p38 Mitogen-Activated Protein Kinase Regulates Bax Translocation in Cyanide-Induced Apoptosis

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Execution of cyanide-induced apoptosis is mediated by release of cytochrome c from mitochondria. To determine how cyanide initiates cytochrome c release, Bax translocation was investigated in primary cultures of cortical neurons. Under nonapoptotic (control) conditions, Bax resided predominantly in the cytoplasm. After 300-μM cyanide treatment for 1 h, Bax translocated to the mitochondria, as shown by immunocytochemical staining and subcellular fractionation; Western blot analysis confirmed "cytosol-to-mitochondria" translocation of Bax. Temporal analysis showed that Bax translocation preceded cytochrome c release from the mitochondria, which was initiated 3 h after cyanide treatment. In double-immunofluorescence labeling for both Bax and cytochrome c, it was observed that cytochrome c was released only in cells showing Bax in mitochondria. The role of p38 mitogen-activated protein (MAP) kinase in Bax translocation was studied. The p38 MAP kinase was activated 30 min after cyanide, and its phosphorylation level of activity began to decrease 3 h later. SB203580, a p38 MAP kinase inhibitor, blocked translocation of Bax to mitochondria, whereas SB202474, a control peptide, had no effect on translocation. Inhibition of p38 MAP kinase by SB203580 blocked all downstream effects of Bax translocation, including cytochrome c release, caspase activation, and internucleosomal DNA fragmentation. These results demonstrated that Bax translocation is critical for cyanide-induced cytochrome c release and that p38 MAP kinase regulates Bax translocation from cytosol to mitochondria.

Key Words: cyanide; apoptosis; Bax; p38 MAP kinase; cytochrome c.

Cyanide induces apoptotic death in cortical neurons through the cytochrome c-activated caspase cascade. Our studies have shown that the Bcl-2 protein family has an important regulatory role in release of cytochrome c from the mitochondrial intermembrane space (Shou et al., 2002). Upregulation of two proapoptotic Bcl-2 members, Bcl-X, and Bax, contributes to the release of cytochrome c 6–12 h after cyanide treatment. However, the early events that initiate cyanide-induced cytochrome c release are still not clear.

Bax, a proapoptotic member of the Bcl-2 family, normally resides in the cytosol and translocates to mitochondria in response to a variety of apoptotic stimuli (Khaled et al., 1999; Murphy et al., 1999; Putcha et al., 1999; Wolter et al., 1997). In mitochondria, Bax exerts a proapoptotic action by disrupting mitochondrial membrane potential, leading to release of cytochrome c, and by heterodimerizing with antiapoptotic Bcl-2 proteins to neutralize their actions. The conformation of Bax is critical for its subcellular location. A single transmembrane domain at the carboxy terminus of Bax is responsible for its membrane insertion (Goping et al., 1998; Nechushtan et al., 1999). Under normal conditions, this transmembrane domain is masked by the amino terminus domain. Deletion of N-terminus or point mutations in the C-terminus domain can target Bax to mitochondria, suggesting that a specific Bax conformation regulates mitochondrial docking. Upstream events that induce a Bax conformational change are still unclear. Recent studies suggest that caspase activation, Bid association, and p38 mitogen-activated protein (MAP) kinase initiate changes in Bax conformation, followed by mitochondrial translocation (De-sagher et al., 1999; Ghatan et al., 2000; Putcha et al., 1999).

p38 MAP kinase is a member of the MAP kinase family that is involved in the cell death cascade and is activated by phosphorylation on Thr-180 and Tyr-182 (Noh et al., 2000; Yamagishi et al., 2001). Activation of p38 MAP kinase has been observed in Alzheimer’s disease (Zhu et al., 2000), in transient forebrain ischemia (Takagi et al., 2000), and in apoptosis induced by various stimuli, including hypoxia (Chae et al., 2001), glutamate (Kawasaki et al., 1997), and nerve growth factor (NGF) withdrawal (De Zutter and Davis, 2001), and low potassium (Yamagishi et al., 2001). p38 MAP kinase appears to have multiple targets in the apoptotic pathway. In NGF withdrawal-induced apoptosis in PC12 cells, the kinase induces expression of monoamine oxidase (MAO) (De Zutter and Davis, 2001). In nitric oxide (NO)-induced neuronal apoptosis, p38 MAP kinase initiates Bax translocation, followed by caspase activation (Ghatan et al., 2000).

Previous studies show that in cerebellar granule and cortical cells, cyanide-mediated activation of the N-methyl-D-aspartate
(NMDA) glutamatergic receptor activation leads to a marked elevation of cytosolic-free Ca$^{2+}$ and reactive oxygen species generation, which in turn are intracellular initiators of apoptosis (Shou et al., 2000; Sun et al., 1997). It is conceivable that p38 MAP kinase might mediate Bax translocation and the subsequent activation of the apoptotic cascade. In this study, the relationship of p38 MAP kinase activation with Bax translocation and the subsequent release of cytochrome c from mitochondria during cyanide-induced apoptosis was determined.

**MATERIALS AND METHODS**

**Primary cortical cell culture.** Primary cortical cells were prepared as previously described (Shou et al., 2000). In brief, cerebral cortices were dissected from embryonic day-16 Sprague-Dawley rats and dissociated in 0.025% trypsin at 37°C for 15 min. Trypsin digestion was stopped by trypsin inhibitor and DNase I. After passage through a Pasteur pipette several times, cortical cells were plated at a density of 5 × 10^3 cells/cm² onto 6 well plates precoated with 10 µg/ml poly-L-lysine. Cells were grown in 80% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 22 mM glucose, 2 mM glutamine, and 100 U/ml penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂. Cells were trypsinized with 0.025% trypsin at 37°C for 15 min. Trypsin digestion was stopped by trypsin inhibitor and DNase I. After passage through a Pasteur pipette several times, cell pellets were resuspended in PBS and centrifuged at 500 g for 5 min. Cell pellets were resuspended in 12% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. After blocking with PBS containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was probed with anti-phospho-p38 MAP kinase (Tyr 182) antibody (New England Biolab, Beverly, MA) and anti-p38 MAP kinase antibody (New England Biolab) diluted 1:100 in PBS. Reactions were detected with the fluorescein-linked antimouse Ig (second antibody) conjugated to horseradish peroxidase using a Storm 860 fluorescence-PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Densitometry analysis was performed using the ImageQuant software (Molecular Dynamics). To determine whether caspase-3 was activated after inhibition of p38 MAP kinase, cells were pretreated with SB203580 (p38 MAP kinase inhibitor), followed by KCN (300 µM). Whole cell lysates were subjected to SDS–PAGE, and blots were probed with anti-caspase-3 antibody (Santa Cruz Biotechnology), which binds both procaspase and cleaved (activated) product. Procesase-3 conversion to caspase-3 was used as an index of caspase activity (Shou et al., 2002).

**Immunochemistry.** For immunochemical analysis, cortical neurons were grown on poly-L-lysine-coated coverslips. After cyanide treatment, cells were labeled with 500 nM mitotracker red (Molecular Probes, Eugene, OR) for 30 min at 37°C. After three washes with PBS, cells were fixed with 5% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. Cultures were then washed with PBS and exposed to blocking solution (5% goat serum in PBS). After PBS washing, cells were incubated with mouse monoclonal anti-Bax antibody (1:100 diluted in PBS) at room temperature for 3 h. Cells were washed twice in PBS and incubated with Alexa Fluor 488-conjugated goat antimouse secondary antibody (Molecular Probes) for 1 h at room temperature in the dark. Coverslips were then mounted onto glass slides and examined by confocal microscopy.

**Internucleosomal DNA fragmentation.** Genomic DNA was extracted according to the protocol described previously (Shou et al., 2000). Cells were collected in PBS and lysed in lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA; 0.5% SDS) with RNase A (final concentration 100 µg/ml) for 1 h at 37°C. Then 100 µg/ml protease K was added and incubated for 4 h at 50°C. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of precooled ethanol, then resuspended in Tris-EDTA buffer. Ten micrograms of DNA sample were separated on a 1.2% agarose gel containing 0.5 µg/ml ethidium bromide for 2 h and visualized under ultraviolet light.

**Statistics.** Each experiment was repeated a minimum of three times, and data were expressed as mean ± SEM. One-way ANOVA with a Tukey-Kramer procedure for multiple comparisons was used to examine the statistical differences between treatments. Differences were considered as significant at p < 0.05.

**RESULTS**

Cyanide Induces Bax Cytosol-to-Mitochondria Translocation

The cellular distribution of Bax was examined using both subcellular fractionation and immunochemical analysis. Under control conditions, Bax resided primarily in the cytosol, although a small portion of Bax could be detected in the mitochondrial fraction (Fig. 1A). Densitometric analysis indicated that under control conditions the cytosolic Bax level averaged 6.5 times greater than mitochondrial levels. Previous studies showed that in control cells, Bax is located in the cytosol and in association with intracellular membranes, including mitochondria (Goping et al., 1998; Wolter et al., 1997). Bax redistribution from cytosol to mitochondria was observed within 1 h after cyanide treatment (Fig. 1A). Based on densitometric analysis of the bands, equal levels of Bax were observed in cytosol and mitochondria at 1 h. Over the next 12 h, cytosolic Bax levels gradually decreased, and mitochondrial levels increased correspondingly (Fig. 1A). Densitometric analysis of the bands showed that at 24 h of treatment, the mitochondrial Bax levels were 1.8 times greater than control levels and 1.2 times greater than the 12-h levels. Cyanide-
induced Bax translocation exhibited a concentration-dependent pattern. Bax translocation was observed after 100 μM cyanide, and the extent of translocation was concentration-dependent from 100 to 500 μM cyanide (Fig. 1B). Bax translocation was further confirmed by immunofluorescence analysis. As shown in Figure 2B, Bax was visualized diffusively in the cytosol. One hour after cyanide treatment, cells exhibited a punctate membrane staining pattern, and Bax co-localized extensively with the specific mitochondria marker (mitotracker red), showing the distribution of Bax to mitochondria (Fig. 2E). The extent of Bax translocation was time-dependent (Figs. 2E, 2H, and 2K).

p38 MAP Kinase Was Activated by Cyanide

Because Bax translocation is involved in cytochrome c release from mitochondria (Finucane et al., 1998; Jurgensmeier et al., 1998; Narita et al., 1998), Bax and cytochrome c subcellular distribution was examined simultaneously during cyanide-induced apoptosis. In control neurons, Bax had a diffusely staining pattern, and cytochrome c exhibited a punctate staining pattern in close association with the mitochondrial membrane, which was labeled by mitotracker red (Figs. 3A–3D). After 300 μM cyanide treatment for 1 h, Bax staining exhibited a punctate pattern that co-localized to mitochondria (Fig. 3G), whereas cytochrome c remained associated with mitochondria (Fig. 3F). As expected, Bax translocation preceded cytochrome c release from mitochondria. At 3 h, cytochrome c staining in the cytosol was observed in cells in which Bax had translocated to mitochondria (Fig. 3L). In cells where cytochrome c was released into the cytosol, Bax displayed a punctate mitochondrial-associated staining pattern. A diffuse cytochrome c staining pattern was not observed in the absence of Bax mitochondrial translocation, suggesting that Bax translocation to mitochondria was responsible for initiating cytochrome c release.

p38 MAP Kinase Activation Is an Upstream Event in Cyanide-Induced Apoptosis

Because p38 MAP kinase activation occurs in some forms of apoptosis (Chae et al., 2001; De Zutter and Davis, 2001; Yamagishi et al., 2001), its level of activation was determined in cyanide-induced apoptosis. p38 MAP kinase activation was detected as the phosphorylated form in immunoblots. As early as 30 min after cyanide treatment, p38 MAP kinase was activated, and activity remained elevated for up to 4 h (Fig. 4A). The increase of p38 MAP kinase phosphorylation at peak level was about 300% of control (Fig. 4B), and activation of p38 MAP kinase by cyanide exhibited a concentration-dependent pattern (Fig. 5A). After 300-μM cyanide treatment for 30 min, there was a twofold increase in the phosphorylation of p38 MAP kinase. NMDA receptor activation and subsequent Ca^{2+} influx were upstream of p38 MAP kinase activation, because selective NMDA receptor antagonist MK801 (1 μM) and intracellular Ca^{2+} chelator BAPTA (20 μM) blocked p38 MAP kinase phosphorylation 51% and 44%, respectively, based on densitometric analysis. Reactive oxygen species also played a role in cyanide-induced p38 MAP kinase activation because PBN (N-t-butyl-n-phenyl-nitrone), a free-radical scavenger, and NAC (N-acetyl-cysteine) inhibited p38 MAP kinase phosphorylation. SB203580, a specific p38 MAP kinase inhibitor, inhibited p38 MAP kinase activation by cyanide, whereas SB202474, the inactive compound, had no effect (Figs. 5B and 5C).

FIG. 1. Cyanide-induced cytosol-to-mitochondria translocation of Bax in cortical neurons. (A) Time course of Bax redistribution after 300 μM cyanide treatment. Cells were treated with cyanide (1–24 h), then cytosolic and mitochondrial Bax levels were determined by Western blot analysis. To show equal protein loading, the blots were stripped and reprobed with antibodies against β-actin or cytochrome c oxidase subunit IV (COX IV). (B) After cyanide treatment (100–500 μM) for 6 h, cytosolic and mitochondria Bax levels were determined by Western blot analysis.
in cyanide-induced apoptosis was examined by using the specific p38 MAP kinase inhibitor, SB203580. Cortical cells were treated with 300 μM cyanide in the presence or absence of 20 μM SB203580 for various times, and the Bax subcellular distribution was examined. SB203580 partially inhibited Bax cytosol-to-mitochondria translocation at various times after cyanide treatment (Fig. 6). Based on densitometric analysis of the bands, a decrease of approximately 25% in Bax translocation was produced by SB203580 over the 1–12 h of cyanide treatment.

Because p38 MAP kinase mediates Bax translocation, its relationship with cytochrome c release and caspase activation was examined. Blockade of p38 MAP kinase activation by SB203580 partially inhibited cytochrome c release from 3 h to 12 h after cyanide treatment (Fig. 7), consistent with p38 MAP kinase activation being upstream to cyanide-induced cytochrome c release. Previous results showed that the apoptotic pathway is mediated by mitochondrial release of cytochrome c and subsequent activation of the caspase cascade (Shou et al., 2002). By blocking the Bax-mediated cytochrome c release, SB203580 treatment also prevented caspase-3 activation and the characteristic internucleosomal DNA laddering observed in cyanide-induced apoptosis (Figs. 7B and 7C).

**DISCUSSION**

Cyanide neurotoxicity in cortical neurons is manifested as apoptotic death that is mediated by cytochrome c release from mitochondria, followed by execution of cell death by the caspase cascade (Mills et al., 1999; Prabhakaran et al., 2002; Shou et al., 2000). The processes underlying the release of cytochrome c from the mitochondrial intermembrane space...
into the cytosol have not been elucidated in detail in this type of cell death. In mitochondria-mediated apoptosis, the Bcl-2 protein family modulates release of cytochrome c (Finucane et al., 1998; Jurgensmeier et al., 1998; Narita et al., 1998; Yin et al., 2002). This study shows that Bax translocation from cytosol to mitochondria occurs early in the cytotoxicity to trigger cytochrome c release. Activation of p38 MAP kinase precedes Bax translocation, and its inhibition significantly reduced cyanide-induced Bax translocation, cytochrome c release, and subsequent level of apoptotic death.

Cyanide enhances NMDA receptor functions to initiate influx of Ca$^{2+}$ (Patel et al., 1992; Sun et al., 1997) and mobilizes intracellular calcium stores (Yang et al., 1997). In cultured neurons, cyanide-induced cytotoxicity is linked to the NMDA receptor-mediated rise in cytosolic Ca$^{2+}$ that in turn activates a series of biochemical reactions, leading to generation of reactive oxygen species (ROS) and NO. From this study, it is concluded that the apoptotic initiation stimuli produced by cyanide are elevation of cytosolic Ca$^{2+}$ following NMDA receptor activation and generation of excess intracellular ROS. These stimuli appear to initiate p38 MAP kinase activation, which in turn mediates Bax translocation, followed by cytochrome c release and execution of apoptosis.

The Bcl-2 gene family regulates mitochondria-mediated apoptosis (Gross et al., 1999; Shinoura et al., 1999). The anti-apoptotic protein regulators Bcl-2 and Bcl-X$_L$ reside in the outer mitochondrial membrane and inhibit cytochrome c release. Increasing Bcl-2 expression confers resistance to apoptosis, whereas decreasing expression enhances apoptotic cell death. Proapoptotic Bcl-2 family members include Bax, Bad, Bid, Bcl-X$_S$, and Bim proteins that translocate from cytoplasm to mitochondria on an apoptotic signal to stimulate release of cytochrome c. Bax expression is regulated by the redox-sensitive transcription factor NF$\kappa$B; oxidative stress increases Bax expression via NF$\kappa$B activation and produces a proapoptotic state (Shou et al., 2002; Wiu et al., 2001). The ratio of Bax/Bcl-2 regulates the cellular state, and death-promoting pathways are activated if the ratio increases. In the case of...
cyanide, cellular Bcl-2 levels influence the apoptotic response (Jensen et al., 2002) and overexpression of Bcl-2 protected hypothalamic cells, preventing changes in theΔψm (Myers et al., 1995; Shimizu et al., 1996). We have demonstrated that regulation of the Bax protein family expression level has a marked influence on cyanide-induced apoptosis in cortical cells (Shou et al., 2002). Cyanide produces an NFκB-mediated upregulation of Bcl-XL and Bax expression, which in turn stimulates cytochrome c release from mitochondria, followed by caspase-3 activation (Shou et al., 2002; Wiu et al., 2001). Inhibition of NFκB activation with SN50 (a specific inhibitor) or kB decoy DNA suppressed upregulation of Bcl-XL and Bax and decreased apoptosis. In the presence of cyanide, increased cytosolic Ca2+ is a signal that initiates Bad (proapoptotic Bcl-2 family member) translocation to mitochondria, thus linking the apoptotic response to Ca2+ influx (Shou et al., 2003, unpublished data).

Bax is a proapoptotic member of the Bcl-2 protein family involved in regulating cytochrome c release in apoptotic cell death. Overexpression of Bax initiates cytochrome c release and subsequent cell death (Rosse et al., 1998). Addition of Bax to a cell-free system can induce cytochrome c release from isolated mitochondria (Jurgensmeier et al., 1998). It has been proposed that Bax interacts with components of the mitochondrial permeability transition (MPT) pore to promote opening of the pore, which ultimately causes outer mitochondria membrane rupture and cytochrome c release (Marzo et al., 1998; Shimizu et al., 1999). Alternatively, Bax could form a channel in the mitochondrial membrane that directly releases cytochrome c. The three-dimensional structure of Bcl-2 family members bears a striking structural similarity to the pore-forming domains of certain bacterial toxins (Muchmore et al., 1996). Moreover, Bax has pore-forming capability in vivo (Antonsson et al., 1997).

Under normal, basal conditions, Bax resides mainly in the cytosol, and in order for Bax to induce cytochrome c release,
an association of Bax with the mitochondrial membrane is necessary. Upon activation of extracellular membrane receptors or intracellular targets by an apoptotic stimulus, translocation of Bax to the mitochondrion is initiated (Murphy et al., 1999; Wolter et al., 1997). In this study, cyanide initiated a subcellular cascade of reactions, resulting in Bax translocation to the mitochondrion prior to cytochrome c release. Double-immunofluorescence staining showed that Bax association with mitochondria is an initiating event for mitochondrial release of cytochrome c and subsequent caspase activation. The signal that induces movement of Bax from the cytosol to mitochondria following an apoptotic stimulus may involve a conformational change to expose a masked C-terminal transmembrane domain of the protein (Goping et al., 1998; Nechushtan et al., 1999). The conformational change may be triggered by a rise in intracellular pH (Khaled et al., 1999) or by Bid (Eskes et al., 2000). Alternatively, phosphorylation of critical amino acid residues in either the N- or C-terminal domains of Bax may also induce a conformational change. p38 MAP kinase may mediate this phosphorylation. p38 MAP kinase has been reported to regulate Bax translocation in apoptosis induced by NO (Ghatan et al., 2000) and by T cell receptor stimulation (Yoshino et al., 2001). This study shows that cyanide-induced p38 MAP kinase activation is likely an upstream signal for Bax translocation.

p38 MAP kinase is a member of the stress-activated protein kinase family. It is phosphorylated in numerous cell types following treatment with a variety of toxic agents, and its activation is associated with induction of apoptosis (De Zutter and Davis, 2001; Noh, 2000; Yamagishi et al., 2001). In this study, it was shown that p38 MAP kinase was activated by the intracellular Ca$^{2+}$ overload resulting from cyanide-induced NMDA receptor activation. This is supported by the observation that the NMDA receptor antagonist MK801 and the intracellular Ca$^{2+}$ chelator BAPTA attenuated cyanide-induced p38 MAP kinase activation. These findings are consistent with previous observations showing that glutamate activates p38 MAP kinase in cerebellar granule neurons (Kawasaki et al.,

![FIG. 6. Blockade of cyanide-induced Bax translocation by SB203580, a specific inhibitor for p38 MAP kinase. Cortical cells were pretreated with SB203580 (20 μM) for 30 min, then subjected to 300-μM cyanide treatment for 1–12 h. Cytosolic and mitochondrial Bax levels were determined by Western blot analysis.](https://academic.oup.com/toxsci/article-abstract/75/1/99/1696244)

![FIG. 7. p38 MAP kinase activation contributes to mitochondria release of cytochrome c, caspase activation, and internucleosomal DNA fragmentation induced by cyanide. (A) Cortical cells were treated with cyanide (300 μM) in the presence or absence of a p38 MAP kinase inhibitor (20 μM SB203580) for 1–12 h, and cytosolic and mitochondrial fractions were prepared. Fractions were separated by SDS–PAGE and cytochrome c level detected by anticytochrome c antibody. Blot was stripped and reprobed with antibody against β-actin or cytochrome c oxidase subunit IV to demonstrate equal protein loading. (B) p38 MAP kinase activation contributed to caspase-3 activation. Cells were treated with cyanide (300 μM) in the presence or absence of SB203580 (20 μM) for 24 h, whole cell lysates were subjected to SDS–PAGE, and the blot was probed with anticaspase-3 antibody. (C) p38 MAP kinase activation contributed to internucleosomal DNA fragmentation induced by cyanide. Cells were treated with 300 μM cyanide in the presence or absence of SB203580 or SB202474 (inactive, control compound) for 24 h, then genomic DNA was extracted and subjected to agarose gel electrophoresis. The lanes shown represent: DNA marker (1); control cells (2); 300 μM cyanide-treated cells (3); cells pretreated with either 20 μM SB202474 (4); or 20 μM SB203580 (5).](https://academic.oup.com/toxsci/article-abstract/75/1/99/1696244)
In addition to intracellular Ca\(^{2+}\) overload, it appears that oxidative stress produced by cyanide also contributed to p38 MAP kinase activation, because the antioxidants PBN and NAC inhibited cyanide-induced p38 MAP kinase activation.

In summary, this study shows that in cyanide-induced apoptosis, p38 MAP kinase activation is upstream of Bax translocation. Association of Bax with the mitochondrion promotes cytochrome c release from the intermembrane space into the cytosol to execute apoptosis by activating the caspase cascade.

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REFERENCES


