Role of Phospholipase in the Genesis of Doxorubicin-induced Cardiomyopathy in Rats

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ABSTRACT

The role of phospholipase on the mechanism of doxorubicin-induced cardiomyopathy was investigated in the heart mitochondria of Wistar rats.

In the in vitro study, rats were divided into 3 groups: 1, the control group, untreated; 2, the doxorubicin 1-day group, in which doxorubicin (4 mg/kg) was injected s.c. once; and 3, the doxorubicin 4-day group, in which doxorubicin (4 mg/kg) was injected once a day for 4 consecutive days. In each group, the level of lipid peroxides and the phospholipase activity, the phospholipid content, and the enzymatic activities in the respiratory chain were measured. The doxorubicin 4-day group showed significant increases of lipid peroxide level and phospholipase activity and an inhibition of mitochondrial respiratory function compared with the control group, while the doxorubicin 1-day group showed no significant difference.

In the in vitro study, Experiment 1, intact rat heart mitochondria were incubated with 0.1 unit of phospholipase A2. After the incubation, the enzymatic activities of the respiratory chain were disturbed in the same manner as in the in vitro experiment. In Experiment 2, rat heart mitochondria were incubated with ascorbate and ferrous sulfate. The experiment demonstrated the elevation of phospholipase activity associated with lipid peroxidation.

These results suggested that the enhanced phospholipase activity caused by lipid peroxidation is responsible for the mechanism of doxorubicin-induced cardiomyopathy.

INTRODUCTION

In 1967, Di Marco et al. (1) developed a new antitumor drug, doxorubicin, which has been widely used because of its effectiveness on many kinds of human neoplasm. Unfortunately, the dose-dependent and lethal cardiotoxic effect induced by doxorubicin has limited its clinical use. Minow et al. (2) demonstrated that cardiomyopathy was seen in up to one-third of the patients receiving more than 600 mg/m2 of the drug. Many investigators have proposed that the lipid peroxidation of cardiac membrane played an important role in generating the cardiac dysfunction caused by doxorubicin (3–5). Doroshow (6) has established that the proximal portion of the NADH dehydrogenase complex [NADH: (acceptor) oxidoreductase, EC 1.6.99.3] was the site of anthracycline reduction in mitochondria and that the superoxide formation was in a dose-dependent manner. As biomembrane consists of phospholipids and protein, it is deduced that the peroxidation of the fatty acids of the membrane phospholipids deteriorates the cellular dysfunction. Indeed, Dobretsov et al. (7) reported that the lipid peroxidation increased the phospholipid bilayer rigidity and modified the physical state of the membrane resulting in the loss of its function, whereas Iwata et al. (8) reported that when using rats breathing pure oxygen, activation of phospholipase induced by lipid peroxidation is responsible for the genesis of oxygen-induced lung injury. Thus, it is suggested that not only lipid peroxidation but also the activation of phospholipase is involved in the development of cellular dysfunction. In this study, we investigate the mechanism of doxorubicin-induced cardiomyopathy, especially in relation to phospholipase activity.

MATERIALS AND METHODS

In Vivo Study

Experiments were carried out on male Wistar rats weighing 200 ± 30 (SD) g.

Measurement of Concentrations of Doxorubicin in Heart and Plasma. We determined the doxorubicin concentration in the heart and plasma by use of high performance liquid chromatography with fluorescence detector (9, 10). To measure the heart doxorubicin concentration, 5 rats were given injections of doxorubicin (4 mg/kg); doxorubicin-HCl; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) once s.c. and 5 other rats were given injections of doxorubicin (4 mg/kg) s.c. once a day for 4 consecutive days. Twelve h after the last injection of doxorubicin, each rat was killed and the heart was removed. The heart was homogenized and extracted with a mixture of butyl alcohol and toluene. The extract was evaporated in a vacuum, and the residue was dissolved with a mixture of phosphate buffer and methanol. An aliquot of this solution was injected onto a reverse-phase ODS column and eluted with a mixture of formic acid and methanol. The fluorescence signal was monitored at excitation wavelength 470 nm and emission wavelength 585 nm.

To measure the plasma doxorubicin concentration, doxorubicin (4 mg/kg) was injected s.c. Blood samples were withdrawn into heparinized syringes at 0.5, 1, 3, 10, and 24 h after doxorubicin administration. The plasma samples adjusted to about pH 3 by 1 M H3PO4 were injected directly onto the column and eluted stepwise with phosphate buffer and a mixture of the same buffer and acetonitrile. The chromatograms were monitored at excitation wavelength 470 nm and emission wavelength 585 nm.

Preparation of Rat Heart Mitochondria. Rats were divided into three groups: the control group, untreated; the doxorubicin 1-day group, doxorubicin (4 mg/kg) was s.c. injected once; and the doxorubicin 4-day group, doxorubicin (4 mg/kg) was injected once a day for 4 consecutive days. Rats were killed 12 h after the last doxorubicin injection, and the hearts were removed. The cardiac mitochondria fraction was prepared by the method of Hatefi et al. (11) and finally suspended in 0.25 μmol sucrose/10 mM Tris-HCl (pH 7.8) buffer (5–6 mg protein/ml). Due to the large amount of protein required for measurement of lipid peroxide level, phospholipase activity, electron-transport activity, and phospholipid content, segments from three hearts were combined for preparation of the mitochondria fraction; that is, each group consisted of 24 rats, and 8 samples were obtained from each group.

Measurement of Mitochondrial Lipid Peroxides. Mitochondrial lipid peroxides were determined by the method of Ohkawa et al. (12). The outline of the procedure used is as follows: 0.2 ml mitochondria suspension was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution of pH 3.5, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butyl alcohol and pyridine (15/1, v/v) were added, then the mixture was shaken vigorously. After centrifugation at 3000 rpm for 15 min, the absorbance of the organic layer (upper layer) was measured at 532 nm. As an external standard, tetraethoxypropane was used, and lipid per-
Phospholipase Activity. Phospholipase activity of heart mitochondria was measured by the amount of fatty acids released from the substrate in the reaction mixture. One ml of mitochondria suspension was incubated at 37°C with 40 nmol of 1,2-dipalmitoyl-snglycero-3-phosphocholine (Funakoshi Pharmaceutical, Tokyo, Japan) as the substrate. After incubation for 30, 60, and 90 min, linoleic acid was extracted by the method of Bligh and Dyer (13). The extract was then separated by thin-layer chromatography. The specific activity of phospholipase A2 was determined by the method of Hatefi (15). The reaction mixture consisted of 0.06 ml K2HPO4-HCl (100 mM, pH 7.0), 0.1 ml of NaN3 (0.1 M), 0.06 ml of EDTA (1 mM), 5 ml of 1% deoxycholic acid (pH 8.0), 0.18 ml of 1% ferricytochrome c (Sigma Chemical Co., St. Louis, MO), and 2.6 ml of distilled water. The reaction was initiated by adding 10 µl of mitochondria suspension and 75 µl of 10 mM NADH. After 15 s incubation at 30°C, the reaction rate was followed for 1 min by recording the increase in absorbance of cytochrome c at 550 nm. The activity of NADH-cytochrome c reductase, the succinate-cytochrome c reductase, and the cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) were measured.

In Vivo Study
Cardiac mitochondrial suspensions (5-6 mg protein/ml) were prepared from intact rats and used in the following experiments.

Experiment 1, Effect of Phospholipase A2 on Mitochondrial Function. Using cardiac mitochondrial suspension, changes in the electron-transport activity induced by 0.1 unit of phospholipase A2 (Naja naja venom; Sigma) were determined after 10 min of incubation with phospholipase A2 at 30°C. The activities of the electron-transport chain divided into three segments (the NADH-cytochrome c reductase, the succinate-cytochrome c reductase, the cytochrome c oxidase) were determined by the same methods used in the in vivo study.

Experiment 2, Effect of Lipid Peroxidation on Phospholipase Activity. Rat heart mitochondria were incubated at 37°C for 60 min. Incubations were performed in 0.15 M KCl/20 mM Tris-HCl (pH 7.4) buffer containing 0.1 mM ascorbate and 0.04 mM FeSO4. Control samples contained neither ascorbate nor FeSO4. After the incubation, the phospholipase activity and the lipid peroxide content were measured by the same methods used in the in vivo study.

Proteins were determined by the biuret reaction (19) using bovine serum albumin as a standard. All results in this paper are presented as a mean ± SD. Analysis of variance with Dunnett's test was used for statistical analyses of the data and was considered significant when P was less than 0.05.

RESULTS

In Vivo Study
Concentrations of Doxorubicin in Heart. The concentrations of doxorubicin in each group are shown in Table 1. Doxorubicin concentration in the doxorubicin 4-day group was markedly higher compared with the doxorubicin 1-day group.

Concentrations of Doxorubicin in Plasma. Plasma concentration of doxorubicin is shown in Table 2. Doxorubicin concentration reached its peak at 30 min after the injection and decreased time dependently. Negligible concentration was observed 24 h after the injection.

Levels of Lipid Peroxides. The level of lipid peroxides in heart mitochondria in each group is shown in Fig. 1. In the control group, it was 3.86 ± 0.75 nmol malondialdehyde/mg protein. In the doxorubicin 4-day group, there was a significant increase in the level of lipid peroxides (6.78 ± 1.10) compared with doxorubicin 1-day group.

Table 1 Concentrations of doxorubicin in the heart

<table>
<thead>
<tr>
<th>Time after injection (h)</th>
<th>Concentration (µg of compound/g tissue, wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.179 ± 0.032a</td>
</tr>
<tr>
<td>1</td>
<td>0.077 ± 0.035</td>
</tr>
<tr>
<td>3</td>
<td>0.039 ± 0.006</td>
</tr>
<tr>
<td>10</td>
<td>0.006 ± 0.011</td>
</tr>
<tr>
<td>24</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Table 2 Concentrations of doxorubicin in the plasma

<table>
<thead>
<tr>
<th>Time after injection (h)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.155 ± 0.27a</td>
</tr>
<tr>
<td>1</td>
<td>6.28 ± 1.30a</td>
</tr>
</tbody>
</table>

* Statistically significant, at P < 0.01, compared with doxorubicin 1-day group.

** Mean ± SD.
with the control group \( (P < 0.01) \), although the level of lipid peroxides in the doxorubicin 1-day group was not significantly increased compared with that of the control group.

**Phospholipase Activity.** Fig. 2 illustrates the phospholipase activity in heart mitochondria, which was measured by the amount of linolenic acid released from dilinolenoyl phosphatidylcholine. There was a linear relationship between the release of linolenic acid and the incubation time in each group. The releasing rate of linolenic acid as phospholipase activity decreased from 60-min values was 311 ± 40 (ng/mg protein/h) in the control group. In the doxorubicin 4-day group, marked elevation in phospholipase activity was observed with values of 521 ± 160 \( (P < 0.01) \), although there were no significant changes in the doxorubicin 1-day group.

**Mitochondrial Function.** The activities of the electron-transport chain were determined as described in "Materials and Methods." Number of experiments in each group was eight.

**Table 3** Electron-transport activities in each group

<table>
<thead>
<tr>
<th></th>
<th>NADH-cytochrome c reductase (pH 8.0, 30°C)</th>
<th>Succinate-cytochrome c reductase (pH 7.4, 30°C)</th>
<th>Cytochrome c oxidase (pH 7.0, 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>426 ± 56( ^* )</td>
<td>377 ± 56</td>
<td>2494 ± 216</td>
</tr>
<tr>
<td>Doxorubicin, 1 day</td>
<td>507 ± 121</td>
<td>406 ± 49</td>
<td>2102 ± 434</td>
</tr>
<tr>
<td>Doxorubicin, 4 days</td>
<td>205 ± 29( ^* )</td>
<td>358 ± 51</td>
<td>1624 ± 125( ^* )</td>
</tr>
</tbody>
</table>

\( ^* \) Mean ± SD.

**Table 4** Electron-transport activities with phospholipase A\( _2 \) pretreatment

<table>
<thead>
<tr>
<th></th>
<th>NADH-cytochrome c reductase (pH 8.0, 30°C)</th>
<th>Succinate-cytochrome c reductase (pH 7.4, 30°C)</th>
<th>Cytochrome c oxidase (pH 7.0, 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>413 ± 62( ^* )</td>
<td>385 ± 50</td>
<td>2404 ± 203</td>
</tr>
<tr>
<td>Phospholipase A( _2 ) pretreatment</td>
<td>273 ± 70( ^* )</td>
<td>358 ± 56</td>
<td>1439 ± 383( ^* )</td>
</tr>
</tbody>
</table>

\( ^* \) Mean ± SD.

was a significant decrease in the doxorubicin 4-day group when compared with the control group.

**In Vitro Study**

Experiment 1. The activities of three segments in the mitochondrial electron-transport chain with phospholipase A\( _2 \) pretreatment are shown in Table 4. The activities of NADH-cytochrome c reductase and cytochrome c oxidase were decreased significantly compared with the control group without phospholipase A\( _2 \) pretreatment. The activity of succinate-cytochrome c reductase showed a tendency to decrease, but the decrease was not significant.

Experiment 2. The level of lipid peroxides and the activity of phospholipase in mitochondria incubated with ascorbate and FeSO\( _4 \) for 60 min at 37°C are shown in Fig. 4. After incubation, the phospholipase activity and the level of lipid peroxides were elevated significantly compared with the control.

**DISCUSSION**

Cardiotoxicity caused by doxorubicin has been studied extensively by the morphological approach. Mettler *et al.* (20) re-
ported that histological changes in doxorubicin-treated rats consisted of myocyte vacuolation and degeneration, interstitial edema, and mild fibroplasia, and that these changes were commonly observed in other species. These findings almost agree with those of other reports. On the contrary, the biochemical approach has not yet fully clarified the mechanism of doxorubicin-induced cardiomyopathy. In the present study, rat heart mitochondria were used for analyzing the mechanism of cardiotoxicity. Mitochondria play a fundamental role as an energy-generating site in cells through oxidative phosphorylation. Taking this important function of mitochondria into consideration, it is probable that their dysfunction would directly lead to cardiac dysfunction. In our study, the doxorubicin 4-day group showed a significant inhibition of the activity of NADH-cytochrome c reductase and cytochrome c oxidase. Mailer and Petering (21) reported an inhibition of oxidative phosphorylation in bovine heart mitochondria by doxorubicin. Ogura et al. (22) also demonstrated that oxidative phosphorylation in rat heart mitochondria was inhibited and the lipid peroxide level in mitochondria was increased. Since mitochondrial phospholipids are essential components in maintaining electron-transport activity (23, 24), peroxidation of mitochondrial phospholipids has been believed to have a primary importance in the genesis of doxorubicin-induced cardiotoxicity. In the present study, the doxorubicin 4-day group showed a high tissue doxorubicin concentration and a significant elevation of lipid peroxide level, although the doxorubicin 1-day group showed a low tissue doxorubicin concentration and no significant elevation of lipid peroxide level. Accordingly, lipid peroxidation might be linked with the genesis of doxorubicin-induced mitochondrial dysfunction. On the contrary, many investigators emphasized recently that the enhanced activation of phospholipase induced various pathological conditions associated with the breakdown of membrane phospholipids (25–28). In our in vitro experiment 1, we obtained the result that the disturbance of the respiratory chain induced by phospholipase occurred mainly in NADH-cytochrome c reductase, and cytochrome c oxidase as we observed in the in vivo study, whereas Yasuda and Fujita (29) pointed out that the phospholipase that might bind to the membrane structures are released by alterations in the phospholipid structure of the membranes after lipid peroxidation. In the present study, the doxorubicin 1-day group without increasing lipid peroxide content showed no significant changes of phospholipase activity, while in the doxorubicin 4-day group, phospholipase activity elevated remarkably associated with the increase of lipid peroxide content. In our in vitro experiment 2, we also confirmed that the release of linolenic acid from 1-α-phosphatidylcholine dilinolenoyl increased associated with the increase of lipid peroxide content. Therefore, it can be surmised that the alterations of membrane phospholipid structures caused by lipid peroxidation decrease the resistance from the phospholipase attack, resulting in degrading the mitochondrial membrane. Indeed, the doxorubicin 4-day group, the phospholipid content was decreased. Olson et al. (30) reported that accelerated calcium influx plays an important role in the genesis of doxorubicin-induced cardiotoxicity. It is interesting that calcium is the essential factor in the activation of phospholipase. These results indicate that doxorubicin-induced mitochondrial dysfunction is based, at least in part, on the degradation of mitochondrial phospholipids by phospholipase, which in turn leads to cardiac dysfunction.

REFERENCES

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