Dynamic Microstructure and Hydration of Peroxidized Membrane of Rat Cardiac Mitochondria and Effects of Adriamycin

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Abstract Nanosecond time-resolved fluorometry of diphenyl hexatriene, DPH, fluorescence was used to study the effects of lipid peroxidation caused by NADH or adriamycin treatment on the dynamic microstructure of mitochondrial membranes from rat myocardium. Isolated mitochondria were incubated with NADH, FeCl_3 , and ADP, or with adriamycin. Parameters for microdynamics were calculated from the fluorescence intensity and anisotropy decay curves for DPH fluorescence. Peroxidized lipids were measured as malondialdehyde (MDA) resulting from the thiobarbiturate reaction. As peroxidized lipids accumulated, the membrane viscosity increased and the wobbling angle of the phospholipids decreased. The structural changes induced in unsaturated phospholipids by peroxidation probably increased the friction of neighboring phospholipids and restricted the range of their wobbling motion. The fluorescence intensity and fluorescence lifetimes decreased significantly when MDA was higher than 10 nmol/mg protein. These alterations in the behavior of DPH fluorescence strongly suggest that the hydration of the phospholipid layer of the mitochondria is occurring as a consequence of lipid peroxidation, since the fluorophore, DPH, is hydrophobic and its fluorescence is known to be quenched by increasing the dielectric constant of the surrounding media. The present results provide experimental supports to the hypothesis of membrane hydration induced by lipid peroxidation.

Key words: microdynamics of membrane, cardiac mitochondria, peroxidation, hydration, adriamycin.

A deviation of the respiratory chain from the normal cycle causes an accumulation of anionic oxygen and peroxidized lipids in tissues. About 2% of the oxygen consumed in state 4 mitochondria is utilized for the production of hydrogen

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peroxide (BOVERIS *et al.*, 1972). The oxygen reduction forms are closely related to lipid peroxidation which causes a decrease in membrane fluidity (CURTIS *et al.*, 1984). Lipid peroxidation is more easily induced in tissues of aged rats in *in vitro* tests than in young ones (PLAYER *et al.*, 1977; NOHL and HEGNER, 1978). Ischemia-reperfusion results in peroxidation of phospholipids in heart (ROMASCHIN *et al.*, 1987), liver (MARUBAYASHI *et al.*, 1982), and intestine (OTAMORI *et al.*, 1988). Reoxygenation of the heart causes peroxidation of mitochondria (GUANIERI *et al.*, 1980). Administration of adriamycin, which is widely used as an antitumor agent, causes lipid peroxidation of cardiac tissues as a side effect *in vivo* (MYERS *et al.*, 1977; THAYER *et al.*, 1977; POLLAKIS *et al.*, 1983; PRAET *et al.*, 1984). As the activity of catalase, which reduces hydrogen peroxide to water, is low in cardiac tissues (THAYER *et al.*, 1977) and as there are high levels of polyunsaturated acyl chains in mitochondrial cardiolipin (BINDOLI, 1988), cardiac mitochondria are easily peroxidized. Since peroxidation causes a decrease in membrane fluidity, accompanied by mitochondrial dysfunction, the fluidity of these membranes has been studied by many investigators by means of electron spin resonance or steady state fluorescence. The fluidity (microviscosity) has been reported as order parameter or steady state fluorescence anisotropy (or polarization). Fluidity is one of the factors postulated to play a role in controlling the activity of membrane proteins (SHINITZKY, 1984). The study of the dynamic microstructure of peroxidized cardiac mitochondrial membrane is, therefore, of importance from both the physiological and pathophysiological points of view.

Membrane fluidity consists of two parameters of dynamic microstructure, i.e., the viscosity of the membrane and the wobbling angle of phospholipids (KAWATO *et al.*, 1977; KINOSITA *et al.*, 1977). The two terms of dynamic microstructure of lipid membranes can be separately measured by time-resolved fluorometry (KAWATO *et al.*, 1977; Araiso and KOYAMA, 1989) and studied in mitochondria from bullfrog heart (KOYAMA *et al.*, 1990a, b) and from rat heart (KINJO *et al.*, 1988). In the present study, isolated rat cardiac mitochondria were peroxidized by application of NADH or adriamycin, and their dynamic microstructure was studied with a nanosecond time-resolved fluorometer.

Another interest in the present study was the effect of peroxidation on the hydration of mitochondrial membranes. Lipid peroxidation increases ion permeation through biomembranes (CHIEN *et al.*, 1979). It is hypothesized that the generation of polar group on the unsaturated acyl chain of phospholipids by oxidation makes the hydrophobic regions of phospholipid bilayers accessible to water and introduce water molecules into intraphospholipid spaces (MEERSON *et al.*, 1982). This succession of events may open channels permeable to water and ions, disturb ion distribution across membranes, and cause fatal dysfunction of cells and mitochondria (MEERSON *et al.*, 1982). However, since no evidence for this hypothesis has been provided, studies on hydration of mitochondrial membranes induced by lipid peroxidation may be physiologically relevant.

METHODS

Male Wistar rats (7 weeks old) were sacrificed by instantaneous decapitation. Mitochondria were prepared by the method described by TYLER and GONZE (1967) with some modifications. Hearts were rapidly excised and ventricular muscles from two rats were pooled and minced with scissors into 2 mm cubes. The minced muscle was suspended in buffer (10 mM Tris, 250 mM sucrose, pH 7.2), homogenized with a polytron (PT 10-35, Kinematica, Switzerland) three times at scale 5 for 1 s and centrifuged at $700 \times g$ for 5 min to sediment nuclei. Mitochondrial pellets were obtained by centrifugation at $7,000 \times g$ for 10 min and suspended in 12 ml of KCl buffer (10 mM Tris, 150 mM KCl at pH 7.2) and washed three times by suspending and centrifugation. The application of protease, which was recommended by the above authors, was discarded in the present study, because they described also that the prolonged exposure to proteinase results in the yield of mitochondria of inferior quality (TYLER and GONZE, 1967). The obtained mitochondria showed a respiratory control ratio (RCR) of 3.2–3.5 and a ratio of inorganic phosphate molecules esterified per oxygen atom utilized (P/O) of 2.5–3.1 when assayed polarographically with succinate as substrate. The mitochondria suspension was divided into four portions, of 3 ml each. All manipulations were carried out at 4°C.

Peroxidation was induced by incubation of 3 ml of mitochondrial suspension with NADH (50 μ M), ADP (2 mM), and FeCl_3 (200 μ M) for 15 min at 25°C. The peroxidation reaction was stopped by addition of 10 volumes of ice-cold KCl buffer solution in some cases (referred to as peroxidation (KCl)). In other experiments peroxidation was accelerated and stopped by the addition of 200 μ l of 35% perchloric acid solution (referred to as peroxidation (HClO_4)). The suspension was then centrifuged, and mitochondria were washed three times by centrifugation as above. Mitochondria were treated with HClO_4 also in control measurements.

The effects of adriamycin were studied in another series of experiments. Adriamycin was dissolved in ethanol at a concentration of 1% (w/v) and added to portions of mitochondrial suspension to give final concentrations of 0 mM, 0.1 mM, 0.3 mM, and 1 mM. Portions of the suspension were incubated for 30 min at 25°C. The suspensions were then centrifuged and washed three times. The final treatment with HClO_4 was not used in the adriamycin experiments.

One ml from each treatment group of control was used for the measurement of lipid peroxide (UCHIYAMA and MIHARA, 1978) and protein (LOWRY *et al.*, 1951). The amount of lipid peroxide was measured by the thiobarbiturate reaction and expressed as amount of malondialdehyde (MDA).

Two ml of each treatment group were used for steady state and time-resolved measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence and its anisotropy (KAWATO *et al.*, 1977; KINOSITA *et al.*, 1977; ARAISO *et al.*, 1986; KINJO *et al.*, 1988). Briefly, the suspension of mitochondria of which membranes were made to contain DPH was illuminated with a pulsed light from a hydrogen spark gap pulsed lamp. The pulsed light was made monochromatic to 340 nm and polarized

to the vertical direction. DPH molecules whose molecular axes were oriented parallel to the polarization of the light for excitation, entrapped in the mitochondrial membranes, absorb the light energy and emit fluorescent light. The vertical and perpendicular components of the fluorescent light, $I_{\parallel}(t)$ and $I_{\perp}(t)$, respectively, were separately measured with a single photon counting system controlled with a personal computer to obtain intensity decay curves. Anisotropy decay curve, $r(t)$, was constructed from the intensity decay curves. The lifetime of fluorescence (τ), anisotropy at infinite time (r_{∞}), membrane viscosity (η), and wobbling angle of phospholipids (θ_w) were calculated from these measurements. Steady state anisotropy was measured with a spectrofluorometer (Shimadzu RF-50, Shimadzu Inc., Kyoto, Japan). Control measurements were also made in mitochondria that were left at 25°C for the corresponding time periods without application of NADH, adriamycin, or HClO_4 . These measurements were referred to as "intact."

For comparison of DPH fluorescence spectra, protein contents of each suspension were adjusted to be 0.5 mg/ml by adding proper amounts of KCl buffer and repeating protein measurement.

Mitochondrial membranes contain different microenvironments. Anisotropy decay curves were assumed to represent an average time course for multicomponent decays and used for calculating average values for parameters of dynamic microstructure. Adriamycin, when administered *in vivo*, distributes preferentially in the inner membrane (GOORMAGHTIGH *et al.*, 1982) of which the dynamic properties are different from those of the outer membrane (KOYAMA *et al.*, 1990b). Adriamycin may peroxidize the inner membrane more strongly than the outer one. Incubation of mitochondria with oxidant agents, on the other hand, probably affects the outer membrane. Thus, the effects of peroxidation on mitochondrial membranes should be studied in the separately sampled outer and inner membranes. Unfortunately, the isolation of the substantial membrane fraction exposed to peroxidation is impossible in rat liver because of changes in the physical properties of the membranes by ischemia (FREDERIKS *et al.*, 1984). In the present study, the two membranes were not separated. It is assumed that the present results represent the mean of the overall effects of peroxidation on the mitochondrial membranes.

All measurements were made at 37°C. The significance of the difference was checked by the Student's non-paired *t*-test.

RESULTS

Examples of DPH fluorescence spectra from peroxidized mitochondria are shown in Fig. 1. The intensity of fluorescence spectrum was lowered by peroxidation (KCl) and by the addition of HClO_4 . Examples of recordings of DPH fluorescence decay curves in the control (A) and in the peroxidation (HClO_4) sample (B) are shown in Fig. 2. It can be seen that the curves for the parallel, $I_{\parallel}(t)$, and the perpendicular, $I_{\perp}(t)$, intensity components were more similar in the control than in the peroxidation sample. The anisotropy, $r(t)$, calculated as $\{I_{\parallel}(t) - I_{\perp}(t)\} / \{I_{\parallel}(t) + I_{\perp}(t)\}$

Fig. 1. Recording of DPH fluorescence spectra from rat cardiac mitochondria washed after incubations with a solution containing NADH, FeCl_3 , and ADP. Peroxidation was stopped by addition of ice-cold KCl buffer or accelerated by an addition of HClO_4 .

$+2 \times I_{\perp}(t)$ }, fell to a lower value in the control than in the peroxidized samples. Concurrently, the values for steady state anisotropy, r_s , reported in the upper right corner of the figure, were found to be low in control samples and high in the peroxidized mitochondria. The fluorescence intensity in **B** decayed more slowly than in **A**. MDA was found to be 15 nmol/mg protein in the former case.

Parameters for fluorescence decay and calculated values for MDA are reported in Table 1 as average and S.D. Minor alterations in parameters were observed in the cases of peroxidation (KCl) samples. More marked changes were obtained in peroxidation (HClO_4) samples, which showed higher MDA values compared with peroxidation (KCl) treatment. The wobbling angle of phospholipid, θ_w , decreased and viscosity, η , increased as a result of peroxidation. The fluorescence lifetime of

Fig. 2. Examples of decay curves of parallel and perpendicular components of DPH fluorescence, $I_{\parallel}(t)$ and $I_{\perp}(t)$, and anisotropy decay curve, $r(t)$, in rat myocardial mitochondria (A) in control and (B) after peroxidation (HClO₄). The horizontal thick line on the anisotropy decay curve indicates the anisotropy value at infinite time, r_{∞} .

Table 1. Parameters for fluorescence, anisotropy, wobbling angle, viscosity, and peroxidized lipids after peroxidation in rat cardiac mitochondria.

	Time (ns)	r_s	r_∞	θ_w (degree)	η (P)	MDA (nmol/mg protein)
Intact	6.7 ± 0.3	0.126 ± 0.002	0.053 ± 0.010	59 ± 2	0.46 ± 0.04	4.4 ± 1.8
Control (HClO ₄)	6.4 ± 0.4	0.126 ± 0.005	0.063 ± 0.014	57 ± 2	0.43 ± 0.06	7.5 ± 2.9
Peroxid. (KCl)	5.9 ± 0.4	$0.140 \pm 0.003^*$	0.065 ± 0.010	55 ± 2	$0.49 \pm 0.01^*$	9.7 ± 4.0
Peroxid. (HClO ₄)	$5.1 \pm 0.8^*$	$0.182 \pm 0.019^{**}$	$0.118 \pm 0.026^{**}$	$46 \pm 4^{**}$	$0.64 \pm 0.18^*$	$25.9 \pm 10.3^{**}$

Mean $\pm$ S.D. ($n=5$). τ , lifetime; r_s , steady state anisotropy; r_∞ , anisotropy at infinite time; θ_w , mean wobbling angle of phospholipids; η , membrane viscosity; MDA, lipid peroxidation expressed with malondialdehyde production by thiobarbiturate reaction. $^*p<0.05$; $^{**}p<0.01$ vs. control.

Table 2. Parameters for fluorescence, anisotropy, wobbling angle, viscosity, and peroxidized lipid after adriamycin treatment.

	Time (ns)	r_s	r_∞	θ_w (degree)	η (P)	MDA (nmol/mg protein)
Control	7.0 ± 1.0	0.122 ± 0.001	0.053 ± 0.003	58 ± 1	0.47 ± 0.06	4.5 ± 6.3
Adriamycin						
0.1 mM	5.8 ± 0.3	0.134 ± 0.008	0.064 ± 0.008	$56 \pm 1^*$	0.47 ± 0.06	12.0 ± 5.8
0.3 mM	$5.3 \pm 0.3^*$	$0.146 \pm 0.013^*$	0.073 ± 0.015	$54 \pm 2^*$	0.51 ± 0.06	$14.6 \pm 5.5^*$
1 mM	$4.5 \pm 0.7^*$	$0.164 \pm 0.025^*$	$0.097 \pm 0.012^{**}$	$48 \pm 3^{**}$	$0.57 \pm 0.14^*$	$22.9 \pm 6.4^*$

Mean $\pm$ S.D. ($n=3$). Abbreviations are the same as in Table 1. $^*p<0.05$; $^{**}p<0.01$ vs. control.

Fig. 3. Examples of recordings of DPH fluorescence spectra from rat cardiac mitochondria incubated with adriamycin of different doses at 25°C for 15 min. The numbers in parentheses represent the sensitivity scale of fluorometer employed at each recording. Small numbers are associated with low sensitivity and vice versa.

the probe; τ , also decreased significantly. It was anticipated that proteins are destroyed by HClO_4 and an overestimation of membrane viscosity might be caused in mitochondrial membranes. The application of HClO_4 in the control measurements, however, resulted in a decrease in viscosity despite the increase in MDA. Moreover, an addition of a proteolytic agent, papain, to mitochondria resulted in a decrease in membrane viscosity (data are not shown). The protein destruction seems to reduce the effects of protein on lipids and membrane viscosity. The measured increase in membrane viscosity in peroxidation (HClO_4) is probably underestimated.

The intensity of DPH fluorescence spectra in mitochondria was decreased in a dose-dependent fashion by adriamycin, as shown in Fig. 3. Examples of recordings of DPH fluorescence and anisotropy decay curves are shown in Fig. 4. The parallel ($I_{\parallel}(t)$) and perpendicular ($I_{\perp}(t)$) decay curves were more similar in the control

Fig. 4. Examples of decay curves of parallel and perpendicular components of DPH fluorescence, $I_{||}(t)$ and $I_{\perp}(t)$, and its anisotropy decay curve, $r(t)$, in rat myocardial mitochondria. (A) mitochondria incubated for 30 min at 25°C with solvent, (B) with 1 mM adriamycin. The horizontal thick line on the anisotropy decay curve indicates the estimated anisotropy value at infinite time.

measurements (Fig. 4A) than in those obtained in adriamycin-treated mitochondria (Fig. 4B). The wide separation of the two components of the fluorescence decay curves in the latter case indicates that the anisotropy at infinite time, r_{∞} , was increased by the treatment of adriamycin. The fluorescence lifetime was shorter in the adriamycin-treated mitochondria than in the control. The obtained values are listed in Table 2. The values for the steady state anisotropy, r_s , and the viscosity, η , increased with increasing dose of adriamycin. The wobbling angle of phospholipids, θ_w , decreased as in the case of peroxidation.

DISCUSSION

The increase in steady state anisotropy caused by peroxidation is consistent with the anisotropy increase of DPH fluorescence in liposomes prepared from extracted beef liver (SEVANIAN *et al.*, 1988) and with the order parameter increase in rat liver microsomes (OHKI *et al.*, 1984) after peroxidation. The increase in the steady state anisotropy was accompanied with a decrease in the wobbling angle and an increase in viscosity in the present study.

In a previous study it was demonstrated that DPH molecules entrapped in lipid bilayers wobble together with the surrounding oscillating phospholipids and that the orientational alteration in DPH is mainly caused by the phospholipid oscillation. The wobbling angle, therefore, represents the range of wobbling motion of phospholipids in the lipid bilayer (ARAI and KOYAMA, 1989). The decrease in the wobbling angle observed in the present study indicates that lipid peroxidation caused a reduction in the range of molecular motions of phospholipids in the bilayer structure. Peroxidation of lipids produces hydroperoxide side chain, cyclisation of lipid chains (PORTER *et al.*, 1981), and cross-linking of phospholipids (MASOTTI *et al.*, 1988). These changes in lipid structure limit the molecular motion of phospholipids and other molecules within lipid bilayer.

The viscosity of lipid bilayers is caused by the mutual collision and friction of oscillating phospholipids. The friction between phospholipids is probably increased by the changes in their structure resulting from peroxidation. The viscosity of the bilayer is consequently increased. The reduction in the wobbling motion of phospholipids may be understood by a graphic illustration, shown in Fig. 5. The hydrophilic hydroperoxide branch will be situated away from acyl chains and limit the range of wobbling motion of phospholipids.

The fluorescence of DPH is easily quenched by an increase in the dielectric constant. If DPH molecules are situated in polar environments, their fluorescence intensity decays quickly (WRATTEN *et al.*, 1989). The DPH fluorescence decay curves in Fig. 2B show an increase in the rate of decrease of both the parallel and perpendicular components of the fluorescence. The fluorescence lifetime was significantly reduced by peroxidation, as shown in the first column of Table 1. This result, together with the decrease in the DPH fluorescence spectra in Fig. 1, suggests that the DPH fluorescence was quenched, possibly due to water dipole which

Fig. 5. Speculative illustration for the possible cause of the reduction in wobbling angle of peroxidized phospholipids induced by lipid peroxidation. The hydroperoxide branch is situated away from acyl chains, since it is hydrophilic as illustrated by MEERSON *et al.* (1982). Assuming that the range of wobbling motion, θ_w , is permitted to phospholipid itself, the actual range of wobbling motion of acyl chains will be reduced to a limited one, $\theta_w' (< \theta_w)$ by the presence of hydroperoxide branch. In this figure, two acyl chains are illustrated as if they wobble together. But in actual living tissues or in liposomes above the phase transition temperature, their motions would be somewhat desynchronized (see Fig. 9 in reference ARAISO *et al.*, 1990).

penetrated into the lipid bilayer after peroxidation treatment. WRATTEN *et al.* (1989) found increases in DPH fluorescence lifetimes when a small fraction of peroxidized phospholipids was added to liposomes. SEVANI *et al.* (1988), who worked with liposomes prepared with lipids extracted from beef liver, however, found a remarkable decrease in DPH fluorescence intensity after lipid peroxidation of more than 5–10 mol% of the liposome phospholipids. Since the level of MDA in mitochondria in the present study reached the level of 26 nmol/mg protein, the present results are consistent with the peroxidation and hydration hypothesis of MEERSON *et al.* (1982) and with the results obtained in liposomes by SEVANI *et al.* (1988). Hydration of the membrane is postulated to result in loss of the role of the membrane in the isolation of the interior side of mitochondria from the cytosol. The ion distributions of calcium, potassium, and protons would then be disturbed across the mitochondrial membranes.

The increase in the steady state anisotropy caused by adriamycin is consistent with the increase in polarization of DPH fluorescence in the cardiac mitochondria from mice that received intraperitoneal injection of adriamycin (PRAET *et al.*, 1984). In our study, the viscosity increased and the wobbling angle decreased as the applied adriamycin increased. These alterations in dynamic microstructure are similar to

those seen following the above lipid peroxidation. The MDA value in adriamycin-treated mitochondria increased to a similar level as in the case of peroxidation (HClO_4) (see last columns of Tables 1 and 2). The accumulation of lipid peroxide after adriamycin application is consistent with the increase in lipid peroxidation in cardiac tissues from mice injected with adriamycin (MYERS *et al.*, 1977; PRAET *et al.*, 1988). The DPH fluorescence yield was decreased (Fig. 3) and the decay time of fluorescence intensity became shorter (Fig. 4) following adriamycin treatment of mitochondria. The remarkably concave decay curve indicates the development of a strong inhomogeneity in the lipid bilayer of the membrane. These results suggest that the hydration of lipid bilayers was also induced by adriamycin application. These alterations are proposed to be produced mainly by lipid peroxidation induced by adriamycin treatment.

The effects of lipid peroxidation on the boundary lipids and on the lipid-protein interaction could not be analyzed. However, it seems reasonable to speculate that similar alterations in dynamic microstructure were induced to boundary lipid surrounding membrane proteins. The increased viscosity, reduced molecular motion of lipids and their hydration would seriously affect conformational changes and function of proteins. Moreover, the effects of fluidity on receptor function and collisional coupling of receptors with second messengers through lateral diffusion (SHINITZKY, 1984) also will be suppressed by increases in microstructure rigidity.

In a previous study the diffusion coefficient for the lateral diffusion of the band 3 proteins in erythrocyte membranes was estimated from viscosity measurements obtained by time-resolved fluorometry (ARAI and KOYAMA, 1987). The estimated value for lateral diffusion rates was found to be consistent with its diffusion coefficient, which was measured with photobleaching technique by BLOOM and WEBB (1983). It seems probable that the observed increase in viscosity which we observed following lipid peroxidation could reduce the lateral diffusion rates of membrane proteins. Such an effect also may be a factor in reduction of the mitochondrial function.

The effects of lipid peroxidation and the antitumor activity of adriamycin are said to be independent (MYERS *et al.*, 1977). Administration of large amounts of antioxidants, i.e., α -tocopherol and/or coenzyme Q_{10} provides a partial protection against chronic cardiotoxicity of adriamycin (MYERS *et al.*, 1976; CORTES *et al.*, 1978; VAN VLEET and FERRANS, 1980). The basis for these clinical observations is probably due to suppression of membrane hydration and alterations in dynamic microstructure of mitochondrial membranes via the reduction in the level of lipid peroxidation.

In conclusion, exposure of cardiac mitochondria to oxidizing conditions, or to adriamycin, caused the formation of lipid peroxides within the membrane leading to increases in membrane viscosity, hydration, and to decreases in wobbling motion of phospholipids.

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