Mechanisms of Adriamycin-Dependent Oxygen Activation Catalyzed by NADPH-Cytochrome *c*-(Ferredoxin)-Oxidoreductase

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Under aerobic conditions, O_2 uptake and production of $O_2^{\cdot -}$ and H_2O_2 by isolated NADPH-cytochrome *c*-(ferredoxin)-oxidoreductase from *Euglena gracilis* was strongly stimulated by adriamycin. Further stimulation was not observed with 0.1 mM Fe^{3+}-EDTA. Methionine fragmentation (measured as ethylene release), as a reliable indicator for the formation of $OH^{\cdot}$ radical-like oxidants under aerobic conditions (100 µmol O_2 in a 10 ml reaction vessel) was strongly stimulated by 0.1 mM Fe^{3+}-EDTA or, in the absence of iron, by partial anaerobiosis (1 µmol O_2 per vessel). The highest rate of methionine fragmentation was observed under anaerobic conditions in the presence of both reduced adriamycin and added H_2O_2.

Aerobic methionine fragmentation in the presence of adriamycin and Fe^{3+}-EDTA was inhibited by superoxide dismutase and catalase by more than 90%, while methionine fragmentation under semianaerobiosis in the absence of Fe^{3+}-EDTA was inhibited by superoxide dismutase to only about 50%, while catalase again inhibited by more than 90%. These results indicate that the adriamycin-catalyzed production of a strong oxidant appears to be governed by different mechanisms depending on oxygen availability; namely the production of a Fenton-type oxidant driven by adriamycin-catalyzed superoxide formation and also, the formation of the "crypto-$OH^{\cdot}$ radical" by direct electron donation from the adriamycin semiquinone radical to H_2O_2 under oxygen limiting conditions.

Introduction

The anthracycline adriamycin (ADR) belongs to the rhodomycin group of naturally occurring quinone antibiotics which possess outstanding antitumour properties [1–3]. In the tumour cell, it appears to produce DNA strand scissions and also to bind to DNA by intercalation [4–6]. The most important organo-specific side effect observed after prolonged treatment with ADR is a severe dose-dependent cardiomyopathy [7]. Oxygen activation and free radical formation during redox cycling of ADR have been proposed [6, 8–13] as a mechanism to explain both the antineoplastic and cardiotoxic activities. In this context, interactions with flavin coenzymes and chelated iron have been described [14, 15], where one-electron reduction by NADPH via several different flavoproteins (NADPH cytochrome c/P_{450}/quinone reductases) seems to play a dominant role in the activation of these anthracenediones [4, 16]. Activated oxygen species such as $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ radicals and ADR radicals themselves have been suggested [6, 11, 13] as the agents ultimately responsible for both the antineoplastic activity and cardiotoxicity, since scavengers such as α-tocopherol exhibit an amelioration of the destructive effects, *e.g.* lipid peroxidation [17]. Drug-induced DNA strand scission in model reactions was decreased by catalase, SOD and free radical scavengers [6]. Since both catalase and SOD activities seem to be strongly decreased in heart tissue as compared to certain other mammalian tissues (*e.g.* liver, kidney, red blood cells), lack of protection against the action of these active species of oxygen may be at least partly responsible for the observed heart toxicity of these drugs [18].

From the above considerations, the question arises as to whether ADR toxicity in aerobic heart tissue and in oxygen limited tumour tissue may be accounted for by a single mechanism or if separate destructive reactions are operating. The present study represents an attempt to investigate redox reactions of ADR in model systems designed to

Abbreviations: ADR, adriamycin; SOD, superoxide dismutase; NADPH-OR, NADPH-cytochrome *c*-(ferredoxin)-oxidoreductase.

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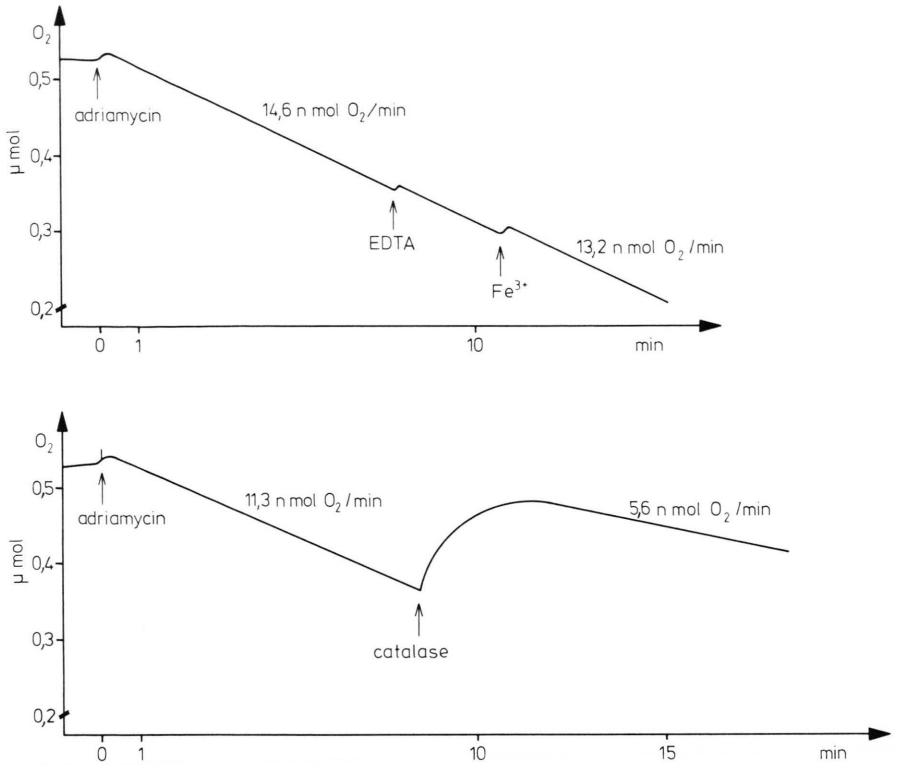

Fig. 1. Time course of adriamycin-catalyzed oxygen reduction. The reaction mixture contained in 2 ml: 0.1 M Chelex-treated phosphate buffer, pH 7.8; 10 µmol glucose-6-phosphate; 50 µg glucose-6-phosphate dehydrogenase; 1 µmol NADP; 30 µg NADPH-OR; 0.18 µmol adriamycin; 100 units catalase and 0.01 µmol Fe^{3+}-EDTA where indicated. O_2 uptake was followed polarographically in a Hansatech oxygen electrode thermostated at 22 °C.

mimic relevant *in vivo* conditions, in order to determine criteria for oxygen activation by ADR.

Materials and Methods

NADPH-cytochrome *c*-(ferredoxin)-oxidoreductase (NADPH-OR) and superoxide dismutase (SOD) were prepared from autotrophically grown *Euglena gracilis* cells [19] and from dried green peas [20, 21] respectively. Catalase and glucose-6-phosphate dehydrogenase were from Boehringer, Mannheim. Adriamycin (Adriablastin®) from Farmitalia Carlo Erba GmbH, Freiburg was maintained as a 1.8 mM solution in the dark at 0 °C. Chelex-treated phosphate buffer, pH 7.8 was used in all experiments. The individual incubation conditions are given in the tables and figures.

Oxygen consumption was determined polarographically with a Hansatech oxygen electrode (Bachofer, Reutlingen). Superoxide production was monitored as nitrite formation from hydroxylamine [22, 23]. Methionine fragmentation (ethylene formation from methionine) was followed by gas chromatography [24] and anaerobic and semianaerobic conditions were maintained as described previously [25].

Results

NADPH-cytochrome *c*-(ferredoxin)-oxidoreductase (NADPH-OR) isolated from *Euglena gracilis* in the presence of an electron donor system consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP showed no measurable oxygen exchange [19], whereas the addition of 0.1 mM ADR resulted in an uptake of oxygen after a negligible lag phase (Fig. 1). This oxygen uptake (approx. 15 nmol O_2 min^{-1}) was only slightly affected by the further addition of 0.1 mM Fe^{3+}-EDTA (new rate; about 13 nmol O_2 min^{-1}). Incuba-

tion of ADR or Fe^{3+}-EDTA alone or in combination did not result in oxygen uptake in the absence of the enzyme system (data not shown). ESR studies have shown that ADR in aqueous solution produces no signal in the absence of an activating system (H. Nohl, personal communication).

As shown in Fig. 1b, the addition of catalase resulted in a rapid oxygen release, followed by a resumption of oxygen uptake at 50% of the initial rate, indicating that H_2O_2 was a product of ADR-catalyzed oxygen reduction. This reaction also yielded intermediary superoxide ($O_2^{\cdot -}$), as measured by nitrite formation from hydroxylamine [22, 23]. Both SOD (data not shown) and Fe^{3+}-EDTA (at a concentration greater than 5 μM) inhibited nitrite formation, indicating a reaction between O_2^- and Fe^{3+}-EDTA (Fig. 2), since oxygen uptake was scarcely affected (Fig. 1a).

Methionine fragmentation yielding ethylene as one of the products, [24] is a reliable indicator for the production of a strong oxidant other than $O_2^{\cdot -}$ or H_2O_2 [25–27]. As shown in Fig. 3, increasing concentrations of ADR only very slightly stimulated methionine fragmentation. However, in the presence of 0.1 mM Fe^{3+}-EDTA, ethylene formation was dependent upon the ADR concentration. Conversely, when the ADR concentration was maintained at 0.1 mM, a sigmoidal increase in methionine fragmentation was observed, which was dependent upon the Fe^{3+}-EDTA concentration. The Fe^{3+}-EDTA concentration range which resulted in the greatest stimulation of methionine fragmentation coincided with that which led to the inhibition of superoxide-dependent nitrite formation from hydroxylamine, namely 10^{-5} M – 10^{-4} M. This indicates the involvement of $O_2^{\cdot -}$ in ADR and Fe^{3+}-EDTA catalyzed methionine fragmentation and was confirmed by the inhibitory effect of increasing amounts of SOD, whereby 100 units gave about 90% inhibition as compared to about 98% inhibition with 100 units of catalase (Fig. 4).

The oxygen requirement of the ADR-catalyzed methionine fragmentation reaction is shown in Table I. Under partially anaerobic conditions (*viz.* 1 μmol O_2 per reaction vessel), the rate of ethylene formation was higher than those obtained under either anaerobic or aerobic conditions and approached the rates in the presence of Fe^{3+}-EDTA. In contrast to methionine fragmentation in the presence of both ADR and Fe^{3+}-EDTA, which was inhibited by SOD and catalase by about 90% and 98% respectively, the reaction under partial anaerobiosis in the absence of Fe^{3+}-EDTA was inhibited by 100 units SOD to only about 50%. Further inhibition was not observed at higher (200 units) SOD concentrations (Table II). As shown

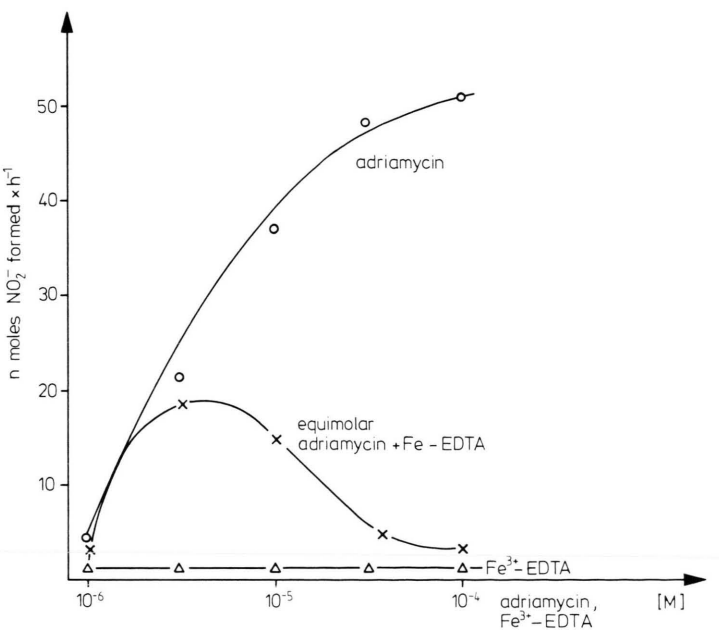

Fig. 2. Effect of adriamycin and Fe^{3+}-EDTA on nitrite formation from hydroxylamine as an indicator of superoxide (O^{2-}) production. The reaction conditions were as described for Fig. 1 with the addition of 1 μmol NH_2OH to each reaction vessel and the indicated amounts of ADR and Fe^{3+}-EDTA. Nitrite was determined after 20 min reaction using sulphanilamide and naphthylethylene diamine. The extinction was determined at 540 nm [23, 34].

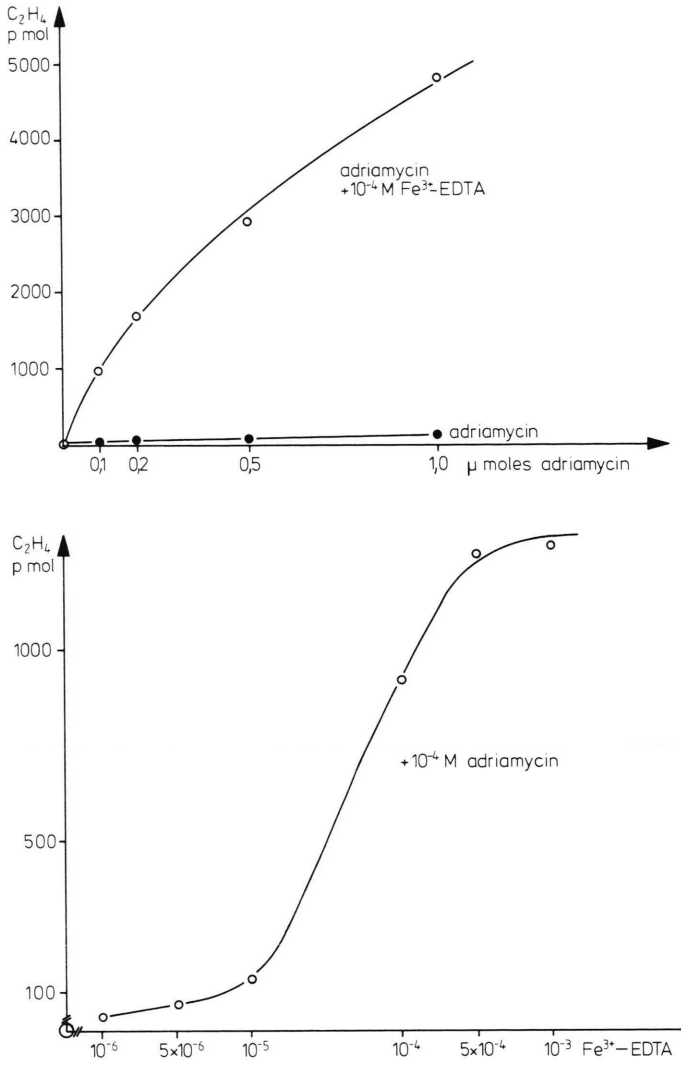

Fig. 3. Effect of increasing amounts of adriamycin and Fe^{3+}-EDTA on ethylene formation from methionine. The reactions were carried out under aerobiosis under the conditions outlined for Table I.

Table I. Effect of oxygen concentration on ethylene formation from methionine. Reaction conditions: The reaction mixture contained in 2 ml: 10 µmol glucose-6-phosphate; 50 µg glucose-6-phosphate dehydrogenase; 1 µmol NADP; NADPH-OR containing 30 µg protein: 0.18 µmol ADR; 20 µmol methionine; 0.2 µmol pyridoxal phosphate; 100 µmol phosphate buffer, pH 7.8. Oxygen tensions were achieved and maintained as described [25]. Reactions were conducted for 45 min in 10 ml Fernbach flasks at 22 °C. The experiments were repeated four times. Results from typical experiments are shown.

Conditions	pmol C_2H_4/45 min
Aerobiosis	78
Partial anaerobiosis	2078
Anaerobiosis	20

Table II. Effect of superoxide dismutase and catalase on ethylene formation from methionine under partial anaerobiosis. Reaction conditions were as outlined for Table I; partial anaerobiosis is regarded as 1 µmol O_2 present in a 10 ml reaction vessel at the start of the experiment.

Additions	pmol C_2H_4/45 min
No ADR	5
ADR	4790
ADR + 100 units SOD	2350
ADR + 200 units SOD	2105
ADR	4740
ADR + 100 units catalase	102
ADR + 200 units catalase	85

Table III. Effect of reduced adriamycin and H_2O_2 on ethylene formation from methionine under anaerobic conditions. Reaction conditions were as outlined for Table I. 1 μmol H_2O_2 was present as indicated.

Conditions	pmol C_2H_4/45 min
ADR	20
H_2O_2	110
ADR + H_2O_2	45 540

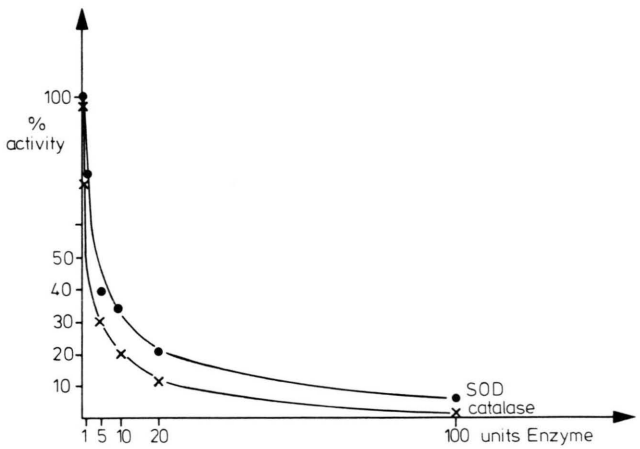

Fig. 4. Inhibition of ethylene formation from methionine by superoxide dismutase (SOD) and catalase. Reaction conditions (aerobic) were as outlined for Table I with 10^{-4} M ADR, 5×10^{-5} M Fe^{3+}-EDTA and the indicated amounts of SOD and catalase.

in Table III, under anaerobic conditions high rates of methionine fragmentation were observed in the presence of both 0.1 mM ADR and 0.5 mM H_2O_2, whereas ADR or H_2O_2 alone in the presence of the electron donor system yielded very little ethylene.

Discussion

Previous investigations have shown that ADR toxicity is manifested by lipid peroxidation and DNA damage [28–31]. There is a general agreement that these effects are due to a flavoprotein-catalyzed redox cycling of ADR, leading to the production of reactive semiquinone radicals [4, 5, 12] which upon autoxidation, yield superoxide [8, 13] and H_2O_2 after its dismutation.

Initiation of lipid peroxidation in both model reactions [15] and *in vivo* [31] was observed to be strongly stimulated by (chelated) iron. From these and other studies, a model for ADR toxicity was proposed in which at least two roles of activated ADR have to be envisaged, namely 1) interference with nucleic acid metabolism and 2) initiation of lipid peroxidation through the production of oxygen radicals. Since it is likely that cellular flavoproteins (with a requirement for NADPH as the electron donor) represent a major pathway of ADR activation in the above systems, we investigated possible mechanisms of ADR-dependent oxygen activation by NADPH-cytochrome *c*-(ferredoxin)-oxidoreductase.

The aim of the present investigation was to determine the conditions required for the activation of ADR by the above mentioned ubiquitous flavoprotein which might produce aggressive oxygen species of a similar reactivity to the frequently proposed (but never proven) *free* $OH^{\cdot}$ radical. The test system we used was the release of ethylene from methionine. This reaction is driven by radiolytically produced *free* $OH^{\cdot}$ radicals [32], but not by $O_2^{\cdot -}$, H_2O_2 or both in combination, nor by the xanthine oxidase reaction alone [25–27].

In biological systems, methionine fragmentation occurs readily in the absence of oxygen, but in the presence of H_2O_2 and appropriate one-electron donors such as certain semiquinones, reduced paraquat, reduced ferredoxin, Fe^{2+} ions and reduced nitrofurantoin [25–27, 33–34]. Thus, this system is a reliable indicator for an extremely reactive oxygen species which we have termed the "crypto-$OH^{\cdot}$ radical" [25]. The differences between the reactions of the crypto-$OH^{\cdot}$ radical and *free* $OH^{\cdot}$ radicals have recently been discussed [34].

The formation of the methionine fragmenting oxygen species by the NADPH-OR/ADR system was strongly dependent upon the reaction conditions. Under aerobic conditions, only negligible ethylene formation was observed, similar to the recently investigated paraquat system [25], whereas in the presence of ADR plus Fe^{3+}-EDTA, methionine fragmentation was dependent upon both the ADR and Fe^{3+}-EDTA concentrations (Figs. 3 and 4). This aerobic reaction was inhibited by both SOD and catalase, indicating that $O_2^{\cdot -}$ as well as H_2O_2 was involved in the reaction.

The involvement of $O_2^{\cdot -}$ in the aerobic system containing both ADR and Fe^{3+}-EDTA was also indicated by the results shown in Fig. 2, where ADR-stimulated $O_2^{\cdot -}$ formation (measured as

nitrite from hydroxylamine) was inhibited by the same concentrations of Fe^{3+}-EDTA which were active in stimulating methionine fragmentation. Together with the fact that Fe^{3+}-EDTA did not seem to strongly interfere with the basic mechanism of monovalent oxygen reduction by the NADPH-OR/ADR system (Fig. 1 a), the above mentioned results suggest that $O_2^{\cdot-}$ reduced Fe^{3+}-EDTA to Fe^{2+}-EDTA which in the presence of H_2O_2, formed a Fenton-type oxidant via a catalysed Haber-Weiss reaction [35–37]. Under oxygen-limiting conditions, Fe^{3+}-EDTA is not necessarily a prerequisite for the production of a strongly oxidising species, capable of fragmenting methionine. Under these conditions, catalase still inhibited almost quantitatively ethylene formation, whereas SOD gave only partial inhibition (Table II). Autoxidation of ADR$^{\cdot}$ would be limited under partial anaerobiosis and thus electron transfer from ADR$^{\cdot}$ to H_2O_2 would be favoured. This agrees with the respective results with catalase and SOD.

The observation that under anaerobic conditions the combined presence of both reduced ADR and exogenously supplied H_2O_2 was required supports the view that both ADR$^{\cdot}$ and H_2O_2 are necessary for the formation of the ultimate destructive species, in agreement with the results of Winterbourn [38]. The results presented here may cast some new light on the mechanism of ADR toxicity as well as its antineoplastic activity. In neoplastic tissues, as well as in other tissues with either a limited oxygen supply or with oxygen consumption, oxygen limitation could produce conditions favouring electron transfer from ADR$^{\cdot}$ to H_2O_2, since ADR$^{\cdot}$ autoxidation forming $O_2^{\cdot-}$ would be limited.

Thus, the enzymic reducing power would be channelled into the formation of the crypto-OH$^{\cdot}$ radical, an oxidant with similar destructive properties to the free OH$^{\cdot}$ radical [25–27]. It has been recently demonstrated that DNA undergoes strand scission upon exposure to xanthine/xanthine oxidase [39]. The mechanism of generation of the oxygen species which caused strand scission is similar to that reported here for the formation of a strong oxidant by ADR and thus could explain the stimulation of DNA strand scission by ADR reported earlier [6].

In tissues with high oxygen tensions, the iron-catalyzed Haber-Weiss reaction is most likely to represent the predominant reaction responsible for ADR induced tissue damage.

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