Nitric Oxide Suppression of Apoptosis Occurs in Association with an Inhibition of Bcl-2 Cleavage and Cytochrome c Release

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It is now known that caspase-3-like protease activation can promote Bcl-2 cleavage and mitochondrial cytochrome c release and that these events can lead to further downstream caspase activation. NO has been proposed as a potent, endogenous inhibitor of caspase-3-like protease activity. Experiments were carried out to determine whether NO could interrupt Bcl-2 cleavage or cytochrome c release by the inhibition of caspase activity linking these events. NO inhibited the capacity of purified caspase-3 to cleave recombinant Bcl-2. Both Bcl-2 cleavage and cytochrome c release were inhibited in tumor necrosis factor α- and actinomycin d-treated MCF-7 cells exposed to NO donors. The NO-mediated inhibition of Bcl-2 cleavage and cytochrome c release occurred in association with an inhibition of apoptosis and caspase-3-like activation. Thus, NO suppresses a key step in the positive feedback amplification of apoptotic signaling by preventing Bcl-2 cleavage and cytochrome c release.

Recent advances in the study of the signaling pathways involved in apoptotic cell death have begun to elucidate the relationship between caspases, Bcl-2 family members, and the mitochondrion. Key observations include the following: (i) Although Bax induces pore transition by forming anion channels (1) resulting in cytochrome c release (2), it has been hypothesized that Bcl-2 and Bcl-XL inhibit pore transition by forming cation channels (1, 3–5), preventing the mitochondrial release of cytochrome c (6, 7) and apoptosis-inducing factor (8); (ii) Bcl-2 (9) and Bcl-XL (10) are cleaved by caspase-3-like proteases, and cleavage generates Bax-like fragments (9, 10) capable of inducing apoptotic cell death; and (iii) Proteolytic activation of caspase-3-like enzymes is promoted by cytochrome c release from mitochondria (11). Cytochrome c forms a complex with Apaf-1 and pro-caspase-9, leading the activation of caspase-9 that then can cleave the zymogen form of caspase-3 (11). It is unlikely that these pathways can be generalized to all cell types or all inducers of apoptosis. Furthermore, cytochrome c release has been shown to occur independent of pore transition (12), making the relationship between the cleavage of Bcl-2 family members and cytochrome c release unclear in some cells. Nonetheless, these interconnected events do provide both a mechanism for the initial activation of the downstream caspases as well as an amplification scheme to assure the full activation of the execution machinery in a cell committed to apoptosis. Adequate activation of downstream caspases, such as caspase-3, is thought to assure the cleavage of target proteins required to complete the terminal events in apoptosis. For example, cleavage of DNA fragmentation factor (DFF), in humans (13) or the murine analogue inhibitor of caspase-activated DNase (14) by caspase-3-like caspases leads to the release of caspase-activated DNase into the nucleus, resulting in DNA degradation (15).

We (16) and others (17) have proposed that NO prevents apoptosis in some cell types by suppressing increases in caspase-3-like activity. A reaction product of NO can directly inhibit caspase activity by S-nitrosylation of the active site cysteine present in all caspase enzymes (18). In hepatocytes, NO also prevents increases in caspase activity by a cGMP-dependent mechanism that does not involve S-nitrosylation (16–19). It is interesting to note that NO-mediated inhibition of apoptosis in splenocytes is associated with increases in Bcl-2 levels (20). Combined, these observations raise the possibility that NO preserves Bcl-2 levels by preventing increases in caspase activity. We hypothesize that a consequence of suppressed caspase activity would be not only an inhibition of Bcl-2 cleavage but also an inhibition of mitochondrial cytochrome c release. The net effect would be a failure to cleave death substrates, such as DFF, and an inhibition of apoptosis. We tested this hypothesis in MCF-7 adenocarcinoma cells stimulated by TNFα to undergo apoptosis.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), Williams media E, penicillin, streptomycin, l-glutamine, and Hepes were purchased from Life Technologies Inc. Cytochrome c monoclonal antibody was obtained from PharMingen (San Diego, CA), and Bcl-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant TNFα was obtained from R & D Systems (Minneapolis, MN). N-Acetyl-Tyr-Val-Ala-Asp-p-nitro-anilide (Ac-DEVD-pNA) was obtained from Alexis Corporation (San Diego, CA). SNAP was synthesized as described previously (21). Oxidized SNAP (OxSNAP) was prepared by incubating SNAP at room temperature for 48 h to completely liberate NO. Recombinant human caspases-1, -2, -3, -4, -5, -6, -7, and -8 were prepared as described previously (22). Lysate from TNFα- and actinomycin D (ActD)-treated hepatocytes was prepared as described previously (16). All other chemicals were purchased from Sigma unless specified otherwise.

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The abbreviations used are: DFF, DNA fragmentation factor; TNF, tumor necrosis factor; DMEM, Dulbecco’s modified Eagle’s medium; NAP, N-succinyl-N-acetylpenicillamine; OxSNAP, oxidized SNAP; ActD, actinomycin D; Ac-DEVD-CHO, N-acetyl-Tyr-Val-Ala-Asp-p-nitro-anilide; rh, recombinant human; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; mt, mutant.
indicated otherwise.

Cell Culture and Transfection with Bcl-2—Subconfluent cultures of MCF-7 cells were maintained in DMEM containing 5% low endotoxin calf serum supplemented with 10 mM Hepes (pH 7.4), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 12-well plates until 70% confluence. Cells were transfected with pCMV, pCMV-Bcl-2, and pCMV-Bcl-2(D34A) by the lipofectamine method (23). Three stable transfectants were selected by culturing with G418 (800 μg/ml) and procarboxamycin (50 μg/ml) in the presence or absence of Ac-DEVD-CHO. MCF-7 cells were cultured in 6-well plates until 70% confluence. Cells were transfected with pCMV, pCMV-Bcl-2, and pCMV-Bcl-2(D34A) by the lipofectamine method (23). Three stable transfectants were selected by culturing with G418 (800 μg/ml) followed by serial dilution of the cells in 96-well plates. Wild type or mutant Bcl-2 protein levels were comparable by Western blotting in each of the three clones, and a similar response was elicited to TNF-α and Ac-ActinD in each. One of the three clones was then used for all of the experiments.

Measurement of Caspase-3-Like Activity—The cell pellets were washed with ice-cold phosphate-buffered saline and resuspended in 100 mM Hepes buffer (pH 7.4) containing protease inhibitors (5 μg/ml aprotinin and pepstatin, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was lysed by three freeze-thaw cycles and a cytosolic solution (S-100) was obtained by centrifugation at 100,000 × g for 1 h at 4 °C. 80 μg of cytosolic protein was combined with 400 μM of the synthetic substrate Ac-DEVD-pNA in 150 μl of 100 mM Hepes (pH 7.4) containing 20% glycerol and protease inhibitors, and the reaction was carried out at 37 °C for 1 h. Caspase-3-like activity was assayed by measuring the increased absorbance at 405 nm (16).

In Vitro Cleavage of Bcl-2 and DFF—[35S]Methionine-labeled Bcl-2 and DFF were synthesized from pBS-Bcl-2 and pETDFF (cDNA provided by Dr. X. Wang) using a TNT-coupled transcription and translation system (Promega) (16). rh-Caspase-3 (1 μg/100 μl) was pretreated with or without SNAP on ice for 1 h. The enzyme was separated from the excess NO donor through a Sephadex G-25 column. For the Bcl-2 and DFF cleavage assays, 4-μl aliquots of [35S]-labeled proteins were incubated with rh-caspase-3 (18 ng, activity = 0.1 absorbance/h) or other rh-caspases with the same activity as rh-caspase-3 (asayed as described) (18). Cytosol was added using 4 or 2 μg of total protein for Bcl-2 or DFF cleavage assays, respectively. The reaction volume was 10 μl and was carried out for 1 h at 37 °C in the presence or absence of 20 mM DTT. The cleavage reaction was stopped by mixing with an equal volume of 2× SDS-sample buffer and heating the mixture for 2 min. Cleavage profiles of Bcl-2 and DFF were examined by electrophoresis on 15% SDS-PAGE and fluorography.

Western Blot Analysis—Cells were harvested and washed with ice-cold phosphate-buffered saline. Cell pellets were suspended in ice-cold 10× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 5% 2-mercaptoethanol). The cell suspension was lysed by three freeze-thaw cycles and a cytosolic solution (S-100) was obtained by centrifugation at 100,000 × g for 1 h and used for cytosolic cytochrome c release. Some cells were lysed by three freeze/thaw cycles; cell lysate was obtained by centrifugation at 13,000 × g for 20 min and used for Bcl-2 Western blot analysis. Proteins (20 μg) were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was hybridized with cytochrome c antibody or Bcl-2 antibody, and protein bands were visualized by exposing to x-ray film, as described previously (16).

Cytosolic DNA Extraction and Electrophoresis—Cytosolic DNA was prepared by method of Leist et al. (24). Briefly, cell pellets were resuspended in 750 μl of lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0) and occasionally shaken while on ice for 45 min. The cytosolic fraction was collected by centrifugation at 13,000 × g for 20 min at 4 °C, and protein concentrations were determined. Cytosol aliquots containing equal amounts of protein were extracted with a mixture of phenol and chloroform. One-tenth of the volume was precipitated by adding an equal volume of isopropanol. After storing at −20 °C overnight, a DNA pellet was obtained by centrifugation at 13,000 × g for 15 min at 4 °C and was dissolved in 100 μl of 10 mM Tris-HCl (pH 8.0). After digesting DNA with RNase (0.1 mg/ml) at 37 °C for 1 h, samples (15 μl) were electrophoresed through a 1.2% agarose gel in 450 mM Tris borate-EDTA (pH 8.0) buffer. DNA was photographed under visualization with UV light.

Other Analysis—Cell viability was determined by crystal violet staining, as described previously (23). Protein concentration was determined with the BCA assay (Pierce). Nitrite accumulation in culture medium was measured by the Griess reaction (21).

**RESULTS**

Recent evidence has placed the proteolytic cleavage of Bcl-2 by activated caspases as a key event in the amplification of pro-apoptotic signaling (9). Cleavage of Bcl-2 (9) or Bcl-XL (10) may result in acceleration of mitochondrial membrane pore transition and release of cytochrome c, thus further activating downstream caspases leading to the degradation of key death substrates such as DFF (13, 14). We first tested the capacity of caspase-1 through -8 to cleave radiolabeled rh-Bcl-2. Caspases-1, -3, -5, and -8 cleaved Bcl-2, yielding a 24-kDa product (Fig. 1A), consistent with the described cleavage at the loop domain (9, 25). Caspase activity can be reversibly inhibited by S-nitrosylation (16, 18). Therefore, we tested the capacity of NO to inhibit Bcl-2 cleavage by rh-caspase-3. The addition of the NO donor SNAP markedly suppressed Bcl-2 cleavage as did the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 1B). The effects of SNAP were reversed if the reaction mixture...
Inhibition of Apoptosis by Nitric Oxide

Fig. 2. Effect of SNAP on TNF-α- and ActD-induced apoptosis of MCF-7 cells. A, effect of SNAP on TNF-α- and ActD-induced apoptosis. After preculture with 80 μM FeSO₄ for 24 h followed by another 6-h culture with fresh medium, MCF-7 cells were treated with TNFα (32 ng/ml) and ActD (0.2 μM/L) in the presence or absence of Ac-DEVD-CHO (240 μM), SNAP (140 μM), or oxidized SNAP (OxSNAP) for 18 h. Cell viability was determined by crystal violet staining. B, effect of SNAP on TNF-α- and ActD-induced DNA fragmentation. MCF-7 cells were treated the same as in A. Cytosolic DNA was isolated and subjected to 1.2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining. C, SNAP treatment also prevented the cleavage of radiolabeled rh-Bcl-2 by S-100 lysate from TNF-α (Fig. 1b). SNAP treatment also prevented the cleavage of radiolabeled rh-Bcl-2 by S-100 lysate from TNF-α- and ActD-treated hepatocytes (Fig. 1c). Again, the NO-induced inhibition was reversed by DTT. Thus, NO inhibits Bcl-2 cleavage by caspases through a mechanism consistent with S-nitrosylation of the protease.

We next compared the anti-apoptotic actions of NO in intact cells with the capacity of NO to prevent Bcl-2 cleavage and downstream pro-apoptotic signaling events. MCF-7 cells exposed to TNFα and ActD underwent a rapid loss of viability associated with DNA fragmentation that was inhibited by SNAP and the caspase-3-like protease inhibitor (Fig. 2, A and B). Of note, the protection by SNAP only took place if the cells were pre-incubated in Fe₂SO₄ (data not shown). SNAP pre-incubated in solution to exhaust all the NO (OxSNAP) did not protect the cells. Induction of apoptosis was associated with Bcl-2 cleavage and cytochrome c release into the cytosol as determined by immunoblotting (Fig. 2C). Ac-DEVD-CHO and SNAP, but not OxSNAP, blocked both the cleavage of endogenous Bcl-2 and release of cytochrome c. Caspase activation can promote the conversion of Bcl-2 to Bax-like death domain (9), which in turn may lead to cytochrome c release. Cytochrome c release, however, leads to caspase-3-like protease activation following complex formation with Apaf-1 and caspase-9 in the presence of dATP (11). Fig. 2D shows that caspase-3-like activity was elevated in cells undergoing apoptosis and that a reduction in activity correlated with reduced Bcl-2 cleavage and cytochrome c release. A consequence of caspase-3-like activation is the cleavage of DFF (13). Consistent with the caspase-3-like protease activation, lysate from TNF-α- and ActD-treated MCF-7 cells efficiently cleaved radiolabeled rh-DFF (Fig. 2E). SNAP, but not OxSNAP, as well as Ac-DEVD-CHO suppressed the DFF cleavage by the activated lysate. An identical inhibition of both Bcl-2 cleavage and cytochrome c release was observed in hepatocytes stimulated with cytokines to express the inducible NO synthase. Spontaneous apoptosis was prevented in these cells by inducible NO synthase expression, and both the protection and inhibition of Bcl-2 degradation and cytochrome c release were reversed by the NO synthase inhibitor N⁵-monomethyl-L-arginine (data not shown). Thus, the inhibition of apoptosis by NO is associated with inhibition of Bcl-2 cleavage that is in turn associated with suppressed caspase-3-like activity and reduced cytochrome c release.

If Bcl-2 cleavage is required for cytochrome c release, caspase activation, and cell death, the mutant Bcl-2 (mt-Bcl-2) not susceptible to cleavage by caspases (Fig. 1B) should prevent these events. As shown in Fig. 3 (A and B), MCF-7 cells transfected with mt-Bcl-2 were protected from TNF-α- and ActD-induced apoptosis to a much greater degree than cells transfected with a vector carrying wild type Bcl-2. Overexpression of mt-Bcl-2 also efficiently blocked the appearance of cleaved Bcl-2, which is endogenous Bcl-2, and more efficiently prevented the release of cytochrome c than overexpression of wild type Bcl-2 (Fig. 3C). The suppression of Bcl-2 cleavage and cytochrome c release was associated with an inhibition of caspase-3-like protease activation measured as activity (Fig. 3D) and the capacity of cell lysates to cleave DFF (Fig. 3E). Treating cells overexpressing either wild type or mt-Bcl-2 with SNAP or Ac-DEVD-CHO suppressed the residual cell death, Bcl-2 cleavage, cytochrome c release, and caspase-3-like protease activation (Fig. 3, A–E), indicating that some level of caspase activation had still taken place.
Cytosolic proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Bcl-2 and mitochondrial cytochrome c were visualized by Western blot analysis. 

Wild type Bcl-2 or mt-Bcl-2 (D34A) were incubated in 80 μl FeSO4 for 24 h followed by culture with fresh medium for another 6 h. A, cell viability was determined by crystal violet staining. B, DNA fragmentation. Cytosolic DNA was isolated and subjected to 1.2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining.

Panels A and D represent the means ± S.D. from one of three similar experiments performed in triplicate. Panels B, C, and E are representative of two similar experiments.

DISCUSSION

This report further establishes the capacity of NO to prevent certain key events in apoptotic signaling in association with its capacity to suppress increases in caspase-3-like activity. Recent reports indicate that cleavage of Bcl-2 family members by activated caspases is an important step in the apoptosis signaling cascade (9, 10). Here, we confirm that caspases-1, -3, -5, -7, and -8 can cleave Bcl-2, and we provide evidence that NO blocks Bcl-2 cleavage through its capacity to limit caspase activity. By interrupting this step, NO appears to suppress a mechanism for the initiation or amplification of the activation of downstream caspases, involving the release of cytochrome c.

Two pathways for the activation of terminal caspases such as caspase-3 are thought to exist. One pathway involves the direct activation of caspase-3 and other executioner proteases by upstream caspases (e.g. caspase-8), which become activated following association with the cytoplasmic death signaling complex of prodeath receptors (e.g. Fas and TNFα receptors) (26–28). In a second pathway, caspase-3-like protease activation follows a series of events involving the mitochondrion. Cytochrome c is released in a process that may involve loss of mitochondrial membrane potential in association with pore transition (2, 29). In the cytosol, cytochrome c forms a complex with Apaf-1 in the presence of dATP. Interaction of this complex with pro-caspase-9 results in the proteolytic activation of caspase-9, which then cleaves and activates downstream caspases such as caspase-3 (11). Bcl-2 and Bcl-XL prevent mitochondrial cytochrome c release through a mechanism that could involve the capacity of these proteins to form or interact with transmembrane pores (6, 7). Cleavage of Bcl-2 (9) and Bcl-XL (10) results in the formation of Bax-like proteins that are thought to contribute to pore formation and perhaps cytochrome c release. Using the MCF-7 cell line, we show that suppression of caspase activity prevents Bcl-2 cleavage and cytochrome c release following treatment with TNFα and ActD. This indicates that increases in caspase activity are required for both events. Overexpression of a mutant Bcl-2 (mt-Bcl-2) not susceptible to cleavage by caspases also prevented Bcl-2 cleavage and increases in cytosolic caspase-3-like activity. These observations put Bcl-2 cleavage proximal to the release of cytochrome c and the activation of downstream caspase-3-like proteases. Some apoptosis and increases in caspase-3-like activity were seen even with mt-Bcl-2 overexpression, suggesting that other mechanisms for caspase-3-like protease activation exist. However, the presence of low levels of the Bcl-2 cleavage product in mt-Bcl-2-expressing cells and the capacity of NO and Ac-DEVD-CHO to block the remaining cell death suggest that the levels of mt-Bcl-2 were not high enough to completely block this step.

MCF-7 cells do not express a functional caspase-3 protein and have been shown not to exhibit internucleosomal DNA laddering typical of apoptosis (30). Consistent with this observation, we found only very low levels of caspase-3 protein in our cells by Western blot analysis (data not shown); however, we did observe DNA laddering. Variations in the MCF-7 cells used in these two studies may explain these differences. Less likely explanations include the differences in stimuli (TNFα and ActD in this study and TNFα and cycloheximide in Ref. 30) or the influences of the iron loading in our study. Because caspase-3-like activity increases significantly and DNA fragmentation occurs, our results suggest that activation of caspases other than caspase-3 can lead to DNA fragmentation. In addition to recombinant caspase-3, caspase-7 and -8 both cleaved recombinant Bcl-2, demonstrating the capacity of other caspase-3-like proteins to cleave this substrate.

NO has emerged as a potent inhibitor of apoptosis in many cell types (16, 17, 20, 31–33). A dominant mechanism for the inhibition of apoptosis in several cell types is the inhibition of

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Inhibition of Apoptosis by Nitric Oxide

31441
caspase activity by S-nitrosylation (16–18), and in hepatocytes, a cGMP-dependent decrease in caspase activation (16, 19). We did not observe a protective effect of cGMP in MCF-7 cells (data not shown), suggesting that S-nitrosylation is the primary mechanism for the inhibition of caspases in these cells. S-Nitroso compounds such as SNAP release both NO and NO⁻. Only reaction products with the reactivity of NO can nitrosylate thiol (34). Thus, NO most likely reacts with oxygen to form N₂O₃ or iron to form an iron nitrosyl complex prior to the S-nitrosylation step (35, 36). It is interesting to note that we had to pretreat the MCF-7 cells with Fe₂SO₄ to observe the protective effect (data not shown). Thus, the availability of iron may be a key determinant in the fate of NO and its capacity to inhibit apoptosis by S-nitrosylation. It has also been shown that the iron-nitrosyl complex (S-Fe-NO) can modify the thiol of glutathione reductase (36).

NO inhibited the cleavage of Bcl-2 by rh-caspase-3, and reversal of the inhibition by DTT is consistent with S-nitrosylation of caspase-3 by exposure to the NO donor. We have previously shown that NO reversibly inhibits caspases-1 through -8 (18); therefore, NO should inhibit both the caspases that participate in Bcl-2 cleavage and cytochrome c release as well as the proteases that become activated following the release of cytochrome c. This is supported by the capacity of NO to mimic the caspase inhibitor Ac-DEVD-CHO. These data suggest that NO may be produced under certain circumstances to suppress both upstream and downstream caspase activation. Because of the capacity of NO to rapidly diffuse intracellularly and from cell to cell, this would represent an efficient mechanism to guard against the consequences of the activation of caspases, which might result from cell injury or exposure to other activators such as TNFα in the local environment.

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