Electron Spin Resonance Studies on the Spin Label of Rat Cardiac Mitochondria Treated with Adriamycin

KIMINORI KAJIYAMA, MASAYASU SUGIYAMA, HIROSHI IWAHASHI,
TOSHIHIRO HIDAKA, MARK L. ENTMAN*, IKUKO SHIMADA
AND RYOHEI OGURA

*Department of Medical Biochemistry, Kurume University School of Medicine, Kurume, 830 Japan and *Section of Cardiovascular Sciences, Department of Medicine, The Methodist Hospital and Baylor College of Medicine, Houston, Texas, U.S.A.*

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Summary: Adriamycin (ADR), which is a potent antitumor agent, has severe cardiotoxicity; however, the underlying mechanism is not well understood. In our present study, the electron spin resonance (ESR) technique was employed to analyze the physical properties of cardiac mitochondrial membrane treated with ADR. After intercalation of spin probe into the isolated cardiac mitochondrial membrane, mitochondria were incubated with 3.45×10^{-4} M ADR for 10 minutes at 37°C, the membrane fluidity was measured by ESR spin probe; 5-doxyl stearic acid (5-DSA) and 12-doxyl stearic acid (12-DSA). The ADR *in vitro* treatment resulted in an elevation of the order parameter S and S ($T_{\perp}$). It was suggested that the membrane fluidity of cardiac mitochondria decreased in both 5-DSA and 12-DSA regions of the lipid bilayer.

Key words: ESR — spin label — mitochondria — adriamycin — membrane fluidity

Introduction

Adriamycin (ADR) is a widely used antitumor agent in cancer chemotherapy, but the therapeutic usefulness of ADR is limited by a severe side-effect, cardiotoxicity (Lampidis et al. 1980; Lefrak et al. 1973). The pathogenesis of ADR-induced cardiotoxicity is not fully understood, but several effects of ADR on the cardiac mitochondria have been reported (Folkers et al. 1973; Gosálvez et al. 1979; Myers et al. 1977; Ogura et al. 1979, 1986a). Goormaghtigh et al. (1980) reported that ADR binds to the cell membrane by interacting with negatively charged phospholipids. There have been a number of interesting studies showing the formation of semiquinone free radicals in the pres-

ence of ADR (Bachur et al. 1978). As mitochondria are the site of anthracycline (ADR)-mediated free radical generation, the cardiac toxicity of ADR is considered to depend on free radical-mediated lipid peroxidation in the mitochondrial membrane (Doroshov, 1983; Myers et al. 1977; Ogura et al. 1979, 1986a). From this evidence, it has been suggested that the cardiac mitochondrial membrane is affected by ADR. The purpose of the present study was to examine the physical properties of the cardiac mitochondria that were affected by ADR. Electron spin resonance (ESR) was employed to analyze the membrane fluidity of cardiac mitochondria treated with ADR at a concentration of 3.45×10^{-4} M.

Materials and Methods

The paramagnetic spin probes, 5-doxyl stearic acid (5-DSA) and 12-doxyl stearic acid (12-DSA), were purchased from Aldrich. The spin probes were stored in a freezer at -80°C , as a stock solution containing $100\text{ }\mu\text{g/ml}$ in 99.5% ethanol. Adriamycin (ADR) was purchased from Sigma, and was dissolved in 0.9% NaCl to make a concentration of $3.45 \times 10^{-3}\text{ M}$.

Isolation of Mitochondria

Male Wistar rats weighing about 200 g were sacrificed by decapitation. The hearts were removed quickly and placed in an ice-cold solution of 0.07 M sucrose, 0.21 M mannitol and 5 mM 3-N-morpholinopropanesulfonic acid (MOPS) at pH 7.4. Mitochondria were isolated from heart homogenates using differential centrifugation after Nagarse digestion (Ogura et al. 1986b). The mitochondrial pellet obtained was resuspended in a solution of 100 mM KCl, 50 mM MOPS and 0.2 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4 and at a final concentration of 20–25 mg protein per ml. The protein content was determined by the biuret procedure using bovine serum albumin as a standard (Gornall et al. 1949).

Spin labeling

Aliquots of the spin probe ($100\text{ }\mu\text{l}$) were placed in small test tubes and dried to a thin film under a nitrogen stream. The mitochondrial suspension (10 mg in protein) was preincubated with the Tris-HCl buffer containing 0.21 mM mannitol, 10 mM KH_2PO_4 , 10 mM KCl, 2 mM MgCl_2 , and 0.2 mM EDTA at pH 7.4 for 5 minutes at 37°C and then for 10 min in the spin probe test tube to incorporate the spin probe into the mitochondria (Sugiyama et al. 1984). After that, the mitochondrial suspension was treated with ADR at a final concentration of $3.45 \times 10^{-4}\text{ M}$ for 10 min at 37°C . The mitochondria were then sep-

arated by three five-minute centrifugations of $12,000 \times g$ at 25°C .

The mitochondrial pellets were washed twice in the Tris-HCl buffer. The pellet ($50\text{ }\mu\text{l}$) was drawn into a disposable micro-sampling capillary (Corning). The ESR spectra were measured at 37°C with a JOEL X-band spectrometer, model JES-FE3X (JOEL Ltd., Tokyo). Before the ESR measurement, the ESR capillary was placed in the cavity for 5 min, and allowed to reach an equilibrium temperature (Sakanashi et al. 1986a). To determine the membrane fluidity, the outer ($2T_{\parallel}$) and inner ($2T_{\perp}$) hyperfine splitting were measured in gauss on the ESR spectrum. The order parameter (S) in 5-DSA was calculated according to the formula (1) of Gaffney (1975). However, with 12-DSA, the outer hyperfine splitting value could not be measured, since the low-field peak was not resolved. Therefore, the $S(T_{\perp})$ was calculated from the inner splitting value according to the formula (2) of Gaffney (1975).

$$S = (T_{\parallel} - T_{\perp} - C) / (T_{\parallel} + 2T_{\perp} + 2C) \times 1.723 \dots \dots \dots (1)$$

$$\text{where } C = 1.4 - 0.053(T_{\parallel} - T_{\perp})$$

$$S(T_{\perp}) = (43.7 - 3T_{\perp} / 46.1) \times 1.723 \dots \dots (2)$$

Results and Discussion

In the present study, the electron spin resonance (ESR) technique was employed to examine a direct effect of adriamycin (ADR) on the physical properties of the mitochondrial membrane, *in vitro*. The spin probes, 5-doxyl stearic acid (5-DSA) and 12-doxyl stearic acid (12-DSA), were embedded in the mitochondrial membrane, and the degree of motion exhibited by a spin probe in the membrane, was related with the position of the nitroxide ring on the acyl chain. The spin probes, 5-DSA and 12-DSA, each have a nitroxide radical ring at the 5th and 12th carbon position

from the carboxyl group of the acyl-chain, respectively. Therefore, the order parameter S for 5-DSA reflects the fluidity of the hydrophilic region of the membrane, while that of 12-DSA reflects the fluidity of the hydrophobic region of the membrane. Although the order parameter values do not directly represent the molecular motion of the lipid bilayer, they obviously reflect the dynamic state of the membrane fluidity (Hubbell and McConnell, 1971). A spontaneous decay of the intensity of the ESR signal as a result of nitroxide reduction was observed, but this decay in intensity was not observed until after the first 30 minutes of ESR measurement.

The electron spin spectrum of 5-DSA is shown in Fig. 1. This spectrum indicates a typical anisotropic motion. The outer and inner hyperfine splitting were clearly observed, and the order parameter (S) was calculated as shown in Table 1. The order parameter (S) was significantly increased following ADR treatment. The increase of the order parameter (S) after ADR treatment was 2%. The value of the order parameter (S) is known to shift between 0.0 and 1.0. A value near 1.0 indicates a rapid anisotropic motion, that is, less fluidity in the region surrounding

Fig. 1. Electron spin resonance spectra of 5-DSA embedded in cardiac mitochondria. DSA stearic acid spin label probe, $2T_{||}$: outer hyperfine splitting, $2T_{\perp}$: inner hyperfine splitting, $2T_{||}$ and $2T_{\perp}$ were measured in gauss. Temperature: 37°C, Microwave power: 4 mW Field: 3280 ± 50 gauss, Modulation time: 100 KHz, 2.0 gauss, Response: 1S

TABLE 1
Effects of adriamycin on the order parameter S of 5-doxyl stearic acid in cardiac mitochondria

	n	$T_{ }$	order parameter S
Control	5	25.78 ± 0.13	0.598 ± 0.009
ADR treatment	5	25.93 ± 0.08	$0.609 \pm 0.005^*$

Mitochondria were isolated as described in the Materials and Methods. 10 mg of mitochondrial protein were incubated with 10 mg spin probe for 10 minutes at 37°C and then were incubated with 3.45×10^{-4} M ADR for 10 minutes at 37°C. After three five-minute centrifugations at $12,000 \times g$, the 50 μ g mitochondrial pellets were measured by ESR at 37°C. Order parameters were expressed as mean $\pm$ SE, * $p < 0.05$ versus control. $T_{||}$: outer hyperfine splitting in gauss, n: number of animals.

the spin probe. Therefore ADR treatment, *in vitro*, resulted in a decrease of membrane fluidity of cardiac mitochondria.

The electron spin spectrum of 12-DSA is shown in Fig. 2. The outer splitting could not be measured because the low-field peak was not resolved. Therefore, the $S(T_{\perp})$ value was used as an order parameter. Table 2 lists the $S(T_{\perp})$ values obtained from mitochondria before and after ADR treatment. The ADR treatment resulted in a significant increase in $S(T_{\perp})$. The ADR treatment, *in vitro*, resulted in a decrease of membrane fluidity in the 12-DSA region of the lipid bilayer similar to that observed in the 5-DSA region. The elevation of $(T_{\perp})$ from the control was 4 %.

In the present study, the order parameters of mitochondria treated with ADR were found to increase with both the 5-DSA and the 12-DSA spin probes. This evidence indicates that the interaction of ADR with the 5 and 12-DSA regions of the lipid bilayer results in functional and mechanical disorders of the membrane. The 12-DSA region of the lipid bilayer membrane contains a large portion of unsaturated fatty acid double bonds. Unsaturated fatty acid is known to be easily oxidized to lipid peroxide. Studies by several investigators (Myers et al. 1977;

Fig. 2. Electron spin resonance spectra of 12-DSA embedded in cardiac mitochondria. DSA stearic acid spin label probe, $2T_{\perp}$: inner hyperfine splitting, $2T_{\perp}$ was measured in gauss, Temperature: 37°C Microwave power: 4 mW, Field: 3280 ± 25 gauss, Modulation time: 100 KHz, 2.0 gauss, Response: 1 S

TABLE 2
Effect of adriamycin on the order parameter $S(T_{\perp})$ of 12-doxyl stearic acid in cardiac mitochondria

	n	$T_{\perp}$	orderparameter $S(T_{\perp})$
control	6	11.08 ± 0.07	0.392 ± 0.007
ADR treatment	6	$10.93 \pm 0.01^{**}$	$0.407 \pm 0.002^{**}$

See Table 1 $^{**}p < 0.01$ versus control, $T_{\perp}$; inner hyperfine splitting in gauss.

Doroshov 1983; Ogura et al. 1979, 1986a) reported that ADR exposure leads to the accumulation of lipid peroxide in the cardiac mitochondrial membrane. Sakanashi et al. (1986b), using B-16 melanoma cultured cells exposed to ultraviolet light, observed that the membrane fluidity is influenced by lipid peroxide formed in the membrane. These results indicated that the membrane fluidity of the 12DSA spin probe reflects the lipid peroxidation in the membrane.

On the other hand, the change of membrane fluidity in the 5-DSA region shows that the hydrophilic region of the membrane is affected by ADR treatment. Membrane fluidity has been known to be involved in various membrane-bound enzyme activities. Succinate dehydrogenase, which is a typical respiratory enzyme embedded in the inner membrane of mitochondria, was reduced in activity after ADR treatment *in vivo* and *in vitro*, (Ogura et al. 1979, 1986; Kagiya et al. 1985). It was also observed that Na^+ , Ca^{2+} -ATPase activity in cardiac mitochondria was affected by ADR, *in vitro* (Kagiya et al. 1985). These studies confirmed that the phospholipid-sensitive enzyme system in the membrane may be affected by ADR, due to the ability of ADR to form a specific complex with negatively charged phospholipids; a ADR-cardiolipin complex (Goorgahtigh et al. 1980).

From the present study, it can be suggested that adriamycin has a direct effect on the cardiac mitochondrial membrane which is followed by a change in membrane fluidity. Because the lipid composition is the most significant contributing factor to changes in membrane fluidity, further studies concerning the phospholipid composition of mitochondrial membranes are being conducted.

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