Biochemical Pharmacology, Vol. 41, No. 11, pp. 1701–1705, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EVIDENCE FOR SUPEROXIDE FORMATION DURING HEPATIC METABOLISM OF TAMOXIFEN

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(*Received* 8 *August* 1990; *accepted* 13 *December* 1990)

Abstract—Spin trapping of free radicals during the hepatic metabolism of tamoxifen was investigated; the spin trap employed in this study was 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). The spin adduct 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH) was detected in an *in vitro* incubation mixture of phenobarbital-treated rat hepatocytes containing tamoxifen, dimethyl sulfoxide, and DMPO. However, since the spin adduct 2,5,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3) was not observed, the DMPO-OH resulted from the cellular bioreduction of 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH) by glutathione peroxidase. Addition of superoxide dismutase (SOD) to the *in vitro* system indicated that superoxide production was intracellular. When noninduced hepatocytes were utilized, free radical production was not evident. Thus, the cytochrome P450 monooxygenase system was responsible, in part, for the intermediacy of superoxide anion during hepatic metabolism.

Tamoxifen (TAM), the structure of which is given in Fig. 1, is a antioestrogenic drug that has been utilized in the effective clinical treatment of advanced breast cancer [1]. It is available as a pallative drug under the trade name Nolvadex. Many different metabolic routes have been reported for this drug. Fromson *et al.* [2] first reported that TAM was metabolized extensively in laboratory animals, such as the dog, rat, and rhesus monkey; the major metabolite observed was 4-hydroxytamoxifen, derived from aromatic hydroxylation. Kemp *et al.* [3] reported that the primary serum metabolite isolated from humans is the *N*-desmethyl derivative. Further studies from rat hepatic systems indicate the production of tamoxifen *N*-oxide, 4'-hydroxy-tamoxifen, α-hydroxytamoxifen *N*-oxide, 1-[4-(2-hydroxyethoxy)phenyl] - 1 - (4 - hydroxyphenyl) - 2-phenyl-1-butene, and other metabolites [4–7]. Recently, 4-hydroxyl-*N*-desmethyltamoxifen was identified as a metabolite in human bile [8]. However, free radical intermediates have not been reported.

One method that is commonly employed to confirm the presence of transient free radicals in biologic milieu is spin trapping [9]. 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 [10]. 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.

The purpose of this study was to utilize spin-trapping techniques to demonstrate the formation of free radicals during the metabolism of tamoxifen. For this study an *in vitro* cell suspension of isolated rat hepatocytes was utilized. The spin-trapping agent employed was 5,5-dimethyl-1-pyrroline-1-oxide (DMPO).

MATERIALS AND METHODS

General comments

Xanthine oxidase, hypoxanthine, bovine erythrocyte superoxide dismutase (SOD), *N*,*N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (DTPA), *N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), bovine liver catalase (CAT), collagenase, and tamoxifen citrate were obtained from the Sigma Chemical Co. Chelex 100 was purchased from Bio-Rad. Potassium phosphate buffer (pH 7.8) was passed through a Chelex-100 column according to the method of Poyer and McCay [11] to remove trace metal ions. All other reagents were obtained from commercial suppliers unless otherwise noted. ESR spectra were recorded on a Varian Associates model E-9 spectrometer.

Synthesis

DMPO was synthesized according to the method of Bonnett *et al.* [12]. Purification of this spin trap 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 triply

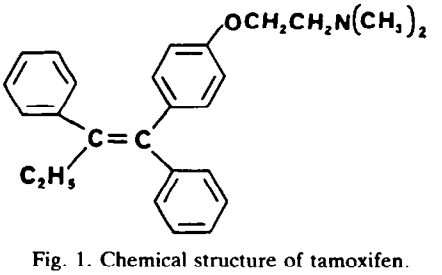

Fig. 1. Chemical structure of tamoxifen.

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Kugelrohr distilled at room temperature. The spin trap was stored at −78° and then Kugelrohr distilled prior to use. The spin trap purified in this manner was devoid essentially of any paramagnetic impurities.

Biochemical studies

Spin trapping of superoxide radical. The superoxide-generating system contained hypoxanthine (400 μM), DTPA (1 mM), DMPO (0.1 M), CAT (300 units/mL), 50 mM potassium phosphate buffer (pH 7.8), and xanthine oxidase such that the rate of superoxide production was 10 μM/min at 25°. The measurement of superoxide was determined optically by the reduction of cytochrome *c* at 550 nm using 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 spin trap did not inhibit the enzyme under these experimental conditions. The reduction of cytochrome *c* by superoxide was prevented completely by the addition of SOD (10 μg/mL). Free radical trapping was not observed if any component of the reaction mixture was omitted.

Spin trapping of hydroxyl radical. The spin trapping of hydroxyl radical was undertaken by the addition of ferric ammonium sulfate (0.1 mM) and SOD (10 μg/mL) to the aforementioned superoxide-generating system. The production of hydroxyl radical was inhibited completely by the addition of catalase (300 units/mL).

Isolation of rat hepatocytes. Male Fischer 344 rats (Kingston Colony, Charles River Breeding Laboratories) were acclimated for at least 7 days prior to being killed. They were housed on corn cob bedding to prevent enzyme induction and fed rodent chow. They were allowed food and water *ad lib.* and were maintained on a 12-hr photo period. For induction of cytochrome P450, rats were pretreated with 0.1% (w/v) phenobarbital in their drinking water (pH 7.4) for 3–5 days prior to being killed.

Hepatocytes were isolated using a modification of the procedure by Seglen [13]. Briefly, rat livers were perfused *in situ* via the inferior vena cava with a chelated calcium-free Krebs–Ringer solution (24 mM $NaHCO_3$, 120 mM NaCl, 4.8 mM KCl, and 1.2 mM KH_2PO_4) at pH 7.4 containing L-lysine (0.1 mM), ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) (0.1 mM), HEPES (5.0 mM), dextrose (20 mM), and concentrated heparin (200 units) at a rate of 20 mL/min for about 15 min. The liver was perfused afterwards with a Krebs–Ringer solution containing $CaCl_2$ (1.0 mM), dextrose (20 mM), bovine serum albumin (15.0 g/L), and collagenase (400 mg/L). The hepatocytes were washed three times in a Krebs–Ringer solution with 1.2 mM $CaCl_2$. After the last wash, the cells were resuspended in Swim's S-77 medium, pH 7.4, supplemented with 26.0 mM $NaHCO_3$, 20.0 mM HEPES, 2.0 mM L-glutamine, and 2.0 mM $CaCl_2$. The cells were typically 90–93% viable as determined by the Trypan blue exclusion method, and the viability remained above 85% for the duration of the studies.

Glutathione peroxidase activity of isolated hepatocytes. The isolated hepatocytes were homogenized in a glucose/phosphate buffer (0.25 M glucose containing 1 mM EDTA and 50 mM potassium phosphate at pH 7.2). The homogenates were centrifuged at 9000 *g* for 20 min, followed by 100,000 *g* for 60 min. The supernatant was used to determine glutathione peroxidase activity as outlined by Flohe and Gunzler [14]. Glutathione peroxidase activity was normalized to protein as measured by the Coomassie blue dye method [15].

Spin trapping of free radicals generated during hepatic metabolism of tamoxifen. Spin-trapping experiments were designed to detect free radicals generated during the hepatic metabolism of tamoxifen. A typical incubation mixture contained 1 million hepatocytes, tamoxifen (0.01 mM) in dimethyl sulfoxide (0.14 M final concentration), DMPO (0.1 M), and sufficient Swim's medium to bring the total volume to 0.5 mL. The incubation mixture was transferred immediately to a flat quartz cell and fitted into the cavity of the ESR spectrometer at room temperature. The scan of the ESR spectrum was begun within 1 min after the addition of tamoxifen.

RESULTS

The spin trapping of superoxide with DMPO was conducted using a superoixde-generating system consisting of hypoxanthine in the presence of the enzyme xanthine oxidase at pH 7.8. The rate of superoxide formation, as measured by the superoxide dismutase inhibitable reduction of cytochrome *c*, was 10 μM/min. The ESR spectrum obtained for 2 - hydroperoxy - 5,5 - dimethyl - 1 - pyrrolidinyloxyl (DMPO-OOH) is illustrated in Fig. 2A (A_N = 14.3 G and A_H = 11.7 G). The ESR signal was inhibited completely by the addition of SOD, confirming that the ESR spectrum resulted from the spin trapping of superoxide.

The spin trapping of hydroxyl radical with DMPO was conducted by the addition of ferric ammonium sulfate (0.1 mM) to the aforementioned superoxide-generating system. The ESR spectrum obtained for 2-hydroxyl-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH) is shown in Fig. 2B ($A_N = A_H$ = 14.9 G). This signal was inhibited completely the addition of catalase (CAT), indicating that the ESR spectrum resulted from the spin trapping of hydroxyl radical. When dimethyl sulfoxide (DMSO) was added to the reaction mixture, the ESR spectrum in Fig. 2C was obtained (A_N = 15.3 G and A_H = 22.0 G). This spectrum is characteristic of 2,5,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3); the intensity of the signals from DMPO-OH was diminished markedly.

When phenobarbital-induced rat hepatocytes were incubated with tamoxifen (0.01 mM) in DMSO (0.14 M final concentration), the ESR spectrum in Fig. 3A was obtained ($A_N = A_N$ = 14.9 G); this tracing is characteristic of DMPO-OH [16]. The addition of SOD (10 μg/mL) or CAT (300 units/mL) did not affect the intensity of the ESR signal; the addition of *n*-octylamine completely inhibited the ESR signal. When tamoxifen was omitted from the incubation mixture, the baseline in Fig. 3B resulted. Free radical generation required all components listed in the aforementioned incubation

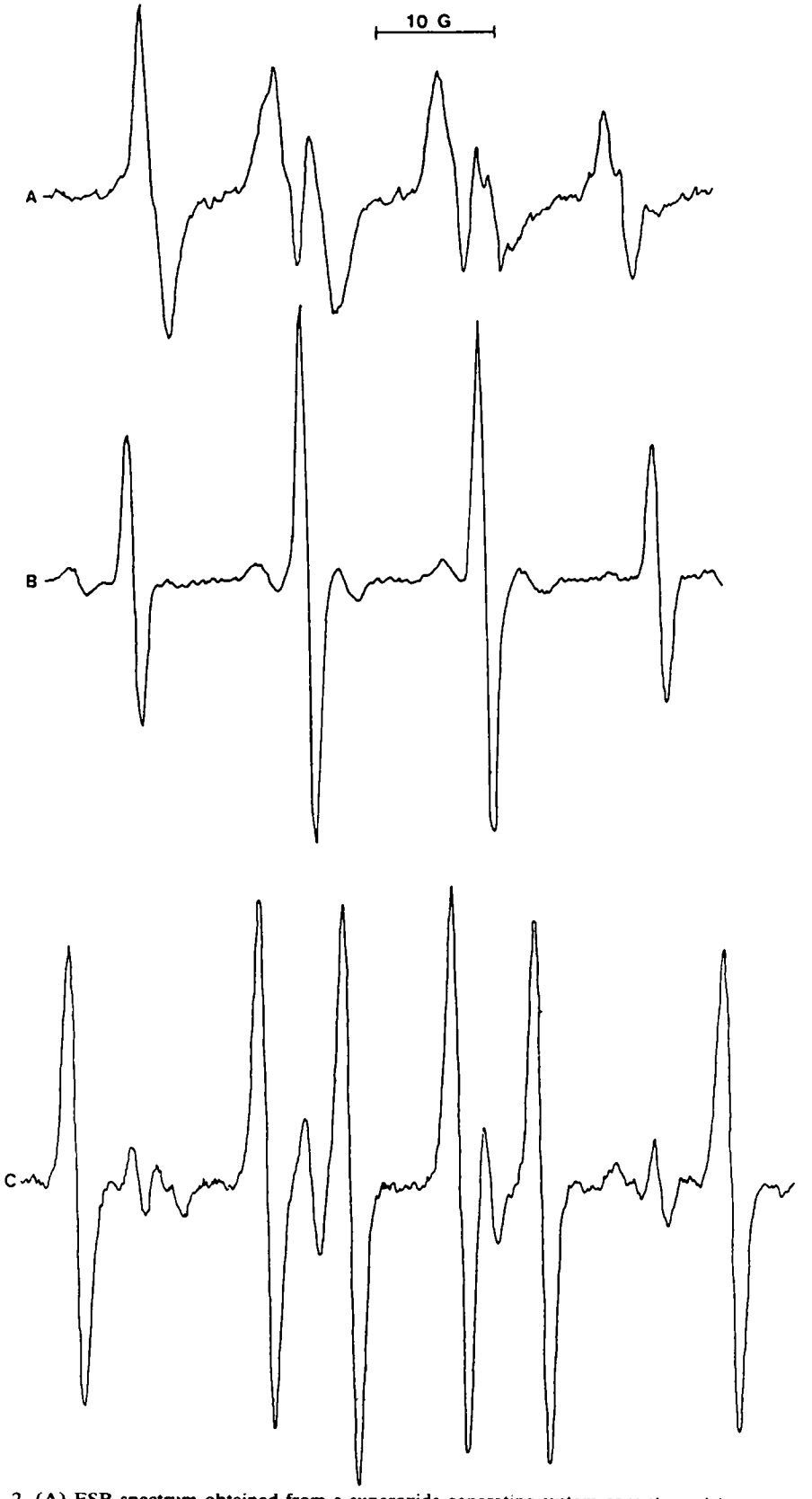

Fig. 2. (A) ESR spectrum obtained from a superoxide-generating system consisting of hypoxanthine and xanthine oxidase in the presence of DMPO (0.1 M). A_N = 14.3 G and A_H = 11.7 G. (B) ESR spectrum obtained when ferric ammonium sulfate (0.1 mM) was added to the aforementioned superoxide-generating system. $A_N = A_H$ = 14.9 G. (C) ESR spectrum obtained when DMSO (0.14 M) was added to B. A_N = 15.3 G and A_H = 22.0 G. The microwave power was 20 mW and the modulation frequency was 100 kHz with an amplitude of 1.0 G. The sweep time was 12.5 G/min and the receiver gain was 8.0×10^3 with a response time of 1.0 sec.

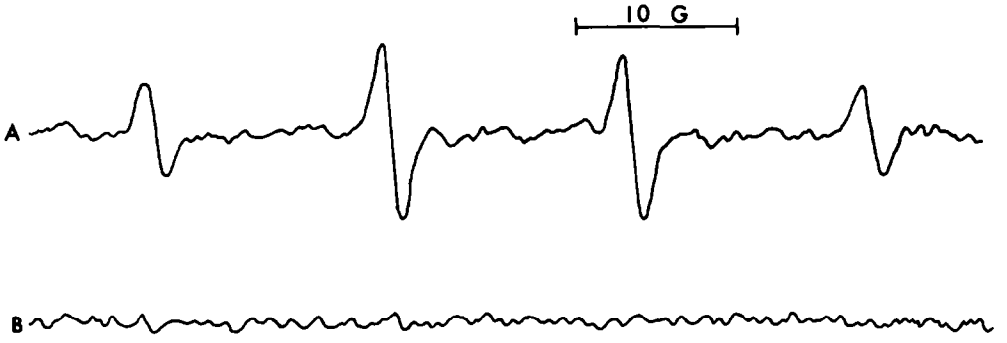

Fig. 3. (A) ESR spectrum obtained when hepatocytes (1×10^6) were incubated with TAM (0.01 mM) and DMSO (0.14 M) in the presence of DMPO (0.1 M). $A_N = A_H = 14.9$ G. (B) ESR spectrum obtained when TAM was omitted from A. Scanning conditions were the same as in Fig. 2 except that the receiver gain was 1.25×10^4.

mixture. In addition, when non-induced hepatocytes were incubated with tamoxifen and DMSO in the presence of DMPO, a baseline similar to the one depicted in Fig. 3b was obtained.

DISCUSSION

Comparison of Figs. 2B and 3A first suggests that hydroxyl radical is being generated during the hepatic metabolism of tamoxifen. However, it is known that the spin adduct DMPO-OOH can decompose into several species, one being DMPO-OH [17]. 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 [18]. Since DMPO-OOH was not observed, then either superoxide radicals are 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 [19], whose physiological function is the reduction of organic hydroperoxides to alcohols [20]. The glutathione peroxidase activity of these cells was determined to be 21 + 1 nmol/min/mg protein. Since DMSO was used as a vehicle to solubilize the drug, then the adduct DMPO-CH_3 should be observed at the expense of DMPO-OH if hydroxyl radical was being generated directly [9]. However, comparison of Figs. 2C and 3A reveals that hydroxyl radical is not being produced during hepatic metabolism of tamoxifen. Thus, the adduct DMPO-OH primarily arose from the bioreduction of DMPO-OOH by glutathione peroxidase, and not from the direct spin trapping of hydroxyl radical.

When tamoxifen was omitted from the incubation mixture, the ESR spectrum in Fig. 3B was obtained. This result indicates that the spin trap was not metabolized by the hepatocytes to produce nitroxide free radical intermediates, and that the spin adduct arose from the hepatic metabolism of the drug.

When non-induced rat hepatocytes were utilized in this study, a baseline tracing similar to the one illustrated in Fig. 3B was obtained. Since phenobarbital is known to induce the cytochrome P450 monooxygenase system, then this enzymatic pathway must be partly responsible for the transformation of tamoxifen during phase I hepatic metabolism to afford free radical intermediates. This is supported further by the fact that *n*-octylamine (0.1 mM) completely inhibited the ESR signal when induced hepatocytes were incubated with tamoxifen; *n*-octylamine is an effective inhibitor of cytochrome P450 [21].

The experimental results mentioned above raise the question of whether superoxide is being generated intracellularly or extracellularly, both. To answer this query, induced hepatocytes were incubated with tamoxifen (0.01 mM), DMSO (0.14 M), and SOD (10 μg/mL) in the presence of DMPO (0.1 M). The resulting ESR spectrum was identical to the one depicted in Fig. 3A. Since SOD does not diffuse across the plasma 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 the hepatic metabolism of tamoxifen. These data suggest that superoxide is spin trapped initially by DMPO to afford the spin adduct DMPO-OOH, which is subsequently bioreduced to give DMPO-OH. Since it has been demonstrated that superoxide can mediate cell damage without the intermediacy of hydrogen peroxide and the subsequent formation of hydroxyl radical [22], then the cytotoxic effects of tamoxifen [23] may be due in part to the formation of this reactive intermediate. Finally, it is important to note that these conclusions may be limited to this cell type and to these experimental conditions; the generality of these results is being explored.

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