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Mitochondrial sulfhydryl group modification by adriamycin aglycones

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Induction of Ca^{2+} release from isolated, preloaded rat heart mitochondria by low concentrations ($<5\ \mu M$) of adriamycin aglycones, has recently been reported [(1988) Biochem. Pharmacol. 37, 803]. Ca^{2+} release occurs via a generalized, Ca^{2+}-dependent increase in the permeability of the inner mitochondrial membrane to small molecules. The process is antagonized by dithiothreitol, suggesting thiol involvement. This communication demonstrates modification of mitochondrial sulfhydryl groups, detected as decreased 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reactivity, by adriamycin aglycones. Ca^{2+} release and sulfhydryl modification are shown to depend similarly on aglycone concentration and on the C-7 substituent of the anthracycline ring. In addition, DTNB elicits Ca^{2+} release. It can therefore be proposed that adriamycin aglycones alter mitochondrial membrane permeability by altering mitochondrial thiol status.

Adriamycin; Mitochondria; Sulfhydryl group; Ca^{2+}; Membrane permeability; (Rat heart)

1. INTRODUCTION

Adriamycin (Doxorubicin) is a potent antineoplastic agent [1,2], but its therapeutic usefulness is limited by cumulative and irreversible cardiotoxicity [3,4]. Several biochemical mechanisms have been proposed to account for the toxic effects of the drug (review [5]). Perhaps foremost among them are schemes (e.g. [6]) involving redox reactions of the anthraquinone ring, leading in turn to adriamycin-free radicals, activated oxygen species, and lipid peroxidation. None of these hypothetical mechanisms has been unequivocally confirmed. Most importantly, peroxidation of cardiac lipids is not always a consequence of adriamycin exposure [7,8].

This laboratory has recently reported that low concentrations ($<5\ \mu M$) of aglycone metabolites of adriamycin trigger the release of Ca^{2+} from isolated, preloaded rat heart mitochondria [9]. Aglycone-induced Ca^{2+} release is independent of Na^+ and is accompanied by oxidation of pyridine nucleotides, membrane potential collapse, mitochondrial swelling, and a transition of the mitochondria from the condensed to the orthodox configuration. Ca^{2+} release is inhibited by dithiothreitol and ATP. These properties are sufficient to demonstrate that aglycone-induced Ca^{2+} release is representative of a previously identified phenomenon, namely, a generalized, Ca^{2+}-dependent increase of inner membrane permeability to small (<1000 daltons) solutes [10,11].

This communication demonstrates that adriamycin aglycones modify mitochondrial sulfhydryl groups. Sulfhydryls have been implicated in maintenance of inner membrane permeability properties [12,13]. It can therefore be proposed that the aglycones elicit Ca^{2+} release by altering mitochondrial thiol status. Consistent with this proposal, Ca^{2+} release and sulfhydryl modification are found to depend similarly on

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Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Mops, 3-(*N*-morpholino)propanesulfonic acid

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aglycone concentration and on the C-7 substituent on the anthracycline ring.

2. MATERIALS AND METHODS

Mitochondria were isolated from the hearts of male Sprague-Dawley rats by a modification [9] of the procedure of Sordahl [14] which yields a mixed population of interfibrillar and subsarcolemmal organelles. All measurements were carried out in a standard resin (Chelex-100)-treated buffer consisting of sucrose, 100 mM; KCl, 50 mM; Mops-KOH, pH 7.2, 20 mM; and KH_2PO_4, 1.7 mM to which was added 0.8 μM rotenone and mitochondrial protein equivalent to 0.2 mg/ml. Temperature was maintained at 30°C.

Ca^{2+} uptake and release were followed by means of a Ca^{2+}-selective electrode (Radiometer F2112Ca, K8040 reference). Mitochondrial sulfhydryl groups were measured using 100 μM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [15]. Production of the nitrothiobenzoate anion was followed at the wavelength pair 520–412 nm [16] in an SLM-Aminco DW-2000 spectrophotometer and quantitated using a differential extinction coefficient of 1.15×10^{-4} M^{-1} determined by calibration with cysteine. All data shown are representative of multiple experiments. Protein was determined as in [17].

The 7-hydroxy and 7-deoxy aglycones of adriamycin were prepared, as described [9], according to Bachur [18] and Smith et al. [19]. Adriamycin hydrochloride was generously supplied by Adria Laboratories, Columbus, OH. Sucrose, Mops, cysteine, NADH, DTNB, rotenone, and succinate were purchased from Sigma. Chelex 100 was obtained from Bio-Rad (Richmond, CA) and NADH dehydrogenase from Boehringer Mannheim (Indianapolis, IN). All other reagents were of the highest quality available.

3. RESULTS

The time course of the reaction of the thiol-specific reagent DTNB (100 μM) with the sulfhydryl groups of isolated rat heart mitochondria is shown in fig.1. In 11 experiments, the probe reacted with 37.4 ± 2.9 nmol/mg protein over a period of 30 min. This agrees well with the value of 39.8 ± 3.0 reported for pig heart mitochondria [20]. The slow time course is consistent with prior interpretation of the DTNB reaction as an indicator of protein thiols [16].

In the presence of the 7-hydroxy aglycone of adriamycin (17.3 μM), sulfhydryl detection was markedly reduced (fig.1). An average of only 17.6 ± 2.1 nmol/mg (n = 11) sulfhydryls reacted with the probe, under these conditions, in 30 min, corresponding to a 52.6 ± 6.5% decrease in sulfhydryl reactivity. This effect is significant at the $P \ll 0.001$ level (Student's t-test). Mitochondrial sulfhydryl reactivity was never completely

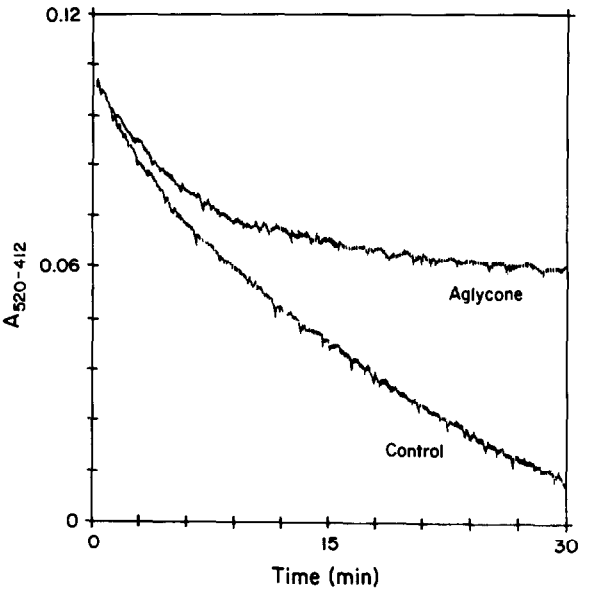

Fig.1. Effect of 7-hydroxy adriamycin aglycone on mitochondrial sulfhydryl group detection by DTNB. Mitochondria were added to standard buffer supplemented with rotenone, 5 mM succinate, 100 μM DTNB, and either 17.3 μM aglycone or an equivalent volume of the solvent dimethylsulfoxide.

eliminated by adriamycin aglycone. Experiments in which inhibition was enhanced either by increasing aglycone concentration or by decreasing DTNB concentration suggested maximal inhibition of 65%. Inhibition of the DTNB reaction occurred with a lag of 3–6 min (fig.1). The lag was not altered by incubation of the mitochondria with aglycone for 5 min prior to the addition of DTNB. The lag therefore reflects interaction of aglycone with thiol groups which are relatively inaccessible to DTNB and not the time course of aglycone action.

Several observations indicate that adriamycin aglycone chemically alters mitochondrial sulfhydryls rather than simply decreasing probe access. (i) Aglycone effects were partially overcome by increasing the DTNB concentration. At 2 mM DTNB, 17.3 μM aglycone inhibited sulfhydryl group detection only 34%. (ii) Addition of Triton X-100 (0.16%) after 30 min increased by 53% the number of sulfhydryl groups detected by DTNB but failed to overcome inhibition by the aglycone. (iii) In mitochondria, adriamycin is reduced by NADH dehydrogenase [21]. In a model system

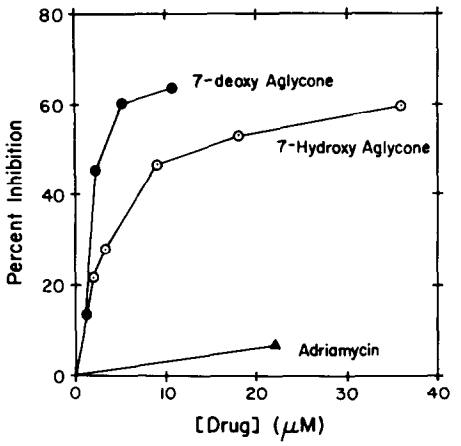

Fig.2. Effect of adriamycin and its aglycone metabolites on mitochondrial sulfhydryl group detection by DTNB. Measurements were conducted as shown in fig.1.

consisting of cysteine, NADH, and NADH dehydrogenase, where probe access is not an issue, pre-incubation with adriamycin or with the 7-hydroxy aglycone decreased the subsequent reaction of cysteine with 100 μM DTNB.

Ca^{2+} release from isolated rat heart mitochondria is difficult to quantitate but is clearly seen at 5 μM hydroxy aglycone. Furthermore, both the 7-hydroxy and 7-deoxy aglycones are potent releasing agents whereas the parent molecule is ineffective [9]. Mitochondrial sulfhydryl groups responded similarly (fig.2). Inhibition of sulfhydryl group detection by DTNB was evident at 1–2 μM aglycone and was half-maximal at 1.7 μM and 4.5 μM 7-deoxy and 7-hydroxy aglycone, respectively. In contrast, >20 μM adriamycin had little effect on mitochondrial thiols.

If aglycone-induced Ca^{2+} release can be attributed to modification of DTNB-reactive thiol groups, then DTNB itself should also induce Ca^{2+} release. This is indeed the case (fig.3). 100 μM DTNB and 17.3 μM hydroxy aglycone are similarly potent releasing agents.

4. DISCUSSION

The data summarized above suggest that adriamycin aglycones modify mitochondrial sulfhydryl groups and in so doing induce an increase in inner membrane permeability which can be observed as Ca^{2+} release. Sulfhydryl modification, either oxidation or arylation and either direct or mediated by activated oxygen species [22], is a logical chemical consequence of generation of the aglycone-free radical. It can therefore be suggested that the critical event in adriamycin cardiotoxicity is loss of subcellular compartmentation resulting not from lipid peroxidation but from modification

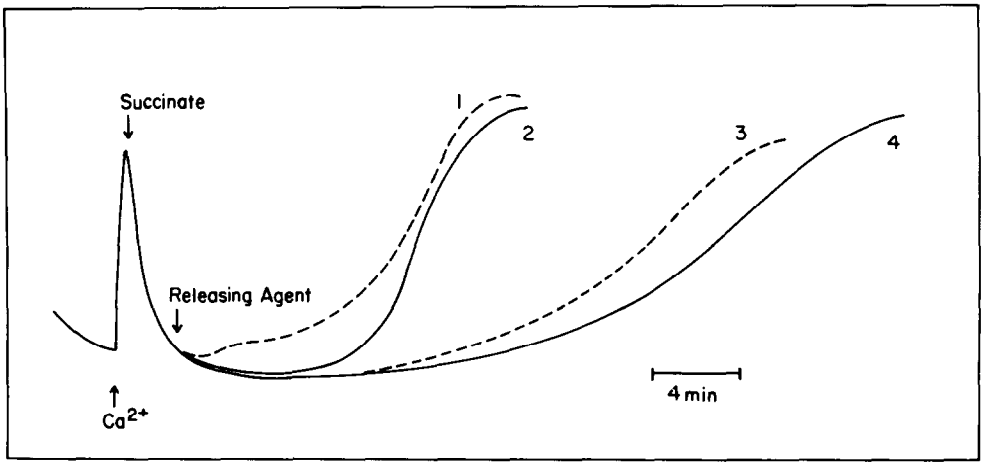

Fig.3. Effect of DTNB and 7-hydroxy adriamycin aglycone on Ca^{2+} retention by isolated, preloaded rat heart mitochondria. Mitochondria (0.4 mg protein) were preincubated for 3 min in 2 ml standard buffer supplemented with rotenone. At the points indicated, Ca^{2+} (60 nmol), succinate (5 mM), and either 17.3 μM aglycone (trace 1) or 100 μM DTNB (trace 2) were added. Traces 3 and 4 are solvent controls for the aglycone and DTNB, respectively.

of mitochondrial thiols. This is consistent with the report that sulfhydryl-containing reagents, in some instances, reduce anthracycline cardiotoxicity [23].

Sulfhydryl modification is induced in this system by 7-hydroxy adriamycin aglycone. The 7-deoxy compound, which is physiologically more important [24], is also more potent. Adriamycin itself is without marked effect in this system. Enhanced potency of the more hydrophobic aglycones may reflect increased access to key membrane thiols. Recent reports have identified 7-deoxy adriamycin aglycone as a major metabolite in rat hearts [25], in aerobic rat hepatocytes [26] and in some humans [27] after adriamycin exposure.

Adriamycin elicits Ca^{2+} release from sarcoplasmic reticulum [28], and sulfhydryl reagents have also been found to alter Ca^{2+} sequestration by liver microsomes [16] and by vesicles of both liver plasma membrane [29] and skeletal muscle sarcoplasmic reticulum [30–32]. In the latter case, as in mitochondria, a generalized permeability increase is induced [30]. Alteration of the permeability of cellular membranes mediated by thiol modification may thus represent a general mechanism of adriamycin toxicity, with the relative effectiveness of the parent compound and various metabolites on any particular membrane system governed by their access to membrane sulfhydryls.

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