Neural progenitor cells (NPC) can proliferate, differentiate into neurons or glial cells, or undergo a form of programmed cell death called apoptosis. Although death of NPC occurs during development of the nervous system and in the adult, the underlying mechanisms are unknown. Here we show that nitric oxide (NO) can induce death of C17.2 NPC by a mechanism requiring activation of p38 MAP kinase, poly(ADP-ribose) polymerase, and caspase-3. Nitric oxide causes release of cytochrome c from mitochondria, and Bel-2 protects the neural progenitor cells against nitric oxide-induced death, consistent with a pivotal role for mitochondrial changes in controlling the cell death process. Inhibition of p38 MAP kinase by SB203580 abolished NO-induced cell death, cytochrome c release, and activation of caspase-3, indicating that p38 activation serves as an upstream mediator in the cell death process. The anti-apoptotic protein Bel-2 protected NPC against nitric oxide-induced apoptosis and suppressed activation of p38 MAP kinase. The ability of nitric oxide to trigger death of NPC by a mechanism involving p38 MAP kinase suggests that this diffusible gas may regulate NPC fate in physiological and pathological settings in which NO is produced.

This is despite the fact that considerable death of NPC occurs during development of the nervous system (1, 9–11) as well as in the adult nervous system in the process of NPC turnover (12, 13). Recent findings suggest that NPC may undergo a form of programmed cell death called apoptosis in which DNA damage (14), release of cytochrome c from mitochondria (15), and activation of members of the caspase family of proteases (13, 16) play important roles.

Nitric oxide (NO) is an intra- and intercellular signaling molecule which plays important roles in regulating synaptic plasticity and cell survival in the adult nervous system in both physiological and pathological settings (17, 18). Roles for NO in the development of the nervous system are suggested from studies showing that NO can regulate neurite outgrowth (19, 20) and synaptogenesis (21, 22). Interestingly, NO can also induce apoptosis of a variety of types of cultured cells including neurons (23–25) and may contribute to the death of neurons in several disorders including ischemic stroke (26) and Alzheimer's disease (27). As evidence, nNOS-deficient mice exhibit significant protection against cerebral ischemia (28) and N-methyl-d-aspartate-mediated excitotoxicity (29, 30). The cytotoxic effects of NO may result from its interaction with the superoxide anion to form peroxynitrite and other reactive oxygen radicals (29, 31–37). Oxidative damage to cellular proteins and nucleic acids can, in turn, trigger an apoptotic cascade involving activation of poly(ADP-ribose) polymerase (PARP), mitochondrial membrane permeability transition, and release of cytochrome c, and activation of caspases that execute the cell death process (25, 38–43). Several different protein kinases have been reported to act at one or more steps in the apoptotic pathway, with c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase being two prominent examples (44–46). It was recently reported that p38 MAP kinase can induce translocation of the proapoptotic protein Bax from the cytosol to mitochondria in mature neurons undergoing NO-induced apoptosis (47).

Although NO is a prominent messenger in the brain, its effects on NPC are unknown. To determine whether NO regulates NPC survival or death and to explore the underlying mechanisms, we examined the effects of NO on C17.2 cells, a clonal line of NPC that can differentiate into neurons or glia when co-cultured with primary neurons or transplanted into the adult rodent brain (48, 49). Our data demonstrate that NO can induce apoptotic death of NPC by a p38 MAP kinase-dependent mechanism: p38 MAP kinase acts at early step prior to dysfunction of mitochondria and caspase activation, and overexpression of Bel-2 significantly attenuates the activation of p38 MAP kinase and protects the NPC against NO-induced death. These findings suggest roles for NO in regulating NPC fate in physiological and pathological settings.
EXPERIMENTAL PROCEDURES

Culture and Experimental Treatment of Neural Progenitor Cells—The C17.2 NPC line (a generous gift from C. Cepko) was maintained in plastic culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Life Technologies, Inc.) and 5% horse serum (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂, 95% air at 37 °C. For experimental analyses, cells were grown on poly-L-lysine-coated culture dishes or glass coverslips. Experimental treatments included: sodium nitroprusside (SNP) and 3-aminobenzamide (Sigma) which were prepared as 200–500 × stocks in culture medium; zVAD-fmk (Biomol Research Labs, Inc.) and SB 203580 (Sigma), which were prepared as 500 × stocks in dimethyl sulfoxide. Treatments were administered by direct dilution into the culture medium, and an equivalent volume of vehicle was added to control cultures.

Assessment of Cell Survival and Apoptosis—After exposure to experimental treatments, cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature and then washed with PBS. Cells were then either stained with the DNA-binding dye Hoechst 33258 (5 μg/ml in PBS for 2 h at room temperature or overnight at 4 °C) or membranes were permeabilized with 0.2% Triton X-100 and the cells were stained with propidium iodide (5 μg/ml for 10 min at room temperature). Coverslips were mounted onto glass slides and examined under epifluorescence illumination using a ×40 objective lens. Cells were considered “apoptotic” if their nuclear chromatin was condensed or fragmented, whereas cells were considered viable if their chromatin was diffuse and evenly distributed throughout the nucleus. In preliminary studies we determined the integrity of the plasma membrane by staining cells with the dye trypan blue. Essentially no cells were stained with trypan blue (data not shown), indicating that the cells with condensed and fragmented nuclei have intact membranes and were therefore not undergoing necrosis.

Immunoblot Analysis—After experimental treatment the cells (~2 × 10⁴ cells) were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer, and the protein concentration in each sample was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard. Proteins (50 μg of protein per lane) were then resolved on 7.5–12% SDS-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 10% nonfat milk in TBST (Tris-HCl based buffer with 0.2% Tween 20, pH 7.5), and then incubated for 2 h in the presence of primary antibody. Cells were then incubated for 1 h in the presence of a 1:5000 dilution of secondary antibody (IgG) conjugated to horseshadish peroxidase. Reaction product was visualized using an Enhanced Chemiluminescence (ECL) Western blot detection kit (Amersham Pharmacia Biotech, United Kingdom). The primary antibodies included anti-PARP (mouse, 1:1000, PharMingen, San Diego, CA), anti-caspase-3 (rabbit, 1:1000, PharMingen), anti-tubulin (mouse, 1:5000, Sigma), anti-phospho-p44/42 MAP kinase (mouse, 1:1000, New England Biolabs), and anti-p44/42 MAP kinase (rabbit, 1:1000, New England Biolabs), anti-phospho-JNK (rabbit, 1:1000, Cell Signaling, Inc.), anti-phospho-JNK (mouse, 1:1000, Santa Cruz Biotechnology), anti-JNK (rabbit, 1:1000, Santa Cruz Biotechnology), anti-Bcl-2 (rabbit, 1:1000, StressGen, Inc.), and anti-cytochrome c antibody. After experimental treatment, cells were incubated with 100 nM Mitotracker Red CMX Ros for 30 min at 37 °C (the dye is taken up by mitochondria where it forms thiol conjugates with peptides and is thereby trapped in the mitochondria), washed with PBS, and fixed with 4% paraformaldehyde in PBS at 37 °C for 30 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min at 4 °C, followed by a 2-h incubation at room temperature in blocking solution (2% normal goat serum, 0.1% Triton X-100 in PBS, pH = 7.4) containing primary monoclonal cytochrome c antibody (10 μg/ml, PharMingen, San Diego, CA). After washing, cells were incubated for 2 h in PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100, Vector Laboratories). Cells were then imaged in dual-scan mode on a Zeiss CLSM 510 confocal microscope using a ×40 water immersion objective (N.A. = 1.4). The excitation and emission wavelength for Mitotracker CMX Ros were 510 and 590 nm, respectively, and for fluorescein isothiocyanate were 488 and 510 nm, respectively.

The second method involved immunoblot analysis of cytochrome c in cytosolic and mitochondrial fractions of cells. At designated time points following exposure to experimental treatments, cells (~1 × 10⁴) were trypsinized and then washed with ice-cold buffer A (250 mM sucrose, 20 mM HEPES-KOH, 1 mM EDTA, 1 mM EGTA, 2 μg/ml leupeptin, and 1 μg/ml pepstatin, pH 7.4). Cells were resuspended in 200 μl of buffer A and carefully homogenized using a Dounce homogenizer. The homogenates were separated into cytosol (supernatant) and mitochondrial fractions by differential centrifugation. Cytosolic (300 μg) and mitochondrial (50 μg) proteins were then subjected to immunoblot analysis using a monoclonal cytochrome c antibody as described above.

Transient Transfection Assay—Two expression plasmids were employed for transient transfections: the pBabe Puro vector (5.1 kilobase) contained the complete coding sequence of human Bcl-2 cDNA (a generous gift from D. Bredesen) and the pEGF-P1 plasmid contained the green fluorescence protein (GFP) cDNA (CLONTECH). Cells were transfected with 5 μg of pBabe-Puro-Bcl-2 plasmid or co-transfected with 5 μg of pEGF-P1 plasmid using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the instructions of the manufacturer. Experiments were performed 24 h after transfection.

RESULTS

NO Induces Caspase Activation and Nuclear DNA Damage in Neural Progenitor Cells—To determine the possible involvement of NO in the regulation of NPC survival, we exposed C17.2 cells to increasing concentrations of the NO donor SNP and quantified the percentage of cells exhibiting apoptotic morphological alterations and nuclear chromatin condensation/fragmentation (Fig. 1A). Essentially no cell death occurred in control cultures or cultures exposed to 50 μM SNP (Fig. 1B). However, at concentrations from 100 to 400 μM SNP induced cell death in a concentration- and time-dependent manner such that 50 and 70% of the cells were killed within 10 h of exposure to 200 μM and 500 μM SNP, respectively (Fig. 1B). Essentially all cells with apoptotic morphologies excluded trypan blue indicating that their plasma membranes were intact and that they were therefore not necrotic (data not shown).

We next assessed caspase activity levels by performing immunoblot analyses of lysates from cells that had been exposed to SNP for increasing time periods. The blots were probed using antibodies against caspase-3 and the caspase-3 substrate, PARP. SNP induced cleavage of procaspase-3 into the active form of caspase-3, with cleavage of procaspase-3 occurring within 4 h of exposure to SNP (Fig. 2A). When cells were treated with the caspase inhibitor zVAD-fmk, cleavage of pro-caspase-3 was largely prevented (Fig. 2B). SNP also induced PARP cleavage which was evident within 4 h (Fig. 2C). To further examine caspase-3 activity, we exposed cells to SNP for 4, 7, and 10 h and then processed the cells for in situ localization of activated caspase-3 using fluorescein-conjugated avidin (Fig. 2D). Cells in which DEVD-biotin is introduced into cells and then detected with fluorescein-tagged avidin. Levels of activated caspase-3 were markedly increased in cells within 4 h of exposure to SNP and remained elevated at 7 h (Fig. 2D). The activated caspase-3 was localized mainly to cytoplasmic compartments at the 4-h time point, but was also present in the nucleus at the 7-h time point.

Caspase Activity Assays—Caspase-3 activity was assayed by two methods. First, the extent of cleavage of procaspase-3 into the truncated active caspase-3 fragments, and the extent of cleavage of the caspase-3 substrate PARP, was determined by immunoblot analysis. Second, to localize activated caspase-3 in situ, a protocol that employs biotinylated DEVD, a pseudosubstrate and inhibitor of caspase-3 was used (50). At designated time points following exposure of cells to experimental treatments, they were exposed for 10 min to Locke’s buffer containing 0.01% digitonin. Cells were then incubated for 20 min in the presence of 10 μg/ml DEVD-biotin (Calbiochem), washed three times with PBS, and fixed for 30 min in a cold solution of 4% paraformaldehyde in PBS. Cells were incubated for 5 min in PBS containing 0.2% Triton X-100, followed by a 30-min incubation in PBS containing 5 μl/ml Oregon Green streptavidin (Molecular Probes, Inc.). Cells were then washed twice with PBS and then incubated with 0.1 μg/ml fluorescein-conjugated avidin (Molecular Probes, Inc.) for 15 min. Cells were then washed twice with PBS and mounted onto glass slides and examined using epifluorescence microscopy (Zeiss CSML 510, Germany) with excitation at 488 nm and emission at 510 nm.

Evaluation of Cytochrome c Release from Mitochondria—Two methods were used to determine the release of cytochrome c from mitochondria. The first method involved confocal imaging of cells double-labeled with Mitotracker Red CMX Ros (Molecular Probes, Inc.) and a cytochrome c antibody. After experimental treatment, cells were incubated with 100 nM Mitotracker Red CMX Ros for 30 min at 37 °C (the dye is taken up by mitochondria where it forms thiol conjugates with peptides and is thereby trapped in the mitochondria), washed with PBS, and fixed with 4% paraformaldehyde in PBS at 37 °C for 30 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min at 4 °C, followed by a 2-h incubation at room temperature in blocking solution (2% normal goat serum, 0.1% Triton X-100 in PBS, pH = 7.4) containing primary monoclonal cytochrome c antibody (10 μg/ml, PharMingen, San Diego, CA). After washing, cells were incubated for 2 h in PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100, Vector Laboratories). Cells were then imaged in dual-scan mode on a Zeiss CLSM 510 confocal microscope using a ×40 water immersion objective (N.A. = 1.4). The excitation and emission wavelengths for Mitotracker CMX Ros were 510 and 590 nm, respectively, and for fluorescein isothiocyanate were 488 and 510 nm, respectively.
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Recent studies have established a mechanism for activation of caspase-3 that involves mitochondrial alterations that result in release of cytochrome c. Activation of caspase-3 involves mitochondrial release of cytochrome c, which is a key step in the apoptotic process. Mitochondria are the site of the mitochondrial membrane, which is the location of the cytochrome c release. In the absence of SNP, cytochrome c was present at high levels in the mitochondrial fraction, and a corresponding increase in the amount of cytochrome c was present in the mitochondrial fraction, and a corresponding increase in the amount of cytochrome c in the cytosolic fraction. In the presence of SNP, cytochrome c was released from mitochondria, indicating that cytochrome c was released from mitochondria. Recent studies have established a mechanism for activation of caspase-3 that involves mitochondrial alterations that result in release of cytochrome c into the cytosol. The release of cytochrome c into the cytosol allows for the activation of caspase-3, which is a key step in the apoptotic process. The release of cytochrome c into the cytosol allows for the activation of caspase-3, which is a key step in the apoptotic process.

**Fig. 1.** Nitric oxide induces death of C17.2 neural progenitor cells. A cells were exposed for 7 h to vehicle (Control) or 200 or 500 μM SNP. Cells were then stained with the fluorescent DNA-binding dye Hoechst 33258; phase-contrast micrographs (upper panels) and images of Hoechst fluorescence (lower panels) of representative microscope fields are shown. B, cells were exposed for the indicated time periods to the indicated concentrations of SNP and the percentage of cells that were dead in each culture were determined. Values are the mean and S.E. of determinations made in four to six cultures.

**Fig. 2.** Nitric oxide induces caspase 3 activation and proteolytic cleavage of PARP in neural progenitor cells. A, cells were exposed to 200 μM SNP for 4, 7, or 10 h in the absence (left) or presence (right) of 50 μM zVAD-fmk (additional cultures were left untreated (0 time point)). Proteins in cell lysates were separated by electrophoresis and subjected to immunoblot analysis using antibodies against caspase-3 or tubulin. Note that SNP induced cleavage of caspase-3, which was largely prevented by treatment with zVAD-fmk. B, subcellular localization of activated caspase-3 in NP after exposure to SNP. Confluent laser scanning microscope images showing fluorescence corresponding to activated caspase-3 bound to the substrate DEVD. Cultures had been left untreated (control), or exposed to 200 μM SNP for the indicated time periods in the absence or presence of 50 μM zVAD-fmk. C, cells were exposed to 200 μM SNP for 4, 7, or 10 h, or were left untreated (0 time point). Proteins in cell lysates were separated by electrophoresis and subjected to immunoblot analysis using a PARP antibody. Note that SNP induced cleavage of PARP.

**NO-induced Death of NPC Involves Release of Cytochrome c from Mitochondria**—Recent studies have established a mechanism for activation of caspase-3 that involves mitochondrial alterations that result in release of cytochrome c into the cytosol where it forms a complex with Apaf-1 and caspase-9 resulting in caspase-9 activation which then cleaves and activates caspase-3 (51). To determine whether NO induces cytochrome c release from mitochondria in NPC, we performed double labeling confocal fluorescent imaging using the mitochondrial marker dye Mitotracker red in combination with immunostaining with an antibody against cytochrome c. In untreated control cells, cytochrome c immunoreactivity was co-localized with Mitotracker red fluorescence indicating that the cytochrome c was confined to mitochondria (Fig. 3A). After exposure to SNP, many cells exhibited cytochrome c immunoreactivity diffusely distributed throughout the cytoplasm and a decrease in mitochondria-associated cytochrome c immunoreactivity indicating that cytochrome c was released from mitochondria (Fig. 3A). Whereas no cells (zero of 1000 cells examined in three separate cultures) exhibited this cytochrome c release pattern in control cultures, 20% to 5% of the cells exhibited cytochrome c release in cultures that had been exposed for 4 h to 200 μM SNP. We next isolated mitochondrial and cytosolic fractions from control and SNP-treated cells and performed immunoblot analyses to determine the relative content of cytochrome c in mitochondria versus the cytoplasm. In the absence of SNP, cytochrome c was present at high levels in the mitochondrial fraction and was not detected in the cytoplasm (Fig. 3B). SNP caused a progressive decrease in the amount of cytochrome c present in the mitochondrial fraction, and a corresponding increase in the amount of cytochrome c in the cytosolic fraction (Fig. 3B).

**NO Induces p38 MAP Kinase Activation, but Does Not Activate JNK, ERK-1, and ERK-2**—Mitogen-activated protein (MAP) kinases function in a variety of signal transduction pathways and play important roles in regulating cell growth, differentiation, and programmed cell death. Members of the MAP kinase family include the extracellular signal-regulated kinases (ERK-1 or p42 MAP kinase; and ERK-2 or p44 MAP kinase), JNK, and p38 MAP kinase (52). ERKs are typically activated by growth factor stimulation whereas JNK and p38 MAP kinases are strongly activated by a variety of cellular stresses including ultraviolet radiation, hyperosmolarity, heat shock, and proinflammatory cytokines. p38 MAP kinase and JNK have recently been shown to be involved in cell death induced by nerve growth factor deprivation in a neuronal cell line (53). In addition, p38 MAP kinase and JNK activities are also implicated in developmental neuronal cell death, and in cell death associated with axotomy (54, 55). We therefore determined whether one or more of these kinases was phospho-
rlylated and activated in response to NO in NPC. Exposure of cells to SNP resulted in a marked (5-fold) increase in the level of phosphorylated p38 MAP kinase within 1 h with a further increase in phospho-p38 MAP kinase levels by 4 h; the increase in phospho-p38 MAP kinase occurred without a change in the overall level of p38 MAP kinase protein during the 4-h period of exposure to SNP (Fig. 4). In contrast, SNP treatment did not affect phosphorylation of JNK, ERK-1, or ERK-2 during a 4-h exposure period (Fig. 4). In an additional experiment in which cells were treated for 12 h with SNP, increased phosphorylation of p38 MAP kinase was evident, whereas there was no change in the phosphorylation of JNK, ERK-1, and ERK-2 (data not shown).

Activation of PARP, Caspases, and p38 MAP Kinase Are Each Required for NO-induced Death of NPC—To determine whether caspase activation, PARP activity, and/or p38 MAP kinase activation are required for NO-induced death of NPC, we pretreated cells with a cell-permeant caspase inhibitor (zVAD-fmk), an inhibitor of PARP (3-aminobenzamide) (56, 57), or an inhibitor of p38 MAP kinase (SB 203580) (58). Preliminary studies showed that none of the three inhibitors affected cell viability under basal culture conditions (data not shown). Cell death caused by SNP was significantly attenuated in cells pretreated with zVAD-fmk, 3-aminobenzamide, and SB 203580 (Fig. 5). At a dose of 500 μM SNP that killed 50% of the cells within 7 h, ~25% of the cells were killed in the presence of zVAD-fmk or 3-aminobenzamide, and only 15% of the cells were killed in the presence of SB 203580. A combined treatment with zVAD-fmk and 3-aminobenzamide provided no additional protection beyond that obtained with either compound alone, whereas a combined treatment with zVAD-fmk and SB 203580 resulted in additive protection such that only 6% of the

FIG. 3. Nitric oxide induces cytochrome c release from mitochondria in neural progenitor cells by a mechanism requiring membrane permeability transition pore formation. A, confocal laser scanning microscope images showing Mitotracker red fluorescence (red), cytochrome c immunoreactivity (green), and merged images (yellow indicates sites of co-localization) in NPC cells. The cells were either untreated (control) or exposed to 200 μM SNP for 4 h. Note localization of cytochrome c in mitochondria in all NPC in control cultures and in some cells in SNP-treated cultures, and diffuse localization of cytochrome c in the cytoplasmic compartment of some NPC in SNP-treated cultures (arrows). B, cells were exposed to 200 μM SNP for 4, 7, or 10 h. Mitochondrial and cytosolic fractions of the cells were prepared and 300 μg of protein were subjected to immunoblot analysis using a cytochrome c antibody. Note that SNP induced cytochrome c release from mitochondria.

FIG. 4. The nitric oxide donor, SNP selectively activates p38 MAP kinase in neural progenitor cells. Cells were treated with 500 μM SNP, and protein extracts were prepared at the indicated time points to assess the activation of p38 MAP kinase, JNK, Erk1, and Erk2. Level of total (p38, JNK, Erk1, and Erk2) and phosphorylated MAP kinase (pp38, p-JNK, p-Erk1, and p-Erk2) were determined by Western blotting using specific antibodies. Arrows indicate the position of specific immunoreactive bands corresponding to distinct isoforms of JNK and Erk.

FIG. 5. Inhibitors of capasases, PARP, and p38 MAP kinase protect neural progenitor cells against nitric oxide-induced death. Cells were pretreated for 1 h with the indicated concentrations of the caspase inhibitor zVAD-fmk, the PARP inhibitor 3-aminobenzamide (3-AB), and the p38 MAP kinase inhibitor SB203580. Cells were then exposed to SNP or vehicle as indicated and cell death was quantified 7 h later. Values are the mean and S.E. of determinations made in at least 4 cultures. *, p < 0.01; **, p < 0.001 compared with value for cells exposed to SNP alone (ANOVA with Scheffe post-hoc tests).
mediated death of NPC, but did not establish where in the apoptotic cascade p38 MAP kinase acted. To determine whether p38 MAP kinase activation is required for mitochondrial events in the cell death cascade we pretreated NPC with SB203580 or vehicle, exposed the cells to SNP for 7 h, and then examined levels of mitochondrial and cytosolic cytochrome c by immunoblot analysis. SNP-induced cytochrome c release in cell pretreated with vehicle, but caused little or no cytochrome c release in cells pretreated with SB203580 (Fig. 6A). SB203580 also prevented activation of caspase-3 in NPC exposed to SNP (Fig. 6, B and C). These data indicate that p38 MAP kinase acts at a premitochondrial step in the apoptotic cascade triggered by NO.

Bcl-2 is an anti-apoptotic protein that can prevent cell death by suppressing oxyradical-mediated membrane damage, stabilizing mitochondrial membrane potential, and preventing release of cytochrome c (59, 60). To determine whether Bcl-2 could protect NPC against NO-induced cell death, we transiently transfected C17.2 cells with an expression plasmid containing cDNA encoding Bcl-2. The transfection efficiency was ∼30% as determined by co-transfection with a green fluorescent protein reporter plasmid (Fig. 7A). Immunoblot analysis demonstrated a marked increase in levels of Bcl-2 protein in cells 24 h after transfection with the bcl-2 expression plasmid (Fig. 7B). Control cultures were transfected with GFP plasmid alone. Twenty-four hours after transfection, cells were exposed to SNP, and 7 h later were fixed and stained with the fluorescent DNA-binding dye propidium iodide. In control cultures transfected with GFP alone, an identical percentage (53%) of the transfected cells (GFP+) and nontransfected cells (GFP−) exhibited apoptotic nuclei (Fig. 7C). In contrast, only 7% of the cells overexpressing Bcl-2 underwent apoptosis when exposed to SNP. Bcl-2 may prevent apoptosis by associating with mito-
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**DISCUSSION**

Death of NPC occurs during development of the nervous system (1, 9–11, 61), and is also believed to occur in populations of NPC located in the dentate gyrus of the hippocampus (62–64) and the subventricular zone (13) in the adult brain. In contrast to the considerable data available on the cellular and molecular mechanisms that regulate the survival of postmitotic neurons (65, 66), the mechanisms that determine whether NPC live or die are largely unknown. The ability of NO to induce apoptosis has been documented in previous studies of non-neuronal cells (24), and may play roles in the deaths of neurons that occur in a variety of neurological disorders (23, 67). Although the present findings provide the first evidence that nitric oxide can induce apoptosis of NPC, previous observations are consistent with a role for nitric oxide in the regulation of the survival/death decision of NPC and their immediate progeny. Nitric-oxide synthase is expressed in cells throughout the cerebral cortex, hippocampus, and other brain regions during the highly proliferative period of brain development (68). The neuronal isoform of nitric-oxide synthase is also expressed in presumptive NPC in the subventricular zone and olfactory bulb of the mature rodent brain (69, 70), and in cells immediately adjacent to NPC in the adult subventricular zone (71). Thus, NPC are likely to be exposed to nitric oxide in the developing and adult brain.

To determine whether excessive generation of NO can cause NPC death, and to elucidate the underlying mechanisms, we employed C17.2 cells, a clonal line of multipotent NPC. These cells have been extensively characterized in previous studies in which it was shown that they can differentiate into functional neurons and glial cells when transplanted into the developing mouse cerebellum (48, 72). Another property of C17.2 cells that is shared with endogenous NPC is their mitogenic response to bFGF and EGF (4, 73, 74). The data obtained in the present study allow the following conclusions regarding the regulation of C17.2 NPC survival by NO: 1) NO can induce apoptosis of NPC in a time- and concentration-dependent manner. 2) NO induces apoptosis by a mechanism involving mitochondrial dysfunction and release of cytochrome c. 3) NO-induced death of NPC requires PARP activation. 4) NO-induced death of NPC requires caspase activation. 5) NO-induced death of NPC requires p38 MAP kinase activation, and p38 MAP kinase acts at a site(s) upstream of mitochondrial alterations. 6) Bcl-2 can attenuate p38 MAP kinase activation and can protect NPC against NO-induced death.

Because cells overexpressing Bcl-2 and cells pretreated with zVAD-fmk plus SB 203580 exhibited similar resistance to NO-induced apoptosis, we determined whether overexpression of Bcl-2 affected p38 MAP kinase activation. To this end, we transfected cells with Bcl-2 expression plasmid or empty vector as a control for 24 h and then treated the cells with SNP for 1 or 4 h. Levels of phosphorylated p38 MAP kinase in cell lysates were then assessed by immunoblot analysis. Overexpression of Bcl-2 resulted in an attenuation of p38 MAP kinase activation without affecting the total amount of p38 MAP kinase (Fig. 8).

 **Fig. 8.** Overexpression of Bcl-2 attenuates the activation of p38 MAP kinase induced by SNP. A, cells were transfected with Bcl-2 expression plasmid or empty vector and then exposed to 500 μM SNP for the indicated time periods. Cell lysates (50 μg of protein/lane) were subjected to immunoblot analysis using antibodies against phospho-p38 MAP kinase or total p38 MAP kinase. B, data from densitometric analysis of immunoblots (mean and S.E. from three separate experiments). ***, p < 0.001 compared with value for vector-transfected cells (ANOVA with Scheffe post-hoc tests).

**Fig. 9.** Model showing pathways that mediate nitric oxide-induced apoptosis of neural progenitor cells. Nitric oxide induces activation of p38 MAP kinase by a mechanism that can be inhibited by Bcl-2 and that likely involves oxidative stress. p38 MAP kinase induces mitochondrial membrane alterations resulting in release of cytochrome c and activation of caspases. See text for discussion.
tyrosine residues by upstream dual-specificity kinases. However, the different MAP kinase members are activated in response to different extracellular stimuli and have different downstream targets, and thus serve different roles in cellular responses. ERKs are activated by growth factors by means of a Ras-Raf-1-dependent cascade (82–85), whereas JNK and p38 MAP kinase are activated by various cellular stressors including UV irradiation, heat shock, and proinflammatory cytokines (86–90). Our finding that NO selectively activates p38 MAP kinase without affecting JNK indicates that p38 MAP kinase and JNK contribute to distinct signaling pathway of apoptosis. The latter interpretation is consistent with a recent report that a kinase inhibitor can prevent activation of caspases and apoptotic machinery. NO-induced cytochrome c release and caspase activation (Fig. 9). However, the additive effects of p38 MAP kinase upstream of mitochondrial changes and caspases are unclear. It was recently reported that a p38 MAP kinase inhibitor can prevent activation of caspases and apoptosis in neurons (98, 99) and can block Bax translocation to mitochondria (47). Our data are consistent with a site of action of p38 MAP kinase upstream of mitochondrial changes and caspase activation (Fig. 9). However, the additive effects of caspase and p38 MAP kinase inhibitors in protecting NPC against NO-induced death suggests that at least some activation of caspases may occur independently of p38 MAP kinase in nitric oxide-induced NPC death.

We found that the p38 MAP kinase inhibitor attenuated NO-induced cytochrome c release and caspase activation, demonstrating an action of this kinase upstream of mitochondrial alterations. Previous studies have provided evidence that p38 MAP kinase is activated by oxidative stress, including the oxidative stress induced by nitric oxide, and this mechanism is also likely to occur in NPC. The ability of Bcl-2 to attenuate nitric oxide-induced activation of p38 MAP kinase in NPC is consistent with a role for oxidative stress in that Bcl-2 has been shown to function in antioxidant pathways (100) and can suppress oxidative damage to cell membranes (60). On the other hand, it has been proposed that Bcl-2 can prevent apoptosis by heterodimerizing with Bax and modulating pore formation in mitochondrial membranes (102–104). A similar mechanism of protection of NPC against nitric oxide-induced death is likely because NO-induced cytochrome c release was suppressed in NPC overexpressing Bcl-2. However, we also found that Bcl-2 overexpression attenuated the activation of p38 MAP kinase suggesting that, in addition to being activated upstream of mitochondrial changes, p38 MAP kinase may also be activated in response to mitochondrial changes. Bcl-2 is expressed at high levels during neurogenesis in the developing brain (105), rodent subventricular zone cells positive for NPC markers also express Bcl-2 in vivo (106), and it has been reported that Bcl-2 is present in NPC from the subventricular zone of the adult human brain (101). It is therefore conceivable that signals that regulate NO production and Bcl-2 expression might interact in the regulation of survival of NPC in the developing and adult nervous system. Our data suggest a role for nitric oxide in regulating survival of the NPC, although further work will be required to establish the specific roles of nitric oxide in determining NPC fate in vivo.
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