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Reactive Oxygen Species Participate in Peroxynitrite-Induced Apoptosis in HL-60 Cells

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Peroxynitrite ($ONOO^-$) is a physiological product generated by the interaction of superoxide ($O_2^{\cdot-}$) and nitric oxide ($^{\cdot}NO$). We have previously shown that peroxynitrite induces apoptosis in HL-60 cells. In the present study, we demonstrated that peroxynitrite generates reactive oxygen species (ROS) in HL-60 cells. Brief exposure of HL-60 cells to $ONOO^-$ induced elevation of lucigenin chemiluminescence, indicating generation of superoxide anion. Exogenous superoxide dismutase (SOD), a scavenger of $O_2^{\cdot-}$, fully abolished the chemiluminescence response, further supporting this notion. Following $O_2^{\cdot-}$ generation, the accumulation of hydrogen peroxide (H_2O_2) was observed. The addition of SOD exacerbated but that of catalase attenuated peroxynitrite-induced DNA fragmentation, suggesting that this H_2O_2 production contributes to the apoptotic process. In addition, pre-treatment of HL-60 cells with N-acetyl-L-cysteine (15 mM), a ROS scavenger, fully scavenged peroxynitrite-elicited ROS generation and effectively inhibited $ONOO^-$-induced apoptosis, further enforcing this hypothesis. In summary, our results suggest that $ONOO^-$-stimulated ROS formation may serve as a mechanism for the propagation of peroxynitrite-mediated apoptotic cell death in an intact cell system.

With the recognition that peroxynitrite ($ONOO^-$), a reaction product of nitric oxide ($^{\cdot}NO$) and superoxide anion ($O_2^{\cdot-}$), is an endogenous mediator of various forms of tissue injury (1-5), the biochemical mechanisms by which peroxynitrite elicits its cytotoxicity are attracting considerable attention and investigations. As a strong oxidant, peroxynitrite can oxidize protein and non-protein sulfhydryls (6), initiate lipid peroxidation (7), inactivate enzymes involved in the mitochondrial electron transport chain (8), and cause DNA breakage (9). Recently, we demonstrated that $ONOO^-$ induces apoptosis in human leukemia HL-60 cells in a concentration- and time-dependent manner (10). While free radical intermediates have been proposed and detected in peroxynitrite-mediated oxidation of substrates in vitro (11), the response of the intact cell to peroxynitrite is more com-plicated and the intracellular sites and action of peroxynitrite have not been identified. The precise mechanism involved in peroxynitrite-induced apoptosis is still unclear. In this report, we demonstrated that a brief exposure of HL-60 cells to peroxynitrite, triggered superoxide anion generation and consequently caused the accumulation of H_2O_2. This H_2O_2 formation may amplify the peroxynitrite-induced oxidative damage through secondary antioxidant-depletion oxidative stress and contribute to the peroxynitrite-induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Cell culture. The human promyelocytic leukemia HL-60 cell line was purchased from ATCC and grown in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotic/antimycotics (10). All cells were maintained at a density of 0.2-1 $\times$ 10^6 cells/ml in a culture incubator at 37°C, under a 5% CO_2 humidified atmosphere. Experiments were conducted during the exponential phase of growth. Cell counts were performed on a routine basis to maintain low population density, and viability was examined by trypan blue exclusion.

LDH release. Cell viability was also determined by measuring lactate dehydrogenase (LDH) activity in the cell media as described by Vassault (12). Briefly, cells were harvested, and centrifuged at indicated time. The pellet was lysed with 1% (v/v) NP-40 in 50 mM Tris-HCl buffer (pH 7.5). The percentage of LDH activity was calculated from the ratio of LDH activity in the supernatant to the total.

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Abbreviations used: $^{\cdot}NO$, nitric oxide; ONOO, peroxynitrite; $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; D-PBS, Dulbecco's phosphate-buffered saline; LDH, lactate dehydrogenase; GSH, reduced glutathione; TCA, trichloroacetic acid; NAC, N-acetyl-L-cysteine; SOD, superoxide dismutase; ROS, reactive oxygen species; DDC, diethyldithiocarbamate; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; lucigenin, bis-N-methylacridinium nitrite.

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Preparation of peroxynitrite. Peroxynitrite was prepared from H_2O_2 and 2-methoxyethyl nitrite as described in our previous report (10).

Peroxynitrite treatment. Cell suspensions were treated with various concentrations of peroxynitrite as previously described (10,13). Briefly, HL-60 cells were suspended in 5 ml of Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4 without Ca^{2+} and Mg^{2+}, Life Technologies, Inc.) (1×10^6 cells/ml). Various stock solutions of peroxynitrite were freshly prepared in 0.1 N NaOH to achieve the indicated concentration. Five μl of each stock was added to separate cell suspensions and incubated for 10 min at 37°C. Control and vehicle incubations were carried out by exposing cell suspensions to 5 μl of D-PBS and 0.1 N NaOH, respectively. To ascertain that the observed effect was actually due to peroxynitrite but not secondary to trace residual synthetic reagents or its decomposition products, the cells were resuspended in the buffer in which indicated concentration of peroxynitrite was added 2 min (i.e. to ensure the complete decomposition of $ONOO^-$) prior to the treatment. The exogenous SOD (100 U/ml), catalase (200 U/ml) or both were added into the cell suspensions immediately after the peroxynitrite treatment and were also present in the subsequent culture condition. For NAC pre-treatment, HL-60 cells were pretreated with 15 mM of NAC for 3 h, and then washed twice with D-PBS priot to the peroxynitrite treatment. NAC-containing medium was titrated to pH 7.2 with 0.5 N NaOH priot to the pretreatment.

Measurement of reactive oxygen species (ROS). Generation of reactive oxygen species was measured by using an oxidation-sensitive fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which oxidized form (2′,7′-dichloro-fluorescein, DCF) is highly fluorescent (14). After washed with D-PBS twice, HL-60 cells were resuspended in 5 ml of D-PBS (1×10^6/ml) and treated with indicated concentrations of $ONOO^-$ or H_2O_2 for 5 min at 37°C. Then, the cells were centrifuged and resuspended in Krebs buffer (pH 7.4; NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 1.5 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 1.1 mM; KH_2PO_4, 1.2 mM). DCFH-DA (final concentration: 1 μg/ml) was added into each well following cells (1×10^6/ml/well) were plated in a 24-well plate (Falcon). Plates were kept at dark and read on a Cytofluor 2350 plate reader (PerSeptive Biosystems, Inc. MA) at indicated time points, with an excitation wavelength of 485 nm and an emission wavelength of 530. Usually, 30 min interval was needed from peroxynitrite treatment to DCFH-DA addition.

Measurement of superoxide anion. Generation of $O_2^{\cdot-}$ was measured by chemiluminescence probe, bis-N-methyl-acridinium nitrite (lucigenin, Sigma) (15,16). The chemiluminescence of lucigenin appears to be associated with the $O_2^{\cdot-}$-mediated generation of excited N-methylacridone, via a stepwise univalent reduction of lucigenin to the corresponding cation radical which then reacts with $O_2^{\cdot-}$ to yield a dioxetane intermediate. The dioxetane intermediate consequently disintegrates yielding one ground and one excited state of N-methylacridone which will lead to light emission (15). The chemiluminescence elicited by superoxide in the presence of lucigenin was measured using a Mark 5303 scintillation counter (TM Analytic, Elk Grove Village, IL) (17). HL-60 cells were resuspended in 5 ml of D-PBS (1×10^6/ml) and treated with indicated concentrations of $ONOO^-$ or H_2O_2 for 2 min at 37°C. The cells were centrifuged, resuspended in Krebs buffer and, then, transferred to plastic scintillation vials. After the addition of lucigenin (final concentration: 0.25 mM), the vials were counted at indicated time and continuously for an additional 90 min. Usually, 25 min interval was needed from peroxynitrite treatment to lucigenin addition. The zero time is the time of lucigenin addition. In case of exogenous SOD treatment, SOD was added immediately after $ONOO^-$ addition, and was also present in the Krebs buffer.

DNA fragmentation and quantitative assay. The extent of DNA fragmentation was determined by a method adapted from that of Sellins and Cohen (18). The cell pellets were lysed with 0.3 ml hypotonic lysing buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 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% trichloroacetic acid (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 (19). The percentage of fragmentation was calculated from the ratio of DNA in the supernatant to the total DNA.

Statistical analysis. Experimental results are reported as means ± SEM. Significance of difference between means was analyzed by unpaired two-tailed Student's t-test for two-sample comparisons (Sigma Stat program, Jandel Scientific).

RESULTS AND DISCUSSION

Treatment of HL-60 cells with peroxynitrite, resulted in dose-dependent increase of ROS as measured by the fluorescent DCF (Fig 1A). The DCF fluorescence did not increase, instead, it slightly decreased when cells were treated with H_2O_2 under the same experimental conditions. Interestingly, when HL-60 cells were pretreated with diethyldithiocarbamate (DDC), a specific inhibitor of SOD (20), the $ONOO^-$ elicited elevation of DCF fluorescence was completely abolished (Fig. 1B). This effect was not due to DDC itself competing with DCFH for ROS (data not shown). These results raise the possibility that peroxynitrite-mediated ROS formation initially comes from the generation of superoxide. Since, the ROS detected by oxidation of DCFH was mostly H_2O_2 and hydroxyl radical ($^{\cdot}OH$) but not $O_2^{\cdot-}$ (14), we employed a highly specific chemiluminescence probe, lucigenin (15-17), to test whether superoxide anion is formed by the peroxynitrite treatment. As shown in Figure 2, the generation of superoxide anion in HL-60 cells detected by the lucigenin chemi-luminescence, dose-dependently increased after cells were exposed to peroxynitrite. Peroxynitrite-elicited superoxide anion generation was completely abolished by exogenous SOD, a scavenger of $O_2^{\cdot-}$, which was added into the cell suspension immediately after peroxynitrite treatment. In contrast, the increase of $O_2^{\cdot-}$ was not observed after 100 μM of H_2O_2 treatment under the same experimental conditions, indicating that the effect is specific for peroxynitrite. This notion was further supported by the failure of inducing $O_2^{\cdot-}$ generation after treatment of cells with decomposed peroxynitrite (100 μM). Taken together, these results clearly indicated that exposure of HL-60 cells to peroxynitrite evoked the $O_2^{\cdot-}$ generation.

The increase of superoxide anion in biological systems can elicit the following responses. 1) $O_2^{\cdot-}$ can directly exert detrimental effects, such as inactivation of catalase and peroxidase (21); 2) $O_2^{\cdot-}$ can rapidly interact with $^{\cdot}NO$ to form $ONOO^-$ in conditions when large amounts of $^{\cdot}NO$ are produced (1-5, 22-24); 3) $O_2^{\cdot-}$ can be dismutated by SOD to form H_2O_2 (21). The release of H_2O_2 may not only serve as a second messenger (25) due to its stable and freely diffusible properties but also participate with $O_2^{\cdot-}$ and subsequently engender the highly toxic species, $^{\cdot}OH$, by iron-catalyzed Haber-

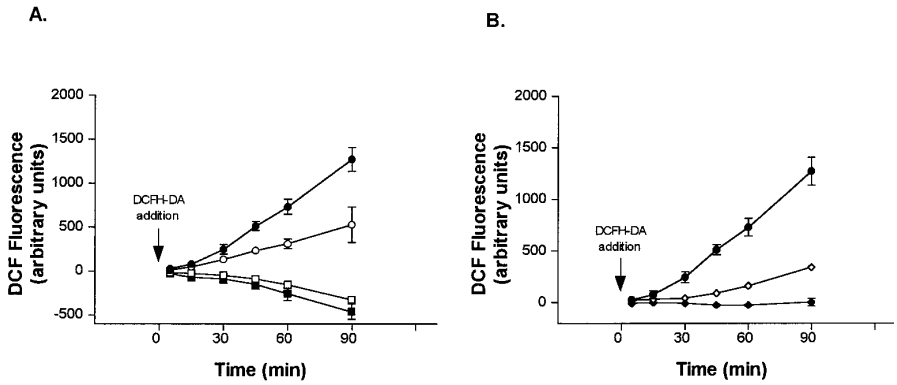

FIG. 1. Peroxynitrite stimulates the generation of reactive oxygen species in HL-60 cells. (A) Exponentially growing HL-60 cells were exposed to various concentrations of peroxynitrite or hydrogen peroxide as described in "Materials and Methods". The relative rate of increase of DCF fluorescence was obtained by subtracting the DCF fluorescence elicited by vehicle from that elicited by indicated concentrations of peroxynitrite (100 μM,●; 50 μM,○) or hydrogen peroxide (10 μM, □; 100 μM, ■) at each indicated time point, respectively. (B) HL-60 cells were pretreated with 50 μM (◇) or 100 μM (◆) of DDC for 1 h prior to 100 μM of peroxynitrite treatment as described in A. Points are mean ± SEM (n=4).

Weiss reaction (21). To demonstrate the contribution of ROS elicited by $ONOO^-$ to the $ONOO^-$-induced apoptotic cell death, exogenous SOD (100 u/ml), catalase (200 u/ml) or both SOD and catalase (SOD/catalase) were added into the cell suspensions immediately after the addition of 100 μM $ONOO^-$. The same amount of SOD, catalase or SOD/catalase were also resupplemented into the culture medium after the washout of post-treated $ONOO^-$. As shown in Figure 3, $ONOO^-$ caused significant DNA fragmentation, an indication of the apoptotic cell death, but decomposed peroxynitrite had no apoptotic effect in HL-60 cells at 4 h after treatment. The addition of catalase or SOD/catalase significantly attenuated the peroxynitrite-induced apoptotic effect. In contrast, the addition of exogenous SOD did not block, instead, slightly exacerbated the peroxynitrite-induced apoptosis. These results indicated that H_2O_2 formation produced from the dismutation of $ONOO^-$-elicited superoxide anion contributes to apoptosis in HL-60 cells. To further test this hypothesis, HL-60 cells were pretreated with cell permeable ROS scavenger, N-acetyl-cysteine (15 mM, 3 h). NAC completely abolished the $ONOO^-$-mediated DCF fluorescence (Fig. 4A). Associated with the effect of scavenging ROS, NAC attenuated the apoptotic response of peroxynitrite in HL-60 cells as determined by DNA fragmentation assay (Fig. 4B) and LDH release assay

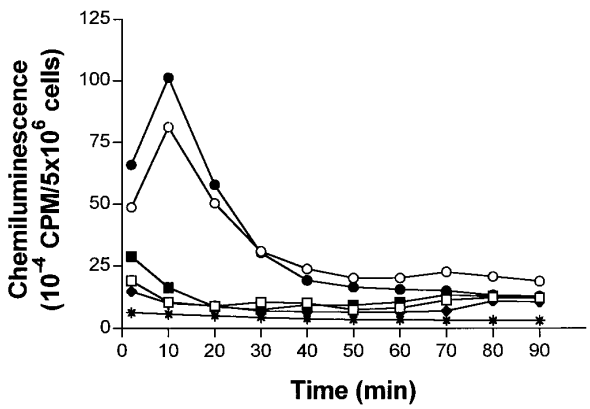

FIG. 2. Peroxynitrite induces superoxide anion generation in HL-60 cells. After exposure of HL-60 cells to vehicle (■), decomposed peroxynitrite (100 μM, □), 100 μM H_2O_2 (◆), peroxynitrite (100 μM, ●; 50 μM, ○) or 100 μM of peroxynitrite in the presence of SOD (100 U/ml) (*) for 2 min at 37°, chemiluminescence of lucigenin was recorded at indicated time. Figure shown is a representative of five separate reproducible results.

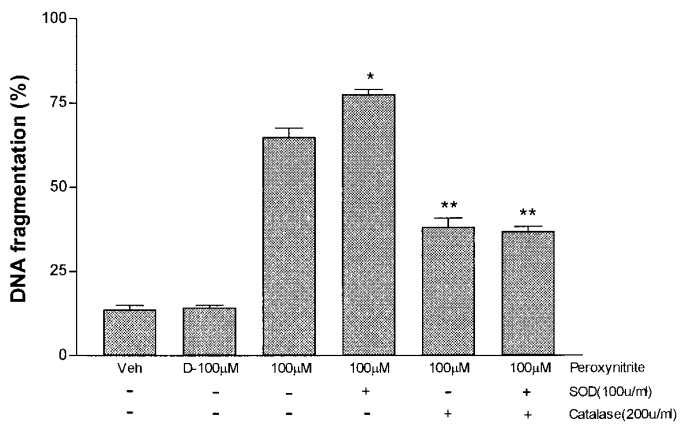

FIG. 3. Effects of exogenous SOD or catalase on peroxynitrite-induced apoptosis in HL-60 cells. Exogenous SOD, catalase or combination of both were added into cell suspension immediately after exposure of HL-60 cells to 100 μM of peroxynitrite, and subsequently supplemented in the post-treatment culture media for an additional time. Cells were harvested 4 h after $ONOO^-$ addition, DNA fragmentation was quantified by diphenylamine assay as described in "Materials and Methods". D-100 μM: decomposed 100 μM of peroxynitrite. (* $p < 0.05$; ** $p < 0.001$ *vs.* treatment of HL-60 cells with 100 μM peroxynitrite alone).

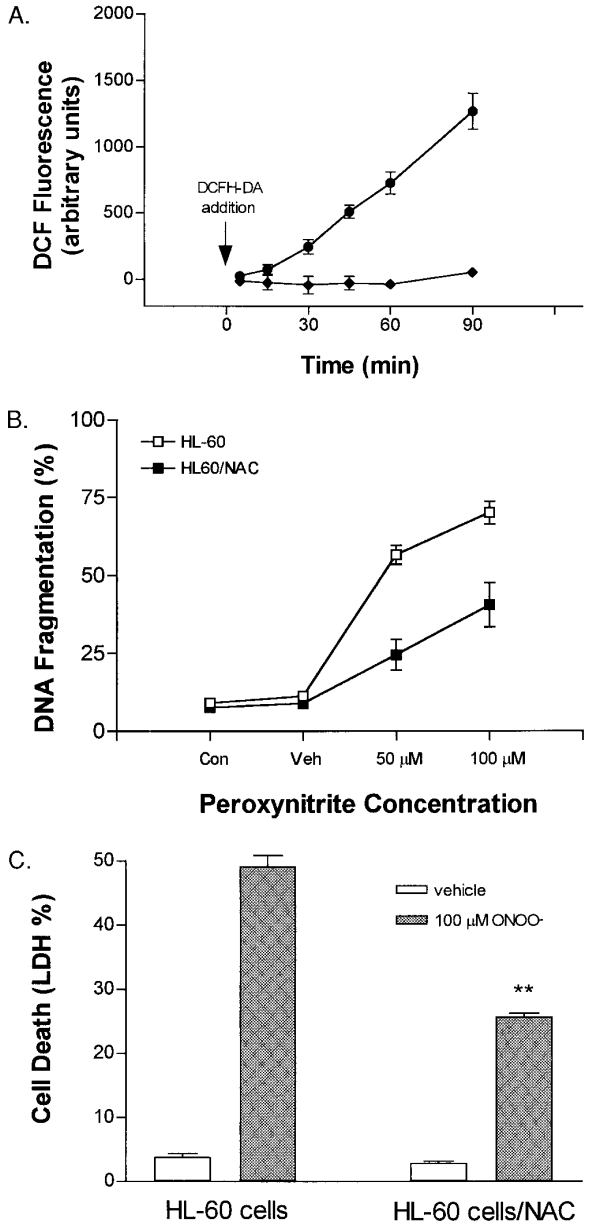

FIG. 4. Influence of NAC on peroxynitrite-induced apoptosis. (A) ROS generation: HL-60 (●)or NAC-pretreated HL-60 (♦) cells were exposed to 100 μM of peroxynitrite for 5 min and DCF fluorescence was read on a Cytofluor 2350 plate reader as described in figure 1. Values are means ± SE, n=4. (B) DNA fragmentation: Cells were harvested 5 h after peroxynitrite exposure, and DNA fragmentation was quantified as described in figure 3. Results of incubations carried out with cells exposed to D-PBS or 0.5 N NaOH were labeled Con or Veh, respectively. (□) HL-60 without NAC pretreatment; (■) HL-60 cells pretreated with 15 mM of NAC for 3 h. (C) After exposure of HL-60 cells pretreated with or without NAC to 100 μM of peroxynitrite, Cells were harvested at 24 h and LDH release was determined as described in the "Materials and Methods" (** $p < 0.001$ *vs.* exposure of HL-60 cells without NAC to 100 μM of peroxynitrite).

(24 hr, Fig 4C). Taken together, these results indicated that the generation of ROS elicited by $ONOO^-$ contributes to the peroxynitrite-induced apoptosis in HL-60 cells. This ROS formation may amplify the peroxynitrite induced oxidative damage through secondary antioxidant-depletion oxidative stress.

ROS are naturally produced in biological systems and their concentrations are tightly controlled by endogenous antioxidant systems including superoxide dismutase, catalase, glutathione peroxidase and others. When the cellular level of ROS exceeds the capacity of natural antioxidative defense system, the condition is known as "oxidative stress" (26). This oxidative stress can be aggravated when the cell encounters a secondary oxidative attack, that further depletes the antioxidant protection and exacerbates cell damage. We postulate that peroxynitrite-induced superoxide generation in HL-60 cells can initiate a cyclic mechanism for the propagation of apoptotic cell death. Since peroxynitrite has already compromised the cell's antioxidant defense, therefore, the peroxynitrite-triggered generation of superoxide anion can extend and amplify oxidative stress and apoptotic cell death by either interacting with nitric oxide to generate additional peroxynitrite or dismutating to H_2O_2 through SOD. In our experimental system, H_2O_2 appeares to be the predominant secondary oxidant in $ONOO^-$-elicited apoptotic cell death. This notion is supported by our observation that SOD potentiated and SOD/catalase attenuated the $ONOO^-$-induced apoptotic cell death. However, in pathophysiological conditions, such as in active sites of inflammation sites or ischemic-reperfusion injury, nitric oxide synthesis was greatly increased (22-24). Therefore, the cyclic propagation of the strong oxidant peroxynitrite due to superoxide generation may aggravate the existing oxidative stress.

It should be noted that the cell generated superoxide anion is the major chemical species of lucigenin chemiluminescence. There was no direct interaction between peroxynitrite and lucigenin under the present experimental condition. Furthermore, HL-60 cells exposed to decomposed $ONOO^-$ failed to induce chemiluminescence (Fig. 2). The fact that chemiluminescence of lucigenin can be inhibited by SOD further proves that the superoxide anion is a major intermediate for this chemi-excitation. Superoxide anion can be produced in eukaryotic cells via several biological pathways. In neutrophils and other leukocytes, a membrane bound multicomponent enzyme complex, NADPH oxidase, is the major source of $O_2^{\cdot -}$ generation (27). In other eukaryotic cells, superoxide anion sometimes leaks out from the damaged electron transfer reactions in mitochondria and the endoplasmic reticulum (28). Because of its distinct chemistry, peroxynitrite may activate these two enzymatic pathways and produce $O_2^{\cdot -}$ as we have observed. Additional mechanisms could also be responsible for generating $O_2^{\cdot -}$. These possible mechanisms include: 1) interaction of peroxynitrite with cellular bicarbonate to produce $O_2^{\cdot -}$ (29); 2) interaction of peroxynitrite with GSH or thiol-containing compounds

to initiate self-sustaining free radical chain reactions which form superoxide anion in the presence of molecular oxygen (11,30). Our observation that Bcl-2 expressing HL-60 cells which exhibit GSH levels 1.5 fold higher than that of normal HL-60 cells (31) produced higher level of superoxide anion than that of normal HL-60 cells (unpublished results), suggests that the latter pathway may contribute to the generation of $O_2^{\cdot -}$. In this study, we presented evidence suggesting that, in an intact cell system, peroxynitrite can mediate free-radical generation and inhibition of these free-radical generation can attenuate apoptotic cell death.

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