

The Exogenous NADH Dehydrogenase of Heart Mitochondria Is the Key Enzyme Responsible for Selective Cardiotoxicity of Anthracyclines*

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The molecular mechanism of the anthracycline-dependent development of cardiotoxicity is still far from being clear. However, it is generally accepted, that mitochondria play a significant role in triggering this organ specific injury. The results presented in this study demonstrate that, in contrast to liver mitochondria, isolated heart mitochondria shuttle single electrons to adriamycin, giving rise to oxygen radical formation via autoxidation of adriamycin semiquinones. This one electron reduction of anthracyclines is catalyzed by the exogenous NADH dehydrogenase associated with complex I of heart mitochondria, an enzyme which is lacking in liver mitochondria. Upon addition of NADH heart mitochondria generate significant amounts of adriamycin semiquinones while liver mitochondria were ineffective. Adriamycin semiquinones undergo both autoxidation leading to superoxide radical release and complex reactions under formation of adriamycin aglycone. Due to the high lipophilicity adriamycin aglycones accumulate in the inner mitochondrial membrane where they interfere with electron carriers of the respiratory chain. Adriamycin aglycone semiquinones emerging from an interaction with complex I were found to trigger homolytic cleavage of H_2O_2 which results in the formation of hydroxyl radicals. As demonstrated in this study the activation of adriamycin by the exogenous NADH dehydrogenase of cardiac mitochondria initiates a cascade of reaction steps leading to the establishment of oxidative stress. Our experiments suggest the exogenous NADH dehydrogenase of heart mitochondria to play a key role in the cardiotoxicity of adriamycin. This organ-specific enzyme initiates a sequence of one electron transfer reactions ending up in the establishment of oxidative stress.

Introduction

In the USA the majority of tumor patients is treated with anticancer drugs of the anthracycline family. The development of dose-dependent cardiomyopathy is the main limitation in the treatment with anthracyclines (adriamycin, AQ) (Carter, 1975). Although much literature exists on this problem it is still unclear why the heart is particularly susceptible to AQ. There is general agreement that heart mitochondria are the major toxicological target for AQ (Sokolove, 1994). This becomes apparent by the morphological alterations which occur in hearts subjected to AQ treatment. Considering that heart mitochondria

which account for 40% of the total mass of the cardiac muscle supply the organ with energy in the form of ATP for physiological and biochemical activities, it is clear that any harm to these vital organelles has severe consequences for the function of the heart. Thus, most research efforts made to clarify the pathogenesis of cardiomyopathy were focused on the response of cardiac mitochondria to AQ.

Table I lists some pertinent mitochondrial alterations collected from the literature, each of which being suggested to be the key event in AQ-induced cardiomyopathy (Carter, 1975; Sokolove *et al.*, 1993; Youngman *et al.*, 1984; Monti *et al.*, 1986; Matsumura *et al.*, 1994; Demant, 1991; Iwamoto *et al.*, 1974; Ji and Mitchell, 1994). Although all of these alterations reported may have severe consequences on heart function, evidences are lacking which exclude that these events could occur with mitochondria of other tissues as well. Most of the proposed mechanisms can be directly or indirectly related to the establishment of oxida-

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Table I. Proposed causes of adriamycin-cardiotoxicity.

– inhibition of ADP/ATP translocase	– decrease of lipid-phase transition
– complexation of cardiolipin	– inhibition of succinate-dependent oxidative phosphorylation (involvement of hexokinase)
– inhibition of cytochrome oxidase (via cardiolipin-binding)	– covalent protein binding
– redox-cycling of complexed iron (copper) – GSH	– inhibition of carnitine/palmitoyl transferase
– oxidation of thiol-groups	– inhibition of pyruvate carrier
– opening of Ca^{2+} pores	– antioxidant status of the heart
– permeability increase for H^+	– elevated catecholamine oxidation
– inhibition of ATP-ases	– DNA alkylation
– inhibition of coenzyme Q function	– covalent binding to nuclear DNA
– inhibition of respiration at site II	– stimulation of adenylate cyclase
– inhibition of $\text{Na}^+/\text{Ca}^{2+}$ pumping system	– inhibition of cytochrome c
– inhibition of FAD-biosynthesis (energy metabol.)	– inactivation of complex I–III
– lipid peroxidation without reductive cycling	– inhibition of complex III and IV
– delocalization of tissue iron	

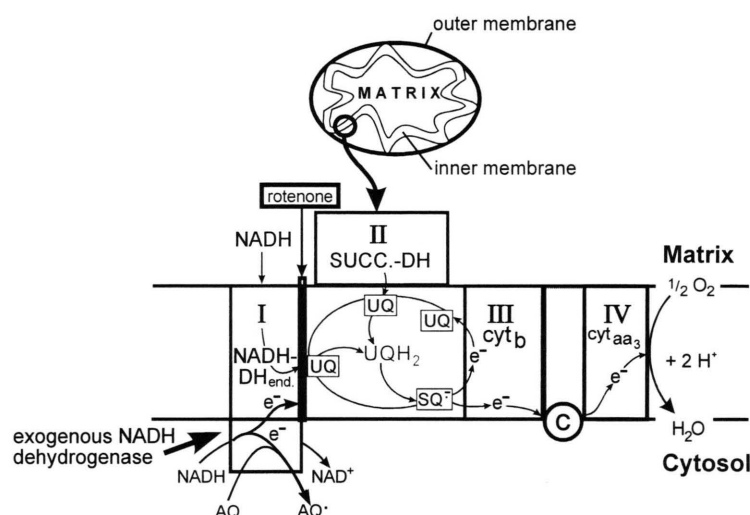


Fig. 1. Flux of reducing equivalents from cytosolic NADH through components of the respiratory chain. Adriamycin (AQ) can directly accept electrons from the exogenous NADH dehydrogenase. (UQ, ubiquinone; SQ^- , ubisemiquinone; Succ.-DH, succinate dehydrogenase; $\text{NADH-DH}_{\text{end}}$, endogenous NADH dehydrogenase, cytochrome c).

tive stress, which is generally accepted to play a role.

It is also generally accepted that oxidative stress is developed via one-electron redox cycling of AQ and that NADH must be added to the mitochondrial suspension to start this toxicogenetic metabolism (Davies and Doroshov, 1986a; Nohl and Jordan, 1983; Nohl, 1988).

Progress in the understanding of the cardiosensitive toxicology of AQ was made by a report on a heart-specific mitochondrial enzyme which catalyzes the oxidation of cytosolic NADH (Fig. 1) (Nohl, 1987). The enzyme introduces reducing equivalents from cytosolic NADH into complex I

exhibiting a strong negative redox potential. The redox potential is about -360 mV, which is negative enough to reduce most anticancer drugs of the AQ family to the semiquinone form (AQ^\cdot) (Nohl, 1988).

Results and Discussion

Our experiments revealed that AQ^\cdot were formed by rat heart mitochondria (RHM) when external NADH was present (AQ^\cdot : $g=2.0039$, $\Delta H_{\text{pp}} = 3.75$ G, isotropic, at 200 K and 5mW microwave power), while succinate was ineffective (ESR signal intensity was about 20 times lower).

During control experiments carried out in the absence of AQ no significant ESR signal intensity was observed excluding the contribution of ubisemiquinones to the signal observed in presence of AQ. AQ seems to directly interact at the oxidant site of the exogenous NADH dehydrogenase. Evidence for this assumption comes from the use of rotenone, an inhibitor which blocks out input of reducing equivalents from NADH into the respiratory chain. In contrast rotenone-inhibited mitochondria were even more active in generating AQ· in the presence of NADH. Since rotenone excludes any other component of the respiratory chain from the transfer of reducing equivalents it was assumed that AQ can serve as one-electron acceptor at the oxidant site of the exogenous NADH dehydrogenase. Rat liver mitochondria (RLM) suspensions mildly catalyzed one-electron reduction of AQ both with succinate and NADH (about 4 times less than NADH-supplemented RHM) due to the adherence of a small portion of damaged mitochondria (<5%). This, however, can be excluded to occur with intact RLM since liver mitochondria do not have an exogenous NADH dehydrogenase (Nohl, 1987), and due to the high polarity NADH and AQ compounds do not have access to the respective catalysts of intact mitochondria. The amount of AQ· obtained in RHM was dependent on the presence or absence of oxygen illustrating the involvement of O₂ in redox cycling of AQ.

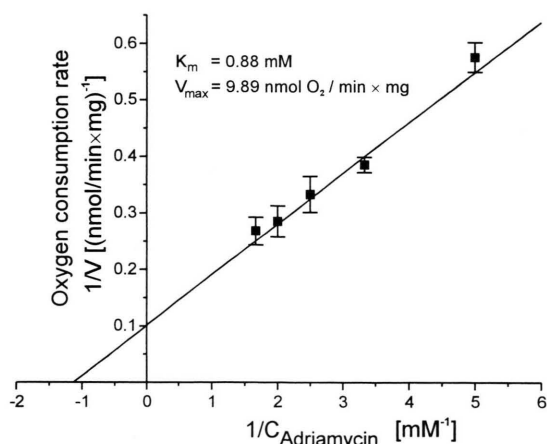


Fig. 2. Lineweaver-Burk plot of adriamycin (AQ) related O₂·⁻ generation in RHM supplemented with NADH. The O₂·⁻ generation was assessed from the O₂ consumption in the presence of rotenone and CN⁻.

clung of AQ. Oxygen serves as an one-electron acceptor of AQ· and forms O₂·⁻ radicals.

The rate of O₂·⁻ formation evolving from this cycle is hard to follow by classical detection methods. Adrenochrome absorbs light in the range of anthracyclines, cytochrome *c* is not able to penetrate into the intramembrane space of mitochondria where O₂·⁻ release was expected to occur. A more reliable method is the determination of O₂ consumption in mitochondria in which electron flow to cytochrome oxidase was inhibited with rotenone and in addition cytochrome oxidase was knocked out as the major mitochondrial O₂ sink by CN⁻ ligand binding (Fig. 2). Under these conditions autoxidation of AQ· is the only way by which oxygen is consumed. With this system it was possible to follow indirectly O₂·⁻ formation from autoxidizing AQ· with increasing AQ concentrations. K_m and V_{max} values were calculated from the double reciprocal plot of Fig. 2. Based on V_{max} values one can estimate that 3–5% of the total mitochondrial oxygen consumption runs into O₂·⁻ formation via autoxidizing AQ·. The K_m value was 10 times above the one reported for submitochondrial particles (SMP) of RHM (Doroshov and Davies, 1986) which stresses the involvement of the exogenous NADH dehydrogenase in AQ redox-cycling. Tissue concentrations established during AQ treatment are considerably below K_m values (15 times). This means that the toxicity of the drug increases exponentially when approaching the K_m level (Sokolove *et al.*, 1993).

Using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) to spin trap radicals formed we obtained

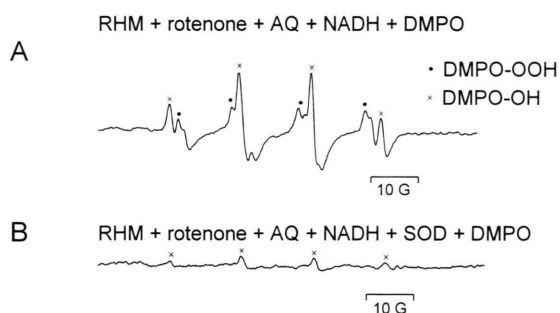


Fig. 3. NADH-induced AQ activation in heart mitochondria gives rise to O₂·⁻ and HO· formation detected as DMPO spin adducts (A) and response to the addition of SOD (B) (ESR spin trapping). (DMPO, 5,5-dimethyl-1-pyrroline N-oxide; SOD, superoxide dismutase).

a mixed ESR spectrum of overlapping DMPO-OOH ($a_N = 14.1$ G, $a_H = 11.3$ G) and DMPO-OH ($a_N = 14.9$ G, $a_H = 14.9$ G) adducts (Fig. 3A). The presence of HO· radicals became visible when O₂^{-·} adduct formation was prevented by superoxide dismutase (SOD) (Fig. 3B). To understand the mechanism of HO· formation we became interested in the existence of a catalyst among split products formed from native AQ-glycosides. Using apolar solvents (Folch extraction) we were able to

extract two major metabolites which were separated by means of thin layer chromatography (Fig. 4). The R_f values of the metabolites moving with the mobile phase (CHCl₃, CH₃OH, CH₃COOH; 100:2:5, on Kieselgel 60) revealed the loss of the sugar residue (Takanashi and Bachur, 1975) whereas the hydrophilic parent compound remained at the starting line.

The major product was subjected to infrared spectroscopy which revealed the loss of the carbonyl group absorption at 1710 cm⁻¹ present in the parent compound (Fig. 5). According to the IR-, UV/VIS-spectra, the high lipophilicity, and the chromatographic retention behavior this metabolite was identified as a 7-deoxyadriamycinol aglycone.

About 25% of AQ (625 μM) were converted by NADH-respiring RHM (5.25 mg/ml) into 7-deoxyadriamycinol aglycone within 30 minutes. The lipophilic metabolite was not obtained under identical conditions with succinate or when liver mitochondria were used as catalysts. However, again focusing electron transfer from NADH via the exogenous NADH dehydrogenase of RHM to AQ by the use of rotenone, stimulated the yield of 7-deoxyadriamycinol aglycone (AglAQ) significantly (65% conversion). When increasing oxygen tension a complete suppression of the aglycone formation occurred while in contrast the removal of oxygen further stimulated the yield of the lipophilic split product by a factor of 1.5.

Based on these observations we designed a metabolic cycle in which AQ· formed from NADH-respiring mitochondria were suggested to undergo disproportionation, resulting in the existence of unstable hydroquinones which subsequently cleave off the sugar residue and form aglycone

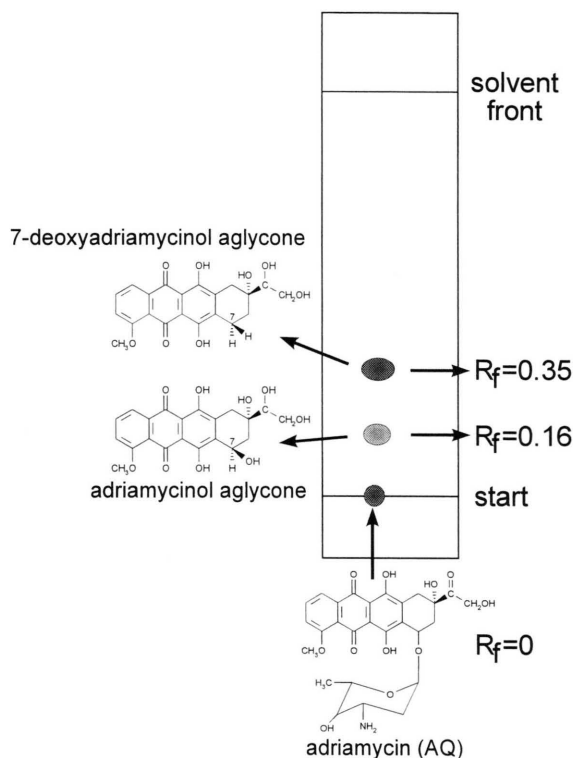


Fig. 4. Thin layer chromatography of AQ metabolites obtained from NADH-respiring RHM.

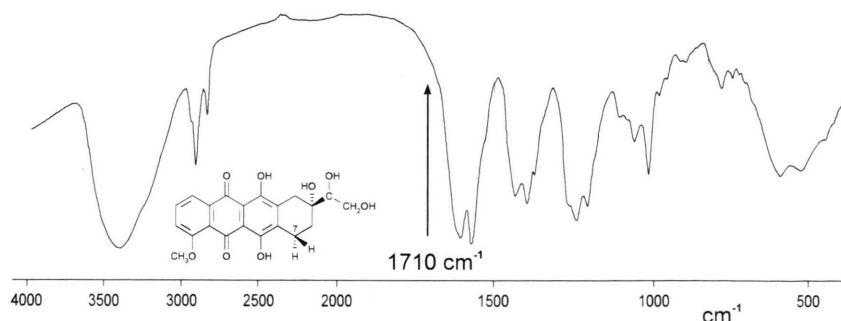


Fig. 5. Infrared spectrum of the major AQ metabolite extracted from the thin layer plate by chloroform.

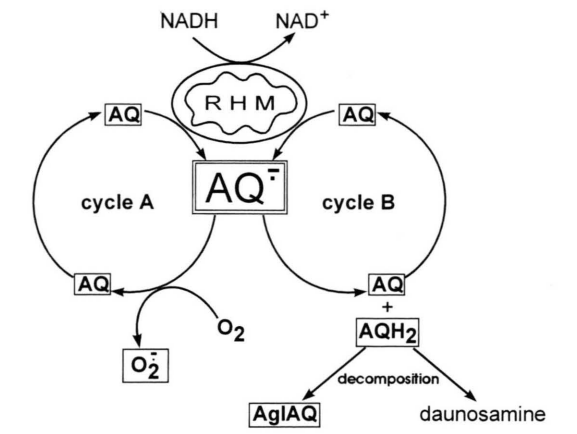


Fig. 6. Metabolic pathways of AQ initiated by NADH-respiring heart mitochondria. (AglAQ, 7-deoxyadriamycinol aglycone; AQH₂, adriamycinol).

split products (Fig. 6). The latter decomposition step was described elsewhere (Lea *et al.*, 1990). Cycle B is stimulated when the competition for the common precursor AQ^{•-} was reduced (Fig. 6). This was possible by slowing down autooxidation rates of AQ^{•-} via reduction of oxygen tension. Due to the lipophilic character of aglycones formed we expected accumulation of these split products in the lipid phase of the inner mitochondrial membrane. The presence of these metabolites became visible when mitochondria were supplemented with substrates for respiration. Irrespectively whether NADH or succinate were used AglAQ semiquinones (AglAQ^{•-}) were formed.

The use of ESR technique revealed that NADH-related AglAQ^{•-} formation most likely occurred by free collision reaction because the ESR signal indicated the existence of non-restricted motions of this radical (Fig. 7A). In contrast, the anisotropic shape of succinate-derived AglAQ^{•-} ESR signal indicated a physical interaction most likely with succinate dehydrogenase (Fig. 7B). The latter appears to stabilize this AglAQ^{•-} species because in contrast to the freely moveable AglAQ^{•-} formed from a collision with the NADH-related electron-transfer pathway the former was totally resistant to the presence of H₂O₂. HO[•] radicals were found to result from an interaction of H₂O₂ and NADH-related AglAQ^{•-}. Fig. 7C shows the respective spin adduct obtained from a radical reaction of HO[•] with dimethyl sulfoxide (DMSO) in the presence of DMPO. Succinate could not sub-

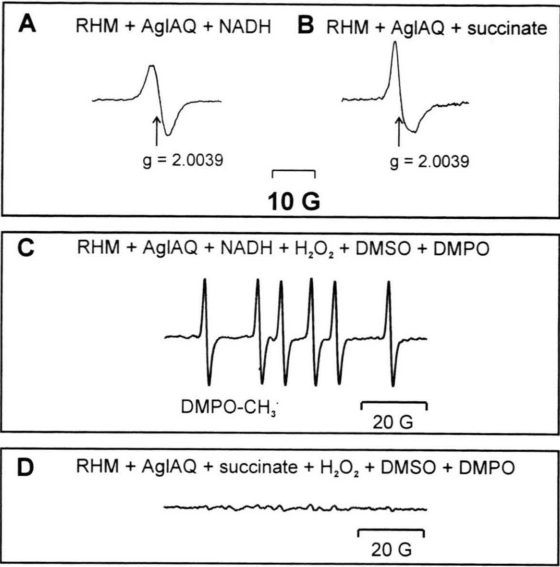


Fig. 7. AglAQ interferes in mitochondrial respiration. NADH-related AglAQ^{•-} formation catalyzes homolytic cleavage of H₂O₂. (A, B) Formation of AglAQ^{•-} radicals in NADH- or succinate-supplemented RHM. (C, D) Interaction of this radical species with H₂O₂ in presence of DMSO and DMPO. (C) The reaction of HO[•] with DMSO liberates [•]CH₃ radicals giving rise to the formation of DMPO-CH₃ adducts (*a*_N = 16.3 G, *a*_H = 23.5 G). (DMSO, dimethylsulfoxide; DMPO, 5,5-dimethyl-1-pyrroline N-oxide).

stitute for NADH in this reaction demonstrating that only AglAQ^{•-} formed on the NADH pathway may cycle electrons out of the respiratory chain to H₂O₂ (Fig. 7D). The disappearance of the semireduced form of AglAQ in the presence of H₂O₂ and the formation of HO[•] radicals in this reaction strongly suggested AglAQ^{•-} to catalyze the following sequence of electron cycling from the NADH pathway to H₂O₂ (Fig. 8).

The presence or absence of diethylenetriaminopentaacetic acid (DETAPAC) was not critical for

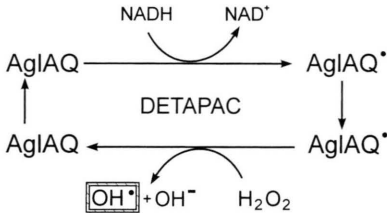


Fig. 8. AglAQ^{•-}-catalyzed reductive homolytic cleavage of H₂O₂. (DETAPAC, diethylenetriaminopentaacetic acid).

this cycle excluding the catalytic involvement of contaminating traces of iron (Fig. 8). This observation is in line with earlier reports in which transition metals were also excluded as being involved in the transfer of single electrons from adriamycin semiquinones or ubisemiquinones to H_2O_2 (non-iron catalyzed reductive homolytic cleavage of H_2O_2) (Winterbourn, 1981; Nohl and Jordan, 1984).

Fig. 9 summarizes the toxicogenetic cycle of AQ activation which is initiated by one-electron reduc-

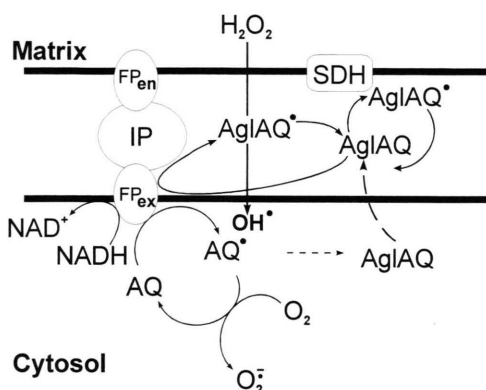


Fig. 9. Proposed mechanism of heart-selective toxicity of AQ. (FP_{en} and FP_{ex} , endogenous and exogenous flavoprotein of complex I; IP, iron-sulfur protein of complex I; SDH; succinate dehydrogenase).

tion of AQ catalyzed through the exogenous NADH dehydrogenase of heart mitochondria.

AQ activation through this enzyme can be considered as the key event in cardioselective toxicogenesis of anthracyclines. AQ^\bullet originating from this catalyst drives both aglycone formation and $\text{O}_2^{\bullet-}$ release from autoxidizing AQ^\bullet . The latter is likely to undergo rapid dismutation and form H_2O_2 due to the presence of cytosolic SOD. Accumulation of aglycones in the inner mitochondrial membrane results from the second pathway. Aglycones interfere in the NADH-related mitochondrial electron transfer pathway and deviate electrons to H_2O_2 out of sequence. HO^\bullet resulting from this AglAQ•-catalyzed cleavage of H_2O_2 may be one major factor which initiates and propagates oxidative impairment of the mitochondrial energy production. Ischemic hearts seem to further stimulate the toxicity of AQ by two mechanisms. Due to the anaerobic glycolysis compensating for reduced mitochondrial ATP production NADH tissue levels increase which drives AQ activation. Depression of oxygen tension favors AglAQ accumulation in the respiratory chain, thereby stimulating reductive homolytic cleavage of H_2O_2 . We, therefore, expect a stimulation of oxidative stress in hearts with low oxygen supply. We actually have work in progress which seems to support this concept.

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