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α-Phenyl-*N*-*t*-butylnitrone Protects Oxidative Damage to HepG2 Cells

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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. Recently, there has been considerable interest in the antioxidant nature of PBN on degenerative diseases, presumably related to oxidative stress. In the present study, the protective effect of PBN on the HepG2 cell line under oxidative stress was investigated. When the HepG2 cells were exposed to oxidant, such as hydrogen peroxide, menadione, or ethanol, the protective role of PBN was manifested as a reduction in trypan blue uptake and a decrease in the endogenous production of oxidants, as measured by the oxidation of 2',7'-dichlorodihydrofluorescin. The modulation of activity of major antioxidant enzymes, such as superoxide dismutase and catalase, was not significantly different either in the presence or in the absence of PBN. This indicates that PBN acts as a direct scavenger of reactive oxygen species.

Keywords: HepG2 cell line, α-Phenyl-*N*-*t*-butylnitrone, Reactive oxygen species.

Introduction

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH), are generated *in vivo* from the incomplete reaction of oxygen during aerobic metabolism, stimulated host phagocytes, or from exposure to environmental agents such as radiation, redox cycling agents (Ames, 1983; Cerutti, 1985; Babior, 1992). These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks (Halliwell and Gutteridge, 1984; Storz *et al.*, 1987; Lee *et al.*, 1998). Oxidative stress and ROS-mediated cell damage have been implicated in aging and a variety of human diseases; including alcohol-mediated organ damage, various forms of neurodegenerative diseases, many types of cancers, cardiovascular diseases, lung diseases, and UV-mediated skin diseases (Ames *et al.*, 1993).

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 (Oliver *et al.*, 1990) and delays senescence, it also alleviates oxidative damage from ischemia/reperfusion injury (Nakashima *et al.*, 1999). This phenomenon was accounted for by the fact that PBN protected biologically important molecules from oxidative damage by efficiently trapping ROS, including O_2^- (Carney *et al.*, 1991).

In the present study, the role of PBN in cellular defense against oxidative stress after exposure to oxidant, such as hydrogen peroxide, menadione, or ethanol, was investigated using the HepG2 cell line. To determine the differences between the HepG2 cells in the absence and presence of PBN, the viability and intracellular peroxide generation were examined upon exposure to oxidant. We report here that PBN protects the HepG2 cells from oxidative stress and acts as a direct scavenger of ROS.

Materials and Methods

Materials PBN, menadione, hydrogen peroxide (30% solution), trypan blue, and pyrogallol were obtained from the Sigma Chemical Co. (St. Louis, USA). The 2',7'-dichlorofluoroscin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, USA).

Cell culture The human hepatoma HepG2 cell line (ATCC) was grown in a Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (w/v) fetal bovine serum (Hyclone, Logan, USA) and 1% (w/v) penicillin-streptomycin (GIBCO/BRL, Gaithersburg, USA). Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity Oxidative stress was exposed as cells were treated with various concentrations of oxidant for 1 hr at 37°C. To test the

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protective effect of PBN, cells were pre-treated with PBN for 1 hr at 37°C. Cell viability was determined at the end of each treatment using the trypan blue exclusion assay (Samali *et al.*, 1999). The plasma membrane integrity of cells was tested by staining with 0.2% trypan blue. After 5-10 min of incubation, the number of cells excluding or staining positively for uptake of trypan blue was counted under a light microscope. Cell viability was expressed as a percentage of the counts relative to the untreated controls.

Measurement of intracellular ROS Intracellular peroxide production was measured using the oxidant-sensitive probe DCFH-DA. Fluorescence was measured using a Shimadzu RF5301 PC spectrofluorophotometer set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm (Royall and Ischiropoulos, 1993). Cells were exposed to oxidant, and a 5-mM stock solution of DCFH-DA dissolved in ethanol (20 μl) was added to each culture 30 min before the assay and allowed to incubate at 30°C. They were washed twice in ice-cold distilled water, resuspended in 200 μl of water, and disrupted by three cycles of sonication for 10 s at low output. The supernatant was obtained after centrifugation in a microcentrifuge at a maximum speed for 10 min and crude extract (500 μg protein) was suspended in distilled water, and then the fluorescence was recorded.

Activity of antioxidant enzymes Cells were collected at 10,000 × g for 10 min at 4°C and were washed once with cold PBS. Cell-free extracts in a sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4) were prepared by sonication in an ice-water bath for three 10 s bursts with 30 s cooling intervals. The resulting suspension was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was collected and the protein levels were determined by the method of Bradford. Catalase activity was measured in terms of the decomposition of hydrogen peroxide, which was followed directly by a decrease in absorbance at 240 nm (Beers and Sizer, 1952). The SOD activity in cell extracts was assayed spectrophotometrically using the pyrogallol assay (Marklund and Marklund, 1974), where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%.

Results and Discussion

The aim of this work was to evaluate the role of PBN in the protection of the HepG2 cells from oxidative stress, which was introduced by exposure to menadione, hydrogen peroxide, or ethanol. The HepG2 cells exhibited different sensitivity to extracellular agents known to produce oxidative stress. While the peroxides may cause oxidative injury from both outside and inside the cells, menadione may act as intracellular sources of free radicals and ROS by redox cycling. Menadione (2-methyl-1,4-naphthoquinone) is a synthetic naphthoquinone derivative, and the cytotoxic effects of menadione are thought to be mediated through one-electron reduction to semiquinone radicals, which can rapidly reduce O_2 to O_2^- and regenerate the quinone (Thor *et al.*, 1982), or react with thiol-containing compounds such as glutathione. In addition, autoxidation of glutathionyl-hydroquinones and generation of O_2^- can also occur (Powis, 1987). Either type of redox cycling may thus result in the intracellular formation of large amounts of O_2^-, and H_2O_2 by O_2^- dismutation. A large part of the cytotoxic effects of these oxidants could be mediated by the formation of hydroxyl radicals via Fenton-type reactions. The reduced toxicity of such oxidants on cells can be achieved by removal of a potential precursor of hydroxyl radicals, namely O_2^- or H_2O_2 (Lee and Park, 1998). There is considerable interest in the role of oxidative stress and the ethanol generation of ROS in the mechanisms by which ethanol is hepatoxic (Nordmann *et al.*, 1992). Ethanol is an oxidative agent known to cause dose- dependent depletion of glutathione in plasma (Milakofsky *et al.*, 1986) and a significant change in the intracellular thiol pool in the liver and brain (Uysal *et al.*, 1989). As shown in Fig. 1, when cells were exposed to hydrogen peroxide, menadione, or ethanol a dose-dependent decrease in cell viability was

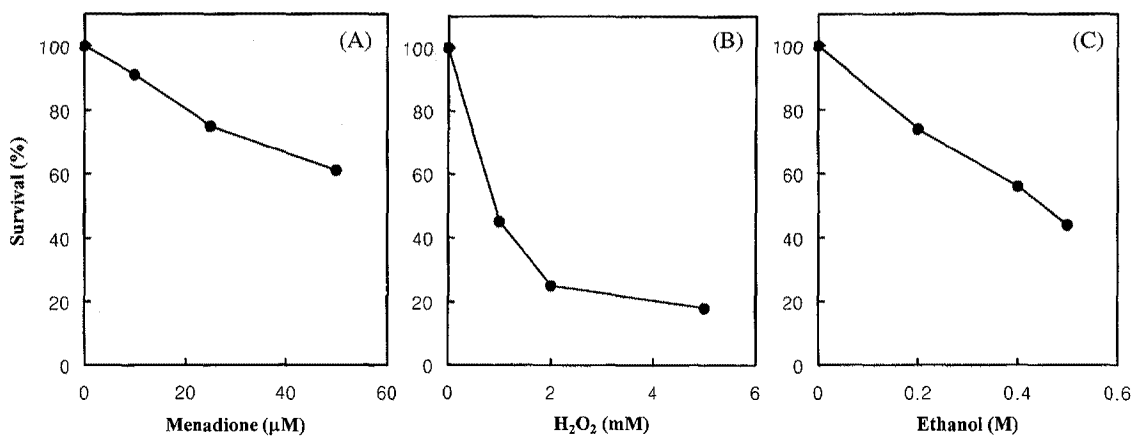

Fig. 1. Viability of HepG2 cells, as determined by trypan blue exclusion, after treatment with oxidants, such as menadione (A), hydrogen peroxide (B), or ethanol (C) for 1 hr. Cells were counted twice and the average shown as a percentage of the untreated control, which was taken as the 100% value. The results are representative of three separate experiments.

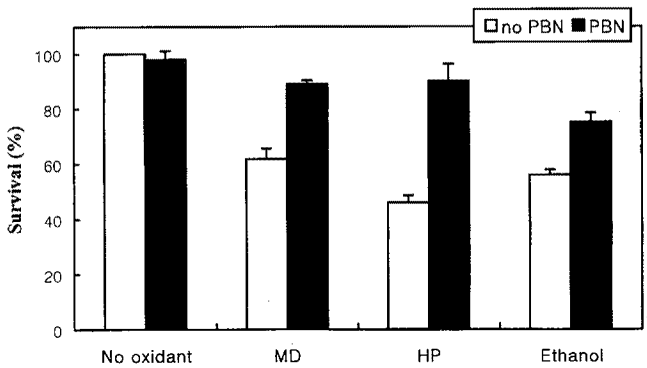

Fig. 2. Effect of PBN on cell viability upon exposure to oxidants. Cells were pre-treated with 2 mM PBN for 1 hr, and then exposed to oxidants. Viability of cells was determined by a trypan blue exclusion assay. Cell viability was expressed as a percentage of counts relative to untreated control cells. The values are the mean ± S.D. of three separate experiments. The viability of PBN-treated cells was statistically different from corresponding untreated cells upon exposure to the oxidant ($P<0.01$). MD, menadione; HP, hydrogen peroxide.

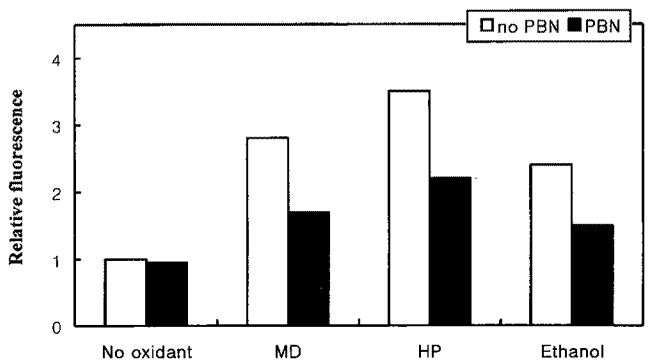

Fig. 3. Effect of PBN on generation of intracellular peroxides upon exposure to oxidant. Cells were treated with 2 mM PBN for 1 hr prior to expose oxidant. DCF fluorescence was measured in cell-free extracts from HepG2 cells. Fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. The results shown are representative of two similar experiments.

observed. However, the cells treated with 2 mM PBN were significantly ($p<0.01$) more resistant when compared to the control cells that were not treated with PBN. More than 90% of the PBN-treated cells survived; whereas 46% of the cells died at 1 mM H_2O_2 in the absence of PBN. A similar protective effect of PBN was observed when the HepG2 cells were exposed to either menadione or ethanol (Fig. 2). The protective effect of PBN was concentration-dependent (data not shown). PBN by itself up to 10 mM had no effect on the viability of the HepG2 cells.

PBN, a lipophilic spin-trapping agent, has been known to protect tissues against oxidative injury from various degenerative and pathological conditions. PBN alleviates the oxidative damage to the brain during ischemia/reperfusion-

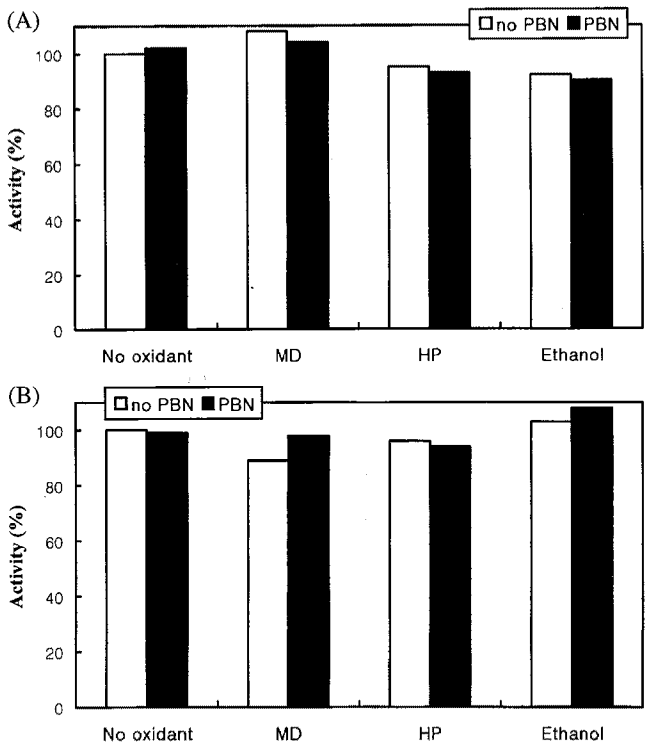

Fig. 4. Effect of PBN on the activity of SOD (A) and catalase (B) upon exposure to oxidant. Activity of non-oxidant-treated cells is expressed as 100%. The results are representative of three separate experiments.

induced injury to the gerbil brain (Oliver *et al.*, 1990), and is effective in the reversal of age-related increases of oxidative damage in the brain (Carney *et al.*, 1991). PBN also renders a protective effect against the acetaminophen toxicity in MVh2E1-9 that constitutively expresses human CYP2E1 cells (Dai and Cederbaum, 1995). It also protects the synaptosomal membrane structure from oxidative stress in accelerated senescence (Butterfield *et al.*, 1997).

To investigate whether or not the difference in viability of the HepG2 cells upon exposure to oxidants is associated with ROS formation, the levels of intracellular peroxides in the HepG2 cell line were measured by fluorescence spectroscopy with the oxidant-sensitive probe DCFH-DA. Deacylation by esterase to dichlorofluoroscin occurs within the cells, and the nonfluorescent dichlorofluoroscin is subsequently oxidized in the presence of intracellular hydroperoxides and peroxides to highly fluorescent dichlorofluorescein (Bass *et al.*, 1983). As shown in Fig. 3, a 2.5- to 3.5- fold increase in fluorescence was observed in the HepG2 cells when they were exposed to 100 μM menadione, 1 mM H_2O_2, or 0.5 M ethanol. This increase in fluorescence was reduced by 36 to 40% in cells pretreated with 2 mM PBN for 1 hr. These data strengthen the conclusion that PBN provides protection from the cytotoxic actions of menadione, H_2O_2, and ethanol by decreasing the steady-state level of intracellular oxidants.

Despite their role in the cellular defense mechanism, the

antioxidant enzymes are susceptible to inactivation by ROS. Previous studies demonstrated that oxidative processes result in the loss of key antioxidant enzymes, which may exacerbate oxidative stress-mediated cytotoxicity. However, it is also possible that procaryotes and eucaryotes compensate for inactivation of antioxidant enzymes by the enhanced expression of SOD, catalase, and other antioxidant enzymes. Therefore, whether or not the presence of PBN induced concomitant alterations in the activity of major antioxidant enzymes was investigated. We measured the activity of O_2^--dismutating enzyme SOD and the activity of H_2O_2-detoxifying enzyme catalase. As shown in Fig. 4, the activity of antioxidant enzymes was not significantly decreased upon exposure to oxidants, and PBN did not affect the activity of antioxidant enzymes. These results indicate that PBN acts as a direct scavenger of ROS.

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