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# Comparison of Neurotoxicity Induced by Some Glutathione Depletors in Mouse Cortical Cell Cultures

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We examined the neurotoxic effects of 3 glutathione (GSH) depletors, buthionine sulfoximine (BSO), diethyl maleate (DEM) and phorone, under the presence of trolox, cycloheximide (CHX), pyrrolidine dithiocarbamate (PDTC) or MK-801 in primary mouse cortical cell cultures. All three depletors induced neuronal death in dose and exposure time dependent manner, and decreased total cellular GSH contents. The patterns of the neuronal death and the GSH decrements were dependent on the individual agents. DEM (200 $\mu$M) induced rapid and irreversible decrement of the GSH. BSO (1 mM) also decreased the GSH irreversibly but the rate of decrement was more progressive than that of DEM. Phorone (1 mM) reduced the GSH content to 40% by 4 hr exposure, that is comparable to the decrement of BSO, but the GSH recovered and reached over the control value by 36 hr exposure. BSO showed a minimal neurotoxicity (0~10%) at the end of 24 hr exposure, but marked neuronal cell death at the end of 48 hr exposure. The BSO (1 mM)-induced neurotoxicity was markedly inhibited by trolox or CHX and partially attenuated by MK-801. DEM induced dose-dependent cytotoxicity at the end of 24 hr exposure. Over the doses of 400 $\mu$M, glial toxicity also appeared. DEM (200 $\mu$M)-induced neurotoxicity was markedly inhibited by trolox or PDTC. Phorone (1 mM) induced moderate neurotoxicity (40%) at the end of 48 hr exposure. Only CHX showed significant inhibitory effect on the phorone-induced neurotoxicity. These results suggest that the GSH depletors induce neuronal injury via different mechanisms and that GSH depletors should be carefully employed in the researches of neuronal oxidative injuries.

Key Words: Glutathione depletor, Neuronal death, Oxidative injury, Buthionine sulfoximine, Diethylmaleate, Phorone

## INTRODUCTION

The nervous system has many biochemical, physiological and anatomical reasons for its special vulnerability to oxidative stress (Evans, 1993). In fact, there is increasing evidence to indicate that oxidative stress is fundamental to the pathogenesis of wide range of inflammatory, ischemic, metabolic, and degenerative neurological diseases (Browne et al, 1999; Cookson & Shaw, 1999; Koedel & Pfister, 1999; Love, 1999; Markesbery & Carney, 1999; Smith et al, 1999; Taylor et al, 1999). Glutathione (GSH) is major endogenous cellular antioxidant. Depletion of GSH has been shown to induce oxidative stress or to enhance the cytotoxicity of various oxidative insults (Reed & Fariss, 1984; Miccadei et al, 1988). GSH depletors have been used to induce oxidative stress in vivo or in vitro study (Redegeld et al, 1992). Buthionine sulfoximine (BSO) depletes GSH by inhibiting $\gamma$-glutamylcysteine synthetase, a key enzyme in GSH synthesis (Meister, 1995). Diethyl maleate (DEM) and phorone deplete GSH by conjugating with GSH (Oguro et al, 1987).

To gain information on the mechanisms underlying the oxidative neuronal injury induced by GSH depletion, we examined the neurotoxic effects of

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BSO, DEM or phorone under the presence of trolox (TLX; water and lipid soluble vitamin E analog antioxidant), cycloheximide (CHX; protein synthesis inhibitor), pyrrolidine dithiocarbamate (PDTC; antioxidant) or MK-801 (NMDA receptor antagonist) in primary mouse cortical cell cultures.

## METHODS

### *Cell culture*

Mixed cortical cell cultures containing both neurons and glia were prepared by plating fetal mice cortical cells onto an established monolayer of glia as previously described (Rose et al, 1993). To establish glial bed, the glial cultures were prepared from neocortices of 1- to 3-day-old pups. Cortical cells were plated on 24-well multiwell plate (Primaria, Falcon) at a density of 0.5 hemispheres per plate, and grown for 2~4 weeks at 37°C and 5% $CO_2$ in medium consisting of Eagles medium (MEM; Earles salts, glutamine free, GIBCO) supplemented with 21 mM glucose, 2 mM glutamine, 26.5 mM bicarbonate, 10% fetal bovine serum (FBS), 10% horse serum (HS) and 10 ng/ml epidermal growth factor (EGF). To establish neuronal element, cortical cells from fetal mice at 14~17 days gestation were plated onto the established glial bed at a density of about 2.75 hemispheres per 24-well plate in MEM supplemented with 21 mM glucose, 2 mM glutamine, 26.5 mM bicarbonate, 5% FBS and 5% HS. After 3~5 days, non-neuronal cell division was halted by 2-day exposure to 10 $\mu$M cytosine arabinoside (Ara-C); subsequently medium was exchanged twice a week with one lacking fetal serum. Cultures were used for experiments at 13~15 days in vitro (DIV).

### *Drug exposure*

After a triple exchange wash of the cell cultures with MEM (supplemented with 21 mM glucose), all exposures to drugs were performed at room temperature in same serum-free culture medium. The stock solutions of drugs were usually made at ×100 concentration of working solution in distilled water or dimethylsulfoximine (DEM and phorone), and then the drugs were diluted in MEM immediately before experiments. The cultures were then returned to the humidified 37°C, 5% $CO_2$ incubator.

### *Assessment of cell death*

Neuronal cell death was morphologically estimated by examination of cultures under phase-contrast microscope and quantified by measuring lactate dehydrogenase (LDH) released from damaged cells to bathing medium after 24 or 48 hr of the drug exposure (Koh & Choi, 1987). To yield the LDH signal specific to experimental injury, the mean value of background LDH, determined in sister cultures subjected to sham wash within each experiment, was subtracted from values obtained in experimental conditions. To normalize the LDH values, observed LDH values were scaled to those in sister cultures exposed to 500 $\mu$M NMDA for 24- or 48-hr (=100), which result in nearly complete neuronal death without injurying glia (Choi et al, 1987).

### *Glutathione measurement*

Total glutathione levels (GSSG+GSH) were measured using a modification of the method of Tietze (1969) as described in Murphy et al (1989). Mixed cortical cells were plated on 6-well multiwell vessels and grown as described above. The cells were washed once with HBSS, collected in 1 ml of 3% perchloric acid and removed to an eppendorf tube. The tubes were centrifuged (10 min at 7,400×g at 4°C) to remove precipitated protein, and the supernatants were transferred to fresh test tubes containing 0.5 M KOH and 9 mM $Na_2B_4O_7$ (0.8~0.9 ml, adjusted to pH 7.5). The tubes were placed on ice for 10 min to allow precipitation and settling of $KClO_4$. An aliquot (0.25 ml) of the neutralized supernatant was added to 0.25 ml of 0.1 M potassium phosphate and 5 mM EGTA (pH 7.5) in a 1 ml plastic cuvette, then 0.4 ml of a solution containing 0.1 mg/ml DTNB, 0.32 mg/ml NADPH, and 0.1 M phosphate buffer (pH 7.5) was added. GSH+GSSG was determined spectrophotometrically by measuring the change in $A_{412}$ upon addition of 100 $\mu$l of GSSG reductase (8 U/ml). Experimental rates were compared with those determined using GSH standards treated in the same manner. Precipitated protein pellets were air-dried and resuspended in 0.1 M NaOH for protein determination using the bicinchoninic acid reagent method (Smith et al, 1985).

*Materials*

PDTC, CHX, DEM, BSO, trypsin, Ara-C, EGF and trolox were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). NMDA and MK-801 were obtained from Research Biochemical International (Natick, MA, USA). Phorone was from Fluka (Buchs, Swiss). FBS and HS were purchased from Hyclone (Logan, UT, USA). Other cell culture products were from Gibco BRL Products (Gaithersburg, MD, USA).

*Statistical analysis*

The data were expressed as the mean±standard error of the mean. To compare multiple means of % LDH releases, Analysis of variance (ANOVA) followed by Student-Neuman-Keuls multiple comparison test was used. Probability value of less than 0.05 was considered to be statistically significant.

## RESULTS

*Neuronal death induced by treatment with BSO, DEM or phorone*

We examined the effects of the concentration and the exposure-duration of the three drugs on neuronal cell death to compare the neurotoxic effects of the drugs. BSO in the concentration ranges of 30 $\mu$M to 1 mM produced minimal neuronal death (~10%) with 24 hr exposure, while the BSO-induced neuronal deaths were markedly increased with 48 hr exposure (50±1.5% at 30 $\mu$M, n=12; 90±6.5% at 1 mM, n=16) without any toxicity to glial cells. DEM (100 $\mu$M ~1 mM) produced cell death in a concentration-dependent manner at the end of 24 hr exposure. DEM over the concentration of 400 $\mu$M induced not only neuronal death but also glial death. Phorone produced minimal neuronal death at the concentration of 1 mM or 3 mM with 24 hr exposure (11±2.6% and 12± 2.4% respectively, n=12). The increment of exposure duration to 48 hr increased the phorone-induced neuronal death (40±1.9% and 32±4.5% respectively, n=16)(Fig. 1).

*Changes of glutathione levels by treatment with BSO, DEM or phorone*

As all three drugs induced neuronal cell death, we examined the effects of the drugs on glutathione levels. Cellular levels of glutathione (oxidized and reduced, GSH+GSSG) decreased in a time-dependent manner during the exposure of each drug (Fig. 2). After 30 min of 200 $\mu$M DEM, 1 mM BSO or 1 mM phorone exposure, GSH+GSSG levels were reduced by 70%, 50% or 40%, respectively. After 4 hr of 200 $\mu$M DEM, 1 mM BSO or 1 mM phorone

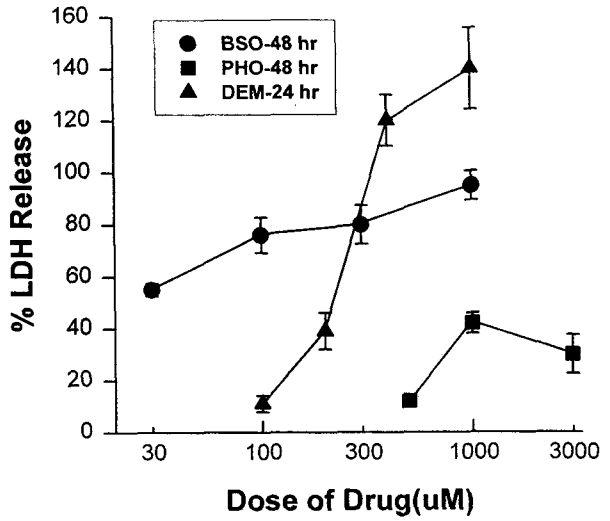


**Fig. 1.** Concentration-dependent neurotoxic effect of BSO (48 hr exposure, ●), phorone (48 hr exposure, ■) and DEM (24 hr exposure, ▲) in murine cortical cell culture. Each point and vertical bar represent the mean with SEM from 8~16 wells, respectively.

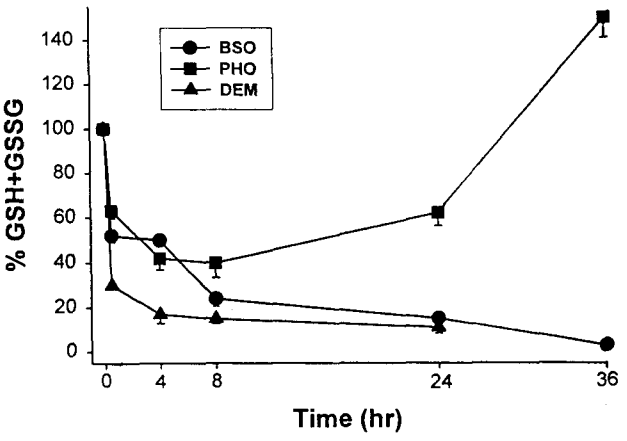


**Fig. 2.** The effect of BSO (1 mM, ●), phorone (1 mM, ■) and DEM (200 $\mu$M, ▲) on % total cellular GSH plus GSSG level in murine cortical cell culture. The control (t=0) GSH plus GSSG level was 12±2.1 nmol/mg protein (n=10). Each point represents mean±SEM of duplicate incubations with 2~3 batches of cultures.

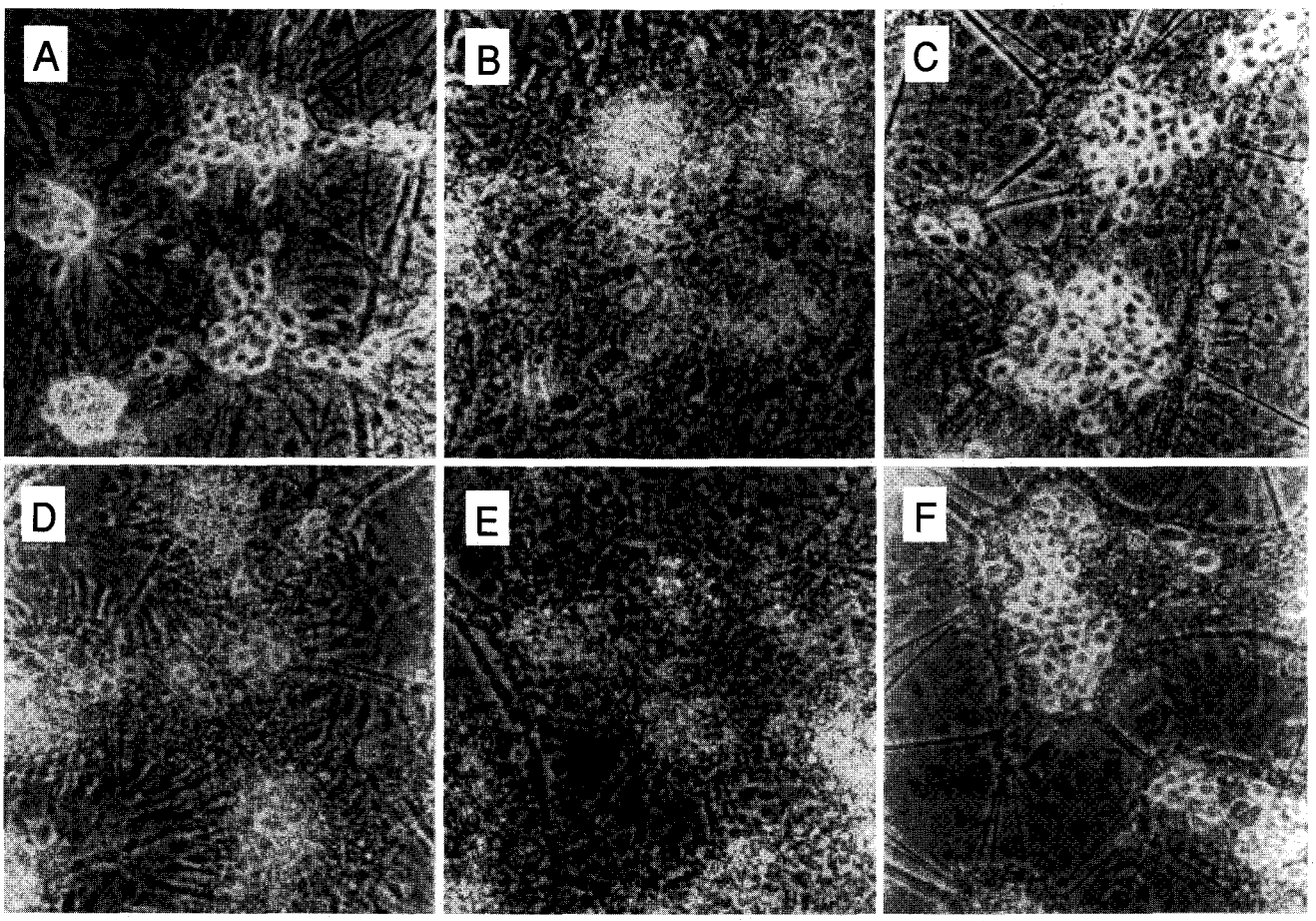


**Fig. 3.** Phase contrast photomicrographs of murine cortical neuronal death induced by GSH depletors (×200 fields). A: 48 hr after sham wash, B: 48 hr exposure to 1 mM BSO, C: 48 hr exposure to 1 mM BSO plus CHX, D: 24 hr exposure to 200 μM DEM, E: 24 hr exposure to 200 μM DEM plus CHX, F: 24 hr exposure to 200 μM DEM plus trolox. BSO-induced neuronal death was prevented by CHX (1 μg/ml). DEM-induced neuronal death was prevented by trolox (100 μM) but not by CHX.

exposure, GSH+GSSG levels decreased to 15%, 50% or 40%, respectively. At the end of 24 hr exposures, GSH+GSSG levels decreased to 10~15% in DEM- or BSO- treated group. However, GSH+GSSG levels in phorone-treated group were recovered to 70% of control levels. Interestingly, GSH+GSSG levels in 36 hr exposure of phorone increased to 150% of control value, while the levels in BSO-treated group decreased to less than 5% (Fig. 2).

Effects of trolox, CHX, PDTC or MK-801 on the BSO-, DEM- or phorone-induced neuronal death BSO (1 mM) induced a minimal neuronal cell death (11±1.8%, n=16) at the end of 24 hours exposure, and a marked neuronal cell death (90±6.5%, n=16) at the end of 48 hr exposure. The BSO-induced death with 48 hr exposure was almost abolished by co-treatment with 100 μM trolox or 1 μg/ml CHX and also partially attenuated by co-treatment with 10 μM MK-801. However, PDTC (50 μM) did not affect the BSO-induced cell death (Fig. 3, 4). DEM (200 μM) induced moderate neuronal death (39±7.2%, n=16) at the end of 24 hr exposure and a severe neuronal death (97±4.9%, n=16) at the end of 48 hr exposure. The DEM-induced neuronal death was markedly inhibited by 100 μM trolox or 50 μM PDTC. Neither CHX nor MK-801 affected the DEM- induced death (Fig. 5). Phorone (1 mM) induced minimal neuronal death (11±2.6%, n=12) at the end of 24 hr exposure and moderate neuronal death (40±1.9%, n=16) at the end of 48 hr exposure. Only the co-treatment with 1 μg/ml CHX significantly inhibited the phorone-induced neuronal death (Fig. 6).

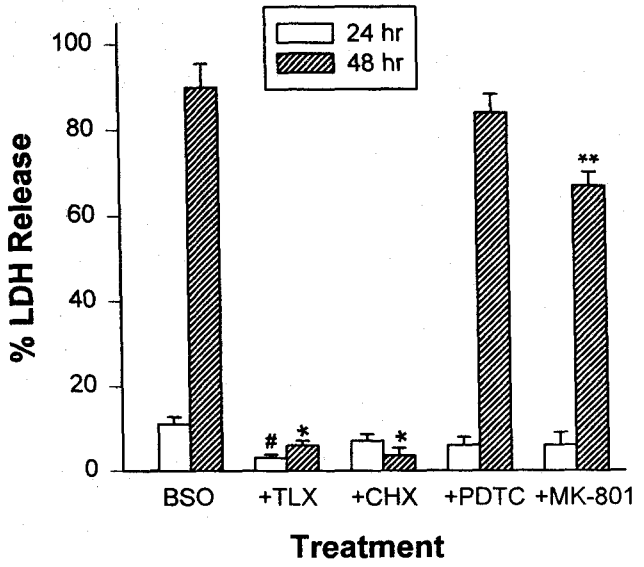


**Fig. 4.** Effect of trolox (TLX, 100 $\mu$M), cycloheximide (CHX, 1 $\mu$g/ml), pyrrolidine dithiocarbamate (PDTC, 50 $\mu$M) or MK-801 (10 $\mu$M) on 1 mM BSO-induced neuronal death at the end of 24 hr (□) and 48 hr (▨) exposure. Mean±SEM from 12~16 wells. #: Significantly different from 24 hr-control ($p<0.01$). *: Significantly different from 48 hr-control (*; $p<0.01$, **; $p<0.05$).

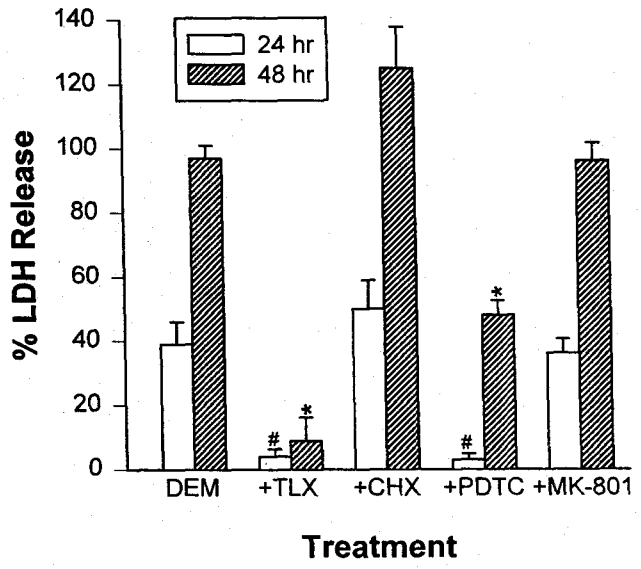


**Fig. 5.** Effect of trolox (TLX, 100 $\mu$M), cycloheximide (CHX, 1 $\mu$g/ml), pyrrolidine dithiocarbamate (PDTC, 50 $\mu$M) or MK-801 (10 $\mu$M) on 200 $\mu$M DEM-induced neuronal death at the end of 24 hr (□) and 48 hr (▨) exposure. Mean±SEM from 12~16 wells. #: Significantly different from 24 hr-control ($p<0.01$). *: Significantly different from 48 hr-control ($p<0.01$).

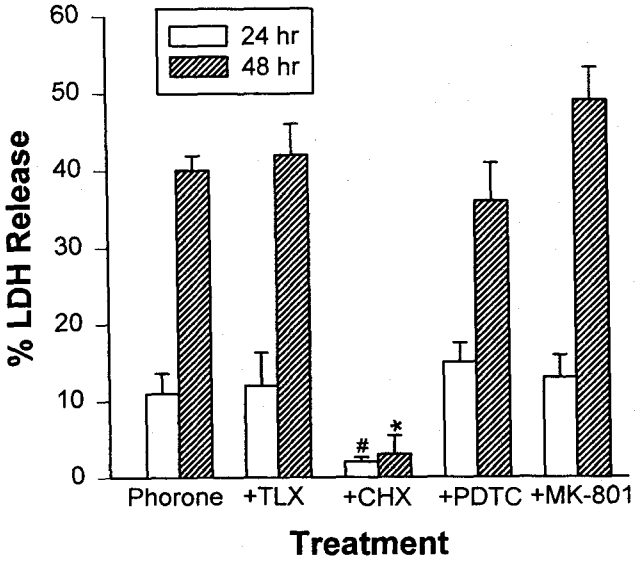


**Fig. 6.** Effect of trolox (TLX, 100 $\mu$M), cycloheximide (CHX, 1 $\mu$g/ml), pyrrolidine dithiocarbamate (PDTC, 50 $\mu$M) or MK-801 (10 $\mu$M) on 1 mM phorone-induced neuronal death at the end of 24 hr (□) and 48 hr (▨) exposure. Mean±SEM from 12~16 wells. #: Significantly different from 24 hr-control ($p<0.01$). *: Significantly different from 48 hr-control ($p<0.01$).

## DISCUSSION

All of 3 GSH depletors used in this study produced neuronal cell deaths, while the characteristics of the neuronal cell deaths were dependent on the individual agents. They decreased the cellular GSH, but the patterns of GSH decrement also depended on the individual agents. All 3 drugs used in this study have been used for potent depletors of cellular GSH (Meister, 1991; Redegeld et al, 1992). DEM and phorone conjugate cellular GSH via reaction with thiol group of GSH (Oguro et al, 1987; Meister, 1991), while BSO inhibits $\gamma$-glutamylcysteine synthetase, a key enzyme for synthesis of GSH (Meister, 1991).

In terms of the extents of neuronal cell death or the length of time to evolve neuronal cell death, DEM is most potent or rapid, while phorone is the least (Fig. 1). DEM decreased the cellular GSH content rapidly and irreversibly, while the pattern of GSH decrement by BSO was progressive and irreversible. However the decrement by phorone was slow and reversible, even the GSH content reached over the control value at 36 hr after phorone treatment (Fig. 2). This finding suggests that there is some correlation

between the extents and/or the evolving time of neuronal death and the patterns of GSH depletion.

In present study, DEM and phorone showed different abilities to deplete the GSH. However many studies have demonstrated that both drugs completely depleted cellular GSH contents in hapatocyte cultures and also decreased GSH in liver and brain in vivo study (Costa & Murphy, 1986; Meister, 1991; Redegeld et al, 1992). It has been also reported that both drugs have various additional effects such as inhibition of protein synthesis, inhibition or activation of some enzymes (Meister, 1991). Taken together, the discrepancy between effects of both drugs in this study might be derived from their effects on the additional actions besides depleting GSH.

The neuronal cell deaths induced in present study showed distinct responses to some pharmacological interventions. Trolox is a vitamin E analog antioxidant and has been used to inhibit lipid peroxidation induced by various oxidative injuries (Massey & Burton, 1990; Silver et al, 1992; Chow et al, 1994). Present study demonstrated that treatment with trolox almost abolished the neuronal death induced by either BSO or DEM, but failed to attenuate phorone-induced neuronal death. These data indicated that the membrane lipid peroxidation would be associated with the neuronal death induced by either BSO or DEM, whereas the phorone would not induce the lipid peroxidation in the process of its neurotoxicity. In accordance with our data, some results have been reported that trolox prevented BSO-induced neuronal cell death (Gwag et al, 1995; Kim et al, 1997). CHX, a protein synthesis inhibitor, markedly inhibited the neuronal death induced by either BSO or phorone but did not affect the DEM-induced neurotoxicity in this study. CHX has been reported as an anti-apoptotic agent in various neuronal apoptosis-related in vitro and in vivo experiments (Martin et al, 1988; Oppenheim, 1991; Koh et al, 1995; Choi, 1996). Present data implied that apoptosis might be involved in the neuronal death induced by either BSO or phorone. PDTC is a thiol antioxidant, and have been used to inhibit the activation of NF-$\kappa$B (Schreck et al, 1992; Orrenius et al, 1996). PDTC inhibited the DEM-induced neurotoxicity but had no effect on BSO- or phorone-induced neurotoxicity in this study. Interestingly MK-801, an NMDA receptor antagonist, partially but significantly inhibited the neuronal death induced by BSO. It is difficult to explain this finding. BSO inhibits cellular GSH synthesis by inhibiting $\gamma$-glutamylcysteine synthetase which mediates the synthesis of $\gamma$-glutamylcysteine from glutamate and cysteine (Meister, 1995). Therefore, it is supposed that the inhibition of the enzyme by BSO might increase the intracellular glutamate which led to the increase of glutamate release. And increased glutamate release could induce excitotoxicity.

Taken together, these results suggest that the GSH depletors induce neuronal death via different mechanisms, depending on the characteristics of the individual agents, which may differ in the rate and the extent of cellular GSH depletion, and/or the additional actions besides depleting GSH.

Conclusively, present study suggests that GSH depletors should be carefully employed in the researches of neuronal oxidative injuries.

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