

# Mechanism of Pyrethroid Pesticide–Induced Apoptosis: Role of Calpain and the ER Stress Pathway

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Exposure to the pyrethroid pesticide deltamethrin has been demonstrated to cause apoptosis both *in vitro* and *in vivo*. However, the molecular pathways leading to deltamethrin-induced apoptosis have not been established. To identify these pathways, SK-N-AS neuroblastoma cells were exposed to deltamethrin (100nM–5 $\mu$ M) for 24–48 h. Deltamethrin produced a time- and dose-dependent increase (21–300%) in DNA fragmentation, an indicator of apoptosis. Data demonstrate that the initiation of DNA fragmentation resulted from interaction of deltamethrin with Na<sup>+</sup> channels and consequent calcium influx, as tetrodotoxin and the intracellular Ca<sup>2+</sup> chelator BAPTA-AM completely prevented apoptosis. DNA fragmentation was accompanied by increased caspase-9 and -3 activities and was abolished by specific caspase-9 and -3 inhibitors. However, deltamethrin did not increase cytosolic cytochrome *c* levels, indicating that the mitochondrial pathway was likely not involved. Additional studies demonstrated that deltamethrin exposure activated caspase-12 activity and that pharmacological inhibition and siRNA knockdown of calpain prevented deltamethrin-induced DNA fragmentation, thus indicating a role for the endoplasmic reticulum (ER) stress pathway. This was confirmed by the observation that inhibition of eIF2 $\alpha$  abolished deltamethrin-induced DNA fragmentation. Together, these data demonstrate that deltamethrin causes apoptosis through its interaction with Na<sup>+</sup> channels, leading to calcium overload and activation of the ER stress pathway. Because ER stress and the subsequent unfolded protein response have been observed in a number of neurodegenerative diseases, these data provide mechanistic information by which high-level exposure to pyrethroids may contribute to neurodegeneration.

**Key Words:** pyrethroid; pesticide; neurodegeneration; ER stress; siRNA; calpain; caspases; apoptosis; eIF2 $\alpha$ ; cytosolic calcium; sodium influx; sodium channel.

Pyrethroid insecticides are widely used to control a broad range of insect-pests in agriculture, public health, veterinary medicine, and residential settings, accounting for about 25% of the worldwide insecticide market (Casida and Quistad, 1998). In

recent years, the use of pyrethroids has been increasing because restrictions have been placed on many of the organophosphorus insecticides. Although exposure of the general population is thought to be generally low, high levels of exposure have been observed in pesticide applicators (Calvert *et al.*, 2003; Kimata *et al.*, 2009), and pyrethroid-related poisoning reports to poison control centers have increased over recent years (Power and Sudakin, 2007). Furthermore, residual neurological symptoms in individuals highly exposed to pyrethroids have been reported (Muller-Mohnssen, 1999). These findings raise concern over the potential long-term neurotoxic effects of high-level exposures.

Pyrethroids have been generally classified into two subclasses, type I and type II, based on high dose exposure. Type I pyrethroids produce the T-syndrome, which is characterized by whole body tremor and type II pyrethroids produce the CS-syndrome, which is characterized by choreoathetosis and salivation (Breckenridge *et al.*, 2009; Soderlund *et al.*, 2002). The mechanism by which pyrethroids are thought to exert neurotoxicity is by prolonging the opening of Na<sup>+</sup> channels (Narahashi, 1996). This prolonged opening of Na<sup>+</sup> channels results in persistent depolarization, leading to repetitive firing, and if the exposure is high enough, seizures, paralysis, and death (Bradberry *et al.*, 2005). However, recent attention has focused on potential alternative targets that may be involved in pyrethroid toxicity (Breckenridge *et al.*, 2009). Specifically, various lines of evidence have suggested possible roles for voltage-gated calcium channels, chloride channels, GABA<sub>A</sub> receptors, and the mitochondrial electron transport chain in the acute manifestation of neurotoxicity elicited by pyrethroids (Breckenridge *et al.*, 2009; Soderlund *et al.*, 2002). Although these studies have provided important insight into potential mechanisms by which pyrethroids produce their characteristic syndromes following high-level exposure, little attention has focused on the long-term neurological and pathological effects of such exposures.

Recently, attention has focused on the potential relationship between pyrethroid exposure and neurodegeneration

(Abdel-Rahman *et al.*, 2004; Bloomquist *et al.*, 2002; Elwan *et al.*, 2006; Tayebati *et al.*, 2009). As with the classic pyrethroid poisoning-induced syndromes, the mechanism(s) by which pyrethroids result in neurodegeneration is not clear. However, higher level, but nonlethal, exposure to the type II pyrethroid insecticide deltamethrin has been shown to cause apoptosis both *in vitro* (Elwan *et al.*, 2006; Wu *et al.*, 2003) and *in vivo* (Wu and Liu, 2000a,b). Although apoptosis is important for the regulation of normal physiological function-throughout life, excessive apoptosis contributes to pathological cell death observed in several neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Arduino *et al.*, 2009). Thus, the ability of pyrethroid insecticides to cause apoptosis may contribute to the potential for high-level exposures to contribute to neurodegeneration. However, the mechanism by which pyrethroids, and in particular deltamethrin, induces apoptosis has not been established.

Here, we sought to identify the molecular mechanism and pathway by which deltamethrin causes apoptosis. The data reveal that deltamethrin initiates the apoptotic cascade through interaction with Na<sup>+</sup> channels, leading to calcium overload and initiation of the apoptotic cascade. However, the mitochondrial-mediated pathway did not appear to be involved as there was no release of cytochrome *c* release from the mitochondria or alteration of mitochondrial membrane potential. Further studies identified the role of calpain and the endoplasmic reticulum (ER) stress pathway as mediators of deltamethrin-induced apoptosis. Given the prominent role of apoptosis, calpain, and the ER stress pathway in neurodegeneration (Culmsee and Landshamer, 2006; Vosler *et al.*, 2008), these data provide mechanistic information as to how high-level exposure to pyrethroids could result in neurodegeneration.

## MATERIALS AND METHODS

**Materials.** Deltamethrin (lot # 392-7B, purity: 99%) was purchased from Chem Service Inc. (West Chester, PA). Cell culture supplies, including minimum essential medium (MEM) with Earl's salts and L-glutamine, phosphate-buffered saline (PBS) 1× without calcium and magnesium, MEM nonessential amino acids 100×, sodium pyruvate (100mM), trypsin EDTA (0.05% trypsin, 0.53mM EDTA in Hank's Balanced Salt Solution (HBSS), without sodium bicarbonate, calcium, and magnesium) 1×, and fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Herndon, VA). Cytotoxicity Detection Kit (lactate dehydrogenase, LDH) and the Cell Death Detection ELISA<sup>PLUS</sup> assay kit were obtained from Roche Applied Science (Indianapolis, IN). Caspase-9 substrate (Ac-LEHD-7-amino-4-methylcoumarin [AMC]), specific caspase-3 inhibitor (Z-DEVD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), and calpain inhibitor (PD150606) were obtained from Alexis Biochemicals (San Diego, CA). Caspase-3 substrate (Ac-DEVD-AMC) was purchased from Bachem Americas, Inc. (Torrance, CA). Digitonin, Ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA), Tris-HCl, selective intracellular Ca<sup>2+</sup> chelator (BAPTA-AM), and EDTA 2Na were obtained from Sigma (St. Louis, MO). The ER stress inhibitor salubrinal (eIF2 $\alpha$  inhibitor) was obtained from EMD chemicals (Gibbstown, NJ). The caspase-12 activity assay kit was purchased from BioVision, Inc. (Mountain View, CA) and mitochondrial isolation kit was purchased from Pierce (Rockford, IL).

Materials for Western blot were purchased from Invitrogen (Carlsbad, CA). Purified mouse anti-cytochrome *c* was purchased from BD Biosciences-Pharmingen (San Diego, CA). All other chemicals and reagents were obtained from Fisher Scientific (Pittsburgh, PA).

**Cell culture.** SK-N-AS human neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in MEM containing 10% fetal bovine serum, 2mM L-glutamine, 5mM sodium pyruvate, 5mM nonessential amino acids, 50 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin. SK-N-AS cells were chosen as a model based on previous observations that deltamethrin targets the dopamine system (Bloomquist *et al.*, 2002; Elwan *et al.*, 2006), and these cells have dopaminergic characteristics, including expression of tyrosine hydroxylase and the dopamine transporter (Wang and Bannon, 2005). SK-N-AS cells also express several Na<sup>+</sup> channel isoforms that are sensitive to deltamethrin (data not shown) and have been used to investigate mechanisms of apoptosis (Day *et al.*, 2009).

Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Three days after plating in a flask, cells were resuspended in MEM medium and then plated at  $2 \times 10^4$  cells per well into 96-well plate. A stock solution of 10mM deltamethrin was prepared in absolute ethanol (EtOH), and dilutions of deltamethrin were made in MEM medium and added to the cells. The final concentration of EtOH was less than 0.5% for all experiments. Cells were exposed to EtOH in concentration equivalent to that used in highest concentration of deltamethrin as a vehicle control. This concentration had no effect on any of the parameters studied.

**LDH assay.** The cytotoxic effect of deltamethrin on SK-N-AS cells was monitored as previously described (Elwan *et al.*, 2006) by measuring LDH leakage into the culture media with a cytotoxicity detection kit (Roche Applied Scientific). Cells were incubated with different concentrations of deltamethrin (0–50 $\mu$ M) in MEM medium for 24 and 48 h at 37°C. After the treatment, 100  $\mu$ l of cell-free media was added to 100  $\mu$ l of the catalyst-dye mixture (supplied with kit) in a 96-well plate and then incubated at room temperature for 30 min. The LDH activity was measured at 490 nm with Spectramax microplate reader (Molecular Devices).

**Determination of DNA fragmentation.** DNA fragmentation was determined with the Cell Death Detection ELISA<sup>PLUS</sup> assay kit (Roche Applied Science) according to Elwan *et al.* (2006). Briefly, SK-N-AS cells ( $2 \times 10^4$  cells/well) were exposed to 0–5 $\mu$ M deltamethrin for 24 and 48 h in the presence or absence of Z-DEVD-FMK (50 $\mu$ M), Z-LEHD-FMK (50 $\mu$ M), tetrodotoxin (TTX) (1 $\mu$ M), BAPTA-AM (5 $\mu$ M), PD150606 (10 $\mu$ M), or salubrinal (50 $\mu$ M). At the end of treatment, media was removed, cells were washed once with PBS and lysed. The supernatants were applied to a streptavidin-coated 96-well microtiter plates and incubated with 80  $\mu$ l of immunoreagent (mixture of anti-histone-biotin and anti-DNA-POD). Following incubation, the amount of nucleosomes retained by anti-DNA-POD in the immunocomplex was spectrophotometrically determined with 2,2'-azino-di(3-ethoxybenzyl thiazoline sulfonate) at 405nm using Spectramax microplate reader (Molecular Devices).

**Caspase activity assay.** Caspase-3 and caspase-9 activities were determined according to Kaul *et al.* (2003) with some modifications. Briefly, SK-N-AS cells ( $1 \times 10^6$  cells/ml) were grown in 6-well plate and treated with 0–5 $\mu$ M deltamethrin for 24–48 h. After treatment, cells were lysed with 0.8 ml lysis buffer (50mM Tris-HCl, 1mM EDTA, 10mM EGTA, 10 $\mu$ M digitonin, pH 7.4) for 20 min at 37°C. Lysates were then centrifuged at 10,000 rpm for 15 min and supernatants were incubated with 50 $\mu$ M Ac-DEVD-AMC or Ac-LEHD-AMC at 37°C for 60 min. Caspase activity was then determined by measuring the levels of released AMC using a Spectramax spectrofluorometer with excitation at 380 nm and emission at 460 nm. The activity was calculated as fluorescence units/milligram protein/hour and expressed as percentage of control. Activity of caspase-12 was assayed with kit and protocol supplied by BioVision, Inc.

**Measurement of intracellular Na<sup>+</sup> ([Na<sup>+</sup>]).** Cells were rinsed once with PBS and then incubated with 10 $\mu$ M CoroNa<sup>TM</sup> Green-AM (catalog # C36676, Invitrogen) in oxygenated Krebs-Ringer-HEPES buffer (125 mM NaCl,

4.8 mM KCl, 2.6 mM CaCl<sub>2</sub>, 1.2 mM Mg SO<sub>4</sub>, 25 mM HEPES, 0.1% sucrose and 0.1% BSA, pH 7.4) for 1 h at 37°C. Dye loaded cells were rinsed twice with sucrose and bovine serum albumin (BSA)-free KRH buffer and then Na<sup>+</sup> influx was determined after adding deltamethrin by measuring fluorescence intensity every 30 s over 60 min with excitation 485 nm and emission 515 nm.

**Measurement of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>).** SK-N-AS cells were seeded on a 96-well plate at a density of 2 × 10<sup>4</sup> cells per well. After 12 h, cells were rinsed once with PBS and then incubated with 5 μM Calcium Green-1-AM ester (catalog # C3012, Invitrogen) in oxygenated KRH buffer for 1 h at 37°C. After loading the calcium dye, cells were rinsed twice with sucrose and BSA-free KRH buffer then warmed up in a Spectramax spectrofluorometer for 10 min at 37°C. The test compounds were added to the cells after obtaining a stable baseline. Intracellular Ca<sup>2+</sup> was determined by measuring fluorescence intensity in every 5 s over 60 min at excitation 506 nm and emission 531 nm.

**Measurement of mitochondrial membrane potential (ΔΨ<sub>m</sub>).** Rhodamine 123 (Rh123) (catalog # R302, Invitrogen), a cell-permeant cationic green-fluorescent dye was used to determine mitochondrial ΔΨ<sub>m</sub>. Briefly, cells were plated at 2.5 × 10<sup>4</sup> cells per well in a black clear bottom 96-well plates and treated with 0, 1, 5, 10, and 25 μM deltamethrin for 1, 12, 24, and 48 h. At the end of treatment, cells were incubated with 10 μM Rh123 in phenol red free MEM medium for 30 min at 37°C and then washed once with PBS. The fluorescence intensity was measured with excitation at 485 nm and emission at 530 nm using a Spectramax microplate reader (Molecular Devices).

**Western immunoblotting.** SK-N-AS cells (2 × 10<sup>7</sup>) were exposed to deltamethrin (0–5 μM) for 24 and 48 h at 37°C. Cells were washed once with PBS and then cytosolic fractions were extracted using mitochondria isolation kit (Pierce) for cytochrome *c* expression. For CHOP/GADD153 expression, cells were harvested in lysis buffer (catalog # 1067-100, BioVision, Inc.) with 0.1% protease inhibitor and kept on ice for 20 min. Samples were centrifuged at 14,000 × g for 10 min and supernatants were collected. Protein concentrations were quantified using the bicinchoninic acid assay (Smith *et al.*, 1985), and 20 μg of protein sample was loaded per lane on 4–12% NuPAGE Novex Bis-Tris Mini Gels (Invitrogen). After electrophoresis, proteins from the gels were blotted to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated in 7.5% nonfat milk in 0.1% Tween 20 containing Tris buffered saline (TTBS) for 1 h at room temperature to block nonspecific protein binding sites. The membranes were then incubated overnight at 4°C with 1:2000 diluted anti-cytochrome *c* monoclonal primary antibody (catalog # 556433, BD Pharmingen) or a polyclonal antibody to CHOP/GADD153 (catalog # sc-575, Santa Cruz, CA). After being washed three times with TTBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The specific antibody bound protein was detected by SuperSignal<sup>®</sup> West Dura Extended Duration Substrate (Thermo Scientific Pierce) using Alpha Innotech Fluorochem (San Leandro, CA) imaging system and stored as a digital image. Membranes were then stripped for 15 min at room temperature with Pierce Stripping Buffer and re-probed with a monoclonal α-tubulin antibody to confirm equal protein loading in each lane.

**siRNA transfection.** Transfection was performed according to the manufacturer's (Invitrogen) protocol. Twenty-four hours after culturing in antibiotic free MEM media, cells (50–60% confluence) were transfected with 15 nM siRNA (Santa Cruz, CA) using a lipid-based transfection reagent (Lipofectamine RNAiMAX, Invitrogen) in order to knockdown calpain-1. A scrambled siRNA sequence was employed in parallel wells to control for nonspecific effects of transfection. At 24 h posttransfection, cells were exposed to 5 μM deltamethrin for an additional 24 h and used for Western blot and DNA fragmentation assays.

**Statistical analysis.** Statistical analysis was performed using Prism 5.01 software (Graphpad software, San Diego, CA). All experiments were performed at least 3 times on separate day using different vials of frozen cell stocks. On individual days, experiments were performed in duplicate or triplicate and averaged to form a single experimental unit.

Data are presented as mean ± SEM. All analyses were performed on raw data using analysis of variance. Bonferroni's or Tukey's post hoc multiple comparison tests were performed where appropriate. Statistical significances were determined at level of *p* < 0.05.

## RESULTS

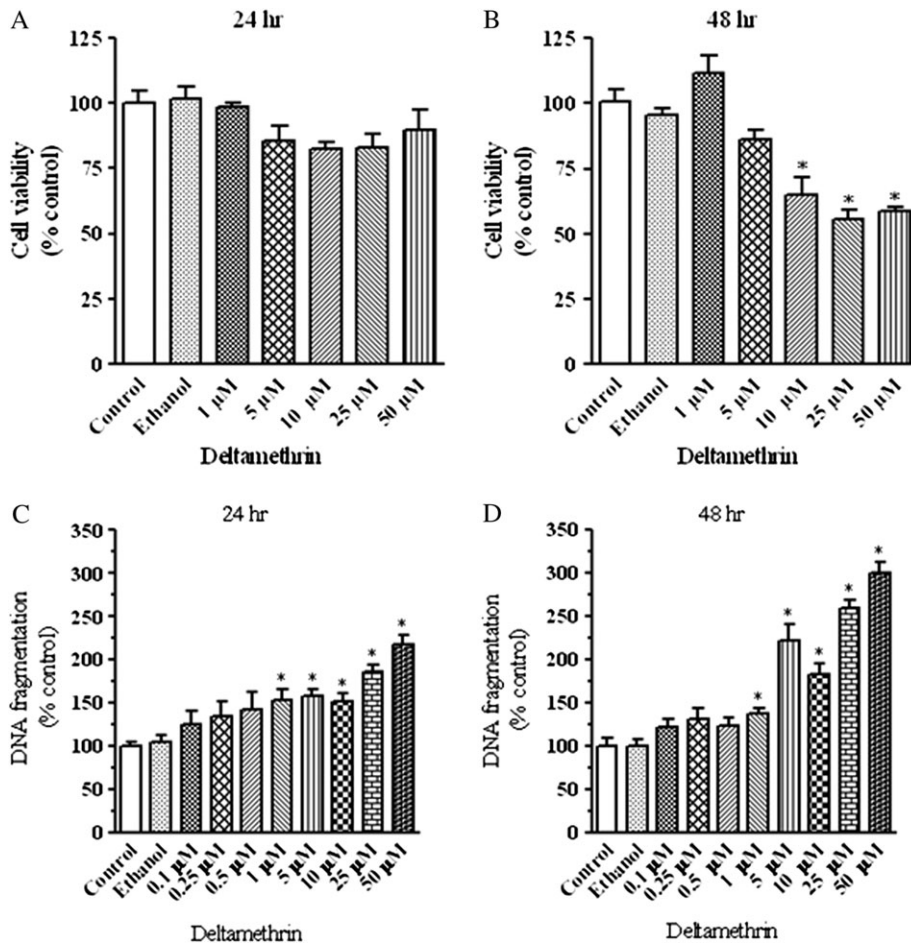
### *Deltamethrin Induces Dose-Dependent Cell Death and Apoptosis in SK-N-AS Neuroblastoma Cells*

To determine the time-course and dose-response of deltamethrin-induced cell death, SK-N-AS neuroblastoma cells were exposed to various concentrations (0–50 μM) of deltamethrin for 24 or 48 h. Overt cell death was determined by measuring LDH release from cells into incubation medium. Exposure to deltamethrin resulted in time- and dose-dependent cell death at concentrations of 10 μM and above (Figs. 1A and 1B). Similar results were observed using alamar blue as an indicator of cell death (data not shown). However, DNA fragmentation, an indicator of apoptosis, increased in a dose- and time-related manner following exposure to deltamethrin (100 nM–50 μM), ranging from 24 to 218% at 24 h after exposure (Fig. 1C) and from 21 to 300% after 48 h of exposure (Fig. 1D).

### *Role of Sodium Channels and Calcium Homeostasis in Deltamethrin-Induced Apoptosis*

Because prolonged opening of Na<sup>+</sup> channels by pyrethroids increases cellular Ca<sup>2+</sup> influx and subsequent cell depolarization (Narahashi, 1996), and elevation of intracellular Ca<sup>2+</sup> concentration has been reported to cause neuronal cell death (Arduino *et al.*, 2009), we sought to assess the relative contributions of sodium channel activation and calcium influx in deltamethrin-induced apoptosis. To determine whether deltamethrin increased cytosolic Na<sup>+</sup> and Ca<sup>2+</sup>, cells were treated with 5 μM deltamethrin, and Na<sup>+</sup> and Ca<sup>2+</sup> influx was measured every 5 s with CoroNa Green-AM and Calcium Green-1-AM, respectively. Deltamethrin (5 μM) caused a rapid increase in Na<sup>+</sup> (40 ± 8%, *p* < 0.05) and Ca<sup>2+</sup> influx (21 ± 0.3%, *p* < 0.05), which remained elevated over the course of monitoring (Figs. 2A and 2B).

To determine the role of Na<sup>+</sup> flux in deltamethrin-induced apoptosis, cells were cotreated with TTX, a selective Na<sup>+</sup> channel blocker, and DNA fragmentation was determined following deltamethrin exposure for 24 and 48 h. Cotreatment with 1 μM TTX completely blocked the deltamethrin-induced DNA fragmentation (Fig. 2C), demonstrating the requirement of deltamethrin interaction with Na<sup>+</sup> channels for the initiation of apoptosis. To determine the role of intracellular Ca<sup>2+</sup> in deltamethrin-induced apoptosis, cells were pretreated with 5 μM of BAPTA-AM, a chelator of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) for 30 min before adding the deltamethrin. Pretreatment with BAPTA-AM completely abolished DNA fragmentation induced by deltamethrin (Figs. 2D and 2E), demonstrating the role of elevated [Ca<sup>2+</sup>]<sub>i</sub> in deltamethrin-induced apoptosis.



**FIG. 1.** Deltamethrin produces a dose- and time-related increase in cell death and apoptosis. Deltamethrin-induced cell death at 24 (A) and 48 h (B), as determined by LDH release and apoptosis at 24 (C) and 48 h (D), as determined by DNA fragmentation ELISA in SK-N-AS neuroblastoma cells. Data represent mean  $\pm$  SEM from three to six separate experiments, each performed in triplicate and presented as percentage of control. \* indicates significantly different from control ( $p < 0.05$ ).

*Activation of Caspase-3 and -9 by Deltamethrin in SK-N-AS Neuroblastoma Cells*

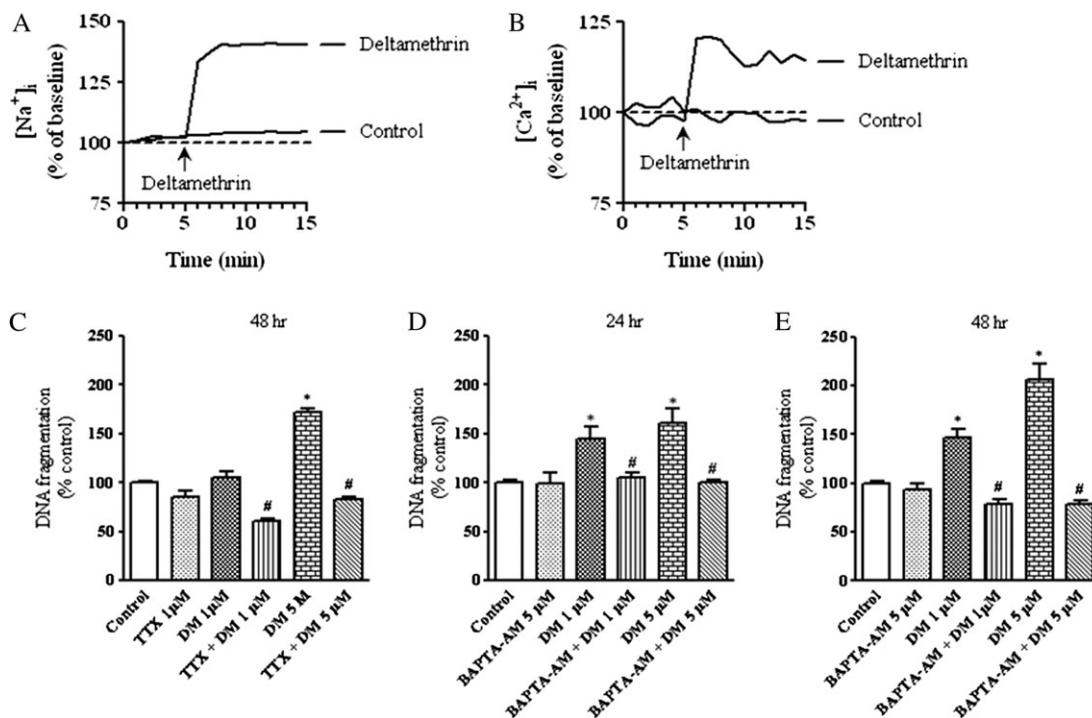
To assess the downstream mediators of deltamethrin-induced DNA fragmentation, we first examined the involvement of caspase-3, which has been recognized to play an important role in the execution phase of neuronal and nonneuronal cell apoptosis (Cohen, 1997). Deltamethrin exposure caused a modest dose- and time-dependent increase in caspase-3 activity (Figs. 3A and 3B). At 24 h postexposure to 5  $\mu$ M deltamethrin, there was a 17% increase in caspase-3 activity, whereas 21 and 26% increases were observed with 1 and 5  $\mu$ M deltamethrin at 48 h following exposure (Fig. 3B). To directly assess the role caspase-3 in deltamethrin-induced apoptosis, cells were cotreated with 50  $\mu$ M Z-DEVD-FMK, a caspase-3 inhibitor, which completely abolished deltamethrin-induced DNA apoptosis (Figs. 3C and 3D).

Caspase-9 is an intracellular cysteine proteases known to cleave and activate caspase-3, resulting in DNA fragmentation and eventual apoptosis (Cohen, 1997; Kitazawa *et al.*, 2003).

At 24 and 48 h after treatment, SK-N-AS cells exposed to deltamethrin showed a time- and concentration-dependent activation of caspase-9 activity (Figs. 4A and 4B). Treatment with 1 or 5  $\mu$ M deltamethrin for 24 h increased caspase-9 activity by 24 and 36%, respectively (Fig. 4A). In contrast, exposure to 1 and 5  $\mu$ M deltamethrin for 48 h resulted in 56 and 63% increase in caspase-9 activation, respectively (Fig. 4B). When cells were cotreated with 50  $\mu$ M Z-LEHD-FMK, a caspase-9 inhibitor, deltamethrin-induced apoptosis was prevented (Figs. 4C and 4D).

*Deltamethrin Does Not Cause Mitochondrial Cytochrome c Release*

Based on the ability of deltamethrin to induce caspase-9 and -3 activity, we measured the release of cytochrome *c* from mitochondria into cytosol, which is an important signal for apoptosome formation and caspase-9 activation (Gorman *et al.*, 2000). No changes in the levels of cytosolic cytochrome *c* were observed at 24 or 48 h after deltamethrin exposure



**FIG. 2.** Requirement of sodium channel activation and intracellular calcium for deltamethrin-induced apoptosis. Representative traces of  $[Na^+]_i$  (A) and  $Ca^{2+}$  (B) influx in SK-N-AS cells. Pretreatment with the  $Na^+$  channel antagonist TTX ( $1\mu M$ ) prevented deltamethrin-induced DNA fragmentation (C). Chelation of intracellular calcium by BAPTA-AM prevented deltamethrin-induced DNA fragmentation (D and E). Data represent mean  $\pm$  SEM of three to five individual experiments, each performed in triplicate and expressed as percentage of control. \* indicates significantly different from control and # indicate significant differences between deltamethrin-treated cells and deltamethrin plus TTX or BAPTA-AM-treated cells ( $p < 0.05$ ).

(Figs. 5A–C). Furthermore, we observed no significant alteration in mitochondrial membrane potential (Figs. 5D and 5E), suggesting an alternate pathway not initiated by mitochondrial cytochrome *c* release was likely responsible for deltamethrin-induced apoptosis.

#### Activation of Caspase-12 and Calpain by Deltamethrin in SK-N-AS Cells

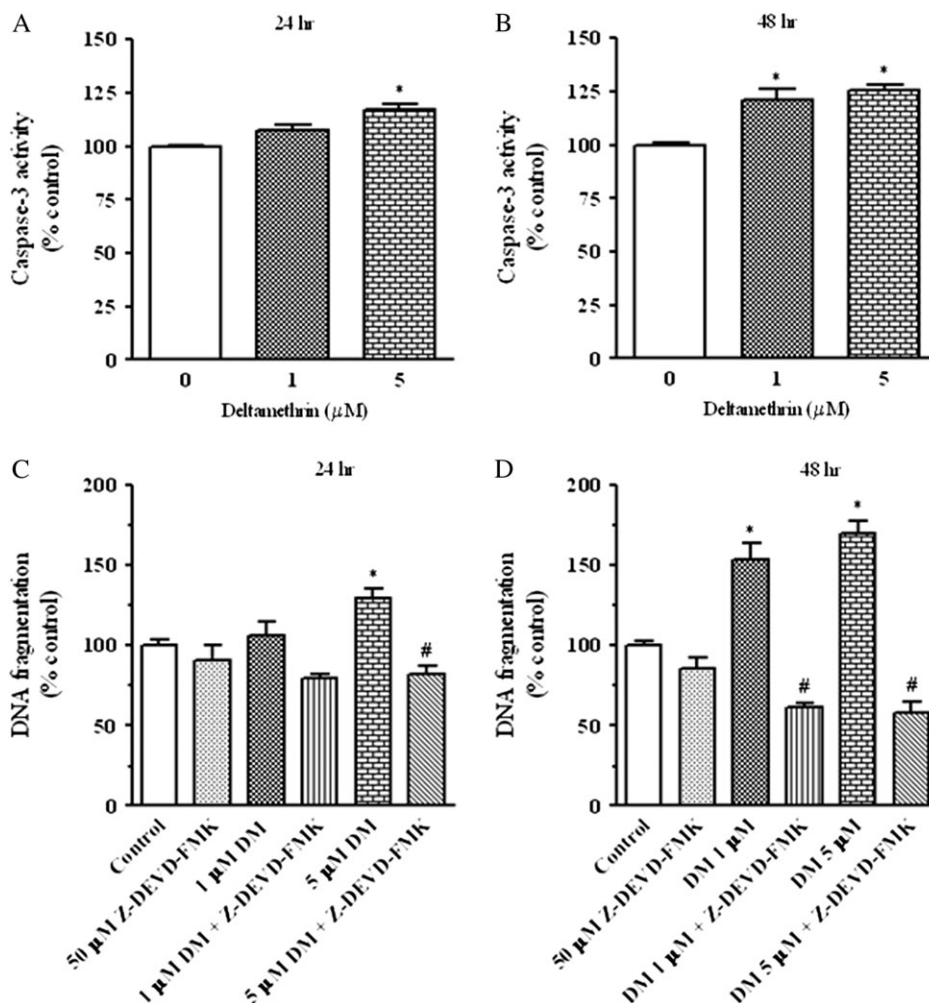
As an alternate mechanism, calcium-induced caspase-12 can directly activate caspase-9, without release of cytosolic cytochrome *c* from mitochondria, as part of the ER stress pathway leading to apoptosis (Morishima *et al.*, 2002; Nakagawa and Yuan, 2000; Rao *et al.*, 2001). Therefore, we examined whether caspase-12 was activated during deltamethrin exposure to SK-N-AS cells. As shown in Figures 5F and 5G, deltamethrin significantly increased the activation of caspase-12 in a dose- and time-dependent manner. Caspase-12 was activated (28–45%) as early as 12 h after deltamethrin exposure (Fig. 5F) and was increased to 30–58% following 24 h of exposure (Fig. 5G).

Activation of calpain, a calcium-dependent cysteine protease, is known to activate caspase-12 when stimulated by release of intracellular calcium from the ER (Nakagawa and Yuan, 2000). To determine whether calpain was activated

during deltamethrin-induced apoptosis, DNA fragmentation was measured following cotreatment with PD150606, a specific calpain inhibitor. PD150606 completely prevented deltamethrin-induced DNA fragmentation (Figs. 6A and 6B). To further substantiate the functional role of calpain in deltamethrin-induced apoptotic cell death, we examined the effects of siRNA knockdown of calpain-1 on deltamethrin-induced DNA fragmentation. Treatment of SK-N-AS cells for 24 h with calpain-1 siRNA reduced calpain-1 protein expression by 71% (Fig. 6C). Knockdown of calpain-1 by siRNA prior to treatment with deltamethrin abolished deltamethrin-induced DNA fragmentation (Fig. 6D), confirming an integral role for calpain in deltamethrin-induced apoptosis and providing additional evidence for a role of the ER stress pathway.

#### Activation of the ER Stress Pathway by Deltamethrin Leads to Apoptosis

To confirm that deltamethrin-induced apoptosis via the ER stress pathway, we determined the effect of deltamethrin on the induction of CHOP/GADD153, an indicator of ER stress (Xu *et al.*, 2005). Western blot analysis revealed the significant induction of CHOP/GADD153 protein at both 24 and 48 h after deltamethrin exposure (Figs. 7A–C). Cotreatment of SK-N-AS cells with salubrinal, an inhibitor of eIF2 $\alpha$ , which is an



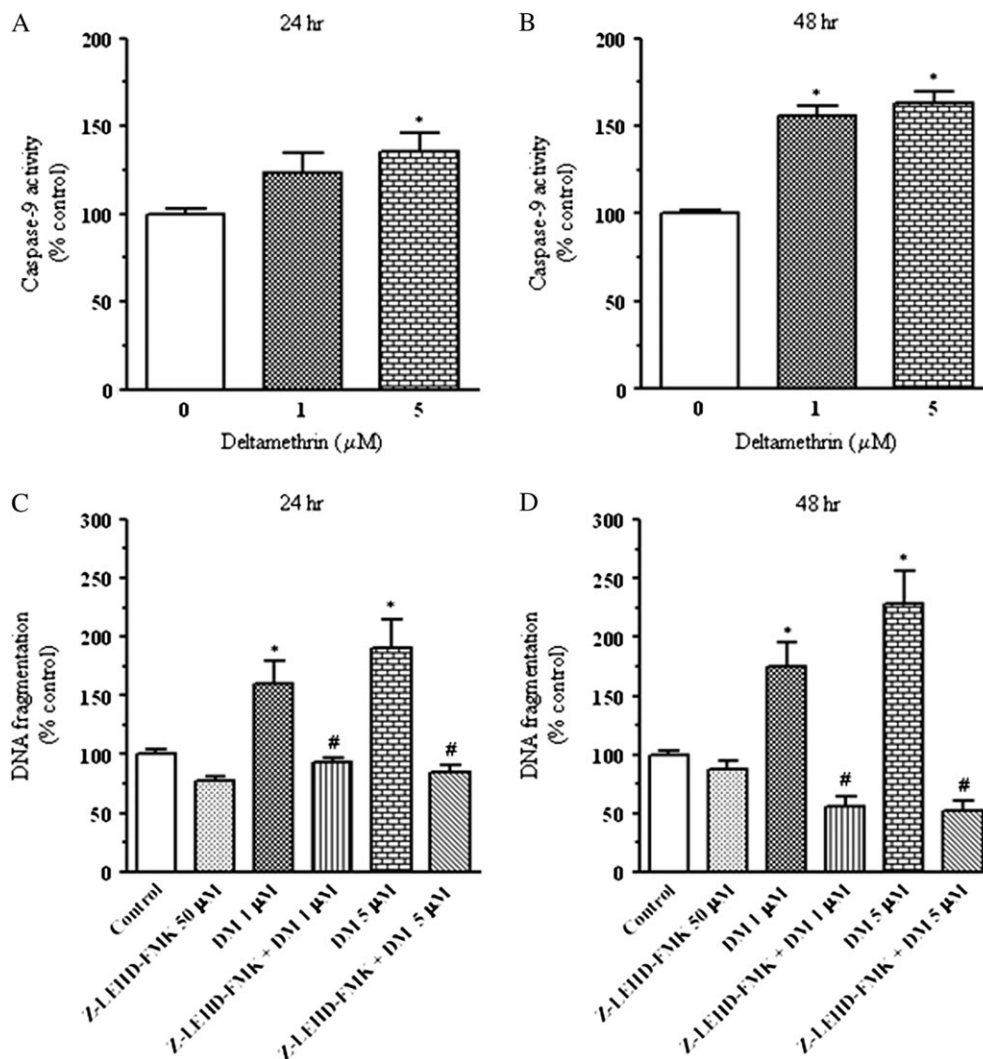
**FIG. 3.** Role of caspase-3 in deltamethrin-induced apoptosis. Exposure to deltamethrin increased the activation of caspase-3 in a dose- and time-dependent manner (A and B). Deltamethrin-induced DNA fragmentation was completely prevented by the caspase-3 inhibitor Z-DEVD-FMK (C and D). Data represent mean  $\pm$  SEM of four individual experiments, each performed in triplicate and expressed as percentage of control. \* indicates significantly different from control and # indicate significant differences between deltamethrin-treated cells and deltamethrin plus Z-DEVD-FMK-treated cells ( $p < 0.05$ ).

integral component of the ER stress pathway (Boyce *et al.*, 2005), prevented DNA fragmentation, confirming that deltamethrin causes apoptosis through activation of the ER stress pathway (Figs. 7D and 7E).

## DISCUSSION

In this study, we investigated the molecular mechanisms by which exposure to the pyrethroid insecticide deltamethrin induces apoptosis. The results demonstrate that deltamethrin-induced apoptosis in SK-N-AS cells through the ER stress pathway. Specifically, deltamethrin exposure initiated a series of cell death signaling events that are dependent on interaction with voltage-gated  $\text{Na}^+$  channels, resulting in elevation of intracellular  $\text{Ca}^{2+}$  and activation of calpain and caspase-12 leading to DNA fragmentation (Fig. 8).

Previously, it has been reported that deltamethrin causes apoptosis in primary cortical neurons and SK-N-MC neuroblastoma cells (Wu and Liu, 2000a,b; Wu *et al.*, 2000; Elwan *et al.*, 2006). However, the mechanism(s) and apoptotic pathways by which deltamethrin causes apoptosis has not been established. Here, we demonstrate that exposure of SK-N-AS dopaminergic cells to higher concentrations ( $10\mu\text{M}$  and above) for 48 h led to overt cell death. However, lower concentrations ( $100\text{nM}$ – $5\mu\text{M}$ ) of deltamethrin caused DNA fragmentation, a hallmark of apoptosis, at 24 h of exposure, similar to that observed in the aforementioned studies. Although there are few studies that report brain levels of deltamethrin following *in vivo* exposures, two recent papers describing pharmacokinetic modeling of deltamethrin in rats found that brain levels of deltamethrin following oral gavage of 2–10 mg/kg deltamethrin ranged from 0.1 to 0.4  $\mu\text{g/g}$  (Godin *et al.*, 2010; Kim *et al.*, 2010). These doses are below those which cause overt

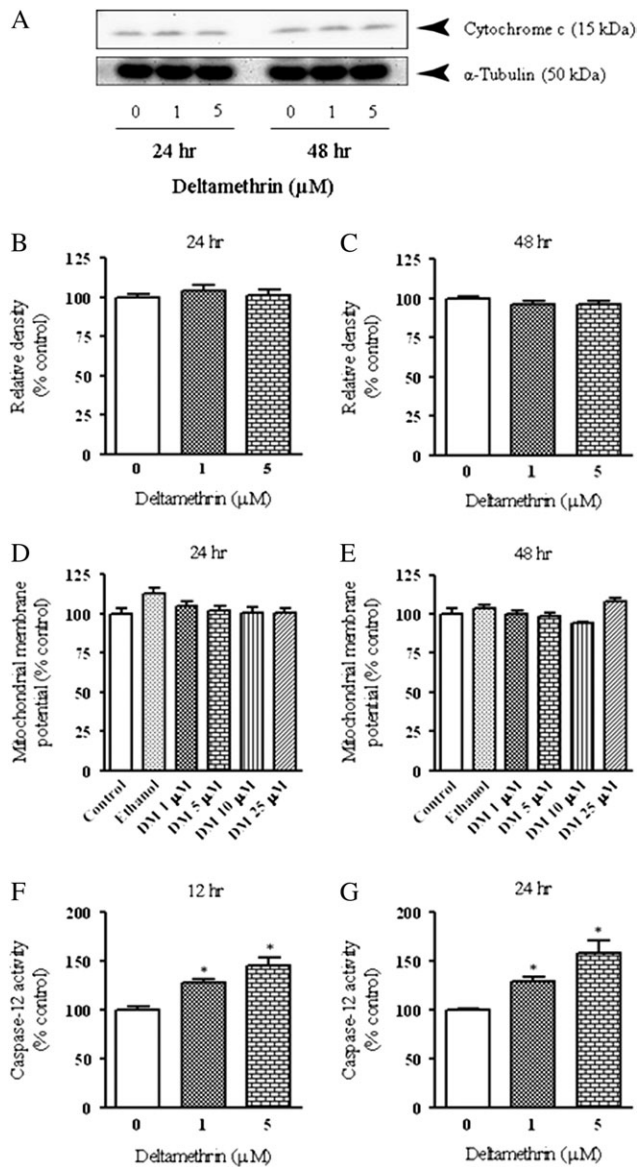


**FIG. 4.** Role of caspase-9 in deltamethrin-induced apoptosis. Exposure to deltamethrin increased the activation of caspase-9 in a dose- and time-dependent manner (A and B). Inclusion of the caspase-9 inhibitor, Z-LEHD-FMK (50 $\mu\text{M}$ ), prevented deltamethrin-induced DNA fragmentation (C and D). Data represent mean  $\pm$  SEM of four individual experiments, each performed in triplicate and expressed as percentage of control. \* indicates significantly different from control and # indicate significant differences between deltamethrin-treated cells and deltamethrin plus Z-LEHD-FMK-treated cells ( $p < 0.05$ ).

symptoms of pyrethroid intoxication and the highest brain concentrations produced are roughly in the low nanomolar range. However, higher doses would be expected to produce levels closer to the range employed in this study. Furthermore, the presence of serum in our media, which may reduce the amount of deltamethrin available to interact with the cell, makes it likely that the dose of deltamethrin that reaches the cell is lower than what we applied. It should be noted that the pharmacokinetic studies found that the models predicted twofold greater peak deltamethrin concentration in humans compared to rats (Godin *et al.*, 2010) and that developing rats accumulated twice as much deltamethrin in the brain compared to adults (Kim *et al.*, 2010). This increased accumulation in the developing animal became even more evident as the dose

increased. These findings suggest that in high-level exposure situations, developing animals and humans may be more at risk than is predicted based on rodent studies.

As a first step in identifying the mechanism and pathways by which deltamethrin causes apoptosis, we focused on the role of  $\text{Ca}^{2+}$ . In neuronal cells,  $\text{Ca}^{2+}$  plays an integral role in activation and/or enhancement of cell death pathways, including those involved in apoptosis (Berliocchi *et al.*, 2005). Pyrethroids, including deltamethrin, have been demonstrated to depolarize cells by prolonging the opening of voltage-gated  $\text{Na}^+$  channels (Narahashi, 1996), subsequently increasing  $[\text{Ca}^{2+}]_i$ . Similarly, recent data in primary cortical neurons using sodium-binding benzofuran isophthalate as the  $\text{Na}^+$  indicator and fluo-3 as the  $[\text{Ca}^{2+}]_i$  indicator reported that



**FIG. 5.** Deltamethrin activates caspase-12 without altering the function of mitochondria. Western blot analysis of cytochrome *c* with  $\alpha$ -tubulin immunoblot as loading control (A). Representative blots are presented and data are expressed as relative pixel density in bar graphs (B and C). Effects of deltamethrin on mitochondrial membrane potential at 24 (D) and 48 h (E). Activation of caspase-12 at 12 (F) and 24 h (G) after deltamethrin exposure. The values represent mean  $\pm$  SEM from at least three individual experiments, each performed in triplicate. Data are expressed as percentage of vehicle control. \* indicates significantly different from control ( $p < 0.05$ ).

pyrethroids, including deltamethrin, significantly increased  $[Na^+]_i$  and  $[Ca^{2+}]_i$ , which could be blocked with the sodium channel blocker TTX (Cao *et al.*, 2008, 2011). Here, acute application of deltamethrin (5 $\mu$ M) rapidly caused increased  $Na^+$  and  $Ca^{2+}$  influx into the SK-N-AS cells and cotreatment with TTX completely prevented DNA fragmentation. This finding is in agreement with studies demonstrating that hypoxia and veratridine application activate  $Na^+$  channels, leading to

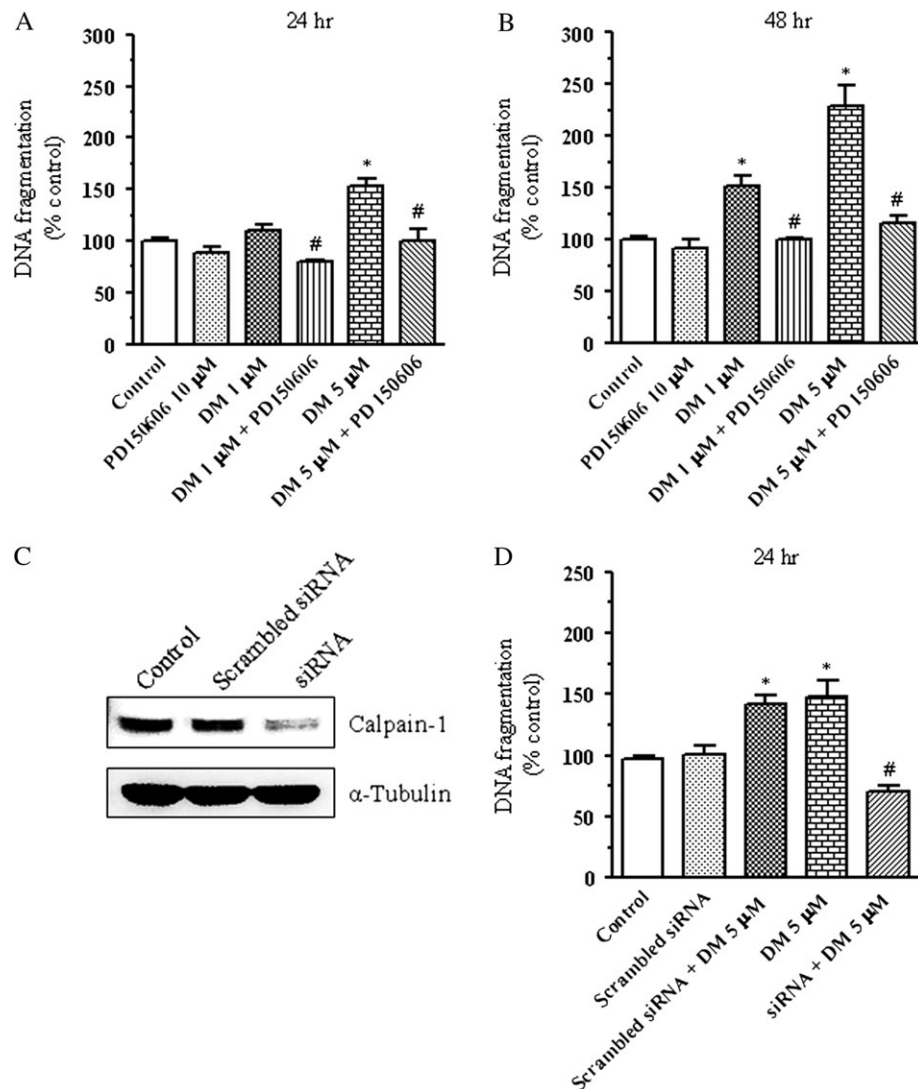
activation of caspase-3 and apoptosis that can be blocked by TTX (Banasiak *et al.*, 2004). We also found that the intracellular  $Ca^{2+}$  chelator BAPTA-AM completely prevented the deltamethrin-induced DNA fragmentation, similar to that observed in veratridine-induced apoptotic cell death in bovine chromaffin cells (Jordan *et al.*, 2000). Although there is some evidence that deltamethrin can directly interact with  $Ca^{2+}$  channels and increase their function (Breckenridge *et al.*, 2009; Clark and Symington, 2008), our data are in agreement with those of Cao *et al.* (2011) that the rise in  $Ca^{2+}$  is secondary to the activation of  $Na^+$  channels because TTX blocks deltamethrin-induced apoptosis. Taken together, these data suggest that application of  $Na^+$  channel agonists, such as deltamethrin and veratridine, causes apoptosis through activation of  $Na^+$  channels and subsequent elevation of  $[Ca^{2+}]_i$ .

Activation of caspase-3 is a critical event in the execution phase of apoptosis involving pesticide exposure (Choi *et al.*, 2010; Kitazawa *et al.*, 2003; Ramachandiran *et al.*, 2007; Sherer *et al.*, 2003). Although the activation of caspase-3 by pyrethroids has not been studied in detail, one study found that very high doses (100–200 $\mu$ M) of deltamethrin increased caspase-3 activity in HepG2 cells (Das *et al.*, 2008), suggesting that caspase-3 may be the final effector caspase in deltamethrin-induced apoptosis. Here, we found that deltamethrin only modestly increased caspase-3 activity. However, pharmacological inhibition of this activation by z-DEVD-fmk prevented apoptosis. This discrepancy may be explained by the fact that z-DEVD-fmk can inhibit additional proteases, such as caspase-9 and calpain (Berger *et al.*, 2006; Knoblach *et al.*, 2004). Thus, the protective effect of z-DEVD-fmk against deltamethrin-induced apoptosis may be the result of its inhibitory effects on upstream proteases.

In the classic intrinsic pathway, caspase-9 is activated by release of cytochrome *c* from the mitochondria into the cytosol, and caspase-9 can then activate caspase-3 as the final effector of apoptosis (Gorman *et al.*, 2000; Lee and Wei, 2000). Here, we observed increased caspase-9 activity following deltamethrin exposure and found that pharmacological inhibition of caspase-9 prevented DNA fragmentation. However, we also found that caspase-9 activation occurred in the absence of elevated levels of cytochrome *c* in the cytosol, indicating that deltamethrin-induced apoptosis is likely initiated through a mitochondrial-independent pathway. Previously, one study found that *in vivo* deltamethrin exposure reduced mitochondrial cytochrome *c* levels (Chen *et al.*, 2007). However, levels in the cytosol were not determined and there were no loading control included in the Western blots. Thus, the potential role of cytochrome *c* release in deltamethrin-induced apoptosis requires additional study.

In addition to the classic intrinsic and extrinsic pathways of apoptosis, it has become recognized that activation of the ER stress pathway can contribute to initiation of apoptosis (Verkhratsky, 2005). The ER plays an integral role in the storage of  $Ca^{2+}$  to regulate intracellular  $Ca^{2+}$  homeostasis, and



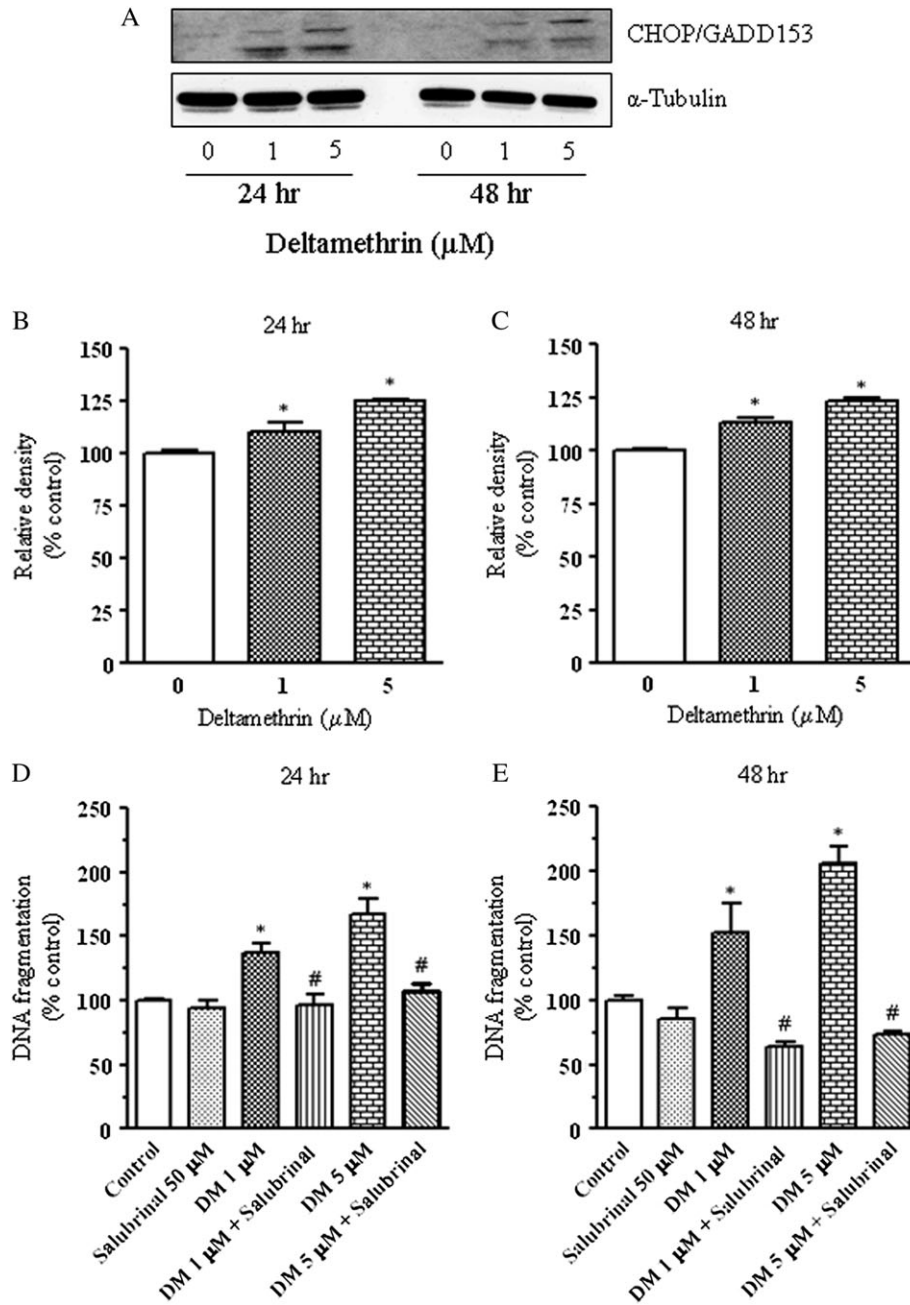


**FIG. 6.** Role of calpain in deltamethrin-induced apoptosis. Pretreatment with the calpain inhibitor PD150606 (10 $\mu$ M) prevented deltamethrin-induced DNA fragmentation (A and B). Treatment of SK-N-AS cells for 24 h with calpain-1 siRNA reduced calpain-1 protein expression (C). Knockdown of calpain-1 by siRNA prior to treatment with deltamethrin abolished deltamethrin-induced DNA fragmentation (D). Data present mean  $\pm$  SEM of four individual experiments, each performed in triplicate and expressed as percentage of control. \* indicates significantly different from control and # indicate significant differences between deltamethrin-treated cells and deltamethrin plus PD150606-treated cells or deltamethrin-treated siRNA transfected cells ( $p < 0.05$ ).

release of calcium stores from the ER can lead to activation of caspase-12, which can then activate caspase-9 and -3, leading to apoptosis (Verkhatsky, 2005). Here, we found that deltamethrin exposure increased caspase-12 activity in a dose- and time-related manner, suggesting that the ER stress pathway may play a role in deltamethrin-induced apoptosis. Additionally, the increased caspase-12 activity occurred earlier and at a greater magnitude than caspase-9, suggesting that activation of the ER stress pathway. Although there are currently no data in the literature pertaining to Na<sup>+</sup> channel agonists and activation of caspase-12, data have demonstrated that conditions such as *in vitro* neuronal ischemia results in a parallel rise in cytosolic Na<sup>+</sup> and Ca<sup>2+</sup> leading to activation

of caspase-12 (Chen *et al.*, 2008). Additionally, the Na<sup>+</sup> channel agonist veratridine has been found to cause release of ER Ca<sup>2+</sup>, suggesting that Na<sup>+</sup> influx may play an important role in regulating intracellular calcium stores (Nikolaeva *et al.*, 2005). Our data support these observations and suggest that activation of Na<sup>+</sup> influx and the subsequent rise in cytosolic Ca<sup>2+</sup> following deltamethrin activates caspase-12 through liberation of ER Ca<sup>2+</sup> stores.

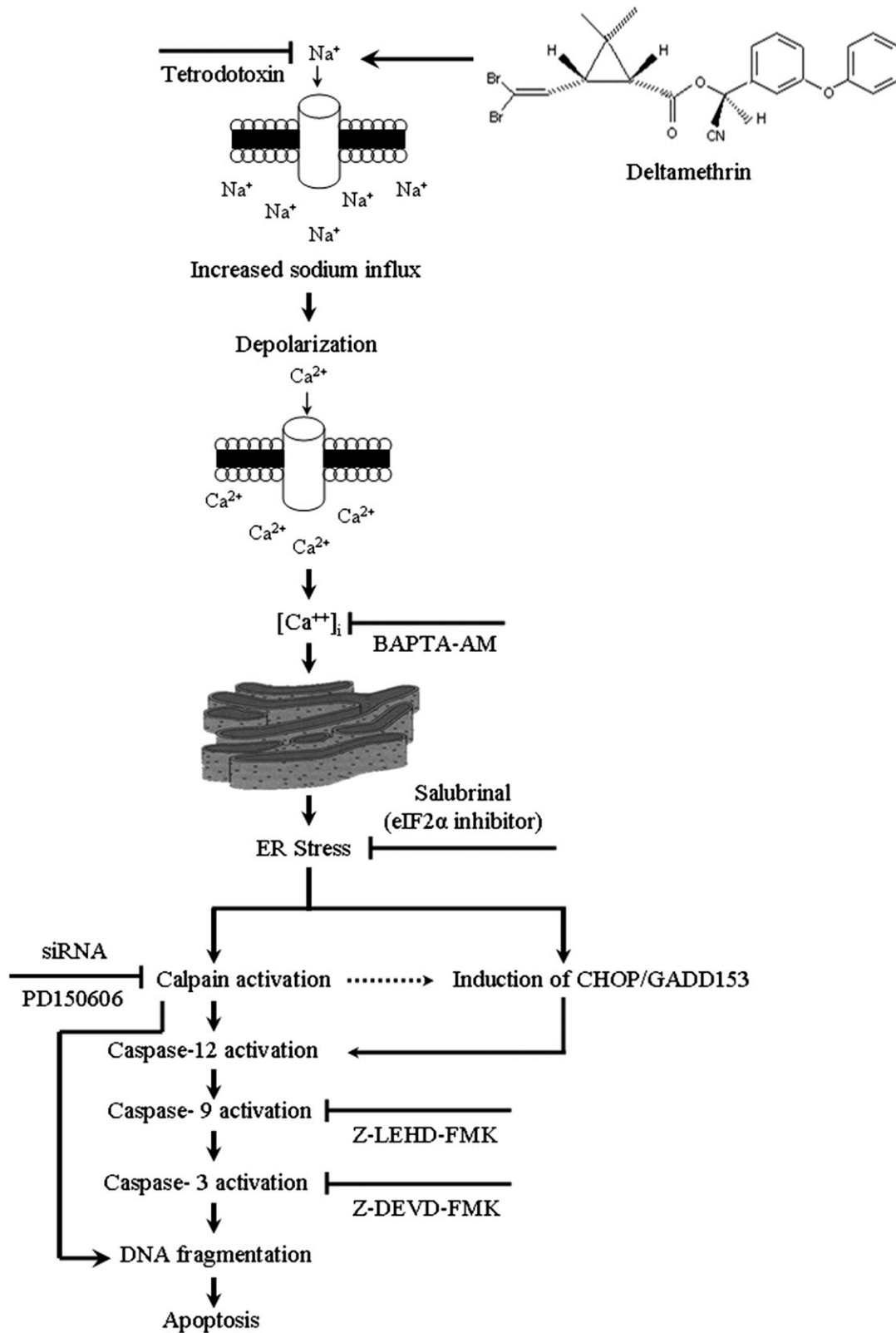
Release of Ca<sup>2+</sup> from the ER can result in activation of caspase-12 following cleavage by calpain initiating apoptosis in the ER stress pathway (Morishima *et al.*, 2002; Nakagawa and Yuan, 2000). Furthermore, a study in *Caenorhabditis elegans*, demonstrated that overexpression of the *C. elegans*



**FIG. 7.** Role of the ER stress pathway in deltamethrin-induced apoptosis. CHOP/GADD153 expression following deltamethrin exposure was determined by Western blot analysis (A). Relative pixel densities from representative blots are presented in bar graphs (B and C). Deltamethrin-induced DNA fragmentation was completely prevented by salubrinal (eIF2 $\alpha$  inhibitor) (D and E). Data represent mean  $\pm$  SEM of four individual experiments, each performed in triplicate and expressed as percentage of control. \* indicates significantly different from control, and # indicates significant differences between deltamethrin-treated cells and deltamethrin plus salubrinal-treated cells ( $p < 0.05$ ).

Na<sup>+</sup> channel induced Ca<sup>2+</sup> release from the ER, calpain activation, and cell death (Bianchi *et al.*, 2004). In this study, we found that the calpain inhibitor PD150606 and the knockdown of calpain-1 by siRNA completely prevented deltamethrin-induced DNA fragmentation, indicating calpain-dependent apoptotic cascade in deltamethrin-induced apopto-

sis. Calpain inhibition has also been demonstrated to protect against veratridine-induced apoptosis in bovine chromaffin cells (Jordan *et al.*, 2000). Over activation of calpain due to ER stress has been reported to cause neuronal cell death in ischemic brain injury, neuromuscular degeneration, and AD (Saido *et al.*, 1994; Saito *et al.*, 1993; Tsuji *et al.*, 1998; Van



**FIG. 8.** Schematic model describing the mechanism of deltamethrin-induced apoptosis. Deltamethrin increases Na<sup>+</sup> influx by delaying the closing of Na<sup>+</sup> channels, leading to depolarization and increased Ca<sup>2+</sup> influx. This initiates the ER stress pathway as evidenced by induction of CHOP/GADD153 and activation of calpain. Calpain may translocate to the nucleus and initiate DNA fragmentation and also activate caspase-12, which in turn activates caspase-9 and caspase-3, leading to DNA fragmentation and apoptosis.

den Bosch *et al.*, 2002). Thus, high-level exposure to deltamethrin may have the potential to cause neurodegeneration through activation of calpain.

The data generated thus far in this study have indicated that deltamethrin induces apoptosis through the ER stress pathway. Disruption of the delicate  $\text{Ca}^{2+}$  balance in the ER can cause transcription of genes to activate the ER stress-mediated apoptotic pathway (Xu *et al.*, 2005). The induction of the transcription factor CHOP/GADD153 has been observed during unfolded protein response and is associated with induction of ER stress-mediated apoptosis (Oyadomari and Mori, 2004). CHOP, a member of the C/EBP family of bZIP transcription factors, is typically expressed at very low basal levels but increased under conditions of ER stress, where it can decrease expression of the anti-apoptotic molecule Bcl-2 (Xu *et al.*, 2005). We found that deltamethrin treatment caused an early significant induction of CHOP that paralleled DNA fragmentation, providing further evidence that deltamethrin causes apoptosis through the ER stress pathway. During initiation of the ER stress pathway, CHOP can be induced by eIF2 $\alpha$ , is phosphorylated by PKR-like ER kinase, and leads to inactivation of mRNA translation in an attempt to reduce ER stress (Xu *et al.*, 2005). Here, we found that sulubrin, an inhibitor of eIF2 $\alpha$ , abolished deltamethrin-induced DNA fragmentation, providing further evidence to support the role of the ER stress pathway in deltamethrin-induced apoptosis.

Taken together, the data presented in this study establish a pathway of apoptosis initiated by deltamethrin exposure (Fig.8). Specifically, deltamethrin activates the ER stress pathway through its interaction with  $\text{Na}^{+}$  channels and subsequent calcium overload, leading to initiation of the apoptotic cascade through calpain-1 activation of caspase-12. We also observe a later increase in caspase-9 and a modest increase in caspase-3. These data suggest that deltamethrin-induced DNA fragmentation and apoptosis may occur in two stages, the first of which involves ER stress and calpain activation and the second involving activation of the caspase-cascade. Indeed, calpain has been found to translocate to the nucleus and initiate DNA fragmentation, possible by activating a DNase (Rami *et al.*, 2000). This may also explain why complete protection from deltamethrin-induced apoptosis was observed with the peptide-based inhibitors employed here because these compounds can also inhibit calpain activity (Knoblach *et al.*, 2004).

Because the ER stress pathway and calpain activation has been implicated a number of neurodegenerative diseases including prion disease, AD, amyotrophic lateral sclerosis (ALS), and PD, these data provide insight into the mechanism by which high-level exposure to pyrethroid pesticides may cause long-term neurological effects and may have the potential to cause neurodegeneration. Although there are few data available on the association between high-level pyrethroid exposure and neurodegeneration in the human population, there is a case report indicating that chronic high-level inhalation pyrethroids produced a motor neuron disorder simulating ALS (Doi *et al.*,

2006). Thus, additional epidemiological and clinical data are needed to determine what association, if any, such exposures have with the development of neurodegenerative diseases.

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