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

KING-TEH LIN,1,2 JI-YAN XUE,1,2 MARIE C. LIN,1 ERIC G. SPOKAS,1 FRANK F. SUN,1 AND PATRICK Y.-K. WONG1,2

1Department of Cell Biology, School of Osteopathic Medicine, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084; and 2Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

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⁻) is chemically reactive species exerting 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, 19, 42). PARP is an important nuclear enzyme participating in DNA repair and genome surveillance (13, 31).

Peroxynitrite (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 retinal 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 Techno-

0363-6143/98 $5.00 Copyright © 1998 the American Physiological Society C855
otics/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 × 10⁷ 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 pelleted 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% bromophenol blue, and 5% ß-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/ml in sample buffer (125 mM Tris·HCl, pH 6.8, 5% glycerol, 2% SDS, 0.003% bromophenol blue, and 1% ß-mercaptoethanol) and then sonicated for 15 s. A small aliquot of each sample was analyzed for protein concentration using the biocinchnonic 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), or anti-caspase-3 mouse monoclonal antibody (a 24.7-kDa fragment corresponding to amino acids 1–219 of 277 human procaspase-3 was used as immunogen; 1:1,000 dilution; Transduction Laboratories) for 2 h. Antibody binding was detected using peroxidase-conjugated anti-mouse IgG (1:4,000 dilution; Sigma) and visualized by a standard chemiluminescence method (reagents from DuPont NEN) performed according to the manufacturer's instructions.

Pretreatment of HL-60 cells with inhibitors of ICE (caspase-1) and of caspase-3. The ICE inhibitor, N-acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO, Biomol), or caspase-3 inhibitor, N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO, 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 co-inubcated 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 Cytomax 2350 (excitation wavelength, 400 nm; emission wavelength, 505 nm).

RESULTS

Proteolytic cleavage of PARP during peroxynitrite-induced apoptosis. Proteolytic cleavage of PARP by 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). However, the latent 32-kDa proenzyme was clearly observed even 5 h after treatment in untreated and vehicle-treated HL-60 cells (Fig. 2). This Western blot analysis demonstrated that caspase-3 was indeed proteolytically activated at the onset of peroxynitrite-induced apoptosis.

The activity of caspase-3 was further investigated using a specific fluorogenic substrate of caspase-3 protease, Ac-DEVD-AFC (30, 35). Peroxynitrite caused a time- and concentration-dependent increase of AFC fluorescence (Fig. 3), indicating activation of caspase-3. 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 AFC fluorescence 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
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-ribosylation) 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 a 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 implicated in internucleosomal DNA fragmentation (41). If such is the case, the proteolytic cleavage of PARP in response to peroxynitrite may serve to activate the endonuclease responsible for peroxynitrite-induced apoptotic DNA fragmentation (nucleosomal fragments).

The results of the present study demonstrate that activation of caspase-3 precedes the activation of endonuclease at the onset of 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.

We thank 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.

Address for reprint requests: P. Y.-K. Wong, Dept. of Cell Biology, School of Osteopathic Medicine, University of Medicine and Dentistry of New Jersey, Stratford, NJ 08084.

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

REFERENCES


