Fenretinide-induced Caspase 3 Activity Involves Increased Protein Stability in a Mechanism Distinct from Reactive Oxygen Species Elevation

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Abstract

Fenretinide (4-HPR) is a synthetic retinoid that displays a broad range of biological effects and has also demonstrated clinical efficacy as a chemopreventative agent. One cellular activity of 4-HPR is its ability to induce apoptosis. This effect has been proposed to relate to changes in intracellular reactive oxygen species. We show herein that a 1-h treatment of HL-60 cells with 4-HPR led to a dose-dependent increase in hydroperoxides. Pretreatment of cells with the antioxidant vitamin C abolished apoptosis, measured as the appearance of the sub-G1 peak, in 4-HPR-treated cells. The retinoid also elicited a 3.6-fold increase in caspase 3 activity; however, this increase was not affected by vitamin C treatment. Analysis of caspase 3 protein expression by Western blot analysis revealed that 4-HPR resulted in a significant increase in the appearance of the active p17 subunit without effecting a concomitant change in p32 procaspase 3 levels. Studies on de novo synthesis and stability of caspase 3 by pulse-chase and immunoprecipitation methods show that 4-HPR-treated samples had decreased incorporation of radioactive amino acid precursors into newly synthesized procaspase 3 but, during the chase (for up to 9 h), had more labeled caspase 3 remaining when compared with controls. These studies suggest that 4-HPR may effect changes in caspase 3 activity by modulating changes in zymogen stability by a mechanism distinct from the retinoid-elicited increase in reactive oxygen species.

Introduction

The synthetic retinoid 4-HPR has reported efficacy as a chemopreventive agent for breast and prostate cancer and is currently being investigated for treatment of bladder cancer (1–4). Due to its reduced toxicity and effectiveness as a chemopreventative agent in comparison with other retinoids, the mechanism of action of 4-HPR is of great interest. Studies in vitro have demonstrated that 4-HPR induces apoptosis in numerous carcinoma cell lines. Recent reports suggest that this effect may be mediated through increases in ROS that result from a perturbation between complex II and complex III of the mitochondrial respiratory chain (5, 6). In addition, elevations in the levels of the lipid second messenger ceramide as well as increased caspase 3 activity have also been reported in cells treated with 4-HPR (7, 8). All three molecular events (ROS, ceramide elevation, and caspase 3 activity changes) appear to be critical in the cell death pathway triggered by this retinoid because inhibitors of each of these events block 4-HPR-induced apoptosis (6–8). Due to the importance of caspase 3 in the execution phase of apoptosis and because the mechanism of caspase 3 modulation by 4-HPR remains ill-defined, we investigated regulation of caspase 3 by 4-HPR. Our results suggest that this retinoid affects the stability of procaspase 3 by a mechanism independent of ROS elevation.

Materials and Methods

Chemicals. All-trans-RA (Sigma Chemical Co.) and 4-HPR (supplied by the Johnson Pharmaceutical Research Institute) were dissolved in absolute ethanol at 10 and 1 mM stock, respectively. The cell-permeable caspase inhibitors DEVD-CHO and YVAD-CHO and the caspase colorimetric substrates Ac-DEVD-pNA and Ac-YVAD-pNA were purchased from Biomol and dissolved in DMSO as stock solutions.

Cell Culture. HL-60 cells (American Type Culture Collection, Rockville, MD) were cultured as described previously (7, 9, 10). In a typical experiment, 2 × 10⁶ HL-60 cells/ml were seeded in T-75 flasks as 10-ml cultures. At the indicated times, cells were harvested, washed twice with PBS, and counted. Cell viability was determined by trypan blue exclusion.

Flow Cytometry. Control and treated cells were washed twice with PBS and fixed in 10 mL of ice-cold 70% ethanol. After overnight incubation at −20°C, samples were washed twice with PBS and resuspended in 1 mL of PBS containing 10 μg/mL propidium iodide and 100 μg/mL RNase A. Cell cycle phase distribution and the percentage of apoptotic cells were determined on a FACScan flow cytometer, and the data were analyzed using cellFit software (9).

Caspase 3 Activity and Protein Expression. Cell lysates were prepared as described previously (11). Briefly, the cell pellet was resuspended in hypotonic lysis buffer (50 μL/10⁶ cells) containing 10 mM HEPES (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin. The cell suspension was incubated on ice for 15 min, passed five times through a 27-gauge needle, and centrifuged at 14,000 rpm for 15 min at 4°C. For Western blot analysis, 30 μg of cell lysate were separated on a 15% gel, and immunoreactivity with anti-caspase 3 (Santa Cruz Biotechnology) was demonstrated by enhanced chemiluminescence or color reaction. Protease activity was assayed as detailed previously (12), with the following modifications. Cell lysates (50 μg) were added to 148 μL of reaction buffer [100 mM HEPES (pH 7.5), 20% glycerol, 0.5 mM EDTA, and 5 mM DTT] and 2 μL of substrate DEVD-pNA (final concentration, 100 μM), followed by incubation at 30°C for 6 h. The enzyme-catalyzed release of pNA was monitored at 405 nm in a microtiter plate reader (model Elx 800; Bio-Tek Instruments). Specificity of caspase 3 assay was validated by showing that the release of pNA was inhibited by a 30-min preincubation with 100 nM inhibitor DEVD-CHO at room temperature.

Pretreatment with Antioxidants. NAC (Sigma Chemical Co.) was prepared in media immediately before use, with the pH adjusted to 7.4. Vitamin C (Sigma Chemical Co.) was dissolved in H₂O and stored at 4°C. Cells were pretreated for 3 h with either antioxidant before the addition of 4-HPR.

Measurement of ROS. The probe DCFH-DA (Molecular Probes) was dissolved in DMSO as a 50 mM stock. HL-60 cells were washed with PBS and resuspended in PBS at a density of 1 × 10⁶ cells/ml as 1-ml cultures. Cultures were incubated with 100 μM DCFH-DA for 15 min before the addition of

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3 The abbreviations used are: 4-HPR, fenretinide; ROS, reactive oxygen species; RA, retinoic acid; NAC, N-acetyl cysteine; DCFH-DA, dichlorofluorescein-diacetate; PARP, poly(ADP-ribose) polymerase.
4-HPR. All samples were incubated at 37°C for 1 h, and absorbance was measured at 504 nm.

**Labeling of Proteins.** To determine new protein synthesis, HL-60 cells were incubated with 20 μCi/ml [35S]methionine/cysteine before the addition of 4-HPR. Relative stability of newly synthesized proteins was monitored by labeling cells overnight with 20 μCi/ml [35S]methionine/cysteine. The label was removed by extensive washing with media, and cells were adjusted to a fixed density and treated with 4-HPR. Aliquots of treated or control cells were harvested at the time points indicated. Radioactive caspase 3 was immunoprecipitated and analyzed by autoradiography, as described below. The same membranes were probed by Western blot analysis to determine caspase 3 expression.

**Immunoprecipitation.** Cell lysates were obtained as described and further processed at 4°C. Lysates (250 μg) were brought up to 1 ml with hypotonic lysis buffer and incubated, with shaking, with 20 μl of protein A-agarose (Santa Cruz Biotechnology). After centrifugation (5 min in a microcentrifuge), the supernatant was transferred to a new tube and incubated overnight with 1 μg of anti-caspase 3 (Santa Cruz Biotechnology), followed by an additional 2-h incubation with 20 μl of protein A-agarose. The agarose beads were washed three times by repeated centrifugation and resuspension in PBS containing 1 mM DTT, 0.5% NP40, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin. After the final wash, the beads were resuspended in 25 μl of SDS-loading buffer [100 mM Tris-HCl (pH 6.8), 4 mM EDTA, 20% glycerol, 4% SDS, 0.01% bromphenol blue, and 5% β-mercaptoethanol], boiled for 5 min, and centrifuged, and the supernatant was subjected to 15% SDS-PAGE.

**Results**

**Determination of ROS.** To determine whether 4-HPR treatment induced the formation of ROS, we used two probes, DCFH-DA and lucigenin, to measure intracellular hydrogen peroxide and superoxide, respectively. A 1-h treatment of HL-60 cells with 1, 3, 5, and 10 μM 4-HPR increased hydroperoxide levels by 170%, 270%, 540%, and 617%, respectively, when compared with controls (Fig. 1A). The dose-dependent elevation in hydrogen peroxide was not matched by changes in superoxide, as measured by the lucigenin assay (data not shown).

**Treatment with Antioxidants.** To test whether the 4-HPR-elicited increase in ROS is related to its ability to induce apoptosis, we measured apoptosis-characteristic internucleosomal cleavage of DNA appearing as an additional peak in flow cytometry analysis, using cells pretreated with the antioxidants NAC or vitamin C. NAC, which had previously been reported to inhibit apoptosis (6), did not affect 4-HPR-induced DNA fragmentation, whereas pretreatment with 100 μM vitamin C abolished the appearance of the sub-G1 peak, which is associated with cells undergoing apoptosis (Fig. 1B).

**Activation of Caspase 3 by 4-HPR.** Previous studies in our laboratory have demonstrated the specific cleavage of the DNA repair enzyme PARP after treatment with 4-HPR (7). Also, PARP processing and apoptosis were similarly inhibited by the caspase 3 inhibitor DEVD-CHO (7, 10). These studies suggested the involvement of caspase 3, known to be critically involved in the execution phase of apoptosis, in the mechanism of 4-HPR. To test this possibility, activation of this enzyme was monitored by Western blot analysis and activity assays. Conceivably, the generation of active caspase-3 would be accompanied by the conversion of the 32-kDa procaspase 3 into p17 and p10 subunits. As shown in Fig. 2A, Western blot analysis revealed the appearance of the p17 subunit at 6–9 h after treatment with 4-HPR, which is absent in control samples. Incubation of cell extracts from control cells and cells treated with the caspase 3 color-
metric substrate DEVD-pNA showed that 9–12-h treatment with 4-HPR resulted in a 3.6-fold increase in caspase activity when compared with control samples (Fig. 2A) or 10 mM NAC, followed by the addition of 4-HPR, and caspase 3 activity was measured 9 h thereafter. Neither antioxidant affected the increase in caspase activity elicited by 4-HPR (Fig. 3).

New Synthesis and Stability of Caspase 3. The conversion of procaspase 3 into its active subunits should accompany decrease of the p32 precursor. Quantification of the caspase 3 zymogen, however, showed no significant change in its expression between control and 4-HPR-treated cells (Fig. 4A, bottom panel). To further explore the mechanisms that underlie the precursor/product relationship of caspase 3, we evaluated its de novo synthesis. In these experiments, cells were incubated with [35S]methionine/cysteine, and procaspase 3 was immunoprecipitated at the time points indicated. An increase in time of labeling resulted in a proportionately greater incorporation of radioactive precursor amino acids into immunoprecipitated procaspase 3 in control samples (Fig. 4A, top panel). In 4-HPR-treated samples, little to no increase in labeling of procaspase 3 was observed.

**Fig. 2.** 4-HPR treatment induces caspase 3 activity. A, the processing of the procaspase was monitored by Western blot analysis. The arrow indicates the p17 form of caspase 3. B, caspase activity was measured at the time points indicated in control and treated cells. Time-matched control values were set at 1. Data represent the mean ± SD from three separate experiments.

**Fig. 3.** Pretreatment with antioxidants does not affect caspase activity. Cells were pretreated for 3 h with 100 μM vitamin C or 10 mM NAC, and caspase activity was measured 9 h after the addition of 4-HPR. Results are the mean ± SD from three separate experiments.

**Fig. 4.** 4-HPR increases caspase 3 protein stability. A, decreased synthesis of procaspase 3 in response to 4-HPR. Top panel, autoradiograph of immunoprecipitated and SDS-PAGE-separated 35S-labeled procaspase 3. Western blot analysis of the same membrane shown in the bottom panel illustrates that the expression of total procaspase remained unchanged as a result of treatment with 4-HPR. B, the decay of labeled caspase 3 over the course of 9 h in control cells. Western blot analysis was performed on the same membranes used for autoradiography to determine total protein expression. Quantification was performed using Jandel Scientific. C, labeled caspase 3 remaining after 6 h of treatment, with control values set at 100.
over the same period of labeling; at all time points assayed, labeling of procaspase 3 in 4-HPR-treated samples was significantly less than that in control samples (Fig. 4A, top panel). Analysis of the steady-state level of procaspase 3 by Western blot analysis shows that its expression remained unchanged over the same duration (Fig. 4A, bottom panel).

We next considered whether 4-HPR affected caspase 3 stability. To test this possibility, cells were labeled with [35S]methionine/cysteine, washed, and chased with unlabeled media. The decrease in immunoprecipitated procaspase 3 was monitored over a 9-h chase. As expected, the amount of label in procaspase 3 decreased over time of chase in control samples (Fig. 4B). Unexpectedly, 4-HPR-treated samples had significantly greater amounts of labeled procaspase 3 remaining 6–9 h after the chase (Fig. 4C). Together, these results suggest that 4-HPR affects procaspase 3 stability but not de novo synthesis.

Discussion

ROS are important mediators and regulators of apoptosis. Various apoptosis stimuli such as tumor necrosis factor, irradiation, and chemicals such as etoposide increase the levels of intracellular ROS (13, 14). Apoptosis can be abolished by antioxidants (15, 16) and induced by treatment with ROS such as H₂O₂ (17, 18).

In agreement with a previous report (6), we show in this study that 4-HPR elicited a concentration-dependent elevation of hydroperoxides (Fig. 5A). Moreover, 4-HPR-induced apoptosis is blocked by prior treatment of cells with the antioxidant vitamin C (Fig. 1B) and not NAC, as has been reported previously by others (6). Thus, although the involvement of ROS in 4-HPR-induced apoptosis appears to be cell line specific (5–8), in HL-60 cells, it is likely to play a critical role in mediating 4-HPR-induced apoptosis.

To further investigate the integral link between caspase 3 and induction of apoptosis by 4-HPR, we monitored both the expression and activity changes of caspase 3. Treatment with 4-HPR and not RA led to the processing of p32 caspase 3 into a p17 fragment (Fig. 2A), which was paralleled by increased caspase activity at 9–12 h post-treatment (Fig. 2B). Pretreatment with the antioxidant vitamin C inhibited apoptosis (Fig. 1C) but had no effect on 4-HPR-induced caspase activity (Fig. 3). These results suggest that caspase activation is mechanistically distinct from increased intracellular per-oxides. Although these events appear to involve separate mechanisms, both mediate the induction of apoptosis because inhibition of either pathway inhibited DNA fragmentation. Fig. 5 summarizes our hypothesis that 4-HPR elicits apoptosis in responsive cells both by ROS-dependent and ROS-independent mechanisms. We further suggest that the latter mechanistic scheme links the elevation in ceramide (7) to subsequent caspase activation, culminating in PARP processing and the eventual establishment of apoptosis. Therefore, 4-HPR as well as other agents can elicit several pathways simultaneously, which ultimately can lead to apoptosis. Whether there is a convergence of the signaling events remains to be determined.

An important contribution of this study was an understanding of the modulation of caspase 3 by 4-HPR. Despite the critical roles that caspases play in the apoptosis cascade, very little is known about the regulation of this family of proteins. Various pathways leading to proteolytic activation of the zymogen have been described. One pathway of caspase activation involves receptor-mediated recruitment of adaptin protein, leading to subsequent activation of caspase 8. Another pathway requires mitochondria-to-cytoplasmic relocation of cytochrome c, followed by caspase 9 activation (19). Considerably less is known about the regulation of the enzymatic activity, although in recent studies, modification by phosphorylation has been proposed to regulate the active caspase (20).

In this study, 4-HPR treatment led to the cleavage of caspase 3 into its p17 form, and, as expected, this resulted in increased enzymatic activity. However, there was no observable difference in the expression of the p32 zymogen. Evaluation of new protein synthesis and procaspase 3 stability using labeled amino acids revealed decreased protein turnover in response to 4-HPR. These results suggest an additional level of caspase control through the regulation of protein turnover. The relative levels of the enzyme may play a role in its activation. Studies have demonstrated that increasing the local concentration of caspases through chemically induced dimerization or overexpression results in caspase activation independent of apoptotic stimuli (21, 22). Therefore, decreasing turnover of the procaspase may allow the concentration of the zymogen to remain unchanged, although there is processing of caspase 3 into the active p17 form. To our knowledge, this is the first study that demonstrates increased caspase 3 stability in cells undergoing apoptosis and raises the possibility of yet another mechanism, namely, one involving increased protein stability, for effecting caspase activation. The mechanism by which 4-HPR affects protein turnover warrants further investigation.

Fig. 5. Proposed mechanism of 4-HPR-induced apoptosis in HL-60 cells. Treatment with 4-HPR increases ceramide levels and caspase 3 activity in HL-60 cells. Inhibition of either ceramide elevation or caspase activity prevents the characteristic DNA fragmentation of cells undergoing apoptosis. 4-HPR-induced ROS appears to be distinct from this mechanism because pretreatment with antioxidants, which effectively blocked apoptosis, had no effect on caspase activity.

References


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