Biochimica et Biophysica Acta 848 (1986) 312–316
Elsevier

BBA 41924

Correlation between outer-membrane lysis and susceptibility of mitochondria to inhibition by adriamycin and polyamines

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(Received September 5th, 1985)

Key words: Adriamycin; Polyamine; Respiration; Membrane lysis; Mitochondria membrane; (Rat liver mitochondria)

Pretreatment of rat liver mitochondria with digitonin or osmotic shock increases their susceptibility to respiratory inhibition by adriamycin and polyamines. Since enhanced inhibitor sensitivity coincides in each case with lysis of the mitochondrial outer membranes, the possibility is raised that this membrane represents a permeability barrier to certain polar, organic cations.

Introduction

Adriamycin is an anthracycline antibiotic effective in the treatment of cancer whose clinical value is seriously compromised by its cytotoxicity, in particular to host cardiac tissue [1]. There is evidence that the toxic side-effects of adriamycin arise from impairment of oxidative phosphorylation, e.g., the uncoupling and inhibitory effects of the drug on isolated mitochondria are well-documented [2–4]. The molecular basis of mitochondrial insult may involve interaction of the drug with cardiolipin in the mitochondrial inner membrane [5,6].

In studying the effects of adriamycin and other organic cations on rat liver mitochondrial respiration, we have found that treatments which lyse outer membranes render the mitochondria more susceptible to the actions of several of these compounds. This report summarizes these observations and presents a possible explanation for their molecular basis.

Materials and Methods

Mitochondria were isolated from livers of Wistar rats (killed by decapitation) as described before [7]. Succinate : oxygen oxidoreductase activity was monitored at 27°C with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) in medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7.0) and 2–4 mg/ml mitochondrial protein; reactions were initiated with 12 mM sodium succinate. Cytochrome *c* : oxygen oxidoreductase activity was also measured polarographically in the same reaction medium containing additionally 100 mM KCl, 1 μM antimycin A and either 5 μM CCCP or 10 μM FCCP; reactions were initiated with 0.125 mM reduced cytochrome *c*. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Digitonin was recrystallized from hot ethanol prior to use.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MGBG, methylglyoxal-bis(guanylhydrazone).

Results and Discussion

Steady-state rates of succinate oxidation (v) are plotted in Fig. 1A–C as a function of inhibitor concentration (i) for mitochondria pretreated with increasing levels of digitonin, a detergent which selectively lyses mitochondrial outer membranes in the concentration ranges used in these experiments [8–10]. In each experiment, the extent of outer membrane lysis corresponding to the different digitonin levels is indicated by the relative accessibilities of exogenous cytochrome *c* to inner-membrane reduction sites (i.e., relative rates of cytochrome *c*: oxygen oxidoreductase, Fig. 1D–F) *. Clearly, susceptibility of mitochondria to the effects of these cationic compounds increases markedly with increasing digitonin pretreatment. Both adriamycin and the polyamines are weak, incomplete inhibitors of the respiration of unpretreated mitochondria over the concentration ranges used. With increasing digitonin pretreatment, the v vs. i curves change systematically: slopes increase to maximum, limiting values and respiratory inhibition is total at relatively low drug levels. That these effects are associated with outer-membrane lysis is indicated by the fact that the limiting shapes of the v vs. i curves are attained at digitonin levels just below those required to make outer mitochondrial membranes maximally leaky to cytochrome *c*.

Another indication that changes induced by digitonin in mitochondrial sensitivity to cationic inhibitors are associated with outer-membrane lysis is that similar changes are effected by suspending the mitochondria in hypoosmotic media. (This treatment causes swelling of the mitochondrial matrix space and subsequent rupture of the outer membrane [15,13].) Table I compares drug concentrations required for 50% inhibition of succinate oxidation for rat liver mitochondria before and after lysis of their outer membranes by digitonin or by osmotic shock. In both kinds of experiments, pretreatments were used that were just sufficient to maximally rupture outer mitochondrial membranes, based on unmasking of cytochrome *c*: oxygen oxidoreductase. In general, 50% respiratory inhibition occurs at organic cation concentrations 2–10-times lower for mitochondria with fully broken outer membranes than for unpretreated mitochondria. Also, the inhibitory effects of the organic cations are considerably more variable with unpretreated than with fully lysed mitochondria as indicated by the experimental spreads in the values in Table I.

In addition to causing lysis of outer mitochondrial membrane, both digitonin and hypoosmotic treatments of mitochondria lead to varying degrees of uncoupling of substrate oxidation from energy transduction reactions. Therefore, the effects of

TABLE I

EFFECTS OF DIFFERENT MITOCHONDRIAL PRETREATMENTS ON INHIBITION OF SUCCINATE OXIDASE BY CATIONIC INHIBITORS

The values presented are inhibitor concentrations (mM) for 50% inhibition of succinate oxidase; ranges represent 3–8 different determinations for each experimental condition. Prior to adding cationic inhibitors to the reaction chamber, the mitochondria were incubated (a) for 1 min in the reaction medium (no pretreatment), (b) for 5 min in 35 mosM mannitol/sucrose ('osmotic shock'), (c) for 2 min in reaction medium containing 0.04–0.06% digitonin, or (d) for 1 min in reaction medium containing 5 μM CCCP or 10 μM FCCP ('uncoupler'). In some experiments, mitochondria were incubated with the cationic inhibitors for several (2–7) minutes before initiating the succinate oxidase reaction; in others, the effects of serial addition of equal aliquots of inhibitor to the same reaction mixture after initiating the reaction were monitored. Similar results were obtained with either procedure.

Drug	Pretreatment			
	none	osmotic shock	digitonin	uncoupler
Adriamycin	0.4–1.0	0.1–0.2	0.1–0.2	>1.0
Spermidine	12–50	8–12	6–10	15–30
Spermine	10–40	4–6	4–7	10–20
MGBG	20–80	8–10	6–8	8–15

* The outer mitochondrial membrane is impermeable to holocytochrome *c*, which is best demonstrated by the fact that this protein can be trapped indefinitely inside sonicated vesicles of this membrane [11,12]. Consequently, in whole mitochondria, rates of electron transfer between externally added cytochrome *c* and redox sites on the inner membrane are controlled by the intactness of the outer membrane [10,13,14]. We performed parallel electron microscopic observations in several of the experiments summarized in this report, which confirmed the close correlation between stimulation of cytochrome *c* oxidation rates by digitonin and disruption of the mitochondrial outer membranes.

chemical uncoupling on mitochondrial susceptibility to the same organic cations were determined. (Mitochondria were uncoupled by inclusion of 5–10 μM carbonyl cyanide phenylhydrazone in the reaction medium; see Table I and Fig. 1C.) The effects of spermine and spermidine on mitochondria were found to be independent of the initial degree of coupling of the mitochondria. The susceptibility of unlysed mitochondria to inhibition by the polyamines is not increased by chemical uncoupling. Furthermore, the same changes in mitochondrial susceptibility to polyamines are elicited by digitonin pretreatment in the presence of chemical uncoupler as in its absence. Neither were the effects of hypoosmotic and digitonin treatments on susceptibility of mitochondria to adriamycin mimicked by prior chemical uncoupling of the mitochondria. In fact, adriamycin was found to be a weaker inhibitor of the succinate oxidase activity of mitochondria in the presence of chemical uncouplers.

The inhibitory effects of a fourth organic cation have also been studied as a function of mitochondrial outer-membrane intactness and coupling state: methylglyoxal-bis(guanylhydrazone), or MGBG, a multivalent cationic anti-cancer drug with known mitochondrial toxicity [16,17]. As indicated in Table I, initially coupled mitochondria display increased susceptibility to MGBG inhibition after osmotic shock or digitonin treatment. Unlike the situation with the other inhibitors, chemical uncoupling of mitochondria increases their susceptibility to MGBG to almost the same extent, although further increase is usually

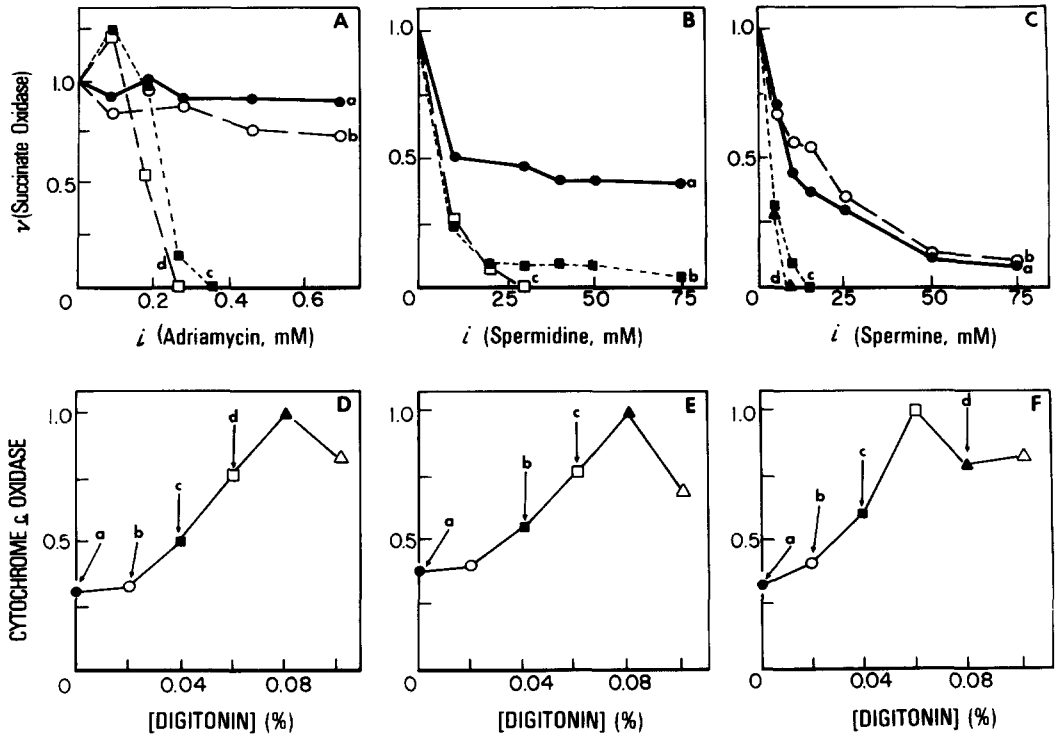

Fig. 1. Correlation between outer mitochondrial membrane intactness and respiratory inhibition by adriamycin, spermidine and spermine. (A–C) Plots of steady-state rates of succinate:oxygen oxidoreductase as a function of inhibitor concentration for mitochondria pretreated with different levels of digitonin. (D–F) Plots of cytochrome *c*:oxygen oxidoreductase vs. digitonin concentration for the mitochondrial preparations used in the experiments of A–C, respectively. The digitonin levels corresponding to curves a–d in the experiments of A–C are indicated by the same letters and symbols in D–F. In A–C, v is the steady-state succinate oxidase rate obtained following incubation of the mitochondria for 1–2 min with the indicated concentration of inhibitor (i) divided by the rate obtained with mitochondria exposed to the same digitonin level in the absence of inhibitor. (5 μM CCCP was included in the reaction medium in the experiments of C.)

elicited by digitonin treatment. Thus, the uncoupling effects of hypoosmotic and digitonin treatments are alone insufficient to account fully for the observed changes in mitochondrial inhibition by MGBG. (Increased sensitivity of mitochondria to MGBG following uncoupling of oxidative phosphorylation suggests that the drug acts at an electron transfer step whose rate is closer to being rate-controlling in the uncoupled than in the coupled state.)

In the case of respiratory inhibition of unpretreated mitochondria by polyamines, plots of v vs. i are often biphasic, containing a 'plateau' of resistant succinate oxidase activity that is eliminated by digitonin and hypoosmotic treatment of the mitochondria (as in the case of spermidine in Fig. 1B). Such behavior is consistent with the presence of multiple types of inhibitor-binding sites on the mitochondria, with the highest-affinity (lowest K_i) sites accessible on only a fraction of the mitochondria. The effects of digitonin and osmotic shock might then be explained in terms of unmasking the high-affinity sites on the other mitochondria. Adriamycin (Fig. 1A) slightly stimulates respiration of lysed mitochondria at low levels (less than 200 μM) and inhibits completely at slightly higher levels. The drug has little net effect on respiration of unpretreated mitochondria in the same concentration range. This could indicate either total or partial inaccessibility of the drug to its binding site(s) in unpretreated mitochondria, with net respiratory rates in the latter case reflecting an average of unaffected, stimulated and inhibited rates at low drug levels. In either event, the dramatic effects of digitonin and osmotic shock on the inhibitory characteristics of adriamycin are again consistent with increased accessibility of the drug to its site(s) of action.

As noted above, the changes in mitochondrial sensitivity to inhibitors occur at digitonin and osmotic-shock levels just below those which maximally permeabilize the outer membranes to cytochrome *c*. This raises the possibility that the outer mitochondrial membrane itself may prevent access of these compounds to their primary sites of action on the inner membrane. By this interpretation, the variable fraction of accessible high-affinity inhibitor-binding sites in unpretreated mitochondria could correspond to the fraction of mitochondria with initially broken outer membranes (which varies typically from 10 to 40% in our preparations, see Figs. 1D–F). Low-affinity binding sites inferred for the polyamines might be on the outer membrane itself, with the weak respiratory inhibition observed resulting, for example, from polycation-induced aggregation of the mitochondria [18]. It is also possible, of course, that the observed effects of digitonin and hypoosmotic treatments on mitochondrial inhibitor susceptibility arise from structural changes in the inner membrane (e.g., conformational changes or unmasking of inhibitor-binding sites.) Such an interpretation would require that these inner-membrane alterations occur coincidentally with outer-membrane lysis for each pretreatment and for each type of inhibitor.

The suggestion that the mitochondrial outer membrane may be a permeability barrier to some cationic inhibitors is contrary to conventional wisdom, which says that this membrane is freely permeable to all solutes smaller than 5 kDa. In fact, recent results from other laboratories indicate that substrate diffusion through the outer mitochondrial membrane may be rate-controlling for reactions catalyzed by enzymes in the intermembrane space [19,20]. Furthermore, the channels that appear to be the main permeability pathway for polar solutes through the outer mitochondrial membrane exhibit permeabilities in vitro that decrease with increasing solute size and net positive charge [21]. Since the inhibitors in this study are all large cations (adriamycin, M_r 544, +1; spermidine, M_r 145, +3; spermine, M_r 202, +4), their apparent inability to cross the outer membrane would be consistent with the selectivity of the channels in this membrane for anions.

Limited permeability of the outer mitochondrial membrane to adriamycin would also provide a basis to explain the observation that the susceptibility of heart mitochondria to respiratory inhibition by adriamycin is greatly increased when hexokinase is present in the assay media [22]. There is evidence that the outer-membrane channels are also the mitochondrial receptors for hexokinase [23,24]. Thus, it is possible that the 'hexokinase effect' involves mediation by the protein ligand of the interactions between its receptor (the channels) and adriamycin, increasing the

drug's ability to penetrate the outer mitochondrial membrane.

Clearly, the above results points to the need to extend permeability studies of the outer mitochondrial membrane and its isolated channels to a wider range of polar organic solutes, in particular, those with multiple positive charges.

The kinetics of uptake of radiolabelled spermidine into rat liver mitochondria have been monitored in recent experiments [25]. Lysis of the outer membranes by digitonin causes a rapid jump in spermidine uptake followed by increased respiration-dependent uptake. Such biphasic kinetics suggest rapid penetration of the polyamine into a previously inaccessible compartment (the intermembrane space) followed by uptake into the matrix, i.e., consistent with the outer mitochondrial membrane representing a permeability barrier to the polyamine.

Acknowledgements

The authors thank Drs. Joyce Diwan (Rensselaer Polytechnic Institute), Robert MacColl and Donald S. Berns (Wadsworth Laboratories) for evaluating the manuscript. This material is based on work supported by grant PCM-8315666 from the National Science Foundation.

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