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IDENTIFICATION OF THE SITE OF ADRIAMYCIN-ACTIVATION IN THE HEART CELL

HANS NOHL

Institute of Pharmacology and Toxicology, Veterinary University of Vienna, Linke Bahngasse 11, A-1030 Vienna, Austria

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Abstract— Based on the assumption, the selective cardiotoxicity of anthraquinone antibiotics is due to peculiarities concerning their metabolism in the heart, we have investigated the exogenous NADH oxidoreductase, a heart-specific enzyme recently described (H. Nohl, *Eur. J. Biochem.* **169**, 585 1987) for its possible role in the development of cardiotoxic effects. Cytosolic anthraquinones have direct access since the enzyme was shown to be associated with the cytosolic face of the inner mitochondrial membrane. Redox properties, kinetic data and the poor substrate selectivity suggest the exogenous NADH-oxidoreductase to be involved in the activation of cellular anthraquinones. According to this concept, a direct single electron-shuttle from exogenous NADH to the anthraquinone adriamycin was demonstrated by the detection of adriamycin-semiquinone-related ESR signals. Activation of adriamycin to its semiquinone state at the expense of NADH was also observed with the solubilized NADH-oxidoreductase of heart mitochondria. Microsomal activation of adriamycin was found to result from contaminating exogenous NADH-oxidoreductase of heart mitochondria attached to microsomal membrane fractions. Based on these findings, it was concluded that adriamycin activation in heart cells is due to the existence of the heart specific exogenous NADH-oxidoreductase. Considering the physiological function of this enzyme, activation of cellular adriamycin also appears to be regulated by metabolic changes of cytosolic NADH/NAD ratios.

It is generally accepted that cardiotoxic effects of adriamycin are linked to a one electron reduction step of this anthraquinone antibiotic (AQ), leading to the generation of AQ-semiquinone radicals ($AQ^{\cdot}$). However, conflicting theories exist about the locus and the pathway of AQ-mediated electron shuttle from cellular redox systems to the final electron acceptor, the metabolite supposed to be responsible for the development of cardiotoxicity. Cardiac mitochondria [1, 2], submitochondrial particles [3] and microsomal membrane fractions [1, 4] have been reported to activate AQ at the expense of reduced pyridine nucleotides. The formation of univalently reduced AQ in heart mitochondria supplemented with NADH has been demonstrated with ESR [2]. Toxic oxygen species such as $O_2^{\cdot-}$ [3, 1] and $OH^{\cdot}$ [2] were also observed to be generated under these conditions. These findings were all made upon the addition of exogenous NADH, indicating the involvement of an NADH-consuming enzyme unit accessible from the cytosol where anthraquinones are solubilized. Such an enzyme was recently described to exist in heart mitochondria but not in liver mitochondria [5]. Thus, it was of interest to study whether or not this enzyme could play a role in the cardioselective toxicity of AQ-antibiotics. Our results show that toxic activation of these anticancer drugs in the heart tissue is exclusively linked to the activity of the novel enzyme. This finding could be a rationale to understand why the heart is especially sensitive to this type of antibiotics.

MATERIALS AND METHODS

The anthraquinone antibiotic adriamycin (Adriblastin®) was purchased from Farmitalia, Carlo Erba GmbH (Freiburg, F.R.G.). NADH came from Boehringer (Mannheim, F.R.G.). Other biochemicals were obtained from Sigma Chemical Co (St. Louis, MO). Mitochondria were isolated from male Wistar rats according to [6]. After sedimentation of mitochondria at 9000 *g*, the remaining supernatant was centrifuged for 1 hr in a Beckman ultracentrifuge, model L8 55M. Microsomal pellets obtained at 28,000, 48,000, 78,000, 90,000 or 105,000 *g* were suspended in ice-cold sucrose (heart microsomes: 0.3 mole/l, liver microsomes: 0.25 mole/l), EDTA (2 mmole/l) triethanolamine–HCl buffer (20 mmole/l) pH 7.4 and immediately used for experiments. Oxygen consumption was measured with a micro Clark-type electrode of own design. ESR measurements were conducted with a Bruker ER 200 D-SRC 9/2.7 spectrometer at room temperature using a quartz flat cell.

RESULTS

The role of the exogenous NADH-oxidoreductase of heart mitochondria in cellular activation of adriamycin

Electron transfer to AQ by intact heart mitochondria was followed by means of ESR. $AQ^{\cdot}$-related single line ESR spectra were obtained in RHM when supplemented with AQ and NADH (Fig. 1A). The detection of the semiquinone radical shows that heart mitochondria are capable of mediating a single electron transfer from exogenous NADH to AQ. The single line spectrum was also obtained in the presence of rotenone, which inhibits

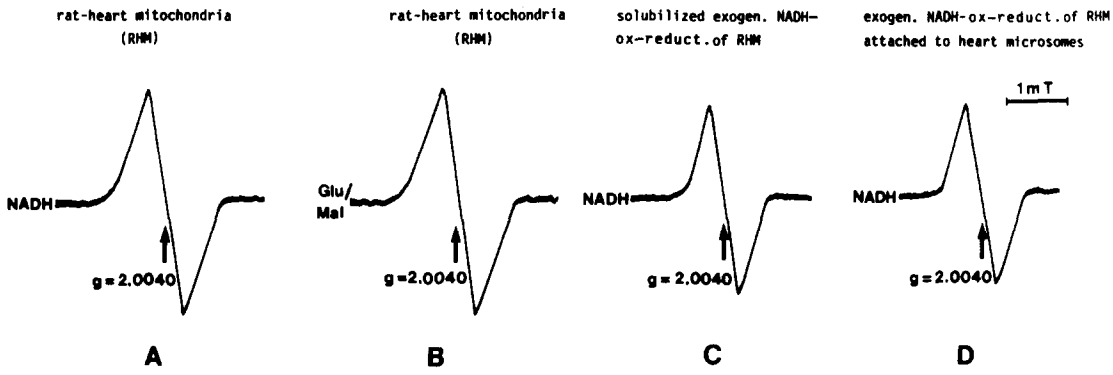

Fig. 1. The role of the exogenous NADH-oxidoreductase in the formation of AQ·-related single line ESR-spectra. AQ· formation was followed in a 0.3 ml quartz flat cell 4 min after the addition of NADH (1.25 mM final conc.) or glutamate/malate (30 mM final conc.) to 6.7 mg of rat heart mitochondria in the presence of 1.25 mM of adriblastin^R. The reaction buffer (125 mM KCl, 50 mM HEPES, pH = 7.4) was saturated with oxygen-free nitrogen. The AQ·-related ESR signal in (D) was obtained from heart microsomal fractions prepared as in [1]. Similar spectra were observed with all NADH-oxidizing active microsomal preparations as used in Fig. 2. The solubilized exogenous NADH-oxidoreductase was obtained as previously described [5]. Protein concentration of the enzyme used for C was 1 mg; concentration of the heart microsomal fraction (90,000 *g*) was: 1.2 mg. Final concentration of rotenone 200 μM. Microwave power: 0.63 mW; modulation amplitude: 0.1 mT; gain: 10^6.

electron flux from the first section of the respiratory chain (complex I) to the rest (experiment not shown). This indicates that exogenous NADH oxidoreductase activates AQ by a direct electron transfer, without the implication of other components of the respiratory chain (see Fig. 4). This concept was also supported by the observation that rotenone-inhibited oxidation of exogenous NADH could be abolished to some extent by the addition of AQ.* Figure 1B shows that AQ can also be reduced to its semiquinone state when endogenous substrates for mitochondrial respiration (glutamate plus malate) were used instead of external NADH. Again rotenone was without effect, thus demonstrating the involvement of complex I in the electron shuttle to exogenous AQ (see Fig. 4). The requirement of the exogenous NADH-oxidoreductase in mediating this electron branching became evident by the fact that liver mitochondria which do not have this type of enzyme, were unable to form AQ· semiquinones during glutamate/malate respiration in the presence of rotenone (data not shown).

These experiments strongly support the idea that exogenous NADH-oxidoreductase of complex I is involved in AQ-activation, independent as to whether reducing equivalents are introduced from cytosolic NADH or from endogenous substrates for complex I.

AQ· formation can also be observed with the isolated exogenous NADH-oxidoreductase when reducing equivalents were fed from NADH (Fig. 1C). This finding further supported the assumption that the organ-specific enzyme can form a redox couple with AQ at the oxidant side. Univalent reduction of AQ to its semiquinone form was also observed when suspensions of heart-microsomal fractions were supplemented with NADH (Fig. 1D).

* H. Nohl, unpublished results (1987).

This observation which is in agreement with the literature [1] prompted us to investigate more thoroughly the role of these subcellular fragments in the toxic activation of cytosolic AQ.

Identity of NADH-oxidizing units associated with microsomal heart fractions

Microsomal fractions from rat heart tissue homogenates were prepared by ultracentrifugation of the 9600 *g* supernatant at 28,000 *g*, 48,000 *g*, 78,000 *g*, 90,000 *g* or 105,000 *g*. The specific activity of the exogenous NADH-oxidoreductase of mitochondria was related to that detected in the various microsomal fractions (Fig. 2). Specific oxidation rates of NADH were found to increase as a function of *g*-values applied for sedimentation of microsomal fractions. Furthermore, all microsomal preparations exhibited contamination by mitochondrial cytochromes. Correlation of NADH-oxidizing activities with the degree of mitochondrial cytochromes attached to microsomal membrane fractions exhibited quantitative similarities, suggesting that the NADH-oxidizing activity also originates from mitochondria. Similar contaminations of liver microsomes with mitochondrial cytochromes could be observed, however, according to the lack of NADH consumption by intact liver mitochondria, also liver microsomes exhibited no NADH-oxidizing activities. Contamination of microsomal fractions with mitochondrial compounds was also observed when applying isolation methods used by other groups for studying AQ activation [1, 4].

Due to the linear increase of microsomal contamination with mitochondrial enzymes, increasing sedimentation velocities were accompanied by decreased activities of native microsomal enzymes when based on contaminating mitochondrial cytochrome levels. Figure 3B shows that this was the case for NADPH-cyt-P450 oxidoreductase (EC 1.6.2.4)

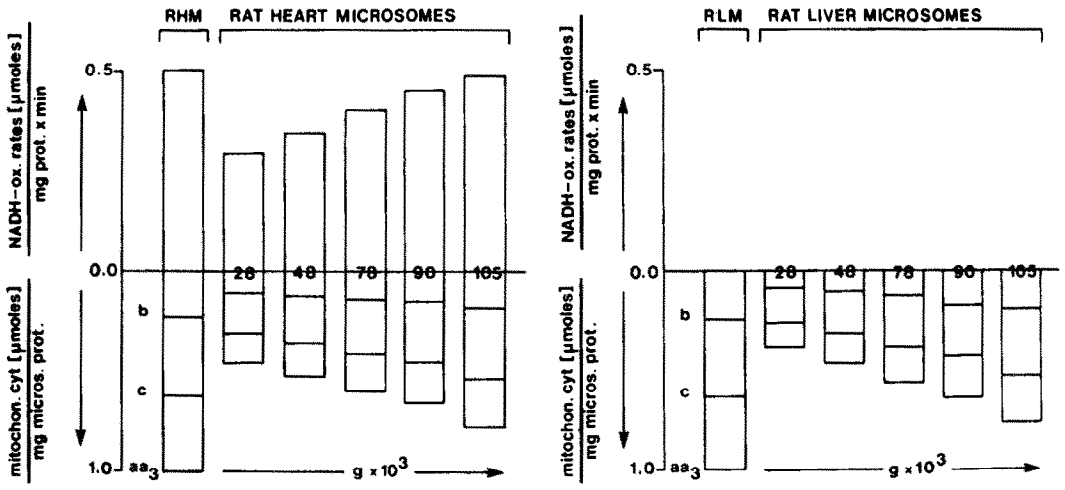

Fig. 2. Assignment of heart microsomal NADH consumption to the activity of contaminating exogenous NADH-oxidoreductase of heart mitochondria. NADH consuming activities were determined as in [5]. Contents of mitochondrial cytochromes were calculated from the respective redox difference spectra using the following extinction coefficients: $\varepsilon_{562} = 28.5$; $\varepsilon_{550} = 27.7$; $\varepsilon_{604} = 19.4\ cm^{-1} \times mM^{-1}$. Final concentrations: heart mitochondria: 0.5 mg/ml; liver mitochondria: 0.6 mg/ml, NADH: 250 µM. The bars represent the mean values of five experiments respectively. Standard deviation (± SEM) were between 2% and 7% of the means.

a native enzyme of liver microsomes, while NADH oxidizing activities of heart microsomes remained unchanged under these conditions (Fig. 3A). These observations further support the concept that microsomal NADH-oxidation activities result from contamination by solubilized exogenous NADH oxidoreductase of heart mitochondria, rather than from the activity of a native heart microsomal enzyme.

DISCUSSION

The present work provides a biochemical basis for an understanding of the specific sensitivity of the heart to treatment with AQ-antibiotics, leading to severe ultrastructural and functional disorders. Our experiments demonstrate the central role of the exogenous NADH oxidoreductase of heart mitochondria in initiating toxic activation of AQ antibiotics in heart tissue. The incapacity of liver mitochondria to transform AQ to the respective semiquinone state, a prerequisite for the toxicity of this drug [2] accords with our earlier observations, that exogenous NADH oxidoreductase is not present in liver mitochondria [5]. Due to the localization of this enzyme at the cytosolic face of the inner mitochondrial membrane [5], it is accessible from the cytosol and may transfer electrons to appropriate

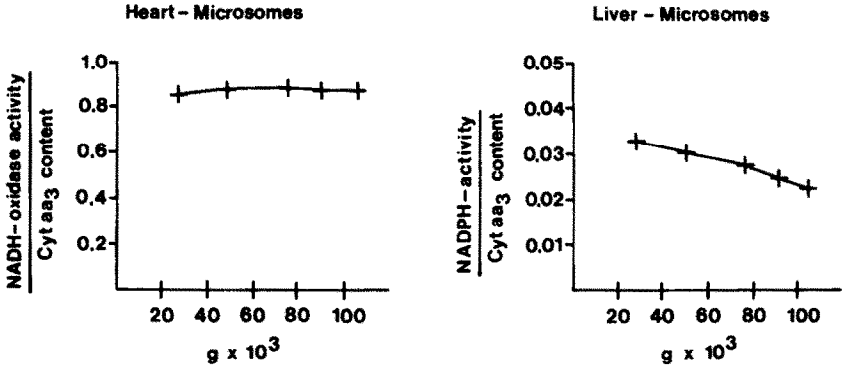

Fig. 3. Influence of microsomal membrane contaminations with mitochondrial constituents on the specific activities of the native liver microsomal enzyme NADPH-cyt P-450 oxidoreductase, as well as NADH-oxidoreductase activities associated with heart microsomal fractions. NADH-oxidoreductase activities were determined as in [5]. The activity of NADPH-cyt P-450 oxidoreductase was measured according to the method described for NADH-oxidoreductase in [5]. The curves were drawn through the mean values of five sets of experiments respectively. The SEM of all measured points ranged between 3.5 and 8.0% of the mean.

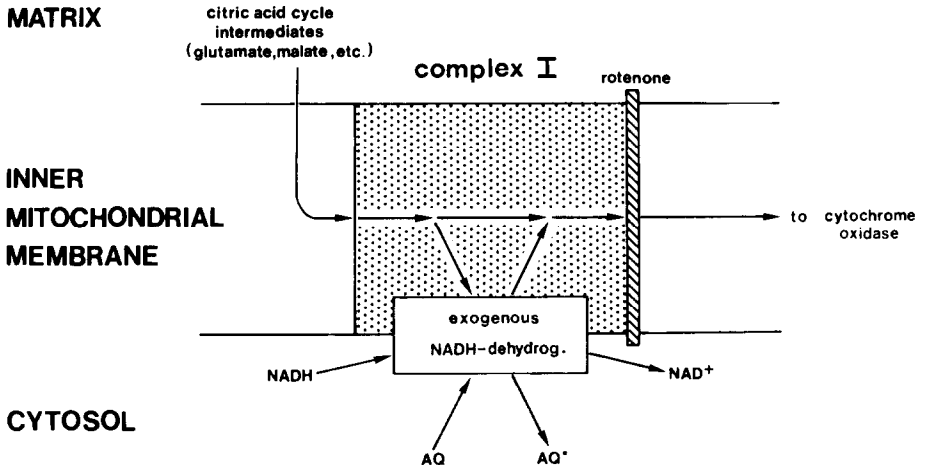

Fig. 4. Schematic presentation of the anthraquinone activator in heart mitochondria. Exogenous NADH-oxidoreductase can mediate electron flow to AQ both from exogenous NADH and from complex I under condition of respiration of citric acid cycle intermediates. In the absence of exogenous electron acceptors reducing equivalents from NADH are also introduced into the respiratory chain [1] thus being sensitive to inhibitors of the respiratory chain (e.g. rotenone).

oxidants which normally do not penetrate the inner membrane (see Fig. 4). Accessibility of the respective activator from the cytosol is a prerequisite for an initiation of AQ metabolism in the cell, since it was shown that the glycosidic drug is not soluble in the lipid bilayer of the mitochondrial membrane [7]. The possible role of the exogenous NADH-oxidoreductase as the cellular AQ-activator could also be expected from the earlier observation that besides complex I also molecular oxygen or ferricyanide may serve as electron acceptors for this enzyme [8, 5]. This indicates a poor selectivity at the oxidant side, also suggesting that AQ accepts electrons from this enzyme. The existence of a direct redox shuttle from exogenous NADH to AQ via NADH-oxidoreductase (without the involvement of other components of the respiratory chain) can be concluded from the observations that a transition of AQ to its semiquinone state, following the addition of NADH also occurs in the presence of rotenone or when using solubilized NADH-oxidoreductase which was free from mitochondrial enzymes (see Figs 1 and 4).

Exogenous NADH-oxidoreductase was earlier shown to introduce reducing equivalents into complex I [5]. This means that the redox potential of the enzyme should exhibit negative values around −300 mV to permit an electron flux from NADH to components of complex I. Since the one electron reduction potential of AQ is reported to be E_0 −292 mV at pH 7 [9], the assumed redox potential of exogenous NADH-oxidoreductase would also explain the observed one electron reduction step of AQ on a thermodynamic basis. These considerations are also valid for electron carriers of complex I. However, univalent reduction of exogenous AQ was shown to depend on the existence of the exogenous NADH-oxidoreductase, since liver mitochondria which do not contain this enzyme were unable to reduce exogenous AQ although components of complex I were kept in a reduced state. This may be explained by the inaccessibility of adequate electron donors to exogenous AQ.

Heart microsomal fractions prepared under various conditions (including those of the literature reported to investigate AQ metabolism) have all been found to reduce AQ at the expense of NADH. Our experiments have shown that the responsible enzyme cannot be a natural constituent of these membrane fractions. Due to the fact that the exogenous NADH oxidoreductase is only loosely bound to the inner membrane of heart mitochondria [5], contamination of microsomal fragments cannot be avoided when applying frequent isolation procedures. The lack of NADH-oxidizing units in liver microsomes despite the simultaneous contamination with mitochondrial marker enzymes fits in with this concept. Thus, it can be concluded that AQ-activation in heart cells is exclusively performed by the exogenous NADH-oxidoreductase associated with mitochondria. The specific activity of exogenous NADH-oxidoreductase was found to be in the range of other enzymes supplying reducing equivalents to the respiratory chain [5]. Thus, although the enzyme is likely to serve as an efficient redox shuttle from exogenous NADH sources to complex I [5], its turnover rate appears to be high enough also to account for total activation of cellular AQ. The relatively low K_m (1 μM) [5] suggests a high sensitivity of the enzyme activity with respect to NADH/NAD changes in the cell reflecting metabolic changes of the lactate/pyruvate ratio. Reduction of oxygen supply to the heart tissue causing anaerobic metabolism with high NADH/NAD levels would therefore increase the activity of the exogenous NADH-oxidoreductase and thereby stimulate the transformation of cellular AQ to the activated form. The existence of such an interaction remains to be established by experimental evidence. The understanding of the physiological role of the organ-specific exogenous NADH-oxidoreductase will help to further elucidate

conditions regulating the toxicity of AQ in the heart tissue.

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