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Satratoxin G–Induced Apoptosis in PC-12 Neuronal Cells is Mediated by PKR and Caspase Independent

Zahidul Islam,*,†,‡ Colleen C. Hegg,*,§ Hee Kyong Bae, and James J. Pestka*,†,‡,[1]

*Center for Integrative Toxicology; †Department of Microbiology and Molecular Genetics; ‡Department of Food Science and Human Nutrition; and §Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824-1224

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Satratoxin G (SG) is a macrocyclic trichothecene mycotoxin produced by *Stachybotrys chartarum*, a mold suggested to play an etiologic role in damp building-related illnesses. Acute intranasal exposure of mice to SG specifically induces apoptosis in olfactory sensory neurons of the nose. The PC-12 rat pheochromocytoma cell model was used to elucidate potential mechanisms of SG-induced neuronal cell death. Agarose gel electrophoresis revealed that exposure to SG at 10 ng/ml or higher for 48-h induced DNA fragmentation characteristic of apoptosis in PC-12 cells. SG-induced apoptosis was confirmed by microscopic morphology, hypodiploid fluorescence and annexin V-fluorescein isothiocyanate (FITC) uptake. Messenger RNA expression of the proapoptotic genes p53, double-stranded RNA–activated protein kinase (PKR), BAX, and caspase-activated DNAse was significantly elevated from 6 to 48 h after SG treatment. SG also induced apoptosis and proapoptotic gene expression in neural growth factor-differentiated PC-12 cells. Although SG-induced caspase-3 activation, caspase inhibition did not impair apoptosis. Moreover, SG induced nuclear translocation of apoptosis-inducing factor (AIF), a known contributor to caspase-independent neuronal cell death. SG-induced apoptosis was not affected by inhibitors of oxidative stress or mitogen-activated protein kinases but was suppressed by the PKR inhibitor C16 and by PKR siRNA transfection. PKR inhibition also blocked SG-induced apoptotic gene expression and AIF translocation but not caspase-3 activation. Taken together, SG-induced apoptosis in PC-12 neuronal cells is mediated by PKR via a caspase-independent pathway possibly involving AIF translocation.

Key Words: **apoptosis; PC-12; PKR; C16; Satratoxin G; Stachybotrys; cell culture; cytotoxicity; RT-PCR; natural products.**

The black mold *Stachybotrys chartarum* grows on cellulose-containing building materials such as gypsum board, ceiling tiles and wood following water damage and is detectable in indoor air samples taken during air quality investigations (Pestka *et al*., 2007). Chronic indoor exposures to *S. chartarum* in water-damaged homes and workplaces following water damage have been postulated to contribute to debilitating respiratory (Croft *et al*., 2002; Hodgson *et al*., 1998; Johanning *et al*., 1996) and nonrespiratory symptoms involving immune and neurological impairment (Gordon *et al*., 2004; Johanning *et al*., 1996). Although *in vitro* and *in vivo* research on *S. chartarum* and its mycotoxins suggests that adverse effects in humans are biologically plausible, establishing an etiologic role in building-related illnesses requires further investigation of mechanisms of action and dose response relationships as well as accurate measurement of exposure in water-damaged buildings (Institute of Medicine, 2004).

The trichothecenes are a family of over 200 fungal sesquiterpenoid metabolites that are extremely potent translational inhibitors (Grove, 2007). Trichothecenes can further activate mitogen-activated protein kinases (MAPKs) and initiate both inflammatory gene expression and apoptosis *in vitro* and *in vivo* in a process known as the ribotoxic stress response (Pestka *et al*., 2004). Because *Stachybotrys* conidiospores and submicron mycelial fragments contain satratoxin G (SG) and other macrocyclic trichothecenes (Brasel *et al*., 2005; Gregory *et al*., 2004), understanding the cellular targets and mechanisms of these toxins is of critical importance.

We have recently observed that single intranasal instillations with SG or two other related macrocyclic trichothecenes, isosatratoxin F, and roridin A, cause apoptosis in the murine nose after 24 h with olfactory sensory neurons (OSNs) and the olfactory bulb (OB) being prominent targets (Islam *et al*., 2006, 2007). The onset of OSN apoptosis and atrophy correspond with increases of proapoptotic gene expression in the nasal turbinates, but the upstream mechanisms remain unclear.

Double-stranded RNA (dsRNA)–activated protein kinase (PKR) is a widely expressed dual specificity (serine/theonine and tyrosine) protein kinase that is activated by dsRNA, interferon, trichothecene mycotoxins and other agents (Williams, 2001; Zhou *et al*., 2003). PKR associates with the ribosome (Wu *et al*., 1998) and can selectively shut down translation via phosphorylation of eukaryotic initiation factor 2α as well as activate nuclear factor kappa B (NF-κB) (Garcia *et al*., 2007). Furthermore, PKR has been demonstrated to mediate apoptosis induced by dsRNA, lipopolysaccharide (LPS), and tumor necrosis factor (TNF)-α (Der *et al*., 1997;

[1] To whom correspondence should be addressed at 234 G.M. Trout Building, Michigan State University, East Lansing, MI 48824. Fax: (517) 353-8963. E-mail: pestka@msu.edu.

Gil and Esteban, 2000; Yeung and Lau, 1998; Yeung *et al*., 1996). Our laboratory has previously shown that MAPK activation and apoptosis induction by trichothecene deoxynivalenol (DON) and other translational inhibitors is downregulated in monocyte and macrophage cultures treated with PKR inhibitors as well as in PKR-deficient monocyte cultures (Zhou *et al*., 2003). These findings imply that a potential critical role exists for this kinase in trichothecene-induced apoptosis. Interestingly, following intranasal instillation of mice to SG (Islam *et al*., 2006) or roridin A (Islam *et al*., 2007), PKR messenger RNA (mRNA) concentrations in nasal turbinates are upregulated in parallel with OSN apoptosis.

The observation that macrocyclic trichothecenes can selectively target OSN and OB is of particular interest because diminution of olfactory function has been associated with early stages of neurodegenerative illnesses such as Parkinson's and Alzheimer's diseases (Demarquay *et al*., 2007; Hawkes, 2003; Takeda *et al*., 2007). Several investigations of SG's effects in vitro have been conducted in leukocytes (Chung *et al*., 2003; Gregory *et al*., 2004; Sorenson *et al*., 1987; Yang *et al*., 2000), however, understanding the mechanisms for induction of OSN death by macrocyclic trichothecenes requires study of their direct effects in neuronal cell cultures.

In general, primary cell neuronal cultures present major problems relative to their inability to maintain cell division and heterogeneity (Slotkin *et al*., 2007). Although there have been several reports of cloned OSN cell cultures (Coronas *et al*., 1997; Illing *et al*., 2002; Lakard *et al*., 2007), these are as yet not generally available and their robustness for mechanistic toxicology studies has not been established. The neural crest-derived pheochromocytoma cell line (PC-12) (Greene and Tischler, 1976) is well-characterized and is widely employed to model undifferentiated, dividing neuronal cultures and in differentiated cells with phenotypic and functional characteristics of sympathetic and sensory neuronal cells (Aykin-Burns and Ercal, 2006; Hegg and Miletic, 1998; Henck *et al*., 2001; Westerink and Ewing, 2008). PC-12 cells have been useful in studies of neurotoxic chemicals (Brenneman *et al*., 2000; Walkinshaw and Waters, 1994) and recently, Nusuetrong *et al*. (2005) reported that satratoxin H (SH)–induced apoptosis occurs in PC-12 cells.

The purpose of this study was to characterize mechanisms of SG-induced apoptosis in PC-12 cultures relative to gene expression and intracellular signaling. The results strongly suggest that SG-induced neuronal cell death is mediated by PKR via a caspase-independent pathway.

MATERIALS AND METHODS

Cells and reagents. PC-12 cells were obtained from American Type Culture Collection (Manassas, VA). All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise noted. SG was purified from *S. chartarum* cultures as previously described (Hinkley and Jarvis, 2001) and identity confirmed by electrospray ionization/collision-induced dissociation tandem mass spectroscopy (Tuomi *et al*., 1998). Inhibitors of oxidative stress (L-N-acetylcysteine (NAC), L-N-nitro-arginine methyl ester (NAME), quercetin), MAPKs (SB203580, p38; PD98059, extracellular-signal regulated kinases (ERK); C-jun N-terminal kinases (JNK) inhibitor I, JNK), PKR (C16 and PKR inhibitor negative control), and caspase-3 (cell-permeable, DEVD-CHO) were obtained from Calbiochem (San Diego, CA).

Experimental design

PC-12 cells were cultured in 100 mm × 20 mm dishes (Corning, NY) with 10 ml of F-12K medium (ATCC) supplemented with 2.5% (vol/vol) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 15% (vol/vol) horse serum (Atlanta Biologicals), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Rockville, MD) at 37°C with 6% CO_2. Cells were harvested by centrifugation at 250 × g for 10 min and resuspended in 10 ml of culture medium in a 50-ml culture tube (Corning, NY). The cell suspensions were passaged through a 22-gauge 1.5-inch needle four to five times to yield single cells and these were then reconstituted in culture medium to 5×10^5 cells/ml.

For experiments with naïve (undifferentiated) PC-12 cells, single cell suspensions (5×10^5/ml) were transferred to six-well (2 ml/well) or 12-well (1 ml per well) collagen-coated plates (BD Biosciences Pharmingen, San Diego, CA) where they grew adherently. Prior to toxin treatment, cells were cultured for 24 h to minimize background stress activation. SG was dissolved in pyrogen-free water (Sigma Chemical Co.) and added to supplemented F-12K medium for addition to cell cultures. For inhibition studies, cells were preincubated with various pharmacologic inhibitors for 30 min prior to SG treatment. For some experiments, PC-12 cells were differentiated to the neuronal phenotype by incubation with 100 ng/ml of nerve growth factor (NGF) for 5 days prior to treatment with SG and/or inhibitors (Levi *et al*., 1985).

DNA fragmentation analysis by agarose gel electrophoresis. PC-12 cells were extracted and analyzed for DNA fragmentation as described previously (Islam *et al*., 2002). In brief, cells were harvested by scraping with a disposable cell lifter (Fisher brand), suspended in phosphate-buffered saline (PBS), centrifuged for 10 min (250 × g) at 4°C and the pellet suspended in 0.1 ml of hypotonic lysing buffer (10mM Tris, pH 7.4, 10mM ethylenediaminetetraacetic acid [EDTA], pH 8.0, 0.5% [vol/vol] Triton X-100). Cells were incubated for 10 min at 4°C and the resultant lysate was centrifuged for 30 min (13,000 × g) at 4°C. The supernatant, which contained fragmented DNA, was digested for 1 h at 37°C with 0.4 μg/ml of RNase A (Roche, Indianapolis, IN) and then incubated 1 h at 37°C with 0.4 μg/ml of proteinase K (Roche). DNA was precipitated with 50% (vol/vol) isopropanol in 0.5M NaCl at −20°C overnight. The precipitate was centrifuged at 13,000 × g for 30 min at 4°C. The resultant pellet was air dried and resuspended in 10mM Tris (pH 7.4), 1mM EDTA (pH 8.0). An aliquot equivalent to 1×10^6 cells was electrophoresed at 70 V for 2 h in 2% (wt/vol) agarose gel in 90mM Tris–borate buffer containing 2mM EDTA (pH 8.0). After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml), and the nucleic acids were visualized with a ultraviolet transilluminator. A 100-bp DNA ladder (GIBCO-BRL) was used for molecular sizing.

Light microscopy. Cells were fixed with 4% (vol/vol) formaldehyde in Dulbecco's phosphate buffer (PBS) (Sigma) for microscopic visualization of apoptotic morphology. Light microscopic photographs were obtained using a Nikon epifluorescent microscope equipped with a SPOT-RT digital camera (Diagnostic Instruments, Detroit, MI).

Flow cytometry. Apoptotic cells were assayed by flow cytometric measurement of hypodiploid cell fluorescence following propidium iodide (PI) staining (Islam *et al*., 2002). Culture media was repeatedly pipetted onto plate surface to release cells and cells centrifuged for 10 min (250 × g) at 4°C. Cells (1×10^6) were resuspended in 0.2 ml of PBS, mixed with 0.2 ml of heat-inactivated fetal bovine serum, and fixed immediately by dropwise addition of 1.2 ml of ice-cold 70% (vol/vol) ethanol with gentle mixing. Cells were held at 4°C overnight, washed and incubated in 1 ml PI DNA staining reagent (PBS containing 50 μg/ml PI, 50 μg/ml RNase A, 0.1mM EDTA disodium, and 0.1%

[vol/vol] Triton X-100) on ice until analysis. Cell cycle distribution for single cells was measured with a Becton Dickinson FACS Vantage (San Jose, CA). Data from 5000 cells were collected in list mode. The 488 line of an argon laser was used to excite PI and fluorescence was detected at 615–645 nm. The cell cycle of individual cells was performed using doublet discrimination gating to eliminate doublet and cell aggregate based on DNA fluorescence. A gate was selected to include hypofluorescent cells. Cells in the DNA histogram with hypofluorescent DNA were designated apoptotic. All other cells distributed in a normal cell cycle profile.

Apoptosis was further assessed by a second flow cytometric method employing an annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD-Pharmingen, San Diego, CA) which detects early redistribution of phosphatidylserine from the inner to the outer layer of the cell membrane of apoptotic cells. Briefly, treated cells were released from the plate surface, washed twice with ice-cold PBS and cells (1×10^5) then resuspended in 100 μl of binding buffer. Annexin V-FITC (5 μl) and PI (5 μl) solutions were added, and the cell suspension vortexed gently. The cell suspension was held on ice for 10 min, and then mixed with 400 μl of binding buffer. Stained cell suspensions were immediately analyzed by flow cytometry using the Becton Dickinson FACS Vantage.

Real-time PCR. Total RNA was isolated from PC-12 cells using RNeasy Protect Mini kit (Qiagen Inc. Valencia, CA). Real-time PCR for apoptosis-related genes (Caspase-3, p53, PKR, BAX, CAD) was performed on an ABI PRISM 7900HT Sequence Detection System using Taqman One-Step RT-PCR Master Mix and Assays-on-Demand primer/probe gene expression products according to the manufacturer's protocols (Applied Biosystems, Foster City, NY). Relative quantification of apoptotic and cytokine gene expression was carried out using an 18S RNA control and an arithmetic formula method (Audige *et al*., 2003).

Caspase-3 assay. PC-12 cells were suspended in 200 μl of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer (100mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [pH 7.5] containing 10% [vol/vol] sucrose, 0.5% [wt/vol] CHAPS, 1mM EDTA, 10mM dithiotreitol, and 1% [vol/vol] protease inhibitor cocktail [Sigma]) and subjected to four repeated freeze-thaw cycles. Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C. Following protein assay using a DC Protein Quantitation Kit (Bio-Rad), lysates were adjusted to 50 μg/100 μl CHAPS buffer and then incubated at 37°C for 30 min with an equal volume of fluorogenic substrate consisting of 25μM DEVD-AMC (Calbiochem, San Diego, CA) dissolved in CHAPS buffer. Substrate cleavage was measured using a Cyto Fluor II microplate fluorescence reader (Biosearch, Bedford, MA) at excitation and emission wavelengths of 360 and 460 nm, respectively.

Confocal microscopic analysis of apoptosis-inducing factor translocation. PC-12 cells were grown to approximately 80% confluency on collagen-coated eight-well slide (BD Biosciences Pharmingen, San Diego, CA) containing 400 μl of medium. Cells were then incubated with PKR C16 inhibitor, PKR negative inhibitor or DMSO vehicle. SG (10 ng/ml) was added 30 min later and the culture incubated for 12–24 h. Cells were washed twice with PBS and fixed with 3% (vol/vol) formaldehyde in PBS for 15 min at room temperature. Cells were washed twice with PBS and permeabilized by incubation with 400 μl of Triton (2% Triton X-100 in PBS) per well for 15 min at 25°C. The cells were blocked with 400 μl of Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) per well for 1 h. For immunostaining, slides were incubated with rabbit AIF polyclonal antibody (eBioscience Inc., San Diego, CA) diluted (1:100) in Odyssey blocking buffer (100 μl per well) at 25°C for 1 h. Wells were washed four times with PBS and then incubated with 100 μl per well FITC-conjugated goat anti-rabbit antibody (Sigma) diluted (1:100) in Odyssey blocking buffer for 1 h at 25°C in the dark. Wells were washed four times with PBS and then incubated with 100 μl of 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml), a marker for cell nuclei, for 5 min. Unbound DAPI was washed away and slides were mounted in Vectashield mounting media for fluorescence (Vector labs, Burlingame, CA). Control slides included omission of the primary antibody or omission of the secondary antibody. PC-12 cells were visualized on a multichannel confocal laser-scanning microscope (Olympus FluoView 1000 LSCM; LUMPlanFl × 40W water-immersion objective; NA 0.8) equipped with imaging analysis software (FV10-ASW 1.5; Olympus). FITC dye was excited at 488 nm and band pass filtered from 505 to 605 nm, and DAPI was excited at 405 nm and band pass filtered from 430 to 470 nm. Images were collected sequentially to minimize fluorescent bleedthrough between emission channels.

PKR siRNA transfection. Rat PKR and control siRNA cocktails (PKR ON-TARGETplus SMARTpool or ON-TARGETplus siCONTROL Nontargeting Pool) were purchased from Dharmacon RNA Technologies (Chicago, IL). The four target sequences for the PKR pool were (1) sense-GAUGGAAAUCCUCGAACAAUU and antisense 5′-PUUGUUCGAGGAUUUCCAUCUU, (2) sense-GGAUUAUAQUACACUCGAAAUU and antisense-5′-PUUUCGAGUGUAUAUAAUCCUU, (3) sense-GAACAAAAGUCAUCGUUAGUU and antisense-5′-PCUAACGAUGACUUUUGUUCUU, (4) sense-GGAAAAGAGAAAUCGGAGUUU and antisense-5′-PACUCCGAUUUCUCUUUUCCUU. Cells were cultured on 100 mm × 20 mm dishes with 10 ml of F-12K medium as described above until approximately 80% confluency was reached and then collected, centrifuged (250 × g) for 10 min at 25°C and resuspended in 10 ml of culture medium. Cell suspensions were passed through 22G 1.5-inch needle four to five times and then diluted in culture medium (2×10^6 cells/ml) for nucleofection. Cells were centrifuged and resuspended in 100 μl of Nucleofector solution, mixed with siRNAs and electroporated using program U-029 and the Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD). Transfected cells were cultured for 48 h on collagen-coated plates as described above prior to SG treatment.

Detection of PKR protein. Cells were collected by centrifugation, washed with PBS, lysed in 50 μl of hot lysis buffer (1% [wt/vol] sodium dodecylsulfate, 1mM sodium *ortho*-vanadate and 10mM Tris, pH 7.4) and then boiled for 5 min. The lysate was vortexed and centrifuged at 12,000 × *g* for 15 min at 4°C. Total cellular proteins were resolved by 12% (wt/vol) acrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). Blots were incubated in Odyssey blocking buffer (LI-COR Biosciences) for 1 h at room temperature with gentle shaking. The membrane was then incubated for another 1 h with primary mouse anti-rat PKR monoclonal antibody (B-10; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse anti-rat β-actin monoclonal antibody (Sigma) diluted in Odyssey blocking buffer (1:1000 and 1:10,000, respectively). The blot was washed four times for 5 min each at 25°C in 0.1% Tween-20 in PBS and then incubated for 1 h with IRdye 800CW-labeled secondary goat polyclonal anti-mouse IgG (LI-COR Biosciences). The membrane was washed four times for 5 min each at 25°C in 0.1% Tween-20 in PBS, rinsed with PBS to remove residual Tween-20 and then scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences). Anti-PKR and anti-actin antibodies binding evoked fluorescent bands that resolved at 68 and 42 kDa, respectively.

Statistics. Data were statistically analyzed with SigmaStat v 3.1 (Jandel Scientific, San Rafael, CA) with the criterion for significance set at $p < 0.05$. Morphometric and RT-PCR data were compared using one-way ANOVA with Student-Newman-Keuls post-test.

RESULTS

SG Induces Apoptosis in Undifferentiated PC-12 Cells

The capacity of SG to induce apoptosis in undifferentiated PC-12 cells was first assessed by monitoring DNA fragmentation. SG concentrations of 10 ng/ml (18.4nM) or higher of SG after 48 h induced DNA fragmentation into 200-kb fragments (Fig. 1A). The characteristic morphological features of apoptosis were detectable microscopically 48 h after SG treatment (Fig. 1B). When frequencies of hypodiploid fluorescent apoptotic cells were quantitated following PI

staining of DNA, apoptotic cell percentages were also found to be significantly increased after 48 h incubation with SG at 10 ng/ml or higher (Fig. 1C). Annexin V-FITC/PI staining of live cells revealed that the number of annexin V-FITC$^+$/PI$^-$ cells increased (lower right quadrant, Fig. 1D) by 10-fold following SG treatment compared with control cells, thus suggesting the presence of the apoptotic marker phosphoserine. Taken together, the resultant data from these four approaches suggested that SG induced characteristic features of apoptosis in undifferentiated PC-12 neuronal cells.

SG Induces Apoptotic Gene Expression in Undifferentiated PC-12 Cells

Expression of mRNAs for the proapoptotic genes caspase-3, p53, PKR, BAX, and CAD were measured by real-time PCR in control and SG-treated cells at several time intervals (Fig. 2). The tumor suppressor gene p53, which is involved in cell cycle arrest after DNA damage, was significantly upregulated from 6 to 48 h after SG treatment, as was CAD, which targets and damages DNA, and PKR. Expression of BAX, which induces mitochondrial-related proteins with proapoptotic activity, was upregulated at 18 and 48 h. mRNA expression for caspase-3, which activates CAD, was not significantly affected by the treatment at any time during the 48 h period.

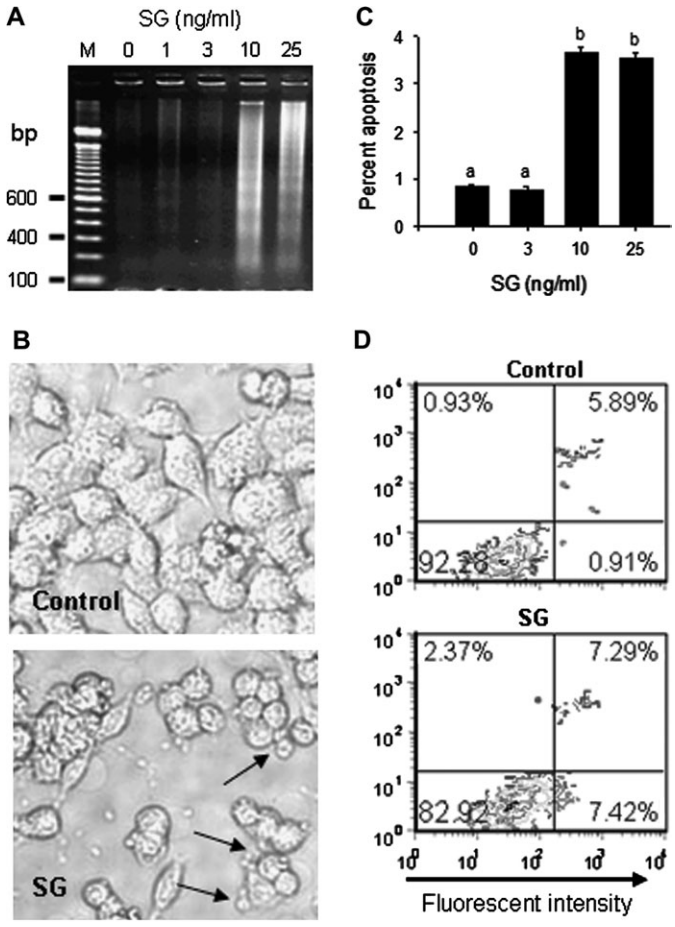

FIG. 1. SG induces apoptosis in undifferentiated PC-12 cells. Cells were grown on collagen-coated plates, treated with SG for 48 h and assessed for apoptosis by four methods. Panels demonstrate: (A) concentration-dependent induction of DNA fragmentation; (B) SG (10 ng/ml) induction of vesicles morphologically consistent with apoptosis; (C) concentration-dependent induction of hypofluorescent DNA in PI-stained cells. Data are mean ± SEM ($n = 3$). Bars marked with different letters in C, differ ($p < 0.05$); and (D) SG (10 ng/ml) induction of FITC-annexin-V uptake. Results are representative of three independent experiments.

SG Induces Apoptosis and Proapoptotic Gene Expression in NGF-Differentiated PC-12 Cells

The ability of SG to evoke apoptosis in NGF-differentiated PC-12 cells was also determined. SG induced DNA fragmentation in naïve PC-12 cells at 5, 10, and 25 ng/ml and NGF-differentiated cells at 10 and 25 ng/ml (Fig. 3A). SG upregulated expression of two representative proapoptotic genes, p53 and CAD similarly in naïve and differentiated PC-12 cells (Fig. 3B). Thus, NGF-differentiated PC-12 cells were also susceptible to SG-induced apoptosis and proapoptotic gene expression. Based on these findings, undifferentiated cells were used for remaining experiments unless otherwise noted.

SG-Induced Apoptosis is Caspase-3 Independent

The role of caspase-3 in SG-induced apoptosis was assessed in PC-12 cells. Incubation with 10 ng/ml SG markedly induced caspase-3 activity after 18 h and thereafter (Fig. 4A). However, although incubation with the caspase inhibitor DEVD-CHO effectively blocked caspase-3 activation (Fig. 4B), SG-induced apoptosis was unaffected (Fig. 4C). These results suggest that SG-induced apoptosis was caspase-3-independent.

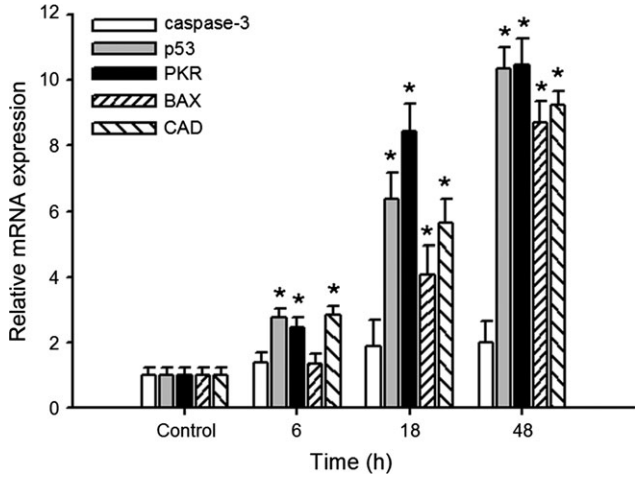

FIG. 2. SG induces apoptotic gene expression in undifferentiated PC-12 cells were incubated with SG (10 ng/ml) for various time intervals and analyzed for apoptotic gene expression by real-time PCR. Data are mean ± SEM ($n = 3$). Asterisks indicate significant differences from control group ($p < 0.05$). Results are representative of three independent experiments.

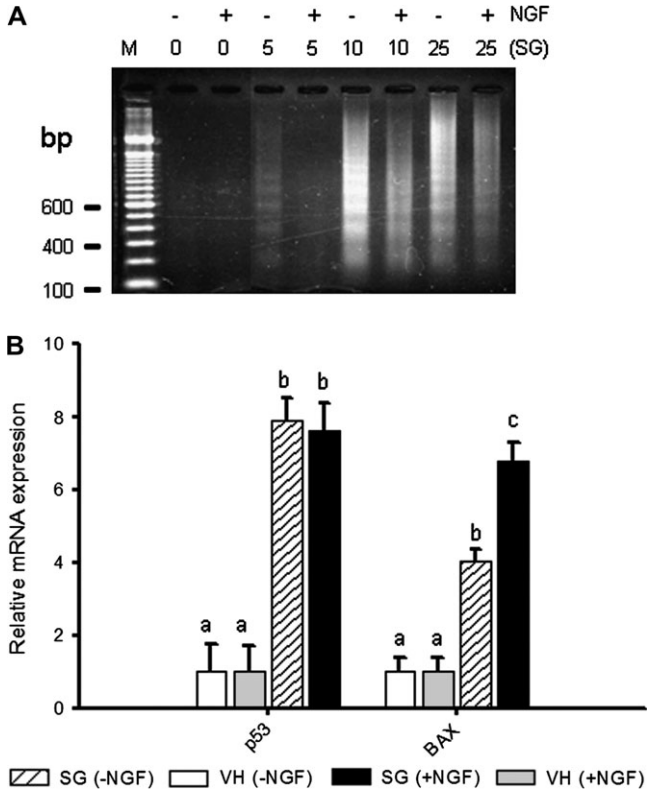

FIG. 3. SG induces apoptosis and proapoptotic gene expression in NGF-differentiated PC-12 cells. Cells were differentiated by incubating with 100 ng/ml of NGF for 5 days. Panels show: (A) concentration-dependent DNA fragmentation in differentiated and undifferentiated PC-12 cells treated for 48 h with or without SG (10 ng/ml) and (B) p53 and BAX mRNA expression in differentiated and undifferentiated PC-12 cells treated with SG (10 ng/ml) or its vehicle (VH) for 18 h. Data are mean ± SEM ($n = 3$). Bars without the same letter within a group differ ($p < 0.05$).

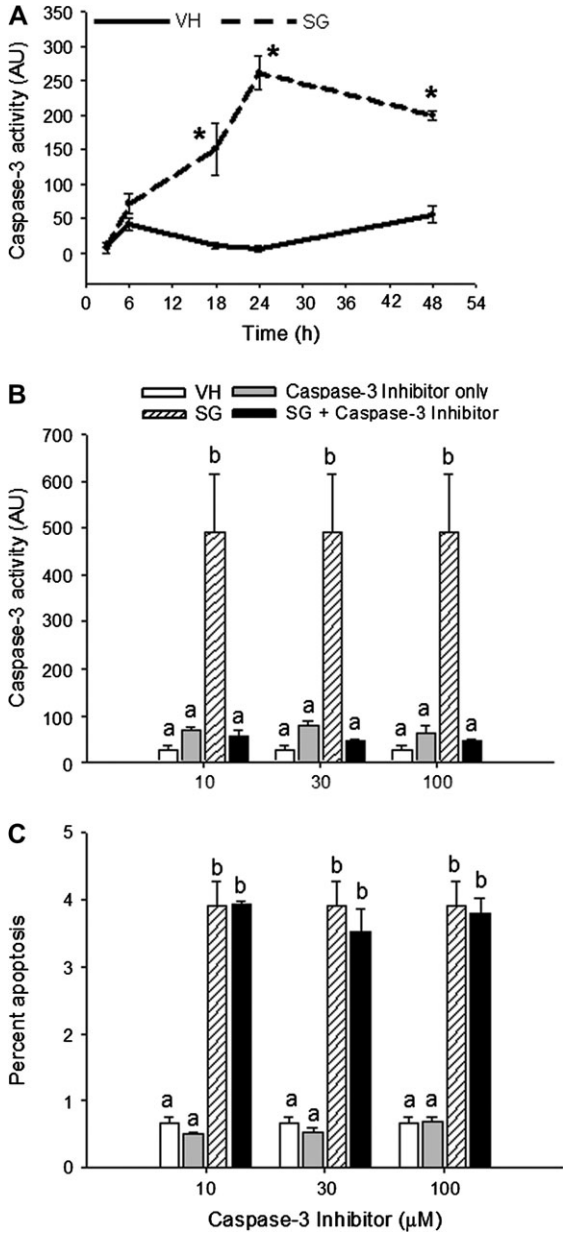

FIG. 4. SG-induced apoptosis is caspase-3 independent. (A) PC-12 cells were cultured with SG (10 ng/ml) and analyzed at intervals for caspase-3 activity. (B, C) Cells were treated with cell-permeable caspase-3 inhibitor I (DEVD-CHO) for 30 min prior to SG (10 ng/ml) treatment. Cells were harvested after 48 h and analyzed for (B) caspase-3 activity or (C) apoptosis by PI uptake. Data are mean ± SEM ($n = 3$). Asterisks indicate significant difference from vehicle control ($p < 0.05$). Bars without same letter differ ($p < 0.05$). Results are representative of three independent experiments.

SG Induces AIF Translocation

Nuclear translocation of AIF reportedly contributes to caspase-independent apoptosis in PC-12 neuronal cells (Liou *et al.*, 2005) as well as in mouse neurodegeneration models (Wang *et al.*, 2003). Immunofluorescence detection of AIF in conjunction with confocal microscopy revealed markedly increased AIF immunoreactivity in the nuclei of PC-12 cells 12 h after SG addition as compared with vehicle-treatment (Fig. 5). Elevated AIF immunoreactivity in SG-treated cells was still detectable after 24 h but at markedly lower levels than at 12 h. These data indicate that SG induced AIF translocation into the nucleus by 12 h and to a lesser extent after 24 h.

SG-Induced Apoptosis and Proapoptotic Gene Expression is Suppressed by PKR Inhibition

PC-12 cells were pretreated with a panel of pharmacologic inhibitors to assess potential upstream roles of oxidative stress, MAPKs and PKR in SG-induced apoptosis. Cells were harvested 48 h after SG treatment and hypodiploid fluorescent cells measured by flow cytometry (Fig. 6A). Neither inhibitors of oxidative stress, (L-NAC, L-NAME, and quercetin) nor inhibitors of p38, ERK, and JNK modulated SG-induced apoptosis. In contrast, SG-induced apoptosis was completely inhibited by the PKR inhibitor C16 (Jammi *et al.*, 2003). When a structural analog of the PKR inhibitor was used as a negative

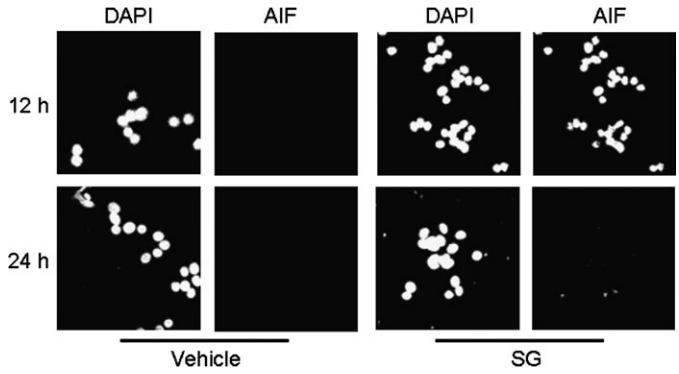

FIG. 5. SG induces AIF translocation. PC-12 cells were grown on eight-well culture slides and treated with or without SG (10 ng/ml) for 12 and 24 h. Cell nuclei were detected with DAPI and subjected to immunohistochemistry with anti-AIF antibody followed by FITC-conjugated secondary antibody.

control, SG-induced apoptosis was not affected (Fig. 6B). Consistent with the aforementioned findings, C16 suppressed expression of the proapoptotic genes p53, PKR, BAX, and CAD at 18 h (Fig. 7A). SG-induced proapoptotic gene expression in NGF-differentiated PC-12 cells was similarly suppressed by the PKR inhibitor (Fig. 7B).

PKR siRNA Knockdown Inhibits SG-Induced Apoptosis

The role of PKR in SG-induced PC-12 cell apoptosis was confirmed by knockdown with PKR siRNAs. Electroporation with a PKR siRNA cocktail at 0.1, 1, 5, and 10μM followed by a 48 h incubation markedly suppressed expression of PKR protein as compared with that for negative control siRNA (Fig. 8A). SG-induced apoptosis was significantly reduced in cells treated with PKR-siRNA at all concentrations (Figs. 8B and 8C). SG similarly induced apoptosis in cells electroporated in the presence and absence of negative control siRNA suggesting the absence of off-target effects (Fig. 8C). Collectively, the siRNA data indicated that SG-induced apoptosis in PC-12 cells is likely to be a PKR-dependent process.

PKR Mediates SG-Induced AIF Translocation but not Caspase-3 Activation

Preincubation with C16 did not inhibit SG-induced caspase-3 activity indicating that activation of this enzyme was a PKR-independent effect (Fig. 9). However, PKR inhibition blocked AIF translocation to the nucleus (Fig. 10) at 12 h, whereas the PKR negative inhibitor control or vehicle alone had no effect. PKR-dependent SG-induced apoptosis in PC-12 thus appeared to be linked to AIF nuclear translocation but not caspase-3 activation.

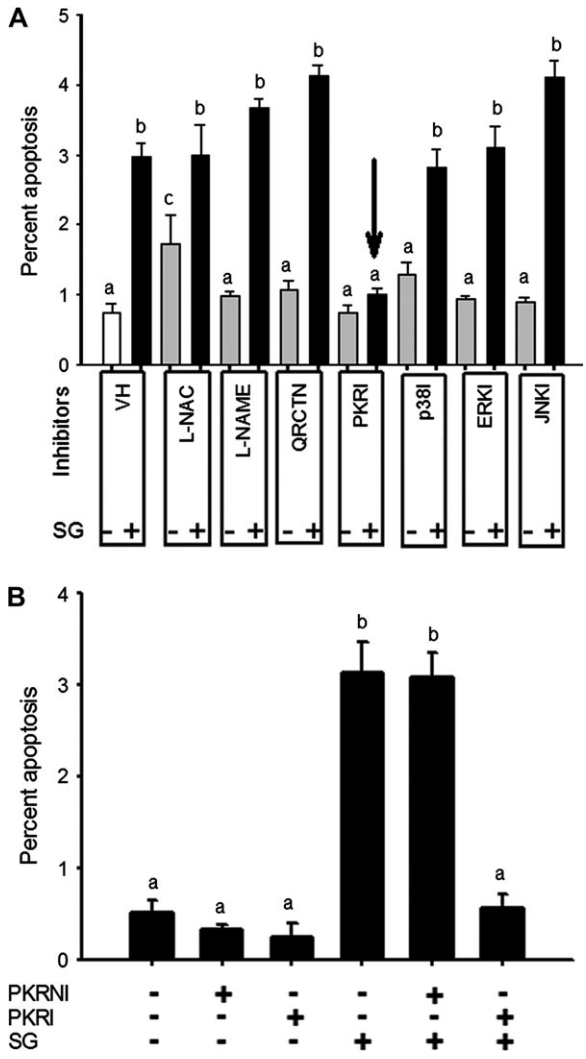

FIG. 6. SG-induced apoptosis is blocked by inhibition of PKR but not oxidative stress or MAPK. (A) PC-12 cells were treated with inhibitor for 30 min before SG (10 ng/ml) treatment. Inhibitors included L-NAC at 1mM, L-NAME at 5mM, quercetin at 10μM, PKR inhibitor (PKRI) C16 at 2.5μM, p38 inhibitor SB203580 (p38I) at 2.5μM, ERK inhibitor PD98059 (ERKI) at 50μM and JNK inhibitor I (JNKI) at 2.5μM. Cells were harvested 48 h after SG treatment and apoptosis assessed by PI uptake. Arrow indicates that SG-induced apoptosis was completely inhibited by PKR inhibitor. Several concentrations of each inhibitor were used but only the highest dose is shown. (B) Cells were treated with PKRI at 2.5μM and nonfunctional PKR inhibitor negative control (PKRNI) at the same concentration 30 min before SG (10 ng/ml) treatment. Apoptosis was measured after 48 h. Data are mean ± SEM ($n = 3$). Bars without the same letter differ ($p < 0.05$). Results are representative of two independent experiments.

DISCUSSION

The capacity of macrocyclic trichothecenes to selectively target neurons in the nose and in the brain of mice following intranasal instillation is of fundamental importance because olfactory function loss often occurs with early stages of neurodegenerative illnesses such as Parkinson's and Alzheimer's diseases (Demarquay *et al.*, 2007; Hawkes, 2003; Takeda *et al.*, 2007). Understanding how SG causes apoptosis in PC-12 cells can provide insight how this and other macrocyclic trichothecenes induce apoptosis in OSNs

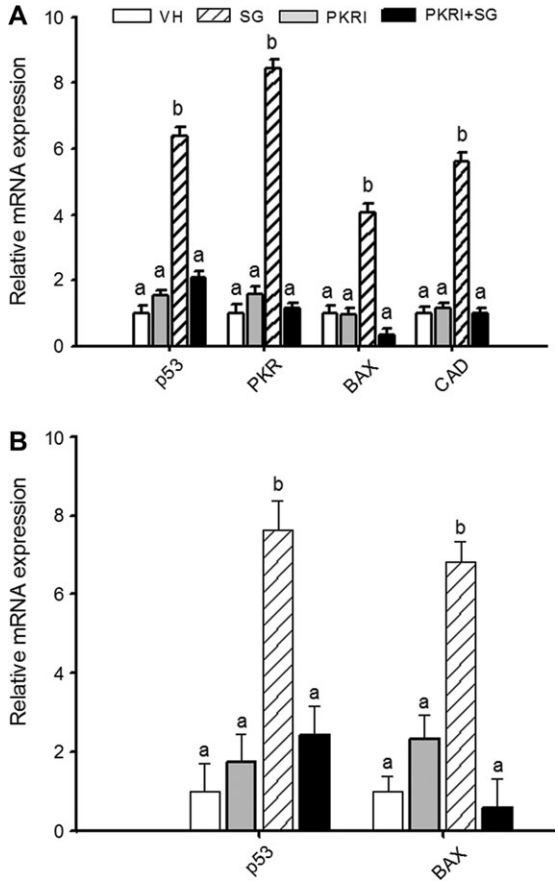

FIG. 7. PKR inhibitor blocks SG-induced proapoptotic genes in undifferentiated and NGF-differentiated PC-12 cells. (A) Undifferentiated and (B) NGF-differentiated PC-12 cells (5×10^5/ml) were treated with or without PKR inhibitor (PKRI) C16 at 2.5μM for 30 min prior to addition of SG (10 ng/ml) or its vehicle (VH). Relative mRNA expression of proapoptotic genes was measured 18 h later. Data are mean ± SEM ($n = 3$). Bars without the same letter differ ($p < 0.05$).

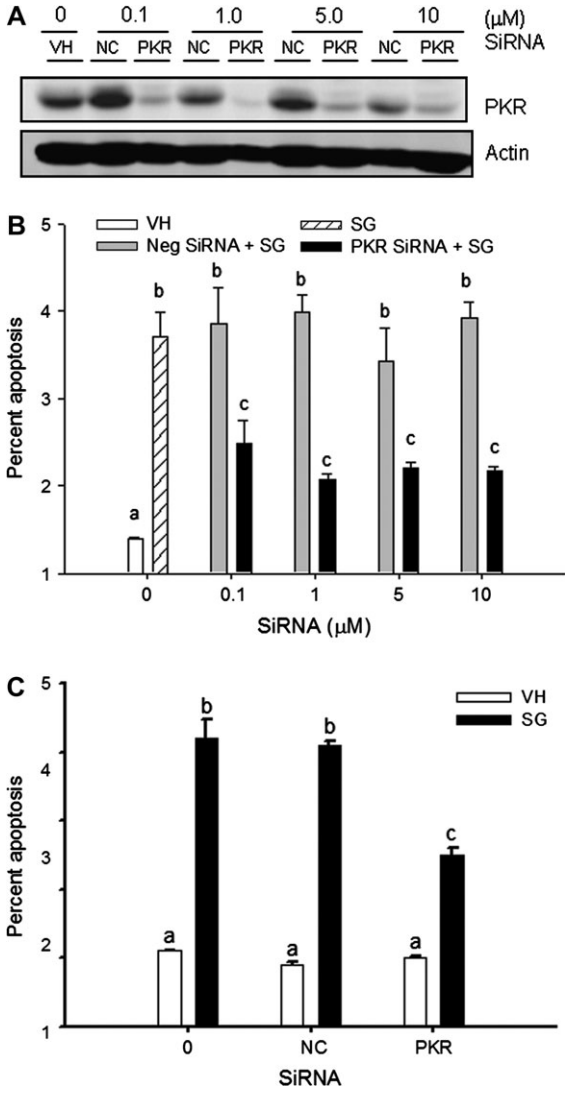

FIG. 8. PKR siRNA inhibits SG-induced apoptosis. (A, B) PC-12 cells were electroporated with PKR-siRNAs or nonspecific siRNAs as negative control (NC) at the indicated concentrations for 48 h and then cells were either (A) subjected to Western blot analysis for PKR and actin or (B) treated with SG (10 ng/ml) or vehicle (VH) for an additional 48 h and apoptosis quantitated by flow cytometry of hypofluorescent cells. (C) Cells were electroporated without siRNA, with 5μM nonspecific siRNAs or with 5μM PKR-siRNAs and then treated with SG (10 ng/ml) or vehicle (VH) for 48 h. Data are mean ± SEM ($n = 3$). Bars without the same letters differ ($p < 0.05$).

following intranasal instillation (Islam *et al.*, 2006, 2007). The results presented here indicate that SG induced apoptosis in both naïve and differentiated PC-12 cells within 48 h. Prior to and during apoptosis onset, SG markedly upregulated expression of PKR, BAX, and p53, all three of which have been previously reported to mediate apoptosis in murine OSNs (Chang *et al.*, 2002a; Ge *et al.*, 2002; Huang *et al.*, 1995). Expression of these genes is consistent with observations made previously in turbinates of mice within 24 h after single nasal instillations with macrocyclic trichothecenes (Islam *et al.*, 2006, 2007). Although oxidative stress and MAPK were not observed here to contribute to SG-induced apoptosis, the process appeared to be mediated by PKR in a caspase-independent manner.

Relatively rapid apoptotic cell death (6–24 h) in PC-12 cells has been previously reported to be induced by SH, a macrocyclic trichothecene closely related to SG, at concentrations approximating 5–50 ng/ml (Nusuetrong *et al.*, 2005). SH exposure induced phosphorylation of ERK 1/2, p38, and JNK 1/2, but in contradistinction to our findings, inhibition of p38 and JNK 1/2 blocked apoptosis. In further contrast, SH-induced p38 activation and apoptosis were suppressed by NAC, glutathione and glutathione reductase suggesting oxidative stress to be an underlying mechanism for the toxin's effects. A fundamental difference between the two studies was that the PC-12 cells were subjected to 24 h serum deprivation prior to inhibitor and SH treatment, whereas our study did not employ serum deprivation. Serum deprivation alone can evoke

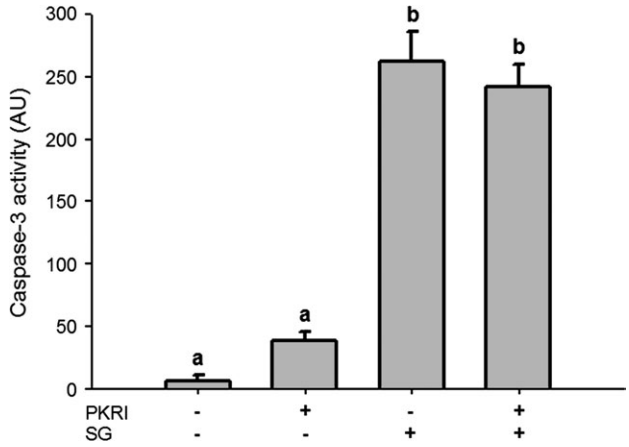

FIG. 9. SG-induced caspase-3 activation is PKR-independent. PC-12 cells were cultured with 2.5μM of PKRI C16 or vehicle for 30 min prior to incubation with SG (10 ng/ml) or vehicle. After 48 h, cells were harvested and analyzed for caspase-3 activity. Bars without the same letters differ ($p < 0.05$).

PC-12 apoptosis and this is mediated by oxidative stress (Ferrari *et al*., 1995; Liu *et al*., 2003; Maroto and Perez-Polo, 1997). Thus, oxidative stress might have exacerbated SH effects, making it difficult to discern the toxin's underlying mechanisms. Nevertheless, the aforementioned findings are important because they demonstrate that macrocyclic trichothecenes have the potential to interact with other stressors to evoke neuronal death.

Consistent with SG's effects on PC-12 cells, our laboratory has previously demonstrated in cloned macrophages that induction of apoptosis by the simple trichothecene DON is regulated by PKR (Pestka *et al*., 2004; Zhou *et al*., 2003). Although the role of PKR in mediating apoptosis in virus-infected cells is well-established (Garcia *et al*., 2007), PKR can be activated in the absence of viruses by the PKR-activating

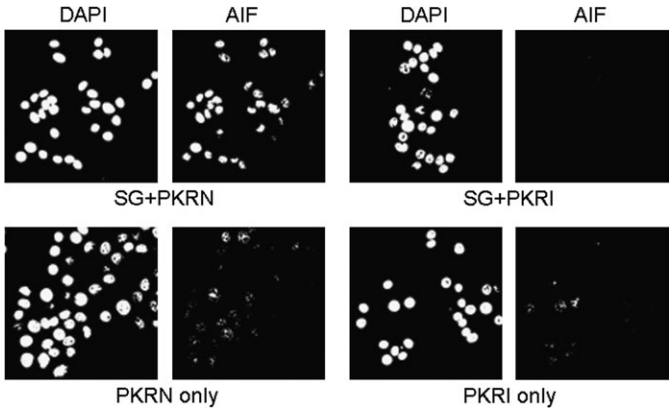

FIG. 10. SG-induced AIF translocation is PKR-dependent. PC-12 cells were grown in eight-well culture slides and incubated with 2.5μM PKRI C16 or PKRI negative control (PKRN) for 30 min and then treated with SG (10 ng/ml) for 12 h. AIF immunoreactivity and DAPI staining of cell nuclei were detected with immunofluorescence microscopy.

protein PACT and by its mouse homologue RAX (Bennett *et al*., 2004; Ito *et al*., 1999; Patel and Sen, 1998). PKR also mediates apoptosis induction by dsRNA, TNF-α, LPS, tunicamycin, or serum starvation (Cole, 2007).

Of particular significance to the present study, PKR has been linked to neuronal apoptosis. Chang *et al*. (2002a) demonstrated that PKR and eIF2α phosphorylation play a significant role in apoptosis of neuroblastoma cells and primary neuronal cultures induced by β-amyloid peptides, calcium ionophores and flavanoids. PKR activation has been associated with degenerating neurons in Alzheimer's (Chang *et al*., 2002b; Onuki *et al*., 2004; Peel, 2004), Parkinson's and Huntington's (Bando *et al*., 2005) diseases as well as amyotrophic lateral sclerosis (Garcia *et al*., 2006; Hu *et al*., 2003), HIV-associated dementia (Alirezaei *et al*., 2007), and retinal ganglion cell death (Shimazawa *et al*., 2007).

There are several possible downstream mechanisms by which PKR might mediate apoptosis. First, phosphorylation of eIF2α with accompanying translational inhibition have been proposed to be sufficient for PKR-induced apoptotic responses simply by inhibiting synthesis of anti-apoptotic factors such as inhibitor of protein synthesis (IAP) (Scheuner *et al*., 2006). Second, PKR-mediated phosphorylation has been demonstrated to induce the eIF2α/ATF4/CHOP pathway which drives expression of apoptotic genes (Lee *et al*., 2007). Finally, NF-κB activation by PKR has also been suggested to mediate apoptosis (Gil and Esteban, 2000). Proapoptotic genes regulated by NF-κB include p53, caspase 1, IRF-1, Fas L, and Fas (Garcia *et al*., 2006). Understanding involvement of NF-κB in the PC-12 model described here is complicated by this transcription factor's capacity to regulate expression of both proapoptotic and anti-apoptotic genes. Overall, clarification is needed on the mechanisms by which PKR mediates SG-induced apoptosis in neuronal cells.

Conventional apoptosis is generally considered to involve initiation of execution resulting from activation of caspases (Krantic *et al*., 2007). Caspase-3 is widely recognized to be a critical central mediator of apoptosis in many types of cells (Turk and Stoka, 2007) and has been specifically shown to mediate apoptosis in OSNs (Cowan and Roskams, 2004). Although caspase-3 is inactive in adult neuronal cells, it is reactivated upon injury and can contribute to normal apoptotic death (Stoka *et al*., 2006; Yakovlev *et al*., 2001). Caspase-3 and CAD can be activated by binding of death ligands to TNF-receptor family or in response to stress-induced mitochondrial proteins such as BAX (Green, 2000). Although SG did not induce caspase-3 gene expression, it did activate caspase-3. Thus, it was surprising that caspase-3 inhibition by DEVD-CHO did not inhibit SG-induced apoptosis. Relatedly, it was observed that the PKR inhibitor C16 did not inhibit SG-induced caspase-3 activity, even though it suppressed SG-induced apoptosis. Because DEVD-CHO also blocks caspase-6, caspase-7, caspase-8, and caspase-10 activity, caspases per se do not appear to be integral to SG-induced apoptosis.

Both caspase-dependent and caspase-independent neuronal cell death pathways have been described (Stefanis, 2005). The mitochondrial flavoprotein AIF is a primary mediator of caspase-independent apoptosis-like programmed cell death and has been linked to neurodegeneration (Cao *et al*., 2007; Krantic *et al*., 2007). Translocation of AIF from mitochondria to the nucleus acts as a proapoptotic trigger possibly by binding to DNA, stimulating DNAse activity and evoking DNA fragmentation (Cande *et al*., 2002; Martinou and Green, 2001; Ye *et al*., 2002). Both p53 and BAX have been shown to have upstream roles in AIF translocation (Cheung *et al*., 2005; Stambolsky *et al*., 2006). The possibility therefore exists that SG-induced p53 and BAX gene expression drives AIF translocation in PC-12 cells.

Taken together, the results presented herein indicate that SG induces PKR-dependent cell death in PC-12 cells that has the characteristics of apoptosis but does not appear to require caspases. Consistent with these observations, SG induces nuclear translocation of AIF, a known mediator of caspase-independent apoptosis-like programmed cell death. There is a need for improved understanding of both the mechanisms by which PKR drives SG-induced cell death and the role of AIF in this process. The relevance to these findings to previously observed SG-induced OSN cell death in mouse nasal turbinates will require further exploration using specific *in vitro* and *in vivo* models for this neuronal cell-type.

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