Protective Role of α-Phenyl-N-t-butylnitrone against Ionizing Radiation in U937 Cells and Mice

Jin Hyup Lee and Jeen-Woo Park

Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

ABSTRACT

Ionizing radiation (IR) induces the production of reactive oxygen species (ROS), which play an important causative role in radiation damage. 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 protective role of PBN against IR in U937 cells and mice. On exposure to IR, there was a distinct difference between the control cells and the cells pretreated with PBN in regard to viability, cellular redox status, and oxidative damage to cells. Lipid peroxidation, oxidative DNA damage, and protein oxidation were significantly lower in the cells treated with PBN when the cells were exposed to IR. Although the activities of antioxidant enzymes were comparable in PBN-treated and control cells, 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 with PBN-treated cells. The IR-induced mitochondrial damage reflected by the altered mitochondrial permeability transition, the increase in the accumulation of ROS, the reduction of ATP production, and the morphological change were significantly higher in control cells compared with PBN-treated cells. PBN administration for 14 days with a daily dosage of 30 mg/kg provided substantial protection against killing and oxidative damage to mice exposed to whole body irradiation. These data indicate that PBN may have great application potential as a new class of in vivo, nonsulfur-containing radiation protector.

INTRODUCTION

The damaging effect of IR on living cells is predominantly attributable to ROS, including O2−, OH−, and H2O2, generated by the decomposition of water. The secondary radicals formed by the interaction of 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).

The search for agents that protect against IR is important to those at risk by virtue of environmental exposure or health-related treatment and scientific study of the mechanism of radiation injury and cytotoxicity (4). Although no radioprotective drug available today has all of the requisite qualities to be an ideal radioprotector, sulphydryl radioprotectors such as cysteine, cysteine, cystamine, aminooxy-hydroxyl dihydrobromide, and mercaptoethyl guanidine are the best radioprotectors known today. However, their use encounters two great difficulties: their toxicity and the short period during which they are active (5).

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, including O2− (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, the role of PBN in the cellular and in vivo defense against IR was investigated using the U937 cells and mice. There is mounting evidence that human monocytic U937 cells are highly susceptible to many types of stresses. They also have a variety of functions against external stresses. PBN-treated and untreated U937 cells were expected to exhibit differences in sensitivity to the toxic effects of IR. To determine whether such differences exist between cells treated and untreated with PBN, viability, cellular redox status, oxidative damage to cells, and mitochondrial damage were examined on their exposure to IR. PBN was also administered to mice, and in vivo radioprotective effect was assessed. This study indicates that PBN may play an important role in regulating the damage induced by IR, and PBN may have great application potential as a new class of in vivo, nonsulfur-containing radiation protector.

MATERIALS AND METHODS

Materials. Hydrogen peroxide, PBN, pyrogallol, glutathione reductase, G6PDH, tert-butylnitropropene-β-NADP+, β-NADPH, GSSG, DNPH, DTNB, xylene orange, antirabbit IgG TRITC-conjugated secondary antibody, and antirabbit IgG FITC-conjugated secondary antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Antihuman HNE-Michael adduct antibody and antihuman DNPH antibody were obtained from Calbiochem (La Jolla, CA). DCFH-DA, CMAC, DPP, DHR 123, and the LIVE/DEAD viability/cytotoxicity kit were purchased from Molecular Probes (Eugene, OR).

Cell Culture. Human premonocytic U937 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 culture supplemented with 10% (v/v) fetal bovine serum, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37°C in a 5% CO2/95% air humidified incubator.

Microscopy. Adult male ICR mice were supplied through the production. The animals were housed five per cage in a climate-controlled, circadian rhythm-adjusted room and allowed food and water ad libitum. The animals were, on average, 50–70 days old and weighed between 20 and 30 g at the time of irradiation. Experiments on mice were conducted according to the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (Washington, DC).

Irradiation and Cytotoxicity Assays. U937 cells were first grown on a 96-well plate at a density of 2 × 104 cells/well, until 80% confluence before IR, and cell viability after IR was assessed by a novel tetrazolium compound, MTS, and an electron coupling reagent, PMS. After culture for confuence...
optimization, various concentrations of PBN were applied to the cells and cells were incubated for an 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 was 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 137Cs at a dose rate of 1 Gy/min. After 48 h of irradiation to cells, 20 µl of MTS/PSM solution was added and incubated for another 4 h at 37°C in a humidified, 5% CO2 atmosphere. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The absorbance was read in an ELISA plate reader at 490 nm with a 620 nm reference. Cell viability is expressed as a percentage of the absorbance seen in the untreated control cells. Cell viability was also observed using a fluorescent LIVE/DEAD viability assay, following the manufacturer’s protocol. Cells were double-stained with calcein-AM and ethidium homodimer-1 and observed with a fluorescence microscope.

Toxicology of PBN and Whole Body Irradiation. To determine its maximal tolerated dose, solutions of PBN were freshly prepared in 0.9% NaCl. Two groups of 15 mice each received either PBN or 0.9% NaCl. PBN was administered before irradiation at a dose of 30 mg/kg in volumes equivalent to 1% of each animal’s weight to once daily for 2 weeks. Control mice were given 0.9% NaCl, and all injections were administered i.p. Survival was assessed up to 30 days after injection without irradiation. To determine survival after whole body irradiation, the same protocol for the PBN administration was applied, and then the groups of 15 mice were transferred to round Plexiglas containers (30.5 cm in diameter and 10.5 cm in height) with holes for ventilation. After irradiation with a 137Cs source at a dose rate of 1 Gy/min, the mice were returned to climate-controlled cages for observation. Survival was assessed 30 days after irradiation.

Preparation of Tissue Extracts. The tissue portions were homogenized with a solution containing 25 mM PIPES, 1 mM EDTA (pH 7.2) containing 8.5 µl leupeptin and 100 µg/ml aprotinin, using a homogenizer at maximum speed for 15 s. Each sample was then centrifuged at 4000 × g for 15 min at 4°C, and the resulting supernatants were stored at −20°C and used for the assays. The protein concentration in the supernatant was measured by the Bradford method using the Bio-Rad Protein Assay kit.

Enzyme Assay. Cells were collected at 1,000 × g for 10 min at 4°C and were washed once with cold PBS. Cells were homogenized with a Dounce homogenizer in sucrose buffer [0.32 M sucrose and 10 mM Tris-Cl (pH 7.4)]. Cell homogenates were centrifuged at 1,000 × g for 5 min, and the supernatants were centrifuged further at 15,000 × g for 30 min. The supernatants were used to measure the activities of several cytosolic enzymes. Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the decrease in absorbance at 240 nm (12). Superoxide dismutase activity in cell extracts was assayed spectrophotometrically using a homogenate at maximum speed for 15 s. Each sample was then centrifuged at 4000 × g for 15 min at 4°C, and the resulting supernatants were stored at −20°C and used for the assays. The protein concentration in the supernatant was measured by the Bradford method using the Bio-Rad Protein Assay kit.

DNA Damage Analysis. 8-OH-deoxyguanosine levels in U937 cells were assessed using a fluorescent binding assay, as described by Struthers et al. (25). 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. For in vivo visualization of lipid peroxidation in tissues, tissues were perfused with 5 µM DPPP for 30 min and the cryosections of tissues were prepared. The images of DPPP fluorescence by reactive species were analyzed by the Zeiss Axiovert 200 inverted microscope at fluorescence 4′,6-diamidino-2-phenylindole region (excitation, 351 nm; emission, 380 nm).

Nitrosothiolchemistry. Tissues from saline or PBN-administered mice (n = 10, each) after irradiation were fixed by retrograde perfusion via the aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). For paraffin sections, tissue blocks containing all kidney, liver, and lung zones were dehydrated and embedded in paraffin. The sections were dewaxed and rehydrated. For immunofluorescence labeling, the staining was performed using fluorescein tag-conjugated secondary antibodies. To reveal antigens, sections were put in a 1-mM TRIS solution (pH 9.0) supplemented with 0.5 mM EGTA and heated in a microwave oven for 10 min. Nonspecific immunoglobulin binding was prevented by incubating the sections in 50 mM NH4Cl for 30 min, followed by blocking with PBS supplemented with 1% BSA, 0.05% saponin,
and 0.2% gelatin. Sections were incubated overnight at 4°C with antihuman HNE-Michael adduct antibodies for lipid peroxidation, antihuman DNP adduct antibody for protein oxidation, and avidin-TRITC conjugated for DNA base modification conjugation in PBS supplemented with 0.1% BSA and 0.3% Triton X-100 (1:200 dilution). After rinsing with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin, labeling was visualized with the fluorescence tag-conjugated antirabbit IgG TRITC conjugate (1:200 dilution) as a secondary antibody for antihuman HNE-Michael adduct antibody and antirabbit IgG FITC conjugate (1:200 dilution) as a secondary antibody for anti-DNP antibody diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. Microscopy was performed using a Zeiss fluorescence microscope.

Cryosection Preparation. Adult mice were anesthetized with i.p. injections of pentobarbital sodium (1 ml/kg body weight). Tissues were quickly dissected from mice. Tissues were rinsed briefly in cold PBS three times before freezing onto metal chuck for cryosectioning (26). Tissues were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). After snap-freezing, a block was obtained without changing the shape of the sample. The block was then transferred into a cryostat chamber. A precooled adhesive tape (Instrumedics, Hackensack, NJ) was used to support a 5-mm-thick section of tissue. The block was then transferred into a cryostat chamber. A precooled adhesive tape (Instrumedics, Hackensack, NJ) was used to support a 5-mm-thick section of tissue. The still frozen section, which adhered to the tape, was then laminated to the cold adhesive-coated slide (Instrumedics). An UV flash was used to polymerize the adhesive coating into a hard solvent-resistant plastic to tightly anchor the section to the slide. Finally, the tape was removed and the slide was immersed in cold acetone (–20 to –25°C), in which the ice was dissolved but not melted (freeze substitution). The slide was then transferred to 10% formaldehyde/0.25% glutaraldehyde/75% alcohol for 10 min at room temperature.

MPT. Mitochondrial membrane potential was measured by the incorporation of rhodamine 123 dye into the mitochondria, as described previously (27). Cells (1 × 10⁶) grown on poly-L-lysine-coated slide glasses were exposed to IR. Cells were then treated with 5 μM rhodamine 123 for 15 min and excited at 488 nm with an argon laser. Cells were double-stained with 100 nm MitoTracker Red, which is a morphological marker of mitochondria. The fluorescence images at 520 nm were simultaneously obtained with a laser confocal scanning microscope.

Mitochondrial ROS. Cells were grown on an easy flask 75 FILT at a density of 1 × 10⁶ cells/well, until 80% confluence before IR. After culture for confluence optimization, 2 mM PBN were applied to the cells, which were then incubated for an additional 2 h at 37°C. After additional incubation, DHR 123 was used to detect mitochondrial ROS. U937 cells in PBS were incubated for an additional 40 min. DHR 123 and Mitracker Red fluorescence were visualized by fluorescence microscope.

Transmission Electron Microscopy. Cells grown to 80% confluence were either treated or untreated with PBN and exposed to IR, rinsed twice with PBS (pH 7.3), and centrifuged at 50 × g for 5 min. Cell pellets were immediately fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C. Cells were postfixed in osmium tetroxide (1%) for 30 min, washed with water, and then subjected to a dehydration procedure using graded ethanol series. For preparing the specimen, cells were embedded in Epon812 (Electron Microscopy Sciences, Fort Washington, PA), and two random areas were cut and processed. The sections (60–70 nm) were cut with an ultramicrotome (Soya MT-7000), transferred to copper grids, and stained with uranyl acetate and lead citrate. At least 40 cells of each sample were observed and photographed using Hitachi H-7100 transmission electron microscope (Hitachi Co., Hitachi, Japan) at 75 kV.

Measurement of ATP Level. Intracellular ATP levels were determined by using luciferin-luciferase (28). Cells (5 × 10⁶) were collected by centrifugation, resuspended in 250 μl of extraction solution [10 mM KH₂PO₄ and 4 mM MgSO₄ (pH 7.4)], heated at 98°C for 4 min, and placed on ice. For ATP measurement, an aliquot of a 50-μl sample was added to 100 μl of reaction solution [50 mM NaAsO₂ and 20 mM MgSO₄ (pH 7.4)] containing 800 μg of luciferin-luciferase (Sigma Chemical Co.). Light emission was quantitated in a Turner Designs TD 20/20 luminometer (Stratec Biomedical Systems, Birkenfeld, Germany). For all experiments, ATP standard curves were run and were linear in the range of 5–2500 nm. Concentrations of ATP stock solution were calculated from spectrophotometric absorbance at 259 nm using an extinction coefficient of 1.54 × 10⁻³ M⁻¹ cm⁻¹.

RESULTS

Cell Killing. As shown in Fig. 1, when cultured U937 cells were exposed to γ-irradiation, a dose-dependent increase in cell viability was observed. However, the cells pretreated with 2 mM PBN for 2 h were significantly more resistant than control cells untreated with PBN. The protective effect of PBN was concentration dependent, and PBN itself up to 10 mM was without effect on the viability of U937 cells (data not shown). The protective effect of PBN against IR was also confirmed by dual staining with calcine-AM and ethidium homodimer-1. IR caused lethal injury to U937 cells, and their nuclei were mostly stained with ethidium homodimer-1 to exhibit a red fluorescence. PBN (2 mM) pretreatment for 2 h decreased the proportion of red fluorescent nuclei of dying cells in γ-irradiated cultures (data not shown).

Cellular Redox Status. To investigate whether the difference in viability of U937 cells after exposure to IR is associated with ROS formation, the levels of intracellular peroxides in the U937 cells were evaluated by confocal microscopy with the oxidant-sensitive probe DCFH-DA. As shown in Fig. 2A, an increase in DCF fluorescence was observed in U937 cells when they were exposed to 20 Gy of γ-irradiation. The increase in fluorescence was significantly reduced in cells pretreated with 2 mM PBN for 2 h. Similar results were also observed by FACS analyses (Fig. 2B). We also demonstrated the level of intracellular H₂O₂ in cells irradiated in the presence and absence of PBN. The pretreatment of PBN resulted in a significantly lower intracellular level of H₂O₂ as compared with that of untreated control with the exposure of 20 Gy of irradiation (Fig. 2C). These data strengthen the conclusion that PBN provided protection from the cytotoxic actions of γ-irradiation by decreasing the steady-state level of intracellular oxidants.

GSH is one of the most abundant intracellular antioxidants, and determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. It has been shown that the GSH-sensitive fluorescent dye CMAC can be used as a useful probe to evaluate the level of intracellular GSH (20). Cellular GSH

![Fig. 1](https://cancerres.aacrjournals.org)
levels in U937 cells treated with 20 Gy of γ-irradiation were significantly decreased (Fig. 3A). However, the depletion of GSH was significantly protected by the pretreatment of 2 mM PBN for 2 h. One important parameter of GSH metabolism is the ratio of GSSG:total GSH (GSHt), which may reflect the efficiency of GSH turnover. When the cells were exposed to 20 Gy of γ-irradiation, the ratio of cellular [GSSG]:[GSHt] was significantly higher in control cells than in PBN-treated cells (Fig. 3B). These 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 20 Gy of γ-irradiation, however, the decrease in this ratio was much less pronounced in PBN-treated cells (Fig. 3C). Despite their role in the cellular defense mechanism, the antioxidant enzymes are susceptible to inactivation by ROS. Previous studies have demonstrated that oxidative processes result in the loss of key antioxidant enzymes, which may exacerbate oxidative stress-mediated cytotoxicity. Whether the presence of PBN induced concomitant alterations in the activity of major antioxidant enzymes
was investigated. The activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, G6PDH, and glutathione reductase was decreased after exposure to /H9253-irradiation, however, PBN did not significantly affect the activity of antioxidant enzymes (Table 1). These results indicate that PBN may act as a direct scavenger of ROS.

**Cellular Damage.** 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 pretreatment decreased the sensitivity to protein damage, we performed carbonyl content measurements for protein oxidation after cellular exposure to IR. Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins (23). Control cells elicited an approximately 2-fold increase of carbonyl groups, as compared with unirradiated cells when 20 Gy of IR were exposed. Although the carbonyl content of PBN-treated cells also increased with irradiation, the increase was significantly lower than that of control cells (Fig. 4A). Protein oxidation was also visualized by immunocytochemical method using anti-DNP antibody. As shown in Fig. 4B, the fluorescent intensity that reflects the endogenous levels of carbonyl groups in proteins was significantly increased when U937 cells were exposed to 20 Gy of /H9253-irradiation, and the increase of protein oxidation 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. DPPP reacts with lipid hydroperoxides stoichiometrically to give highly fluorescent product DPPP oxide.

**Table 1. Effect of PBN on the antioxidant enzyme activities of U937 cells**

<table>
<thead>
<tr>
<th>Antioxidant enzyme</th>
<th>Control</th>
<th>Control/PBN</th>
<th>Irradiated</th>
<th>Irradiated/PBN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>1.58 ± 0.12</td>
<td>1.52 ± 0.08</td>
<td>1.26 ± 0.11</td>
<td>1.31 ± 0.16</td>
</tr>
<tr>
<td>Catalase</td>
<td>131 ± 14</td>
<td>133 ± 4.6</td>
<td>92.4 ± 11</td>
<td>94.3 ± 5.1</td>
</tr>
<tr>
<td>Glutathione peroxidasec</td>
<td>9.3 ± 0.3</td>
<td>9.0 ± 0.2</td>
<td>6.9 ± 0.2</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>Glutathione reductasec</td>
<td>52.6 ± 2.7</td>
<td>51.1 ± 3.6</td>
<td>43.6 ± 1.4</td>
<td>43.1 ± 2.9</td>
</tr>
<tr>
<td>G6PDHc</td>
<td>138 ± 9.5</td>
<td>141 ± 8.5</td>
<td>102 ± 12</td>
<td>106 ± 14</td>
</tr>
</tbody>
</table>

* U937 cells were treated with 2 mM PBN for 2 h before 20 Gy of /H9253-irradiation. Each value represents the mean ± SD from five independent experiments.

b Enzyme activity represents units/mg protein.

c Enzyme activity represents units/g protein.
were exposed to ultrasound. Cells untreated and treated with PBN rhodamine 123 dye into the mitochondria. B, effect of PBN on mitochondrial ROS generation. DHR 123 was used to detect mitochondrial ROS. DHR 123 fluorescence was visualized by a fluorescence microscope. C, effect of PBN on mitochondrial ultrastructure. Cells untreated and treated with PBN were exposed to γ-irradiation, and their mitochondrial structures were then examined under transmission electron microscopy. D, 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.

Fig. 5. Effects of PBN on mitochondrial structure and function. A, effect of PBN on MPT. MPT of U937 cells was measured by the incorporation of rhodamine 123 dye into the mitochondria. B, effect of PBN on mitochondrial ROS generation. DHR 123 was used to detect mitochondrial ROS. DHR 123 fluorescence was visualized by a fluorescence microscope. C, effect of PBN on mitochondrial ultrastructure. Cells untreated and treated with PBN were exposed to γ-irradiation, and their mitochondrial structures were then examined under transmission electron microscopy. D, 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.

(25). DPPP fluorescent intensity was increased markedly in untreated cells, whereas it was increased slightly in PBN-treated cells after exposure to IR (Fig. 4D). 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. Because 8-OH-dG causes misreplication of DNA (30), it has been implicated as a possible cause of mutation and cancer. Therefore, 8-OH-dG has been used as an indicator of oxidative DNA damage in vivo and in vitro (31). Recently, it has been shown that the 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC (25). The fluorescent intensity that reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in untreated cells on exposure to IR. In contrast, the overall DNA appeared to be markedly protected in PBN-treated cells, even after exposure to the same dose of IR (Fig. 4E). These results indicate that PBN seems to protect cells from oxidative damage caused by IR.

Mitochondrial Damage. MPT is a very important event in both necrosis and apoptosis, and ROS is one of the major stimuli that change MPT (32). To answer whether PBN modulates the MPT on exposure to IR, 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 (Fig. 5A). 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 Fig. 5B, the intensity of fluorescence was significantly lower in cells pretreated with 2 mM PBN for 1 h when compared with that in the mitochondria of untreated cells when U937 cells were exposed to 20 Gy of γ-irradiation. MPT precedes the cellular injury accompanied with significant changes in mitochondrial structures (33). Therefore, we also examined the mitochondrial morphology of different U937 cells under electron microscopy (Fig. 5C). Normal shapes of mitochondrial cristae in PBN-treated cells were observed, whereas abnormal or substantially damaged mitochondrial cristae were evident in untreated cells on exposure to IR. Mitochondria of untreated cells were extremely swollen and frequently lacked typical cristae but contained their vesicular remnants and severely collapsed membranes. These results indicate that IR most likely leads to increased mitochondrial injury, whereas PBN protects mitochondria from oxidative damage. Mitochondrial injury is often followed by the depletion of intracellular ATP level. As shown in Fig. 5D, when U937 cells were exposed to 20 Gy of γ-irradiation, the ATP level was decreased only by 19% in PBN-treated cells, whereas it was reduced by 51% in cells not treated with PBN, suggesting a protective role of PBN against the loss of intracellular ATP levels.

In Vivo Radioprotection. Mice treated with PBN at 30 mg/kg daily for 2 weeks survived with no apparent adverse effects after 30 days. ICR male mice received i.p. injections of 0.9% NaCl or PBN at 30 mg/kg daily for 14 days, and then they were subjected to 8 Gy of whole body irradiation. Survival was monitored for 30 days, and the results are shown in Fig. 6. PBN administration before irradiation provided significant protection compared with control animals receiving 0.9% NaCl.

Mouse Redox Status. The livers from PBN-administered mice before γ-irradiation showed the lower level of hydrogen peroxide (Fig. 7A), the lower level of DCF fluorescence (Fig. 7B), and suppressed depletion of the GSH level (Fig. 7C) compared with mice not administered PBN. The ratio of [GSSG]:[GSH] was lower (Fig. 7D) and the ratio for [NADPH]:[NADPH + NADP+] was higher (Fig.

![Image](https://cancerres.aacrjournals.org/)

Fig. 6. PBN effect on the radiation sensitivity of ICR mice. Groups of 15 ICR mice received an injection of either PBN (30 mg/kg, i.p.; ▲) or 0.9% NaCl (○) for 2 weeks, were exposed to 8 Gy of whole body irradiation, and were then observed over 30 days for survival.
Tissue Damage. In the livers of untreated mice, carbonyl groups in proteins were increased significantly after whole body irradiation. Administration of PBN markedly prevented the increase in carbonyl groups in proteins (Fig. 8A). The increase in DPPP fluorescence was also inhibited by the treatment with PBN (Fig. 8B). Sections of liver were immunohistochemically stained for HNE- and DNP-adduct using polyclonal HNE- and DNP-specific antibodies. The livers from PBN-administered mice showed much less staining for both DNP- and HNE-adducts (Figs. 8C and 8D). Staining for these adducts was similar in the sections of kidneys and lungs. As another marker of oxidative damage in the liver, we measured the formation of 8-OH-dG in the liver DNA. The result showed that the lower amount of 8-OH-dG reflected by the lower level of avidin-TRITC fluorescence in PBN-administered mice than in non-PBN-administered mice (Fig. 8E). As shown in Fig. 8F, the livers from non-PBN-administered mice showed the lower level of ATP compared with untreated mice, suggesting the role of PBN against the loss of mitochondrial function caused by IR. The kidneys and the lungs showed the similar results.

DISCUSSION

IR 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 IR caused by formation of •OH, which reacts with almost all cellular components to induce oxidative damage to DNA, lipid peroxidation, and protein oxidation (3, 34, 35). DNA is a particularly important target, suffering double- and single-strand breaks, deoxyribose damage, and base modification (36). Of the total damage to DNA caused by IR, 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 IR can reduce it to O2−. O2− can dismutate to H2O2 with the possibility of extra •OH production by metal-catalyzed Fenton reaction (2). Therefore, there is considerable literature to suggest that free radical scavengers can be used to prevent oxidative damage caused by IR. GSH, GSH precursors, and thiol compounds have been used as radioprotectors. However, toxicities including the possible biological actions of sulfur-centered radicals produced by loss of H from thiols must not be ignored (36).

PBN, a lipophilic nitron compound, has been used widely 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. 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 oxidative damage caused by IR in...
cells and animals. The aim of the present work was to evaluate the role of PBN in protecting U937 cells and mice from IR in regard to cell death, animal mortality, cellular redox status, and oxidative damage to cells and tissues.

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. The antioxidant enzymes were susceptible to inactivation by ROS, however, the activity of antioxidant enzymes were not significantly affected by PBN. GSH is known to play a role in protecting cells against IR. Treatment with buthionine sulfoximine to inhibit GSH synthesis increases radiosensitivity (37). The depletion of intracellular GSH and the increase in the ratio of [GSSG]:[GSH], which reflects the efficiency of GSH turnover, were significantly reduced by PBN. The ratio for [NADPH]:[NADP⁺ + NADPH], the other parameter that reflects the cellular redox status and the avail-

---

**Fig. 8. Effects of PBN on oxidative damage of the livers from mice exposed to IR.**

**A**, protein carbonyl content after IR in mice received an injection of either PBN or 0.9% NaCl. Each value represents the mean ± SD from three independent experiments. **B**, visualization of lipid peroxidation in the livers from mice exposed to γ-irradiation. The livers were perfused with 5 μM DPPP for 30 min, and the cryosections of tissues were prepared. Fluorescence images were obtained under microscopy. **C**, lipid peroxidation in the livers from irradiated mice was detected with polyclonal anti-HNE-Michael adduct antibody and antihuman IgG TRITC conjugate. **D**, protein carbonyl content of the livers from irradiated mice detected with DNP-specific antibody and antihuman IgG FITC conjugate as a secondary antibody. **E**, 8-OH-dG levels in the livers from irradiated mice. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope. **F**, changes in the ATP content of the livers after γ-irradiation. Control and PBN-treated mice were irradiated and assayed for intracellular ATP content from livers. Each value represents the mean ± SD from five independent experiments.
ability of the reducing equivalent for GSH turnover by glutathione reductase, was significantly increased by PBN. These results indicate that IR results in the perturbation of cellular redox balance presumably by depletion of GSH and NADPH pools and PBN may shift the balance to antioxidant condition.

It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states. All of the changes caused by IR are compatible with mitochondrial failure, encompassing reduced production of ATP, generation of ROS, accumulation of rhodamine 123 that reflect mitochondrial swelling or changes in the mitochondrial inner membrane, and changes in mitochondrial morphology. A clear suppression of such damages indicates that PBN prevents a deterioration of the bioenergetic state.

PBN is not only an in vitro but in vivo radioprotector. The radioprotective effect of PBN reflected by suppression of lethality was evident 30 days after exposure to radiation. The measurement of lipid peroxidation, protein oxidation, and oxidative DNA damage in livers, kidneys, and lungs from mice exposed to γ-irradiation indicates that the damage caused by IR was similar in these tissues and that PBN protects damage in tissues in a similar manner. To confirm oxidative damage in irradiated mice because of the pro-oxidant status, the formation of ROS in tissues was measured. PBN administration showed the tendency to lower the formation of ROS. The observed beneficial effects of PBN in mice supported here offer the possibility of developing antioxidant approaches to treating damage caused by IR. In conclusion, PBN is effective in protecting cells and mice from oxidative stress caused by IR, and alleviated damage suggests that further study of PBN or similar compounds is warranted.

ACKNOWLEDGMENTS

We are grateful to Drs. I. S. Kim and J. S. Suh (Kyungpook National University) for help.

REFERENCES

12. Stanton, R. C., and Seifert, J. L. Epidermal growth factor rapidly activates the hoxo

Protective Role of α-Phenyl-N-t-butylNitroprne against Ionizing Radiation in U937 Cells and Mice

Jin Hyup Lee and Jeen-Woo Park


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/20/6885

Cited articles
This article cites 32 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/20/6885.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/20/6885.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/63/20/6885. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.