A novel pharmacological action of ET-1 to prevent the cytotoxicity of doxorubicin in cardiomyocytes

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Suzuki, Takaaki, and Takashi Miyachi. A novel pharmacological action of ET-1 to prevent the cytotoxicity of doxorubicin in cardiomyocytes. Am J Physiol Regulatory Integrative Comp Physiol 280: R1399–R1406, 2001.—We previously reported that cardiomyocytes produce endothelin (ET)-1 and that the tissue level of ET-1 markedly increased in failing hearts in rats with chronic heart failure. Because the level of plasma ET-1 also increased progressively in patients with breast cancer who received doxorubicin (Dox; Adriamycin), which possesses cardiotoxicity, we hypothesized that ET-1 plays a role in the pathophysiology of cardiomyocytes injured by Dox. In this study, we investigated the effect of ET-1 on the cytotoxicity of Dox in primary cultured neonatal rat cardiomyocytes. The results showed that ET-1 effectively attenuated Dox-induced acute cardiomyocyte cytotoxicity (24-h incubation with Dox) evaluated by in vitro cell toxicity assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and lactate dehydrogenase release). The cytoprotective effect of ET-1 was mediated via ET_A receptors, because pretreatment with the ET_A-receptor antagonist BQ123 completely suppressed the cytoprotective effect of ET-1, whereas the ET_B-receptor antagonist BQ788 did not. The cytoprotective effect of ET-1 was abolished by pretreatment with cycloheximide or staurosporine. These results suggest that a protein molecule(s), which is synthesized de novo by the stimulation of protein kinase pathway, is involved in the cytoprotective effect of ET-1. ET-1 increased the expression of an endogenous antioxidant, manganese superoxide dismutase (Mn-SOD), in the cardiomyocytes, as demonstrated by a Western blotting analysis. Pretreatment with an antisense oligodeoxyribonucleotide of Mn-SOD markedly attenuated the cytoprotective effect of ET-1 on the Dox-induced cytotoxicity. However, under conditions of prolonged incubation with Dox (48 h), ET-1 did not affect Dox-induced cardiomyocyte cytotoxicity in culture. These results suggest that ET-1 prevents the early phase of Dox-induced cytotoxicity via the upregulation of the antioxidant Mn-SOD through ET_A receptors in cultured cardiomyocytes.

cultured cardiomyocytes; antioxidant; endothelin type A-receptor antagonist; protein kinase C; antisense oligodeoxyribonucleotide

ENDOTHELIN (ET)-1 is a potent vasoconstrictor peptide first identified from the conditioned medium of vascular endothelial cells (61). We previously demonstrated in primary cultures that ET-1 is synthesized and secreted by cardiac myocytes (47). ET-1 acts not only on vascular smooth muscles, but also on myocardium. ET-1 has been shown to have a positive inotropic effect on the myocardium (12, 15, 29, 40, 49) through activation of phospholipase C and hydrolysis of phosphatidylinositol (18, 51). Furthermore, we and other groups have reported that ET-1 induces hypertrophy of cardiomyocytes (28, 41, 46). Receptors for ET peptides have been subclassed as ET_A and ET_B receptors (26, 37), and the above cardiac effects of ET-1 are mediated primarily by ET_A receptors (25, 26).

We previously reported that tissue levels of both ET-1 peptide and mRNA markedly increased in failing hearts in rats with chronic heart failure (CHF) (38, 39) and in hamsters with cardiomyopathy (59). Moreover, we and other groups have reported that plasma ET-1 levels are increased in patients with CHF (8, 10, 14, 22, 36). These findings suggest a possibility that myocardial ET-1 is involved in the pathophysiology of heart diseases. Because ET-1 increases cardiac muscle contractility (12, 15, 29, 40, 49), an increase in ET-1 expression in the failing heart may possess some adaptive aspect of supporting contractility in the failing heart (39). However, persistent and progressive increase in cardiac ET-1 expression in the failing heart (27) possesses a maladaptive aspect. Indeed, it has been reported that cardiac ET-1 is involved in the progression of heart failure (25, 26, 38). There are several reports that show the effectiveness of the blockade of ET receptors in improving survival (16, 30, 38, 59) and hemodynamic features (5, 30, 38, 52, 53, 59) in heart failure models. We also reported that repeated treatment with an ET_A-receptor antagonist improved the survival and hemodynamics of rats with CHF when started 10 days after the onset of myocardial infarction (38). On the other hand, Nguyen et al. (31) reported that the effect of an ET_A-receptor antagonist initiated immediately after the onset of myocardial infarction was detrimental in cardiac dysfunction in rats. Thus the increase in myocardial ET-1 in heart diseases seems to have an adaptive (beneficial)
role and a maladaptive (harmful) role in various stages of heart diseases.

Doxorubicin (Dox; Adriamycin), an anthracycline anticancer drug, is widely used for the treatment of various human malignancies, including several leukemias, lymphomas, and solid tumors (64). However, the clinical use of Dox is limited because of its serious cumulative dose-dependent cardiotoxicity, which leads to irreversible degenerative cardiomyopathy (42). There are at least two major processes causing cardiotoxicity of Dox. One is free-radical formation in cardiomyocytes (1, 17, 32), and the other is direct effect on the DNA and other cellular components in cardiomyocytes (9, 19–21, 23, 43, 44). It has been reported that the level of plasma ET-1 increased progressively in patients with breast cancer who had received Dox (57, 58). However, the pathophysiological effect of increasing the plasma level of ET-1 in patients who receive Dox treatment has not been elucidated.

Because it has been reported that plasma ET-1 becomes a marker for Dox-induced cardiotoxicity in patients with breast cancer in whom congestive heart failure developed (58), we hypothesized that ET-1 plays a role in the pathophysiology of cardiomyocytes injured by Dox. To test this hypothesis, in the present study, we investigated the effect of ET-1 on the cytotoxicity of Dox in primary cultured cardiomyocytes. We herein report a new pharmacological action of ET-1 on cardiomyocytes: ET-1 rescues the cultured cardiomyocyte from the Dox-induced early phase of cardiotoxicity via upregulation of manganese superoxide dismutase (Mn-SOD), an endogenous antioxidative molecule, through ETA receptors, suggesting that myocardial ET-1 has an adaptive (beneficial) aspect in injured cardiomyocytes in the early phase of Dox-induced cytotoxicity in culture.

MATERIALS AND METHODS

Isolation and primary culture of cardiac myocytes. Ventricular cardiomyocytes were isolated from 2- to 3-day-old Sprague-Dawley rats as described previously (48). For the purification of myocytes, the isolated heart cells were suspended in a culture medium [DMEM-Ham’s F-12 medium supplemented with 5% FBS and preincubated in a culture medium (19, 21, 23, 43, 44)]. It has been reported that the level of plasma ET-1 increased progressively in patients with breast cancer who had received Dox (57, 58). However, the pathophysiological effect of increasing the plasma level of ET-1 in patients who receive Dox treatment has not been elucidated.

Because it has been reported that plasma ET-1 becomes a marker for Dox-induced cardiotoxicity in patients with breast cancer in whom congestive heart failure developed (58), we hypothesized that ET-1 plays a role in the pathophysiology of cardiomyocytes injured by Dox. To test this hypothesis, in the present study, we investigated the effect of ET-1 on the cytotoxicity of Dox in primary cultured cardiomyocytes. We herein report a new pharmacological action of ET-1 on cardiomyocytes: ET-1 rescues the cultured cardiomyocytes from the Dox-induced early phase of cardiotoxicity via upregulation of manganese superoxide dismutase (Mn-SOD), an endogenous antioxidative molecule, through ETA receptors, suggesting that myocardial ET-1 has an adaptive (beneficial) aspect in injured cardiomyocytes in the early phase of Dox-induced cytotoxicity in culture.
Fig. 1. Effect of endothelin (ET)-1 on the doxorubicin (Dox)-induced cytotoxicity evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (see MATERIALS AND METHODS). ET-1 was added 24 h before Dox, and MTT assay was performed 24 h and 48 h after the addition of Dox. The average of optical densities at 584 nm in control wells is expressed as 100%. Each column and bar represents the mean ± SD of relative activity in 6 samples.

Materials. ET-1 was purchased from Peptide Institute (Osaka, Japan). Dox was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). BQ123 and BQ788 were a generous gift from Banyu Pharmaceutical (Tokyo, Japan). Cycloheximide and staurosporine were obtained from Sigma Chemical (St. Louis, Mo).

Statistical analysis. All data except those of Western blotting are expressed as means ± SD. Statistical analysis was carried out by ANOVA followed by Scheffe’s test for multiple comparisons with a commercially available statistical package (StatView, version 5.0; Abacus Concepts, Berkley, CA). The results were considered statistically significant at P < 0.05.

RESULTS

Cardiomyocyte cell toxicity of Dox and the effects of ET-1 on Dox-induced cytotoxicity. Dox showed cell toxicity in primary cultured cardiomyocytes. As shown in Fig. 1A, 24-h incubation with Dox ranging from 10 to 20 μM inhibited MTT activity in a dose-dependent manner. Twenty-four-hour treatment before Dox with ET-1 ranging from 0.1 to 10 nM reversed the MTT activity inhibited by 20 μM of Dox dose dependently (Fig. 1A). Furthermore, we also observed that the duration of survival term of the cardiomyocytes was greatly improved by the ET-1 treatment under the 24-h Dox-treated cultures (data not shown). However, no dose of ET-1 inhibited the decrease of MTT activity induced by a further 24 h incubation with Dox (total, 48 h; Fig. 1B). Conversely, LDH released from the cardiomyocytes increased after treatment with Dox. LDH release from the cardiomyocytes markedly increased after 12 h of incubation with 20 μM Dox. At the same time point, pretreatment with ET-1 inhibited LDH release from the cells by Dox. After 24 h of incubation with Dox, the LDH release increased about sixfold compared with the control and increased about threefold even with existing ET-1 (Table 1). Figure 2 shows morphological changes in cardiomyocytes induced by 20 μM of Dox and the effect of 10 nM of ET-1 on Dox-induced cell toxicity. Pretreatment with ET-1 attenuated the cytotoxic effect of Dox when the cells were incubated with Dox for 24 h. However, many vacuoles were formed in the myocytes by Dox treatment despite the existing ET-1 (Fig. 2, bottom left). A further 24 h of incubation (total, 48 h) of the cells with Dox eventually caused cell death, even with existing ET-1 (Fig. 2, bottom right).

Determination of the ET-receptor subtype involved in the cytoprotective effect of ET-1. To determine which ET-receptor subtype is involved in the cytoprotective effect of ET-1, we examined the effects of ET-receptor antagonists on the cytoprotection of ET-1 against Dox-induced cell toxicity. An ETA-receptor antagonist, BQ123, attenuated the cytoprotective effect of ET-1, and 10 μM of BQ123 completely suppressed the effect of ET-1. On the other hand, an ET type B (ETB)-receptor antagonist, BQ788, did not show any significant effect on the cytoprotective effect of ET-1 on the Dox-induced cell toxicity estimated by MTT assay (Fig. 3).

Protein synthesis and protein kinase inhibition. Cytoprotection of ET-1 was effective when the ET-1 was added 24 h before the Dox treatment of the cardiac myocytes in the former studies. Pretreatment with 1 μM cycloheximide, a protein synthesis inhibitor, markedly suppressed the cytoprotective effect of ET-1 on Dox-induced cytotoxicity estimated by MTT assay (Fig. 4). On the other hand, a protein kinase inhibitor, staurosporine, also inhibited the cytoprotective effect of ET-1 on Dox-induced cardiototoxicity estimated by MTT assay (Fig. 4).

Induction of Mn-SOD by ET-1. To clarify the mechanism of the cytoprotective effect of ET-1 on Dox-

Table 1. Relative concentrations of LDH in the culture medium of cardiomyocytes

<table>
<thead>
<tr>
<th>Hours Incubation With Dox</th>
<th>6</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00 ± 19.62</td>
<td>112.94 ± 11.81</td>
<td>116.60 ± 30.77</td>
</tr>
<tr>
<td>Dox (20 μM)</td>
<td>147.31 ± 17.52</td>
<td>301.52 ± 42.24*</td>
<td>677.64 ± 97.97*</td>
</tr>
<tr>
<td>ET-1 (1 nM) + Dox (20 μM)</td>
<td>112.84 ± 17.51</td>
<td>140.20 ± 5.82</td>
<td>405.15 ± 22.79*</td>
</tr>
<tr>
<td>ET-1 (10 nM) + Dox (20 μM)</td>
<td>116.78 ± 21.02</td>
<td>126.81 ± 14.35</td>
<td>390.95 ± 46.53*</td>
</tr>
</tbody>
</table>

Values are means ± SD of 4 samples. Average of the concentration of lactate dehydrogenase (LDH) in culture medium of control wells incubated for 6 h is indicated as 100%. Endothelin (ET)-1 was added 24 h before doxorubicin (Dox). *Significant compared with the control at each incubation time (P < 0.01).
induced cardiomyocyte cell death, we investigated whether upregulation of Mn-SOD, a mitochondrial antioxidative molecule, occurred after treatment with ET-1. As shown in Fig. 5A, protein levels of Mn-SOD increased with incubation time by 10 nM of ET-1 estimated by Western blotting analysis. Similar results were obtained in three other independent experiments.

The induction of Mn-SOD by ET-1 was attenuated by the pretreatment with the ET A-receptor antagonist BQ123 but not by the ET B-receptor antagonist BQ788 (Fig. 5B).

Suppression of Mn-SOD by antisense ODN. To suppress the induction of Mn-SOD by ET-1 selectively, we used an AS-ODN, which corresponds to the initiation site of Mn-SOD translation. Pretreatment with the AS-ODN (1.15 μM) attenuated ~80% of the cytoprotective effect of ET-1. However, complete inhibition was not obtained even when the dose of AS-ODN was increased. In contrast, S-ODN showed no effect on the cytoprotective effect of ET-1 (Fig. 6).

DISCUSSION

In the present study, we demonstrated that ET-1 prevented Dox-induced acute cytotoxicity (after 24 h of incubation with Dox) in a dose-dependent manner, as evaluated by MTT assay and LDH release from the
Cells in primary cultured rat cardiomyocytes. The cytoprotective effect of ET-1 was completely suppressed by pretreatment with the ETA-receptor antagonist BQ123 but not with the ETB-receptor antagonist BQ788, indicating that the ETA-receptor system is involved in the cytoprotective effect of ET-1 on the cytotoxicity of Dox. The cytoprotective effect of ET-1 was abolished by pretreatment with cycloheximide and staurosporine. These results suggest that some protein molecule(s), which is synthesized de novo by the stimulation of the protein kinase pathway, is involved in the cytoprotective effect of ET-1.

It has been reported that the formation of free radicals is an important process that contributes to cardiotoxic effects of Dox (1, 17, 32). Moreover, Dox-induced acute cardiotoxicity is attenuated in Mn-SOD transgenic mice (62). Therefore, we hypothesized that ET-1 affects the expression of an endogenous antioxidant molecule(s) in cardiomyocytes. Then, we investigated whether ET-1 regulates the expression of Mn-SOD in the cultured cardiomyocytes. The results showed that ET-1 increased the expression of Mn-SOD in the cardiomyocytes, as demonstrated by Western blotting analysis. Moreover, the increased expression of Mn-SOD by ET-1 was suppressed by BQ123, an ETA-receptor antagonist. Then, to investigate whether this upregulation of Mn-SOD is actually involved in the cytoprotective effect of ET-1, we investigated whether the transfection of the AS-ODN, designed to work against rat Mn-SOD mRNA to cultured cardiomyocytes, affects the cytoprotective effect of ET-1 on Dox-induced cardiotoxicity. Pretreatment with the AS-ODN markedly attenuated the cytoprotective effect of ET-1, whereas the S-ODN did not affect this effect of ET-1.
These results suggest that ET-1 has a cytoprotective effect mainly via the upregulation of Mn-SOD levels in the cardiomyocytes. However, because complete inhibition was not obtained by the pretreatment with AS-ODN, these results also imply that the cardioprotective effect of ET-1 is mediated through some other mechanism(s) in addition to that mediated by Mn-SOD.

Recently, it has been reported that ET-1 stimulates c-fos gene expression via the Ras pathway (7) and also activates the phosphorylation cascade of Raf-1 and the extracellular signal-regulated kinase of mitogen-activated protein kinase (MAPK) (60) in neonatal rat cardiomyocytes. Moreover, ET-1 also activates phosphorylation of MAPK/Jun amino-terminal kinase in cardiomyocytes (3, 4). It is known that c-fos and c-jun make the heterodimer complex activating protein-1 (AP-1), which preferentially binds to many genes that have a 12-O-tetradecanoyl-phorbol-13-acetate-responsive element in their promoter region (2). Rat Mn-SOD gene contains an AP-1 binding site in its regulatory sequence (11). Therefore, ET-1 could upregulate the transcription of Mn-SOD via the stimulation of the AP-1 site in the gene. Yamashita et al. (54–56) demonstrated in their series of reports that Mn-SOD was induced by treatment with ischemic preconditioning, norepinephrine, and heat shock and these upregulations of Mn-SOD contributed to tolerance to hypoxia or hypoxia-reperfusion injury in cardiomyocytes. Furthermore, they also suggested that the Mn-SOD induction observed in their system was due to C-kinase activation and subsequent phosphorylation of AP-1. Our present findings that some protein molecule(s), which is synthesized de novo by the stimulation of the protein kinase pathway, is involved in the cytoprotective effect of ET-1 on injured cardiomyocytes by Dox and that Mn-SOD is the most important candidate for this molecule are consistent with the above reports.

However, although ET-1 prevented Dox-induced acute cytotoxicity, ET-1 did not affect the cytotoxicity under conditions of prolonged incubation with Dox over 48 h. These results suggest that ET-1 blocked the early phase of cytotoxicity induced by Dox in culture. Cardiotoxicity induced by Dox has multiple steps, including free-radical formation (1, 17, 32), and complicates direct effects on DNA and other cellular components (9, 19–21, 23, 43, 44). Therefore, ET-1 may have suppressed the free radicals produced by Dox through the upregulation of Mn-SOD and reduced the cytotoxicity of Dox. However, ET-1 could not antagonize the other effects of Dox on the cellular components that gradually progress in the cells.

In summary, the present study revealed a new pharmacological action on cardiomyocytes: ET-1 rescued cultured cardiomyocytes from the early phase of Dox-induced cardiotoxicity and, furthermore, revealed the molecular mechanism that this cytoprotective action was mainly attributable to upregulation of the antioxidant Mn-SOD through ETA receptors. Therefore, it is possible that this action of ET-1 on cardiomyocytes would affect the pathophysiological condition of injured hearts in Dox-treated patients with cancer.

**Perspectives**

We and other groups have reported an increase in plasma levels of ET-1 in patients (8, 10, 14, 22, 36) and experimental animals (6, 24, 38, 39, 50) with CHF and also reported a marked increase in tissue levels of ET-1 in failing hearts in patients (35) and in experimental animals (38, 39, 59) with CHF, suggesting that the heart is one of the major origins of the increase in plasma ET-1 in CHF. It has also been reported that plasma ET-1 was progressively increased in Dox-treated patients with breast cancer in whom congestive heart failure developed (58). Therefore, it can be speculated that myocardial ET-1 plays a role in failing hearts in these patients. As described in the introduction, the increase in myocardial ET-1 in heart diseases seems to have an adaptive (beneficial) role and a maladaptive (harmful) role in various stages of heart diseases. The present study suggests that ET-1 has a beneficial aspect in injured cardiomyocytes in the early phase of Dox-induced cytotoxicity in culture.

Although there are several reports showing the effectiveness of the blockade of ET receptors on CHF in experimental animals (5, 16, 30, 38, 52, 53, 59) and in patients (34), there is no report on how the blockade of ET receptors affects (deteriorates or ameliorates) pathophysiological conditions in vivo of experimental animals or patients with CHF caused by Dox treatment. In other words, there is a possibility that the ET-1 in cardiomyocytes induced by Dox attenuates the acute cardiotoxicity of Dox, and the blockade of ET receptors at early stages of the Dox treatment suppresses the antioxidative effect of ET-1 and accelerates cardiotoxicity. In this regard, an in vivo study that investigates the effects of an ET-receptor antagonist initiated at different stages of heart disease on hemodynamics and survival of CHF animals caused by Dox treatment is considered to be very important. Furthermore, because oxidative stress in the myocardium has been reported to play an important role in the pathogenesis of CHF by various etiologies (13), it is interesting and important to determine whether upregulation of myocardial Mn-SOD by ET-1, as shown in this study, occurs in the diseased heart at some stage of CHF by various etiologies (ischemic heart diseases, valvular heart diseases, cardiomyopathy, etc.) in animals and patients.

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