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The effect of α-phenyl-*N*-*t*-butylnitrone on ionizing radiation-induced apoptosis in U937 cells

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Abstract
Ionizing radiation induces the production of reactive oxygen species (ROS), which play an important causative role in apoptotic cell death. α-Phenyl-*N*-*t*-butylnitrone (PBN) is one of the most widely used spin-trapping compounds for investigating the existence of free radicals in biological systems. We investigated the effects of PBN on ionizing radiation-induced apoptosis in U937 cells. Upon exposure to 2 Gy of γ-irradiation, there was a distinct difference between the control cells and the cells pre-treated with 2 mM PBN for 2 h in regard to apoptotic parameters, cellular redox status, mitochondria function and oxidative damage to cells. PBN effectively suppressed morphological evidence of apoptosis and DNA fragmentation in U937 cells exposed to ionizing radiation. The [GSSG]/[GSH + GSSG] ratio and the generation of intracellular ROS were higher and the [NADPH]/[$NADP^+$ + NADPH] ratio was lower in control cells compared to PBN-treated cells. The ionizing radiation-induced mitochondrial damage reflected by the altered mitochondrial permeability transition, the increase in the accumulation of ROS, and the reduction of ATP production were significantly higher in control cells compared to PBN-treated cells. PBN pre-treated cells showed significant inhibition of apoptotic features such as activation of caspase-3, up-regulation of Bax and p53, and down-regulation of Bcl-2 compared to control cells upon exposure to ionizing radiation. This study indicates that PBN may play an important role in regulating the apoptosis induced by ionizing radiation presumably through scavenging of ROS.

Keywords: *Ionizing radiation, apoptosis, antioxidant, cellular redox status, reactive oxygen species*

Introduction

The damaging effect of ionizing radiation on living cells is predominantly due to reactive oxygen species (ROS) including O_2^-, $^{\cdot}OH$, and H_2O_2, generated by the decomposition of water. The secondary radicals formed by the interaction of $^{\cdot}OH$ with organic molecules may also be of importance [1,2]. These oxygen free radicals have the potential to damage critical cellular components such as DNA, proteins and lipids and eventually results in physical and chemical damage to tissues that may lead to cell death or neoplastic transformation [3]. In many cases, ionizing radiation-induced cell death has been identified as apoptosis [4,5].

As ROS appear to be mediators of the apoptotic pathways induced by ionizing radiation, compounds that regulate the fate of such species may be of great importance in the regulation of cells against radiation-induced apoptosis. α-Phenyl-*N*-*t*-butylnitrone (PBN) is one of the most widely used spin-trapping compounds for investigating the existence of free radicals in biological systems. PBN reverses the age-related oxidative changes in the brains of old gerbils [6], delays senescence in senescence-accelerated mice and normal mice [7,8], and it alleviates oxidative damage from ischemia/reperfusion injury [9]. This phenomenon was accounted for by the fact that PBN protected biologically important molecules from oxidative damage by efficiently trapping ROS,

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including O_2^- [10]. From a study concerning the tissue distribution, excretion and metabolism of PBN, it was shown that this is rapidly absorbed, widely distributed inside the body and remains for a long period in many tissues when injected i.p. into rats [11].

In the present study, we investigated the effect of PBN in the ionizing radiation-induced apoptosis using the U937 cells. There is mounting evidence that human monocytic U937 cells are highly susceptible to many types of stresses. They also have variety of functions against external stresses. PBN-treated and untreated U937 cells were expected to exhibit differences in sensitivity to the ionizing radiation-induced apoptosis. To determine if such differences exist between cells treated and untreated with PBN, morphological evidence of apoptosis, DNA fragmentation, cellular redox status, mitochondrial dysfunction, oxidative damage to cells and changes in apoptotic pathway proteins and p53 were examined upon their exposure to ionizing radiation. This study indicates that PBN may play an important role in regulating the apoptosis induced by ionizing radiation.

Materials and methods

Materials

PBN, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), pyrogallol, xylenol orange, propidium iodide (PI), avidin-conjugated tetramethylrhodamine isothiocyanate (TRITC), 4′6-dianidino-2-phenylindole (DAPI), anti-rabbit IgG TRITC conjugated secondary antibody and anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugated secondary antibody were obtained from Sigma Chemical Co. (St Louis, MO, USA). Anti-human 4-hydroxynonenal (HNE)-Michael adduct antibody and anti-human dinitrophenyl (DNP) antibody were obtained from Calbiochem (La Jolla, CA). 2′,7′-dichlorofluoroscin diacetate (DCFH-DA), diphenyl-L-pyrenylphosphine (DPPP), dihydrorhodamine (DHR) 123 and rhodamine 123 were purchased from Molecular Probes (Eugene, OR, USA). Antibodies against Bcl-2, Bax, lamin B, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP) and p53 were purchased from Santa Cruz (Santa Cruz, CA, USA).

Cell culture

Human premonocytic U937 cells (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37°C in a 5% CO_2/95% air humidified incubator. After culture for confluence optimization, 2 mM PBN were applied to the cells and cells were incubated for additional 2 h at 37°C. PBN was prepared in 0.1% ethanol and then diluted 100-fold in complete media. To control for 0.1% ethanol in the pretreatment, a control group of cells were incubated in fresh complete media with 1/100 vol of 0.1% ethanol for 2 h. After incubation, cells were irradiated at room temperature with ^{137}Cs source at a dose rate of 1 Gy/min.

Apoptosis assay

DAPI staining was used for apoptotic nuclei determination. U937 cells were collected at 2000*g* for 5 min, washed once with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, and stained with 0.8 mg/ml DAPI in the dark state [12]. The morphological changes of apoptotic cells were analyzed by the Zeiss Axiovert 200 microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). ApoAlert Annexin V apoptosis detection system (CLONTECH, Palo Alto, CA, USA) was used to measure the relative distribution of apoptotic and necrotic cells. The cell suspension was double-stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Cell membrane permeability was determined by YO-PRO-1/PI staining and flow cytometry. Cytoplasmic histone-associated DNA fragments were measured according to the instruction manual of the Cell Death Detection ELISA plus kit (Roche, Mannheim, Germany).

DNA fragmentation

DNA fragmentation in U937 cells was evaluated by TdT-mediated dUTP nick end labeling (TUNEL) assay. After the TUNEL reaction, cells were evaluated with confocal microscopy. The green fluorescence of FITC was recorded with excitation at 488 nm through a 515 nm band pass, together with the transmission image. DNA fragmentation induced by apoptosis was also assessed by the Photometric ELISA for Detection of 5 -bromo- 2 -deoxy-uridine (BrdU)-Labeled DNA Fragments (Roche, Mannheim, Germany).

Cellular redox status

NADPH was measured using the enzymatic cycling method as described by Zerez et al. [13] and expressed as the ratio of NADPH to the total NADP pool. The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\,M^{-1}\,cm^{-1}$) as described by Akerboom and Sies [14], and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine [15]. Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with fluorescence-activated cell sorter (FACS) analyses. Measurement of 2′,7′-dichlorofluorescein (DCF) fluorescence in cells was made at least

10,000 events/test using a FACS caliber flow cytometer (Becton Dickinson) with a fluorescein isothiocynate filter. Intracellular hydrogen peroxide concentrations were determined using a ferric sensitive dye, xylenol orange, as described [16].

Cellular oxidative damage

The protein carbonyl content was determined spectrophotometrically using the DNPH labeling procedure as described [17]. The protein carbonyl contents in U937 cells also determined with DNP-specific antibody (1:200 dilution) and anti-human IgG FITC (excitation, 488 nm; emission, 520 nm) conjugate (1:800 dilution) as a secondary antibody, and then fluorescence was observed using a fluorescence microscope. The lipid peroxidation in U937 cells was determined with rabbit polyclonal anti-HNE Michael adduct antibody (1:200 dilution) and anti-human IgG TRITC conjugate (1:800 dilution). Lipid peroxidation was also estimated by using a fluorescent probe DPPP as described by Okimoto et al. [18]. After U937 cells (1×10^6 cells/ml) were incubated with 5 μM DPPP for 15 min in the dark, cells were exposed to ionizing radiation. 8-Hydroxy- 2′-deoxyguanosine (8-OH-dG) levels of U937 cells were estimated by using a fluorescent binding assay as described by Struthers et al. [19]. After U937 cells were exposed to ionizing radiation, cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) for fluorescent microscope with 540 nm excitation and 588 nm emission.

Mitochondrial damage

Mitochondrial membrane permeability transition (MPT) was measured by the incorporation of rhodamine 123 dye into the mitochondria, as previously described [20]. Cells were exposed to ionizing radiation and then treated with 5 μM rhodamine 123 for 15 min and excited at 488 nm with an argon laser. FACS was used for fluorescence intensity quantification. To evaluate the levels of mitochondrial ROS U937 cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 and cells were washed, resuspended in complete growth media, and ionizing radiation was applied to the cells. The cells were then incubated for an additional 40 min. FACS was used for fluorescence intensity quantification. Intracellular ATP levels were determined by using luciferin-luciferase as described [21]. Light emission was quantitated in a Turner Designs TD 20/20 luminometer (Stratec Biomedical Systems, Germany).

Immunoblot analysis

Proteins were separated on 10–12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Quantitaion of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described [22].

Statistical analysis

The difference between two mean values was analyzed by Student's *t*-test and was considered to be statistically significant when $p < 0.05$.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

The effects of radiation and PBN on the cellular markers of apoptosis were studied to determine whether these would correlate with changes in the apoptotic pathways. When U937 cells were irradiated at the clinically relevant dose of 2 Gy, shrinkage of the cell and plasma membrane blebbing was apparently observed by light microscopy (data not shown). To assess whether these changes were attributable to apoptotic changes, nuclear morphology was assessed by fluorescence microscopy using DAPI. As shown in Figure 1A, nuclear condensation and fragmentation were apparent in U937 cells exposed to ionizing radiation. However, the cells pre-treated with 2 mM PBN for 2 h were significantly more resistant than untreated cells to radiation-induced apoptosis. PBN by itself did not induced apoptosis at this concentration. The proportion of cell death was detected by annexin V/PI staining and YO-PRO-1/PI staining. As shown in Figure 1B and C, a substantial increase in the number of apoptotic cells was observed upon exposure to 2 Gy of γ-irradiation. PBN pre-treatment decreased the population of apoptotic cells in γ-irradiated cultures. The protective effect of PBN against ionizing radiation-induced apoptosis was also confirmed by the cell death detection ELISA plus, as shown in Figure 1D.

Ionizing radiation-triggered apoptosis in U937 cells was determined by the measurement of DNA fragmentation using TUNEL assay and evaluated by confocal microscopy (Figure 2A). DNA fragmentation was further confirmed by the Photometric ELISA for Detection of 5-bromo-2-deoxy-uridine

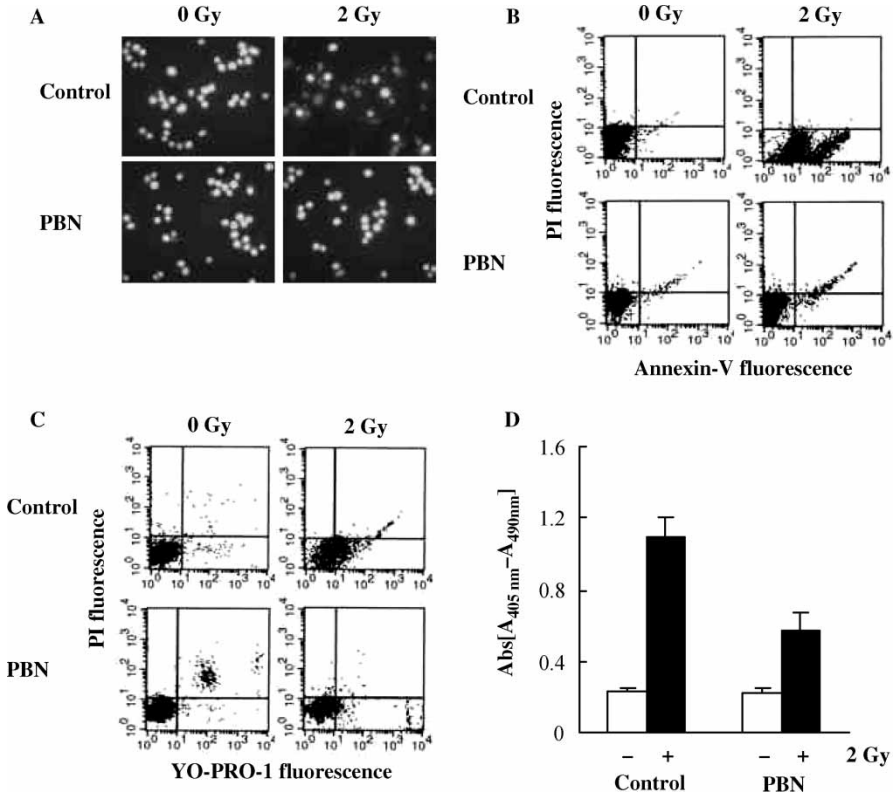

Figure 1. PBN blocks ionizing radiation-induced apoptosis. (A) Ionizing radiation-induced nuclear condensation and fragmentation in U937 cells. Cells untreated and treated with 2 mM PBN for 2 h were exposed to 2 Gy of γ-irradiation, and then harvested, fixed, permeabilized and loaded with 0.8 μg/ml DAPI for 5 min. The morphological changes of cells were analyzed by fluorescence microscopy. (B) Expression of phosphatidylserine on the plasma membrane was measured by staining cells with FITC-labeled annexin V, in conjunction with PI. Cells were analyzed by flow cytometry. (C) Cells were stained by YO-PRO-1/PI followed by flow cytometric analysis. (D) Apoptosis in U937 cells were determined using cell death detection ELISA plus kit. Each value represents the mean ± SD from five independent experiments.

(BrdU)-Labeled DNA Fragments (Figure 2B). Results of DNA fragmentation assay for U937 cells treated with 2 Gy of γ-irradiation showed that apoptosis was the main mechanism of cell death observed, since DNA fragments were present only inside the cells. The DNA fragmentation was significantly reduced in PBN-treated cells compared to untreated control cells upon exposure to ionizing radiation.

To investigate whether the difference in apoptotic cell death of U937 cells upon exposure to ionizing radiation is associated with ROS formation, the levels of intracellular peroxides in the U937 cells were evaluated by confocal microscopy with the

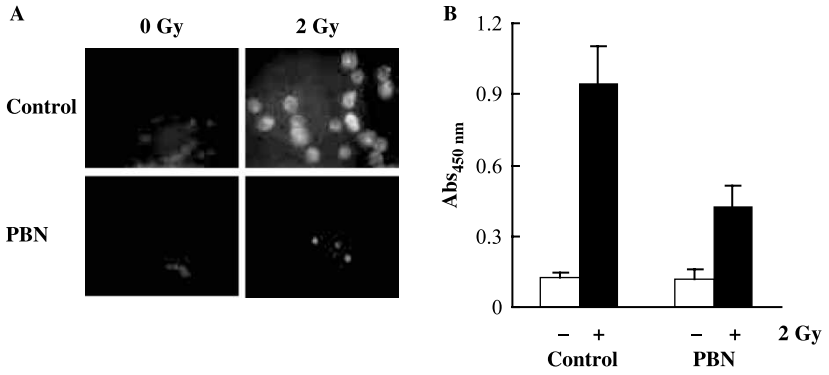

Figure 2. Effects of PBN on ionizing radiation-induced DNA fragmentation. (A) Characteristic DNA fragmentation in γ-irradiated cells determined by TUNEL assay and examined by confocal microscopy. TUNEL positive cells were shown by bright FITC staining of nuclei shown by the confocal image. (B) DNA fragments were detected by Photometric ELISA for Detection of BrdU-labeled DNA Fragments on the cell extracts from U937 cells exposed to ionizing radiation. Each value represents the mean ± SD from five independent experiments.

oxidant-sensitive probe DCFH-DA. As shown in Figure 3A, an increase in DCF fluorescence was observed in U937 cells when they were exposed to 2 Gy of γ-irradiation. The increase in fluorescence was significantly reduced in cells pre-treated with 2 mM PBN for 2 h. We also demonstrated the level of intracellular H_2O_2 in cells irradiated in the presence and absence of PBN. The pre-treatment of PBN resulted in a significantly lower intracellular level of H_2O_2 as compared to that of untreated control with the exposure of 2 Gy of γ-irradiation (Figure 3B). These data strengthen the conclusion that PBN provided protection from the ionizing radiation-induced apoptosis by decreasing the steady-state level of intracellular oxidants. One important parameter of GSH metabolism is the ratio of GSSG/total GSH (GSH_t) which may reflect the efficiency of GSH turnover. When the cells were exposed to 2 Gy of γ-irradiation, the ratio of cellular [GSSG]/[GSH_t] was significantly higher in control cells than in PBN-treated cells (Figure 3C). This data indicate that GSSG in control cells was not reduced as efficiently as in PBN-treated cells. These results suggest that decrease in the efficiency of GSH recycling may be responsible for the higher concentration of intracellular peroxides. NADPH, required for GSH generation by glutathione reductase, is an essential factor for the cellular defense against oxidative damage. The ratio for [NADPH]/[$NADP^+$ + NADPH] was significantly decreased in cells treated with 2 Gy of γ-irradiation, however, the decrease in this ratio was much less pronounced in PBN-treated cells (Figure 3D).

As indicative markers of oxidative damage to cells, the occurrence of oxidative DNA damage, protein oxidation and lipid peroxidation were evaluated. To determine whether PBN pre-treatment decreased the sensitivity to protein damage, protein oxidation was visualized by immunocytochemical method using anti-DNP antibody. As shown in Figure 4, the fluorescent intensity which reflects the endogenous levels of carbonyl groups in proteins was significantly increased when U937 cells were exposed to 2 Gy of γ-irradiation and the increase of protein oxidation was markedly reduced in PBN-treated cells. It is well established that oxidative stress in various cells usually leads to accumulation of potent, cytotoxic lipid peroxides such as malondialdehyde and HNE [23]. Lipid peroxidation was visualized by immunocytochemical method using anti-HNE antibody. As shown

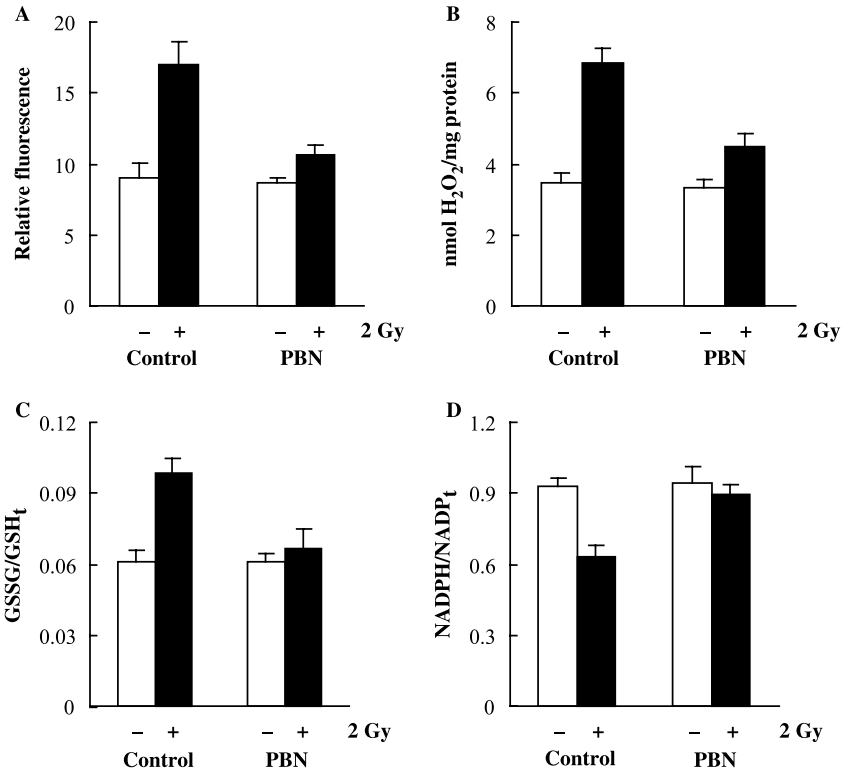

Figure 3. Effects of PBN on the cellular redox status of U937 cells exposed to ionizing radiation. (A) Measurement of *in vivo* molecular oxidation. DCF fluorescence was measured in U937 cells exposed to ionizing radiation. Fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. The averages of fluorescence intensity were calculated as described [22]. Each value represents the mean ± SD from three independent experiments. (B) Production of hydrogen peroxide in U937 cells exposed to ionizing radiation was determined by the method described under "Materials and methods". Each value represents the mean ± SD from five independent experiments. Ratio of GSSG versus total GSH pool (C) and NADPH versus total NADP pool (D) in U937 cells. Each value represents the mean ± SD from five independent experiments.

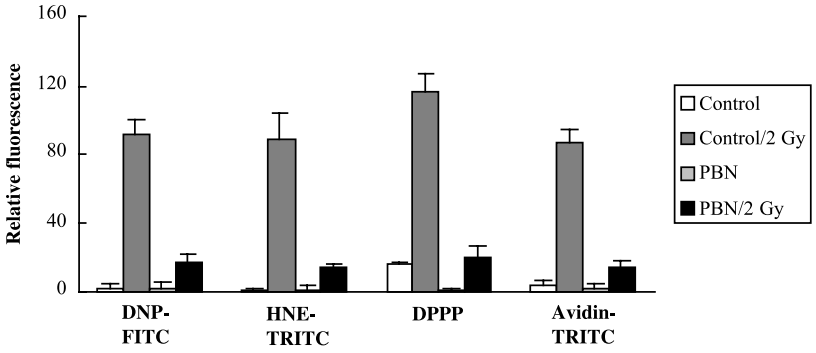

Figure 4. Cellular oxidative damage in U937 cells. Relative intensities of DNP-FITC, HNE-TRITC, DPPP and avidin-TRITC fluorescences in U937 cells. Protein carbonyl content of U937 cells detected with DNP-specific antibody and anti-human IgG FITC conjugate as a secondary antibody. Lipid peroxidation in U937 cells was detected with polyclonal anti-HNE Michael adduct antibody and anti-human IgG TRITC conjugate. To visualize lipid peroxidation in U937 cells exposed to γ-irradiation, cells (1×10^6/ml) were stained with 5 μM DPPP for 15 min. Fluorescence images were obtained under microscopy. In order to estimate 8 -OH-dG levels in U937 cells, the cells were fixed and permeabilized immediately after exposure to γ-irradiation. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope. The averages of fluorescence intensity were calculated as described [22]. Each value represents the mean ± SD from three independent experiments.

in Figure 4, the fluorescent intensity which reflects the endogenous levels of HNE adducts in proteins was significantly increased when U937 cells were exposed to 2 Gy of γ-irradiation and the increase of lipid peroxidation was markedly reduced in PBN-treated cells. Recently, it has been shown that DPPP is a suitable fluorescence probe to monitor lipid peroxidation within cell membrane specifically [18]. DPPP fluorescent intensity was increased markedly in untreated cells, whereas, it was not significantly increased in PBN-treated cells after exposure to ionizing radiation. The reaction of intracellular ROS with DNA resulted in numerous forms of base damage, and 8-OH-dG is one of the most abundant and most studied lesions generated. 8-OH-dG has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* [24]. Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC [19]. The fluorescent intensity, which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in untreated cells upon exposure to ionizing radiation. In contrast, the overall DNA appeared to be markedly protected in PBN-treated cells even after exposure to the same dose of ionizing radiation. These results indicate that PBN appears to protect cells from oxidative damage caused by ionizing radiation.

Alterations in mitochondrial integrity and function may play an important role in the apoptotic cascade. MPT, associated with the opening of large pores in the mitochondrial membranes, is a very important event in apoptosis, and ROS is one of the major stimuli that change MPT [25]. To answer whether PBN modulates the MPT upon exposure to ionizing radiation, we determined the change in MPT by intensity of fluorescence emitting from a lipophilic cation dye, rhodamine 123. Significantly less rhodamine 123 dye was taken up by the mitochondria of untreated cells, compared with PBN-treated cells (Figure 5A).

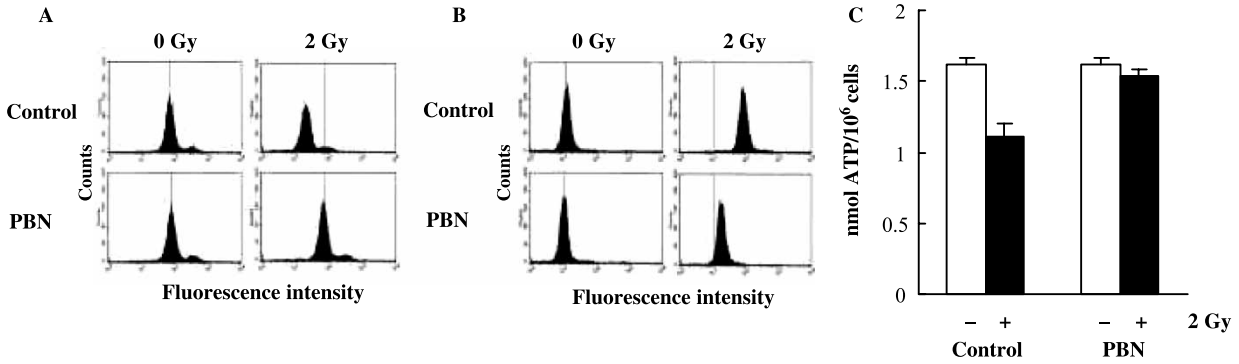

Figure 5. Effects of PBN on mitochondrial function. (A) Effect of PBN on MPT. MPT of U937 cells was measured by the incorporation of rhodamine 123 dye into the mitochondria. Fluorescence was measured by flow cytometry. (B) Effect of PBN on mitochondrial ROS generation. DHR 123 was employed to detect mitochondrial ROS. Fluorescence was measured by flow cytometry. (C) Effect of PBN on the levels of intracellular ATP. Control and PBN-treated U937 cells were irradiated and assayed for intracellular ATP content. Each value represents the mean ± SD from five independent experiments.

To determine if changes in MPT were accompanied by changes in intracellular ROS, The levels of intracellular peroxides in the mitochondria of U937 cells were evaluated by confocal microscopy with the oxidant-sensitive probe DHR 123. As shown in Figure 5B, the intensity of fluorescence was significantly lower in cells pre-treated with 2 mM PBN for 2 h when compared to that in the mitochondria of untreated cells when U937 cells were exposed to 2 Gy of γ-irradiation. These results indicate that ionizing radiation most likely leads to increased mitochondrial injury while PBN protects mitochondria from oxidative damage. Mitochondrial injury is often followed by the depletion of intracellular ATP level. As shown in Figure 5C, when U937 cells were exposed to 2 Gy of γ-irradiation the ATP level was not significantly decreased in PBN-treated cells whereas, it was reduced by 30% in cells not treated with PBN, suggesting a protective role of PBN against the loss of intracellular ATP levels.

We evaluated changes in the apoptotic marker proteins as a result of ionizing radiation and the influence of PBN on these proteins. Caspase-3 activation in U937 cells was assessed by immunoblot analysis of lysates from cells that had been exposed to 2 Gy of γ-irradiation, with and without PBN (Figure 6). Ionizing radiation induced cleavage of caspase-3, however, the cleavage was significantly reduced by PBN. Ionizing radiation also induced the formation of fragments which represents proteolytic cleavage of PARP and lamin B, indicates an oncoming apoptotic process. The cleaved products of PARP and lamin B increased markedly in control cells compared to PBN pre-treated cells upon exposure to ionizing radiation (Figure 6). Taken together, ionizing radiation-induced cleavage of procaspase-3 into the active form of caspase-3 and caspase-3 induces degradation of PARP or lamin B. The results also indicate that PBN exhibits a protective effect on the ionizing radiation-induced apoptosis. The role of mitochondrial pathway of apoptosis in the ionizing radiation-induced death of U937 cells were examined by immunoblot analysis of the abundance of Bcl-2, an antiapoptotic protein and of Bax, an proapoptotic protein. As shown in Figure 6, the abundance of Bcl-2 in U937 cells was significantly decreased in control cells as compared to that of PBN pre-treated cells when exposed to ionizing radiation. The amount of Bax was increased after exposure to ionizing radiation, and it was significantly increased in control cells as compared to that of PBN pre-treated cells. We determined the changes in levels of the p53 tumor suppressor protein, which leads either to cell cycle arrest and repair or to apoptosis. The results in Figure 6 compare the changes in p53 in U937 cells induced by radiation with and without PBN. Levels of p53 increased as a result of radiation treatment and PBN inhibited the radiation-induced change in p53 levels.

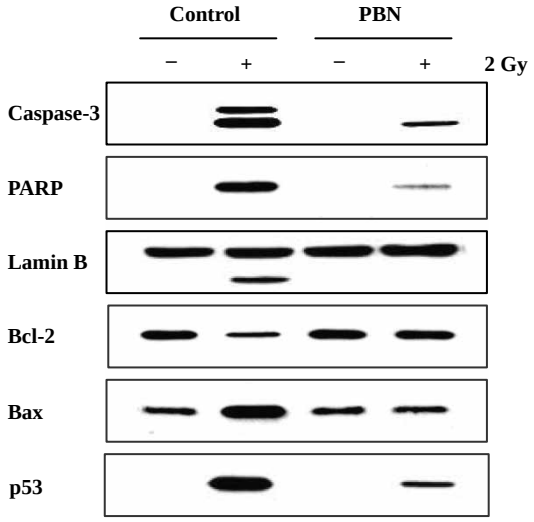

Figure 6. Immunoblot analysis of apoptotic marker proteins in U937 cells exposed to ionizing radiation. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3, cleaved PARP, lamin B, Bcl-2, Bax and p53.

Discussion

Ionizing radiation is toxic to living cells and organisms because it induces deleterious structural changes in essential biomolecules. A significant part of the initial damage done to cells by ionizing radiation is due to formation of $^{\cdot}OH$, which reacts with almost all cellular components to induce oxidative damage to DNA, lipid peroxidation and protein oxidation [3,26,27]. DNA is a particularly important target, suffering double- and single-strand breaks, deoxyribose damage and base modification [28]. Of the total damage to DNA caused by ionizing radiation, as much as 80% may result from radiation-induced water-derivative free radicals and secondary carbon-based radicals [1]. In addition to the generation of hydroxyl radicals, the hydrated electrons formed by ionizing radiation can reduce it to O_2^{-}. O_2^{-} can dismutate to H_2O_2 with the possibility of extra $^{\cdot}OH$ production by metal-catalyzed Fenton reaction [2]. Free radicals can also initiate a variety of cellular signal transduction pathways that may either aid the cell in coping with the excess oxidative stress resulting from radiation or set into motion pathways that lead to the destruction of cells damaged beyond the repair capabilities of the cell [29,30]. Therefore, there is considerable literature to suggest that free radical scavengers can be used to prevent oxidative damage and cell death including apoptosis caused by ionizing radiation.

PBN, a lipophilic nitron compound, has been widely used as a spin trap *in vitro*. PBN not only effectively scavenges ROS but also suppresses the chain reactions leading to lipid peroxidation by trapping lipid radicals [31]. PBN is not acutely toxic

and thus has been used in animal studies [11]. There is mounting evidence that PBN has an ameliorative effect on a variety of functions under acute oxidative stress conditions [6,9,10]. Therefore, it was plausible to assume that PBN may play a role in preventing apoptosis caused by ionizing radiation in cells. The aim of the present work was to evaluate the role of PBN in protecting U937 cells from ionizing radiation in regards to apoptotic cell death, cellular redox status, mitochondrial dysfuntion and oxidative damage to cells. In the present study, a temporal pattern of events reflect ionizing radiation-induced apoptosis was observed, starting from the elevation of ROS level, followed by MPT alteration, caspase-3 activation and DNA fragmentation. The pre-treatment with PBN significantly reduced ROS level and inhibited the whole apoptotic.13 pathway.

Biological systems have evolved to develop an effective and complicated network of defense mechanisms including antioxidant enzymes and small molecular weight antioxidants to cope with lethal oxidative environments. GSH is known to play a role in protecting cells against ionizing radiation. Treatment with buthionine sulfoximine to inhibit GSH synthesis increases radiosensitivity [32]. The depletion of intracellular GSH and the increase in the ratio of [GSSG]/[GSH_t] which reflects the efficiency of GSH turnover were significantly reduced by PBN. The ratio for [NADPH]/[$NADP^+$ + NADPH], the other parameter which reflects the cellular redox status and the availability of the reducing equivalent for GSH turnover by glutathione reductase, was significantly increased by PBN. These results indicate that ionizing radiation results in the perturbation of cellular redox balance presumably by depletion of GSH and NADPH pools and PBN may shift the balance to anti-oxidant condition.

The involvement of mitochondria in apoptosis has been extensively discussed [33]. All the changes caused by ionizing radiation are compatible with mitochondrial failure, encompassing reduced production of ATP, generation of ROS and accumulation of rhodamine 123 which reflect mitochondrial swelling or changes in the mitochondrial inner membrane. A clear suppression of such damages indicates that PBN prevents a deterioration of bioenergetic state.

Cleavage of caspase-3 and its target proteins such as PARP and lamin B, a signature event of apoptosis, was induced by ionizing radiation. In the meantime, the Bcl-2 protein is a suppressor of apoptosis that homodimerizes with itself and forms heterodimers with a homologous protein Bax, a promoter of cell death [34]. PBN effectively suppressed programmed cell death by decreasing apoptotic features including caspase activation, increasing anti-apoptotic molecules (Bcl-2), and decreasing pro-apoptotic molecules (Bax), presumably via preservation of redox status. Wild-type p53 tumor suppressor protein has been shown to be functionally necessary for growth inhibition and apoptosis following exposure to ionizing radiation [35]. Levels of p53 were increased in U937 cells after radiation treatment. The presence of PBN inhibited the radiation-induced increase in p53 levels, which correlates with a reduction in radiation-induced DNA damage.

In conclusion, the present study demonstrates that PBN abrogates the ionizing radiation-induced early production of ROS, leading to protection against apoptotic cell death.

Acknowledgements

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