Calcineurin-mediated Bad translocation regulates cyanide-induced neuronal apoptosis

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In cyanide-induced apoptosis, an increase in cytosolic free Ca^{2+} and generation of reactive oxygen species are initiation stimuli for apoptotic cell death. Previous studies have shown that cyanide-stimulated translocation of Bax (Bcl-associated X protein) to mitochondria is linked with release of cytochrome *c* and subsequent activation of a caspase cascade [Shou, Li, Prabhakaran, Borowitz and Isom (2003) *Toxicol. Sci.* **75**, 99–107]. In the present study, the relationship of the cyanide-induced increase in cytosolic free Ca^{2+} to activation of Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) was determined in cortical cells. Bad is a Ca^{2+} -sensitive pro-apoptotic Bcl-2 protein, which on activation translocates from cytosol to mitochondria to initiate cytochrome *c* release. In cultured primary cortical cells, cyanide produced a concentration- and time-dependent translocation of Bad from cytosol to mitochondria. Translocation occurred early in the apoptotic response, since mitochondrial Bad was detected within 1 h of cyanide treatment. Mitochondrial levels of the protein continued to increase up to 12 h post-cyanide exposure. Concurrent

with Bad translocation, a Ca^{2+} -sensitive increase in cellular calcineurin activity was observed. Increased cytosolic Ca^{2+} and calcineurin activation stimulated Bad translocation since BAPTA [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid], an intracellular Ca^{2+} chelator, and cyclosporin A, a calcineurin inhibitor, significantly reduced translocation. BAPTA also blocked release of cytochrome *c* from mitochondria as well as apoptosis. Furthermore, treatment of cells with the calcineurin inhibitors cyclosporin A or FK506 blocked the apoptotic response, linking calcineurin activation and the subsequent translocation of Bad to cell death. These observations show that by inducing a rapid increase in cytosolic free Ca^{2+} , cyanide can partially initiate the apoptotic cascade through a calcineurin-mediated translocation of Bad to mitochondria.

Key words: apoptosis, Bcl-2/Bcl-X_L-antagonist, causing cell death (Bad), calcineurin, cyanide, cytochrome *c*.

INTRODUCTION

Pro-apoptotic members of the Bcl-2 gene family promote apoptosis by regulating cytochrome *c* release from mitochondria. Since most pro-apoptotic Bcl-2 proteins are localized in the cytosol under normal conditions, cytosol-to-mitochondria translocation of these proteins is an important apoptotic regulatory mechanism. Previous studies have shown that Bax (Bcl-associated X protein) is translocated from cytosol to mitochondria by a p38 MAPK (mitogen-activated protein kinase)-dependent process in cyanide-induced apoptosis [1]. In addition to Bax, another member of the pro-apoptotic Bcl-2 protein family, Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death), undergoes translocation in apoptosis induced by various stimuli [2–5].

Bad is a 'BH3 (Bcl-2 homology 3)-only' pro-apoptotic Bcl-2 protein, which displays sequence homology only within the BH3 domain. Bad lacks a C-terminal hydrophobic domain essential for targeting membranes such as the mitochondrial outer membrane [6]. Thus Bad exerts a pro-apoptotic action through heterodimerization with other Bcl-2 proteins. Using the yeast two-hybrid protein interaction assay, Bae et al. [7] showed that Bad interacts selectively with Bcl-2, Bcl-X_L and Bcl-w to inhibit their anti-apoptotic actions. The interaction of Bad with pro-apoptotic Bcl-2 proteins is negligible.

The subcellular location of Bad is determined by its phosphorylation status at four serine residues (Ser-112, Ser-136, Ser-155 and Ser-170). The initial study by Zha et al. [8] identified Ser-112 and Ser-136 as two major phosphorylation sites in response to interleukin-3. A third site, Ser-155, located at the centre of the BH3 domain, was shown to be phosphorylated in response to growth factor [9,10]. Recently, Dramsi et al. [11] identified Ser-170 as a novel phosphorylation site in response to cytokines and a critical regulator of Bad pro-apoptotic activity. When Bad is phosphorylated at one or more of the four serine residues in response to survival factors, it is sequestered in the cytosol, complexed with signal transducer protein 14-3-3 to form an inactive heterodimer [8]. Death-inducing stresses, such as growth-factor deprivation, result in dephosphorylation of Bad, dissociation from 14-3-3 and then translocation to mitochondria where it binds to Bcl-2 and Bcl-X_L. Thus mitochondria-localized Bad promotes cell death by neutralizing the anti-apoptotic actions of Bcl-2 and Bcl-X_L.

Bad dephosphorylation and translocation have been observed during apoptosis induced by depriving cells of growth factor, such as interleukin-3 [8], or in apoptosis resulting from a sustained increase in cytosolic free calcium in prostate cancer cells or neurons [2]. In interleukin-3-deprivation-induced apoptosis, Bad is dephosphorylated by the specific serine/threonine phosphatase,

Abbreviations used: AMC, 7-amino-4-methylcoumarin; Bad, Bcl-2/Bcl-X_L-antagonist, causing cell death; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; BAPTA/AM, BAPTA tetrakis(acetoxymethyl ester); Bax, Bcl-associated X protein; BH3, Bcl-2 homology 3; CsA, cyclosporin A; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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PPI α , whereas Ca²⁺-activated calcineurin is the phosphatase that dephosphorylates Bad in glutamate-induced apoptosis [2]. Calcineurin is a Ca²⁺/calmodulin serine/threonine phosphatase that on activation predisposes to apoptosis. Constitutive high levels of activated calcineurin and subsequent dephosphorylation of Bad regulates mitochondria-mediated apoptosis in neuronal cells [12] and traumatic spinal cord injury [5].

In cyanide-induced neurotoxicity, increased cytosolic Ca²⁺ is an initiation stimulus linked with generation of reactive oxygen species to stimulate mitochondria-dependent apoptosis [13,14]. In cyanide-induced apoptosis, Bax is translocated to mitochondria, followed by release of cytochrome *c* and subsequent activation of the caspase cascade [15]. In the present study, cyanide-induced apoptosis was associated with Bad dephosphorylation by Ca²⁺-activated calcineurin, followed by translocation to mitochondria to produce cytochrome *c* release and in turn initiation of apoptosis.

MATERIALS AND METHODS

Primary cortical culture

Primary cortical cells were prepared from embryonic-day-16 Sprague–Dawley rats as described previously [16]. Briefly, cerebral cortices were dissected and cells were passed through a Pasteur pipette several times and plated at a density of 5×10^5 cells/cm² on 6-well plates precoated with 10 μ g/ml poly(L-lysine). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) foetal bovine serum, 10% (v/v) horse serum, 22 mM glucose, 2 mM glutamine and 1 ml of penicillin/streptomycin (5000 units/ml) at 37 °C in an atmosphere of 5% CO₂/95% air. Cytosine arabinofuranoside (10 μ M) was added at 7 DIV (days *in vitro*) for 24 h to limit proliferation of non-neuronal cells. The medium was changed twice a week until experiments were performed at 14–16 DIV. At the time of experimentation, the percentage of astrocytes in the cultures was approx. 30% as determined by immunostaining of the astrocyte-specific marker GFAP (glial fibrillary acidic protein) and 70% neurons as measured by immunostaining of the neuron-specific marker microtubule associated protein-2.

Subcellular fractionation and Western-blot analysis

Subcellular fractionation was performed as described by Desagher et al. [17]. After different treatments, cortical neurons were harvested in TBS (Tris-buffered saline) and centrifuged at 500 *g*. Cell pellets were resuspended in isotonic mitochondrial buffer (MB: 210 mM mannitol/70 mM sucrose/1 mM EDTA/10 mM Hepes, pH 7.5), supplemented with a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and homogenized for 30 strokes with a Dounce homogenizer. Samples were centrifuged at 500 *g* for 5 min at 4 °C and the resulting supernatant was centrifuged at 10 000 *g* for 30 min at 4 °C to obtain the heavy membrane pellet enriched for mitochondria. The 10 000 *g* supernatant was used as crude cytosol and the heavy membrane pellet was resuspended in 50 μ l of MB.

Western blotting was performed according to the protocol supplied with the ECF (enhanced chemifluorescence) Western-blot kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). Cell extracts (30 μ g) were boiled for 5 min with loading buffer and then subjected to SDS/PAGE (12% gel). After the proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.), the membrane was preincubated in blocking buffer [TBS containing 5% (w/v) non-fat dried milk] for 1 h at room temperature (26 °C) and then probed with a primary

Figure 1 Cyanide-induced cytosol-to-mitochondria translocation of Bad in cortical neurons

(A) Concentration response of Bad translocation during cyanide-induced apoptosis. Cells were treated with different concentrations of cyanide (100–500 μ M) for 6 h, and cytosolic and mitochondrial Bad levels were determined by Western-blot analysis. To show equal protein loading, the blots were stripped and re-probed with antibodies against β -actin or CYO IV (cytochrome *c* oxidase subunit IV). (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondria as determined by densitometric analysis of the Western blot.

antibody overnight at 4 °C. The membrane was washed three times with 0.1% Tween 20/TBS, then probed with secondary antibody for 1 h at room temperature. Then the membrane was washed again with 0.1% Tween 20/TBS and probed with the third antibody for 1 h at room temperature. After incubating with ECF substrate for 10 min, the immunoblot was detected using a Storm 860 fluorescence phosphorimager. Densitometric analysis was performed using the Image-Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Primary antibodies used were as follows: anti-rat Bad monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-mouse β -actin monoclonal antibody (Sigma, St. Louis, MO, U.S.A.), anti-mouse cytochrome *c* monoclonal antibody (PharMingen, San Diego, CA, U.S.A.) and anti-bovine cytochrome oxidase subunit IV monoclonal antibody (Molecular Probes, Eugene, OR, U.S.A.).

Calcineurin phosphatase assay

After various treatments, cells were collected by centrifugation and total proteins were extracted by lysing the cells in lysis buffer (50 mM Tris/HCl, pH 7.5/10 mM EDTA/100 mM NaCl/0.1% Triton X-100). Phosphate-reduced samples were prepared and calcineurin activity was detected by the serine/threonine Phosphatase Assay System according to the manufacturer's instructions (Technical Bulletin no. 218; Promega, Madison, WI, U.S.A.). On addition of calmodulin to the assay, this procedure measures protein phosphatase 2B activity; calcineurin is also known as protein phosphatase 2B. The amount of free phosphate released from

Figure 2 Time course of Bad redistribution after 300 μ M cyanide treatment

(A) Cells were treated with cyanide (1–24 h), and cytosolic and mitochondrial Bad levels were determined by Western-blot analysis. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot. CYO IV, mitochondrial marker cytochrome *c* oxidase subunit IV.

a phosphopeptide substrate was measured with molybdate dye solution and enzyme activity was expressed as percentage of control.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining

TUNEL was performed on 4% (w/v) paraformaldehyde-fixed cells using the Apoptag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, U.S.A.). Briefly, cells were preincubated in equilibration buffer containing 0.1 M potassium cacodylate (pH 7.2), 2 mM CaCl_2 and 0.2 mM dithiothreitol for 10 min at room temperature. This was then incubated in TUNEL reaction mixture (containing 200 mM potassium cacodylate, pH 7.2, 4 mM MgCl_2 , 2 mM 2-mercaptoethanol, 30 μ M biotin-16-dUTP and 300 units/ml terminal deoxynucleotidyl transferase) in a humidified chamber at 37 °C for 1 h. After incubation in stop/wash buffer for 10 min, the elongated digoxigenin-labelled DNA fragments were visualized using anti-digoxigenin peroxidase antibody solution followed by staining with 0.2 mg/ml diaminobenzidine tetrachloride and 0.005% H_2O_2 in PBS (pH 7.4). Cells were then counterstained with haematoxylin. The selectivity of the assay is based on the presence of 3'-OH DNA fragment ends in apoptotic cells.

Internucleosomal DNA fragmentation

Genomic DNA was extracted according to the method described previously [16]. Cells were collected in PBS and lysed in lysis

Figure 3 Activation of calcineurin by cyanide and inhibition by specific blockers

(A) Cortical cells were treated with cyanide (100–500 μ M) for 12 h and cellular calcineurin activity was determined. (B) Cells were pretreated with 1 μ M CsA, 1 μ M FK506 or 10 μ M BAPTA/AM 30 min before treatment with 300 μ M cyanide. Cellular calcineurin activity was measured 12 h after the addition of cyanide to the cells. Each bar represents means $\pm$ S.E.M. for four or more assays. *Significantly different from control (A) or from cyanide treatment group (B); $P < 0.05$.

buffer (10 mM Tris/HCl, pH 7.4/10 mM EDTA/0.5% SDS) with RNase A (final concentration 100 μ g/ml) for 1 h at 37 °C. Then 100 μ g/ml proteinase K was added and incubated for 4 h at 50 °C. DNA was precipitated with 0.1 vol. of 3 M sodium acetate (pH 5.2) and 2.5 vol. of precooled ethanol and then resuspended in Tris/EDTA buffer. DNA samples (10 μ g) were separated on a 1.2% (w/v) agarose gel containing 0.5 μ g/ml ethidium bromide for 2 h and visualized under UV light. either

Quantification of cellular caspase activity

Caspase-3 activity was determined by measuring the release of AMC (7-amino-4-methylcoumarin) from the caspase tetrapeptide substrate DEVD-AMC (Bachem Biosciences, King of Prussia, PA, U.S.A.) as described previously [16]. Briefly, cells grown in 6-well culture dishes were washed with PBS and lysed with 100 μ l of lysis buffer, containing 0.05% Igepal buffer (Sigma), 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris (pH 7.5), on ice. The lysate (50 μ l) was added to a reaction mixture containing 100 μ l of 2 $\times$ reaction buffer (20 mM HEPES, pH 7.5/50 mM NaCl/2.5 mM dithiothreitol), and 50 μ l of DEVD-AMC (final concentration, 50 μ M) and incubated for 1 h at 37 °C. The reaction was stopped by a 10 $\times$ dilution with ice-cold lysis buffer. Fluorescence of free AMC was determined (excitation and emission wavelengths: 360 and 465 nm respectively) using a fluorescence microplate reader.

Figure 4 Cytosolic Ca^{2+} increase contributes to Bad cytosol-to-mitochondria translocation induced by cyanide

(A) Cortical cells were treated with 300 μM cyanide in the presence or absence of 10 μM BAPTA in Ca^{2+} -free medium for 1–12 h, and cytosolic and mitochondrial fractions were separated by SDS/PAGE; Bad levels were detected by anti-Bad antibody. Blots were stripped and re-probed with antibody against β -actin or cytochrome *c* oxidase subunit IV to demonstrate equal protein loading. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot.

Immunocytochemistry

For immunocytochemical analysis, cortical neurons were grown on poly(L-lysine)-coated coverslips. After cyanide treatment, cells were labelled with 500 nM Mitotracker Red (Molecular Probes) for 30 min at 37 $^{\circ}\text{C}$. After washing three times with PBS, cells were fixed with 4% 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% (v/v) goat serum in PBS] at room temperature for 3 h. Cells were washed twice in PBS and incubated with mouse monoclonal anti-Bad antibody (1:100 dilution in PBS) at room temperature for 3 h. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1 h at room temperature in the dark. Coverslips were then mounted on to glass slides and examined by confocal microscopy.

Statistics

Each experiment was repeated a minimum of three times and results were expressed as means $\pm$ S.E.M. One-way ANOVA with Tukey–Kramer procedure for multiple comparisons was used to examine the statistical differences between treatments. Differences were considered as significant at $P < 0.05$.

Figure 5 Calcineurin activation contributes to Bad cytosol-to-mitochondria translocation induced by cyanide

(A) Cortical cells were treated with 300 μM cyanide in the presence or absence of 1 μM CsA for 1–12 h and cytosolic and mitochondrial fractions were separated by SDS/PAGE; Bad level was detected by anti-Bad antibody. Blots were stripped and re-probed with antibody against β -actin or cytochrome *c* oxidase subunit IV to demonstrate equal protein loading. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot.

RESULTS

Cyanide induces cytosol-to-mitochondria translocation of Bad

Distribution of Bad in cortical neurons was examined by Western-blot analysis. In control cells, Bad was localized exclusively in cytosol and was not detected in the mitochondrial fraction (Figures 1 and 2). This was consistent with previous studies, which showed, under normal conditions, that Bad was phosphorylated at its serine residues and sequestered in the cytosol by complexing with 14-3-3 [2,8]. After treatment with cyanide (100–500 μM) for 6 h, Bad underwent redistribution from the cytosol to mitochondria. Bad translocation increased with concentration from 100 to 300 μM cyanide, but decreased at 500 μM . Previously, we showed that the proportion of apoptotic cells decreases after treatment with 500 μM cyanide and there is a switch from apoptosis to necrosis at this higher cyanide concentration, partially due to depletion of intracellular ATP [14]. Also, the decrease in translocation at 500 μM cyanide suggested that Bad translocation might be ATP dependent and occurs only in apoptosis and not necrosis. Western-blot analysis of the fractions with anti- β -actin antibodies showed a low level of cross-contamination of the mitochondrial fraction, thus explaining the light band in the mitochondrial fraction under control conditions.

Bad translocation was an early event in cyanide-induced apoptosis, as demonstrated by detection of Bad in mitochondria 1 h

Figure 6 NF- κ B activation is not involved in Bad cytosol-to-mitochondria translocation induced by cyanide

(A) Cortical cells were treated with 300 μ M cyanide in the presence or absence of 50 μ g/ml SN50 for 1–12 h and the cytosolic and mitochondrial fractions were separated by SDS/PAGE; Bad level was detected by anti-Bad antibody. Blots were stripped and re-probed with antibody against β -actin or cytochrome *c* oxidase subunit IV to demonstrate equal protein loading. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot.

after cyanide treatment, accompanied by a corresponding decrease in cytosolic Bad level (Figure 2). Time-course studies showed that the cytosol-to-mitochondria redistribution of Bad increased with time from 1 to 12 h after cyanide treatment.

Ca²⁺-activated calcineurin contributes to Bad translocation

Cyanide produced a concentration-related increase in cellular calcineurin activity (Figure 3A), parallel to the concentration response observed with Bad translocation. The increased activity was blocked by CsA (cyclosporin A) or FK506, calcineurin inhibitors (Figure 3B). Calcineurin activation was linked to an increase in cytosolic free Ca²⁺, since BAPTA/AM [bis-(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxy-methyl ester)], a Ca²⁺ chelator that buffers intracellular free Ca²⁺, blocked phosphatase activation.

Bad translocation is controlled by dephosphorylation at one or more of four serine residues and it is dephosphorylated by calcineurin [2,5]. Since cytosolic Ca²⁺ is markedly increased in cyanide-induced apoptosis, we examined the role of Ca²⁺-activated calcineurin in Bad translocation. BAPTA/AM, a cell-permeant intracellular Ca²⁺ chelator, was used to inhibit cytosolic Ca²⁺ increase induced by cyanide. Cortical cells were treated with 300 μ M cyanide in Ca²⁺-free media in the presence or absence of 10 μ M BAPTA for 1–12 h. Cytosolic and mitochondrial fractions were separated, and Bad subcellular distribution

Figure 7 p38 MAPK activation is not upstream to Bad cytosol-to-mitochondria translocation induced by cyanide

(A) Cortical cells were treated with 300 μ M cyanide in the presence or absence of 20 μ M SB203580 for 1–12 h and the cytosolic and mitochondrial fractions were separated by SDS/PAGE; Bad level was detected by anti-Bad antibody. Blots were stripped and re-probed with antibody against β -actin or cytochrome *c* oxidase subunit IV to demonstrate equal protein loading. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot.

was examined by using a Western-blot assay. As shown in Figure 4, 10 μ M BAPTA significantly inhibited Bad translocation after cyanide treatment, showing that an increase in cytosolic Ca²⁺ was upstream of Bad translocation. Note that 10 μ M BAPTA did not completely block Bad translocation induced by cyanide. Probably, the cytosolic Ca²⁺ level may have exceeded the buffering capacity of BAPTA. CsA was used to inhibit calcineurin activity, and subsequent Bad translocation was determined. In the presence of 1 μ M CsA, cyanide-induced Bad translocation was decreased markedly from 1 to 6 h (Figure 5). Both cytosolic and mitochondrial Bad levels at 12 h were significantly increased. It appears that at this time of cyanide exposure the total cellular Bad levels are increased, perhaps reflecting commitment of the cell to apoptosis. Our studies have shown that at 12 h post-exposure to cyanide, the cells are irreversibly committed to apoptosis [15,16].

In addition to calcineurin inhibition, CsA has additional actions, including NF- κ B (nuclear factor κ B) inhibition [18]. To exclude the possibility that CsA inhibited Bad translocation through NF- κ B inhibition, as opposed to calcineurin, SN50, an NF- κ B inhibitor, was used to block NF- κ B activation and the resultant effect on Bad translocation was observed. As shown in Figure 6, SN50 had no effect on cyanide-induced Bad translocation, indicating that NF- κ B was not involved. Scheid et al. [19] have also shown that MAPK phosphorylates Bad on Ser-112. Since p38 MAPK is activated in cyanide-induced apoptosis [1], its role in Bad phosphorylation/translocation was examined. The specific

Figure 8 Immunostaining of Bad subcellular distribution during cyanide-induced apoptosis

After cyanide treatment (300 μ M for 12 h), cortical cells were immunostained for Bad and double-stained with Mitotracker Red for mitochondria. Cells were pretreated with 10 μ M FK506 or CsA, for 30 min before the addition of KCN. Confocal microscopy revealed localization of mitochondria (red) and Bad (green). Co-localization of Bad in mitochondria was shown as yellow coloration in merged images.

p38 MAPK inhibitor SB203580 had no effect on Bad translocation (Figure 7). It was concluded that p38 MAPK did not contribute to Bad phosphorylation in cyanide-induced apoptosis.

Translocation of Bad from cytosol to mitochondria was confirmed further by confocal immunofluorescence microscopy (Figure 8). In control cortical cells, Bad exhibited a diffuse cytosolic staining pattern, but after incubation with 300 μ M cyanide for 12 h, it co-localized extensively with a specific mitochondrial marker, namely Mitotracker Red. After pretreatment of cells with the calcineurin inhibitors FK506 or CsA the translocation of Bad to mitochondria was markedly inhibited, similar to the observation in Western-blot analysis of the cell fractions described above.

Bad translocation, cytochrome *c* release and apoptotic cell death

After translocation to mitochondria, Bad heterodimerizes with Bcl-2/Bcl-X_L to neutralize their anti-apoptotic effect. Since Bcl-2/Bcl-X_L regulates cytochrome *c* release in apoptosis, it seems probable that Bad translocation was also linked to cytochrome *c* release after cyanide treatment. BAPTA pretreatment partly inhibited cytochrome *c* release from mitochondria, suggesting that Ca²⁺-activated calcineurin was involved, presumably by promoting Bad translocation (Figure 9).

To link further calcineurin activation and cell death, the level of apoptosis was quantified. Cyanide produced a significant level of apoptosis, as characterized by positive TUNEL staining and internucleosomal DNA fragmentation (Figure 10). The apoptosis was blocked by CsA or FK506 pretreatment or by buffering intra-

Figure 9 Increase in cytosolic calcium contributes to mitochondrial release of cytochrome *c* induced by cyanide

(A) Cortical cells were treated with 300 μ M cyanide in the presence or absence of 10 μ M BAPTA in Ca²⁺-free medium for 1–12 h and cytosolic and mitochondrial fractions were separated by SDS/PAGE; the cytochrome *c* level was detected by anti-cytochrome *c* antibody. Blots were stripped and re-probed with antibody against β -actin or cytochrome *c* oxidase subunit IV to demonstrate equal protein loading. Results are representative of four separate experiments. (B) Percentage of total Bad (cytosolic + mitochondrial fractions) localized in the mitochondrial fraction as determined by densitometric analysis of the Western blot.

cellular free Ca²⁺ with BAPTA/AM. Treatment of the cells with cyanide induced a marked increase in cellular caspase activity within 6 h (Figure 10D), and pretreatment with the calcineurin inhibitors significantly decreased the level of caspase activation produced by cyanide.

DISCUSSION

Bcl-2 proteins are regulators of cytochrome *c* release from mitochondria in apoptosis [20–22]. We have shown that cytosol-to-mitochondria translocation of Bax initiates cytochrome *c* release 3 h after cyanide treatment, and NF- κ B-mediated up-regulation of Bcl-X_s and Bax further facilitated cytochrome *c* release 6–12 h after cyanide treatment [15]. In the present study, it was determined that Bad dephosphorylation by calcineurin and translocation is an upstream premitochondrial event of cytochrome *c* release in cyanide-induced apoptosis.

Cyanide initiates a rapid and marked increase in cellular Ca²⁺ leading to mitochondrial dysfunction. Cyanide enhances *N*-methyl-D-aspartate receptor function [23,24] and mobilizes intracellular calcium stores [25]. In cultured neurons, cyanide-induced cytotoxicity is linked to *N*-methyl-D-aspartate receptor-mediated increase in cytosolic Ca²⁺ that in turn activates a series of biochemical reactions leading to cell death. Present results link the increase in Ca²⁺ to activation of calcineurin, which in turn

Figure 10 Inhibition of cyanide-induced apoptosis by calcineurin inhibitors and an intracellular Ca^{2+} chelator

(A) Effect of treatments on cyanide-induced TUNEL staining in cortical cells: (a) control (vehicle); (b) 300 μ M KCN; cells pretreated 30 min before KCN with (c) 1 μ M CsA; (d) 1 μ M FK506; (e) 10 μ M BAPTA/AM. Cells were TUNEL-stained 24 h after the addition of KCN and apoptotic positive cells are darkly stained. (B) The level of apoptosis was determined by counting the number of TUNEL-positive cells. Four randomly selected fields of TUNEL-stained cells were counted and the average percentage of apoptotic cells per total was determined. Results are expressed as means $\pm$ S.E.M. for three experiments. *Significant difference from control; + significant difference from the cyanide treatment group ($P < 0.05$). (C) Ca^{2+} -activated calcineurin contributes to DNA fragmentation induced by cyanide. Cells were treated with 300 μ M cyanide in the presence or absence of BAPTA or CsA for 24 h and then genomic DNA was extracted and subjected to agarose-gel electrophoresis. Lane 1, control cells; lane 2, 300 μ M KCN; lane 3, 300 μ M KCN + pretreatment with 10 μ M BAPTA; lane 4, 300 μ M KCN + pretreatment with 1 μ M CsA. (D). Activation of cellular caspase-3 by cyanide. Cells were incubated with 300 μ M KCN for 6 h in the presence or absence of BAPTA, FK506 or CsA, and cellular caspase-3 activity was determined. Results are means $\pm$ S.E.M. of $n = 6$ samples. * $P < 0.05$ versus control; + $P < 0.05$ versus KCN group.

dephosphorylates Bad to stimulate its translocation to mitochondria. The subcellular location of Bad is controlled by its phosphorylation status at several serine residues. It exists in a phosphorylated state in the cytoplasm, bound to 14-3-3, and in a non-phosphorylated state at the mitochondria surface, where it appears to interact with Bcl-2/Bcl-X_L to produce an anti-apoptotic state [8,26].

Bad lacks a C-terminal stretch of hydrophobic domain for membrane association, and hence it exerts its function by heterodimerizing with other Bcl-2 members through its BH3 homology domain [8]. The dimerization partners of Bad are all anti-apoptotic Bcl-2 proteins, primarily Bcl-2 and Bcl-X_L. Thus Bad is considered to be pro-apoptotic by neutralizing the anti-apoptotic effects of Bcl-2 and Bcl-X_L [7]. Agents that blocked Bad translocation to mitochondria prevented cytochrome *c* release and apoptosis. Presumably, the unopposed anti-apoptotic effects of Bcl-2 and Bcl-X_L maintained mitochondrial integrity and aborted cell death under these conditions.

To date, several kinases have been shown to phosphorylate Bad. Akt, a serine/threonine kinase downstream of phosphatidylinositol 3-kinase, is site specific for Ser-136 [27,28]. Mitochondria-bound cAMP-dependent protein kinase specifically phosphorylates Ser-112 [29]. Furthermore, one study demonstrated that Bad was phosphorylated by the MAPK/ERK kinase (where ERK stands for extracellular-signal-regulated protein kinase) to regulate its function [19]. p38 MAPK, related to MAPK, is activated in cyanide-induced apoptosis [1]. In this study, SB203580 was used to block p38 MAPK activation and in turn its effect on Bad translocation was examined. Since inhibition of p38 MAPK did not alter Bad translocation, it was concluded that p38 MAPK was not involved in Bad phosphorylation/translocation.

Dephosphorylation of Bad appears to be a premitochondrial reaction for several apoptotic stimuli. Bad dephosphorylation has been observed during apoptosis induced by growth-factor deprivation or sustained increase in intracellular Ca^{2+} [8]. The serine/threonine-specific phosphatase PPL α is responsible for Bad

dephosphorylation in growth-factor-deprivation-induced apoptosis, whereas calcineurin dephosphorylates Bad in apoptosis resulting from Ca^{2+} increase [2]. The present study showed that Ca^{2+} -activated calcineurin contributes to Bad translocation, since BAPTA and CsA partly inhibited Bad translocation. Similarly, Shibasaki and McKeon [30] linked increased intracellular Ca^{2+} in hamster kidney-21 cells to calcineurin-mediated apoptosis.

In neuronal cells, activation of calcineurin is associated with cell death. In primary cultured cortical cells, high activity of calcineurin produces caspase-3-mediated apoptosis, which is reduced by CsA or FK506 [12]. In studies of traumatic spinal cord injury, pretreatment of animals with FK506 blocked Bad dephosphorylation and prevented the activation of caspase-3-mediated apoptosis [5]. The ability of CsA or FK506 to produce neuronal protection is attributed partially to inhibition of calcineurin [31,32]. However, CsA also interacts with mitochondrial cyclophilin D to prevent mitochondrial permeability transition, a critical step in mitochondria-mediated apoptosis.

In addition to dephosphorylation of Bad, calcineurin may participate in the initiation of apoptosis through interaction with other apoptotic regulators such as Bcl-2. Activated calcineurin can form a complex with Bcl-2, resulting in neutralization of the anti-apoptotic action of Bcl-2 [33]. Calcineurin also dephosphorylates Bcl-2 to modulate its activity in the mitochondria [34]. Recently, Erin et al. [35,36] proposed that the interaction of Bcl-2 with activated calcineurin may serve to regulate apoptotic responses to stressful stimuli.

In summary, cyanide induced cytosol-to-mitochondria translocation of Bad. Increase in intracellular Ca^{2+} and activation of calcineurin were initiators of the translocation, since the Ca^{2+} chelator BAPTA and the calcineurin inhibitor CsA decreased Bad translocation and in turn linked Bad translocation to mitochondrial cytochrome *c* release. Cytosolic Ca^{2+} increase and calcineurin activation are upstream of cytochrome *c* release. Thus, by inducing a rapid increase in cytosolic Ca^{2+} , cyanide can stimulate the apoptotic cascade in cortical neurons that involves dephosphorylation of Bad by calcineurin, translocation of Bad to mitochondria, release of cytochrome *c* from mitochondria and activation of caspases.

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