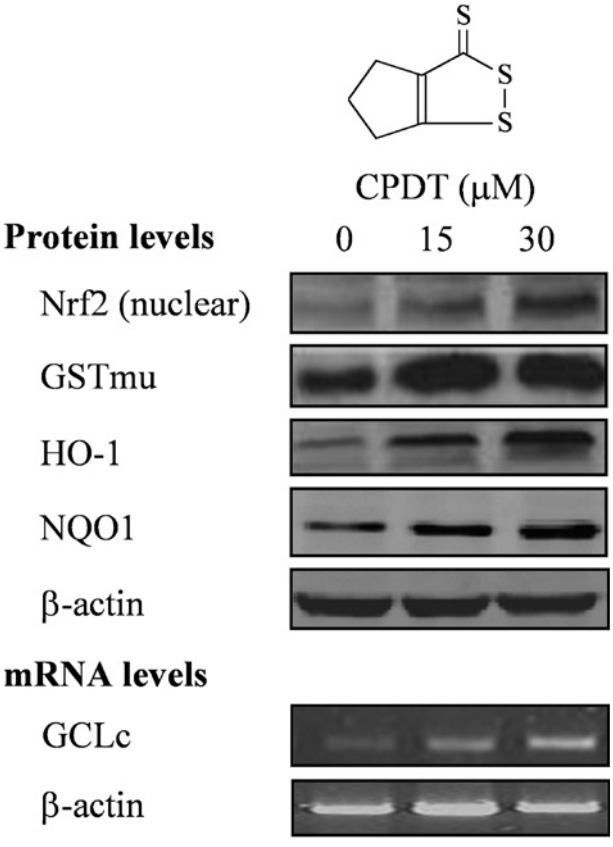


**Figure 1.** Activation of nuclear-factor erythroid 2-related factor 2 (Nrf2) and Nrf2 target genes by 5,6-dihydrocyclopenta-1,2-dithiole-3-thione (CPDT) in rat spinal cord explants. Lumbar spinal cord explants (350-μm sections) were prepared from 7-day-old rats and after 1 week of culture were treated with CPDT (its chemical structure is shown in the figure) at 15 and 30 μM as well as solvent control for 48 h. Cytosolic and nuclear extracts were then prepared from the explants (a pool of 30 explants for each sample) and examined for levels of Nrf2, GSTmu, HO-1 and NQO1 by Western blotting or GCLc mRNA by RT-PCR. β-actin was used as a control. The results are representative of triplicate experiments.

**CPDT protects against glutamate excitotoxicity-induced motor neuron death in spinal cord explants.** We next asked if CPDT could protect motor neurons against glutamate excitotoxicity. Glutamate excitotoxicity was induced by THA treatment. THA is a specific inhibitor of glutamate transporters and raises extracellular glutamate levels by inhibiting glutamate uptake by astrocytes and presynaptic terminals, which

causes over-stimulation of postsynaptic glutamate receptors and motor neuron excitotoxicity [21]. Rat lumbar spinal cord explants, after 1 week in culture, were treated with vehicle (0.1% DMSO), THA (100 μM), or a combination of THA (100 μM) and CPDT (15 and 30 μM) for 3 weeks. Culture media were replaced twice weekly (Monday and Friday) with an equal volume of freshly prepared media containing the test compounds or solvent. In groups involving co-treatment with THA and CPDT, either the two agents were always added together to the medium or the explants were treated with the CPDT without THA for 48 h before being switched to the combination treatment. At the end of the 3-week treatment, the explants were harvested and stained with an anti-neurofilament antibody (SMI-32) for visualization and counting of motor neurons. A typical image of vehicle-treated rat lumbar spinal cord explant is shown in Figure 2a, where motor neurons in the ventral horns are readily visible. Shown in Figure 2b are various treatment schedules. Consistent with a previous report [21], THA treatment markedly reduced the number of motor neurons; the number of motor neurons per explant decreased nearly 66% compared with that in the control (Fig. 2c). THA-induced motor neuron death in this type of culture was previously shown to result from glutamate excitotoxicity [21]. Indeed, THA treatment markedly elevated glutamate levels in culture media (see below). Interestingly, whereas CPDT was only able to offer very limited neuron protection against THA-induced motor neuron death when the two agents were always added together to the culture medium, complete neuron protection was achieved when the explants were pretreated with CPDT for 48 h prior to initiating the combination treatment (Fig. 2c). These results show that the spinal cord culture needs to be primed by CPDT for the motor neurons to become fully resistant to THA-induced excitotoxicity. These results also appear to exclude the possibility that CPDT blocks glutamate toxicity by direct chemical reaction with THA or by interfering with THA interaction with postsynaptic receptors, since these modes of action would permit expression of the effect of CPDT when added to the culture medium simultaneously with THA. Moreover, the observation that CPDT priming was necessary only at the beginning of the 3-week THA treatment suggests that THA-induced motor neuron injury is very rapid and irreversible. It is also of note that CPDT was equally effective at 15 and 30 μM, suggesting that it may offer protection even at lower concentrations.

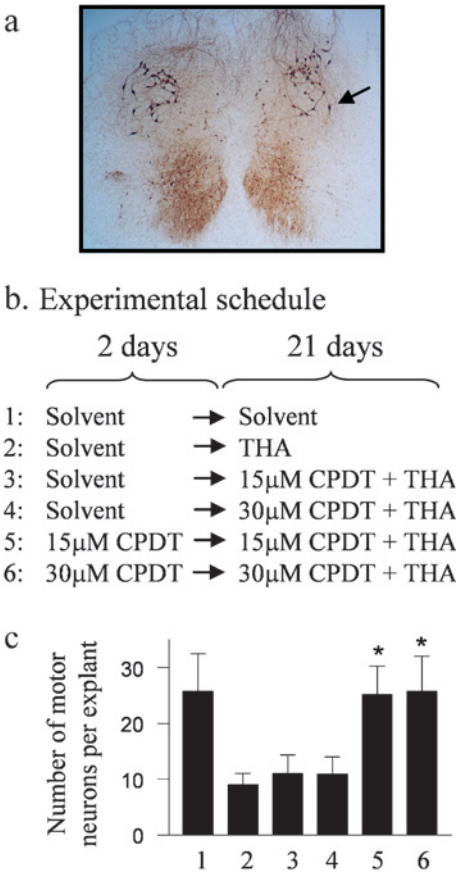


**Figure 2.** Protection of threo-hydroxyaspartate (THA)-induced motor neuron death by CPDT. Spinal cord explants were prepared as described in the legend to Figure 1 and after 1 week of culture were treated with solvent (0.1% DMSO), 100 μM THA or combination of THA (100 μM) plus CPDT (15 or 30 μM) for 3 weeks (see *b* for detail on the treatment schedules). The media as well as the test compounds were changed biweekly (Monday and Friday). At the end of the treatment, the explants were harvested and motor neurons were immunostained using SMI-32 (an anti-neurofilament monoclonal antibody). (*a*) A typical image of SMI-32-stained explants shows motor neurons in dark brown color (the arrow points to one of the neurons). (*c*) Average number of motor neurons per explant (mean ± SD, $n$=10–15) in each treatment group. Each value marked by an asterisk as well as that in the control group (group 1) is significantly different from that in group 2 (THA only, $p<0.05$).

**Increased motor neuron survival against glutamate excitotoxicity in CPDT-treated spinal cord explants is accompanied by activation of Nrf2 signaling and attenuation of oxidative damage**. Having demonstrated the remarkable neuroprotective activity of CPDT, we next sought to confirm the activation of Nrf2 and Nrf2 target genes in the target tissue at the end of the 3-week experimental period. Thus, explants after 1 week of culture were exposed to either solvent, THA or THA plus CPDT. In the THA plus CPDT group, the explants were first treated with CPDT for 48 h before the combination treatment, since this

treatment schedule allowed CPDT to fully protect motor neurons. As shown in Figure 3, exposure of the explants to CPDT in the presence of THA lead to increased nuclear accumulation of Nrf2 and increased expression of all four Nrf2 target genes tested, including *GCLc*, *GSTmu*, *HO-1* and *NQO1*. Interestingly, THA itself also seems to positively modulate some of the genes, *GSTmu* and *HO-1* in particular, perhaps resulting from a cellular response to glutamate-induced oxidative stress, since cellular stress may lead to activation of Nrf2-ARE signaling [29]. However, THA seems to have no effect on *GCLc* expression. A previous study showed that the transcription of *Nrf2* gene itself could be stimulated by a CPDT analog (D3T) in murine cells, presumably due to the presence of a functional ARE in the 5'-flanking region of the gene [30]. We therefore wondered if CPDT could transcriptionally up-regulate Nrf2 in the rat spinal cord tissues. Nrf2 mRNA levels in the explants were measured by RT-PCR. THA alone caused a slight increase in Nrf2 mRNA level, but the combination treatment of THA with CPDT was much more effective, suggesting that CPDT may indeed stimulate Nrf2 transcription in these tissues.

To gain further insight into the neuron-protective activity of CPDT, we measured intracellular $Ca^{2+}$ levels, mitochondrial integrity and tissue GSH contents in spinal cord explants at the end of the 3-week treatment period. As described previously, glutamate excitotoxicity is thought to result mainly from excess $Ca^{2+}$ entry into neurons triggered by over-stimulation of postsynaptic glutamate receptors, and a large rise in intracellular $Ca^{2+}$ level causes rapid mitochondrial $Ca^{2+}$ overload, resulting in mitochondrial damage and mitochondrial generation of ROS. Intracellular $Ca^{2+}$ levels and mitochondrial integrity were measured in cells harvested from the explants, and GSH was measured in tissue homogenates. Although these assays are not specific for motor neurons and non-neuron cells in spinal cord tissues were not excluded in the experiments, our finding that THA treatment significantly increased intracellular $Ca^{2+}$ level and caused loss of mitochondrial transmembrane potential ($\Delta\Psi m$), as shown in Figure 4, is consistent with changes previously observed in motor neurons resulting from excitotoxicity [5, 6]. In the present experiment, the spinal cord tissues were first treated with CPDT at 15 and 30 μM for 48 h before combined treatment of CPDT and THA for 3 weeks. Again, this treatment schedule was selected because it completely prevented THA-induced motor neuron death (see Fig. 2b). We found that CPDT fully prevented both intracellular $Ca^{2+}$ rise and loss of $\Delta\Psi m$ (Fig. 4). Interestingly, CPDT treatment rendered intracellular $Ca^{2+}$ level even lower than in the control. We further

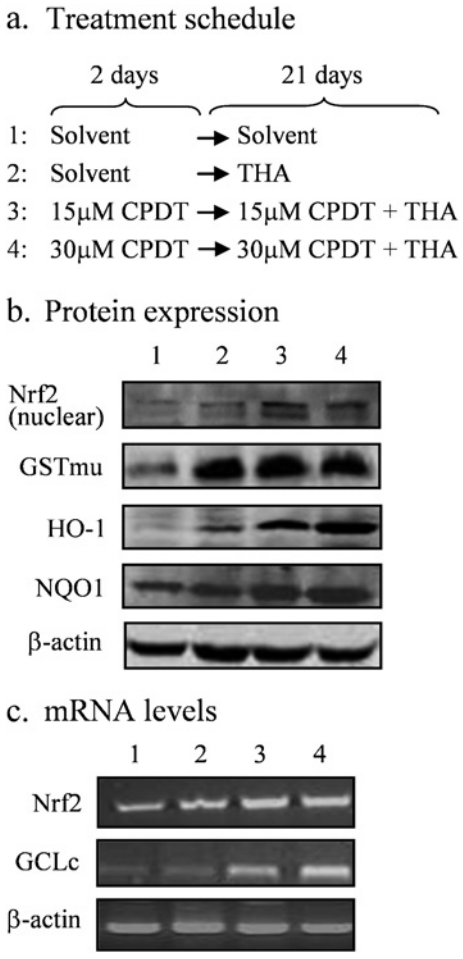


**Figure 3.** Activation of Nrf2 and Nrf2 target genes by CPDT in rat spinal cord explants under chronic challenge with excess extracellular glutamate. Spinal cord explants were prepared as described in Figure 1 and after 1 week of culture were treated with solvent (0.1 % DMSO), 100 μM THA or a combination of THA (100 μM) and CPDT (15 or 30 μM). In the combination treatment, the explants were first incubated with CPDT for 48 h before switching to the combination treatment (see *a* for detail on the treatment schedules). The media as well as the test compounds were changed biweekly (Monday and Friday). Cytosolic and nuclear extracts were then prepared from the explants (a pool of 30 explants for each sample) and were measured for levels of selected proteins (*b*) and mRNAs (*c*) by Western blotting analysis or RT-PCR. β-actin was used as a control. The results are representative of at least three independent experiments.

reasoned that THA treatment might also deplete tissue GSH since excitotoxicity is associated with increased oxidative stress and GSH is a major cellular antioxidant. Indeed, 3-week THA treatment markedly reduced GSH level (Fig. 4). Significantly, CPDT not only prevented THA-induced GSH depletion but actually elevated tissue GSH level three- to fourfold over the control. Elevation of tissue GSH levels by CPDT is consistent with the finding that CPDT up-regulates GCLc (Figs 1 and 3), which is the rate-limiting enzyme in GSH biosynthesis. These results

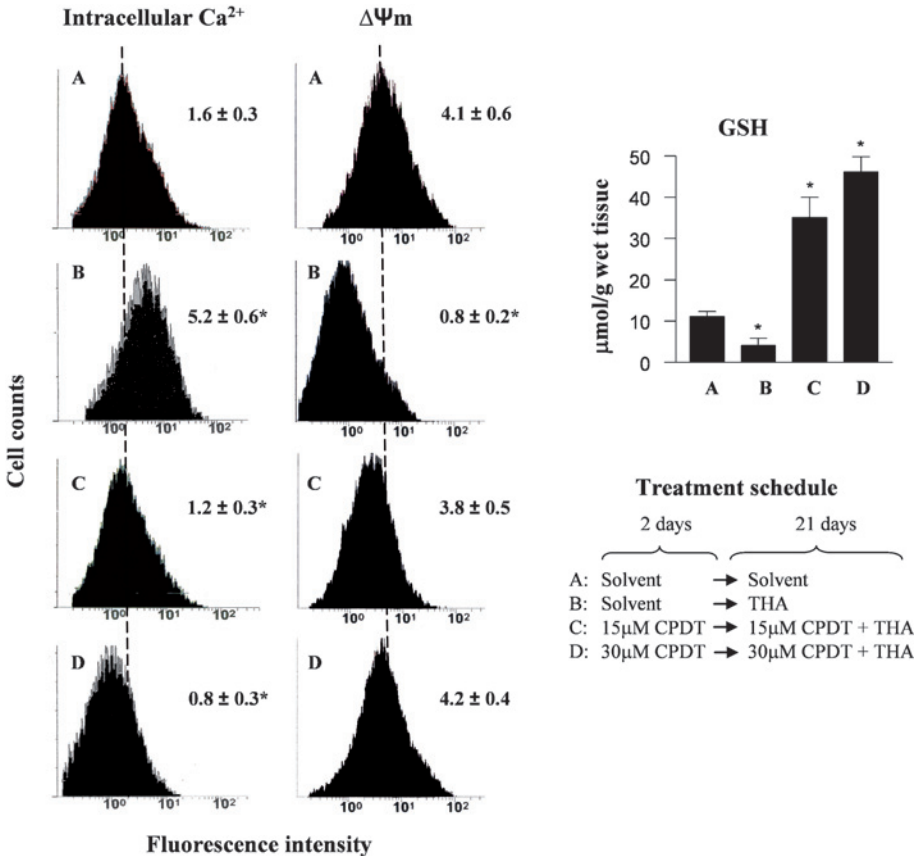


**Figure 4.** Inhibition of glutamate excitotoxicity in spinal cord explants by CPDT. Preparation of rat spinal cord explants and treatment of the explants with the test compounds were the same as described in Figure 3. At the end of the experimental treatment (treatment schedule shown in the figure), the explants were harvested (pool of 30 explants in each sample) and prepared as either a single-cell suspension or tissue homogenates for measurement of intracellular $Ca^{2+}$ levels (using Fluo 3-AM as the probe) and mitochondrial transmembrane potential ($\Delta\Psi m$) (using rhodamine 123 as the probe) by flow cytometry, and for measurement of tissue GSH levels using the monochlorobimane fluorimetric method. Each value is presented as mean ± SD ($n$=3). The values shown for intracellular $Ca^{2+}$ and $\Delta\Psi m$ are average fluorescence intensities of all gated cells. Values marked by an asterisk are significantly different from the corresponding control value ($p$<0.05).

therefore indicate that CPDT elicits a significant and apparently multi-prong defense against excess extracellular glutamate-induced excitotoxicity.

**CPDT does not modulate glutamate transport.** We also examined the impact of CPDT on glutamate transport. At the end of 3-week drug treatment as described above (also see Fig. 5a for treatment schedules), both the explants and the medium were harvested for measurement of tissue GLT1 expression and levels of glutamate in the medium. Several glutamate transporters are known to exist, but GLT1 is expressed mainly in glial cells in nervous tissue and is responsible for up to 90 % of all glutamate transport in adult tissue [31, 32]. While chronic tissue exposure to THA at 100 μM appeared to slightly elevate GLT1 expression, perhaps a tissue response to inhibition of glutamate transporters by THA, combining THA with CPDT at 15 μM caused little change in GLT1 level, indicating that CPDT did not modulate GLT1 expression (Fig. 5b). As expected, chronic treatment of the spinal cord explants with THA (100 μM) elevated medium glutamate level by nearly twofold (Fig. 5c). However, combining THA with CPDT (15 μM) had a negligible impact on THA-induced increase in extracellular glutamate. These results clearly indicate that the protective effect of CPDT against glutamate excitotoxicity did not result from modulation of glutamate transport.

## Discussion

The present study provides several important findings and concepts. First, it shows that transcription factor Nrf2 and many of its target genes, which play an important role in cellular defense against oxidants and other toxic chemicals, can be significantly activated by a single chemical agent in spinal cord tissues. In the case of Nrf2 activation, both gene transcription and nuclear protein accumulation can be stimulated. These results are consistent with literature data showing that Nrf2 activation may involve both increased gene transcription resulting from positive auto-regulation through Nrf2-ARE signaling (presence of ARE in the 5'-flanking region of the gene) and increased nuclear translocation of Nrf2 resulting from dissociation from its cytoplasmic repressor Keap1. Second, we have identified CPDT as a highly promising agent for protection of motor neurons against glutamate-induced excitotoxicity and death. CPDT activates Nrf2 and its target genes in spinal cord tissues

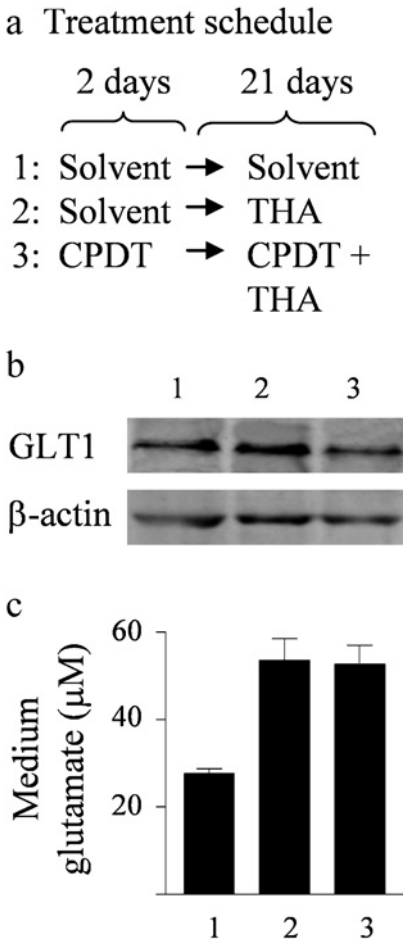


**Figure 5.** Effect of CPDT on glutamate transporter GLT1 and extracellular glutamate levels. Freshly prepared rat spinal cord explants after 1 week of culture were treated with solvent (0.1% DMSO), 100 μM THA or combination of THA (100 μM) and CPDT (15 μM) for 3 weeks (*a*). The media were changed biweekly (Monday and Friday) along with the test compounds. The explants and media were harvested 3 days after last medium change. Thirty explants receiving the same treatment were pooled and homogenized, and the homogenates were measured for GLT1 levels by Western blotting (*b*). β-Actin was used as the loading control, and the results are representative of three independent experiments. Glutamate concentrations in the media were measured using a biochemical assay kit (*c*), based on the glutamic dehydrogenase-catalyzed conversion of glutamate to α-ketoglutarate with concomitant conversion of $NAD^+$ to NADH. Each value is a mean ± SD ($n$=3). Glutamate concentration in the fresh medium was 17 μM, which was approximately twofold higher than that used in the original culture of rat lumbar spinal cord explants [21]. However, the relatively high glutamate concentration was apparently not toxic to the rat spinal cord explants, as the number of motor neurons per explants in the control group in our experiments was comparable to that reported previously.

but does not seem to modulate glutamate transport or glutamate interaction with postsynaptic receptors, thus working through a mechanism different from that of Riluzole, the only clinically available drug for ALS management, which acts by inhibiting glutamate release from glutamatergic nerve terminals and the response of postsynaptic glutamate receptors [33]. Therefore, CPDT emerges as a novel class of anti-excitotoxicity agent that works at steps downstream of postsynaptic glutamate receptor activation. Third, the remarkable neuron-protective activity of CPDT suggests that pharmacological activation of Nrf2-ARE signaling may be an important strategy in the fight against glutamate-induced excitotoxicity. Our observation that the neuron-protective activity of CPDT is greatly enhanced when it is given to cultured spinal cord explants prior to THA exposure is consistent with the working of Nrf2-ARE signaling, as time is needed for signal transduction and gene expression to provide an increased cellular protection. Should the validity of such strategy be confirmed in future *in vivo* studies, it could significantly facilitate the development of anti-excitotoxicity drugs, since many agents including dietary phytochemicals are known to stimulate Nrf2-ARE signaling. Fourth, it is also conceivable that agents like CPDT may synergize with Riluzole in fighting against excitotoxicity since they target different steps of the disease process as mentioned above.

Although the present study strongly suggests that the Nrf2-ARE signaling pathway is important for protection of motor neurons against glutamate-induced excitotoxicity and may be exploited for the development of anti-excitotoxicity drugs, future experiments are necessary to determine which cell types in the spinal cord may respond to CPDT. Measurement of Nrf2 and its target genes in the present study were performed in either whole tissue homogenates or all cells harvested from such tissue. Our results show that CPDT treatment of the spinal cord tissues inhibits glutamate-induced intracellular $Ca^{2+}$ rise and mitochondrial damage as well as elevation of tissue GSH levels. While elevation of GSH level may be attributed to Nrf2-ARE-mediated transcriptional activation of GCL and preservation of mitochondrial integrity may conceivably result from inhibition of intracellular $Ca^{2+}$ accumulation, it is unclear how CPDT inhibits glutamate-induced increases in intracellular $Ca^{2+}$. However, CPDT might induce $Ca^{2+}$-buffering proteins in motor neurons, as a recent study showed that Nrf2 knockout was associated with a significant decrease in mRNA levels of several calcium binding proteins such as calbindin-28 and visinin-like 1 in mouse primary neuronal cultures [34].

While this manuscript was in preparation, three additional known activators of Nrf2-ARE signaling, including 1,2-dithiole-3-thione, *tert*-butylhydroquinone and sulforaphane, were also found to confer protection of spinal cord motor neurons against glutamate-induced excitotoxicity in our preliminary studies (results not shown). The chemical structures of

these compounds, *tert*-butylhydroquinone and sulforaphane in particular, differ greatly from that of CPDT, thus providing further support to the belief that activation of Nrf2-ARE signaling is fundamental to the neuroprotective activity of CPDT.

In summary, CPDT, which significantly activates Nrf2 and Nrf2 target genes in spinal cord tissues, fully protects motor neurons against glutamate-induced excitotoxicity. The prevention of motor neuron death in spinal cord tissue by CPDT is accompanied by inhibition of glutamate-induced intracellular $Ca^{2+}$ rise, mitochondrial damage and GSH depletion. Our data strongly suggest that pharmacological activation of the Nrf2-ARE signaling pathway can sufficiently protect motor neurons against glutamate excitotoxicity.

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