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CAN SPIN TRAPPING COMPOUNDS LIKE PBN PROTECT AGAINST SELF-INFLICTED DAMAGE IN POLYMORPHONUCLEAR LEUKOCYTES?

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Polymorphonuclear leukocytes (PMNs) have been suggested to be damaged by superoxide radical generated on their own. The protective capacity of a spin trapping compound, phenyl-N-*tert*-butyl nitrone (PBN) was evaluated for this damage which occurs after the induction of superoxide generation. The life span of PMNs after superoxide generation was measured in the presence of PBN using the cell counting method, and effects of PBN on the amount of superoxide generated were quantitated using both cytochrome c reduction and spin trapping with DMPO. Results indicated significant extension of life span when PBN was present, and the extension was dose dependent. However, the magnitude of life span extension was not as large as expected from the decrease of superoxide generation. Possible mechanisms for the protection of PMNs by PBN are discussed.

KEY WORDS: spin trapping, PBN, superoxide, leukocytes, oxidative damage

INTRODUCTION

Previous studies revealed that polymorphonuclear leukocytes (PMNs) isolated from blood and suspended in a medium were killed after the respiratory burst is induced by various non-physiological and physiological stimuli.[1-6] Various indications have been found that this cell death is self-inflicted, and in addition, that death is lytic, suggesting that the death-causing damage occurs in the cytoplasmic membrane. The intracellular antioxidant systems of the PMN could provide limited self-defense probably by converting superoxide into less reactive or nonreactive compounds.[7,8] These defenses may not preserve the life of the activated PMNs for an extended period of time, but may permit survival long enough to support PMNs' microbicidal activity against the target. Recent studies have shown that addition of various enzymes and reagents which could decrease superoxide generation elongate the life span of PMNs after stimulation.[9]

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The largest elongation of the life span was achieved in the presence of an NADPH oxidase inhibitor, which completely inhibits superoxide generation. Thus, measurement of the elongation of life span of PMNs after stimulation has been suggested to be a method to evaluate antioxidant activity of the reagent added to the incubation medium.[9]

Phenyl N-*tert*-butyl nitrone (PBN) is a well known synthetic spin trapping compound.[10] Antioxidant-like activity of PBN in the aging study of the Mongolian gerbil model[11] and in lipid peroxidation in rat liver microsomes[12] has been suggested to derive from its trapping reactivity towards oxygen radicals or other free radicals. In biological systems, PBN appears to react with free radicals and transform them into a less reactive form, thus revealing antioxidant activity. Therefore, an experimental attempt was performed using PBN in various concentrations to evaluate the protective capabilities for PMNs against self-inflicted cell death. We monitored PMN's life span and quantitated its superoxide generation in order to discover a correlation between these two parameters.

MATERIALS AND METHODS

Preparation of PMN Suspension

Heparinized venous blood was obtained from healthy human volunteers and was layered on an equal volume of Ficoll-Hypaque solution with a density of 1.112 g/ml.[13] The solution was centrifuged at 1400 × g for 60 min, and the granulocyte layer was removed and suspended in Hanks' balanced salt solution (HBSS). Erythrocytes in the suspension were lysed by suspending the cells in 0.2% saline for 30 sec, followed by washing with HBSS. Viability of the cells immediately after isolation was greater than 98% by the Trypan Blue exclusion method. All steps of PMN preparation were normally performed at room temperature (21 ± 2°C). To avoid loss of cells by their adhesion to the container, plastic vials were pretreated with 200 µl of plasma combined with 1.8 ml of HBSS. This solution was briefly vortexed in the plastic vial and incubated for 30 min in a 37°C warm water bath. Subsequently, vials were washed twice with HBSS before final cell suspension was introduced.

Materials

Granulocyte separation medium was prepared from meglumine sulfate, meglumine disulfate and Ficoll, which were obtained from Sigma Chemical Co. (St. Louis, MO U.S.A.) HBSS, PMA, cytochrome c (horse heart, type IV), trypan blue, 5,5-dimethyl pyrroline N-oxide (DMPO), diethylenetetraamine triacetic acid (DETAPAC), and superoxide dismutase (bovine erythrocyte) were also purchased from Sigma. DMPO was purified by adding an equal volume of HBSS and washing twice with an equal volume of benzene. The aqueous layer was filtered through a column of activated charcoal (15 mm i.d., 20 mm long) which had been previously wetted with HBSS. PBN was synthesized in these laboratories.

Measurement of Survival Rates

Each well in a 96-well tissue culture plate (Becton-Dickinson) was seeded with 250 µl (1×10^4 cells) of PMN suspension and the plate was placed on the stage of an inverted

microscope (Olympus) in a warm room (37 ± 1°C). A single microscope sight was monitored throughout the incubation and the number of cells per unit area was counted using an eyepiece micrometer disc (Fisher).[9] A simultaneous survival rate of control cells was monitored on a second microscope (Zeiss) equipped with the same eyepiece disc. Initial cell count was approximately 110 cells/0.36 mm.[2] Usually, cells sediment on the bottom of the well and adhere, so that the location of the cell in the well did not change throughout the incubation. Because cell death occurred by way of cell lysis or cell rupture, cells which retain their shape or remain intact were counted and percent survival rate was calculated using the initial cell number. A PBN concentration which provides optimal extension of survival time was found by screening the percent survival rate after 150 min incubation using three different PBN concentrations. Then the survival rate curve was created in the presence of several different concentrations of PBN. In order to check the validity of using cell count as live cell count, trypan blue exclusion was used at the termination of the experiment and the viability of countable cells was determined.

Cytochrome c Reduction

Superoxide generation by PMNs was measured by the superoxide dismutase (SOD) inhibitable reduction of cytochrome c.[14] PMNs (1×10^6 cells/ml) were mixed with cytochrome c (0.1 mM) with or without PMA. The cells were incubated at 37°C for 15 min and the reaction was terminated on ice. After brief centrifugation, the supernatant was transferred to an optical cuvette and the absorbance at 550 nm was monitored with a Perkin Elmer Lambda 3 UV/VIS Spectrophotometer at room temperature. Absorbance at 550 nm ($\varepsilon = 21\ 000\ M^{-1}\ cm^{-1}$) was used to determine the superoxide generation rate. In these experiments, cytochrome c reduction was completely inhibited in the presence of SOD (150 U/ml).

Spin Trapping

Although PBN is a spin trapping compound it does not give a stable superoxide spin adduct in superoxide generating systems such as stimulated PMN. Thus, DMPO was used as a spin trap to monitor the amount of superoxide produced. Therefore, in this study, PBN was used as an antioxidant which could modify the concentration of oxygen radicals, and DMPO was used as a spin trap. DMPO (50 mM) and DETAPAC (0.1 mM) were mixed with 0.2 ml (1×10^7 cells) of PMN suspension in HBSS before the addition of PMA (100 ng/ml). DETAPAC was added to suppress the effect of iron on radical generation. Stimulated PMNs were transferred to a flat quartz EPR sample-cell (Wilmad WG814), which was then placed into the spectrometer in a horizontal orientation.[15] EPR spectra were recorded using a Bruker ER 300E EPR spectrometer equipped with 100-kHz field modulation. Other typical EPR operating conditions were microwave power, 20 mW; modulation width, 1.0 gauss; field sweep, 100 gauss/84 s; time constant, 160 ms. Concentration of superoxide was calculated by calibrating the EPR intensity of DMPO-OOH using a solution of a stable radical, 2,2,5,5-tetramethyl piperidine-1-oxyl (TEMPO).

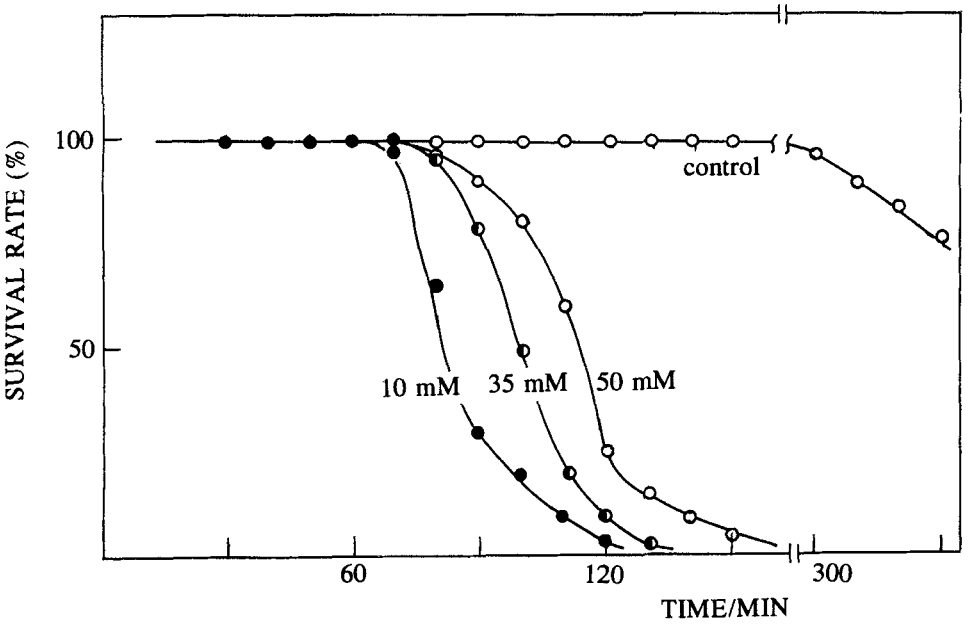

FIGURE 1 Time course of percent cell-count of PMNs incubated under various conditions at 37°C. "control" is in HBSS, from left to right; 100 ng/ml PMA was added in HBSS at time 0 in the presence or absence of 10 mM PBN, 100 ng/ml PMA was added in HBSS at time 0 in the presence of 35 mM PBN. 100 ng/ml PMA was added in HBSS at time 0 in the presence of 50 mM PBN.

RESULTS

Survival Rate Curve

Cells were counted every 10 min, beginning 30 min after the addition of PMA. Cell count obtained was approximately the same as live cell count because countable cells achieved high viability (>95%). In the absence of induced superoxide generation (– (minus) PMA control), the survival rate did not decrease for 5 hrs (Figure 1). Then a gradual decline in cell number occurred over the next hour. Control cells induced with PMA (+PMA control), maintained 100% survival until 80 min, on the average. At this point, an abrupt decrease in cell number occurred with a total death at 120 to 140 min. Average T_{50} (50% survival time) was 100 min.

PBN was used in 10 mM, 20 mM, 35 mM, 50 mM concentrations. In all cases when PBN was present in the medium, the period of initial drop in the cell number was prolonged, but an abrupt drop similar to the control was observed. Survival curves in the presence of PBN paralleled the +PMA control survival curve with a 10 to 30 min life extension (Figure 1). Usually addition of PMA promotes cell adhesion to the bottom of the well and adherent cells visually flatten their shape. At PBN concentrations of 50 mM this cell shape change was not observed, suggesting that PBN interacts with the cell adhesion processes.

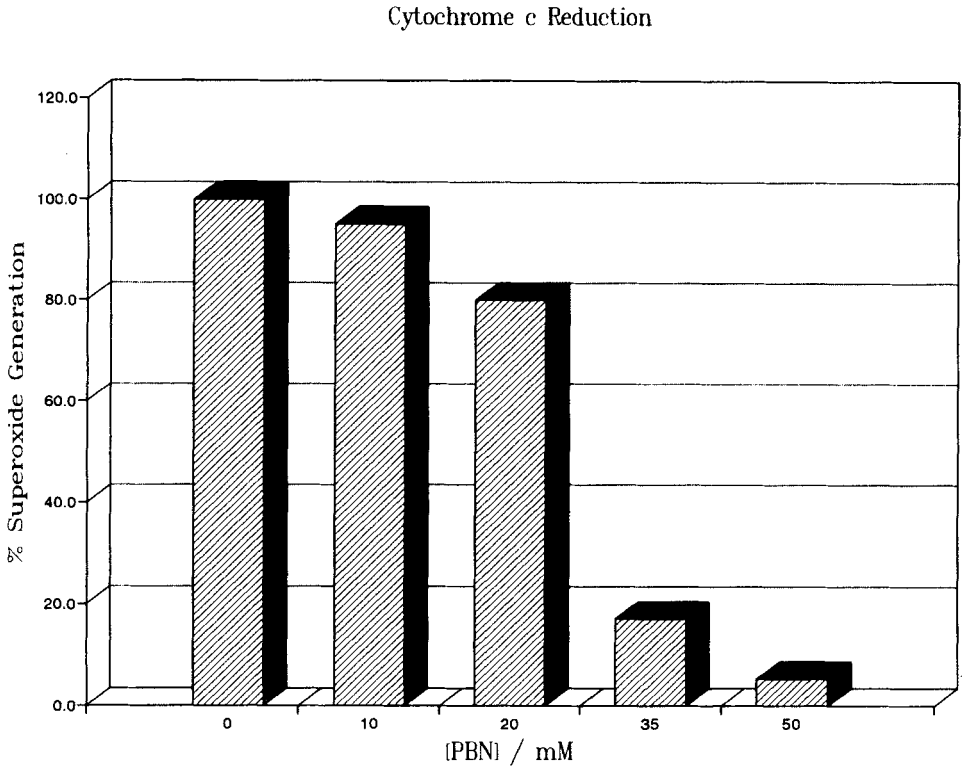

FIGURE 2 Relative superoxide generation quantitated by the cytochrome c reduction method. PMNs were incubated for 15 min at 37°C in HBSS, in the presence of 100 ng/ml PMA, 100 μM cytochrome c, and various concentrations of PBN shown on the x-axis. Each number represents the average of three separate experiments. S.D. is ±20%.

Cytochrome c Reduction

Superoxide obtained from PMA-stimulated PMNs in the presence of PBN was determined using SOD-inhibitable cytochrome c reduction. The +PMA control cells yielded superoxide generation of an average of 4.0 nM/million cells/min. In the presence of PBN in the medium, significant reduction of superoxide was achieved (Figure 2). A concentration of 50 mM reduced superoxide production 95% as compared to control. As PBN concentration decreased, a marked increase in superoxide generation occurred; 35 mM produced 85% reduction, 20 mM a 20% reduction, 10 mM a 5% reduction.

As a test for location uptake of PBN, neutrophils were preincubated with a 50 mM concentration for 30 min in a 37°C warm water bath and subsequently washed. Superoxide generation was again reduced significantly (70%).

Spin Trapping

When neutrophils were stimulated with PMA at room temperature, transferred into a flat EPR cell, an EPR spectrum was obtained approximately 3–4 min following

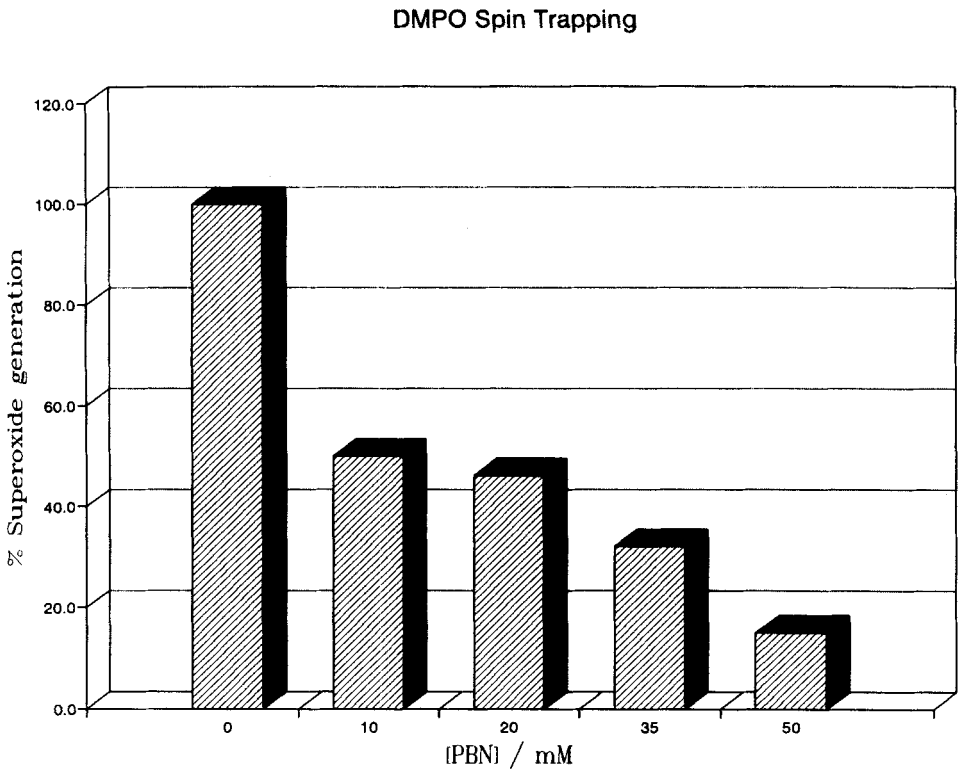

FIGURE 3 Relative superoxide generation quantitated by the DMPO spin-trapping method. HBSS suspension of PMNs (10^7 cells/ml) was stimulated with PMA (100 ng/ml) in the presence of 50 mM DMPO and various concentrations of PBN shown on the x-axis. EPR line height obtained 10 min after stimulation was measured. Each number represents the average of three separate experiments. S.D. is ±28%.

stimulation. The EPR spectrum is characteristic of the superoxide adduct DMPO-OOH. Superoxide was quantitated by measuring the height of the lowest field line of the EPR spectrum of DMPO-OOH, 10 min after stimulation. Superoxide generation as a function of PBN concentration exhibits dose dependent reduction (Figure 3). At PBN concentration of 50 mM it demonstrated approximately 85% decrease, 35 mM-70%, 20 mM-70%, 20 mM-55%, 10 mM-50%. As a test for location uptake of PBN, PMNs were preincubated in the presence of 50 mM PBN. This preincubated cell achieved 57% ± 23% reduction (n = 3) of superoxide generation as compared with control.

DISCUSSION

The immediate outcome of superoxide generation of PMNs may be the damage of membrane components followed by the accumulation of the damaged components in the plasma membrane. If the damaged species accumulates at a rate beyond the PMN's ability to repair itself, cell death could occur. In addition, an abrupt decrease in cell number suggests the presence of the threshold amount of damaged species above which

the mechanical structure of the cell rapidly disintegrates.[9] In fact, microscopic observation indicates that the direct cause of cell death is cell lysis. In these experiments, PBN provided PMNs with moderate lifetime extension after stimulation with PMA. Because PBN is relatively hydrophobic (solubility in water at room temperature is approximately 0.1 M) and thus partitions into the plasma membrane, the mechanism of this lifetime extension could be partial quenching of superoxide within the membrane.

Thus, superoxide generation was quantitated in order to correlate to the life span extension. Although both cytochrome c reduction and spin trapping demonstrated significant reduction in superoxide production in the presence of PBN, a discrepancy is exists: the cytochrome c reduction assay in the presence of 50 mM PBN showed 98% decrease in superoxide generation, but T_{50} in the presence of 50 mM (110 min) did not depict extensive increase of lifetime and the T_{50} was much shorter than the control with no superoxide generation (360 min). Cytochrome c is a protein molecule (molecular weight: 13 000) which may not enter into the cell membrane so that only superoxide present in the media can be detected. Therefore, the 98% decrease in superoxide accessed by cytochrome c reduction does not necessarily mean that superoxide which has a capacity of damaging surrounding molecules is decreased by 98%. In contrast, DMPO (molecular weight: 114) is a small molecule and is likely to be more accessible to the superoxide generation site than cytochrome c.[16] DMPO spin trapping results indicated 50% reduction of superoxide production in the presence of 50 mM PBN, and this reduction could be closer to the actual decrease because PBN may quench superoxide closer to its generation site.

Preincubation of PMNs with PBN provides evidence that plasma membrane intake of PBN is involved in the lifetime extension. Cells washed with HBSS buffer after preincubation with a 50 mM PBN solution still showed lifetime extension similar to that achieved by the cell whose lifetime was measured in the presence of 50 mM PBN in the incubation medium. However, the reduction of superoxide generation from PBN-preincubated PMN was less than that measured in the presence of PBN in the medium. The reason may be attributed to the competitive trapping of superoxide by DMPO and PBN, both present in the medium. These facts support the hypothesis that PBN enters into the plasma membrane in close proximity to the superoxide generation site[17] and subsequently reacts to transform superoxide into less harmful species.

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