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The pesticide deltamethrin increases free radical production and promotes nuclear translocation of the stress response transcription factor Nrf2 in rat brain

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Abstract

The transcription factor NF-E2-related factor 2 (Nrf2) plays a critical role in the mammalian response to chemical and oxidative stress through induction of phase II detoxification enzymes and oxidative stress response proteins. We reported that Nrf2 expression was activated by deltamethrin (DM), a prototype of the widely used pyrethroid pesticides, in PC12 cells. However, no study has examined Nrf2 nuclear translocation and free radical production, two hallmarks of oxidative stress, in the mammalian brain in vivo. To this end, we examined translocation of Nrf2 and production of free radicals in rat brain exposed to DM. Indeed, DM initiated nuclear translocation of Nrf2 in a dose-dependent manner. Furthermore, Nrf2 translocation was accompanied by the expression of heme oxygenase-1 gene, an Nrf2-regulated gene linked to free radical production. Deltamethrin exposure promoted free radical formation in rat brain and reactive oxygen species generation in PC12 cells. Translocation of Nrf2 may be a response to DM-dependent induction of free radicals and DM may act as a mammalian neurotoxin by initiating oxidative stress.

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Conflict of interest

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

Keywords

Deltamethrin; free radical; Nrf2; oxidative stress; neurotoxicity; in vivo

Introduction

The transcription factor NF-E2-related factor 2 (Nrf2), a member of the Cap'n'Collar family of bZIP proteins, is a central mediator of the oxidative stress response. Nuclear translocation of Nrf2 and activation of the antioxidant response element (ARE) has been linked to the enhanced expression of genes encoding antioxidant and phase 2 drug-metabolizing enzymes (Ishii et al., 2002; Nguyen et al., 2003). Toxic insult, glutathione depletion, or chemical activation causes Nrf2 to translocate to the nucleus, where it dimerizes with small Maf proteins to form a transactivation complex that binds to the ARE (Nguyen et al., 2003). Consequently, Nrf2-induced ARE activation coordinates the expression of many genes that combat oxidative stress and chemotoxicity in a wide variety of tissues and cell types (Copple et al., 2008; Moi et al., 1994). In addition to protecting against chemical insults, carcinogenesis, and aging (Copple et al., 2008; Randle et al., 2008; Rojo et al., 2008), Nrf2 has been shown to directly inhibit apoptosis mediated by Fas, a substrate for caspase-3-like proteases and an antagonist of ERK-mediated cell survival (Cullinan et al., 2004; Cullinan et al., 2003; Kotlo et al., 2003; Ohtsubo et al., 1999). A central and critical role for Nrf2 in coordinating the mammalian cellular defense response against a variety of noxious stimuli is well-established. Moreover, Nrf2/ARE signaling confers neuroprotection against several central nervous system diseases (Calkins et al., 2009; Chen et al., 2009; Cuadrado et al., 2009; de Vries et al., 2008; Johnson et al., 2008).

Pyrethroid pesticides, the major class of insecticides, are commonly used in agriculture and urban settings due to their high potency, selectivity as nerve toxins, and low persistence compared to other classes of insecticides (Casida et al., 1983). Human exposure to pyrethroids is widespread. Deltamethrin ((*S*) a-cyano-3-phenoxybenzyl-(1*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate, DM), one of the most potent pyrethroid insecticides with a cyano substituent, produces the prototypical type II neurological syndrome characterized by salivation without lacrimation followed by jerking leg movements and progressive writhing convulsions (or 'choreoathetosis with salivation') (Casida et al., 1983; Soderlund et al., 2002).

We have previously shown that DM inhibited dopamine biosynthesis by reducing tyrosine hydroxylase mRNA and protein expression in PC12 cells (Liu et al., 2006a, b). In addition, DM caused neuronal apoptosis, accompanied by increased expression of proapoptotic p53 and Bax, and decreased expression of antiapoptotic Bcl-2 (Wu et al., 2000; Wu and Liu, 2000a, b). Deltamethrin also selectively increased dopamine release and uptake in the dopaminergic nerve terminals of the striatum, and even altered serotonergic neurotransmission (Kirby et al., 1999; Martinez-Larrañaga et al., 2003). Environmental or occupational exposure to pyrethroid pesticides, therefore, may produce specific damage to dopaminergic neurons, but the full extent of DM-induced neuropathology is still unknown.

It is now widely accepted that the redox-sensitive regulation of Nrf2 represents a convergence point for multiple stress-activated signaling pathways that results in the coordinated up-regulation of a battery of antioxidant proteins involved in cellular defense (Nguyen et al., 2003). The involvement of Nrf2 in defense against DM-induced stress in vitro is largely based on studies in PC12 cell lines and primary astrocytes (Li et al., 2006b; Li et al., 2007a), but there is a paucity of data demonstrating chemically induced Nrf2 activation by DM in vivo. Similarly, while the generation of superoxide (O_2^-), hydroxyl radicals (HO^\bullet), and H_2O_2 have been observed in response to other pyrethroid compounds (Casida et al., 1983; Soderlund et al., 2002), little is known about the effects of DM on free radical generation in neurons.

Our aims were to determine the effects of DM exposure on neuronal free radical generation in vivo and in vitro, to measure Nrf2 nuclear translocation during DM exposure in rat brain, and to examine the downstream expression of Nrf2-regulated genes (Alam et al., 1999). Nuclear localization of Nrf-2 protein and expression of heme oxygenase-1 gene (HO-1) mRNA increased in rat hippocampus and cerebral cortex in response to DM exposure. The Nrf2 gene is a novel target of deltamethrin and possibly a major coordinator of the stress response against pyrethroid toxicity.

Materials and methods

Reagents and instruments

Deltamethrin (98.5% w/w) was obtained from Roussel-Uclaf Corp (Romainville Cedex, France). Hemin, N-acetyl cysteine (NAC), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Trizol reagent and a first-strand cDNA synthesis kit were obtained from Gibco Co. Taq DNA polymerase was obtained from Promega (Shanghai, China). Anti-Nrf2 antibodies (C-20, sc-722, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Nitrocellulose membrane (NC) membranes were purchased from Millipore Company (Bedford, MA). Enhanced chemiluminescence's reagents were from HyClone-Pierce Company (Utah, USA). T-Gradient thermoblock PCR (Biometra Co., Germany), HVE50 gel imaging system, electrophoresis chamber (Bio-Rad Co., USA), ultraviolet spectrophotometer (Shimadzu Co., Japan), Bruker ER-420 spectrometer (Bruker Co., Germany).

Animals and treatment

Sprague–Dawley male rats weighing between 180 and 220 g were obtained from the animal care center of Tongji Medical College. Rats were fed regular rat chow and water ad libitum until the start of the experimental procedures. Four male rats were randomly assigned to 2 groups: (i) control and (ii) DM. Rats were administered vehicle (olive oil) or 12.50 mg/kg DM (i.p.) daily for 5 days and sacrificed at 5 h after the last injection. The hippocampi were isolated and free radicals detected by electron spin resonance (ESR) spectroscopy. In another series of experiments, 18 male rats were randomly assigned to three groups and administered 0 (vehicle), 3.125, or 12.50 mg/kg DM daily for 5 days. Rats were sacrificed at 5 h after the last i.p. injection and cerebral cortices and hippocampi isolated. Levels of Nrf2

protein in the cytoplasmic and nuclear fractions were measured by immunoblotting. For HO-1 gene expression studies, 10 male rats were randomly assigned to two groups and administered vehicle or 12.50 mg/kg DM daily for 5 days. Rats were sacrificed at 5 h after the last i.p. injection and cerebral cortices and hippocampi were isolated. The heme-oxygenase-1 mRNA level was measured by RT-PCR. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of Tongji Medical University.

ESR spectrometry

Isolated rat hippocampal tissue was frozen in liquid nitrogen and cut into 1 mm³ blocks that were transferred to precision ESR tubes (of 3 mm inside diameter). Electron spin resonance spectra were recorded at 77 K on a Bruker ER-420 spectrometer (X-band), with microwave power of 100 mW, modulation frequency of 100 kHz, modulation amplitude at 1.0 G, time constant of 0.5 sec, and scanning time of 300 sec. Each sample was loaded in a quartz glass flat cell, and inserted horizontally into the cavity at room temperature (20°C). Spectral signal intensity was a direct reflection of free radical concentration. Peak signal heights were normalized to the control samples.

PC12 cell culture and treatment

Differentiated PC12 cells were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone-Pierce Company, Utah, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂/95% air. The medium was changed every other day, and cells were plated at an appropriate density according to each experimental scale. After 24 h incubation, cells were switched to serum-free media for treatment. Cells were treated with various concentrations of DM (0, 10, or 100 μM) for various treatment durations (1, 6, 12, or 24 h) in triplicate using orthogonal experimental design.

To examine reactive oxygen species (ROS) production in the presence of an exogenous antioxidant, PC12 cells were pre-incubated with 10 mM *N*-acetyl cysteine (NAC) for 2 h, then exposed to 10 μM DM (or 0.1% DMSO vehicle) for 6 h. After the treatment, cells were washed three times with ice-cold PBS, and ROS production was measured by a molecular probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA). The final concentration of DMSO (0.1%) had no effect on the parameters measured (data not shown).

Assay of the cellular ROS

The intracellular generation of ROS was also measured using 2', 7'-dichlorofluorescein, which is oxidized to the fluorescent 2', 7'-dichlorofluorescein by H₂O₂ and other ROS (Shen et al., 1996). ROS was measured as described previously (Li et al., 2007a) with modifications. Briefly, after treatment with DM or vehicle, cells were washed three times with ice-cold PBS and incubated with 10 μM 2', 7'-dichlorofluorescein diacetate (2',7'-dichlorodihydrofluorescein diacetate; 100 mM in dimethyl sulfoxide) for 30 min at 37°C. The cellular free radical content was assayed by measuring 2',7'-dichlorofluorescein fluorescence in a fluorescent spectrophotometer (excitation at 485 nm, emission at 535 nm,

bandwidth 5 nm). The DCF fluorescence intensity was also recorded by fluorescence and phase contrast combination microscopy.

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

Total RNA was isolated from brain samples using TRIzol reagent according to manufacturer's instructions. Briefly, reverse transcription reactions (RT) were carried out in a 20 μ L reaction volume. The temperature cycling conditions were as follows: incubation at 42°C for 45 min, at 70°C for 15 min, and then at 37°C for 20 min. PCR was carried out sequentially. The primer sequences selected from detected gene for cDNA amplification were shown in Table 1 (sense and antisense primer, respectively). The temperature cycling conditions were as follows: predenaturation at 94°C for 10 min, 35 cycles of (denaturation at 94°C for 60 s, annealing 55°C for 30 s, and extension at 72°C for 60 s) and a final extension at 72°C for 5 min. As an internal control, a 310-bp DNA fragment of a rat housekeeping gene, β -actin was also amplified. An aliquot of each PCR amplified product was resolved by agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. For respective samples, the PCR product values were normalized to the β -actin PCR product values.

Preparation of cytosolic and nuclear extracts

Cells were treated with various chemicals as detailed in the respective figure legends. Nuclear and cytosolic extracts were isolated as described previously (Schreiber et al., 1989) with modifications. Briefly, cells were washed with cold PBS and resuspended in cold buffer A (lysis buffer containing 20 mM HEPES, pH 8.0, 1 mM EDTA, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.5 mg/mL benzamidine, 0.1 mg/mL leupeptin, and 1.2 mg/mL aprotinin). The cells were allowed to swell on ice for 15 min; then 7.5 μ L of 10% (v/v) NP-40 was added and the suspension vortexed vigorously for 10 sec. The homogenate was then centrifuged for 50 sec at 16,000 \times g, and the supernatant was used as cytosolic extract. The nuclear pellet was resuspended in cold buffer B (extraction buffer 20 mM HEPES, pH 8.0, 1 mM EDTA, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.5 mg/mL benzamidine, 0.1 mg/mL leupeptin, 1.2 mg/mL aprotinin, and 20% glycerol). All the protein fractions were stored at -70°C until use, and the protein concentrations were determined by the Bradford method with bovine serum albumin as a standard.

Immunoblot analysis

Briefly, protein samples were resolved on 8% (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and blocked for 1h in Tris-buffered saline (TBS), 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk. The NC membranes were incubated overnight with primary antibody diluted in the same buffer (Nrf2 1:500, GAPDH 1:8000). After washing with 0.1% (v/v) Tween 20 in TBS, the membranes were incubated with peroxidase-conjugated secondary antibody for 1h, and then washed and developed using the ECL chemiluminescent detection system, followed by visualization with X-ray film.

Statistical analysis

Each data bar represents the mean values \pm SD (standard deviations) of at least three independent experiments in all cases. Results were analyzed using SPSS for Windows (version 12.0). Differences between groups were analyzed by a t-test or Univariate Analysis of Variance or one-way analysis of variance (ANOVA). If the F values were significant, LSD post hoc tests were used to compare multiple groups. A P value of < 0.05 was considered statistically significant in all cases.

Results

DM induced free radical production in vivo and ROS generation in vitro

One signal was isotropic with $g = 2.000$. The remaining g values were identical to those of superoxide oxygen, nitrogen-centered free radicals, or carbon-centered semiquinone free radicals. Free radical concentrations derived from the peak amplitudes in the DM-treated group were 2.45 times greater than the untreated group ($p < 0.05$; Figure 1). These experiments directly demonstrated that DM promoted reactive free radical formation in brain.

Fluorescence from DCF oxidation is used to evaluate ROS; DM treatment caused a large shift from the non-fluorescent DCFH to the fluorescent DCF. A cell viability assay indicated that 10 and 100 μM DM doses were not toxic to the cells during the time frame of the experiments (data not shown). Multivariate analysis of variance demonstrated a significant effect of DM concentration ($p < 0.05$) and treatment duration on ROS generation ($p < 0.05$). After 1 h of 100 μM DM exposure, the DCF fluorescence intensity was 1.79 times that of control ($p < 0.05$; Figure 2). After 6 h exposure, DCF fluorescence intensity was enhanced 1.54-fold by 10 μM DM and 1.56-fold by 100 μM DM (Figure 2; $p < 0.05$). At 12 h exposure, 10 μM DM enhanced DCF fluorescence intensity by 2.09 times the control level while 100 μM DM increased DCF fluorescence by 2.74-fold relative to control (Figure 2). Treatment for 6 h and 12 h with 10 μM DM enhanced DCF fluorescence intensity by 1.37 and 1.35 times that of control (Figure 2; $p < 0.05$). Together these results demonstrated that DM induced a dose- and time-dependent increase in ROS production (Figure 2).

In separate experiment, 10 μM DM treatment for 6 h enhanced DCF fluorescence intensity by 2.24 times relative to control (Figure 3). Pretreatment with the antioxidant NAC significantly reduced this DM-induced DCF fluorescence by 88%, indicating that NAC pretreatment attenuate ROS production (Figure 3).

Deltamethrin exposure triggered Nrf2 nuclear translocation in vivo

Protein levels of Nrf2 were measured in both cytoplasmic and nuclear fractions from brain homogenates prepared from rats treated with a range of DM doses (Figure 4). Deltamethrin injection induced a marked increase in Nrf2 protein in the cytoplasmic fraction of cerebral cortical tissue. For the low dose (3.125 mg/kg), cytoplasmic Nrf2 protein was 1.51-fold higher than the control group. After the higher DM dose (12.5 mg/kg), cytoplasmic Nrf2 was 2.29-fold higher than the vehicle-treated group (Figure 4). Similar changes in Nrf2 protein levels were observed in extracts from hippocampus tissue. Protein levels in the

cytoplasmic fraction of hippocampus tissue was increased 2.26-fold in the low dose group and by 3.58-fold in the high dose group (Figure 4). In the nuclear fraction from hippocampal tissue, protein levels were 2.42-fold higher in the low dose group and 2.43-fold higher in the high dose group relative to controls (Figure 4). This substantial increase in nuclear Nrf2 suggests that deltamethrin evoked enhanced nuclear translocation.

Nrf2 nuclear translocation in vivo was accompanied by the induction of HO-1

Transcription of the HO-1 gene is known to depend on Nrf2. The HO-1 mRNA levels were analyzed in brain tissue from rats treated with DM. At a dose of 12.5 mg/kg (a dose that increased nuclear Nrf2, Figure 4), HO-1 mRNA levels were significantly elevated in tissue from both cerebral cortex and hippocampus compared to vehicle-treated controls (Figure 5).

Discussion

To our knowledge, this study is the first to show that DM can promote free radical formation in rat brain (Figure 1). Previous reports indicated that pyrethroids generated O_2^- , HO^\cdot radicals, and H_2O_2 , with concomitant lipid peroxidation (Casida et al., 1983; Soderlund et al., 2002). However, whether DM also induces the generation of free radicals in brain and ROS in dopaminergic neurons was not known. Our results demonstrated increased free radical levels in hippocampus, suggesting that DM exposure resulted in oxidative damage. Generation of free radicals may result from reactive metabolites of DM. We employed PC12 cells, a rat adrenal pheochromocytoma cell line that models dopaminergic neurons, to investigate whether DM exposure induced ROS generation in this cell type. Indeed, an increase in ROS did occur in PC12 cells treated with DM, and this response was effectively inhibited by pretreatment with NAC, a ROS scavenging agent, further supporting that prooxidant effects of DM in rat brains.

Under normal conditions, there is a steady-state balance between prooxidants and antioxidants that protects cellular constituents from oxidative damage. However, when the rate of free radical generation exceeds the capacity of antioxidant defences, oxidative stress ensues. These in vivo and in vitro data indicate that exposure to DM may induce oxidative stress in rat brain (Figures 1, 2, and 3) and is consistent with our previous results that deltamethrin increased lipid peroxidation products and decreased the activities of the antioxidant enzymes superoxide dismutase and glutathione reductase in rat brain (Li et al., 2005).

Previous work also established a relationship between DM exposure in vitro and increased cellular and nuclear accumulation of Nrf2, which in turn activated the expression of Nrf2-regulated oxidative stress response genes, such as HO-1 and gamma-glutamylcysteine synthetase heavy subunit (GCSH; Li et al., 2006b). However, it had not yet been demonstrated whether deltamethrin exposure in vivo actually results in Nrf2 nuclear translocation. Administration of deltamethrin to rat brain tissue did result in a pronounced increase in nuclear Nrf2, probably from translocation from the cytoplasm. Ideally, this translocation would appear as an increase in nuclear Nrf2 and a concomitant decrease in cytosolic protein. Elevated levels of Nrf2 protein in DM-treated rat were, at least in part, due to an increase in Nrf2 gene transcription and cytoplasmic translation that increases Nrf2 in

both cytosolic and nuclear compartments in a coordinated fashion. These results are consistent with our previous findings that demonstrated the appearance of Nrf2 protein in the nuclei concomitant with an increase in the cytosol (Li et al., 2006b).

The *HO-1* gene is a downstream target of Nrf2. Transcription of HO-1 was enhanced in parallel with Nrf2 nuclear translocation. The activity of Nrf2 is normally suppressed in the cytosol by binding to the chaperone Keap1 (Li et al., 2004; Martin et al., 2004; Nguyen et al., 2004). However, upon stimulation by electrophilic agents or ROS that modify thiol groups in Keap1 (Li et al., 2006a; Kobayashi et al., 2004), Nrf2 is liberated and translocates into the nucleus. It was observed that the various dose of DM induced nuclear translocation of Nrf2 in vivo, which supports a recent report on the DM-induced nuclear accumulation of Nrf2 in PC12 cells (Li et al., 2007b). Hence, it appears that DM elicits a signal that presumably passes through the Keap1-Nrf2 complex, resulting in the dissociation of Keap1-Nrf2 followed by nuclear accumulation of Nrf2. The signal that dissociates Keap1-Nrf2 could be free radicals, such as nitric oxide (NO[•]), peroxynitrate, or reactive oxygen/nitrogen species. Peroxynitrite is a strong oxidant produced by rapid interaction between super oxide anion and nitric oxide. There is evidence to suggest that peroxynitrite induces the expression of HO-1 in PC12 cells through Nrf2 activation, nuclear translocation, and selective ARE binding (Dhakshinamoorthy et al., 2004). It was reported that free radicals induced transcriptional up-regulation of protective genes through Nrf2-ARE binding (Buckley et al., 2003; Dhakshinamoorthy et al., 2004). The result from our lab demonstrated that DM can enhance the activity of NOS enzyme in cerebral cortex, hippocampus, and cerebellum, resulting in elevated NO[•] (Niu et al., 1997).

Free radical production (possibly superoxide oxygen, nitrogen-centered, or carbon-centered semiqui-none free radicals) was enhanced in brain by DM exposure. When cells were pretreated with NAC, however, a scavenger of ROS, DM-induced ROS accumulation was suppressed. These results suggest that ROS generation is an upstream mediator of nuclear Nrf2 accumulation (Li et al., 2007b). Collectively, these data support the notion that Nrf2 translocation is probably dependent on free radicals generated by DM. It is still possible, however, that other induction process are involved in the DM response. Further, these responses may be associated but not causatively linked. It is thus critical to provide additional evidence to support the hypothesis that DM induces Nrf2 via ROS/free radicals. One further approach will be to test whether antioxidants are able to block Nrf2 activation in vivo.

Previous studies have demonstrated that Nrf2-dependent transcription can prevent ROS-induced apoptosis in neurons and astrocytes in vitro (Cao et al., 2005; Kraft et al., 2004; Lee et al., 2003a, b; Shih et al., 2003). Several lines of evidence suggest that chemical induction of antioxidants and detoxification genes through Nrf2-ARE signaling protects against a number of different oxidative insults, including H₂O₂, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lee et al., 2003a, b; Cao et al., 2005; Kraft et al., 2004; Shih et al., 2003). Recently, we demonstrated that Nrf2-dependent transcription activated by tert-Butylhydroquinone (*t* BHQ), a known inducer of Nrf2-mediated transcription, can prevent DM-induced oxidative stress in PC12 cells through translocation of Nrf2 and increased HO-1 gene expression (Li et al., 2007a). The present

results showed that HO-1 mRNA expression was induced in rat brain by DM, consistent with previous results demonstrating increases in protein expression and HO-1 activity in rat brain treated with DM. This implies that Nrf2 could confer antioxidant neuroprotection through coordinated induction of ARE-driven genes.

In conclusion, we have detected increases in Nrf2 protein, HO-1 mRNA, and free radicals in vivo in response to deltamethrin, a derivative of the widely used pyrethroid pesticides. These findings demonstrate for the first time that Nrf2 translocation from cytoplasm to nucleus is initiated in vivo and is probably a response to the DM-dependent induction of free radicals. These findings define enhanced Nrf2 expression as a primary molecular response to DM neurotoxicity. This report adds to our understanding of the mechanisms of deltamethrin toxicity and to the molecular mechanisms of cellular antioxidant defense.

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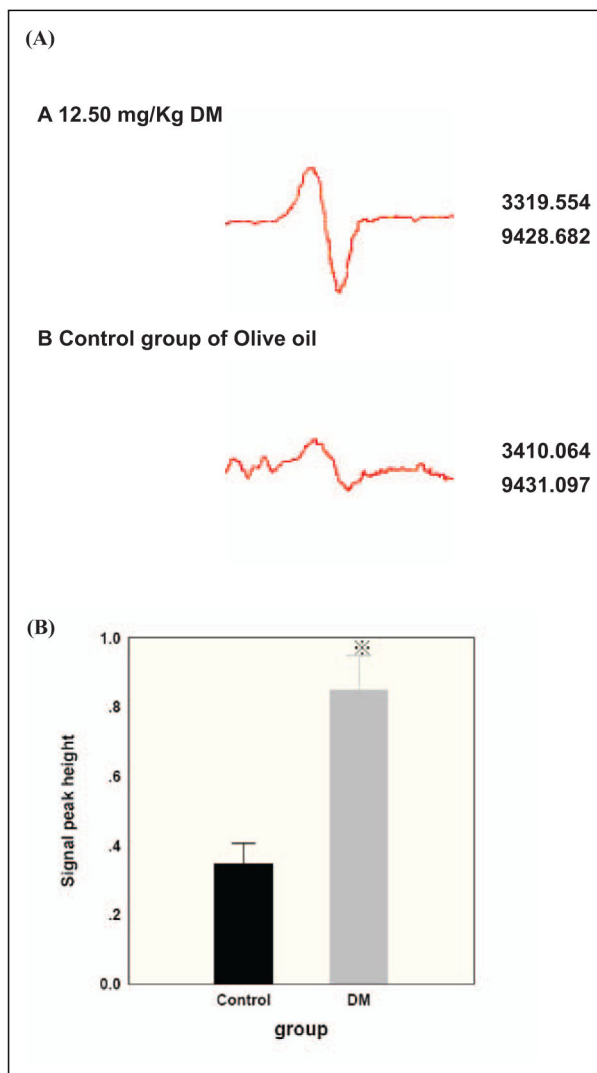


Figure 1.

The induction of free radicals in rat hippocampal tissue during administration of deltamethrin (DM) as detected by electron spin resonance (ESR) spectroscopy. (A) ESR measurements were carried out on an X-band ESR spectrometer under the following experimental conditions: TE = 77 K, SF = 100 KHz, MA = 1 G, CF = 3350 G, SW = 200 G. Magnetic intensity and microwave frequencies are shown by the first and second line in the right upper of each panel. (B) Free radical concentrations derived from the amplitude of signal peaks. Values are mean \pm standard deviation of two determinations. (symbol) $p < 0.05$ relative to the control group.

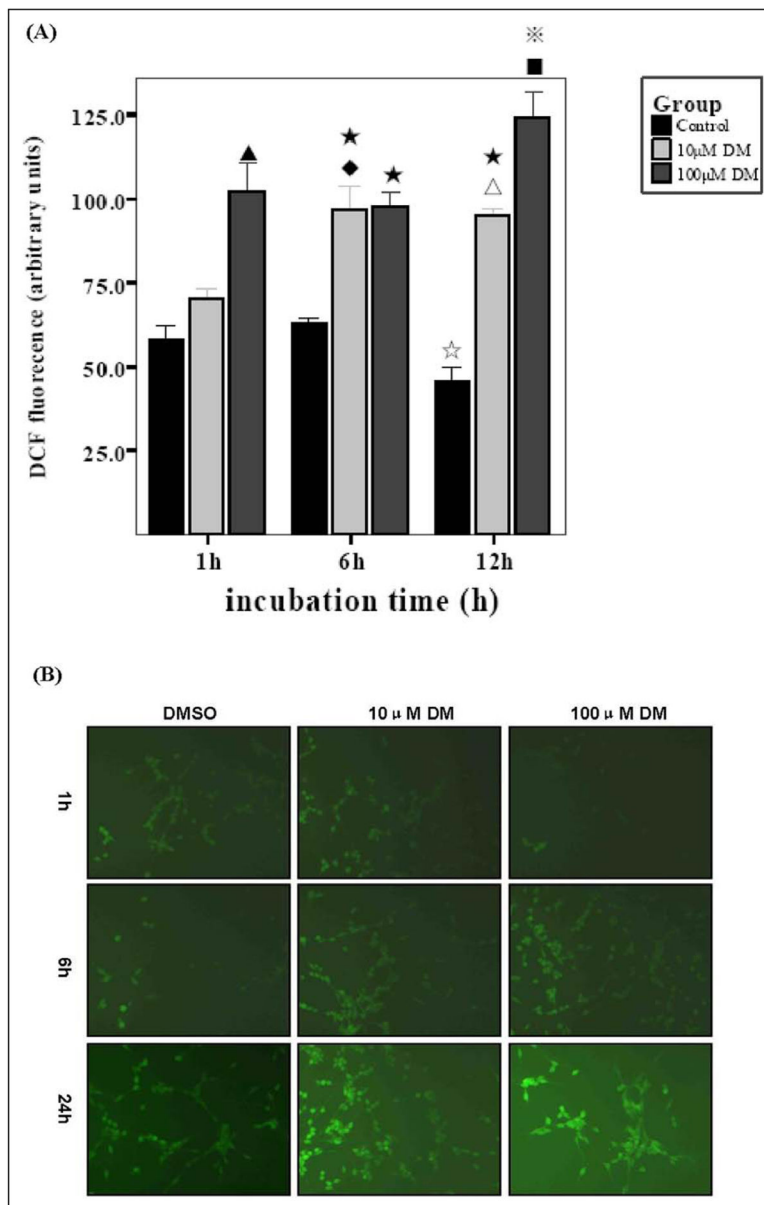


Figure 2.

Reactive oxygen species (ROS) generation in PC12 cells was increased by treatment with deltamethrin (DM). (A) PC12 cells were treated with DM for 1, 6, or 12 h. The treated cells were loaded with H₂DCF-DA (10 μ M) and DCF fluorescence intensity (Arbitrary units of DCF fluorescence) was measured using a fluorescent spectrophotometer. Values are mean \pm standard deviation of three determinations. (B) The cells were treated with various dose of DM (0, 10, or 100 μ M) for various times (1, 6, or 24 h), washed three times with PBS, and loaded with 10 μ M H₂DCF-DA for 30 min. The DCF fluorescence intensity was recorded by fluorescence and phase contrast combination microscopy.

▲ $P < 0.01$ vs. time-matched control cells;

★ $P < 0.01$, ※ $P < 0.001$ vs. time-matched control cells;

■ $P < 0.01$ vs. time-matched cells with 10 pM DM;

☆ $P < 0.05$ vs. treatment-matched cells for 6 h;
△ $P < 0.05$, ◆ $P < 0.01$ vs. treatment-matched cells foil h.

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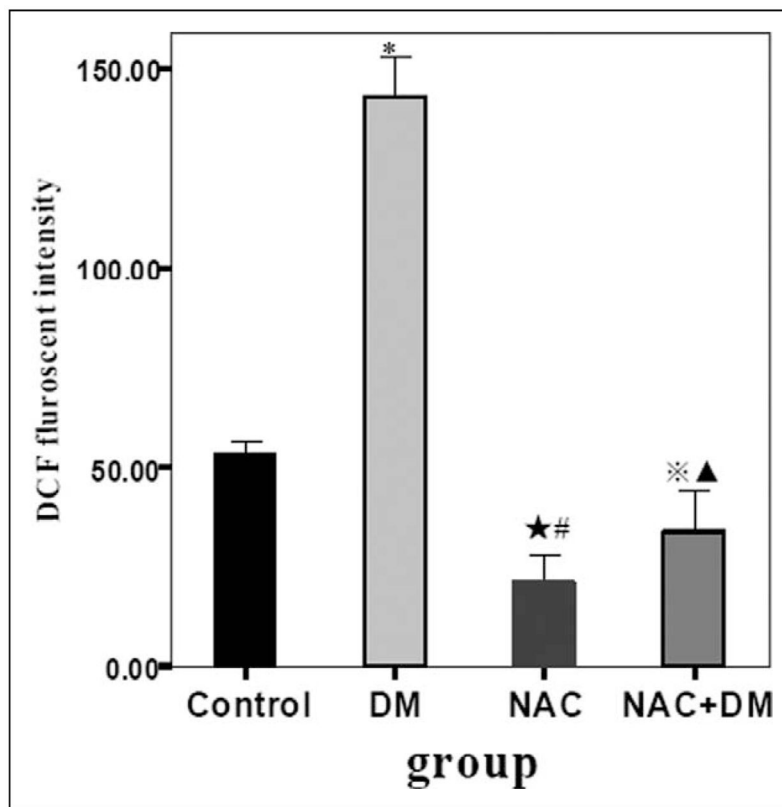


Figure 3.

The deltamethrin (DM)-evoked 2', 7'-dichloro-fluorescein (DCF) fluorescence intensity increase was attenuated by *N*-acetyl cysteine (NAC). PC12 cells were pretreated with or without NAC, followed by incubation with or without 10 μ M DM for 6 h. The treated cells were loaded with H₂DCF-DA (10 μ M) and DCF fluorescence intensity (Arbitrary units of DCF fluorescence) was measured using a fluorescent spectrophotometer. Values are mean \pm standard deviation of three determinations.

* $P < 0.001$, ★ $P < 0.01$, ※ $P < 0.05$ vs. control cells;

$P < 0.001$, ▲ $P < 0.01$ vs. DM treated cells.

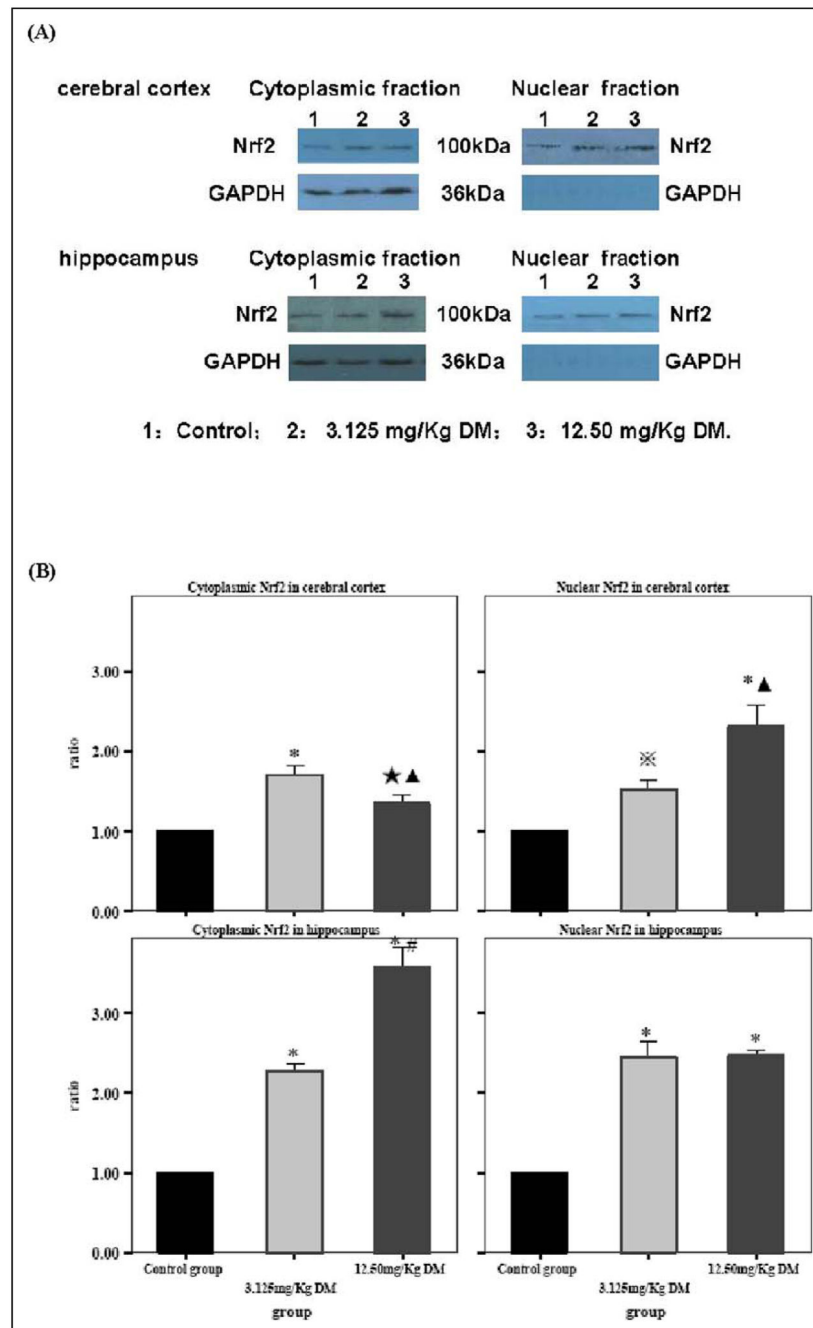


Figure 4. Changes in Nrf2 protein expression in hippocampus and cerebral cortex following deltamethrin (DM) exposure. The rats were exposed to olive oil (control), 3.125 mg/kg DM (low dose), or 12.50 mg/kg DM (high dose) for 5 days. Protein expression was examined by immunoblot analysis. Equal amounts of protein extract (45 μ g) were loaded in each lane of 8.0% (w/v) SDS-PAGE gels, and the blot was probed with the polyclonal anti-NF-E2-related factor 2 (Nrf2) antibody (1:500) overnight. The protein was visualized by chemiluminescence. (A) Representative immunoblot of Nrf2. Immunoblot for GAPDH, a

cytoplasmic marker used as an internal control in detecting cytoplasmic Nrf2 protein, indicated that the nuclear fraction was not contaminated by the cytoplasmic fraction. (B) Quantification of Nrf2 densitometric analysis. Ratio = relative level of target protein expression in experimental group relative to the level of target protein expression in the control group. Values are mean \pm standard deviation of three determinations.

* $P < 0.001$, ★ $P < 0.01$, $P < 0.05$ vs. control group;

$P < 0.001$, ▲ $P < 0.01$ vs. 3.125 mg/kg DM-treated group.

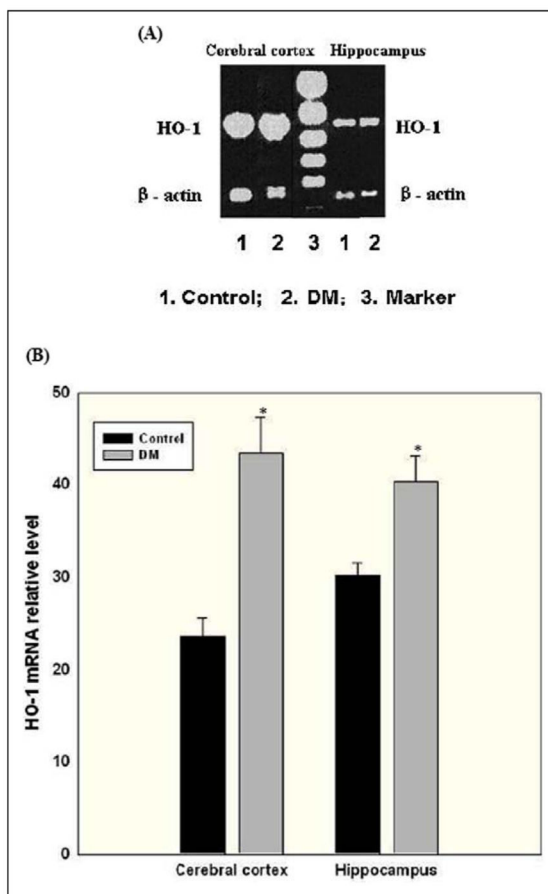


Figure 5. Changes in HO-1 mRNA expression in cerebral cortex and hippocampus following deltamethrin (DM) exposure. (A) The rats were exposed to olive oil (control) or 12.50 mg/kg DM for 5 days. Then total RNA in cerebral cortex and hippocampus was isolated and subjected to RT-PCR. (B) Quantification of HO-1 mRNA levels. Values are mean \pm standard deviation of five determinations. * $P < 0.001$ vs. control group

Table 1

Primer sequences used in RT-PCR analysis

Gene name	Primer sequence for PCR	Size of expected product (bp)
<i>HO-1</i>	5'-GAATTCAGCATGCCCCAGGATTTG-3' 3'-AGGACGAGTTGTAGGTCGATCAGATCT-5'	615
β - <i>actin</i>	5'-GAGCACCTGTGCTGCTCACCGAGG-3' 3'-TCGCACCGATGTCGAAGTGGTGGTG-5'	310

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