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Scientific paper

EFFECT OF GLUTATHIONE DEPLETION ON NRF2/ARE ACTIVATION BY DELTAMETHRIN IN PC12 CELLS

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Transcription factor NF-E2-related factor 2 (Nrf2) is important for cell protection against chemical-induced oxidative stress. Previously, we have reported that in PC12 cells, Nrf2 can be triggered by deltamethrin (DM), a commonly used pyrethroid insecticide. Molecular mechanisms behind Nrf2 activation by DM are still unclear. Here we studied the effects of cell glutathione (GSH) depletion on Nrf2 activation by DM. We found that DM enhanced Nrf2 expression at the mRNA and protein levels and increased nuclear Nrf2 levels. Activation of Nrf2 was associated with activation of its downstream targets, such as heme oxygenase-1 (HO-1) and glutamate cysteine ligase catalytic subunit (GCLC). In contrast, DL-buthionine-[S,R]-sulfoximine (BSO), a known GSH-depleting agent, did not increase Nrf2 protein expression or cause its nuclear accumulation. However, pre-treatment with BSO triggered mRNA expression of HO-1 and GCLC. Furthermore, BSO pre-treatment suppressed DM-induced Nrf2 upregulation and activation and lowered mRNA expression of HO-1 and GCLC upon DM treatment. These data demonstrate that GSH depletion is not necessary for the activation of Nrf2/ARE by DM in PC12 cells, and that GCLC and HO-1 expression can increase through other signalling pathways.

KEY WORDS: *BSO, DL-buthionine-[S,R]-sulfoximine, glutamate cysteine ligase catalytic subunit, GCLC, heme oxygenase, HO-1, pyrethroid insecticide*

Pyrethroids are widely used agricultural pesticides because of their effectiveness as nerve poisons and their low environmental impact (1). The most effective of the group is deltamethrin [(S)a-cyano-3-phenoxybenzyl-(1R)-cis-3-(2.2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, DM], which causes neurological disorders (choreoathetosis with salivation, also known as CS syndrome) (1, 2).

Transcription factor NF-E2-related factor 2 (Nrf2), a part of the Cap'n'Collar family of basic leucine zipper (bZIP) proteins, plays a key role in the activation of multiple genes encoding antioxidants and phase II drug-metabolising enzymes through antioxidant response elements (AREs) (3, 4). AREs are located in the 5'-flanking region of various genes engaged in the cellular protection against oxidative stress, such as the glutamate cysteine ligase modifier subunit (GCLM) (5-7), heme oxygenase 1 (HO-1) (8-11), and the glutamate cysteine ligase catalytic subunit (GCLC).

Glutamate cysteine ligase (GCL) is the first enzyme in the pathway of glutathione (GSH) synthesis. GCL is a heterodimeric enzyme containing a catalytic subunit (GCLC), which is capable of catalysis alone, and a modifier subunit (GCLM), which increases the catalytic efficiency of GCLC and reduces feedback inhibition by GSH. Synchronised induction of these enzymes should protect the cell against the adverse effects of reactive oxygen species (ROS).

We have previously reported that DM enhances the release of free radicals and nuclear translocation of the stress response transcription factor Nrf2 in rat brain and PC12 cells *in vitro* (12, 13). Several mechanisms, including oxidation of redox-sensitive cysteines in Keap1 (14), ubiquitination (15, 16), phosphorylation (17, 18), and nuclear shuttling (19, 20), have been proposed to regulate Nrf2 activity. In spite of their differences, all of these mechanisms seem to involve Keap1 protein-mediated recognition and transduction of signals for Nrf2 activation. A study by Goldring et al. (21) has shown that diethylmaleate (DEM), a GSH-depleting agent, can release Nrf2 *in vivo*, permitting its nuclear translocation and coordinated upregulation of ARE-driven defence genes. These findings imply that a relationship between nuclear translocation of Nrf2 and GSH level depends on whether exposure involves a substance with inherent protein reactivity.

The rationale for testing whether GSH depletion affects Nrf2 induction by DM is to see if DM triggers Nrf2 activation through GSH depletion or the process involves other chemical stressors. With this in mind, we studied the effects of GSH depletion on Nrf2 activated by DM in differentiated PC12 cells, a rat adrenal pheochromocytoma cell line with the phenotypic properties of sympathetic neurons, which are more susceptible to oxidative stress than undifferentiated cells (22).

MATERIALS AND METHODS

Reagents and instruments

Deltamethrin (DM, 98.5 %) was purchased from Roussel-Uclaf Corp. (Romainville Cedex, France). The TRIzol® reagent was purchased from Life Technologies (Foster, CA, USA). rTaq (recombinant Taq) DNA polymerases, ReverTra Ace (MMLV Reverse Transcriptase RNaseH-), and an RNase inhibitor were obtained from Toyobo Co., Ltd. (Osaka, Japan). All primers were synthesized by BIOASIA biologic technology Co., Ltd. (Shanghai, China). Random Primers were purchased from Promega (Shanghai, China) and dNTPs were procured from Shengong Co., Ltd. (Shanghai, China).

Anti-Nrf2 antibodies (C-20: *sc-722*) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were a kind gift from Professor Ma Qiang (NIOSH, USA). Anti-HO-1 antibodies were obtained from Santa Cruz Biotechnology Inc.. Dulbecco's Modified Eagle Medium (DMEM) was procured from HyClone (Logan, UT, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies were obtained from Proteintech Group Inc. (Chicago, IL, USA). DL-buthionine-[S,R]-sulfoximine (BSO), dimethyl sulfoxide (DMSO), RNase A, and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NC membranes were purchased from Millipore (Bedford, MA, USA). The enhanced chemiluminescence reagent was obtained from HyClone-Pierce company. HEPES, EDTA, MgCl2, KCl, DTT, sodium orthovanadate, NaF, PMSF, benzamidine, leupeptin, aprotinin, and NP-40 were obtained from Shanghai Chemical Reagent Company of the Chinese Medical Group (Shanghai, China).

Culture and treatment of PC12 cells

Differentiated PC12 cells were obtained from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). They were cultivated in DMEM supplemented with 10 % heat-inactivated foetal bovine serum at 37 °C in a humidified atmosphere consisting of 95 % air and 5 % CO_2. The medium was replaced every other day, and cells plated at a density adjusted to the experimental needs. After an incubation period of 24 h, cells were transferred to a serum-free culture medium and treated.

Based on previous results (23, 24), in this experiment we used BSO in the concentration of 500 μmol L^{-1} reconstituted with DMSO in the final concentration not over 0.1 % so as not to influence the evaluated parameters (data not shown).

PC12 cells were incubated with 500 μmol L^{-1} BSO for 16 h immediately after they were transferred to a serum-free culture medium. Negative control cultures were not exposed to BSO, and exposure to BSO did not affect cell viability (data not shown).

Following BSO exposure the cells were treated with 10 μmol L^{-1} DM for 1 h.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Reverse transcription PCR was done as reported in our earlier paper (25). Total RNA from PC12 cells was extracted using the TRIzol® Reagent, in line with the manufacturer's instructions. The reaction volumes were 20 μL. Incubations at 42 °C lasted 45 min, at 70 °C 15 min, and finally at 37 °C 20 min. PCR was carried out sequentially. Primer sequences for cDNA amplification are listed in Table 1.

Pre-denaturation was set to 10 min at 94 °C; denaturation (35 cycles) to 60 s at 94 °C, annealing to 30 s at 55 °C, extension to 60 s at 72 °C, while the final single extension at 72 °C lasted 5 min. For internal control we used a 188-bp DNA fragment of the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and amplified it in the same PCR system as the tested gene.

The obtained PCR products were then resolved by agarose gel electrophoresis. After staining with ethidium bromide, DNA in gel was visualised under ultraviolet light. The experimental values were normalised to the corresponding GAPDH values.

Immunocytochemical detection of HO-1

Protein expression of HO-1 was detected by immunocytochemistry. PC12 cells were cultivated on poly-L-lysine-coated coverslips as described by Deptala et al. (26) with slight modifications. After the treatment, the cells were rinsed with PBS twice and fixed in 95 % ethanol at -20 °C for 15 min. After a second wash in PBS, the fixed cells were permeabilised in 0.2 % Triton X-100 in PBS for 15 min, rinsed with pure PBS and blocked with normal goat serum at room temperature for 30 min. The cells were first treated with an anti-HO-1 antibody (in the dilution of 1:50) at 4 °C for 18 h and then incubated with FITC-labelled goat anti-rabbit IgG (H+L) at 37 °C for 30 min.

After the cells were rinsed with PBS, microscope slides were prepared. We used a 50 % glycerin buffer solution as a mounting medium. Slides were examined on a laser scanning confocal microscope equipped with a UPLAPO 100x oil immersion objective lens (Fluoview FV500, Olympus, Tokyo, Japan). The imaging settings were optimised to ensure signal detection and consistency of images. Imaging data were processed using Fluoview version 4.3.53 and analysed using image-Pro Plus version 5 (Media Cyberbetics Inc., Bethesda, MD, USA) and Adobe Photoshop 8.0 (Adobe Inc., San Jose, CA, USA). The images were analysed by an investigator blind to this treatment protocol. HO-1 expression was checked by measuring whole-cell FITC signal intensity.

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were isolated according to Schreiber et al. (27) with some modifications. After rinsing with cold PBS, cells were resuspended in cold lysis buffer A (20 mmol L^{-1} HEPES pH 8.0, 1 mmol L^{-1} EDTA, 1.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl, 1 mmol L^{-1} DTT, 1 mmol L^{-1} sodium orthovanadate, 1 mmol L^{-1} NaF, 1 mmol L^{-1} PMSF, 0.5 mg mL^{-1} benzamidine, 0.1 mg mL^{-1} leupeptin, and 1.2 mg mL^{-1} aprotinin).

They were incubated on ice for 15 min, after which 7.5 μL of 10 % NP-40 detergent was added. Afterwards the cells were intensively stirred in a vortex mixer for 10 s. The homogenate was centrifuged at 16,000 *g* for 50 s, and the supernatant was used as the cytosolic extract. The nuclear pellet was resuspended in cold extraction buffer B (20 mmol L^{-1} HEPES pH 8.0,

Table 1 *Primer sequence used in RT-PCR analysis*

Gene name	Primer sequence for PCR	Size of expected production / bp
Nrf2	5'-CCATTTACGGAGACCCAC-3'	448
	3'-CTTATTTCAACGGCGAGT-5'	
HO-1	5'-ACAGAAGAGGCTAAGACCG-3'	441
	3'-TTACCTTCCTCTACGGAC-5'	
γ- GCLC	5'-CATCTACCACGCAGTCAA-3'	351
	3'-GCTATTGAAGTAAAGGGTC-5'	
γ- GCLM	5'-ACATTGAAGCCCAGGAGT-3'	266
	3'-ACACCACCAAACCGTTAC-5'	
GAPDH	5'-AAATGGGTGATGCTGGTG-3'	188
	3'-TGAGCGAGTTCTAACAGTCG-5'	

bp – base pairs

1 mmol L^{-1} EDTA, 1.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl, 1 mmol L^{-1} DTT, 1 mmol L^{-1} sodium orthovanadate, 1 mmol L^{-1} NaF, 1 mmol L^{-1} PMSF, 0.5 mg mL^{-1} benzamidine, 0.1 mg mL^{-1} leupeptin, 1.2 mg mL^{-1} aprotinin, and 20 % glycerol). All protein fractions were stored at -70 °C until used, and protein concentrations were determined with the Bradford method, using bovine serum albumin as the standard.

Western blotting analysis

Protein samples were resolved on 10 % SDS-polyacrylamide gels and were transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween-20, and 5 % nonfat dry milk for 1 h. After that they were incubated overnight with primary antibodies diluted in the identical buffer. Nrf2 was used in the dilution of 1:500; and GAPDH in the dilution of 1:8,000. After the washing with 0.1 % Tween-20 in TBS, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h and then rinsed and developed using the ECL chemiluminescent detection system. Blots were then visualised with X-ray films. The results were analysed using the Bandscan 4.3 software. Densitometry was normalised against the signal obtained from co-incubation with anti-GAPDH.

Measurement of the reduced GSH content

The reduced *GSH* content was measured using reversed-phase high performance liquid chromatography with o-phthalaldehyde precolumn derivatisation as described elsewhere (13, 28).

Statistical analysis

Data bars correspond to mean values ± SD of minimum three independent experiments. The results were evaluated using SPSS for Windows (version 18.0, Chicago, IL, USA). Differences between the groups were assessed using one-way analysis of variance (ANOVA). If the *F* values were significant, LSD *post hoc* tests were employed for multiple comparisons between the groups. The value of $P \leq 0.05$ was deemed statistically significant.

RESULTS

Effects of DM on the Nrf2/ARE pathway

Using cytosolic and nuclear fractions of PC12 cells to establish the relative distribution of Nrf2 we found that Nrf2 migrated with a molecular weight of around 100 kDa. Such an atypical migration was previously observed in human neuroblastoma cells (29).

As proposed by Shukla et al. (30), samples from the nuclear fraction resolved on parallel gels were stained with Coomassie brilliant blue for protein visualisation, and analysed with Western blot to detect the target protein. Save for the evidently increased signal intensity in the 100 kDa molecular weight fraction, the protein distribution pattern in PC12 cells treated with DM or BSO resembled that of untreated cells. This suggests that the nuclear protein loaded in the gel lanes was of the same amount (data not shown), and that the 100 kDa band corresponded to the specific recognition of Nrf2 antibodies. Protein expression of Nrf2 increased after DM treatment both in the nuclear and the cytosolic fractions compared to control PC12 cells (Figure 1).

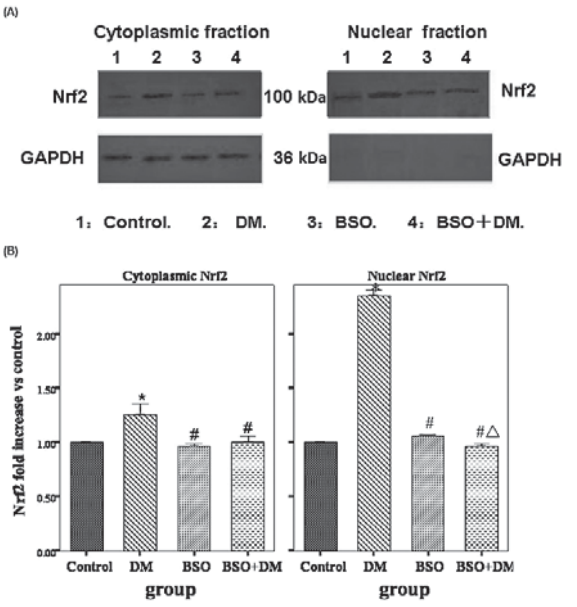

Figure 1 *Effect of BSO pre-treatment on DM-induced expression of the Nrf2 protein in PC12 cells. Cells were incubated with or without 500 μmol L^{-1} BSO for 16 h, followed by exposure to 10 μmol L^{-1} DM for 1 h. Protein expression was examined using the Western blot analysis. Equal amounts of protein (45 μg) extract were loaded onto each lane of 8.0 % SDS-PAGE gels, and the blot was probed with the polyclonal anti-Nrf2 antibody (1:400) overnight and visualized by chemiluminescence. (A) representative Western blot images of Nrf2. Western blot for GAPDH, a cytoplasmic marker, was used as internal control and indicated that the nuclear fraction was not contaminated; (B) immunofluorescent intensity evaluated by quantifying the ratio between the relative level of target protein expression in the experimental group and relative level of target protein expression in the control group, normalised in respect to control. Values are mean ± standard deviation of three determinations.*
** P<0.05 compared to control; # P<0.05 compared to DM; △ P<0.05 compared to BSO*

This implies that DM may induce Nrf2 expression and facilitate Nrf2 activation. To establish the effects of DM on Nrf2 gene transcription, we analysed Nrf2 mRNA expression using RT-PCR. The data showed that the mRNA level of Nrf2 was also elevated upon DM treatment (Figure 2). The enhanced expression and nuclear translocation of Nrf2 indicates that the expression of antioxidant and phase II detoxification genes might also be enhanced through Nrf2-mediated transcriptional activation of ARE-containing genes. To verify this assumption, we assessed the mRNA levels of HO-1 and GCLC, two acknowledged Nrf2-targeted antioxidant genes, and observed that the mRNA level of HO-1 in PC12 cells indeed increased 1.7 fold by DM treatment (Figure 2).

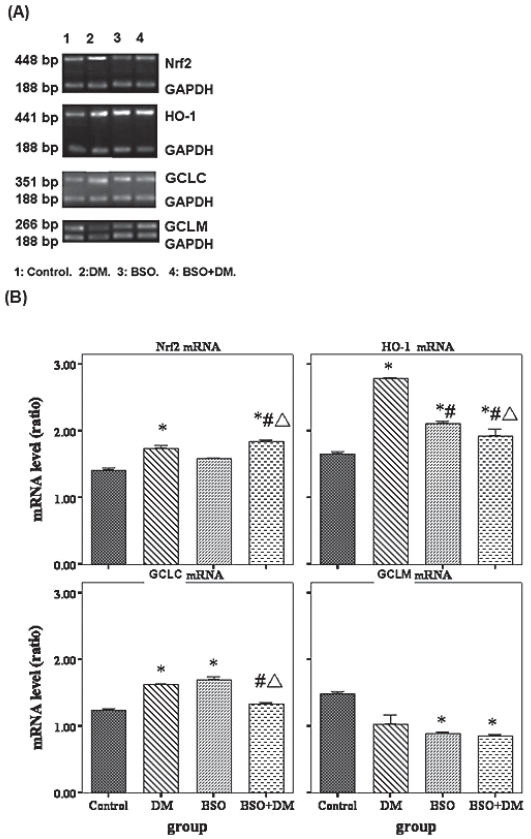

Figure 2 *Effect of BSO on DM-induced expression of Nrf2, HO-1 and mRNA GCLC/M. PC12 cells were incubated with or without 500 μmol L^{-1} BSO for 16 h, followed by exposure to 10 μmol L^{-1} DM for 1 h. Total RNA in cells was isolated and subjected to RT-PCR. mRNA expression was examined by semi-quantitative RT-PCR. (A) agarose gel electropherogram for RT-PCR products from these genes and (B) mRNA expression evaluated by quantifying the ratio between band densitometry of the target gene and GAPDH, normalised in respect to control. Values are mean ± standard deviation of three determinations.*
** P<0.05 compared to control; # P<0.05 compared to DM; △ P<0.05 compared to BSO*

This increase in HO-1 mRNA expression was linked with an even greater, 6.4-fold increase in its protein expression (Figure 3) in respect to untreated cells. In PC12 cells treated with DM, the GCLC mRNA level also increased (Figure 2) compared to control. In summary, our data show that Nrf2 activation by DM induced the expression of several Nrf2-regulated genes in PC12 cells.

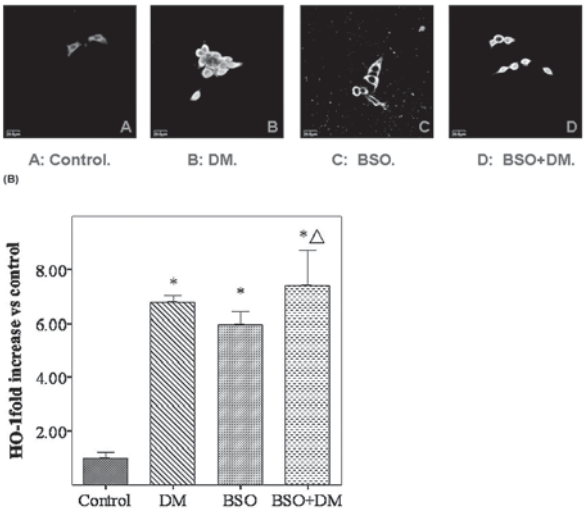

Figure 3 *Effect of BSO on DM-increased HO-1 immunofluorescent intensity. PC12 cells were grown on cover slips and incubated with or without 500 μmol L^{-1} BSO for 16 h, followed by exposure to 10 μmol L^{-1} DM for 1 h. The HO-1 protein level was determined as described in Materials and Methods. Cover slips were mounted on glass slides with fluorescent mounting medium and visualised using a LSCM with a 100x objective. (A) fluorescence images of a typical experiment and (B) ratio between HO-1 in the experimental group and control, normalised in respect to control. Values are mean ± standard deviation of three determinations.*
** P<0.05 compared to control; △ P<0.05 compared to BSO.*

Effects of BSO pre-treatment on the nuclear translocation of Nrf2 induced by DM

GSH depletion triggers oxidative stress and is considered a major activator of the Nrf2-ARE pathway. To further clarify the requirement of GSH depletion in Nrf2 activation, we pre-treated PC12 cells with BSO, a known GSH-depleting agent, and then with DM to assess changes in the expression and subcellular distribution of Nrf2. Compared to vehicle-treated controls, cells treated with BSO alone showed no change in Nrf2 expression or its distribution (Figure 1). However, compared to the DM-treated cells, cells pre-treated with BSO had a lower Nrf2 protein level in both the nucleus and the cytosol (Figure1), indicating

that BSO inhibited DM-induced Nrf2 activation and translocation.

Effects of BSO pre-treatment on DM-increased mRNA expression of GCS, HO-1, and Nrf2

We then measured the effects of BSO pre-treatment on the expression of Nrf2 and Nrf2-regulated genes HO-1, GCLC, and GCLM to explain the molecular mechanism behind Nrf2 activation by DM. The mRNA levels of HO-1, GCLC, and GCLM were measured in PC12 cells treated with DM or BSO. Cells treated with BSO alone showed increased GCLC and HO-1 mRNA expression, but decreased GCLM mRNA expression compared to vehicle-treated controls. However, Nrf2 mRNA expression was unchanged, indicating that BSO promotes gene expression in an Nrf2-independent manner. The alternative explanation is that GSH depletion can directly enhance mRNA expression of GCLC and HO-1. When compared to the DM-treated cells, cells pre-treated with BSO before DM treatment had lower GCS-h and HO-1 mRNA expression and a modest increase in the Nrf2 mRNA level (Figure 2), suggesting that BSO pre-treatment could suppress DM-induced upregulation of GCLC and HO-1 mRNA.

Effects of BSO pre-treatment on DM-increased protein expression of HO-1

Both BSO alone and DM alone increased HO-1 protein expression (Figure 3B). In cells pre-treated with BSO for 16 h followed by DM exposure, HO-1 mRNA expression was slightly lower than in cells treated with DM or BSO alone. Surprisingly, BSO did not suppress DM-induced upregulation of the HO-1 protein. On the contrary, a slight increase was observed (Figure 3B), indicating a synergistic effect of BSO and DM.

Effect of BSO pre-treatment on the GSH content in PC12 cells treated with DM

DM alone had no effect on the GSH content, whereas BSO alone and BSO+DM decreased it significantly by 44.0 % and 68.1 %, respectively (Figure 4). However, the difference in GSH levels between BSO and BSO+DM treatment was not significant (Figure 4).

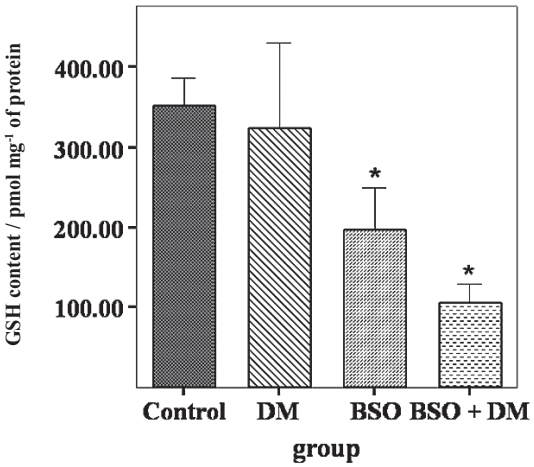

Figure 4 *Effect of BSO pre-treatment on GSH content in PC12 cells treated with DM. PC12 cells were pre-incubated with 500 μmol L-1 BSO for 16 h, followed by exposure to 10 μmol L-1 DM or vehicle for 1 h. Reduced glutathione (GSH) content was determined by reversed-phase HPLC with o-phthalaldehyde pre-column derivatisation.*
** P<0.05 compared to control.*

DISCUSSION

Several studies have shown that oxidants and xenobiotics might activate inactive Nrf2 by facilitating its translocation from the cytoplasmic compartment towards the nucleus (31, 32). To establish whether DM controls Nrf2 activity in such a way, we determined Nrf2 levels in subcellular fractions after DM treatment. The original hypothesis has been that Nrf2 level in the cytosolic fraction would decrease, with a simultaneous increase in the nuclear fraction. However, DM treatment increased Nrf2 protein levels in both the cytosolic and the nuclear fractions at the same time (Figure 1). This DM-induced Nrf2 upregulation was also observed at the mRNA level by RT-PCR, indicating that increased Nrf2 protein level in DM-treated PC12 cells was, at least to some extent, caused by enhanced Nrf2 gene transcription. Our findings undoubtedly confirm the association between DM exposure and elevated cellular and nuclear accumulation of Nrf2, which consequently promotes the expression of Nrf2-regulated oxidative stress response genes (Figure 1).

Generally, in *in vitro* toxicology studies, chemical treatment in culture media should be conducted under serum-free conditions. This is why we transferred the PC12 cells after initial 24-h incubation to a serum-free culture medium for treatment. How serum deprivation affected Nrf2 protein expression or nuclear accumulation was unclear. Nevertheless, the addition of DM or BSO into serum-free culture medium significantly affected Nrf2 protein expression and nuclear accumulation compared to control.

DM treatment had no effect on GSH content, whereas BSO alone and BSO+DM had a similar

significantly decreasing effect on (Figure 4). BSO was used to see whether nuclear Nrf2 level increased by DM in PC12 cells were mediated by GSH. In contrast to DM, BSO did not significantly increase nuclear Nrf2, which suggests that GSH depletion alone could not cause Nrf2 upregulation or activation. It is known that BSO irreversibly binds GCLC and is not likely to produce ROS or chemically reactive metabolites that can modify cellular macromolecules (33). In contrast, DM requires metabolism to induce extensive covalent modification of cellular proteins.

Goldring et al. (21) have proposed that Nrf2 may be activated in mice liver by its dissociation from the cytoplasmic inhibitor Keap1 either through a redox-sensitive mechanism related to GSH depletion or through Michael addition (21).

However, the results we obtained with BSO suggest that lowering the GSH level did not induce nuclear translocation of Nrf2 (Figure 1 and Figure 4). Instead, BSO pre-treatment inhibited Nrf2 activation and nuclear translocation provoked by DM (Figure1), suggesting a mechanism of Nrf2 activation independent of GSH depletion. Therefore, a more direct interaction may be involved, probably related to chemical modification of Nrf2 or Keap1 potentiated by the earlier loss of GSH.

Nguyen et al. (32) identified nine distinct classes of Nrf2 inducers that share several characteristics: a) all are chemically reactive; b) most are electrophiles; c) many are substrates for GSH S-transferases; and d) all can covalently modify sulphydryl groups by alkylation, oxidation, or reduction. So far, various mechanisms have been suggested to explain Nrf2 activation, including oxidation of redox-sensitive cysteine residues in Keap1, phosphorylation of Nrf2, and covalent adduct formation with protein stabilisation (31).

The findings of Goldring et al. (21) imply that changes in the Nrf2 system may not depend on toxicity related to changes in the GSH level, but could be associated with the formation of chemically reactive or electrophilic species. Indeed, Zhang et al. (34) have found that 4-hydroxynoneneal, a lipid peroxidation product, induces Nrf2 at sub-toxic concentrations. This finding supports the idea that chemically reactive electrophilic species can activate Nrf2, and that Keap1 degradation is probably not required for Nrf2 translocation.

In our study, Nrf2 mRNA expression was not suppressed by BSO+DM. However, BSO pre-treatment did inhibit its DM-mediated increase in protein level in the cytoplasm and the nucleus. This inhibitory effect of BSO on Nrf2 activation and translocation may be mediated by the effects of BSO on ROS accumulation. Earlier findings in DM-exposed PC12 cells showed that ROS mediated nuclear accumulation of Nrf2 (25). Moreover, increased nuclear accumulation of Nrf2 after DM exposure was associated with the production of free radicals in rat brain (12). Pre-treatment with BSO for 16 h significantly reduced DM-induced increase in DCF fluorescence intensity (by about 40 %), indicating that BSO could attenuate ROS production induced by DM (35). Taken together, these data suggest that Nrf2 activation may rely on chemically reactive metabolites generated through a DM-mediated protein modification. Therefore, BSO pre-treatment may inhibit DM-induced Nrf2 activation and translocation by decreasing ROS levels in PC12 cells.

Induction of HO-1 by Nrf2 through ARE has recently been established using Nrf2 dominant-negative mutants (Nrf2M) (36). Recent experiments (6, 37-41) have also shown that Nrf2 takes place in both basal and induced expression of multiple ARE-containing genes that encode detoxification enzymes and human GCLC and GCLM.

It has already been established that BSO, an inhibitor of gamma-glutamylcysteine synthetase and the rate-limiting enzyme in the biosynthesis of GSH, increases HO-1 enzyme activity, HO-1 protein expression, and HO-1 mRNA expression in rat brain and liver cell lines (42-46). However, our results have demonstrated that BSO can induce HO-1 and GCLC mRNA expression without affecting Nrf2 translocation (Figure 1 and Figure 2), precluding therefore the involvement of Nrf2-ARE signalling. HO-1 and GCLC expression is, in fact, altered by redox-sensitive transcription factors that recognise particular binding sites within the promoter and distal enhancer regions of these genes (3, 47). These transcription factors include Fos/Jun activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and the newly recognised Nrf2 proteins (48, 49). Additionally, HO-1 is quickly upregulated by oxidative and nitrosative stress and by GSH depletion as well (50-52).

In a study with rat lung epithelial L2 cells exposed to oxidative stress or GSH depletion, Tian et al. (53) have found evidence for enhanced transcription of the regulatory subunit of gamma-glutamylcysteine synthetase. GCS mRNA expression also increased in rat myocardium, although GCS activity is suppressed by BSO (54). Urata et al. (55) have also found that

BSO can trigger the transcription factor NF-κB. We speculate that HO-1 and GCLC upregulation induced by BSO might be mediated by Fos/Jun (AP-1) or NF-κB in PC12 cells. Thus, GCS and HO-1 induction by DM alone or by BSO alone may follow different signalling pathways. We also found that BSO pre-treatment increased HO-1 protein upregulation by DM. It is possible that this upregulation may involve transcription factors other than Nrf2, such as Fos/Jun (AP-1) or NF-κB.

GCS subunits seem ro follow different regulation pathways. However, Gipp et al. (56) did not find a correlation between the steady-state mRNA levels of the two GCS subunits. In our study, the expression of GCS subunits was significantly affected by DM, and GCLC mRNA levels increased (Figure 2), whereas GCLM mRNA levels did not change (Figure 2). This discrepancy might be due to a different response to DM exposure. An additional factor that complicates characterising DM-related decrease in ARE-dependent gene transcription is the effect of other interacting partners on Nrf2, because Nrf2 makes heterodimers with other bZip proteins, for instance the Jun/Fos family, Fra, small Maf, and ATF4 proteins (3, 40, 57, 58).

Partnering factors could influence Nrf2-dependent gene expression, either through stimulating or reducing it. It has not yet been established whether relative proportions of these partner proteins change in DM-treated PC12 cells. It will be interesting to investigate possible DM-associated changes in these factors, above all the Jun/Fos family members and their effects on ARE-mediated gene transcription.

In conclusion, this study has demonstrated that DM can induce Nrf2 nuclear accumulation. Depletion of GSH does not seem necessary for Nrf2 activation. Our results suggest that Nrf2 activation may rely on chemically reactive metabolites derived from covalently modified intracellular molecules that are altered by the highly reactive DM. These data also suggest that GCS and HO-1 induction by DM and BSO follows different signalling pathways.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

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REFERENCES

1. Casida JE, Gammon DW, Glickman AH, Lawrence LJ. Mechanisms of selective action of pyrethroid insecticides. Annu Rev Pharmacol Toxicol 1983;23:413-38.
2. Soderlund DM, Clark JM, Sheet LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 2002;171:3-59.
3. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233-60.
4. Ishii T, Itoh K, Yamamoto M. Roles of Nrf2 in activation of antioxidant enzyme genes via antioxidant responsive elements. Methods Enzymol 2002;348:182-90.
5. Wild AC, Moinova HR, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J Biol Chem 1999;274:33627-36.
6. Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc Natl Acad Sci USA 1999;96:12731-6.
7. Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, Yamamoto M, Hayes JD. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1 Gsta2 Gstm1 Gstm2 Gstm3 and Gstm4 genes in the livers of male and female mice. Biochem J 2002;365:405-16.
8. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2 a Cap'n'Collar transcription factor regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274:26071-8.
9. Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci USA 1997;94:5361-6.
10. Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 2002;62:5196-203.
11. Kwak MK, Akabayashi N, Itoh K, Motohash H, Yamamoto M, Kensler TW. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway: identification of novel gene clusters for cell survival. J Biol Chem 2003;278:8135-45.
12. Li HY, Wu SY, Ma Q, Shi N. The pesticide deltamethrin increases free radical production and promotes nuclear translocation of the stress response transcription factor Nrf2 in rat brain. Toxicol Ind Health 2011;27:579-90.
13. Li HY, Shi N, Chen D, Dai ZH, Lu WH, Wang B, Li YR. [Oxidative stress of deltamethrin on rats nervous system, in Chinese]. Chin J Ind Hyg Occup Dis 2005;23:97-101.
14. Hong F, Sekhar KR, Freeman ML, Liebler DC. Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. J Biol Chem 2005;280:31768-75.

15. Kobayashi A, Kang M, Watai Y, Tong K, Shibata T, Uchida K, Yamamoto M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 2006;26:221-9.
16. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol Cell Biol 2004;24:10941-53.
17. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 2002;277:42769-74.
18. Bloom DA, Jaiswal AK. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2 but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J Biol Chem 2003;278:44675-82.
19. Jain AK, Bloom DA, Jaiswal AK. Nuclear import and export signals in control of NRF2. J Biol Chem 2005;280:29158-68.
20. Li W, Jain MR, Chen C, Yue X, Hebbar V, Zhou R, Kong AN. NRF2 possesses a Redox-insensitive nuclear export signal overlapping with the leucine zipper motif. J Biol Chem 2005:280:28430-8.
21. Goldring CE, Kitteringham NR, Elsby R, Randle LE, Clement YN, Williams DP, McMahon M, Hayes JD, Itoh K, Yamamoto M, Park BK. Activation of hepatic Nrf2 *in vivo* by acetaminophen in CD-1 mice. Hepatology 2004;39:1267-76.
22. Timothy JS, William DA. Transmitter ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. NeuroToxicology 1991;12:473-92.
23. Tyrrell RM, Pidoux M. Endogenous glutathione protects human skin fibroblasts against the cytotoxic action of UVB, UVA and near-visible radiations. Photochem Photobiol 1986;44:561-4.
24. Shen DX, Fu JL, Shi X, Zhou ZC. Toxic effect of inhibition of glutathione synthesis on human embryonic kidney cells. J Health Toxicol 2002;16:150-3.
25. Li HY, Wu SY, Shi N. Transcription factor Nrf2 activation by deltamethrin in PC12 cells: involvement of ROS. Toxicol Lett 2007;171:87-98.
26. Deptala A, Bedner E, Gorczyca W, Darzynkiewicz Z. Activation of nuclear factor kappa B (NF-κB) assayed by laser scanning cytometry (LSC). Cytometry 1998;33:376-82.
27. Schreiber E, Matthias P, Mūller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
28. Yan CC, Huxtable RJ. Fluorimetric determination of monobromobimane and o-phthalaldehyde adducts of gamma-glutamylcysteine and glutathione: application to assay of gamma-glutamylcysteinyl synthetase activity and glutathione concentration in liver. J Chromatogr B Biomed Appl 1995;672:217-24.
29. Lee JM, Moehlenkamp JD, Hanson JM, Johnson JA. Nrf2-dependent activation of the antioxidant responsive element by ter-butylhydroquinone is independent of oxidative stress in IMR-32 Human neuroblastoma cells. Biochem Biophysic Res Commun 2001;280:286-92.
30. Shukla GS, Chiu J, Hart BA. Enhanced expression of pulmonary gamma-glutamylcysteine synthetase heavy subunit in rats exposed to cadmium aerosols. Toxicol Appl Pharmacol 2000;163:249-59.
31. Itoh K,Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999;13:76-86.
32. Nguyen T, Sherratt PJ, Huang H-C, Yang CS, Pickett CB. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element: degradation of Nrf2 by the 26 S proteasome. J Biol Chem 2003;278:4536-41.
33. Randle LE, Goldring CE, Benson CA, Metcalfe PN, Kitteringham NR, Park BK,Williams DP.Investigation of the effect of a panel of model hepatotoxins on the Nrf2-Keap1 defence response pathway in CD-1 mice.Toxicology 2008;20:243:249-60.
34. Zhang H, Court N, Forman HJ. Submicromolar concentrations of 4-hydroxynonenal induce glutamate cysteine ligase expression in HBE1 cells. Redox Rep 2007;12:101-6.
35. Li HY, Shi N, Zhong YF. [Effect of deltamethrin on production of reactive oxygen species in PC12 cells, in Chinese]. Chin J Ind Hyg Occup Dis 2008;26:654-8.
36. Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM, Cuadrado A. Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem 2004;279:8919-29.
37. Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci USA 2001;98:4611-6.
38. Hayes JD, Chanas SA, Henderson CJ, McMahon M, Sun C, Moffat GJ, Wolf CR, Yamamoto M. The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants butylated hydroxyanisole and ethoxyquin. Biochem Soc Trans 2000;28:33-41.
39. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 2000;275;16023-9.
40. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313-22.
41. McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, Hayes JD. The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. Cancer Res 2001;61:3299-307.
42. Oguro T, Yoshida T, Numazawa S, Kuroiwa Y. Possible role of glutatione depletion in the induction of rate-limiting

enzymes involved in heme degradation and polyamine biosynthesis in the liver of rats. J Pharmacobiodyn 1990;13:628-36.
43. Saunders EL, Maines MD, Meredith MJ, Freeman ML. Enhancement of heme oxygenase 1 synthesis by glutathione depletion in Chinese hamster ovary cells. Arch Biochem Biophys 1991;288:368-73.
44. Lautier D, Luscher P, Tyrrell RM. Endogenous glutathione level modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene. Carcinogenesis 1992;13:227-32.
45. Ewing JF, Maines MD. Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. J Nerochem 1993;60:1512-9.
46. Oguro T, Hayashi M, Numazawa S, Asakawa K, Yoshida T. Heme oxygenase-1 gene expression by a glutathione depletor phorone mediated through AP-1 activation in rats. Biochem Biophys Res Commun 1996;221:259-65.
47. Alam J. Heme oxygenase-1: past present and future. Antiox Redox Signal 2002;4:559-62.
48. Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499-511.
49. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 2003;371:887-95.
50. Naughton P, Hoque M, Green CJ, Foresti R, Motterlini R. Interaction of heme with nitroxyl or nitric oxide amplifies heme oxygenase-1 induction: involvement of the transcription factor Nrf2. Cell Mol Biol 2002;48:885-94.
51. Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 2003;23:3394-406.
52. Calabrese V, Scapagnini G, Ravagna A, Colombrita C, Spadaro F, Butterfield DA, Giuffrida Stella AM. Increased expression of heat shock proteins in rat brain during aging: relationship with mitochondrial function and glutathione redox state. Mech Ageing Dev 2004;125:325-35.
53. Tian L, Shi MM, Forman HJ. Increased transcription of the regulatory subunit of γ - glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. Arch Biochem Biophy 1997;342:126-33.
54. Mao LJ, Xu HW. Effect of glutathione depletion on glutathione system in myocadium of rat. Chin J Sports Med 2004;23:266-70.
55. Urata Y, Yamamoto H, Goto S, Tsushima H, Akazawa S, Yamashita S, Nagataki S, Kondo T. Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1beta and tumor necrosis factor-alpha in mouse endothelial cells. J Biol Chem 1996;271:15146-52.
56. Gipp JJ, Bailey HH, Mulcahy RT. Cloning and sequencing of the cDNA for the light subunit of human liver gamma-glutamylcysteine synthetase and relative mRNA levels for heavy and light subunits in human normal tissues. Biochem Biophys Res Commun 1995;206:584-9.
57. Wild AC, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. Free Radic Res 2000;32:281-301.
58. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci USA 1996;93:14960-5.

Sažetak

ULOGA DEPLECIJE GLUTATIONA U AKTIVACIJI NRF2/ARE DELTAMETRINOM U ŠTAKORSKIM PC12-STANICAMA FEOKROMOCITOMA

Transkripcijski čimbenik 2 povezan s NF-E2 (Nrf2) važan je za zaštitu stanice od oksidacijskog stresa uzrokovanog kemijskim spojevima. U prijašnjem smo istraživanju utvrdili da često rabljeni piretroidni insekticid deltametrin aktivira Nrf2 u štakorskim PC12-stanicama feokromocitoma. Još međutim nisu jasni molekularni mehanizmi te aktivacije. U ovome smo istraživanju željeli utvrditi ulogu deplecije staničnoga glutationa (GSH) u aktivaciji Nrf2 od strane DM-a. DM je pojačao ekspresiju Nrf2 u mRNA te povisio razinu proteina i razinu Nrf2 u jezgri. Aktivacija Nrf2 bila je povezana s nizvodnom aktivacijom hem-oksigenaze 1 (HO-1) i katalitičke podjedinice glutamat cistein ligaze (GCLC). DL-butionin-[S,R]-sulfoksimin (BSO), za koji se zna da dovodi do deplecije GSH, nije međutim povećao ekspresiju Nrf2-proteina niti doveo do njegova nakupljanja u staničnoj jezgri. Prethodna primjena BSO aktivirala je međutim ekspresiju HO-1 i GCLC u mRNA. Usto je suprimirala djelovanje DM-a na aktivaciju i regulaciju Nrf2 te smanjila ekspresiju HO-1 i GCLC u mRNA nakon primjene DM-a. Ova saznanja govore da deplecija GSH nije nuždan mehanizam za aktivaciju Nrf2/ARE od strane DM-a u PC12-stanica te da do povećane ekspresije GCLC i HO-1 može doći drugim signalnim putovima.

KLJUČNE RIJEČI: *BSO, DL-butionin-[S,R]-sulfoksimin, katalitička podjedinica glutamat cistein ligaze, GCLC, hem-oksigenaza, HO-1, piretroidni insekticid*

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