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$OH^{\cdot}$-GENERATION BY ADRIAMYCIN SEMIQUINONE AND H_2O_2; AN EXPLANATION FOR THE CARDIOTOXICITY OF ANTHRACYCLINE ANTIBIOTICS

Hans Nohl and Werner Jordan

Institute of Pharmacology, Toxicology and Pharmacy,
Faculty of Veterinary Medicine, University of Munich
Veterinärstrasse 13 D - 8000 München 22 West Germany

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ABSTRACT Anthracycline-induced cardiomyopathy is still a matter of discussion. The many mechanisms proposed cannot explain a selective sensitivity of the heart to these antitumor drugs. The present paper provides experimental evidence which shows that heart tissue has special biochemical conditions which favour an anthracycline-catalysed electron shuttle to H_2O_2. This results in the generation of highly reactive $OH^{\cdot}$-radicals, instead of $O_2^{\cdot-}$-radicals, which are expected to be formed in tissues also supplemented with anthracycline-activating microsomal enzyme systems.

The clinical use of the anthracycline antibiotics for the treatment of a range of neoplasms is severely limited by dose-related cardiotoxicity. While the antineoplastic action of this group of antibiotics is generally accepted to occur at the level of DNA (1-3), various biochemical mechanisms have been described to explain the associated toxic side effects (4-15). Most of these mechanisms suggest anthracyclines as functioning as electron carrier from biological electron transferring enzyme systems to oxygen (4-9) according to the following reaction sequences:

1. $AQ + e^- \longrightarrow AQ^{\cdot-}$

2. $AQ^{\cdot-} + O_2 \longrightarrow AQ + O_2^{\cdot-}$

Dedicated to the memory of Prof. Dr. D. Hegner

ABBREVIATIONS

AQ : anthracycline antibiotics
$AQ^{\cdot}$: anthracycline antibiotic semiquinone
DETAPAC : Diethylenetriamine-penta-acetic acid
DMPO : 5,5-Dimethyl-pyrroline-N-oxide
HEPES : N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
RHM : rat-heart mitochondria
SOD : superoxide dismutase

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Many cytotoxic effects observed during AQ-metabolism may be related to a basic action of activated oxygen. However, this general concept and also others reported in the literature, e.g. destruction of DNA, uncoupling of oxidative phosphorylation, interaction with sodium potassium ATP-ase, inhibition of glutathione peroxidase and ubiquinone-dependent NADH- and succinate- oxidoreductase (10-15), cannot explain the particular susceptibility of the heart tissue to this type of antineoplastic drug. Therefore, a better understanding of the organotropic toxicity of AQ requires more detailed studies on the involvement of the heart in the metabolism of these antibiotics. This has been carried out in the present work by comparing the mode and consequences of biological activation of the anthracycline adriamycin by electron transfer systems from the liver and heart. The results provide evidence for specific cardiotoxic effects due to the formation of $OH^{\cdot}$-radicals via the reaction of adriamycin-semiquinones with H_2O_2.

MATERIALS AND METHODS

Adriamycin (Adriblastin®) was purchased from Farmitalia, Carlo Erba GmbH, Freiburg and NAD(P)H came from Boehringer, Mannheim. 5,5-dimethyl-pyrroline-N-oxide was purchased from Aldrich-Europe, Belgium and purified before use as earlier described (16). Diethylene-triamine-penta-acetic acid and other biochemicals were obtained from Sigma, Chemical Co St. Louis. NADPH-P_{450}-oxidoreductase (from rabbit liver) was a gift from Dr. I. Golly, Munich. Rat-heart mitochondria were isolated as in ref. (17). Microsomes were prepared from the liver and heart of male Wistar rats and from beef heart according to ref. (18). ESR measurements were conducted with a Bruker 418s-maschine at room temperature using a quartz flat cell. Oxygen consumption was measured with a micro Clark-type electrode of own design.

RESULTS

Isolated rat-heart mitochondria were found to catalyse a one electron transfer from glutamate to adriamycin. The resulting semiquinone radical was detected by ESR technique and identified by its characteristic g-value at 2.0027 (Fig. 1). NADH could replace glutamate in the reaction medium, once NADH-dehydrogenase was made

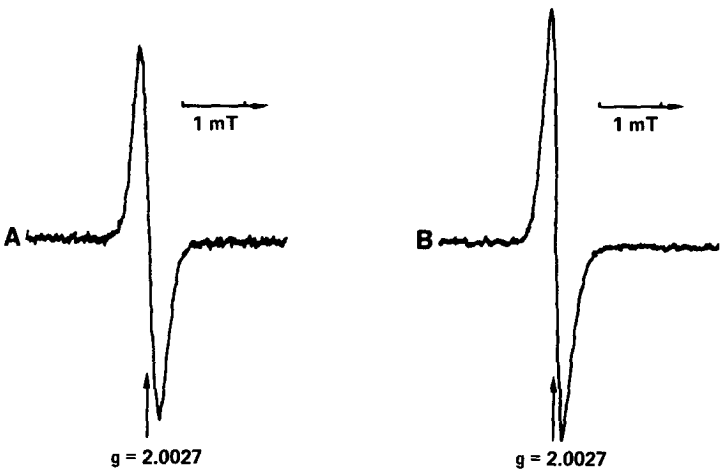

Fig. 1

ESR spectra of anthrasemiquinone metabolites obtained with respiring heart mitochondria (A) and with NADPH-P_{450}-oxidoreductase from liver (B) in the presence of adriamycin (600 µmol/l). 6.5 mg of ultrasonicated RHM or 50 µl of NADPH-P_{450}-oxidoreductase were suspended in 300 µl of a nitrogen saturated KCl (125 mmol/l)-HEPES (50 mmol/l) buffer at pH 7.4. 2.5 mmol/l NADH was added to start mitochondrial electron flow (A) and 235 µmol/l NADPH was present for reaction (B). Microwave power 12 mW, modulation amplitude 0.1 mT.

accessible to pyrimidine nucleotides by ultrasonic treatment. The presence of rotenone, an inhibitor of electron flow from complex I to the rest of the respiratory chain was not critical for electron diversion to adriamycin. This observation indicated that the site of electron shuttle from the respiratory chain to adriamycin is located within respiratory components of complex I. Oxygen was not required for the reaction and had no influence on signal heights of the semiquinone radicals. Adriamycin-semiquinone radicals could also be obtained with NADPH-supplemented NADPH-cytochrome P_{450}-oxidoreductase from liver microsomes (Fig. 1B). However, in contrast to the first activating system, the absence of oxygen was essential.

Fig. 2 shows the influence of oxygen upon ESR-spectra in the presence of the spin trap DMPO. This nitrone compound was added to the reaction medium of fig. 1 to indicate the existence of unstable oxy gen radicals possibly generated during a reaction of molecular oxy-

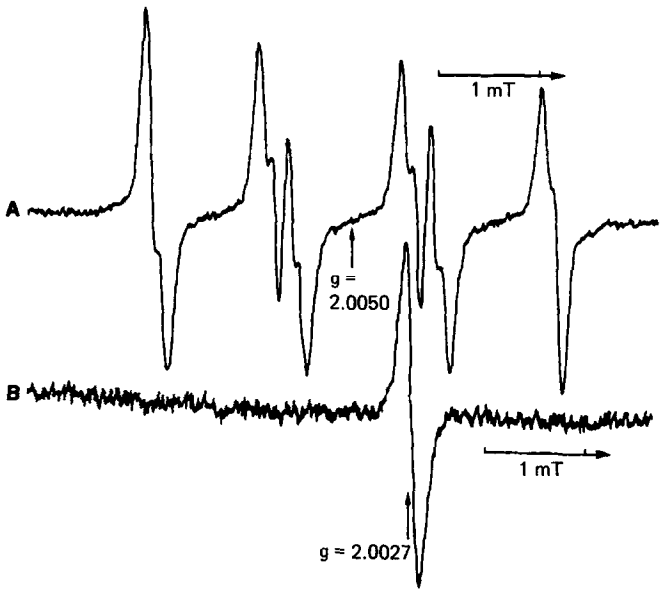

Fig. 2

The reactivity of anthrasemiquinone radicals as generated by NADPH-oxidoreductase from liver (A) or by respiring mitochondria (B) with oxygen. The reaction medium of fig. 1 was saturated with oxygen and contained 160 mmol/l DMPO. The modulation amplitude in (A) was 0.04 mT. 1 mmol/l DETAPAC was added to prevent a possible catalytic function of traces of contaminating iron. Other conditions were those of fig. 1.

gen with the semiquinone. The ESR-spectra obtained with the anticancer semiquinone radical as generated by the microsomal liver enzyme (Fig. 2A) can be assigned to the formation of a DMPO -$HO^{\cdot}_2$ adduct. The g=2.0027 signal was no longer present. This finding strongly indicated a reaction of the semiquinone anticancer agent with oxygen according to reaction 2.

This type of reaction could not be observed using heart mitochondria as the electron donor system for adriamycin (Fig. 2B). The ESR-spectra show the persistence of the semiquinone radical signal despite the presence of oxygen. Results in fig. 2 were in agreement with other assay systems used to study a possible reaction of $AQ^{\cdot}$ as generated by the two activator systems, with oxygen (experiments not shown). Autoxidation resulting in the formation of $O^{\cdot-}_2$-radicals could only be obtained with adriamycin activated by

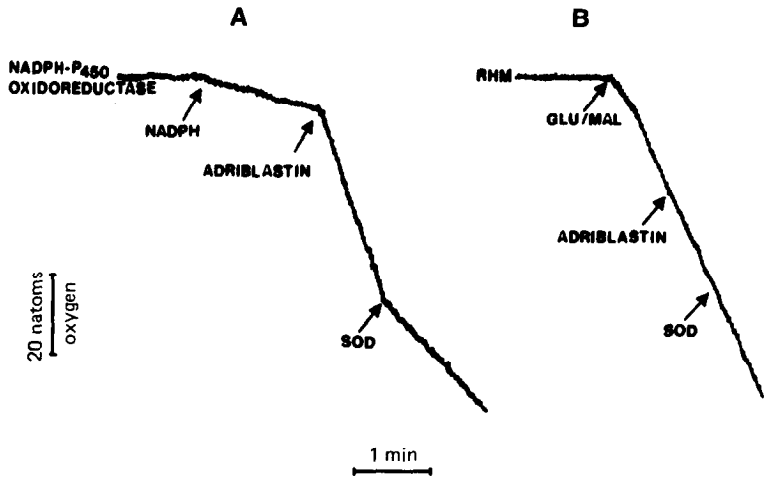

Fig. 3

Influence of adriamycin on oxygen consumption by NADPH-oxidizing NADPH-cytochrome P_{450}-oxidoreductase and glutamate respiring heart mitochondria. 1 mg of intact RHM or 50 µl of NADPH-P_{450}-oxidoreductase were suspended in 600 µl of the KCl-HEPES buffer. Oxygen consumption was measured at room temperature. Glutamate/malate: 600 µmol/l; NADPH: 250 µmol/l; adriamycin: 450 µmol/l; SOD: 5 µmol/l.

NADPH-P_{450}-oxidoreductase. Basic NADPH-dependent oxygen consumption was stimulated 10-fold following the addition of 170 µmol/l adriamycin (Fig. 3A). Oxygen consumption in the presence of adriamycin was sensitive to SOD indicating the generation of $O_2^{\cdot-}$-radicals in this reaction system. Glutamate and NADH-respiring RHM did not show altered respiratory rates in the presence of adriamycin or of SOD. The finding that autoxidation of adriamycin semiquinone requires an electron transfer component from liver microsomes led us to investigate heart microsomal fractions for their capacity to reduce adriamycin. Microsomal preparations from liver (Fig. 4A) were active in transferring electrons from NADPH to adriamycin, while heart microsomal fractions (Fig. 4B) were without effect.

These observations permit the conclusion that in heart tissue, adriamycin is exclusively activated by electron transfer from respiring mitochondria.

While oxygen appears not to react with AQ$^{\cdot}$ generated by an electron shuttle from RHM, Fig. 5A shows that the addition of H_2O_2 cau-

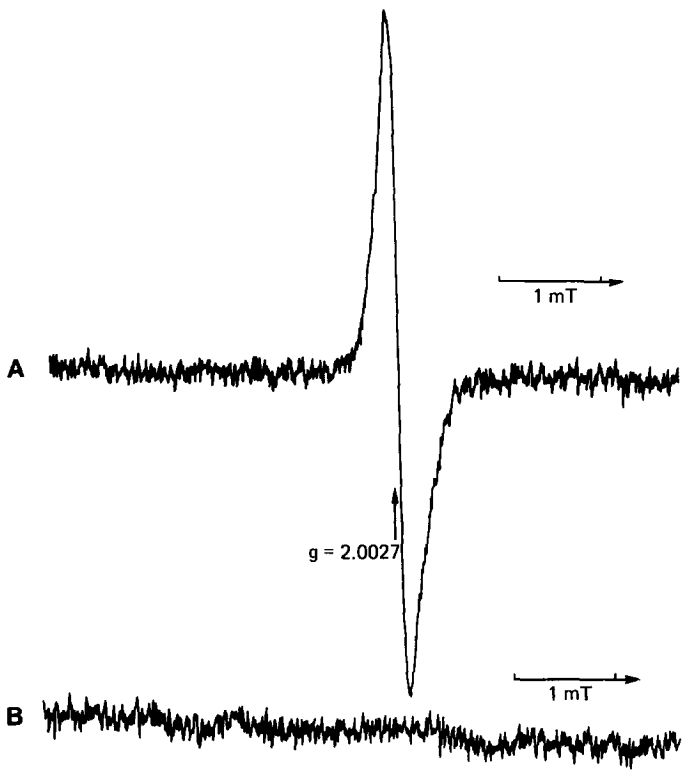

Fig. 4
Reaction of adriamycin with NADPH-supplemented microsomal fractions from rat liver (A) and beef heart (B). 5 mg of microsomal fractions were suspended in 0.3 ml reaction medium of fig. 1. 250 µmol/l NAD(P)H were present to initiate the reaction. ESR spectra were obtained under conditions of fig. 1.

sed the disappearance of the g=2.0027 signal and the simultaneous appearance of an ESR-spectrum characteristic of the formation of the DMPO -OH· adduct. However, when AQ· was formed by the action of either NADPH-supplemented NADPH-P_{450}-oxidoreductase or isolated li ver microsomal membrane fractions (Fig. 5B), only the DMPO -$HO_2^{\cdot}$ spectrum was observed (as in Fig. 2A) despite the presence of both H_2O_2 and oxygen.

DISCUSSION

The present paper provides experimental evidence that adriamycin is able to shuttle electrons from different biological sources to distinct substrates. While both NADPH-P_{450}-oxidoreductase and res-

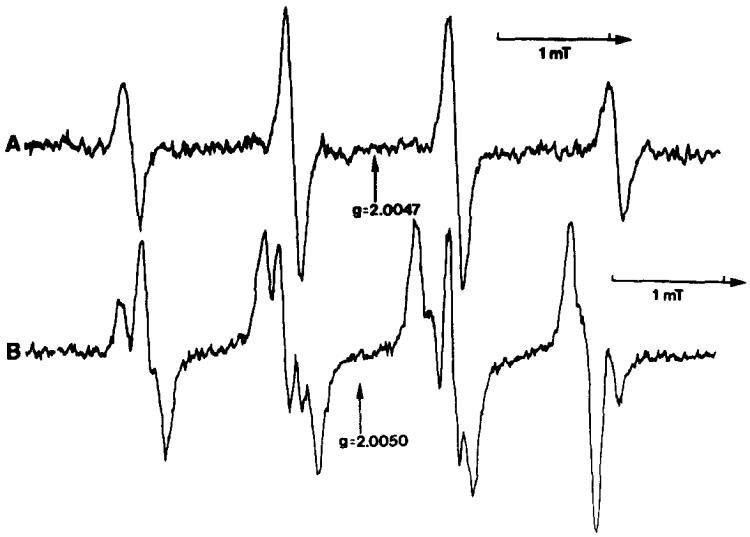

Fig. 5

Competition of oxygen with H_2O_2 for a reaction with adriamycin radicals as generated by respiring mitochondria (A) and NADPH-supplemented microsomal liver fraction (B). 6 mg of mitochondrial or microsomal protein was suspended in the KCl/HEPES buffer of fig. 1 saturated with oxygen. Reaction (A) was started upon the addition of 600 µmol/l glutamate/malate; reaction (B) was initiated by the addition of 265 µmol/l NADPH. H_2O_2: 1 mmol/l; DMPO: 150 mmol/l; DETAPAC: 1 mmol/l. Microwave power 12 mW, modulation amplitude 0.04 mT.

piring RHM can activate adriamycin to the semiquinone state, the subsequent electron flow to either O_2 or H_2O_2 appears to be governed by the type of biological system responsible for the activation of this antibiotic. The lack of change in the oxygen consumption of respiring RHM upon the addition of adriamycin might indicate the involvement of this compound in electron transfer from NADH-ubiquinone-oxidoreductase (complex I) to cytochrome oxidase. The presence of the adriamycin semiquinone in electron transferring RHM further supports the idea that this antibiotic may function as an intermediate in mitochondrial respiration. For kinetic reasons such a function would be expected to occur within the inner membrane where respiratory components are localized. In the absence of protons which normally compensate for the charge of the anion radical, the one electron reduction potential of $O_2/O_2^{\cdot-}$ is about 50 mV more

negative than that of the AQ/AQ$^{\cdot}$ couple (calculated from ref. 19, 20). These conditions, which correspond to the lipophilic phase of the inner membrane, do not allow electron transfer from the semiquinone to oxygen with the concomitant generation of superoxide anion radicals. However, such a reaction is thermodynamically possible as soon as protons are available to form the protonated superoxide radical. In this case the redox potential of $O_2/HO_2^{\cdot}$ is about 400 mV more positive than that of the AQ/AQ$^{\cdot}$ couple (calculated from ref. 20, 21). Autoxidation of AQ$^{\cdot}$ which could be observed when AQ was activated with liver microsomal enzymes might therefore occur in an environment differing from that of the mitochondrial membrane by the presence of protons. Our investigations have demonstrated that the latter mechanism of electron shuttle by adriamycin to oxygen does not exist in heart cells because of the lack of activating microsomal electron carriers. However, the protection of mitochondrially generated AQ$^{\cdot}$ against a reaction with oxygen favours the reaction of the semiquinone radical with H_2O_2. The demonstration of the respective reaction product by spin trapping of OH$^{\cdot}$-radicals exhibits close similarities with ubisemiquinone radical-catalysed OH$^{\cdot}$ formation by respiring RHM (22). H_2O_2, which appears to be capable of oxidizing both types of semiquinones, should be present in relative high steady state concentrations in the heart, as compared to other organs. This is expected from the fact that heart cells do not seem to have significant amounts of catalase (23). The selective sensitivity of the heart towards anthracycline antibiotics must therefore be discussed on the basis of the fact that this tissue has special biochemical conditions which favour an AQ-catalysed shuttle of electrons to H_2O_2. This results in the generation of highly reactive OH$^{\cdot}$-radicals instead of $O_2^{\cdot-}$-radicals which would be expected to be formed in tissues also supplemented with AQ-activating microsomal enzyme systems.

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