p38 MAP Kinase Mediates Bax Translocation in Nitric Oxide–induced Apoptosis in Neurons

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Abstract. Nitric oxide is a chemical messenger implicated in neuronal damage associated with ischemia, neurodegenerative disease, and excitotoxicity. Excitotoxic injury leads to increased NO formation, as well as stimulation of the p38 mitogen-activated protein (MAP) kinase in neurons. In the present study, we determined if NO-induced cell death in neurons was dependent on p38 MAP kinase activity. Sodium nitroprusside (SNP), an NO donor, elevated caspase activity and induced death in human SH-SY5Y neuroblastoma cells and primary cultures of cortical neurons. Concomitant treatment with SB203580, a p38 MAP kinase inhibitor, diminished caspase induction and protected SH-SY5Y cells and primary cultures of cortical neurons from NO-induced cell death, whereas the caspase inhibitor zVAD-fmk did not provide significant protection. A role for p38 MAP kinase was further substantiated by the observation that SB203580 blocked translocation of the cell death activator, Bax, from the cytosol to the mitochondria after treatment with SNP. Moreover, expressing a constitutively active form of MKK3, a direct activator of p38 MAP kinase promoted Bax translocation and cell death in the absence of SNP. Bax-deficient cortical neurons were resistant to SNP, further demonstrating the necessity of Bax in this mode of cell death. These results demonstrate that p38 MAP kinase activity plays a critical role in NO-mediated cell death in neurons by stimulating Bax translocation to the mitochondria, thereby activating the cell death pathway.

Key words: caspase • excitotoxicity • neuronal cell death • p53 • mitochondria

Introduction

Nitric oxide has been implicated as a critical mediator of neuronal injury in association with both acute and chronic neurological insults (Beal, 1996; Dawson and Dawson, 1996; Heales et al., 1999). Pharmacological inhibitors of neuronal nitric oxide synthase (nNOS)[1] have been shown to significantly reduce lesions produced in response to focal ischemia (Yoshida et al., 1994), administration of the neurotoxin MPTP (Schulz et al., 1995a), and intrastriatal injections of NMDA (Schulz et al., 1995b). Moreover, mice that are deficient in the nNOS gene exhibit significant protection against cerebral ischemia (Huang et al., 1994) and NMDA-mediated excitotoxicity (Dawson et al., 1996; Ayata et al., 1997).

The neuroprotection conferred by the absence of nNOS expression may relate to a reduction in oxygen free radicals and related nitric oxide reaction products. For example, nitric oxide can react with superoxide to produce peroxynitrite and other oxygen radicals (Beckman et al., 1990). These reactive agents, in turn, can produce extensive cellular damage by oxidizing DNA, protein, and lipids (Beckman and Crow, 1993). Noted examples of damage include nitrosylation of proteins and oxidative DNA damage with accumulation of strand breaks (Inoue and Kawanishi, 1995). Damage to these cellular substrates is presumed to activate additional downstream signaling pathways, which culminate in apoptosis. However, such signaling pathways that are activated in neurons in response to nitric oxide production have not been identified.

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[1]*Abbreviations used in this paper:* ERK, extracellular signal–regulated kinase; GFP, green fluorescent protein; JNK, c-Jun NH_2-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; SNP, sodium nitroprusside; nNOS, neuronal nitric oxide synthase.

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The mitogen-activated protein (MAP) kinases comprise a family of serine/threonine kinases that function as critical mediators of signal transduction (Cohen, 1997; Ip and Davis, 1998). Members of the MAP kinase superfamily include the extracellular signal–regulated kinases (ERKs), the Jun NH_2-terminal kinase (JNK), and the p38 MAP kinases. ERKs (ERK1 and ERK2) are activated in response to mitogen or growth factor stimulation (Boulton et al., 1991; Qui and Green, 1992; Loeb et al., 1992; Cobb, 1999). In contrast, the JNK and p38 MAP kinases are activated by a variety of cellular stresses including ultraviolet light, hyperosmolarity, heat shock, and proinflammatory cytokines (D'erijard et al., 1995; Galcheva-Gargova et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Rouse et al., 1994). p38 MAP kinase and JNK recently have been shown to be involved in NGF deprivation-induced cell death in a neuronal cell line (Xia et al., 1995; Kummer et al., 1997). In addition, p38 MAP kinase and JNK activity have also been implicated in developmental neuronal cell death (Aloyz et al., 1998; Maroney et al., 1998), as well as cell death associated with axotomy (Glicksman et al., 1998) and excitotoxicity (Kawasaki et al., 1997; Yang et al., 1997).

Nitric oxide production in neurons, as well as in other cell types, may activate caspaselike proteases (Tamatani et al., 1998; Jun et al., 1999). Caspases comprise a family of cysteine proteases that are implicated as cell death effectors in both vertebrate and invertebrate cells (Nicholson and Thornberry, 1997; Porter et al., 1997; Cryns and Yuan, 1998). The release of cytochrome *c* and other apoptogenic factors from injured mitochondria recently have been shown to activate caspases (Kluck et al., 1997; J. Yang et al., 1997). Mitochondrial integrity appears to be regulated, in part, by members of the Bcl-2 family (Reed, 1997). In response to apoptotic signals, Bax, a proapoptotic member of this family, is redistributed from the cytosol to the mitochondria (Hsu et al., 1997; Wolter et al., 1997), where it precipitates a decline in mitochondrial membrane potential followed by cytochrome *c* release and caspase activation (J.G. Xiang et al., 1996; Vekrellis et al., 1997; Eskes et al., 1998; Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999).

Since several neuronal cell types contain caspase proteins that are activated by the same stimuli shown to upregulate p38 MAP kinase activity (Xia et al., 1995; Kawasaki et al., 1997), it is conceivable that the p38 MAP kinase contributes to the activation of caspases. Therefore, we evaluated the relationship between p38 MAP kinase activity, Bax translocation, and caspase activation. We report here that treatment with a nitric oxide donor, sodium nitroprusside (SNP), stimulated Bax translocation to the mitochondria, which was followed by caspase activation. Administration of p38 MAP kinase inhibitors blocked Bax translocation, partially suppressed caspase activation, and significantly enhanced neuronal survival. In contrast, caspase inhibitors completely suppressed SNP-mediated caspase induction, but failed to confer the same degree of protection from cell death as the p38 MAP kinase inhibitors. These findings suggest that nitric oxide–mediated cell death in neurons may occur through a p38 MAP kinase– and Bax-dependent pathway, but in a caspase-independent manner. Moreover, the present results suggest that p38 MAP kinase promotes apoptosis by facilitating Bax translocation to the mitochondria.

Materials and Methods

Materials

Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk), z-Asp-Glu-Val-Asp-fmk (zDEVD-fmk), and the fluorogenic caspase substrate zDEVD-AFC were purchased from Enzyme Systems Product. SNP was obtained from ICN Biomedicals. The MAP kinase kinase (MEK) inhibitor, U0126, was obtained from Promega, and the p38 MAP kinase inhibitor, SB203580, and its structurally related negative control compound, SB202474 (Lee et al., 1994), were obtained from Calbiochem. Propidium iodide and Hoechst 33342 were obtained from the Sigma Chemical Co. A cell lysis buffer for fluorogenic caspase activity assays was obtained from CLONTECH Laboratories, Inc. (ApoAlert CPP32 assay kit).

Cell Culture

SH-SY5Y human neuroblastoma cells were obtained from the American Type Tissue Culture Collection and were routinely maintained in DME/Ham's F12 medium with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. SH-SY5Y cells were plated at 5.0×10^4 cells/35-mm dish for determination of cell number or at 10^6 cells/60-mm dish for Western blot analysis and caspase activity measurements. SH-SY5Y cells were routinely plated 1–2 d before treatment (considered day 0). For determination of viable cell number, triplicate cultures of SH-SY5Y cells were trypsinized, collected by centrifugation (2,000 rpm, 8 min), resuspended in Hanks' balanced salt solution containing trypan blue, and counted using a hemacytometer.

Bax-deficient mice were generated from a 129/Sv × C3H background (Knudson et al., 1995), and p53-deficient mice were generated from a 129/Sv × C57BL/6 background (Donehower et al., 1992) as described previously. The genotypes of the mating pairs and all offspring were determined by PCR, using DNA extracted from the tail (Timme and Thompson, 1994). Cortical neurons from individual animals were separately cultured, and their genotypes were determined before treatment.

Neuronal cultures derived from postnatal day 0 (P0) cortex were established as previously described (H. Xiang et al., 1996; Xiang et al., 1998). In brief, newborn mice were killed by decapitation, and the cortex was dissected free in Hanks' balanced salt solution. The dissected tissues were treated with trypsin for 25 min, washed, and dissociated by trituration. The cells were plated on a poly-D-lysine–coated substrate in Neurobasal medium plus B27 supplements (GIBCO BRL; Brewer, 1997) at 1.25×10^5 cells per 15-mm well for neuronal counting, or at $1.5–2 \times 10^6$ cells/60-mm dish for caspase activity assays. The cultures were maintained at 37°C in a 5% CO_2 atmosphere. Cultures, maintained under these conditions, were previously shown to contain ~95% neurons, as determined by cell morphology and immunocytochemistry for neurofilament and GFAP (H. Xiang et al., 1996).

Assessment of Neuronal Viability

Neurons were routinely maintained in culture for 4 d before treatment. The number of viable neurons was determined by counting cells within four premarked reticules (1 mm^2/well) at the time of treatment and at various times after treatment. Viable neurons were identified according to the following criteria: that neurites were uniform in diameter, smooth in appearance, and at least twice as long as the soma; that somata were normally smooth and round to oval in shape; and that nuclei were normal in appearance, without evidence of condensation or fragmentation. In contrast, degenerating, nonviable neurons possessed neurites that were fragmented and beaded, and the somata were rough, shrunken, vacuolated, and irregular in shape. The nuclei of nonviable neurons were often condensed or fragmented.

Fluorogenic Caspase Assays

Caspase activity was determined by monitoring the cleavage of a specific fluorogenic caspase substrate, zDEVD-AFC (z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin). The cells were plated and maintained as described above. At specific times after treatment, cells were collected by scraping in cold PBS, centrifuged (2,000 rpm, 8 min), and

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lysed on ice for 10 min in the cell lysis buffer provided in the CLONTECH ApoAlert™ CPP32 assay kit. The extracts were kept at −20°C until the time of assay. At that time, the extracts were thawed and reacted with the fluorogenic caspase substrate (zDEVD-AFC, 100 μM) in reaction buffer (0.1 M Hepes buffer, 0.1% Chaps, 1% sucrose, pH 7.4) containing 1 mM DTT. The mixtures were maintained at 37°C for 40 min and subsequently analyzed in a fluorometer (Perkin Elmer) equipped with a 400-nm excitation filter and a 505-nm emission filter. The levels of relative fluorescence were normalized against the protein concentration of each extract, which was determined using the Bio-Rad protein assay reagent. Rabbit IgG was used as a protein standard.

p38 and JNK Western Blot Analysis

SH-SY5Y cells were plated as described above and treated with SNP (500 μM) or UV irradiation (80 J/m^2). Control cultures were sham-treated to exclude the contribution of disturbing the cultures in association with the treatment. At the appropriate time points, the cells were lysed in an extraction buffer as previously described (Xia et al., 1995). Proteins were resolved by SDS-PAGE on 10% gels, and immunoblotting was performed as previously described (Hetman et al., 1999) using a phospho-p38 MAPK (Tyr182) antibody (New England Biolab No. 9211, 1:1,000) and phospho-JNK (Thr183/Tyr185) antibody (Promega No. V7931, 1:5,000) according to the manufacturer's instruction.

Fluorescence-activated Cell Sorting

Human SH-SY5Y cells were plated at 2.0×10^6 cells per 60-mm dish. 1 d after plating, the cells were treated with SNP (500 μM) alone or SNP plus the p38 MAP kinase inhibitor (20 μM, SB203580) or the caspase inhibitor zVAD-fmk (20 μM). At 24 and 72 h after treatment, the cells were harvested by trypsinization. The trypsin action was stopped by adding serum-containing medium. Cells were centrifuged (1,400 rpm, 8 min), and resuspended in PBS to a final concentration of 5×10^5 cells/ml. Hoechst 33342 (20 μl/ml, 1 mM stock) and propidium iodide (5 μl/ml, 5 mg/ml stock) were added sequentially to the cell suspension. The cell suspension was incubated in the dark for 30 min at 37°C in a humidified 5% CO_2 atmosphere. Cells were analyzed using an Epics Elite ESP cell sorter flow cytometer (Coulter Diagnostics Systems). Approximately 2.0×10^4 cells were analyzed per sample. Fluorescence was analyzed and plotted using the Multiplus software package (Phoenix Flow Systems). This protocol determines the absolute number of live (Hoechst 33342-positive/propidium iodide–negative) and dead cells (propidium iodide–positive; Hamori et al., 1980; Poot et al., 1997).

Transfection of SH-SY5Y Cells and Primary Cortical Neurons

An expression construct encoding Bax, which was fused to the enhanced green fluorescent protein (GFP), was created using full-length human Bax cDNA (a gift of S.J. Korsmeyer, Howard Hughes Medical Institute and Dana Farber Cancer Research Center, Boston, MA) inserted into the vector pEGFP-C3 (CLONTECH Laboratories Inc.; Hsu et al., 1997). Expression constructs encoding constitutively active MKK3, (pRc/RSV-Flag-MKK3(Glu)) and dominant negative MKK3 (pRc/RSV-Flag-MKK3(Ala)) have been described (Xia et al., 1995).

SH-SY5Y cells were plated at a density of 5×10^4 cells per well into 2-well LabTekII chambered coverglass (Nalge Nunc International) and maintained at 37°C with 5% CO_2 for 24 h. SH-SY5Y cells were replenished with fresh DME/Ham's F12 culture medium for 1 h at 37°C in 5% CO_2 in preparation for transfection. Postnatal cortical neurons were plated at 2×10^5 cells per well and maintained for 3 d before transfection. DNA transfections were performed using a calcium phosphate precipitation method (Xia et al., 1995). The DNA–Ca_2PO_4 complexes were prepared by mixing 0.75–2.0 μg DNA per 5×10^4 cells with 2.5 M $CaCl_2$ in ddH_2O (final $CaCl_2$ concentration 250 mM). Cotransfection experiments involved the addition of equal amounts of GFP-Bax and MKK3 plasmids. This solution was added dropwise to a solution of 2× Hepes-buffered saline, pH 7.08, and precipitates were allowed to form for 30–60 min. The solution containing DNA precipitates was added to the cultures and gently mixed. After 12 h, the cells were washed three times with PBS, and fresh medium was added. 2 d after transfection, cells were treated as specified in the results. Cells were fixed at appropriate time points with 4% paraformaldehyde and analyzed using confocal microscopy.

Confocal Microscopy

Fixed cells were stained with 2.5 μg/ml Hoechst 33342 in PBS for 30 min to demonstrate nuclear morphology. A subset of cells was treated with MitoTracker red (20 ng/ml; MitoTracker red CMXRos; Molecular Probes Inc.) to visualize mitochondria as previously described (Wolter et al., 1997). Images were collected on a confocal microscope (Nikon Diaphot 300 with a 40× Fluor 0.85 NA objective). The 488-nm line of a krypton/argon laser was used for fluorescence excitation of GFP, the 568-nm line for MitoTracker red and the 364-nm line was used for excitation of Hoechst 33342. Images were processed on the Bio-Rad MRC 1024 UV computer in z-series, with brightest point projections made through NIH Image 1.62f. TIFF files were processed in Adobe Photoshop 5.0 for simultaneous GFP and Hoechst 33342 visualization.

Results

p38 MAP Kinase Activity Is Essential for SNP-induced Cell Death in Human SH-SY5Y Neuroblastoma Cells and Primary Cultures of Cortical Neurons

The involvement of p38 MAP kinase activity in nitric oxide–mediated cell death in neurons was evaluated using both a neuronal cell line and primary cultures of cortical neurons. The addition of SNP (500 μM), a nitric oxide donor, to SH-SY5Y human neuroblastoma cells resulted in significant activation of the p38 MAP kinase (Fig. 1, p-*p*38 MAPK). Activation was detected as early as 3 h after SNP treatment and remained elevated even 24 h after treatment relative to vehicle treated control cells. In contrast to the p38 MAP kinase, SNP treatment did not activate JNK in SH-SY5Y cells (Fig. 1, *p*-JNK). However, both *p*38 MAP kinase and JNK were rapidly and profoundly activated in SH-SY5Y cells in response to UV irradiation, suggesting that JNK can be activated in SH-SY5Y cells in response to the appropriate cellular stress. These results demonstrate that nitric oxide selectively activates the stress-activated p38 MAP kinase in human SH-SY5Y neuroblastoma cells.

The significance of p38 MAP kinase activation was evaluated in SNP-induced cell death by using specific inhibitors for MAP kinases. Under control conditions SH-SY5Y human neuroblastoma cells steadily proliferated, exhibit-

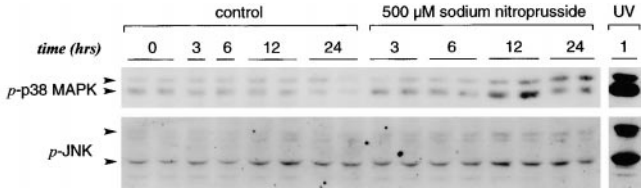

Figure 1. The nitric oxide donor, SNP, activates p38 MAP kinase but not JNK in SH-SY5Y cells. SH-SY5Y cells were treated with 500 μM SNP, and protein extracts were prepared in duplicate (except for the 3- and 6-h time points for control) at the indicated time points to assess the activation of p38 MAP kinase (*p*-p38 MAPK, top) and JNK (*p*-JNK, bottom) by Western blotting using antibodies specific for a phosphorylated (activated) form of the kinases. Protein extracts from UV-irradiated (80 J/m^2) SH-SY5Y cells were also analyzed in parallel as a positive control for activation of both p38 MAP kinase and JNK. Arrowheads indicate the position of specific immunoreactive bands corresponding to distinct phosphorylated p38 MAP kinase and JNK isoforms.

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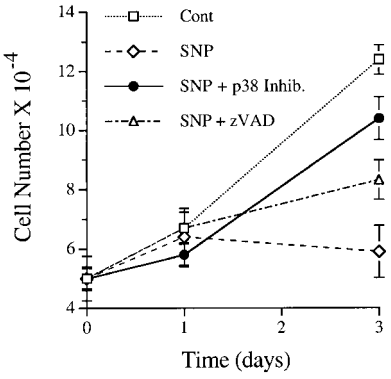

Figure 2. p38 MAP kinase activity is essential for SNP-induced cell death in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were plated as described in Materials and Methods. After 12–18 h in culture, the cells were treated with DMSO (control) or SNP (500 μM) in the presence or absence of a p38 MAP kinase inhibitor (SB203580, 20 μM) or a cell-permeable, irreversible tripeptide caspase antagonist (zVAD-fmk, 20 μM). Cells were trypsinized and counted on day 1 and day 3 using a hemocytometer. Values represent the mean number of cells per culture ± SD of triplicate cultures and are representative of four separate experiments. The number of cells in SNP-treated cultures differed significantly from all other conditions ($P < 0.01$, ANOVA).

ing a doubling time of ~50 h. SNP (500 μM) induced a significant degree of cell loss in SH-SY5Y cells in a time- (Fig. 2) and dose-dependent manner (data not shown). However, the number of SH-SY5Y cells per dish was significantly increased when SNP was added together with the p38 MAP kinase inhibitor, SB203580 (20 μM). In contrast, only a modest increase in cell number resulted from the addition of the cell-permeable, irreversible pan-caspase inhibitor, zVAD-fmk (20 μM).

Since the SH-SY5Y cells used in this study proliferate, it was necessary to determine whether SNP actually induced cell death in SH-SY5Y cells or simply caused cell cycle arrest. Fluorescence-activated cell sorting was used to analyze cells on the basis of propidium iodide uptake and total DNA content, the latter based on the staining intensity of the Hoechst dye. As seen in the control graph (Fig. 3 A), the majority of cells was contained in cubicle 5, which represents viable cells. After SNP treatment (72 h; Fig. 3 B) there was a significant shift in fluorescence up to cubicle 7, reflecting the increase in propidium iodide uptake that occurs with membrane damage. The fluorescence intensity was also shifted to the left on the x-axis, reflecting DNA degradation. The DNA content profile (Fig. 3 B, inset) revealed no sign of cell accumulation in the G1 or G2 phase of the cell cycle, but rather demonstrated a significant shift from a 2N diploid content to a sub-G1 content, which is consistent with DNA degradation, confirming that the SNP effect on cell number was due to the induction of cell death. Addition of the p38 MAP kinase inhibitor (20 μM; Fig. 3 C) significantly shifted the fluorescent signal back to cubicle 5, demonstrating that p38 MAP kinase inhibition maintained cell viability. The size of the sub-G1 peak was also significantly reduced. In contrast, the addition of zVAD-fmk (20 μM, Fig. 3 D) only produced a small shift back to cubicle 5 and failed to reduce the peak associated with the sub-G1 DNA content, corroborating that the caspase inhibitor conferred a small degree of protection from SNP-mediated cell death (Fig. 3 D).

SNP also induced a significant degree of cell loss in primary cultures of cortical neurons. Neuronal survival was reduced by >50% after 48 h of exposure to SNP (Fig. 4). Concomitant addition of the p38 MAP kinase inhibitor protected primary cortical neurons from SNP-mediated cell death. This was not a transient effect, as survival was maintained in the presence of SB203580 for at least 5 d (data not shown). To demonstrate that SNP-mediated cell

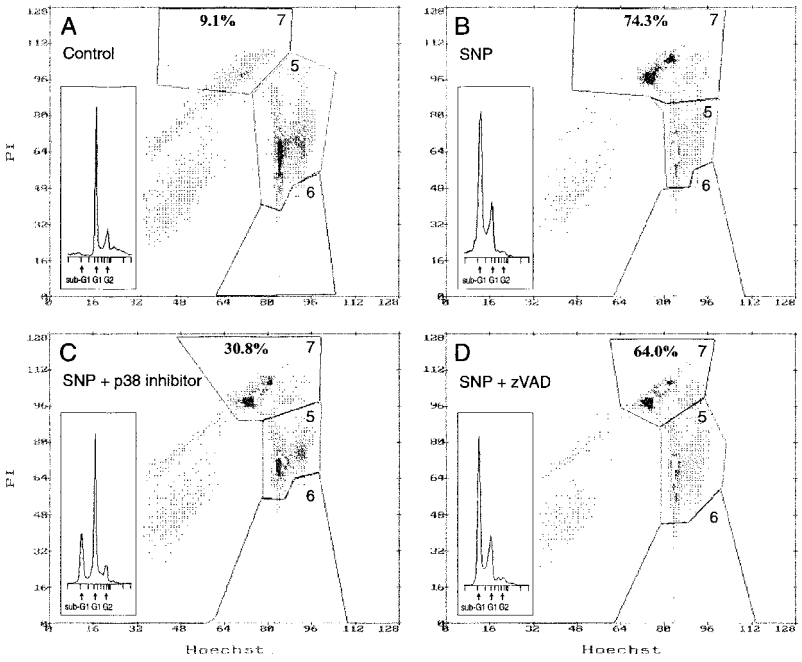

Figure 3. Fluorescence-activated cell sorting analysis revealed cell death, but not cell cycle arrest, in SH-SY5Y human neuroblastoma cells in response to SNP. SH-SY5Y human neuroblastoma cells were plated and maintained in basal culture conditions as described in Materials and Methods. After 12–18 h in culture, the cells were treated with (A) DMSO (vehicle control), (B) SNP (500 μM), (C) SNP plus the p38 MAP kinase inhibitor SB203580 (20 μM), or (D) SNP plus the caspase antagonist zVAD-fmk (20 μM). 3 d after treatment, fluorescence-activated cell sorting was performed. Viable cells are contained in cubicle 5. Nonviable cells are represented in cubicle 7. Everything to the left of cubicles 5–7 represents cellular debris. The insets represent cellular DNA content profiles as measured with the DNA binding dye Hoechst 33342. Positions corresponding to the normal diploid DNA content (2N) in the G1 phase, a 4N content in the G2 phase, and a sub-G1 content (<2N), which are indicative of apoptosis and its associated DNA fragmentation, are shown.

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death was specifically associated with activation of the p38 MAP kinase signal transduction pathway, we also evaluated an inhibitor of the ERK pathway. ERKs are activated by MAP kinase kinase or MEKs in response to growth and differentiating factors (Boulton et al., 1991; Loeb et al., 1992; Qui et al., 1992; Cobb, 1999), in contrast to p38 and JNK, which are activated by cellular stress (Derijard et al., 1994; Galcheva-Gargova et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Rouse et al., 1994; Xia et al., 1995; Kummer et al., 1997). Inclusion of U0126 (20 μM), a selective MEK inhibitor (Favata et al., 1998), actually enhanced neuronal cell death in the presence of SNP (Fig. 4). This is consistent with the demonstration that the ERK pathway mediates neuronal survival in response to trophic factor stimulation (Xia et al., 1995; Hetman et al., 1999). A biologically inactive control compound SB202474 (Inh Control), which is structurally related to both SB203580 and U0126 (Lee et al., 1994), had no influence on neuronal survival, suggesting that the effects observed with SB203580 and U0126 were specifically related to inhibitory actions in their respective pathways.

The inclusion of zVAD-fmk, a broad spectrum caspase inhibitor, had no effect on the survival of primary cortical neurons in culture, in contrast to the modestly protective results obtained with SH-SY5Y cells. The difference between SH-SY5Y cells and primary cortical neurons in relation to the protective effects of zVAD may relate to the embryonic nature of the SH-SY5Y cells (Johnson et al., 1999). These results demonstrate that increased levels of nitric oxide induce cell death in cultured SH-SY5Y cells and primary cultures of cortical neurons by a mechanism involving p38 MAP kinase activation.

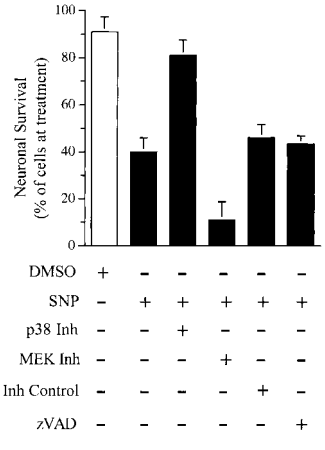

Figure 4. p38 MAP kinase activity is essential for SNP-induced cell death in cultured postnatal murine cortical neurons. Postnatal cortical neurons were plated and maintained in basal culture conditions for 4 d as described in Materials and Methods. Cells were treated with DMSO (vehicle control) or with a single dose of SNP (500 μM) in the presence or absence of the p38 MAP kinase inhibitor SB203580 (p38 Inh, 20 μM), the MEK inhibitor U0126 (MEK Inh, 20 mM), the structurally related negative control compound SB202474 (Inh Control, 20 μM), or the broad spectrum caspase inhibitor zVAD-fmk (zVAD, 20 μM). Neuronal survival was assessed 48 h later by counting the number of viable neurons (H. Xiang et al., 1996). All data are the means ± SD of 12 cultures from three separate experiments. Neuronal survival in cultures treated with SNP differed significantly from survival in non-treated cultures or cultures treated with SNP plus the p38 MAP kinase inhibitor ($P < 0.001$, ANOVA). Neuronal survival in cultures treated with SNP plus the MEK inhibitor differed significantly from survival in all other conditions ($P < 0.001$, ANOVA).

Nitric oxide–inducible Caspase Activity Is Dependent on p38 MAP Kinase Activity

To further characterize the mechanism by which p38 MAP kinase promotes neuronal cell death, we determined if p38 MAP kinase activation leads to mitochondrial dysfunction. To this end, we evaluated the contribution of p38 MAP kinase to caspase induction, which is known to depend on the release of apoptogenic factors from damaged mitochondria (Kluck et al., 1997; J. Yang et al., 1997). Human SH-SY5Y neuroblastoma cells were exposed to SNP (500 μM), and cellular extracts were evaluated for the presence of caspase activity by monitoring the cleavage of the fluorogenic caspase substrate, zDEVD-AFC. Under control conditions (DMSO treatment), basal levels of caspase activity were readily detected. Caspase activity was increased more than sixfold 72 h after exposure to SNP (Fig. 5). Caspase activation was first detected 24 h after treatment and was completely inhibited by the cell-permeable, irreversible caspase inhibitor, zVAD-fmk (20 μM; Fig. 5). Maximum inhibition of DEVD cleavage activity was obtained at concentrations ≥10 μM zVAD-fmk (data not shown). More importantly, caspase activity was also suppressed by concomitant treatment with the p38 MAP kinase inhibitor, SB203580. The p38 MAP kinase inhibitor suppressed caspase activity almost as effectively as zVAD-fmk at 24 h, and reduced caspase activity by almost 80% 72 h after SNP treatment. The results of this study demonstrate that p38 MAP kinase activity is involved in the regulation of caspase induction in SH-SY5Y cells in response to nitric oxide–induced damage, suggesting that p38 MAP

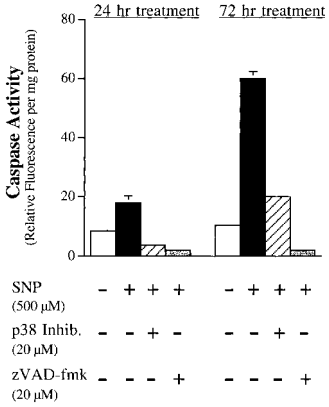

Figure 5. SNP-mediated induction of caspase activity in SH-SY5Y cells requires p38 MAP kinase activity. SH-SY5Y cells were plated in 60-mm dishes as described in Materials and Methods. After 24 h in culture, the cells were treated with DMSO (Control) or SNP (500 μM) in the presence or absence of a cell-permeable inhibitor of p38 MAP kinase 203580 (p38 Inhib, 20 μM), or an irreversible tripeptide caspase antagonist zVAD-fmk (20 μM). The cells were harvested at 24 or 72 h, and cytosolic extracts were prepared and evaluated for zDEVD-AFC cleavage activity as described in Materials and Methods. The extracts were incubated with a fluorogenic substrate, zDEVD-AFC (100 μM final concentration; Enzyme Systems Products) at 37°C. The extent of substrate hydrolysis was determined after a 45-min incubation period (hydrolysis is linear for up to 60 min), and is expressed as arbitrary fluorescence units per milligram of protein. The results represent the mean ± SD ($n = 3$ cultures/condition) and are representative of five separate experiments. Caspase activity in SNP-treated cultures differed significantly from control cultures and cultures treated with SNP plus the p38 MAP kinase inhibitor or zVAD-fmk ($P < 0.001$, ANOVA). Some bars do not express standard error bars because they are small enough to be contained within the symbols.

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kinase activation is linked to a loss of mitochondrial integrity.

Nitric Oxide–induced Cell Death Involves Alterations in the Intracellular Distribution of the Cell Death Activator, Bax

The demonstration that p38 MAP kinase activity was necessary for caspase activation suggested that p38 MAP kinase may regulate some aspect of mitochondrial function, since the release of cytochrome *c* from damaged mitochondria represents an important pathway for activating caspases (Liu et al., 1996; Zou et al., 1997). Overexpressing the Bcl-2 family member, Bax, is sufficient to precipitate a decline in mitochondrial membrane potential, increased free radical production, and caspase activation (J.G. Xiang et al., 1996). These Bax-mediated alterations in mitochondrial function are consistent with the recent demonstration that cell injury promotes Bax translocation from the cytosol to the mitochondria (Hsu et al., 1997; Wolter et al., 1997; Goping et al., 1998).

Therefore, we determined if nitric oxide initiated neuronal damage by stimulating Bax translocation to the mitochondria. The intracellular distribution of the Bax protein was evaluated by transfecting SH-SY5Y cells or primary cortical neurons with a GFP-Bax fusion construct (Hsu et al., 1997; Wolter et al., 1997). DMSO-treated control cells typically displayed a diffuse, cytosolic pattern of fluorescence (Fig. 6, A and H), which was still maintained up to 8 h after SNP treatment (Fig. 6 B). However, SNP treatment (500 μM) produced significant changes in the distribution of fluorescence that was first detected 12 h after treatment and involved a change from a diffuse, cytosolic distribution to a punctate pattern of fluorescence (Fig. 6 C). This change was shown to represent the redistribution of Bax from the cytosol to the mitochondria, as demonstrated by the nearly complete overlap between GFP-Bax fluorescence and the location of mitochondria (Fig. 7), as revealed by staining with MitoTracker red (Wolter et al., 1997). These results are consistent with previous data on Bax translocation demonstrated for many different cell types, including neurons, in response to distinct forms of cell death stimuli (Hsu et al., 1997; Wolter et al., 1997; Youle, R.J., unpublished observations). After 24 h of exposure to SNP, >70% of SH-SY5Y cells and primary cortical neurons displayed this punctate pattern of fluorescence (Fig. 6, D and I). This punctate pattern was not observed in SNP-treated cells that were transfected with GFP alone, in contrast to GFP-Bax, demonstrating that changes in the fluorescence pattern were specifically due to the Bax protein (data not shown).

Bax translocation was generally observed in cells that exhibited retraction or fragmentation of cellular processes and shrinkage of the cell body. In contrast, at 12 h, it was possible to identify cells that displayed a punctate pattern of GFP-Bax fluorescence in combination with a healthy nucleus, suggesting that Bax translocation precedes nuclear fragmentation (Fig. 6 C). We did not observe cells displaying nuclear changes in the absence of Bax translocation, suggesting that changes in the distribution of the Bax protein precede both caspase activation and alterations in nuclear morphology. To ensure that Bax translocation did not simply result from a change in cell shape, cell shrinkage was produced by exposing SH-SY5Y cells to a dilute solution of trypsin (0.125%, 1 min). Although this caused SH-SY5Y cells to retract their processes and round up, thereby reducing their size, the GFP-Bax signal was still diffusely distributed throughout the cytoplasm (data not shown). In marked contrast to SNP treatment alone, the concomitant addition of the p38 MAP kinase inhibitor, SB203580, suppressed Bax translocation in both SH-SY5Y cells and primary cortical neurons, maintaining a diffuse cytoplasmic distribution of fluorescence (Fig. 6, F and J). The addition of U0126, a MEK inhibitor, which actually enhanced neuronal cell death in the presence of SNP (Fig. 4), failed to prevent the intracellular redistribution of Bax (data not shown). Similarly, the cell-permeable caspase inhibitor, zVAD-fmk (20 μM), also had no effect on Bax translocation induced by SNP exposure (Fig. 6 G). These results suggest that p38 MAP kinase activity regulates the translocation of Bax from the cytosol to the mitochondria in response to nitric oxide–induced damage in neurons.

Bax translocation was observed in cells that displayed varying degrees of chromatin condensation and nuclear fragmentation as depicted by the DNA binding dye, Hoechst 33342. The reduction in the number of cells expressing a healthy, diffuse pattern of chromatin staining was consistent with the loss of viability measured by direct cell counting and FACS analysis as described in Figs. 1 and 2. Approximately 63% of DMSO-treated GFP-Bax–transfected control SH-SY5Y cells displayed intact nuclei without any evidence of chromatin condensation or nuclear fragmentation (percentage of cells with intact nuclei ± SD; 63.25 ± 5.1, $n = 4$). The reduction in viable, transfected control cells relative to nontransfected cells is attributed to plasma membrane damage resulting from the transfection process. 24 h after SNP treatment (500 μM), only 32% of transfected cells were viable based on nuclear morphology (percentage of cells with intact nuclei ± SD; 32.70 ± 2.89, $n = 5$). Treatment with the p38 MAP kinase inhibitor (SB203580, 20 μM; percentage of cells with intact nuclei ± SD; 55.67 ± 4.04, $n = 4$) or the broad spectrum caspase inhibitor, zVAD-fmk (20 μM; percentage of cells with intact nuclei ± SD; 50.50 ± 2.12, $n = 3$) significantly reduced chromatin condensation and nuclear fragmentation at 24 h. Interestingly, although zVAD-fmk prevented changes in nuclear morphology, these cells still displayed Bax translocation as depicted by a punctate pattern of GFP fluorescence (Fig. 6 G). The results of this study demonstrate that p38 MAP kinase promotes cell death in response to injury by stimulating Bax translocation to the mitochondria.

The relationship between p38 MAP kinase activity and Bax translocation was directly evaluated by transfecting SH-SY5Y cells with a constitutively active form of the MAP kinase kinase, MKK3. MKK3 specifically phosphorylates and activates p38 MAP kinase (D'erijard et al., 1995; Davis, 1998). In the absence of any treatment, 25% of GFP-Bax–transfected SH-SY5Y cells exhibited evidence of Bax translocation (Fig. 8), with the rest of the cells displaying a diffuse cytoplasmic distribution as described for Fig. 6. In contrast, 75% of GFP-Bax–transfected cells displayed evidence of Bax translocation when cotransfected with a plasmid expressing constitutively ac-

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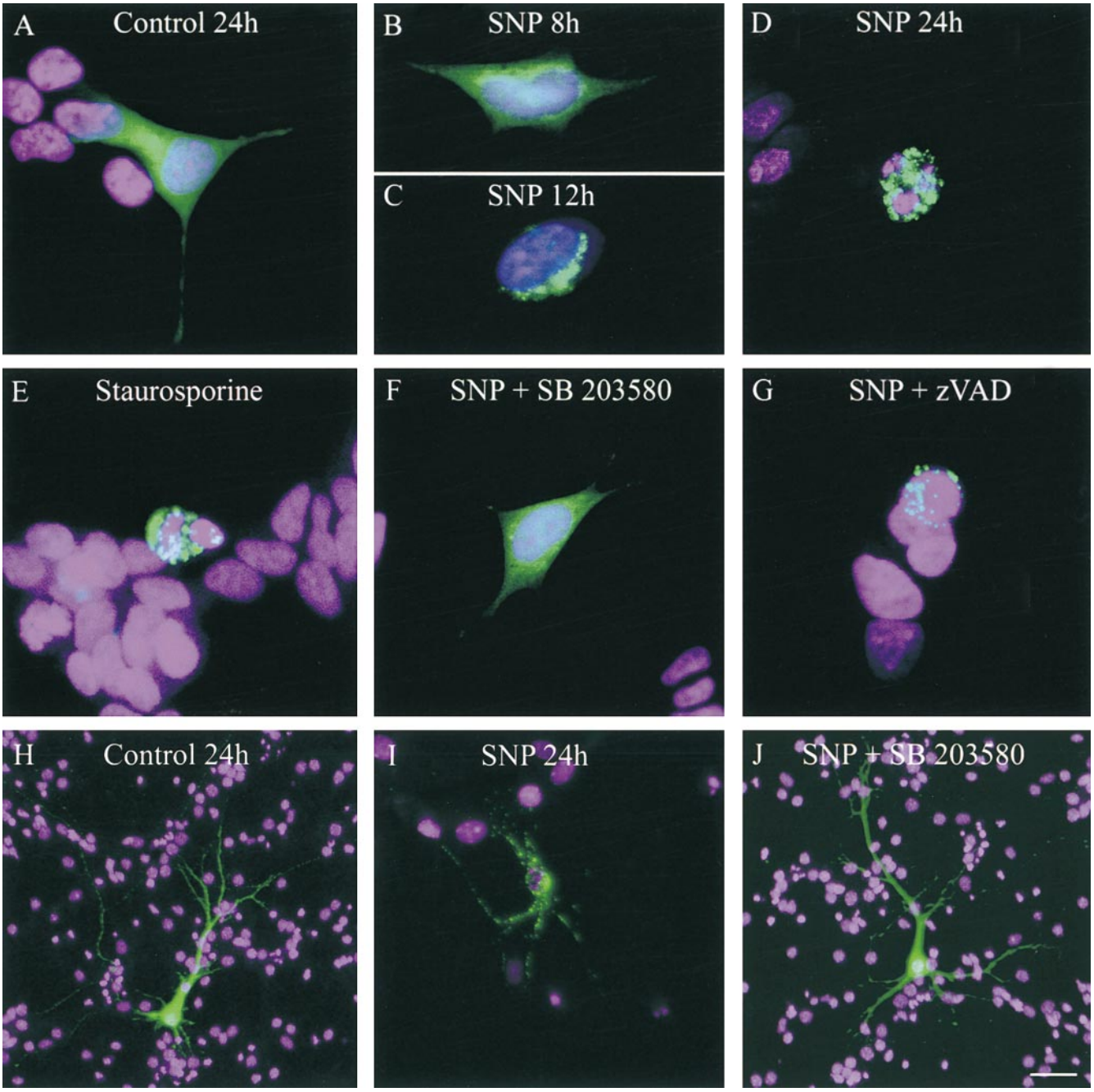

Figure 6. SNP induces p38 MAP kinase–dependent alterations in the intracellular distribution of the cell death activator, Bax. SH-SY5Y cells (A–G) and cortical neurons (H–J) were plated in 2-well LabTek II chambered coverglass as described in Materials and Methods. After 24 h (SH-SY5Y cells) or 72 h (primary neurons) in culture, the cells were transfected with the GFP-Bax fusion construct using a calcium phosphate precipitation method as described in Materials and Methods. 2 d after transfection, the cells were treated with DMSO (Control; A and H), SNP (500 μM; B–D, F, G, I, and J), or staurosporine (0.5 μM; E). A subset of cells treated with SNP were also concomitantly treated with the p38 MAP kinase inhibitor, SB203580 (20 μM; F and J), or the cell-permeable caspase inhibitor, zVAD-fmk (20 μM; G). GFP-Bax fluorescence (green) was assessed at 8 (B), 12 (C), or 24 h (A and D–J) by confocal microscopy as described in Materials and Methods. Nuclear integrity was assessed by staining the cultures with the DNA binding dye Hoechst 33342 (2.5 μg/ml; purple, the blue emission normally associated with this dye was digitally altered to purple to enhance its visualization). Apoptotic cells exhibited chromatin condensation and nuclear fragmentation. The results are representative of six separate transfection experiments for each condition. Bar: (A–G) 10 μm; (I) 20 μm; (H and J) 40 μm.

tive MKK3 (Fig 8). These same cells also exhibited nuclear condensation or fragmentation consistent with the induction of apoptosis (data not shown). However, the p38 MAP kinase inhibitor, SB203580, suppressed Bax translocation induced by activated MKK3 to the level of control cells in the presence (Fig. 8) or absence of SNP (data not shown). Furthermore, expressing constitutively active MKK3 in the presence of a suboptimal dose of SNP

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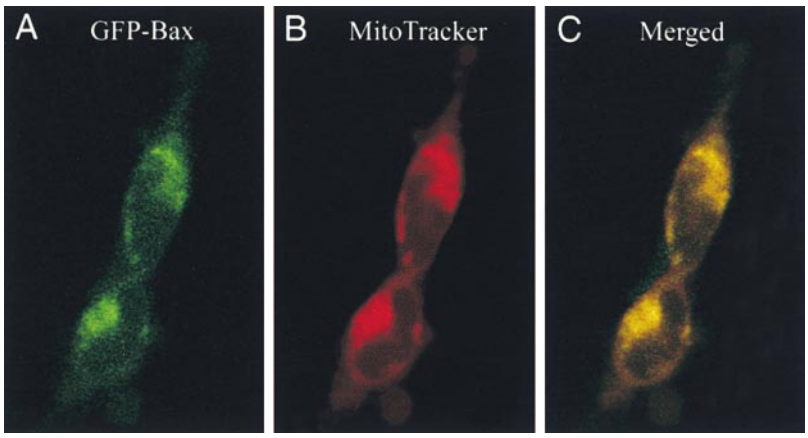

Figure 7. SNP induces Bax translocation to the mitochondria. SH-SY5Y cells were plated and transfected with GFP-Bax using Fugene (Roche Molecular Biochemical) according to the manufacturer's instructions. 1 d after transfection, the cells were treated with SNP (500 μM). The cells were also treated for 30 min before observation with 20 ng/ml MitoTracker red CMXRos to stain mitochondria. GFP-Bax (green) and MitoTracker (red) fluorescence was assessed at 18 h after SNP treatment by laser fluorescence confocal microscopy as described in Materials and Methods. GFP-Bax was diffusely distributed in control cells as seen in Fig. 6 A. However, 18 h after SNP treatment, GFP-Bax (A, green) localizes primarily to mitochondria (B, red) based on the overlap of the GFP-Bax and MitoTracker fluorescent images (C, Merged, yellow). There was no difference in the pattern of mitochondrial distribution between control and SNP-treated cells as assessed by MitoTracker red fluorescence.

showed no additive effect on the number of cells displaying Bax translocation (Fig. 8). These results suggest that activating p38 MAP kinase through MKK3 mimics the pathway stimulated by SNP. As a control for the specificity of the activated MKK3 response, SH-SY5Y cells were also cotransfected with GFP-Bax and a plasmid encoding a dominant negative form of MKK3 (Xia et al., 1995). Cells expressing dominant negative MKK3 did not display evidence of Bax translocation. In fact, the dominant negative MKK3 significantly reduced Bax translocation in cells treated with SNP (Fig. 8). These results demonstrate that p38 MAP kinase activity is sufficient to stimulate Bax translocation to the mitochondria even in the absence of injury and is necessary for SNP-induced Bax translocation. Moreover, these studies suggest that Bax plays an essential role in nitric oxide–mediated cell death in neurons.

Bax Is Required for Nitric Oxide–mediated Cell Death

To determine if Bax was essential for SNP-induced cell death, we evaluated SNP-mediated neurotoxicity in primary cultures of postnatal cortical neurons lacking a functional Bax gene (Knudson et al., 1995). In Bax wild-type (Bax +/+) cortical neurons, SNP exposure produced significant cell death in a dose- and time-dependent manner (Fig. 9, A–D). More than 80% of wild-type cortical neurons were killed 4 d after SNP treatment (Fig. 9 A, 500–1,000 μM). Morphological evidence of neuronal damage was clearly observed 24 h after treatment and was associated with significant loss of viable cell bodies and neurite fragmentation as seen under phase-contrast optics (data not shown). In marked contrast, SNP treatment did not significantly alter the survival of Bax-deficient cortical neurons compared with nontreated or DMSO-treated control neurons (Fig. 9 B, 500 μM). Bax-deficient neurons appeared remarkably healthy 4 d after SNP treatment (500 μM) and showed little morphological evidence of damage. Even at concentrations as high as 1,000 μM SNP, there was a stable population of Bax-deficient neurons

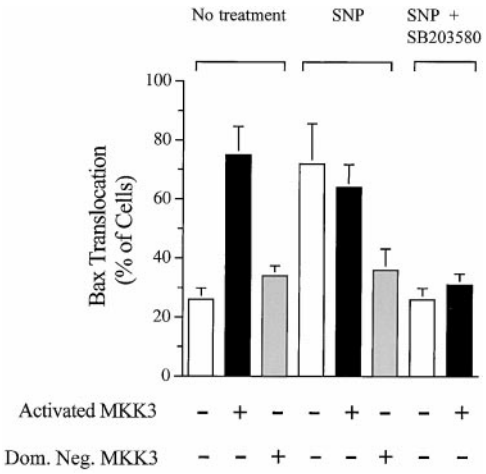

Figure 8. p38 MAP kinase activity is necessary and sufficient to induce Bax translocation. SH-SY5Y cells were plated in 2-well Lab-Tek II chambered coverglass as described in Materials and Methods. After 24 h in culture, the cells were either transfected with the GFP-Bax fusion construct alone or were cotransfected with GFP-Bax and a constitutively active or a dominant negative form of MKK3 using a calcium phosphate precipitation method as described in Materials and Methods. 2 d after transfection, the cells were treated with DMSO (Control) or SNP (500 μM). A subset of cells were also concomitantly treated with the p38 MAP kinase inhibitor, SB203580 (20 μM). The number of transfected cells displaying a cytoplasmic, diffuse distribution or a punctate distribution of GFP-Bax fluorescence was assessed at 24 h by confocal microscopy as described in Materials and Methods, and used to determine the percentage of cells displaying Bax translocation (punctate distribution). The results are the average of six separate transfection experiments for each condition using quadruplicate wells for each experiment. Between 500 and 1,000 GFP-Bax–transfected cells were counted for each condition. The number of cells displaying GFP-Bax translocation in cultures treated with SNP differed significantly from that in cultures treated with DMSO or SNP plus the p38 MAP kinase inhibitor, SB203580 (20 μM; $P < 0.001$, ANOVA). The number of cells displaying Bax translocation in cultures cotransfected with activated MKK3 differed significantly from GFP-Bax–transfected cells, GFP-Bax/dominant negative MKK3–cotransfected cells or GFP-Bax/activated MKK3–cotransfected cells treated with SB203580 (20 μM) ($P < 0.001$, ANOVA).

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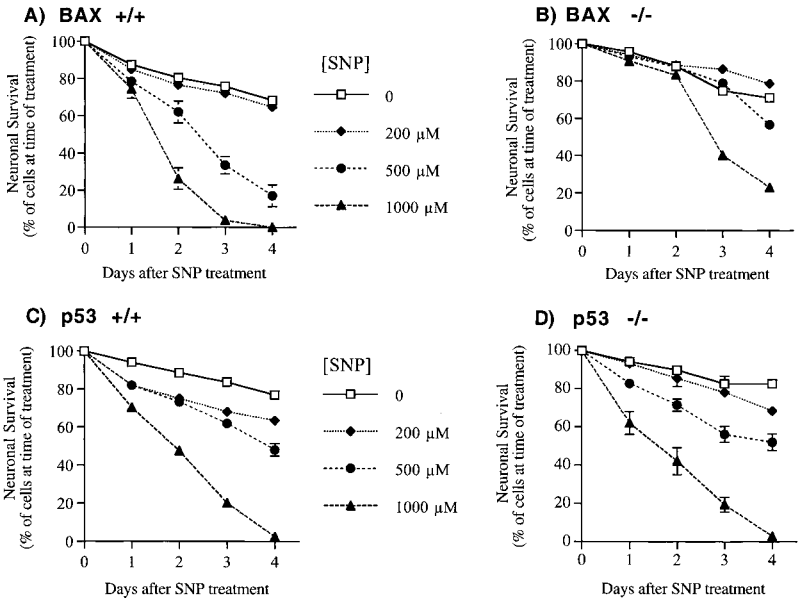

Figure 9. Bax is required for nitric oxide–mediated cell death. Postnatal cortical neurons were plated and maintained in basal culture conditions for 4 d as described in Materials and Methods. Neurons were derived from either the Bax strain of mice (Bax +/+, A; Bax −/−, B) or the p53 strain of mice (p53 +/+, C; p53 −/−, D). Cells were subsequently treated with varying concentrations of SNP (0, 200, 500, and 1,000 μM). Neuronal survival was assessed by counting the number of viable neurons (H. Xiang et al., 1996). All data are the means ± SEM of triplicate cultures, and are representative of three separate experiments with similar results. Neuronal survival in Bax −/− cultures treated with SNP at 500 and 1,000 μM differed significantly from survival in identically treated Bax +/+ cultures ($P < 0.001$, ANOVA). Neuronal survival in p53 −/− cultures treated with SNP did not differ significantly from survival in identically treated p53 +/+ cultures ($P > 0.35$, ANOVA). Most data points do not express standard error bars because they are small enough to be contained within the symbols.

(25%) surviving 4 d after treatment, whereas Bax wild-type neurons were effectively eliminated after 3 d of SNP exposure. In contrast to Bax deficiency, there was no protection conferred against SNP-mediated cytotoxicity by the absence of the p53 gene (Fig. 9 D). p53-deficient neurons exhibited the same sensitivity towards SNP as p53 wild-type (Fig. 9 C) and Bax wild-type neurons (Fig. 9 A). These findings are in direct contrast to our previous results, demonstrating that the absence of either p53 or Bax was sufficient to protect postnatal cortical neurons from cell death induced by camptothecin or glutamate (H. Xiang et al., 1996; Xiang et al., 1998). These results suggest that nitric oxide can induce neuronal cell death by activating a Bax-dependent pathway independently of p53.

Discussion

Excessive generation of nitric oxide has been implicated in neuronal cell death, which is associated with a wide range of neurological disorders including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, and stroke (Heales et al., 1999). Unfortunately, the biochemical mechanisms underlying nitric oxide–mediated toxicity are poorly understood. Excessive stimulation by the excitatory neurotransmitter, glutamate, promotes nitric oxide–mediated neurotoxicity via coupling with the PSD-95 protein (Christopherson et al., 1999; Sattler et al., 1999) and, concomitantly, initiates a stress-activated protein kinase pathway (Kawasaki et al., 1997). These results suggest that increased levels of nitric oxide may be coupled to the activation of downstream transduction cascades. In the present study, we evaluated the signaling pathways associated with nitric oxide–mediated cell death in a neuronal cell line and in primary cultures of murine cortical neurons. The results of this study demonstrate all of the following: (1) that nitric oxide–induced cell death in neurons is mediated through p38 MAP kinase activity; (2) that p38 MAP kinase activity regulates the translocation of the cell death activator, Bax, from the cytosol to the mitochondria; (3) that Bax translocation represents an important step in nitric oxide–mediated cell death, since the p38 MAP kinase inhibitor blocks Bax translocation to the mitochondria while conferring protection from cell death; and (4) that p38 MAP kinase activity facilitates caspase activation, but promotes neuronal cell death by a caspase-independent pathway.

p38 MAP Kinase Is Necessary for Nitric Oxide–induced Cell Death in Neurons

Our results demonstrate that nitric oxide–induced cell death of human neuroblastoma cells and murine cortical neurons in culture is dependent on p38 MAP kinase activity and the presence of a functional Bax gene. p38 MAP kinase activity has been associated with the induction of apoptosis in numerous cell types and in response to a multitude of cellular stresses (Xia et al., 1995; Brenner et al., 1997; Ichijo et al., 1997; Schwenger et al., 1997). However, this is not a universal finding as p38 MAP kinase activation has also been shown to promote cell survival (Nemoto et al., 1998; Roulston et al., 1998; Assefa et al., 1999), suggesting that the role of the p38 MAP kinase pathway in apoptosis/survival is both cell type– and stimulus-dependent. Much of the complexity surrounding the actions of p38 MAP kinase may stem from the presence of distinct p38 isoforms, which have been shown to play different roles in apoptosis (Nemoto et al., 1998).

Stress-activated kinases appear to be critical for induction of apoptosis in neurons. Trophic factor withdrawal (Xia et al., 1995; Kummer et al., 1997) or administration of hypoxia (Conrad et al., 1999) to pheochromocytoma cells (PC12 cells) results in the sustained activation of both p38 MAP kinase and JNK, whereas glutamate administration provokes a similar activation in cultured cerebellar gran-

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ule neurons (Kawasaki et al., 1997). Increased expression of ASK-1, JNK, and p38 MAP kinase has also been detected in apoptotic neurons and glia after spinal cord injury in rats (Nakahara et al., 1999). Consistent with these findings, inhibition of p38 MAP kinase activity suppressed the death of PC12 cells (Xia et al., 1995; Kummer et al., 1997; Le Niculescu et al., 1999) and sympathetic ganglion neurons (Horstmann et al., 1998) after trophic factor withdrawal, and suppressed glutamate-mediated cell death in cerebellar granule cells (Kawasaki et al., 1997). The results from the present study are consistent with reports demonstrating that p38 signaling mediates neuronal cell death in response to a cytotoxic stimulus and suggest that increased levels of nitric oxide may initiate neuronal cell death through p38 MAP kinase signaling.

p38 MAP Kinase Regulates Bax Translocation and Caspase Activation

The downstream signaling events that couple p38 MAP kinase activation with neuronal cell death have not been previously identified. One intriguing possibility is that injury-induced changes in neuronal viability stem from declining mitochondrial function initiated by alterations in the activity of proapoptotic members of the Bcl-2 family. This hypothesis is consistent with the demonstration that mitochondrial dysfunction, including the loss of mitochondrial membrane potential and increased production of reactive oxygen species, plays an obligate role in excitotoxic damage (Ankarcrona et al., 1995; Dugan et al., 1995; Reynolds and Hastings, 1995; Schinder et al., 1996), a stimulus that is also associated with increased production of nitric oxide (Dawson et al., 1996; Ayata et al., 1997). A relationship between the Bcl-2 family member, Bax, and alterations in mitochondrial function would also be consistent with the recent demonstration that Bax translocates from the cytosol to the mitochondria during programmed cell death (Hsu et al., 1997; Wolter et al., 1997). Analysis of various Bax mutations indicated that cell death was only observed when the Bax protein was capable of translocating to the mitochondria (Nechushtan et al., 1999).

The results of the present study suggest that Bax translocation to the mitochondria is subject to regulation by p38 MAP kinase in response to nitric oxide. This finding is consistent with the recent identification of domains in the NH_2 (Goping et al., 1998) and COOH termini (Nechushtan et al., 1999) that regulate Bax targeting to the mitochondria. Eliminating the NH_2-terminal 19 amino acids of Bax enhances membrane integration, suggesting that this domain normally prevents mitochondrial insertion. This domain is rich in glycine and hydroxylated amino acids such as serine and threonine that are potential targets of the p38 MAP kinase. Coincidentally, the Bax COOH terminus contains a serine at position 184 that is critical for regulating the subcellular distribution of Bax (Nechushtan et al., 1999). Phosphorylation of either terminus may precipitate a conformational change, which facilitates membrane insertion. However, evidence for Bax phosphorylation is lacking (Nechushtan et al., 1999), in contrast to the related family members Bcl-2 and Bad (May et al., 1994; Zha et al., 1996; Datta et al., 1997; Haldar et al., 1998; Srivastava et al., 1999). Alternatively, it is conceivable that while Bax is not directly phosphorylated, the phosphorylation of a Bax-binding protein may facilitate membrane targeting and insertion (Desagher et al., 1999).

Bax translocation to the mitochondria has been shown to reduce mitochondrial membrane potential, enhance cytochrome *c* release from the mitochondria, and to activate caspases (J.G. Xiang et al., 1996; Eskes et al., 1998; Jurgensmeier et al., 1998; Finucane et al., 1999; Desagher et al., 1999). The significant reduction in caspase cleavage activity, which is observed in the present study after concurrent treatment with SNP and the p38 MAP kinase inhibitor, is consistent with our demonstration that Bax translocation is regulated by p38 MAP kinase. Thus, we would propose that SNP activates p38 MAP kinase, which promotes Bax translocation to the mitochondria followed by cytochrome *c* release and caspase activation. This is in agreement with the temporal course of p38 activation that was first detected 3–6 h after SNP treatment and before the first evidence of Bax translocation (12 h) and caspase induction (24 h). The residual caspase activity, which remained in the presence of the p38 MAP kinase inhibitor, may reflect a small degree of Bax translocation that was not detectable by confocal microscopy imaging of the GFP-Bax fusion protein. Alternatively, caspase activation may have resulted, in part, from a direct action of nitric oxide or its derivative, peroxynitrite, on mitochondrial permeability with subsequent release of cytochrome *c* (Packer and Murphy, 1994; Vercesi et al., 1997; Brookes et al., 1998).

Irrespective of the mechanism of activation, caspase activity did not play a major role in nitric oxide–induced death of SH-SY5Y cells or primary cortical neurons. Although the pan-caspase inhibitor, zVAD-fmk, blocked DEVD cleavage activity more effectively than the p38 MAP kinase inhibitor, it had no effect on the viability of primary cortical neurons and only marginally delayed the death of SH-SY5Y cells. These results suggest that SNP-induced cell death occurs in a caspase-independent manner, although cells were killed by apoptosis, which is defined as an active process of cell death requiring the activation of discrete biochemical pathways. SNP-induced cell death, as studied in the present report, is considered to be apoptotic based on the following criteria. First, cells exhibit nuclear fragmentation and a significant reduction in volume (Fig. 6), morphological hallmarks of apoptosis. Images contained in Fig. 6 clearly demonstrate that SNP promotes Bax translocation in cells that are significantly shrunken compared with nontreated control cells or cells concomitantly treated with SNP and the p38 inhibitor, SB203580. Necrotic cells typically exhibit swelling, which is not evident in these cultures. Second, SNP-treated cells exhibit activation of two apoptotic signaling pathways, including p38 MAP kinase activity (Fig. 1) and caspase cleavage activity (Fig. 5). Third, cell death is suppressed by inhibiting the p38 MAP kinase cascade or by the absence of the cell death–promoting protein, Bax (Fig. 9). Thus, we would conclude that Bax-mediated changes in mitochondrial integrity could compromise neuronal viability independently of caspase activation as recently suggested by our laboratory (Johnson et al., 1998, 1999) and others (J.G. Xiang et al., 1996; McCarthy et al., 1997; Miller et al., 1997; Bergeron et al., 1998; Kim et al., 1998; Vercammen et al., 1998).

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Bax Translocation Is Essential for Neuronal Cell Death

The present study suggests that Bax-dependent pathways contribute to the death of postnatal neurons after injury. Indeed, there is increasing evidence that Bax may play a central role in both developmental and injury-induced cell death in neurons. For example, targeted disruption of the Bax gene significantly reduces the developmental death of specific populations of peripheral and central nervous system neurons (Deckwerth et al., 1996; Shindler et al., 1997; White et al., 1998). In addition, the absence of Bax reduces neuronal cell death in response to trophic factor deprivation (Deckwerth et al., 1996; Easton et al., 1997), glutamate exposure, and DNA damaging agents (Johnson et al., 1998; Xiang et al., 1998). NGF deprivation recently has been shown to initiate a caspase-independent subcellular redistribution of Bax from the cytosol to the mitochondria (Putcha et al., 1999), which is similar to our observations with SNP. The addition of neuroprotective agents at the time of NGF deprivation prevented Bax translocation. The consistent finding that Bax translocation is proximal to, and independent of, caspases suggests that the capacity of Bax to promote neuronal cell death is likely dependent on its pore forming properties and its ability to increase mitochondrial membrane permeability (Eskes et al., 1998).

The results of the present study indicate that increased levels of nitric oxide, which have been implicated in the pathogenesis of several acute and chronic neurological disorders, may ultimately promote neuronal cell death through mitochondrial dysfunction. Our results also demonstrate that p38 MAP kinase plays an integral role in the regulation of Bax translocation to the mitochondria. Targeting the p38 MAP kinase and interrupting Bax translocation may provide a means for maintaining neuronal viability and metabolic competence following neurotoxic insults.

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