*Biochemical Pharmacology*, Vol. 38, No. 1, pp. 85–90, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00


# EVIDENCE FOR INTRACELLULAR SUPEROXIDE FORMATION FOLLOWING THE EXPOSURE OF GUINEA PIG ENTEROCYTES TO BLEOMYCIN

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(*Received* 3 *November* 1987; *accepted* 20 *June* 1988)

**Abstract**—Spin trapping of free radicals during the exposure of guinea pig enterocytes to bleomycin (BLM) was investigated using an *in vitro* cell suspension. The spin traps employed in this study were 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3-diethyl-5,5-dimethyl-1-pyrroline-1-oxide (DEDMPO). The hydroxyl radical spin-trapped adduct 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH) was observed with DMPO. In the presence of dimethyl sulfoxide (DMSO), the only 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-$CH_3$) observed was that expected from hydroxyl radical formation by the decomposition of the superoxide spin-trapped adduct 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH). Production of hydroxyl radical was not detected in the presence of DEDMPO, which is a nitrone that will spin trap hydroxyl radical, but not superoxide, at cellular concentrations. Thus, these data indicate that superoxide was produced during the exposure of guinea pig enterocytes to BLM and that DMPO-OH resulted from the cellular bioreduction of DMPO-OOH by glutathione peroxidase. Addition of superoxide dismutase to the *in vitro* reaction mixture indicated that superoxide production was intracellular.

Bleomycin (BLM), first isolated by Umezawa *et al.* [1], is a generic name for a group of antibiotics which are derived from *Streptomyces verticillus*. They are water-soluble, basic glycopeptides which differ from one another in their terminal-amine structures [2]. Bleomycins have been shown to mediate single- and double-strand breaks in DNA by intercalation, during which ferrous ion and oxygen are required [3–7]. During this reaction, the strand scission takes place predominantly at guanine-pyrimidine base sequences [8], producing free bases and oligonucleotides as products [3, 9]. As a result of this degradation activity, these antibiotics have been quite useful in the treatment of certain cancers, including squamous cell carcinoma, lymphomas, and testicular carcinoma. However, pulmonary fibrosis has been reported in a number of cases with bleomycin treatment [10].

Many biochemical mechanisms have been proposed to explain the bleomycin induced strand-scission of DNA. A communication by Lown and Sim [11] included a free-radical mechanism in which hydroxyl radical was suggested to be the primary species responsible for the strand-scission of DNA. Hydroxyl radical can arise from the interaction of hydrogen peroxide, formed from the dismutation of superoxide, with ferrous ion via a Haber–Weiss process [12]. The reaction sequence is given opposite. In Lown's proposed mechanism, hydroxyl radical arose by the interaction of hydrogen peroxide with the bleomycin/ferrous ion complex (BLM–$Fe^{2+}$). Hydroxyl radical, which is a highly reactive intermediate and is known to cause extensive membranous injury [13], could conceivably nick DNA since it is in close proximity. However, Low and Sim [11] also confirmed the intermediacy of superoxide radical in the interaction of BLM–$Fe^{2+}$ and oxygen.

One method commonly employed to confirm the presence of transient free radicals in biologic milieu is spin trapping [14]. This technique consists of using a nitrone to "trap" the initial unstable free radical as a "long-lived" nitroxide that can be observed at ambient temperatures using conventional electron spin resonance (ESR) spectrometric procedures [15]. Since the stable nitroxide radical accumulates, spin trapping is an integrative method of measurement and is more sensitive than procedures that determine instantaneous or steady-state concentrations of free radicals.

A review of the literature indicates that several studies have been devoted to the spin trapping of free radicals in an aqueous aerobic system consisting of BLM and ferrous sulfate. Hydroxyl radical was detected in several studies with *N-tert*-butyl-$\alpha$-phenyl nitrone (PBN) [16, 17] and with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) [18, 19]. Superoxide formation was detected by using PBN in the presence of a low concentration of the BLM–$Fe^{2+}$ complex

$$2\,O_2^{\bullet-} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$
$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$
$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$$
$$3\,O_2^{\bullet-} + 2H^+ \longrightarrow 2\,O_2 + OH^- + {}^{\bullet}OH$$

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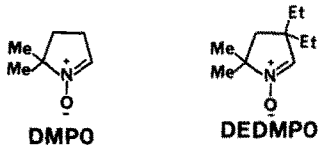


Fig. 1. Structural formulas for 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3-diethyl-5,5-dimethyl-1-pyrroline-1-oxide (DEDMPO).

[16, 17]. In a study published by Sugiura [17], spin trapping of free radicals was inhibited by catalase, a scavenger for hydrogen peroxide, and superoxide dismutase (SOD), which greatly enhances the dismutation rate of superoxide radical [20]. In another work by Sugiura [18], the formation of 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH) was inhibited by the addition of catalase, indicating that hydroxyl radical was being produced. Thus, the intermediacy of both hydroxyl and superoxide radicals in an aqueous aerobic system consisting of BLM and ferrous sulfate has been established by spin-trapping techniques.

Despite the above experimental observations, there are still several important questions that need to be addressed. First, are the same reactive intermediates being generated when BLM and ferrous ion are exposed to cellular systems? There are many cellular components, such as DNA [21], which will react with superoxide. Since there is a short mean free path for superoxide radical to interact with DNA near the site of intercalation, then conceivably hydrogen peroxide may not be produced from the dismutation of superoxide. In this case, hydroxyl radical may not be formed by a Haber–Weiss process in the cytoplasm. Second, are these reactive oxygen radicals being generated extracellularly during BLM treatment, which could induce membranous injury in treated tissues? Third, is the observed spin adduct DMPO-OH [18] simply an artifact due to the reaction of DMPO with oxygen in the presence of trace ferric ion or does it result from degradation of the superoxide spin-trapped adduct 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OOH) [for a discussion see Ref. 22]?

With the above in mind, we decided to examine free radical production in an *in vitro* cell suspension model. For this purpose we utilized guinea pig enterocytes. The spin traps employed in this study were DMPO and 3,3-diethyl-5,5-dimethyl-1-pyrroline-1-oxide (DEDMPO), the structures of which are given in Fig. 1.

## MATERIALS AND METHODS

*General comments.*

Xanthine oxidase, hypoxanthine, bovine erythrocyte superoxide dismutase (SOD), *N*-*N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (DTPA), and bovine liver catalase (CAT) were obtained from the Sigma Chemical Co. Blenoxane, or bleomycin sulfate, was obtained from Bristol Laboratories. Chelex 100 was purchased from Bio-Rad. Phosphate buffers were passed through a Chelex-100 column according to the method of Poyer and McCay [23] to remove trace metal impurities. All other reagents were used as obtained from commercial suppliers unless otherwise noted. Electron spin resonance (ESR) spectra were recorded on a Varian Associates model E-9 spectrometer.

*Syntheses*

DMPO was prepared according to the method of Bonnett *et al.* [24]. DEDMPO was synthesized by a procedure outlined by Rosen and Turner [25]. Purification of these spin traps was 2-fold. First, fractional elution was performed with silica gel (mesh 230–400) using a dichloromethane/methanol solvent system. Second, the purified fraction containing the nitrone was Kugelrohr distilled twice. Nitrones were stored at −70° under argon until use.

*Biochemical studies*

*Spin trapping of hydroxyl radical.* The spin trapping of hydroxyl radical was undertaken by the addition of ferric ammonium sulfate (0.1 nM) to a superoxide-generating system consisting of hypoxanthine (400 $\mu$M), 0.1 M potassium phosphate buffer (pH 7.8), and xanthine oxidase such that the rate of superoxide production was 10 $\mu$M/min at 25°. Measurement of superoxide was determined optically by the reduction of cytochrome *c* at 550 nm with use of an extinction coefficient of 20 $mM^{-1}\,cm^{-1}$. The reaction was initiated by the addition of xanthine oxidase. By monitoring the conversion of xanthine to uric acid at 292 nm, it was determined that the nitrones utilized did not inhibit the enzyme under these experimental conditions. No free radical was spin trapped in the presence of catalase (300 units/ml).

*Isolation of guinea pig enterocytes.* Male Hartley guinea pigs, weighing from 400 to 550 g, from the Hazelton Laboratories were acclimated for at least 3 days prior to use. They were housed on corn cob bedding and fed Purina guinea pig chow. They were allowed food and water *ad lib.* and were maintained on a 12-hr photo period.

Guinea pigs were placed under ether anesthesia. The small intestine was excised and quickly placed in ice-cold saline (0.9% NaCl, w/v, pH 7.4). The intestine was cut into 20-cm lengths and flushed with cold saline to remove fecal material. The intestinal segments were then everted over a glass rod, removed from the rod, and one end was ligated with 3-0 silk. Each intestinal segment was filled until slightly distended with a Krebs CMF (calcium and magnesium free) buffer containing NaCl (120 mM), $NaHCO_3$ (23.8 mM), KCl (4.7 mM), $KH_2PO_4$ (1.2 mM), bovine serum albumin (0.5%, w/v), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, 20 mM), and glucose (5 mM). The filled intestinal lengths were placed in a 250-ml Erlenmeyer flask containing 50 ml of Krebs CMF buffer and 17 mg of protease (Sigma, 7.9 units/mg). The flask was incubated at 37° in a shaker water bath under 95% $O_2$/5% $CO_2$. After 20 min, the Krebs CMF buffer with protease was replaced with fresh Krebs CMF buffer and incubated for another 20 min. Cells were poured off and centrifuged at 1500 rpm for 60 sec. The collected enterocytes were resuspended

in Swim's medium and viability was determined by using the Trypan blue exclusion method, which indicated 90–95% viability.

*Glutathione peroxidase activity of isolated enterocytes.* Cells were homogenized in a sucrose/phosphate buffer (0.25 M sucrose containing 1 mM EDTA and 50 mM potassium phosphate at pH 7.4) by a polytron homogenizer at speed 5 for 3 sec (model PT 10-35 with a PT-20-Ft generator, Brinkmann Instruments Co., Westbury, NY). These homogenates were centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant fraction was then centrifuged at 100,000 *g* for 60 min. The supernatant was used to determine glutathione peroxidase activity as outlined by Flohe and Gunzler [26]. Protein was measured by the method of Lowry *et al.* [27].

*Spin trapping of free radicals generated during BLM exposure to enterocytes.* Spin-trapping experiments were designed to detect free radicals generated during exposure of guinea pig enterocytes to BLM and ferrous sulfate in the presence of oxygen. A typical reaction mixture contained 1 million cells, blenoxane (0.1 mM), ferrous sulfate (0.1 mM), the spin trap (either DMPO or DEDMPO, 0.1 M), and sufficient Swim's medium to bring the total volume to 0.5 ml. Reaction mixtures were then transferred to a flat quartz cell and fitted into the cavity of the ESR spectrometer at room temperature. The ESR spectrum was obtained within 2 min after initiation of the reaction.

## RESULTS

### *Spin trapping of free radicals with DMPO*

When guinea pig enterocytes were incubated with bleomycin (BLM) and ferrous sulfate in the presence of DMPO under aerobic conditions, the ESR spectrum in Fig. 2A was obtained. This spectrum ($A_N$ = $A_H$ = 14.9 G) is characteristic of DMPO-OH [28]. The addition of catalase (300 units/ml) or superoxide dismutase (10 μg/ml) to the reaction mixture did not affect the intensity of the ESR signal, indicating that radical production was intracellular. When ferrous sulfate was omitted from the above reaction mixture and DTPA added, the ESR spectrum in Fig. 2C was obtained. The same spectrum (Fig. 2C) was obtained when BLM was omitted from the above reaction mixture. This indicates that some DMPO-OH was initially present due to the reaction of DMPO with oxygen in the presence of trace metal impurities [22]. Also, free radical generation requires the presence of all components in the reaction mixture described

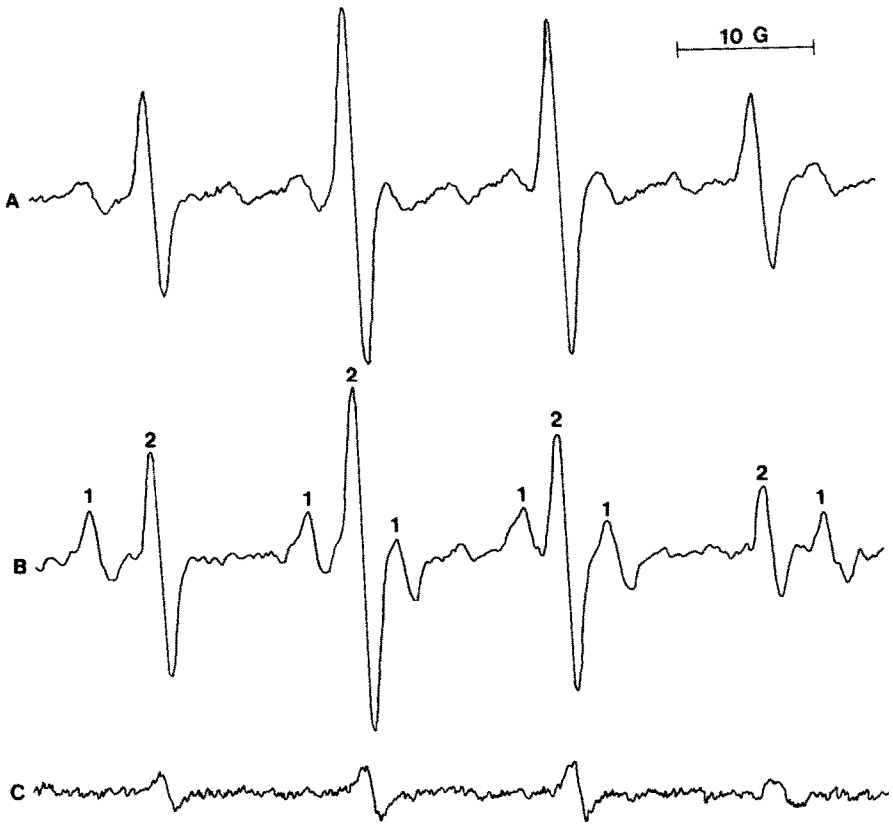


Fig. 2. (A) ESR spectrum obtained when enterocytes ($1 \times 10^6$) were incubated with BLM (0.1 mM) and $FeSO_4$ (0.1 mM) in the presence of DMPO (0.1 M). $A_N = A_H = 14.9$ G. (B) Same as A except that DMSO (0.14 M, final concentration) was added. $A_N = 15.3$ G and $A_H = 22.0$ G (for DMPO-$CH_3$, peaks 1). (C) Same as A except that $FeSO_4$ was omitted and DPTA added. Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 1.0 G. Sweep time was 12.5 G/min and the receiver gain was $4.0 \times 10^3$ with a response time of 1.0 sec.

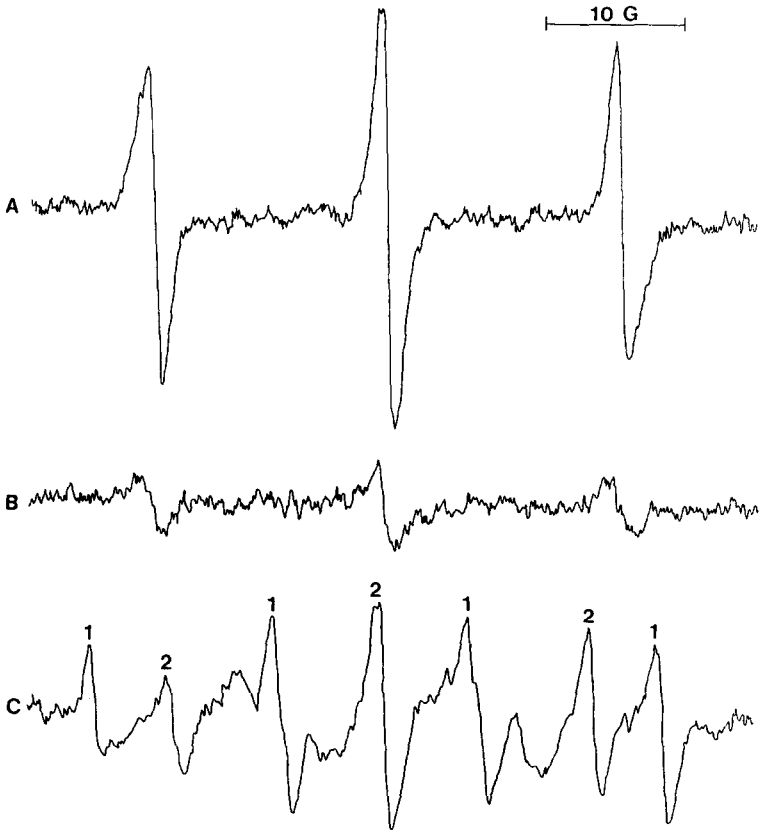


Fig. 3. (A) ESR spectrum obtained when enterocytes ($1 \times 10^6$) were incubated with BLM (0.1 mM) and $FeSO_4$ (0.1 mM) in the presence of DEDMPO (0.1 M). $A_N = 16.5$ G. (B) Same as A except that $FeSO_4$ was not added. (C) Ferric ammonium sulfate (0.1 mM) was added to a superoxide-generating system consisting of hypoxanthine and xanthine oxidase in the presence of DEDMPO (0.1 M). $A_N = A_H = 13.0$ G (for DEDMPO-OH, peaks 1). Scanning conditions were the same as in Fig. 2 except that the receiver gain was $1.25 \times 10^4$.

above. The spectrum in Fig. 2B was obtained when DMSO (final concentration 0.14 M) was added to the system. The peaks labeled 1 arose from the spin adduct 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-$CH_3$) and the peaks labeled 2 from DMPO-OH.

*Spin trapping of free radicals with DEDMPO*

When guinea pig enterocytes were incubated with BLM and ferrous ion in the presence of DEDMPO under aerobic conditions, the ESR spectrum in Fig. 3A was obtained ($A_N = 16.5$ G). The spectrum in Fig. 3B was obtained when ferrous sulfate was omitted from the above reaction mixture. Figure 3C was obtained from the spin trapping of hydroxyl radical to give the spin adduct 3,3-diethyl-2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DEDMPO-OH). Spin trapping of this radical was conducted by adding ferric ammonium sulfate (0.1 mM) to a superoxide-generating system consisting of hypoxanthine (400 $\mu$M) and xanthine oxidase in potassium phosphate buffer (pH 7.8) such that the rate of superoxide production was 10 $\mu$M/min at 25° [25]. The peaks labeled 1 ($A_N = A_H = 13.0$ G) resulted from the spin trapping of hydroxyl radical with DEDMPO, whereas the peaks labeled 2 resulted from the metal-catalyzed aerial oxidation of the spin trap. This nitroxide was also evident in spectrum 2A. Aerial oxidation to this nitroxide occurred rapidly under these conditions since ferrous sulfate was present in a concentration of 1 mM. Thus, this experimental data exclude the production of hydroxyl radical by BLM–$Fe^{2+}$ in the enterocyte system.

## DISCUSSION

Comparison of Fig. 2A and 2C suggests that hydroxyl radical is being generated as a result of the exposure of guinea pig enterocytes to bleomycin and ferrous sulfate under aerobic conditions. However, it is known that the spin adduct DMPO-OOH can decompose into several species, one being DMPO-OH [22]. This decomposition may occur enzymatically or chemically. If the decomposition is primarily chemical, then DMPO-OOH should be observed since the half-life of this adduct is approximately 8 min [29]. Since DMPO-OOH was not observed, then either superoxide radical is not being generated or the decomposition of DMPO-OOH is occurring rapidly by another mechanism. It has been demonstrated that DMPO-OOH is reduced to DMPO-OH by glutathione peroxidase [30], whose physiological function is the reduction of organic

hydroperoxides to alcohols [31]. We determined the glutathione peroxidase activity of the cells to be 19 ± 1 nmol/min/mg protein. To determine whether or not hydroxyl radical is being initially generated, we incubated enterocytes with BLM and ferrous sulfate in the presence of DMSO. If hydroxyl radical is being produced, then the spin adduct DMPO-$CH_3$ should be observed at the expense of DMPO-OH [14; see Ref. 32 for computer-simulated spectra of these adducts]. Comparison of Fig. 2A and 2B reveals that hydroxyl radical was not being produced during the exposure of guinea pig enterocytes to BLM and ferrous sulfate under aerobic conditions. If hydroxyl radical were being generated, then we should observe a marked decrease in the intensity of the DMPO-OH signal (peaks 2, Fig. 2B) and a marked increase in the DMPO-$CH_3$ signal (peaks 1, Fig. 2B). Note that some artifact was initially producing a signal in the DMPO-$CH_3$ region before DMSO was added to the system (Fig. 2A). However, it is evident that some methyl radical was being produced in the presence of DMSO. This could result from the formation of hydroxyl radical by rapid decomposition of DMPO-OOH [22]. Thus, these results indicate that the adduct DMPO-OH primarily arises from rapid cellular bioreduction of DMPO-OOH by glutathione peroxidase and not from the direct spin trapping of hydroxyl radical.

Recently we outlined a synthesis of DEDMPO, which will spin trap hydroxyl radical, but not superoxide, at cellular concentrations [25]. We utilized this spin trap to determine whether hydroxyl radical was being produced in this system. Comparison of Fig. 3A to 3C reveals that hydroxyl radical was not being produced directly. Thus, we conclude that superoxide is generated during the exposure of guinea pig enterocytes to BLM and ferrous sulfate under aerobic conditions.

The experimental results above raise the question of whether superoxide is being generated both intracellularly and extracellularly. To answer this query, we incubated guinea pig enterocytes with BLM, ferrous sulfate, and SOD (10 μg/ml) in the presence of DMPO (0.1 M) under aerobic conditions. The resulting ESR spectrum was identical to that of Fig. 2A. Since superoxide dismutase does not diffuse across the cell membrane, this result suggests that free radical production is primarily intracellular.

In conclusion, there is no evidence from the experimental results given above to indicate that hydroxyl radical is being formed intracellularly during exposure of guinea pig enterocytes to BLM and ferrous sulfate. These data suggest that superoxide is initially spin trapped by DMPO giving DMPO-OOH, which is then subsequently bioreduced to give DMPO-OH. Superoxide radical, and not hydroxyl, is being generated when enterocytes are exposed to BLM and $Fe^{2+}$. And since the generated superoxide is in close proximity to cellular components, such as DNA, during intercalation of the drug, it must interact rapidly with these components so that detectable levels of hydroxyl radical cannot be produced by a Haber–Weiss process. Thus, superoxide must be involved in the free radical mechanism of DNA strand scission during the intercalation of BLM-$Fe^{2+}$. It has been demonstrated that superoxide can mediate DNA damage without the intermediacy of hydrogen peroxide and the subsequent formation of hydroxyl radical [21]. Finally, it is important to note that our conclusions may be limited to this cell type and to these experimental conditions. Thus, we are currently exploring the generality of these findings.

*Acknowledgements*—This work was supported in part by the Wake Forest Research and Publication Fund Grant. The authors wish to thank Dr. Gerald M. Rosen (Duke University Medical Center, Department of Pharmacology) for allowing us the use of his ESR instrument and for his support during this project.

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