The production of hydroxyl radicals by adriamycin in red blood cells

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Spin trapping of the free radicals formed from the interaction between adriamycin and red blood cells resulted in the formation of a hydroxyl spin adduct. The formation of hydroxyl radicals was found to be inhibited by mannitol. Hemoglobin was found not to be obligatory for the formation of hydroxyl radicals which probably result from the reduction of hydrogen peroxide by adriamycin semiquinone.

Adriamycin *Red blood cell* *EPR* *Spin trapping* *Hydroxyl radical*

1. INTRODUCTION

The anticancer activity and cardiotoxicity of adriamycin (doxorubicin) is believed to occur via a free radical mechanism [1,2]. Adriamycin semiquinone radicals are among the metabolites formed following interaction between adriamycin and microsomes derived from heart, liver, spleen, L1210 and V388 murine leukaemias and intact Ehrlich ascites tumor cells [3]. Subsequent autoxidation causes the catalytic production of oxygen-centred radicals [2]. Recently adriamycin semiquinone, generated by the reduction of adriamycin by superoxide radicals formed from the reaction of xanthine oxidase with xanthine, in combination with hydrogen peroxide has been shown to generate hydroxyl radicals [4]. However, despite suggestive evidence using isolated microsomes and enzyme systems, the definite generation of inactive oxygen species in intact cells by adriamycin has not yet been demonstrated. Here, we present for the first time direct evidence for the generation of hydroxyl radicals by adriamycin in intact red blood cells using the electron paramagnetic resonance (EPR) technique of spin trapping.

2. MATERIALS AND METHODS

Adriamycin was obtained from Sigma (Dorset). The spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was prepared and purified as in [5]. All other reagents were of the highest grade available. Red blood cells were obtained from whole human blood after centrifugation to remove plasma and white cells. Sedimented erythrocytes were washed 4-times with isotonic saline. A 10% haematocrit solution of the washed cells was prepared in Krebs-Ringer–phosphate buffer, pH 7.4. Erythrocytes were carbon-monoxylated by slow purging with carbon monoxide. Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E-104X band spectrometer with an E900-3 data acquisition system. EPR spectra were recorded with a field set at 3385 G, modulation frequency 100 kHz, modulation amplitude 1.0 G, microwave power 10 mW and microwave frequency 9.0478 kHz. Computer averaging of 16 scans from $t = 0.5–1.5$ min gave spectra whose intensities represent an estimate of the spin adduct concentrations. Spin adduct concentrations were calculated from a calibrated double integral.

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3. RESULTS AND DISCUSSION

Adriamycin increases oxygen consumption in erythrocyte, resulting in hydrogen peroxide production. This is coupled with an increase in hexose monophosphate shunt activity [6]. Adriamycin stimulation of oxygen uptake is probably linked to the production of oxygen-centred free radicals. The EPR spectrum of the spin adduct formed following interaction between adriamycin and red blood cells is shown in fig.1a. The EPR spectrum was found to have the following spectral characteristics: $g = 2.0050$, $a_N = a_H = 14.9$. These have been assigned to the hydroxyl spin adduct of DMPO, 5,5-dimethyl-2-hydroxy-pyrrolidino-1-oxyl (DMPO-OH) [7]. The supernatant obtained after centrifugation of adriamycin-treated erythrocytes gave no spin adduct spectrum (fig.1b) indicating that the production of DMPO-OH from adriamycin-treated erythrocytes occurs within the cell. Adriamycin treatment of carbonmonoxy hemoglobin-containing erythrocytes, was also found to produce DMPO-OH (fig.1c). The amount of DMPO-OH produced is, however, about 50% less than that produced from normal erythrocytes. This suggests that oxyhemoglobin is involved in DMPO-OH production but that its involvement may not be obligatory. Oxyhemoglobin is considered to be the site of activation of many haemolytic drugs inside the erythrocyte [8]. However, no detectable spin adduct was observed from a solution of 10 μM oxyhemoglobin treated with adriamycin or from adriamycin alone. In [9] it was postulated that the direct reaction of adriamycin with oxyhemoglobin results in semiquinone formation.

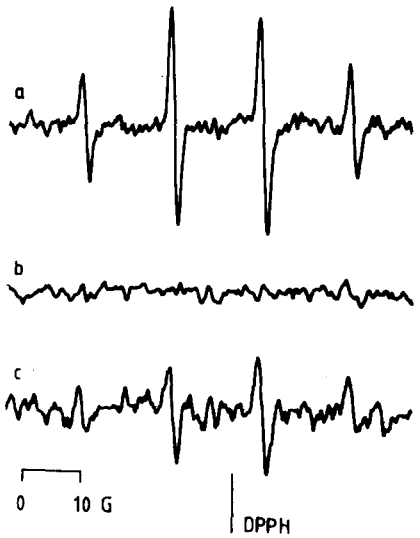

Fig.1. Spin trapping of the free radicals formed in the presence of 100 mM DMPO from 1% red blood cells in Krebs-Ringer–Phosphate buffer (pH 7.4) containing 1 mM DETAPAC after: (a) treatment with 1 mM adriamycin; (b) supernatant of (a) after 15 min incubation; (c) as in (a) but using carbon-monoxylated red blood cells.

The effect of various concentrations of mannitol on the time course of DMPO-OH production from adriamycin-treated erythrocytes is shown in fig.2. The progressive decrease in DMPO-OH production with increasing mannitol concentration clearly demonstrates that the spin adduct is being formed from hydroxyl radicals. Some doubts have been expressed in the literature about the true identity of the hydroxyl spin adduct of DMPO [10]. In [9] it was postulated that the formation of a 'hydroxyl like' radical from the reaction between adriamycin and hemoglobin was also postulated. However, we find no evidence of the formation of any other radical besides the hydroxyl radical.

The mechanism of formation of hydroxyl radicals by adriamycin is still not clear. In red blood cells adriamycin has been shown to stimulate the hexose monophosphate shunt and glutathione

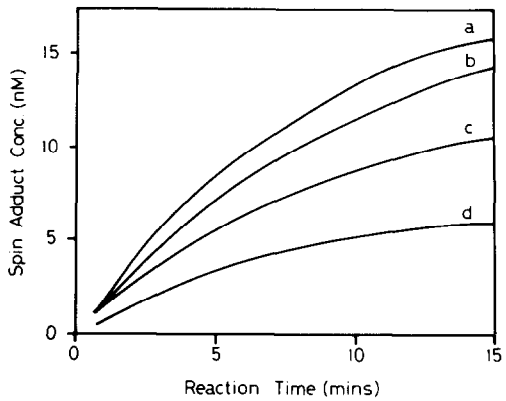

Fig.2. Effect of various concentrations of mannitol on DMPO-OH production from 1 mM adriamycin-treated 1% red blood cells in Krebs-Ringer–Phosphate buffer (pH 7.4) containing 1 mM DETAPAC. (a) Control; (b) 10 mM; (c) 25 mM; (d) 50 mM.

oxidation. Stimulation of the hexose monophosphate shunt is probably occurring because of the oxidation of NADPH by adriamycin, resulting in the formation of hydrogen peroxide and adriamycin semiquinone radicals. These would be unstable to detect [11]. The production of H_2O_2 could be the main cause of glutathione oxidation through increased glutathione peroxidase activity. Adriamycin semiquinone can also reduce H_2O_2, resulting in the formation of hydroxyl radicals. Semiquinones have recently been shown to reduce H_2O_2 to form hydroxyl radicals [12]. We have recently demonstrated the formation of hydroxyl radicals in red blood cells by the antimalarial drug, primaquine, and proposed a mechanism involving oxidations of NADPH leading to the formation of H_2O_2 and the primaquine semiquinone which can then generate hydroxyl radicals from hydrogen peroxide [13]. It appears that in red blood cells adriamycin is acting by a similar mechanism.

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