Possible Involvement of Different Mechanisms in Sudden Death Induced by Endothelin-1 and Big Endothelin-1

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Received June 22, 1994; accepted September 21, 1994

The effects of diltiazem and phosphoramidon on sudden death induced by endothelin (ET)-1 and by big ET-1 were compared in rodents. Diltiazem (2 mg/kg, i.v.) remarkably diminished the lethal toxicity of ET-1 with a reduction in the extent of the rise in plasma immunoreactive ET-1-like activity (IR-ET-1), tissue IR-ET-1 accumulation in the heart and the rise in plasma potassium concentration. In big ET-1-induced lethality, diltiazem only slightly prolonged the latency and did not reduce the mortality. Although diltiazem moderately inhibited the rise in plasma IR-ET-1 and potassium concentration in these mice, it did not affect the accumulation of IR-ET-1 in the heart, lung or kidney. Phosphoramidon (2 mg/kg, i.v.) decreased the lethality of big ET-1 with the decrement in elevation of IR-ET-1 in the heart, lung and plasma as well as with the decrease in plasma potassium concentration, but it failed to improve any parameters in ET-1-induced lethality. In anesthetized rats, ET-1 (5 nmol/kg, i.v.) elevated ST-segment of electromyocardograms, and diltiazem (2 mg/kg, i.v.) significantly reversed this change. Big ET-1 (25 nmol/kg, i.v.) also induced the ST-segment elevation, which was significantly inhibited by phosphoramidon but not by diltiazem. These findings suggest that accumulation of ET-1 in the heart, which may lead to lethal cardiac ischemia, is an important factor in the lethality of ET-1, while additional factors (such as hemococoncentration and bronchoconstriction) may be involved in big ET-1-induced lethality.

Keywords endothelin-1; big endothelin-1; diltiazem; phosphoramidon; tissue accumulation; sudden death

Endothelin (ET)-1 is characterized by its very potent vasoconstrictive activity, so that only a small amount causes potent and long-lasting biological responses when administered to experimental animals. Increasing doses of ET-1 led to sudden death in mice and rats, which was almost completely abolished by the Ca2+ channel blockers diltiazem, verapamil and nifedipine. Since elevation of plasma immunoreactive ET-1-like activity (IR-ET-1) induced by ET-1 was apparently decreased by diltiazem, changes in plasma IR-ET-1 would be an important factor in ET-1-induced lethality. Big ET-1, a precursor of ET-1, also induced sudden death with a slower onset time, and this was effectively inhibited by a metalloproteinase inhibitor, phosphoramidon and by aspergillopeptides in mice via their inhibition against ET converting enzyme (ECE). In big ET-1-induced sudden death, these compounds significantly attenuated the elevation of plasma IR-ET-1 induced by the conversion of big ET-1 to ET-1. Thus, elevated plasma IR-ET-1 caused by either ET-1 or big ET-1 appears to be an important factor in the lethal activity of these two peptides.

Although hypertension induced by big ET-1 is caused by almost the same amount of ET-1 calculated as molar dose, the LD50 value of sudden death induced by big ET-1 is 5.5 times higher than that of ET-1. It remains to be clarified what causes the difference of LD50 values. Different mechanism(s) may be involved between ET-1 and big ET-1-induced sudden death. In the present study, to clarify the characteristics of sudden death in rodents induced by these two substances, we examined the effects of diltiazem and phosphoramidon on IR-ET-1 in the tissue as well as in plasma, and on myocardial ischemia.

MATERIALS AND METHODS

Effects on Sudden Death Induced by ET-1 and Big ET-1 Male ICR mice (6—7 weeks old, Charles River Japan Inc.) were used. Diltiazem (0.125, 0.5 and 2 mg/kg, i.v.) or phosphoramidon (2 mg/kg, i.v.) was administered 10 min before the injection of ET-1 (5 nmol/kg, i.v.) or big ET-1 (25 nmol/kg, i.v.). These doses of ET-1 and big ET-1 were reported to cause almost complete lethality in mice. In control animals, saline containing 0.05% bovine serum albumin (BSA) was given as a vehicle. Mortality (%) and latency (s) to death, as judged by the cessation of respiration, were measured up to 60 min after ET-1 or big ET-1 injection. Eight to 10 mice were used in each group.

Measurement of IR-ET-1 in Plasma, Heart, Lung and Kidney Saline, diltiazem (2 mg/kg) or phosphoramidon (2 mg/kg) was given i.v. to male ICR mice (7—8 weeks old) 10 min before the injection of ET-1 (5 nmol/kg, i.v.) or big ET-1 (25 nmol/kg, i.v.), and thereafter blood samples were collected by cardiac puncture under ether anesthesia at 0, 1, 3 and 5 min for ET-1 and at 0, 5 and 10 min for big ET-1. Organs (heart, lungs and kidneys) were excised simultaneously and frozen immediately on dry ice. The blood samples were put in chilled tubes containing aprotinin (300 KIU/ml) and EDTA (2 mg/ml), and centrifuged at 2000 × g for 15 min at 4°C. Organ samples were homogenized in 1.4 ml of chloroform—methanol (2:1) at 4°C for 2 min. Then, 0.35 ml of water was added to the homogenates followed by centrifugation at 2500 × g for 25 min. The aqueous phase was acidified with acetic acid to a final concentration of 3% and applied to a Sep-Pak C-18 cartridge. The IR-ET-1 fraction was eluted with methanol. After evaporation, the dry residue...
was dissolved in assay buffer (phosphate buffered saline (PBS) containing 1% BSA and 0.1% Triton X-100) to final concentrations of 0.6, 8 and 2 g/ml for the heart, lung and kidney, respectively. Seven to 8 mice were used in each group.

IR-ET-1 was measured by sandwich-enzyme immunoassay (EIA) as previously described. Briefly, the plasma sample, organ extracts or standard ET-1 was added to the rabbit IgG anti ET-1(15-21)-coated micro test plate. After washing with buffer (PBS containing 0.1% Triton X-100), the plate was reacted with rabbit IgG F(ab')2-horse-radish peroxidase (HRP) anti ET-1 at 37 °C for 30 min. The plate was washed with the buffer, and then the bound enzyme activity was measured with o-phenylenediamine as a chromogen. Big ET-1 at a concentration of 100nm showed no cross reactivity in this EIA.

**Measurement of Plasma Potassium Concentration**

Plasma samples obtained as above were applied to a clinical ion meter (Horiba, SERA-520) to measure plasma potassium concentration.

**Effects on the Changes in Electromyocardiogram (EMG) Induced by ET-1 or Big ET-1 in Anesthetized Rats**

Male Sprague-Dawley rats weighing 250–350 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After tracheal intubation, polyethylene catheters were inserted into the left carotid artery and the left femoral vein for the continuous measurement of blood pressure and for drug administration, respectively. A left thoracotomy was performed under artificial ventilation with a respirator (Harvard, Model 683). A fine platinum wire electrode (0.1 mm diameter) was inserted into the myocardial free wall of the left anterior ventricle to measure EMG as previously described. Ten min after the administration of diltiazem (2 mg/kg, i.v.), phosphoramidon (2 mg/kg, i.v.) or saline, ET-1 (5 nmol/kg) or big ET-1 (25 nmol/kg) was injected. Mean arterial blood pressure (MBP) and EMG were continuously recorded on a polygraph system (Nihon Kohden, RM-6000) for 30 min after ET-1 or big ET-1 injection. Four or 6 rats were used in each group.

**Chemicals**

Human ET-1, human big ET-1, and phosphoramidon were supplied by Peptide Institute Inc. (Osaka, Japan); rabbit IgG anti ET-1(15-21) and rabbit IgG F(ab')2-HRP anti ET-1 by International Reagents Corp (Kobe, Japan); Triton X-100 by Wako Pure Chemical Industries (Osaka, Japan); BSA and diltiazem by Sigma (St. Louis, U.S.A.); aprotinin by Seikagaku Corp. (Tokyo, Japan); and EDTA by Dojindo Labs. (Kumamoto, Japan).

Diltiazem and phosphoramidon were dissolved in saline. ET-1 and big ET-1 in saline included 0.05% BSA.

**Statistical Analysis** Data are expressed as mean ± S.E.M. of observations. Mortality for 60 min after ET-1 or big ET-1 injection was evaluated by Fisher's exact probability test. If an animal survived over 60 min, its latency was regarded as 3600 s. Differences in latency and time course of IR-ET-1, potassium concentration and ST-segment changes in EMG among the groups were assessed using an unpaired Student's t-test or one-way analysis of variance with Bonferroni modification. p values less than 0.05 were regarded as statistically significant.

**RESULTS**

**Effects of Diltiazem and Phosphoramidon on Sudden Death Induced by ET-1 and Big ET-1 Intravenous administration of ET-1 (5 nmol/kg) and big ET-1 (25 nmol/kg) to mice caused sudden death with a mean latency to death of 248.3–593.4 s (2 experiments) and 674.2–839.4 s (2 experiments), respectively. Although diltiazem showed dose-dependent (0.125–2 mg/kg, i.v.) and significant inhibition against mortality and latency to sudden death induced by ET-1, it slightly prolonged the latency without any reduction in mortality in big ET-1-induced sudden death (Table I). Phosphoramidon at a dose of 2 mg/kg i.v. did not mitigate ET-1-induced sudden death but markedly attenuated big ET-1-induced sudden death (Table I).**

**Measurement of IR-ET-1 in Plasma, Heart, Lung and Kidney**

Figure 1 demonstrates the effects of diltiazem (2 mg/kg) or phosphoramidon (2 mg/kg) on the time course of changes in plasma IR-ET-1 (a) and in tissue IR-ET-1 of the lungs (b), the kidneys (c) and the heart (d) in mice following i.v. administration of ET-1 (5 nmol/kg, i.v.). Plasma IR-ET-1 (Fig. 1a) in diltiazem-treated mice was significantly lower than that in control at 5 min after ET-1 injection as shown in our previous study. Phosphoramidon, however, did not affect the rise in plasma IR-ET-1 induced by ET-1. Phosphoramidon only slightly yet significantly augmented the accumulation of IR-ET-1 in the lung (Fig. 1b) and the kidneys (Fig. 1c) 5 min after ET-1 administration. Diltiazem also tended to show the same effect. The most remarkable changes were observed in the heart (Fig. 1d), in which diltiazem significantly

### Table I. Effects of Diltiazem and Phosphoramidon on ET-1- and Big ET-1-Induced Sudden Death in Mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg, i.v.)</th>
<th>Mortality % inhibition</th>
<th>Latency % prolongation</th>
<th>Mortality % inhibition</th>
<th>Latency % prolongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diltiazem</td>
<td>0.125</td>
<td>11.1</td>
<td>49.6</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>55.6*</td>
<td>188.6**</td>
<td>0</td>
<td>46.4*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>88.9**</td>
<td>271.7**</td>
<td>0</td>
<td>56.4*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>n.t.</td>
<td>n.t.</td>
<td>10</td>
<td>64.5*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>18.3</td>
<td>80</td>
<td>277.3**</td>
</tr>
</tbody>
</table>

Each drug was administered 10 min prior to i.v. injection of ET-1 (5 nmol/kg) or big ET-1 (25 nmol/kg i.v.). Mortality was expressed as % inhibition against control. Latency was expressed as % prolongation of control. Eight to 10 mice were used in each group. * significantly different from carboxymethyl cellulose-saline control (p<0.05), ** p<0.01, n.t., not tested.
Fig. 1. Changes in IR-ET-1 in Plasma (a), the Lung (b), the Kidney (c) and the Heart (d) after i.v. ET-1 (5 nmol/kg) Injection in Mice (n=7). Saline (○), diltiazem (●, 2 mg/kg) or phosphoramidon (■, 2 mg/kg) was i.v. administered 10 min prior to ET-1 injection. Data are means±S.E.M. of observations. *, significantly different from saline control (p<0.05), **, p<0.01.

Fig. 2. Changes in IR-ET-1 in Plasma (a), the Lung (b), the Kidney (c) and the Heart (d) after i.v. Big ET-1 (25 nmol/kg) Injection in Mice (n=7). Saline (○), diltiazem (●, 2 mg/kg) or phosphoramidon (■, 2 mg/kg) was i.v. administered 10 min prior to Big ET-1 injection. Data are means±S.E.M. of observations. *, significantly different from saline control (p<0.05).

suppressed the accumulation of IR-ET-1, while phosphoramidon did not.

Figure 2 showed the effects of diltiazem or phosphoramidon on the time course of changes in plasma IR-ET-1 (a) and in tissue IR-ET-1 of the lungs (b), the kidneys (c) and the heart (d) in mice following i.v. administration of big ET-1 (25 nmol/kg, i.v.). As compared to ET-1 injection, big ET-1 injection caused a more sustained increase in
tissue IR-ET-1 of the lungs, showing a gradual increase up to 10 min after the injection. Similar to the ET-1 injection, diltiazem lowered the elevation of plasma IR-ET-1 induced by big ET-1 (Fig. 2a). Phosphoramidon significantly attenuated the elevation of plasma IR-ET-1 and a rise in hematocrit (data not shown) as we observed previously.5) Although tissue IR-ET-1 in the kidneys did not differ among the three groups (Fig. 2c), phosphoramidon significantly decreased the elevation of IR-ET-1 of the heart and the lung, while diltiazem did not (Figs. 2b and d).

Measurement of Plasma Potassium Concentration With ET-1 or big ET-1 injection in mice, plasma potassium concentration gradually rose (Figs. 3a and b). The extent of the elevated plasma potassium concentration was much larger with ET-1 (8.3 ± 0.2 mm at 5 min) than with big ET-1 (6.0 ± 0.3 mm at 5 min). Diltiazem (2 mg/kg, i.v.) markedly lowered the elevation of plasma potassium concentration induced by both peptides. Phosphoramidon (2 mg/kg, i.v.) showed an inhibition only against big ET-1-induced response.

Effects on the Changes of EMG Induced by ET-1 or Big ET-1 in Anesthetized Rats Intravenous administration of ET-1 at the lethal dose of 5 nmol/kg to anesthetized rats caused significant hypertension followed by abnormalities of EMG, namely ischemic ST-segment elevation. As shown in Fig. 4a, ET-1 elevated the ST-segment maximally by 12.9 ± 0.7 mV at 15 min after the injection, and the elevation continued throughout the 30 min-experimental period. Pretreatment with diltiazem (2 mg/kg, i.v.) significantly suppressed the ST-segment elevation by ET-1 as reported by Harada et al.11)

In the rats treated with big ET-1 (25 nmol/kg, i.v.), a similar elevation of the ST-segment occurred, but it appeared somewhat slowly and with a far lower amplitude (maximum 4.3 ± 0.4 mV) than that caused by ET-1 (Fig. 4a). Phosphoramidon at 2 mg/kg, i.v. significantly, but not completely, inhibited big ET-1-induced ST-segment elevation. Diltiazem at the same dose resulted in almost no inhibition against the ST-segment elevation within 20 min after big ET-1 injection, but inhibition thereafter was significant (Fig. 4b).
DISCUSSION

Administered exogenously into the coronary arteries, ET caused myocardial ischemia experimentally in rats,1,2 dogs3,13 and pigs4,14 with abnormal electrocardiographic changes. These ischemic changes were mostly attributable to the potent coronary constricting properties of ET. Systemic administration of ET-1 (3–5 nmol/kg, i.v.) provoked sudden death in rats, mice and guinea pigs with fatal ventricular arrhythmias.3,5,15 The cause of the sudden death was due to the potent activities of ET, i.e., vasoconstriction,3,5,15 bronchoconstriction4,15 and the release of active substances such as platelet activating factor.5 In mice treated with a lethal dose of ET-1, a Ca2+-channel blocker, diltiazem, almost completely abolished ET-1-induced lethality with a notable decrease in the plasma level of IR-ET-1.5

The sudden death could also be caused by big ET-1 in mice, accompanied by a significant elevation in plasma IR-ET-1, which was converted from big ET-1 by ECE in vivo.5 Pretreatment with a metalloprotease inhibitor, phosphoramidon, remarkably suppressed the sudden death with an apparent decrement in plasma IR-ET-1.5 Naturally-occurring chelators, aspargillomarasmine, were found to lower the rise in plasma IR-ET-1 by inhibiting ECE, leading to mitigation of big ET-1-induced sudden death in mice.6,7 EDTA counteracted neither the plasma IR-ET-1 elevation nor the sudden death caused by big ET-1 in mice in spite of showing potent ECE inhibition in vitro.7 These facts indicate the importance of the plasma ET-1 level in ET-1- and big ET-1-induced sudden death.

In the present study, diltiazem showed the same inhibitory effects on ET-1-induced sudden death by inhibiting the increase in plasma IR-ET-1 as we recently reported.19 Since ET-1 was considered to be a causative factor in both ET-1- and big ET-1-induced sudden death, diltiazem was assumed to also be effective in the latter case, in which an almost equivalent elevation of plasma IR-ET-1 to that caused by ET-1 injection was observed in control animals (Fig. 2a). Although diltiazem surely lowered plasma IR-ET-1 raised by big ET-1 (Fig. 2a), surprisingly the drug was ineffective against the lethality and accumulation of IR-ET-1 in the heart (Table I and Fig. 2a). This observation may indicate that elevated plasma IR-ET-1 is not necessary for big ET-1 to cause lethality in mice. On the other hand, IR-ET-1 in the heart is closely correlated with either ET-1 or big ET-1-induced sudden death. Diltiazem and phosphoramidon diminished ET-1- and big ET-1-induced sudden death, respectively, with a significant decrease in IR-ET-1 in the heart as the common property. Considering the direct action of ET-1 on the heart,11,16,17 that organ’s accumulation of IR-ET-1 would be one of the crucial factors in sudden death induced by ET-1.

The most remarkable effect of diltiazem in the present study was the mitigation of IR-ET-1 accumulation in the heart in ET-1-induced sudden death, while the drug did not decrease but instead tended to increase IR-ET-1 in the kidney or the lung. These different effects of diltiazem in organs may be due to a difference in the capacity of the uptake of ET-1 between lung or kidney and heart, i.e., much higher uptake of [125I]ET-1 was observed in the former two than in the latter in the rat.19 We previously described the inhibitory effect of diltiazem on the rise in plasma IR-ET-1 by exogenous ET-1 and suspected that the drug enhanced ET-1 clearance from the bloodstream.9 The improvement in regional circulation by diltiazem would enhance the clearance of ET-1 by these organs, leading to a decrease in plasma IR-ET-1. These changes would reduce the amount of ET-1 reaching the heart, resulting in a decreased IR-ET-1 in the heart. We consider that in the heart, decreased plasma IR-ET-1 rather than increased blood flow would affect the heart tissue IR-ET-1 because of there being fewer ET receptors. An alternative possibility is that there is a change in the density of ET receptors in the heart during ischemia induced by ET-1. Since [125I]ET-1 binding in the isolated rat heart was increased by ischemia or ischemia-reperfusion,19 diltiazem may decrease the number of ET-receptors by its anti-ischemic effect. The decrease in IR-ET-1 in the heart by diltiazem (or phosphoramidon) is assumed to lead to alleviation of lethal myocardial ischemia, as evidenced by inhibition against the ischemic myocardial ST-segment elevation of EMG (Fig. 4a).19

Harada et al.11 reported that diltiazem inhibited both ST-segment elevation and arrhythmia induced by intracoronary administration of ET-1 in rats. They stated that the protective effect on ST-segment elevation by diltiazem appeared to be due to the inhibitory action on the constriction of coronary arteries, and that the anti-arrhythmic action was based on a shortening of action potential duration. These observations also suggest the cardio-protective effect of diltiazem without effect on IR-ET-1 in the heart.

We also investigated the effects of diltiazem and phosphoramidon on the change in plasma potassium concentration induced by ET-1 or big ET-1 in mice. A mechanism of arrhythmia due to myocardial ischemia is generally assumed as follows. Lowered coronary blood flow leads to depletion of the production of adenosine 5'-triphosphate (ATP) followed by reduction in Na+-K+ ATPase activity. The reduction in this enzyme activity causes a rise in plasma potassium concentration, or hyperkalemia, which finally induces a disorder of the cardiac conducting system.20 Lin et al. reported that exogenous administration of ET-1 to animals caused hyperkalemia with a change in electrocardiogram, and intraventricular conduction delay.15 In the present study, we showed that ET-1 significantly elevated plasma concentration of potassium, and that diltiazem significantly prevented it (Fig. 3a). This indicates that the vasoconstrictor action of ET-1 may cause development of myocardial ischemia as evidenced by hyperkalemia, and that diltiazem prevented the hyperkalemia through its anti-ischemic activity.

We recently indicated that there was a correlation between the time course change in big ET-1-induced hemoconcentration and that in lung tissue IR-ET-1 which gradually increased, while that in plasma IR-ET-1 did not.9 Thus, when ET-1 or big ET-1 is exogenously given to animals, tissue IR-ET-1 levels may be more important.
than the plasma IR-ET-1 level to exert the activities *in vivo*, *i.e.*, changes in heart tissue IR-ET-1 and lung tissue IR-ET-1 would be involved in sudden death and hemo-
concentration. Considering the large capacity of the lung to bind and to clear exogenously administered ET-1, a large amount of ET-1 converted from big ET-1 may be dominantly trapped in the lung and play an important role in the hemoconcentration and sudden death induced by big ET-1. In the case of big ET-1-induced sudden death, accumulation of IR-ET-1 in the heart tissue tended to be lower than that caused by ET-1 (Figs. 3d and 4d), and ST-segment elevation (Fig. 4b) was much smaller than that caused by ET-1 (Fig. 4a). Additionally, hyperkalemia induced by big ET-1 was also much milder than that caused by ET-1 (Fig. 3b). These results indicate that ischemic insult in the myocardium might be smaller in big ET-1-induced lethality than that in the ET-1-induced one. This phenomenon can be explained as follows: in ET-1-induced sudden death, exogenous ET-1 directly reaches and affects on the heart, while with big ET-1, matured ET-1 is gradually produced during the circulation. Instead of a direct and potent cardiac action, disturbance of the circulation due to sustained hemoconcentration may accelerate ischemia of several organs, finally leading to big ET-1-induced lethality. Thus, ET-1-induced sudden death may be characterized by its accumulation and direct action to the heart, while several additional factors would be involved in big ET-1-induced sudden death. The hypothesis described above may also contribute to the difference in LD50 values of ET-1 and big ET-1.

In conclusion, accumulation of ET-1 in the heart, which may lead to the lethal cardiac ischemia, would be one of the crucial factors in the lethality of ET-1 given exogenously. In big ET-1-induced sudden death, cardiac ischemia was milder than that ET-1-induced, and tissue accumulation of ET-1 in the lung would also be an important causative factor of sudden death, presumably provoking bronchoconstriction and hemoconcentration. The decrease in the plasma level of IR-ET-1 does not always mitigate the toxic effects of ET-1. The elevation of plasma IR-ET-1 observed in ET-1- or big ET-1-induced sudden death may be attributed to a spillover of excessive ET-1 which was not trapped or degraded by the lung, kidney or other organs during circulation. Diltiazem alleviated ET-1-induced sudden death *via* its cardioprotective activity as evidenced by the decreases in accumulation of heart IR-ET-1 and in ischemic ST-segment elevation, but it failed to exert its protective activity in big ET-1-induced sudden death. These results imply that tissue IR-ET-1 level rather than plasma IR-ET-1 level more directly contributes to the responses to diltiazem *in vivo*, and that different mechanism(s) may be involved in ET-1 and big ET-1-induced sudden death.

**Acknowledgment** We express our thanks to the staff members of the Pharmacology Department of our laboratories for their technical assistance.

**REFERENCES AND NOTES**


