Nitric oxide (NO) is implicated in apoptosis and has both cytotoxic and cytoprotective effects. Exogenous NO induced the death of PC12 and HeLa cells via a process showing features of both apoptosis and necrosis, with chromatin condensation, nuclear compaction, and mitochondrial swelling. Activation of caspases was not observed during NO-induced cell death. In addition, cell death was not inhibited by peptide caspase inhibitors or by expression of p35, a baculovirus-encoded caspase inhibitor, indicating that NO-induced cell death was independent of caspases. NO-induced cell death was enhanced by Bax expression in a caspase-independent manner and prevented by the anti-cell death protein Bcl-2. Although Bcl-2 has previously been shown to prevent cell death by inhibiting caspase activation, these results indicate that it can also prevent cell death via a caspase-independent mechanism.

Nitric oxide (NO) is enzymatically generated from L-arginine by constitutive or inducible NO synthase and has a number of physiological roles, including smooth muscle relaxation, and neurotransmission (1, 2). NO has also been implicated in a variety of pathological phenomena, such as septic shock, β-cell destruction, and transplant rejection (1, 2). Some of these pathological events are closely related to apoptotic cell death (3, 4). In many studies, NO has been shown to induce apoptosis, although the precise mechanism involved is still unclear (5–7). In contrast, some investigators have suggested that NO also has the ability to prevent cell death (8–10), which seems to be mediated by the inhibition of caspases (8), common mediators of apoptosis (11). So far, more than 10 caspases have been identified in mammals. Apoptosis is also regulated by Bcl-2 family proteins, including anti-apoptotic proteins such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins such as Bax and Bak (12). Accumulating evidence suggests that Bcl-2 acts upstream of caspase activation to prevent apoptosis (13, 14).

In this study, we analyzed NO-induced cell death, particularly focusing on the role of caspases as well as the influence of apoptosis-regulating molecules such as Bcl-2 family proteins.

EXPERIMENTAL PROCEDURES

Reagents—Caspase inhibitors and substrates were purchased from Peptide Inc. (Minoh, Japan). Other chemicals were purchased from Wako Chemical Co. (Tokyo, Japan).

Cell Lines and Transfection—HeLa cells, a human cervical carcinoma-derived cell line, and PC12 cells, a rat pheochromocytoma cell line, were maintained in RPMI 1640 culture medium, as described elsewhere (15). A stable transfectant of PC12 cells expressing mouse Bax (designated as PC12-Bax) was obtained by infecting retrovirus that was produced from the packaging cell line 22 transfected with the retroviral vector pBLC140 (15) containing mouse bax cDNA. A stable transfectant of PC12 cells expressing human Bcl-2 (designated as PC12-Bcl-2) was obtained by transfecting the pUC-CAGGS vector bearing the human bcl-2 cDNA using electroporation (13). Empty vector-transduced cells were used as the controls (designated as PC12-pBVC and PC12-CAGGS-V, respectively).

Cell Morphology and Cell Death Assay—PC12 and HeLa cell derivatives were maintained in RPMI 1640 medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES/NaOH (pH 7.4), 0.1 mM nonessential amino acids, 0.05 mM 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum. Cells were seeded at 2 × 10⁶ cells in a plastic 6-cm dish 1 day before the experiment, and cell death was induced by addition of nitric oxide donor (SNAP) or hydrogen peroxide (H₂O₂) into media at the indicated concentrations. In experiments using caspase inhibitors, these drugs were added 2 h before the addition of SNAP or H₂O₂ into medium. For electron microscopy, PC12 cells were fixed with 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer containing 7.5% sucrose, cells were postfixed for 1 h with 1% OsO₄, and then examined under a fluorescence microscope (Olympus BHS-RFL-LSM) with excitation at 360 nm, as described previously (15). Cell death was quantitated by counting 1,000 cells. For staining of apoptotic cells by DNA end labeling (TUNEL), cells were fixed in 4% neutral buffered formalin, treated with proteinase K (20 mg/ml) for 15 min at 25 °C, and then stained using ApoptagTM (Oncor). Cell death was also quantitated by counting 1,000 cells.

Western Blotting—HeLa cells were treated with 500 μM SNAP or 1 mM H₂O₂ and suspended in phosphate buffer at the indicated times. After centrifugation, cell pellets were lysed with a buffer containing 50 mM Tris-Cl (pH 8.0), 20 mM EDTA, and 100 mM NaCl. After boiling, 40 μg of total protein was analyzed by Western blotting using an anti-human caspase-3 monoclonal antibody (Transduction Laboratories), which reacted with procaspase-3 (p20) and a large subunit of active caspase-3 with prodomain (p20).

Caspase Activity—Caspase activity was measured as described elsewhere (13). Briefly, cells were suspended in 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 10 mM EGTA, and incubated at 37 °C for 10 min in the presence of 10 mM digitonin. The cleared lysates containing 40 μg protein or recombinant caspase-3 and caspase-6 (10 ng protein each) were incubated at 37 °C for 1 h with 75 nmol of the enzyme substrates YVAD, DEVD, and LEHD, and then assayed using a Hitachi F-3000 spectrophotometer with excitation at 380 nm and emission at 460 nm. One unit was defined as the amount of enzyme required to release 1 pmol of AMC per min at 37 °C.
**Recombinant Caspases—** His₆-tagged caspase-3 and -6 proteins were expressed in the *Escherichia coli* strain XL/C and purified by affinity chromatography on a nickel column (Invitrogen). Fractions showing proteolytic activity (assessed as described above) were pooled and dialedyzed with buffer containing 50 mM Hepes, 0.1 M NaCl, and 10% glycerol (pH 7.5) to remove imidazole. The caspases thus obtained were more than 80% pure, as judged by Coomassie Brilliant Blue staining.

**Plasmid Construction and Transient Expression—** Mouse *bax* (5.7-kb), human *bcl-2* (6.0-kb), and *p35* (5.8-kb) expression plasmids were constructed by inserting the respective cDNA fragment containing the entire coding region into pUC-CAGGS. Using LipofectAMINE, HeLa cells were transfected for 24 h with each expression plasmid (total 1.0 μg) together with 0.3 μg of the 8.5-kb lacZ expression construct. Transfected cells were incubated with or without 500 μM SNAP or 1 mM H₂O₂ for 12 h. Then the cells were fixed in 1% glutaraldehyde for 5 min, rinsed with phosphate-buffered saline, and stained with X-gal buffer for 6 h at 37 °C. The extent of cell death was calculated as the percentage of shrunken blue cells relative to all blue cells (16).

**RESULTS**

**Nitric Oxide Induces Cell Death—** We first examined the morphological characteristics of NO-induced cell death using fluorescence microscopy after staining with Hoechst 33342 (Ho342) and propidium iodide (PI). As described previously (15), viable cells showed round blue nuclei stained with Ho342, necrotic cells showed round pink nuclei stained with both Ho342 and PI, and apoptotic cells showed blue or pink shrunk or fragmented nuclei. Without any treatment, almost all PC12 cells showed round blue nuclei (Fig. 1a), indicating that these cells were viable. After treatment for 24 h with an NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP; 500 μM), PC12 cells with blue or pink shrunk nuclei emerged (Fig. 1b). NO-treated but not intact PC12 cells were positively stained with TUNEL assay (Fig. 1, c and d), indicating NO-treated PC12 cells showed apoptotic manifestations. Electron micrographs of NO-treated PC12 cells also showed evidence of apoptosis, such as chromat condensation and compaction of nuclei, together with features of necrosis such as mitochondrial disruption (Fig. 1e). Similar morphological findings were also observed in NO-treated HeLa cells (data not shown). As shown in Fig. 1, f and g, the proportion of dead PC12 cells increased over time in a concentration-dependent manner as assessed by Hoechst-PI staining and TUNEL assay. Similar results were obtained when HeLa cells were treated with SNAP (Fig. 1, h and i), or were treated with another NO donor, 3-morpholinosydnonimine (SIN-1) (data not shown). These findings indicated that NO induced the unique form of death of PC12 and HeLa cells that was accompanied by apoptotic and necrotic manifestations. This is in contrast to the previous reports that NO induces apoptosis (17, 18), and as described below, the difference might be because of the cell line used.

**Caspase Was Not Involved in NO-induced Cell Death—** Caspases play a crucial role in driving apoptosis (11). Treatment of PC12 cells with apoptotic stimuli such as H₂O₂ (Fig. 2a), VP16, and calcium ionophore (data not shown) resulted in the activation of caspase-3-like protease, a major executioner caspase, and addition of 200 μM of a peptide caspase-3 inhibitor (DEVD-CHO) or a broad caspase inhibitor (z-D-CH₂-DCB) blocked the activation of caspase-3 and prevented cell death.
although this significantly prevented H$_2$O$_2$-induced apoptosis. 

Bax Enhances NO-induced Cell Death—

To further confirm the absence of caspase activation in NO-induced cell death, HeLa cells were transiently transfected with a p35 (a baculovirus broad caspase inhibitory gene) (19, 20) expression construct or vector plasmid (as a control) together with the lacZ expression plasmid, and were treated with 500 μM SNAP. Light microscopy of cells stained with X-gal revealed viable cells as flat and blue, whereas apoptotic cells were round and blue. As shown in Fig. 4, a and b, NO-induced cell death was not prevented by transient expression of p35, although this significantly prevented H$_2$O$_2$-induced apoptosis. These results also suggested that NO-induced cell death was independent of caspase activation.

Bax Enhances NO-induced Cell Death—To further characterize NO-induced cell death, we next examined the influence of two Bcl-2 family proteins, Bax and Bcl-2. Several observations have suggested that Bax induces either caspase-dependent or -independent apoptosis (21, 22). Bax-overexpressing PC12 cells (PC12-Bax) showed significantly increased susceptibility to NO-induced cell death (Fig. 5a). No activity of caspases-1, -3, and -6 was observed during NO-induced death (Fig. 5b and data not shown). Furthermore, addition of 200 μM z-D-CH$_2$-DCB could not prevent NO-induced cell death of PC12-Bax cells (Fig. 5a), indicating that Bax accelerated NO-induced cell death through a mechanism other than caspase activation. To further confirm the absence of caspase activation in the enhancement of NO-induced cell death by Bax, transient transfection with the bax and p35 genes was performed using HeLa cells. In the absence of SNAP, transient expression of bax resulted in apoptosis that was significantly inhibited by coexpression of p35 (Fig. 5d), indicating that p35 prevented bax-induced apoptosis in the absence of SNAP. In contrast, transient expression of bax enhanced NO-induced cell death, and death was not inhibited by coexpression of p35 (Fig. 5c and d), indicating that the enhancement of NO-induced cell death by Bax occurred via a pathway independent of caspase activation.
Bcl-2 Prevents NO-induced Cell Death—Because Bcl-2 has been shown to prevent cell death by blocking activation of the caspase cascade (13, 14), and we found that Bax, a Bcl-2 antagonist, enhanced NO-induced cell death, it was of great interest to determine whether NO-induced cell death was inhibited by Bcl-2. As shown in Fig. 6a, overexpression of Bcl-2 efficiently prevented NO-induced death of PC12 cells, which did not involve caspase activation as described above. Consistent with this finding, transient expression of bcl-2 also prevented NO-induced cell death (Figs. 6, b and c), whereas p35 expression could not prevent it (Fig. 5d). These results indicated that Bcl-2 prevented NO-induced cell death independent of caspase activation.

Fig. 4. NO-induced cell death independent of caspase activation. Panels a and b, transient expression of p35 prevents cell death induced by H₂O₂ but not by NO. HeLa cells were transiently transected with a p35-expression construct (0.5 μg) (p35 in a, and closed columns in b) or vector construct (0.5 μg) (vector in a, and open columns in b) together with 0.3 μg of the lacZ expression construct for 24 h. Transfected cells were treated with (+NO in a and b) or without (−NO in a and b) 500 μM SNAP or with 1 mM H₂O₂ (+H₂O₂ in b) for 12 h after DNA transfection and then stained for 6 h with X-gal as described under “Experimental Procedures” (a). Cell death was assessed as blue shrunken cells relative to all blue cells (b).

Fig. 5. Enhancement of NO-induced cell death by Bax overexpression. Panels a and b, viability and caspase-3-like activity of PC12-pBC-V and PC12-Bax cells treated with SNAP. PC12-pBC-V (open circles) and PC12-Bax (squares) cells were treated with 500 μM SNAP with (closed symbols) or without (open symbols) the caspase inhibitor z-D-CH₂-DCB (200 μM), and cell viability (a) and caspase-3-like activity (b) were measured at the indicated times. Panels c and d, enhancement of NO-induced cell death by transfection of mouse bax. HeLa cells were transiently transfected with a bax-expression (0.5 μg) or vector construct with or without p35-expression construct (0.5 μg) together with 0.3 μg of the lacZ expression construct. Cells were treated with or without 500 μM SNAP for 12 h after DNA transfection and then stained for 6 h with X-gal as described under “Experimental Procedures” (c). The fraction of β-galactosidase-positive dead cells after plasmid transfection with (+NO) or without (−NO) 500 μM SNAP was assessed (d). The amount of transfected plasmid was as follows: vector, vector 0.5 μg plus vector 0.5 μg (open column), vector 0.5 μg plus p35 0.5 μg (closed column); bax, bax 0.5 μg plus vector 0.5 μg (open column) and bax 0.5 μg plus p35 0.5 μg (closed column).
of the caspase cascade, therefore suggesting that Bcl-2 has a unique activity that prevents cell death through a mechanism distinct from blocking caspase activation.

**DISCUSSION**

In the present study, we showed that NO induced a unique form of death in PC12 and HeLa cells, that is accompanied by both apoptotic and necrotic manifestations and is independent of caspsases. Although we cannot exclude the possibility that other caspsases are involved, this is very unlikely because the cell death was not prevented by p35 that is known to inhibit caspase-1, -3, -6, -7, -8, and -10,2 and by z-D-CH$_3$-DCB that inhibits caspases broadly. Our observations are in contrast to the previous reports that NO can induce caspase-dependent apoptosis (17, 18) or inhibit apoptosis (8–10). The discrepancy might be because of the difference in susceptibility of cell lines used to apoptosis as well as caspase-independent cell death. It might be that for cell lines highly susceptible to apoptosis, a small amount of NO that is insufficient to inhibit caspases to trigger apoptosis. In contrast, for cell lines less susceptible to cell death, higher concentrations of NO, which are sufficient to inhibit caspases, might be required to induce a caspase-independent death or might inhibit apoptosis in general through inhibition of caspases.

Although apoptotic hallmarks such as chromatin condensation and nuclear compaction were observed in NO-induced cell death, other apoptotic features like nuclear fragmentation and oligonucleosomal cleavage of genomic DNA were not observed (Fig. 1e and data not shown). The absence of nuclear fragmentation and DNA ladder formation was probably a consequence of direct caspase inhibition, because nuclear fragmentation and DNA ladder are caspase-dependent (23, 24). Interestingly, NO-induced cell death described here morphologically resembled Bax-induced apoptosis in the presence of a caspase inhibitor (21). Given the findings that overexpression of Bax enhanced NO-induced apoptosis without caspase activation (Fig. 5), and previous observations that Bax-induced apoptosis occurs via both caspase-dependent and -independent pathways (21, 22), NO-induced apoptosis might be mediated by Bax through a mechanism other than the caspase activation pathway.

The anti-apoptotic effect of Bcl-2 has already been extensively analyzed. Several mechanisms have been proposed to explain how Bcl-2 prevents apoptosis, including (1) sequestering the proforms of caspases (25–27); (2) inhibiting the release of cytochrome c (28, 29), which leads to caspase activation (30); or (3) preventing mitochondrial dysfunction (31, 32), which induces apoptosis-inducing factor release and thus results in caspase activation (33). All these mechanisms suggest that anti-apoptotic Bcl-2 acts upstream of caspases to inhibit their activation. In the present study, we showed that Bcl-2 prevented cell death occurring independently of the caspase cascade, indicating that Bcl-2 may have the ability to block cell death occurring by a mechanism other than those described above.

Taken together, our results suggested that NO-induced cell death is mediated via a pathway independent of caspase activation, and that NO-induced apoptosis was enhanced or prevented by Bax or Bcl-2, respectively.

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