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# Fluorescent Substances and High Molecular Weight Protein Aggregates Formed in Rat Heart Mitochondria upon Doxorubicin-induced Lipid Peroxidation

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## Abstract

A rat heart mitochondrial suspension was incubated with doxorubicin, $FeCl_3$ and NADH.

Fluorescent substances and high molecular weight protein aggregates were observed in the mitochondrial membranes upon the formation of thiobarbituric acid-reactive substances.

Since both fluorescent substances and high molecular weight protein aggregates are retained in mitochondrial membranes, they can be of use in the clarification of the site of doxorubicin-induced lipid peroxidation.

Doxorubicin-induced cardiotoxicity is characterized by changes in the morphology and function of mitochondria (Bachmann et al 1975; Porta et al 1983). Doxorubicin undergoes one-electron reduction to a semiquinone free radical catalysed by NADH dehydrogenase in mitochondria (Davies & Doroshow 1986). The semiquinone radical is re-oxidized by reacting with molecular oxygen, resulting in formation of superoxide anion radical and other reactive oxygen species (Lown et al 1982; Doroshow & Davies 1986). These reactive free radicals participate in lipid peroxidation of mitochondrial membranes.

Doxorubicin interacts with negatively-charged phospholipids (cardiolipin, phosphatidylserine and phosphatidic acid) and has a particularly high affinity for cardiolipin (Goormaghtigh et al 1980). This phospholipid is exclusively localized in the inner membranes of mitochondria (Nicolay et al 1984). Demant (1983) reported that doxorubicin-induced lipid peroxidation required binding of doxorubicin to the inner mitochondrial membranes. Thus an indicator of lipid peroxidation remaining in the mitochondria could reveal the relationship between doxorubicin binding and lipid peroxidation in the mitochondria.

Lipid peroxidation in biological membranes produces thiobarbituric acid-reactive substances (TBA-RS), fluorescent substances and high molecular weight protein aggregates (Jain & Hochstein 1980; Koster & Slee 1980; Koster et al 1982). TBA-RS produced in the membranes are released into the medium, with little remaining in the membranes (Kunimoto et al 1981; Janero & Burghardt 1988). Whereas fluorescent substances and high molecular weight protein aggregates remain in the membranes after they are formed (Itoh et al 1988, 1990; Minamide et al 1990), although they are rarely used to evaluate doxorubicin-induced lipid peroxidation. Recently, we reported chemiluminescence from the rat heart mitochondria exposed to doxorubicin (Kitada et al 1994). In addition to the chemiluminescence which is highly sensitive to the oxidative stress, both fluorescent substances and high molecular weight protein aggregates would be of great use to indicate doxorubicin-induced lipid peroxidation in rat heart mitochondria. Thus, in the present paper, we attempted to reveal whether fluorescent substances and high molecular weight protein aggregates are formed in rat heart mitochondria upon doxorubicin-induced lipid peroxidation.

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## Materials and Methods

### *Materials*

NADH was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Diethyl maleate was from Wako Pure Chemical Industries Ltd (Osaka, Japan). An electrophoresis calibration kit (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactoalbumin) was from Pharmacia Fine Chemicals (Uppsala, Sweden). Doxorubicin was a gift from the Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan). All other reagents were of the highest grade available.

### *Preparation of rat heart mitochondria*

Heart mitochondria were isolated from male Wistar rats 160–250 g, according to Mela & Seitz (1979), without treatment with alkaline protease before homogenization of the heart. The isolated mitochondria were suspended in a medium containing 150 mM KCl and 50 mM Tris-HCl (pH 7·4) and stored in liquid nitrogen. The mitochondria were used within one week. The protein concentration of mitochondria was determined by the method of Lowry et al (1951), using bovine serum albumin as the standard.

### *Reaction of mitochondria with doxorubicin*

Heart mitochondrial suspension (5 mg protein $mL^{-1}$) was first treated with diethyl maleate, a glutathione-depleting agent, as described previously (Kitada et al 1994). The

treated heart mitochondrial suspension was preincubated at 37°C for 5 min with doxorubicin and 25 $\mu$M $FeCl_3$. The peroxidation reaction was initiated by adding 2·5 mM NADH to the reaction mixture and displacing the air in the cuvette with 95% $O_2$–5% $CO_2$ and was terminated by adding 1 mM EDTA to the reaction mixture at the designated times. KCl (150 mM)-Tris (50 mM) buffer (pH 7·4) gassed with 95% $O_2$–5% $CO_2$ for more than 15 min was used in this reaction. The reaction mixture was protected from light throughout. TBA-RS formed in the reaction mixture were assayed according to Buege & Aust (1978) and expressed as nmol malondialdehyde (mg protein)$^{-1}$.

*Assay for fluorescent substances*

A part of the peroxidation reaction mixture was centrifuged at 7 740 *g* for 7 min and the supernatant was removed. The precipitate (mitochondria) was resuspended in the KCl (150 mM)-Tris(50 mM)-HCl buffer, pH 7·4. The mitochondrial suspension thus obtained was free from NADH which disturbed the fluorescence measurement. The fluorescent substances were detected as reported elsewhere (Itoh et al 1988). Excitation and emission wavelengths were 365 nm and 440 nm, respectively. The samples in cuvettes for fluorescence measurement were irradiated with UV light, using a sterilizing lamp (Toshiba GL 10; wavelength, 253·7 nm; output, 1·8 W), just before the fluorescence measurement. Fluorescence measurement was carried out at 25°C, using a Hitachi fluorescence spectrophotometer 650–60. A cut-off filter (390 nm) was placed on the emission side for each measurement.

*Assay for high molecular weight protein aggregates*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described elsewhere (Itoh et al 1990). The samples for heart mitochondria were applied to 7·5% gels after being mixed for 2 h with 0·2 M phosphate buffer (pH 7·4) containing 5% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0·06% phenol red. The gel bands stained with 0·1% Coomassie brilliant blue were detected using a Shimadzu chromatoscanner CS-9000 by double wavelength detection (550 nm as a sample and 490 nm as a reference). Protein was estimated by integrating the scanning curve with the built-in program in the chromatoscanner.

## Results and Discussion

The concentration dependence of heart mitochondria and doxorubicin on TBA-RS formation was examined. The heart mitochondrial suspension (0·1–1·5 (mg protein) mL$^{-1}$) containing 2·5 mM NADH and 25 $\mu$M $FeCl_3$ was incubated at 37°C for 1 h with 25 $\mu$M doxorubicin. The produced TBA-RS were maximum at 0·5 (mg protein) mL$^{-1}$. Similarly, the TBA-RS formation in heart mitochondrial suspension, at 0·5 (mg protein) mL$^{-1}$, was examined in the presence of 10–100 $\mu$M doxorubicin. As the doxorubicin concentration increased, TBA-RS production increased and reached a plateau at about 50 $\mu$M doxorubicin. Based on these results, the heart mitochondrial suspension at 0·5 (mg protein) mL$^{-1}$ was incubated at 37°C with 50 $\mu$M doxorubicin. TBA-RS formation increased with incubation time and reached a plateau at 1–2 h after the start of the incubation (Fig. 1). Fluorescent substances formed in the same samples were determined. Their formation increased with incubation time and reached a plateau at 1–2 h (Fig. 2).

The heart mitochondrial suspension at 0·5 (mg protein) mL$^{-1}$ was incubated at 37°C for 1 h with 50 $\mu$M doxorubicin and the mitochondrial proteins were separated by SDS–PAGE. The SDS–PAGE chromatoscanning of the mitochondrial proteins is shown in Fig. 3. It is noteworthy that high molecular weight proteins at the top of the gel rods markedly increased in peroxidized mitochondria, accompanied by the decrease of proteins distributed in the middle and lower parts of the gel rods. The pattern of change in the protein molecular distribution in Fig. 3 was similar to that observed in the ascorbic acid/$Fe^{2+}$-induced lipid peroxida-

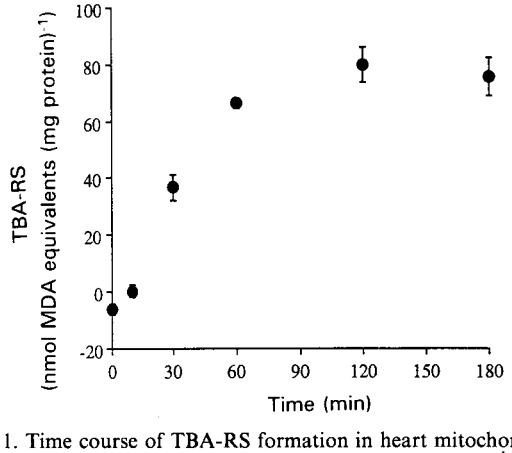


FIG. 1. Time course of TBA-RS formation in heart mitochondria. The heart mitochondrial suspension (0·5 (mg protein) mL$^{-1}$) containing 2·5 mM NADH and 25 $\mu$M $FeCl_3$ was incubated with 50 $\mu$M doxorubicin. The amount of TBA-RS at appropriate incubation time was corrected by subtraction of that of control mitochondria (without doxorubicin) at the same incubation time. The data points represent the means ± s.e. of three experiments.

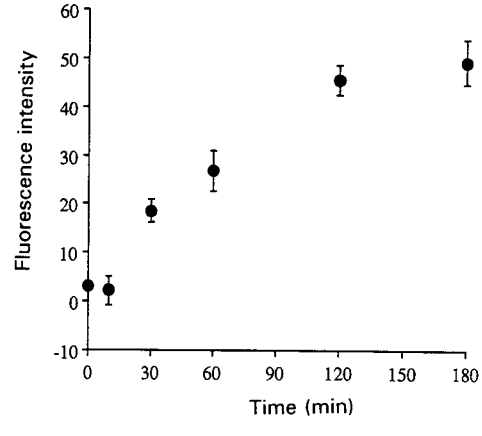


FIG. 2. Time course of fluorescent substances formation in heart mitochondria. Heart mitochondria were incubated with 50 $\mu$M doxorubicin, under the same experimental condition as in Fig. 1. Fluorescence intensity was determined for the chloroform/methanol extracts of peroxidizing mitochondria and plotted against the incubation time. Fluorescence intensity at appropriate incubation time was corrected by subtraction of that of control mitochondria (without doxorubicin) at the same incubation time. The data points represent the means ± s.e. of three experiments.

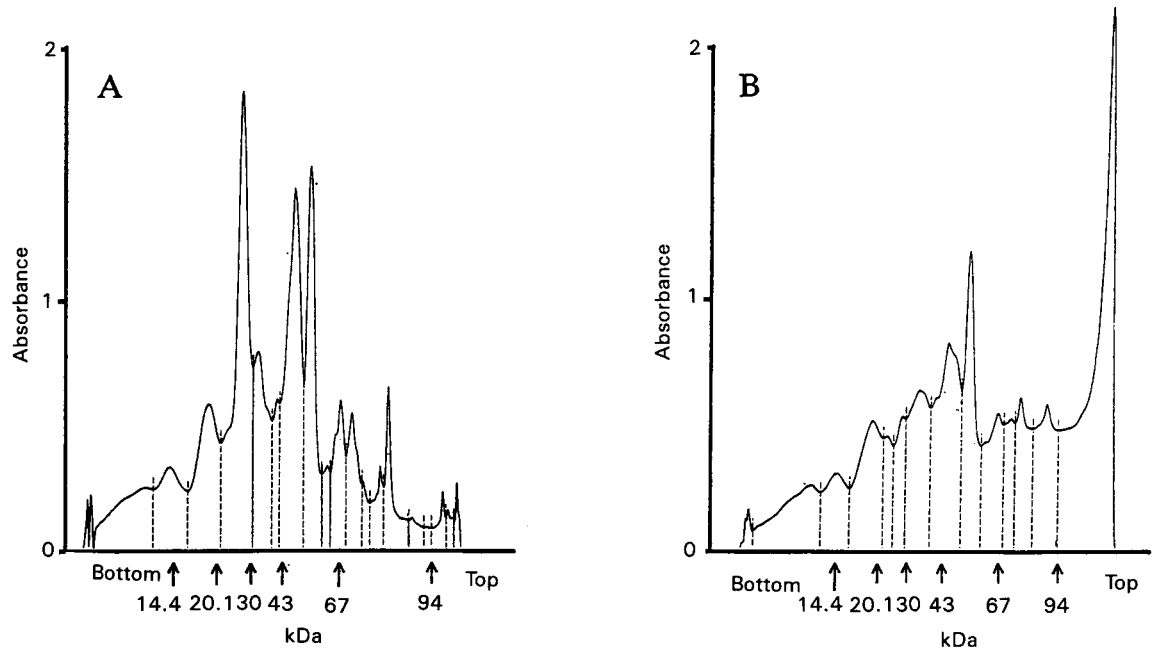


FIG. 3. SDS–PAGE of heart mitochondria. Heart mitochondria were incubated with 50 $\mu$M doxorubicin, under the same experimental condition as in Fig. 1. The mitochondrial proteins were separated by SDS–PAGE and the gels were stained with Coomassie brilliant blue. The stained gels were determined by a chromatoscanner. Heart mitochondria incubated for 0 h (A) and for 1 h (B). Standard proteins: phosphorylase b (molecular weight 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and $\alpha$-lactoalbumin (14·4 kDa).

tion in liver microsomes (Itoh et al 1988). This indicates that the high molecular weight protein aggregates formed in heart mitochondria originated from doxorubicin-induced lipid peroxidation.

The time course of formation of high molecular weight protein aggregates in heart mitochondria with 50 $\mu$M doxorubicin was examined by SDS-PAGE and expressed as the ratio of amount of the protein at the top of the gel rod to that of the total proteins. The protein aggregates increased with incubation time and continued increasing up to 2 h (Fig. 4).

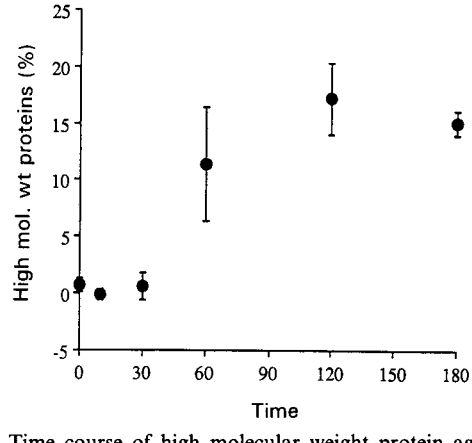


FIG. 4. Time course of high molecular weight protein aggregate formation in peroxidizing heart mitochondria. Heart mitochondria were incubated with 50 $\mu$M doxorubicin, under the same experimental condition as in Fig. 1. High molecular weight proteins which appeared at the top of the gels were expressed as the percentage (%) of total protein, which was corrected by subtraction of the value of control mitochondria (without doxorubicin) at appropriate incubation times. The data points represent the means ± s.e. of three experiments.

Fluorescent substances and high molecular weight protein aggregates are retained in membranes after they are produced (Itoh et al 1988, 1990; Fukuda et al 1992). Fluorescent substances were characterized by analysis of their fluorescence lifetimes (Minamide et al 1990, 1992). For example, the rotational motion of the fluorophores in peroxidized membranes was depressed upon lipid peroxidation. The fluidity of microsomal membranes was shown to decrease upon lipid peroxidation (Eichenberger et al 1982; Gut et al 1985; Itoh et al 1989; Minamide et al 1992). Gut et al (1985) reported that the rotational mobility of cytochrome P450 in microsomal membranes decreased upon lipid peroxidation. We indicated that a part of cytochrome P450 was involved in the high molecular weight protein aggregates formed in peroxidizing microsomes (Itoh et al 1990). Thus, the finding that fluorescent substances and high molecular weight protein aggregates were formed in heart mitochondria exposed to doxorubicin suggests that the function of mitochondria is affected seriously.

In conclusion, fluorescent substances and high molecular weight protein aggregates were found to be formed in rat heart mitochondria upon doxorubicin-induced lipid peroxidation. Further analysis of these products could reveal the influence of the doxorubicin-induced lipid peroxidation on the function of mitochondria.

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