Peroxy nitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease

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Lin, King-Teh, Ji-Yan Xue, Marie C. Lin, Eric G. Spokas, Frank F. Sun, and Patrick Y.-K. Wong. Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. Am. J. Physiol. 274 (Cell Physiol. 43): C855–C860, 1998.—Apoptosis is an active process critical for the homeostasis of organisms. Enzymes of the caspase family are responsible for executing this process. We have previously shown that peroxynitrite (ONOO−), a biological product generated from the interaction of nitric oxide and superoxide, induces apoptosis of HL-60 cells. The aim of this study was to elucidate the mechanisms involved in the execution process of peroxynitrite–induced apoptosis. Proteolytic cleavage of poly(ADP-ribose) polymerase, an indication of caspase-3 family protease activation and an early biochemical event accompanying apoptosis, was observed in a time-dependent manner during peroxynitrite–induced apoptosis of HL-60 cells. Activation of caspase-3 during peroxynitrite–induced apoptosis was substantiated by monitoring proteolysis of the caspase-3 proenzyme and by measuring caspase-3 activity with a fluorogenic substrate. Furthermore, pretreatment of HL-60 cells with N-acetyl-Asp-Glu-Val-Asp-aldehyde, a specific inhibitor of caspase-3, but not N-acetyl-Tyr-Val-Ala-Asp-aldehyde, a specific inhibitor of caspase-1, decreased peroxynitrite–induced apoptosis. These results suggest that the activation of a caspase-3 family protease is essential for initiating the execution process of peroxynitrite–induced apoptosis of HL-60 cells.

Programmed cell death; leukemia cells; reactive nitrogen species; caspase cysteine protease

APOPTOSIS IS A HIGHLY regulated process that is of critical importance for homeostasis of organisms, functioning to eliminate superfluous cells (14, 46). This apoptotic process, aptly termed programmed cell death, normally occurs during development, immune regulation, cell turnover, and tumor suppression. Aberration in this process can disrupt homeostasis and lead to many pathophysiological conditions such as neurodegenerative disorders and cancers (43). It appears that apoptosis can be triggered by either external or internal cues and these various precipitating factors then lead to a characteristic series of morphological and biochemical alterations including cell shrinkage, blebbing, chromatin condensation, and DNA degradation into nucleosomal fragments (14, 47).

Recent evidence suggests that the interleukin-1-converting enzyme (ICE)/CED-3 cysteine protease (caspase) family plays a crucial role in apoptosis (26, 30). Of importance, CPP32/Yama/apopain (caspase-3), a well-characterized member of the caspase family, appears to be the most closely related of mammalian enzymes to CED-3 (29), a gene product necessary for the apoptotic cell death in the nematode Caenorhabditis elegans (26). Activation of caspase-3 occurs during diverse forms of apoptosis and has been proposed to be a crucial step in initiating the execution process of apoptosis (15, 30, 35). Caspase-3 is synthesized as a 32-kDa inactive proenzyme and is proteolytically cleaved to the active form, which, in turn, recognizes and cleaves its corresponding targets (29). The specific cleavage at the sequence Asp-Glu-Val-Asp (DEVD) in poly(ADP-ribose) polymerase (PARP) has been shown to be a common marker of caspase-3 activation at the onset of apoptosis induced by Fas and other stimuli (13, 15, 42). PARP is an important nuclear enzyme participating in DNA repair and genome surveillance (13, 31).

Peroxy nitrite (ONOO−) is a biological product generated from the interaction of nitric oxide (NO) and superoxide anion (O2−), present in a variety of mammalian cells including endothelial cells, neurons, neutrophils, and macrophages (2, 4, 8, 11). It appears that this chemically reactive species exerts a wide profile of biological activities including vasodilation (21, 44), inhibition of platelet aggregation (27, 48), neurotoxicity (4, 20), bacteriostasis (6), inhibition of leukocyte adhesion (16), and induction of apoptosis (4, 18). With the recognition that peroxynitrite is capable of inducing apoptosis, this apoptotic process has been implicated in many pathophysiological conditions including renal damage (3), gastritis (23), enterocolitis (7), fetal growth retardation (25), cardiac allograft rejection (39), and neurodegenerative disorder (4, 40). On the other hand, suppression of tumor malignancy may be viewed as a clinically desirable outcome of this process (9, 10, 18). Therefore, work on the mechanisms of peroxynitrite–induced apoptosis may uncover promising targets for therapeutic intervention. Although DNA strand breaks (33) or reactive oxygen species (ROS) formation (19) elicited by peroxynitrite has been demonstrated to participate in peroxynitrite–induced apoptosis, the execution process of peroxynitrite–induced apoptosis has not been identified. In this report, we demonstrate that a caspase-3 family protease is critical for execution of peroxynitrite–induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Cell culture. The human promyelocytic leukemia HL-60 cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained at a density of 0.2–1 × 10⁶ cells/ml in RPMI 1640 medium (Life Technolo-
cies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and antibiotics/antimycotics (Sigma, St. Louis, MO). Cells were kept in a culture incubator at 37°C under a 5% CO2 humidified atmosphere and used for experiments during the exponential phase of growth. Cell counts were performed routinely to assess population density, and cell viability was assessed by the method of trypan blue exclusion as well as by fluorescence propidium iodide staining (18).

Peroxynitrite treatment. HL-60 cells were resuspended in 5 ml of Dulbecco’s PBS (D-PBS; pH 7.4 without Ca2+ and Mg2+) at 1 × 106 cells/ml. Various stock concentrations of peroxynitrite were freshly prepared in 0.5 N NaOH. Five microliters of each stock were added to separate cell suspensions and incubated for 10 min at 37°C (19). The cells were washed, centrifuged, resuspended in culture medium, and maintained in a culture incubator for the additional time required for each experiment. Five microliters of D-PBS buffer and 0.5 N NaOH were used as control and vehicle, respectively.

Quantitative assay of apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was determined by a method adapted from that of Sellins and Cohen (36). The cell pellets were lysed with 0.3 ml hypotonic lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 0.5% Triton X-100), and the lysates were centrifuged to separate intact and fragmented chromatin. Both pellet and supernatant were precipitated with 12.5% TCA. The DNA precipitate was heated to 90°C for 10 min in 400 µl of 5% TCA, and quantitative analysis was carried out by reaction with diphenylamine (5). The percentage of DNA fragmentation was calculated from the ratio of DNA in the supernatant to the total DNA (supernatant plus pellet).

Western blot analysis. Proteolytic cleavage of PARP and caspase-3 were detected by Western blot analysis as previously described (28). For detection of PARP cleavage, HL-60 cells were treated with 100 µM peroxynitrite and harvested at the indicated times. After one wash with PBS, cells were suspended at 5 × 106 cells/ml in sample buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromphenol blue, and 5% β-mercaptoethanol) and then sonicated for 15 s and incubated at 65°C for 15 min. Fifteen microliters of each cell extract were subjected to 7.5% SDS-PAGE.

For detection of proteolysis of caspase-3 proenzyme, HL-60 cells were treated with 100 µM peroxynitrite and harvested at indicated times. After one wash with PBS, cells were suspended at 5 × 106 cells/0.5 ml in sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.003% bromphenol blue, and 1% β-mercaptoethanol) and then sonicated for 15 s. A small aliquot of each sample was analyzed for protein concentration using the bicinchoninic acid method (Pierce, Rockford, IL). An equal amount (50 µg) of each cell extract was subjected to 12% SDS-PAGE.

After transfer of proteins to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blockade with 5% nonfat dry milk in PBS-0.05% Tween 20, the membranes were incubated with anti-PARP mouse monoclonal antibody that recognizes an epitope at the carboxy end of the DNA-binding domain of PARP (C-2-10; 1:1,000 dilution; Biomol), was added directly to the culture medium 1 h before peroxynitrite treatment. Alternatively, these inhibitors were delivered into the cell by Lipofectin (Life Technologies). In the Lipofectin-enhanced delivery, the inhibitors and Lipofectin were separately diluted in 0.1 ml serum-free, antibiotic-free RPMI 1640 and subsequently coincubated for 20 min at room temperature. After addition of serum-free RPMI-1640 (0.6 ml) to the inhibitor-Lipofectin mixture, the cells were added (5 × 106/0.2 ml) and the cell suspension was incubated at 37°C for 2 h before addition of peroxynitrite. The final concentration of Lipofectin in the mixture was 10 µg/ml. Cells were harvested at selected intervals following peroxynitrite addition, and apoptotic DNA fragmentation was measured as described.

Measurement of caspase-3 activity using the fluorogenic substrate DEVD-7-amino-4-trifluoromethyl-coumarin. The activity of caspase-3 was measured by the fluorometric method described in the ApoAlert CPP32 fluorescence assay kit (Clontech, Palo Alto, CA). Briefly, HL-60 cells were treated with peroxynitrite for 10 min in PBS buffer, resuspended in culture medium, and harvested at the indicated times. The cells (1 × 106 cells) were used to perform the fluorometric assay according to the manufacturer’s instructions. The fluorescence emission of the 7-amino-4-trifluoromethyl-coumarin (AFC), released on proteolytic cleavage of the fluorogenic substrate DEVD-AFC by active caspase-3, was measured using Cytofluor 2350 (excitation wavelength, 400 nm; emission wavelength, 505 nm).

RESULTS

Proteolytic cleavage of PARP during peroxynitrite-induced apoptosis. Proteolytic cleavage of PARP from a 116-kDa polypeptide to an 85-kDa fragment is a sensitive marker at the onset of apoptosis and also reflects caspase-3 activation (13, 15). To ascertain whether these proteolytic events are involved in peroxynitrite-induced apoptosis, HL-60 cells were treated with 100 µM peroxynitrite and harvested at the indicated times. Western blot analysis using a monoclonal antibody (C-2-10) that recognizes an epitope at the carboxy end of the DNA-binding domain of PARP, revealed that the onset of proteolytic cleavage of the 116-kDa PARP holoenzyme into the 85-kDa fragment (Fig. 1A) preceded the appearance of DNA fragmentation (Fig. 1B), a hallmark of apoptotic cell death. The 85-kDa fragment was slightly visible 90 min after peroxynitrite treatment, when no DNA fragmentation could be detected. The intense cleavage at 180 min was correlated with overt DNA fragmentation that resulted from the activation of endonuclease. These results suggested participation of a caspase-3 family protease in executing peroxynitrite-induced apoptosis of HL-60 cells.

Effects of peroxynitrite on the activity of caspase-3 (CPP32/Yama/apopain). To ascertain whether caspase-3 might be proteolytically activated by proteolysis of the inactive proenzyme during peroxynitrite-induced apoptosis, a monoclonal antibody against human caspase-3 proenzyme (32 kDa) was used to detect the levels of caspase-3 proenzyme during peroxynitrite-induced apoptosis by Western blot analysis. The results ob-
tained from Western blot (Fig. 2) lend support to this hypothesis that the levels of caspase-3 proenzyme diminished in a time-dependent fashion due to proteolysis of the proenzyme. As shown in Fig. 2, in response to 100 µM peroxynitrite, the levels of caspase-3 proenzyme were considerably reduced at 3 h and scarcely detectable at 5 h after treatment; this decline in procaspase-3 levels was associated with the appearance of the active caspase-3 subunits, p20 and p12 (20 and 12 kDa). Similar proteolysis was also observed in the HL-60 cells treated with etoposide (10 µg/ml), which was included as a reference because it is well established that etoposide induces apoptosis via caspase-3 activation (Fig. 2). A marked increase of DNA fragmentation was observed at 3 and 5 h after peroxynitrite (Fig. 3), corroborating the results obtained by Western blot analysis (Fig. 2).

Effects of cysteine protease inhibitors on peroxynitrite-induced apoptosis. If caspase-3 is the major cysteine protease responsible for “committing” the HL-60 cells to undergo apoptosis in response to peroxynitrite, a

Fig. 2. Time-dependent proteolysis of caspase-3 proenzyme during peroxynitrite-induced apoptosis of HL-60 cells. HL-60 cells were exposed to vehicle (Veh), 100 µM peroxynitrite, or etoposide (positive control, 10 µg/ml), harvested at indicated times, and lysed with lysis buffer as described in MATERIALS AND METHODS. Con, untreated control. After 12% SDS-PAGE electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and analyzed for caspase-3 using anti-caspase-3 mouse monoclonal antibody. Antibody binding was visualized as described in MATERIALS AND METHODS.

Caspase-3 activity rose progressively over the interval from 90 to 300 min in response to 100 µM peroxynitrite. A smaller time-dependent response was seen with a lower concentration of peroxynitrite (50 µM). Time-dependent activation of caspase-3 was also observed in etoposide-treated HL-60 cells. A marked increase of DNA fragmentation was observed at 3 and 5 h after peroxynitrite (Fig. 3), corroborating the results obtained by Western blot analysis (Fig. 2).

Effects of cysteine protease inhibitors on peroxynitrite-induced apoptosis. If caspase-3 is the major cysteine protease responsible for “committing” the HL-60 cells to undergo apoptosis in response to peroxynitrite, a

Fig. 3. Activation of caspase-3 during peroxynitrite-induced apoptosis. HL-60 cells were treated with 50 or 100 µM peroxynitrite for 10 min in PBS buffer. After being recultured in medium for indicated times, cells were harvested, and cell extracts were obtained as described in MATERIALS AND METHODS. Cytosolic extracts were incubated with the fluorogenic substrate DEVD-7-amino-4-trifluoromethyl-coumarin (DEVD-AFC), and fluorescence of AFC released on proteolytic cleavage by active caspase-3 was measured. Time course data for caspase-3 activity after addition of etoposide (10 µg/ml) are also shown, as positive control. Relative levels of AFC fluorescence in peroxynitrite, etoposide, and vehicle groups were expressed as percent increase of untreated normal HL-60 cells. Values are means ± SE; n = 3.
substance that specifically antagonizes the action of caspase-3 should prevent apoptosis. To test this hypothesis, HL-60 cells were pretreated with either Ac-DEVD-CHO, a potent and selective inhibitor of caspase-3, or Ac-YVAD-CHO, a known specific inhibitor of ICE, 1 h before 100 µM peroxynitrite treatment. As shown in Fig. 4, pretreatment of HL-60 cells with 100 µM Ac-DEVD-CHO significantly (*P < 0.01) attenuated the apoptotic response to 100 µM peroxynitrite, as determined by DNA fragmentation (Fig. 4A) as well as by cell viability assay (Fig. 4B). In contrast, Ac-YVAD-CHO failed to inhibit peroxynitrite-induced apoptosis under the same experimental conditions, indicating that ICE activation is not involved in peroxynitrite-induced apoptosis of HL-60 cells.

Because these inhibitors have poor membrane permeability, it is not surprising that high concentrations (100 µM) of the caspase-3 inhibitor were required to exert effects. To test whether low concentrations of the caspase-3 inhibitor could inhibit peroxynitrite-induced apoptosis, the agents were delivered into the cells by Lipofectin. When delivery was enhanced by this method, the caspase-3 inhibitor Ac-DEVD-CHO was effective against peroxynitrite-induced DNA fragmentation at a concentration of 0.1 µM, 1,000-fold less than previously required. Pronounced inhibition by Ac-DEVD-CHO occurred at higher concentrations (Fig. 5). However, in the case of the ICE inhibitor Ac-YVAD-CHO, a concentration of 100 µM was needed to exert the same degree of inhibition of peroxynitrite-induced DNA fragmentation as 0.1 µM Ac-DEVD-CHO. Taken together, these results strongly indicate that caspase-3 is the key cysteine protease engaged in peroxynitrite-induced apoptotic cell death in HL-60 cells.

DISCUSSION

It has been demonstrated that PARP, an enzyme implicated in DNA repair and genome surveillance, can be activated in response to DNA damage (31). Once recruited to the sites of DNA damage, the activated PARP will catalyze poly(ADP-ribosyl)ation in the process of DNA repair at the expense of NAD⁺, leading to depletion of the cellular ATP energy source (12). Exposure of cultured J774 macrophages to high concentrations of peroxynitrite (500 µM to 1 mM) can cause serious DNA strand breaks and consequent PARP activation (38). The participation of PARP activation in peroxynitrite-mediated cell death was postulated on the basis of the observation that 3-aminobenzamide, a PARP inhibitor, attenuated the cytotoxic effects of peroxynitrite on certain cell types such as rat aortic smooth muscle cells and the human BEAS-2B epithelial cell line (37, 38). It has also been reported that 3-aminobenzamide delays apoptosis in cultured cells (1). However, the inhibitory effect of 3-aminobenzamide on cell death may be due to inhibition of other enzymes besides PARP (32). Furthermore, the relevance of PARP in the progression of apoptosis has been challenged in studies of the susceptibility of cells from wild-type and PARP −/− mice to several apoptosis inducers (includ-
ing peroxynitrite); these studies indicated that neither activation nor cleavage of PARP has a causal role in apoptotic cell death (17). During peroxynitrite-induced apoptotic cell death in our experimental system, PARP appears to be inactivated due to proteolytic cleavage of PARP by caspase-3 family protease rather than activated. Although PARP cleavage may not be a direct cause of apoptosis, proteolytic cleavage of PARP by caspase-3 may function to conserve cellular energy required for the apoptotic process (46), thereby avoiding severe depletion of ATP stores that could cause irreversible structural damage and necrosis (45). In addition, the poly(ADP-ribosylation) can negatively regulate the Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease responsible for peroxynitrite-induced apoptosis, suggesting that the activation of caspase-3 is a critical step in peroxynitrite-induced apoptotic cell death. This notion is supported by the following observations: 1) proteolytic cleavage of caspase-3 proenzyme, as judged by the disappearance of the proenzyme and the corresponding appearance of cleavage subunits, occurs in HL-60 cells undergoing apoptosis after peroxynitrite treatment; 2) the activity of caspase-3 increased in a time- and concentration-dependent manner after peroxynitrite treatment, as determined by fluorometric assay using the caspase-3-specific fluorogenic substrate; and 3) Ac-DEVD-CHO, a specific inhibitor of caspase-3, but not Ac-YVAD-CHO, an agent known to inhibit ICE, effectively suppressed peroxynitrite-induced apoptosis of HL-60 cells. Thus this study provides strong evidence that caspase-3 is the key protease to initiate the execution process in this model of peroxynitrite-induced programmed cell death. Because peroxynitrite-induced apoptosis is likely to be associated with many pathophysiological disorders such as neurological disorders (4, 40), gut inflammatory diseases (7, 23, 34), and cancers (9, 10, 18), manipulating the processes controlling caspase-3 activity has potential as a therapeutic maneuver to influence the progression of the disease states. Because the ICE inhibitor Ac-YVAD-CHO did not appreciably affect peroxynitrite-induced apoptosis, ICE is probably not involved in the upstream event leading to caspase-3 activation. However, we cannot rule out the possibility that other caspase-3-like family proteases, in addition to caspase-3, might be activated concurrently during peroxynitrite-induced apoptosis of HL-60 cells. If multiple proteases in this family operate in a concerted manner to initiate this apoptotic process, identification of these new proteases will help to elucidate the upstream steps that transmit the apoptotic signal of peroxynitrite to the proteolytic machinery (execution process). How peroxynitrite causes activation of the caspase-3 family protease remains to be established. Because PARP cleavage was not apparent until after a considerable delay (90 min after peroxynitrite exposure), it is unlikely that caspase-3 activation is directly due to peroxynitrite per se; rather, it is likely due to activation of upstream events such as release of cytochrome c from mitochondria (22) or generation of ROS (19). It has been suggested that reactive oxygen intermediates are the common mediators of PARP cleavage, DNA fragmentation, and apoptosis in leukemia cells (24). We have previously reported that brief exposure of HL-60 cells to peroxynitrite stimulates the release of ROS in a concentration- and time-dependent manner (19). Whether this ROS generation is indeed primarily responsible for the caspase-3 activation needs further investigation.

Wethank Dr. John Quilley for helpful discussion. This work was supported by National Institutes of Health Grants HL-25316-14 and DK-41747 to P. Y.-K. Wong.

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Received 26 August 1997; accepted in final form 10 December 1997.

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