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ADRIAMYCIN-STIMULATED HYDROXYL RADICAL FORMATION IN HUMAN BREAST TUMOR CELLS

Birandra K. Sinha*, Aspandiar G. Katki, Gerald Batist,
Kenneth H. Cowan and Charles E. Myers
Clinical Pharmacology Branch, National Cancer Institute.
NIH, Bethesda, Maryland 20892, U.S.A

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The anthracycline antibiotic adriamycin (ADR) is one of the most effective drugs in the treatment of a number of human cancers (1). ADR is known to intercalate into DNA, bind to membranes and alter their structure and function, and undergo enzymatic activation to form free radical species (2-10). A range of flavin reductases can reduce ADR to a semiquinone, which, in turn, can react with oxygen to produce $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$. Various investigators have proposed that the antitumor activity of ADR results from drug induced free radical formation (4-6). Sato et al. (5) provided ESR evidence of drug semiquinone radical formation in Ehrlich ascites cells, and Doroshow (6) detected $O_2^{\cdot -}$ and H_2O_2 generation in the presence of ADR in this same murine cell line. In addition, a variety of quenchers of reactive oxygen species, including the hydroxyl radical, have been reported to lessen the cytotoxicity of ADR (11). In the present study, we have examined the formation of these reactive oxygen free radicals using ESR spin trapping techniques in sensitive and resistant human MCF-7 breast tumor cells since ADR is one of the most active agents in the treatment of breast tumors.

MATERIALS AND METHODS

Adriamycin was a gift of the Drug Development Branch of the National Cancer Institute, Bethesda, MD. NADPH and NADH were purchased from the Sigma Chemical Co. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from the Aldrich Chemical Co. and purified by two vacuum distillations before use. The MCF-7 cells, wild type (WT) and resistant cells (ADR^R), were grown in Improved Modified Essential Medium (IMEM) containing 5% fetal bovine serum, 100,000 units/ml penicillin and 10,000 units/ml streptomycin. ADR^R cells were selected in stepwise increasing concentrations beginning at 10^{-8} M ADR. The selection for resistance was continued until the IC_{50} for the resistant cells reached 4.8×10^{-6} M compared to 2.5×10^{-8} M for the WT cells. The resistance was stable for as long as 52 weeks in the absence of the drug. The enzyme activities in the WT and the ADR^R MCF-7 cell lines were examined by washing the cells in cold phosphate-buffered saline (PBS) and sonicating (Heat System Ultrasonics, Inc., New York, model W-225) on ice for 5 sec x 3 at a power setting of 6. The enzyme activities' measurements were carried out on the homogenates.

*Send correspondence to Dr. B. K. Sinha, Bldg. 10, Rm. 6N-119, NIH, Bethesda, MD 20892.

RESULTS AND DISCUSSION

Figure 1A shows a typical electron spin resonance (ESR) spectrum obtained from the WT cells in the presence of ADR and DMPO, a spin-trap. The DMPO-adduct spectrum consists of a quartet (1:2:2:1) with hyperfine splitting constants of $a^N = a^H = 14.9$ G. These splitting constants are characteristic for a DMPO-OH adduct (12), which results from trapping of •OH and required the presence of both the cells and ADR. In the absence of ADR, less than 10% of the adduct was formed. Only traces of the DMPO-OH adduct were detected in the ADR^R cells. To confirm that the DMPO-OH adducts resulted from a reaction between DMPO and free •OH, ethanol or dimethyl sulfoxide (DMSO), known scavengers of •OH, was added. These scavengers react with free •OH to form carbon centered radicals which are then trapped by DMPO. Figure 1B shows the result of an experiment with DMSO ($a^N = 16.5$ G; and $a^H = 23.8$ G for DMPO-CH_3), confirming the presence of free •OH in the MCF-7 cells exposed to ADR. While ADR stimulated the formation of DMPO-OH adducts in a dose-dependent fashion with the WT cells, in contrast, there was little detectable •OH formation with the ADR^R cells. However, significant •OH formation was detectable in the WT cells, even at concentrations of 20-30 μM ADR. Addition of 1 mM NADPH to both cell lines further enhanced the formation of DMPO-OH adducts from ADR. While the ADR^R cells showed an increase in the formation of the •OH in the presence of ADR and NADPH, the relative concentration of the •OH formed with the WT cells was always significantly higher (3- to 4-fold). Addition of NADH also stimulated the formation of •OH with both cell lines; however, NADPH was a better reducing agent for the formation of the •OH with these cells (data not shown). These observations are of considerable interest since both NADH and NADPH cross cell membranes poorly. This supports the concept that ADR can undergo reduction to a free radical at the cell surface.

This difference in free •OH radical formation may result, in part, from decreased net accumulation of the drug in the resistant cells (50% decrease in ADR^R cells for the drug). However, studies in sonicated cell extracts, which eliminates any transport or net drug accumulation problems, also show the same relative difference in •OH formation between these two cell lines. These results clearly show that differences in •OH formation between these two cell lines are not due entirely to differences in drug accumulation. It is possible, however, that the difference between these cell lines may arise from the changes in ADR activation and/or elimination of toxic free radicals in the resistant cells.

At present the enzymes known to activate ADR to a semiquinone free radical which then forms superoxide and OH radicals include: (a) NADPH-cytochrome P-450 reductase (4), (b) NADH-cytochrome-b_5 reductase (6), and (c) xanthine oxidase (13). The detoxifying enzymes which may alter the rate of •OH formation include: (a) superoxide dismutase, (b) catalase, and (c) glutathione peroxidase. Examination of the relative activities of these enzymes in both cell lines shows that there were no significant differences in the activities of the ADR activating enzymes in these cell lines (e.g. 3.86 ± 1.9 vs 3.3 ± 0.9 nmol cytochrome c reduced/min/mg protein for NADPH-cytochrome P-450 reductase, and 129.9 ± 1.9 vs 160.5 ± 13.6 nmol NADH oxidized/min/mg protein for NADH-b_5-reductase in the WT and ADR^R cells respectively). There was no difference in activities of the enzymes superoxide dismutase (SOD) or catalase for WT and ADR^R cells. However, glutathione peroxidase activity was found to be increased in the ADR^R cells, and showed a 4-fold increase when assayed with H_2O_2 in the ADR^R

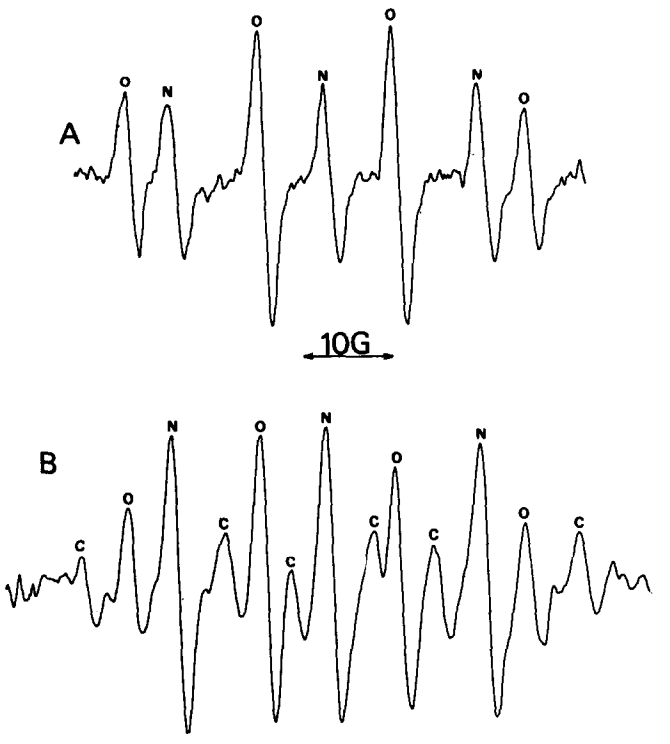

Fig 1. (A) ESR (Varian E-104) spectrum obtained from WT MCF-7 cells in the presence of 330 μM ADR and DMPO (100 mM). For the ESR studies, the IMEM medium was removed, and the cells were washed and suspended in PBS at a cell density of 2.5 x 10^6 cells/ml. (B) identical to (A) except the incubation mixtures contained 50 mM DMSO. Some nitroxide (N) was also present in these spectra.
The ESR settings were: field = 3390 G; microwave power = 20 mW; modulation amplitude = 2 G, and the receiver gain was 5 x 10^4 for (A) and 1 x 10^5 for (B).

Table 1. Effects of scavengers of reactive oxygen species and metal chelator on ADR (100 μM) stimulated hydroxyl radical formation in the WT and the ADR^R cells

System	Addition	% DMPO-OH
WT-Intact	---------	100
	SOD, 500 μg/ml	50
	Catalase, 500 μg/ml	70
	Desferal, 5 mM	55
ADR^R-Lysed	NADPH	100
	+ SOD, 50 μg/ml	0
	+ Catalase, 50 μg/ml	23
	+ Desferal, 5 mM	50

Desferal was preincubated with the cells for 1 hr before adding the drug. SOD and catalase were preincubated with cells for 30 min before adding the drug.

cells (e.g. 4.0 ± 0.9 vs 15.9 ± 2.1 nmol NADPH oxidized/min/mg protein). Organic peroxides such as lipid peroxides are also known to be substrates for glutathione peroxidase, and activity against cumene hydroperoxide, a well characterized substrate for the enzyme, was increased 12-fold in the resistant cell line. The increased activity seen against cumene hydroperoxide as compared to H_2O_2 reflects an increase in non-selenium-dependent peroxidase. The latter has been shown to be a property of glutathione-S-transferase and possesses marked organic peroxidase activity. Glutathione, a key co-substrate for the enzymes, had similar concentrations in the WT and ADR^R cells (9.1 ± 1.7 vs 8.2 ± 2.3 nmol/10^6 cells respectively).

The mechanism of •OH formation in these cells was further characterized by examining the effects of scavengers of reactive oxygen species. Results presented in Table 1 show that SOD at high concentrations inhibited ADR-dependent •OH formation in the intact cells by 50%. Catalase, however, was less effective. It is interesting to note that both SOD and catalase were significantly better in quenching the ADR-dependent •OH formation in the presence of NADPH in both intact and sonicated cells (Table 1). Since neither SOD nor catalase easily penetrates cellular membranes (14), the significant inhibition of •OH formation in the presence or in the absence of NADPH strongly implicates reduction-oxidation of ADR at the cell surface by enzymes requiring NADP/NADPH. Since the •OH formation from peroxides is also metal-dependent, we examined the effects of desferal, a strong chelator of iron. As shown in Table 1, desferal inhibited the ADR-dependent •OH formation by 50% in both intact and sonicated cells. Thus, the formation of •OH in these cells is, at least in part, iron-mediated.

In summary, our studies have shown that ADR stimulates •OH formation only in the wild type cells. In the ADR resistant cell line, •OH formation was suppressed, not by decreases in the activities of these flavin oxido-reductases, but rather by a marked elevation in the activity of the glutathione peroxidases present in the ADR^R cells. Moreover, •OH formation was stimulated by NADPH and NADH and inhibited by SOD and catalase. Work is in progress to evaluate further the sites of •OH generation in these cells and to determine how free radical formation is related to the ability of ADR to kill MCF-7 human breast cancer cells.

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