Peroxynitrite-induced apoptosis involves activation of multiple caspases in HL-60 cells

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Peroxynitrite-induced apoptosis involves activation of multiple caspases in HL-60 cells. Am J Physiol Cell Physiol 279: C341–C351, 2000.—In this study, we show that caspases 2, 3, 6, and 7 were activated during peroxynitrite-induced apoptosis in human leukemia HL-60 cells and that processing of these caspases was accompanied by cleavage of poly(ADP-ribose) polymerase and lamin B. Treatment of cells with DEVD-fluoromethyl ketone (FMK), a selective inhibitor for caspase 3-like proteases, resulted in a marked diminution of apoptosis. VAVAD-FMK, an inhibitor of caspase 2, partially inhibited the apoptotic response to peroxynitrite. However, selective inactivation of caspase 6 by VEID-FMK did not affect apoptosis rates. These data suggest that caspase 3-like proteases and caspase 2, but not caspase 6, are required for peroxynitrite-induced apoptosis in this cell type. Moreover, we demonstrate that peroxynitrite treatment stimulated activation of caspases 8 and 9, two initial caspases in the apoptotic signaling pathway, and preincubation of cells with their inhibitor, IETD-FMK, inhibited activation of caspase 3-like proteases and caspase 2 at the concentration that prevents the apoptosis. These observations, together, suggest that caspase 8 and/or caspase 9 mediates activation of caspase 3-like proteases and caspase 2 during the apoptosis induced by peroxynitrite in HL-60 cells.

Peroxynitrite (ONOO⁻) is a reactive oxidant generated from nitric oxide and superoxide anion that mediates a variety of biological processes, including inhibition of leukocyte adhesion, blockage of platelet aggregation, and induction of vasodilation in mammalian cells (13, 19, 25, 45, 46). Recently, it has been shown that ONOO⁻ can mediate apoptosis and necrosis (2) and that induction of apoptosis by ONOO⁻ is associated with the pathogenesis of diseases such as diabetes (35) and cardiac allograft rejection (38). Although the mechanism by which ONOO⁻ causes apoptosis is not well defined, generation of reactive oxygen species (18) and DNA strand breaks (29) contribute to the peroxynitrite-induced apoptosis. Caspase 3-like proteases are also involved in the apoptosis of HL-60 cells (17, 43).

Caspases are a group of cysteine-dependent aspartate-directed proteases that mediate apoptosis induced by a variety of stimuli. To date, at least 10 mammalian caspases have been identified (44). Based on genetic and biochemical studies, caspases can be tentatively divided into three categories: group I includes caspases 1, 4, and 5; group II includes 3, 6, and 7; and group III includes caspases 2, 8, 9, and 10. Caspases are constitutively present in cells as zymogens and need to be proteolytically cleaved into the catalytic active heterodimer. Although all activated caspases consist of a 17- to 20-kDa large subunit and a small subunit of around 10 kDa, the functions of various caspases differ: group I caspases are generally considered to be required for cytokine maturation; group II caspases are effector caspases that are involved in the execution phase of apoptosis; and group III caspases function as initiator caspases that mediate the early apoptotic signaling (44). It has been shown that caspase 8 is a proximal activator in initiating activation of effector caspases in Fas-induced apoptosis (1). Binding of Fas ligand to the Fas receptor results in autoprocessing and activation of caspase 8. Active caspase 8 directly engages the caspase cascade by activating caspase 3. Alternatively, the release of cytochrome c from mitochondria can initiate a caspase cascade through binding of apoptotic protease-activating factor 1 (Apaf-1), which facilitates the activation of procaspase 9 (15). Active caspase 9 then cleaves and activates caspase 3 and other caspases (20).

Activation of effector caspases induces cleavage of a number of targeted proteins in the progression of apoptosis, such as poly(ADP-ribose) polymerase (PARP), lamins, gelsolin, protein kinase Cδ, and p21-activated kinase 2 (42, 44). Although the biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology is not well known, it has been proposed that the specific cleavage of PARP may interfere with its key homoeostatic function as a DNA repair enzyme and could allow Ca²⁺/Mg²⁺-dependent endonucleases to cause internucleosomal DNA fragmentation (40). The proteolysis of lamins may also be responsible for some of the nuclear changes in cells undergoing apoptosis, because they play a major role in nuclear envelope integrity (12). Previous studies have

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shown that PARP can be cleaved by most caspases under somewhat extreme conditions in vitro, but in vivo PARP is mainly targeted by caspases 3 and 7 (30, 41). So far, caspase 6 is the only caspase known to be able to cleave lamin A (27, 39), although other, as yet untested, caspases have also been suggested to be laminases in vitro (48). Lamin B is structurally related to lamin A (26), and its degradation occurred earlier than that of lamin A in Fas-induced apoptosis of HeLa cells (21).

Although it has been reported that caspase 3-like caspases mediate ONOO\textsuperscript{−}-induced apoptosis (17, 43), whether other caspases are activated and involved in this apoptotic process is not clear. In the present study, we show that ONOO\textsuperscript{−} activates multiple caspases, including caspases 2, 3, 6, and 7, and induces cleavage of PARP and lamin B. Of these activated caspases, however, only caspase 3-like caspases and caspase 2 are involved in the apoptotic response. Furthermore, we show that ONOO\textsuperscript{−} activates caspases 8 and 9, and they are also required for activation of caspase 3-like proteases and caspase 2 and apoptosis, suggesting that caspase 8- and/or caspase 9-mediated activation of caspase 2 and caspase 3-like caspases may contribute to ONOO\textsuperscript{−}-induced apoptosis in human leukemia HL-60 cells.

MATERIALS AND METHODS

Materials. ONOO\textsuperscript{−} and anti-caspases 1, 6, 8, and 10 were purchased from Upstate Biotechnology; clone c 2–10, antipoly (ADP-ribose) antibody was from Biomol; anti-caspases 1, 6, 8, and 10 were obtained from PharMingen; monoclonal anti-actin antibody (clone AC-40) was from Sigma Chemical.

Cell culture. Human promyelocytic leukemia HL-60 cells were grown in suspension in RPMI 1640 medium supplemented with 20% fetal bovine serum in the absence of antibiotics. Cells were passaged twice a week and used between passages 20 and 40.

ONOO\textsuperscript{−} treatment. The procedure for ONOO\textsuperscript{−} treatment was performed as described by Lin et al. (17) with minor modification. Briefly, HL-60 cells were harvested, washed once with PBS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, and diluted to 1 × 10\textsuperscript{6} cells/ml. Stock ONOO\textsuperscript{−} was added to the cells suspended in PBS (pH 8.7), and they were incubated for 15 min at 37°C. Cells were incubated for 15 min in PBS (pH 8.7) without ONOO\textsuperscript{−} as control. After washing with PBS (pH 7.4), treated or untreated cells were suspended in culture medium and then incubated for the indicated times needed for each experiment. To make sure that the observed effects were actually caused by ONOO\textsuperscript{−} rather than its decomposing products, cells were also treated with degraded ONOO\textsuperscript{−} under conditions used for ONOO\textsuperscript{−} in some experiments before incubation in the culture medium. When required, caspase inhibitors were added to cell culture in PBS 1 h before ONOO\textsuperscript{−} treatment.

Nuclear staining assay. After treatment and incubation, cells were harvested, fixed with methanol, and then incubated for 15 min in diamidino phenylindole (DAPI) solution (1 mg/ml). The nuclear morphology of the cells was observed under a fluorescent microscope. Cells with condensed or fragmented nuclei were considered to be apoptotic. Five hundred cells were counted for each sample, and the numbers of apoptotic cells were expressed as the percentage of the total cell population.

DNA fragmentation assay. Detection of DNA fragmentation was performed as described previously (49). Briefly, cells were pelleted and then resuspended in lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, and 1% SDS). After incubation in proteinase K (10 μg/ml) at 56°C overnight, and then in ribonuclease A (10 μg/ml) for 2 h at 37°C, DNA was extracted with 2-propanol. Pelleted DNA was resuspended in Tris-EDTA buffer, separated by horizontal electrophoresis on a 1.5% agarose gel, stained with 0.5% ethidium bromide, and visualized under ultraviolet light.

Western blot analysis. Cells were centrifuged for 5 min at 200 g, washed once with PBS, and then suspended in lysis buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.1 mg/ml bromphenol blue, and 0.5% 2-mercaptoethanol). After sonication for 15 s, equal amounts of the total cellular protein lysates were separated on 10% polyacrylamide gels. The proteins were electrophoretically transferred to a nitrocellulose membrane. After treatment with 5% skim milk at 4°C overnight, the membranes were incubated with various antibodies for 1 h, followed by appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham). Bound antibodies were visualized using standard chemiluminescence on autoradiographic film.

Caspase activity assay. Activity levels of the caspases were measured by a colorimetric assay following the manufacturer’s instructions. Briefly, untreated cells or cells treated with ONOO\textsuperscript{−} were incubated in the complete medium for the indicated times and then harvested. Cells (2 × 10\textsuperscript{6}) were lysed in 0.1 M HEPES buffer (pH 7.4) containing 2 mM dithiothreitol, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, and 1% sucrose. Cell lysates were incubated with a colorimetric substrate, Ac-DEVD-pNA, Ac-VDVAD-pNA, Ac-VEID-pNA, Ac-LEHD-pNA, Ac-LEHD-pNA, or Ac-LEHD-pNA for 30 min at 30°C. The release of chromophore p-nitroanilide was measured with excitation at 400 nm with the use of a fluorescence spectrophotometer (Spectra Max 340 PC).

RESULTS

Induction of apoptosis by ONOO\textsuperscript{−} in HL-60 cells. To test the apoptotic response after ONOO\textsuperscript{−} treatment in HL-60 cells, two procedures were performed. Nuclear staining was done using the chromatin dye, DAPI, and the change in nuclear morphology was observed after ONOO\textsuperscript{−} treatment. As described previously, treatment of cells with ONOO\textsuperscript{−} induced apoptotic morphology characterized by fragmented or condensed nuclei (16) (data not shown). These changes were in a dose-dependent manner, with the maximum induction (36%) at 200 μM ONOO\textsuperscript{−} (Fig. 1A). In contrast, few cells (less
than 5%) showed changes in nuclear morphology in the degraded ONOO\(^{-}\)-treated or the untreated samples (Fig. 1, A and C). Time course analysis showed that apoptotic cells began appearing in significant numbers at 2 h and increased until 4 h after ONOO\(^{-}\) treatment (Fig. 1C). However, the degraded ONOO\(^{-}\) did not lead to significant change of apoptotic rate with increasing incubation time (Fig. 1C).

The induction of apoptosis after ONOO\(^{-}\) treatment was further verified by measuring the formation of DNA fragments using agarose gel electrophoresis. Consistent with the above observation, a DNA ladder pattern was detectable at 2 h after treatment of cells with 200 \(\mu M\) ONOO\(^{-}\) and increased at 3 and 4 h (Fig. 1D). Formation of a DNA ladder was also dependent on the concentrations of ONOO\(^{-}\) administered, detectable at 10 \(\mu M\) ONOO\(^{-}\), and increased at higher concentrations (Fig. 1B). In contrast, no DNA ladder formation was observed in control cells (Fig. 1, B and D, lane 1 for both) and degraded ONOO\(^{-}\)-treated cells (data not shown). These results, together, suggest that ONOO\(^{-}\) induces a quick apoptotic response in HL-60 cells.

ONOO\(^{-}\) induces cleavage of PARP and lamin B. Although the mechanism for formation of the biochemical features of apoptosis is not well defined, it has been proposed that proteolytic cleavage of certain substrates by active caspases are involved in this process. PARP and lamin B are reported to be required for fragmentation of DNA into oligonucleosomes. Thus we examined the effect of ONOO\(^{-}\) on the cleavage of PARP and lamin B. Immunoblot analysis showed that treatment of cells with ONOO\(^{-}\) caused the proteolytic cleavage of PARP with accumulation of an 89-kDa fragment and the concomitant disappearance of the original 116-kDa PARP. Cleaved PARP was detected at 2 h, and most of the original PARP was degraded at 4 h after treatment (Fig. 2, A and B). Lamin B is a 69-kDa protein that was cleaved into 32-kDa frag-
ments in cells treated with ONOO\(^{-}\). Cleavage kinetics of lamin B were similar to those of PARP, although the degree of its cleavage is much lower than that of PARP (Fig. 2, A and B). These results indicate that ONOO\(^{-}\) induces cleavage of at least two substrates in HL-60 cells, implying that caspases able to cleave these two substrates could be activated by this oxidative stress.

**ONOO\(^{-}\) induces activation of caspases 3 and 7.** Caspase 3-like caspases, including caspases 3 and 7, are the major proteases responsible for the cleavage of PARP (30, 41). To assess whether these caspases were activated by ONOO\(^{-}\) in HL-60 cells, we first analyzed the enzymatic activities of caspases 3 and 7 using colorimetric DEVD-pNA as a substrate. DEVD is a synthetic peptide that mimics the PARP site cleaved by these caspases. As shown in Fig. 3A, ONOO\(^{-}\) treatment of HL-60 cells was accompanied by a marked increase in cleaved DEVD-pNA. Compared with controls, there was a twofold increase in DEVD-pNA cleavage within 2 h of incubation of ONOO\(^{-}\) with cells.
and a fourfold increase within 4 h after ONOO\(^{-}\) treatment, indicating that caspase 3-like proteases are activated during apoptosis induced by ONOO\(^{-}\).

We further determined which specific caspases with DEVD-cleavage activity were responsible for the cleavage by using Western blotting. It has been reported that activation of caspase 3 or 7 is accompanied by formation of small active fragments of p17 kDa by caspase 3 and p12 kDa by caspase 7 (5). Figure 3B shows that ONOO\(^{-}\) induced the proteolytic processing of both these caspases in response to ONOO\(^{-}\). The activation of caspase 3, as indicated by the disappearance of full-length caspase 3 and corresponding formation of a p17 fragment, occurred with a time course that parallels the increase in DEVD-pNA cleavage activity and appearance of the cleaved fragment of PARP. Similarly, the decline in procaspase 7 levels was also clearly observed at 2 h after treatment of cells with ONOO\(^{-}\) (Fig. 3B); however, we did not detect its active fragments because this antibody was raised against a domain of the caspase 7 proform. The results were normalized to actin, a housekeeping protein. Desitometry analysis showed that the relative expression of procaspases 3 and 7 consistently decreased with increasing incubation time after ONOO\(^{-}\) treatment (Fig. 3C). These data, together with the finding that ONOO\(^{-}\) induces DEVD-cleavage activity, suggest that ONOO\(^{-}\) activates both caspases 3 and 7 in HL-60 cells.

ONOO\(^{-}\) induces activation of caspases 2 and 6. Although caspase 6 may be a major laminase in cells undergoing apoptosis, in vitro studies suggest that other caspases may also cleave lamins (27, 39). Because lamin is a structural protein of the nuclear envelope, the caspases with an ability to localize in the nucleus may be the best candidates. Caspase 2 is one such protease; both its precursor and processed fragments have been found to be distributed in the nucleus (6). With the use of VDVAD-pNA as an indicator substrate for caspase 2 activation and VEID-pNA for caspase 6, we examined the effect of ONOO\(^{-}\) on the activation of these two caspases and demonstrated that treatment of cells with ONOO\(^{-}\) dramatically increases the levels of VDVAD-pNA cleavage activity, with similar kinetics to DEVD-pNA cleavage (Fig. 4A). VEID-pNA cleavage activity also increased during the course of ONOO\(^{-}\)-induced apoptosis, albeit at much lower levels than VDVAD cleavage (Fig. 4A). These results suggest that ONOO\(^{-}\) also stimulates activation of caspases 2 and 6.

Activation of these two caspases was further confirmed by the cleavage of their proenzymes. A 48-kDa precursor of caspase 2 is proteolytically cleaved to produce a mature enzyme composed of 18-kDa and 12-kDa subunits. A 34-kDa procaspase 6 is cleaved to produce a 19-kDa active fragment. With the use of caspase 2 or caspase 6 antibodies that recognize the full-length proenzymes 2 and 6, respectively, we found that the levels of procaspases 2 or 6 gradually decreased with increasing incubation time after ONOO\(^{-}\) treatment (Fig. 4, B–D). These results were verified by

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**Fig. 4.** Activation and processing of caspases 2 and 6 during ONOO\(^{-}\)-induced apoptosis. After treatment with 200 \(\mu\)M ONOO\(^{-}\) and incubation for the indicated time, cells were harvested and washed once with PBS. A: cytosolic extracts were assayed for ability to cleave VDVAD-pNA or VEID-pNA. Relative levels of pNA absorbance in each sample were expressed as percentage of untreated cells. Values are means \(\pm\) SD for the data from 3 independent experiments. B: cells were lysed in 1× SDS sample buffer, and lysates were subjected to Western blotting with antibodies to caspases 2 or 6, or actin. Results are representative of 3 independent experiments. C: immunoblotted procaspase 2 or 6 was quantified by densitometry, and data are expressed as percentage of expression level in each sample relative to that in control.
reblotting the same membrane with anti-actin antibody.

Caspase 3-like proteases and caspase 2, but not caspase 6, are required for ONOO\textsuperscript{2−}-induced apoptosis. To analyze which caspases contribute to the apoptosis induced by ONOO\textsuperscript{2−}, we investigated effects of several selective caspase inhibitors on ONOO\textsuperscript{2−}-induced apoptosis. Previous studies have shown that a cell-permeable inhibitor of caspase 3-like proteases, DEVD-FMK, inhibited apoptosis induced by most stimuli in various cell types at a concentration of 100 μM (7, 14, 36). When HL-60 cells were treated with this concentration of DEVD-FMK for 1 h and then exposed to ONOO\textsuperscript{2−}, apoptotic cell death was largely inhibited (Fig. 5A), suggesting that caspase 3 and/or caspase 7 may play critical roles in mediating ONOO\textsuperscript{2−}-stimulated apoptosis. DEVD-FMK inhibited the cleavage of PARP and, unexpectedly, lamin B (Fig. 5, B and C), suggesting that this inhibitor may have an ability to inhibit other caspases that mediate lamin B cleavage.

We noticed that inhibition of PARP cleavage by DEVD-FMK was not accompanied by a marked up-regulation of 116-kDa PARP level (Fig. 5B). This may be due to less sample being loaded in this lane. Quantitative analysis of Western blotting results showed that the relative expression of 89-kDa PARP was largely inhibited in DEVD-treated cells, but no inhibition was seen in VDVAD-FMK- or VEID-CH\textsubscript{2}F-treated cells (Fig. 5, B and C). This supports the previous observation that DEVD-sensitive caspase(s) mediate PARP cleavage in HL-60 cells exposed to ONOO\textsuperscript{2−} (17).

To test the roles of caspase 2 and caspase 6 in the apoptosis induced by ONOO\textsuperscript{2−}, we also pretreated HL-60 cells with either VDVAD-FMK, an inhibitor of caspase 2, or VEID-CH\textsubscript{2}F, an inhibitor of caspase 6, before exposing cells to ONOO\textsuperscript{2−}. As shown in Fig. 5A, treatment of cells with VDVAD-FMK led to ~45% inhibition of apoptotic cells as evaluated by nuclear apoptotic morphology. VDVAD-FMK completely inhibited lamin B cleavage and failed to produce an inhibitory effect on PARP degradation (Fig. 5, B and C). In contrast, no inhibition of apoptosis was seen in cells pretreated with VEID-FMK at a concentration sufficient to inhibit lamin B cleavage (Fig. 5). These data suggest that caspase 2, but not caspase 6, partially mediates the apoptosis induced by ONOO\textsuperscript{2−}.

**DEVD-FMK inhibits processing of caspase 2.** Because both caspase 3 and caspase 6 are involved in ONOO\textsuperscript{2−}-stimulated apoptosis, we sought to explain the unexpected result that caspase 3 inhibitor blocks lamin B cleavage by examining interactions between caspases 2 and 3. As shown in Fig. 6, A and B, treatment of cells with DEVD-FMK partially inhibited the processing of caspase 2 as marked by an increase in the procaspase 2 form, whereas loss of procaspase 3 was not affected by VDVAD-FMK. These data correspond to the previous observations that DEVD-FMK blocks lamin B cleavage, whereas PARP degradation is not affected by VDVAD-FMK (Fig. 5, B and C). In contrast, DEVD-FMK failed to inhibit procaspase 6 cleavage. These results suggest that caspase 2 processing may be...
mediated, at least in part, by caspase 3-like proteases in ONOO\(^{-}\)-induced apoptosis. Treatment with various inhibitors and ONOO\(^{-}\) as described in Fig. 5, cells were incubated for 3 h in complete medium and then harvested in sample buffer. A: cell lysates were subjected to Western blotting with antibodies to caspases 2, 3, 6, and 7, respectively. The results are representative of 3 independent experiments. B: immunoblotted procaspases 2, 3, 6, and 7 were quantified by densitometry, and data are expressed as percentage of expression level in each sample relative to that in control.

\[\text{ONOO}^{-} \text{induces activation of caspases 8 and 9. To delineate the early signaling pathway that leads to activation of effector caspases, we investigated the effect of ONOO}^{-} \text{on cleavage of caspases 8 and 9, two initial caspases. Caspase 8 is synthesized as an inactive precursor of 54 kDa, is cleaved to a 44-kDa intermediate product, and is ultimately expressed in its active form as a p18 and p10 heterodimer (32). In untreated cells, the antibody used can detect two protein bands that represent procaspase 8a (55 kDa) and procaspase 8b (54 kDa). Figure 7A shows that treatment of cells with ONOO}^{-} \text{led to formation of a protein doublet, the 44/41-kDa intermediate fragment, which was detectable at 2 h and persisted until 4 h after treatment. However, the level of procaspase 9 was kept intact, and active fragments were not detected even after 4 h incubation, although the antibody used can detect both 46-kDa procaspase 9 and the 37-kDa subunit of active caspase 9 (Fig. 7B). We also examined the effect of ONOO}^{-} \text{on caspase 10 cleavage, though no small active fragment was detected, and the original}

\[\text{effect of ONOO}^{-} \text{on cleavage of caspases 8 and 9, two initial caspases. Caspase 8 is synthesized as an inactive precursor of 54 kDa, is cleaved to a 44-kDa intermediate product, and is ultimately expressed in its active form as a p18 and p10 heterodimer (32). In untreated cells, the antibody used can detect two protein bands that represent procaspase 8a (55 kDa) and procaspase 8b (54 kDa). Figure 7A shows that treatment of cells with ONOO}^{-} \text{led to formation of a protein doublet, the 44/41-kDa intermediate fragment, which was detectable at 2 h and persisted until 4 h after treatment. However, the level of procaspase 9 was kept intact, and active fragments were not detected even after 4 h incubation, although the antibody used can detect both 46-kDa procaspase 9 and the 37-kDa subunit of active caspase 9 (Fig. 7B). We also examined the effect of ONOO}^{-} \text{on caspase 10 cleavage, though no small active fragment was detected, and the original}
form of this caspase remained intact during apoptosis (data not shown).

To further determine whether ONOO\(^-\) induces activation of caspases 8 and 9, we measured their activities using IETD-pNA as a substrate for caspase 8 and LEHD-pNA as a substrate for caspase 9. As shown in Fig. 7C, when extracts from cells exposed to ONOO\(^-\) were incubated with IETD-pNA, a time-dependent increase in IETD cleavage activity was clearly detected, indicating activation of caspase 8. Surprisingly, LEHD cleavage activity was also detected with similar kinetics to IETD cleavage activity, suggesting that caspase 9 is also activated, but not through proteolytic cleavage. This is consistent with one report that caspase 9 can be activated without proteolytic processing (34).

**IETD-FMK inhibits ONOO\(^-\)-induced apoptosis and cleavage of caspases 2 and 3.** IETD-FMK, a selective inhibitor of caspases 8 and 9, was also evaluated for its effect on ONOO\(^-\)-induced apoptosis. Figure 8A shows that pretreatment of cells with IETD-FMK led to dose-dependent inhibition of ONOO\(^-\) stimulated apoptosis. At 100 µM, this inhibitor reduced rates of apoptosis to near control levels. This result suggests that these caspases 8 and/or 9 might mediate initial signaling in ONOO\(^-\)-induced apoptosis.

Because caspase 2 and caspase 3-like proteases mediate the apoptosis induced by ONOO\(^-\), we further explored the possibility that caspases 8 and/or 9 function upstream of these two caspases in a signaling pathway leading to apoptosis after ONOO\(^-\) treatment. As shown in Fig. 8B, 100 µM IETD-FMK treatment...
inhibited ONOO\textsuperscript{-}-induced VDVAD-pNA or DEVD-pNA cleavage activity. Consistent with this result, the expression level in procaspase 2 or that in procaspase 3 was also partially restored in cells treated with IETD-FMK (Fig. 8, C and D), reflecting that cleavage of these two caspases was inhibited. These results suggest that caspases 8 and/or 9 mediate activation of caspase 2 and caspase 3-like caspases during ONOO\textsuperscript{-}-induced apoptosis in HL-60 cells.

**DISCUSSION**

Numerous studies have shown that caspase 3 is a major executioner in the apoptosis induced by various stimuli in most cell types (28). Induction of apoptosis by ONOO\textsuperscript{-} is also mediated by caspase 3 in HL-60 human leukemia cells (17, 43). Recently, it has been reported that other caspases, besides caspase 3, are also expressed in this cell line (22). However, whether they are activated by ONOO\textsuperscript{-} and involved in apoptosis is not clear. In this study, we show that multiple caspases are activated by ONOO\textsuperscript{-}. However, not all these caspases are required for apoptosis.

Consistent with what has been previously reported, caspase 3-like proteases are essential for apoptosis in HL-60 cells exposed to ONOO\textsuperscript{-} (17, 43). Treatment of cells with ONOO\textsuperscript{-} dramatically increases DEVD-pNA cleavage activity (Fig. 3A). However, activation of caspase 3-like proteases may not be the only mechanism leading to apoptosis in HL-60 cells exposed to ONOO\textsuperscript{-}, because complete inhibition of activation of these caspases with DEVD-FMK (by blockade of PARP cleavage) only partially inhibited apoptosis (Fig. 5). In addition, it is not clear whether both caspases 3 and 7 or just one of them are involved in the apoptosis, although we do demonstrate that ONOO\textsuperscript{-}-stimulated apoptosis was accompanied by cleavage of both caspases 3 and 7 (Fig. 3, B and C). Previous studies have shown that caspase 3 and caspase 7 localize in distinct subcellular compartments of cells (4), that caspase 7 can be activated independently of caspase 3, and that there is no significant functional redundancy between these two caspases (10). These findings indicate that caspase 3 and caspase 7 may play distinct roles in the apoptotic signaling pathway. Because analysis of caspase 3 knockout mice has shown that PARP cleavage in apoptotic cells is seemingly unaffected by the loss of caspase 3 (23), caspase 7 activation may compensate for some of the apoptotic biochemical events that are not mediated by caspase 3. The development of an inhibitor specific for caspase 7 would help to further elucidate this point.

Caspase 2 is a long prodomain caspase that is believed to play an early acting role in the apoptosis induced by etoposide, \(\gamma\)-irradiation, or genotoxic agents (9). However, Li et al. (14) have shown that activation of caspase 3 precedes procaspase 2 processing in Fas-induced apoptosis and that caspase 3 has an ability to cleave procaspase 2 to its small, active fragment. Recently, Swanton et al. (37) have provided further evidence, in an in vitro study, that cleavage of caspase 2 is dependent on activation of caspase 3 after cytochrome c addition in cytosolic extracts. In this study, we have demonstrated that caspase 2 mediates ONOO\textsuperscript{-}-induced apoptosis (Fig. 5A). However, we could not discern the kinetics of activation of caspase 2 and caspase 3, because the time course of activation for caspase 2 was similar to that observed for caspase 3 (Fig. 3B and Fig. 4B). Based on the observation that inhibition of caspase 3-like caspases partially attenuated caspase 2 cleavage, whereas caspase 3 processing was not affected by a caspase 2 inhibitor (Fig. 6, A and B), it seems that caspase 3-like proteases may function as upstream activators of caspase 2. However, the possibility could not be ruled out that DEVD-FMK might directly inhibit caspase 2 processing, because this caspase contains DXXD motif, which is the sequence bound by DEVD-FMK (5). Further detailed studies on kinetics of activation for caspases 2 and 3 during ONOO\textsuperscript{-}-induced apoptosis are needed before making any conclusions on the sequence of activation of these caspases.

Although caspase 6 is also activated by ONOO\textsuperscript{-}, it appears that this caspase is not required for apoptosis. This conclusion is based on our observation that pretreatment of cells with VEID-CH\textsubscript{2}F at a concentration of up to 100 \(\mu\)M did not prevent nuclear fragmentation (Fig. 5A). We do not believe the failure of this inhibitor to prevent apoptosis is due to the concentrations used. VEID-CH\textsubscript{2}F is a highly selective inhibitor that was synthesized based on the sequence within lamins cleaved by caspase 6, but not by caspases 3 and 7 (39). The activity of caspase 6 can be inhibited by VEID-CHO at concentrations at least 100-fold lower than those required to inhibit caspases 3, 4, 7, and 8. In Jurkat cells, 10 \(\mu\)M VEID-CHO is enough to completely inhibit Fas-induced caspase 6 activation, as demonstrated by the disappearance of its active fragment (10). In the present study, 100 \(\mu\)M of VEID-CH\textsubscript{2}F blocked the cleavage of lamin B but did not affect PARP degradation (Fig. 5, B and C), revealing its effectiveness and high selectivity. These data, together with the finding that VEID-CH\textsubscript{2}F does not affect caspase 3 processing, suggest that caspase 6 may not be involved in activation of caspase 3 in HL-60 cells. The significance of caspase 6 activation during apoptosis induced by ONOO\textsuperscript{-} is not clear. One possibility is that caspase 6 is merely a bystander in this mode of apoptosis. The findings that no clear role for caspase 6 was seen in Fas-induced apoptosis, and that caspase 6-deficient mice do not display an overt phenotype (23), support this speculation.

Caspase-mediated cleavage of some specific target proteins is associated with apoptotic changes in cellular morphology (23). In this study, we demonstrated that PARP and lamin B were cleaved after treatment with ONOO\textsuperscript{-} before significant increases in numbers of apoptotic cells (Fig. 1, C and D, and Fig. 2). PARP cleavage can be inhibited by DEVD-FMK, and lamin B cleavage blocked by VEID-CH\textsubscript{2}F (Fig. 5, B and C), which is consistent with previous reports that caspase 3-like caspases are proteases for PARP (30, 41), and caspase 6 is a laminase (27, 39). Unexpectedly, treatment of cells with DEVD-FMK also blocked cleavage of lamin B (Fig. 5B,
To our knowledge, this is the first demonstration that lamin B cleavage is regulated by DEVD-sensitive proteases. Previous in vitro studies showed that inactivation of laminases and PARP proteinases are distinct enzymatic activities and that caspases 3 and 7 do not cleave lamin A under conditions where caspase 6 does cleave lamin A (27, 39). Thus caspase 3-like caspases probably do not act as laminases. VDAD-FMK, a potent caspase 2 inhibitor, can block lamin B degradation and apoptosis (Fig. 5, A–C), and DEVD-FMK partially inhibits caspase 2 processing (Fig. 6, A and B). This suggests the possibility that caspase 2 is one of the proteases that mediates action of caspase 3 in cleavage of lamin B. However, whether caspase 2 itself is a laminase or if it further activates other laminase(s) needs to be further defined.

Our data show that ONOO\textsuperscript{−} induces activation of both caspases 8 and 9, and their activation is required for induction of apoptosis and activation of caspases 2 and 3 (Fig. 8). It was previously demonstrated that caspase 8 cleavage is a rapid process in Fas-induced apoptosis, with cleaved products detectable at as early as 5 s after receptor cross-linking in many cell lines (24, 32). However, we did not see the cleaved fragments of caspase 8 until 2 h after ONOO\textsuperscript{−} treatment (Fig. 7A), arguing against the death receptor-mediated mechanism in activation of this caspase by ONOO\textsuperscript{−}. Recently, Scaffidi et al. (31) have reported that delayed cleavage of caspase 8 in some cell types occurs downstream of mitochondrial cytochrome c release. Consistent with this finding, we detected a marked increase in caspase 9 activity at 2 h (Fig. 7C), when DNA fragmentation formed in cells exposed to ONOO\textsuperscript{−}. These data, together with the observation that IETD-FMK inhibits cleavage of caspases 2 and 3, imply that ONOO\textsuperscript{−} may trigger cytochrome c release from mitochondria, resulting in sequential activation of initiator and effector caspases. It has been reported that ONOO\textsuperscript{−} is able to induce release of cytochrome c from heart mitochondria (3). Although caspase 8 has the same ability as caspase 9 in binding Apaf-1, a key step in caspase activation through released cytochrome c from mitochondria, no direct interaction between caspase 8 and Apaf-1 has been demonstrated (11). Further studies suggest that caspase 8 activation may be an event downstream of initiator caspases in cytochrome c-mediated apoptotic pathway, because its activation was diminished in cells from caspase 9\textsuperscript{−/−} and Apaf-1\textsuperscript{−/−} mice in certain contexts (8, 47), and because DEVD-FMK inhibits caspase 8 activation in cytochrome c-mediated apoptosis in HeLa cells after photodynamic therapy (PDT) (7).

Though the activation of both effector caspases and initial caspases induced during the course of apoptosis after ONOO\textsuperscript{−} treatment is easily measured, differentiating the kinetic profiles of their activation is more difficult. Results similar to this were observed in PDT-induced apoptosis, a process involving cytochrome c released mitochondria (7). It is not clear how quickly these caspases are processed in vivo. Sleel et al. (33) have demonstrated in cell-free extracts that most caspases, including caspases 2, 3, 6, 7, and 9, were cleaved and activated within 30 min of addition of cytochrome c, with the exception of caspase 8. The cleavage products of caspase 8 were detectable at 1 h under the same conditions. This may explain our own results, which show that all these caspases are cleaved and activated almost simultaneously at 2 h after treatment. However, our results also seem to indicate that caspase 8 plays a limited role in the cascade, because its maximum processing is delayed relative to effector caspases (Fig. 7, A and C). It is possible that, like caspase 6, this caspase is simply a side product in this process. If this is the case, the inhibition of apoptosis by IETD-FMK observed in this study could be through a blockade of caspase 9 activation. This possibility is worthy of further investigation.

In summary, we have demonstrated that ONOO\textsuperscript{−}-induced apoptosis is associated with activation of caspase 3-like proteases and caspase 2, although multiple caspases that cleave PARP and lamin B are activated in HL-60 cells. The precise sequence of activation of these caspases is not clear; cytochrome c-mediated sequential activation of caspase 9 and caspases 2 and 3 may be involved in this process. Because ONOO\textsuperscript{−}-mediated apoptosis is implicated in the pathogenesis of many disorders, further elucidation of the mechanism of ONOO\textsuperscript{−}-induced apoptosis could provide a basis for the design of therapeutic interventions.

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