The Effects of a Novel Cyclohexane Dicarboximide Derivative, ST-6, on Hypoxia/Reoxygenation Injury in Perfused Rat Heart

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The present study was undertaken to test if some cyclohexane dicarboximide derivatives may have a cardioprotective effect against hypoxia/reoxygenation injury. Isolated rat hearts were subjected to 20-min of hypoxia followed by 45-min reoxygenation, and their recovery of post-hypoxic cardiac contractile function was examined. Treatment with agents was carried out from 3 min after the onset of hypoxia to the end of hypoxia (17 min during hypoxia). Among the 17 compounds, 2-[4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]butyl]hexahydro-1H-isoindol-1,3(2H)-dione (ST-6) showed a significant enhancement of post-hypoxic contractile force. This was associated with attenuation of the releases of creatine kinase and purine nucleosides and bases from the perfused heart. Hypoxia-induced increase in myocardial sodium and decrease in potassium ion content was suppressed by ST-6 treatment. The results suggest that ST-6 is capable of protecting the heart against hypoxia/reoxygenation injury possibly through a mechanism by which sodium overload during hypoxia is suppressed.

Key words: cardioprotection; heart; hypoxia; reoxygenation; sodium channel blockade; sodium overload

Oxygen deficiency and subsequent oxygen replenishment, ischemia/reperfusion or hypoxia/reoxygenation, induce various pathophysiological alterations of the heart, including ionic disturbance,1 energy depletion,2 no or little reflow in the reperfused heart,3 free radical attack,4 and changes in cell membrane permeability.5 These alterations may eventually lead to cardiac cell necrosis and contractile failure. Numerous studies have shown the possibility of various agents for protection of the myocardium from ischemia/reperfusion or hypoxia/reoxygenation injury. In previous studies, we have shown that several class I antiarrhythmic agents, when administered prior to ischemia, protected the myocardium against ischemia/reperfusion injury.6–8 The beneficial recovery of post-ischemic contractile function was associated with reduction in ionic imbalance across the sarcolemma during ischemia as well as reperfusion. Particularly, sodium overload during ischemia seems to play an important role in the genesis of ischemia/reperfusion injury. From these observations, we hypothesized that blockade of sodium overload, particularly during ischemia, may attenuate ischemia/reperfusion injury.9

According to this hypothesis, we tested a series of cyclohexane dicarboximide derivatives, some of which were found to have local anesthetic activity in a preliminary study. This action is, at least in part, related to blockade of a voltage-gated sodium channel.10 The present study was undertaken to find possible agents among these compounds, which may protect the heart from ischemia/reperfusion or hypoxia/reoxygenation injury by preventing sodium overload during ischemia. In the present study we employed a hypoxia/reoxygenation model11,12 because this model is convenient for the application of agents to perfused hearts after the onset of hypoxic episode.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 220–250 g, were used in the present study. The animals were acclimatized at 23±1°C with a constant humidity of 55±5%, a cycle of 12 h-dark and 12 h-light and were given free access to food and tap water according to the Guide lines of the Experimental Animals Care issued by the Prime Minister’s Office of Japan.

Perfusion of Hearts Perfusion of rat hearts was similar to that described in a previous study.13 Rats were anesthetized with ether and their hearts were isolated. The hearts were perfused at 37°C in a Langendorff mode at the flow rate of 9.0 ml/min with the Krebs-Henseleit buffer (KH buffer) of the following composition (mm): NaCl 120, KCl 4.8, KH2PO4 1.2, MgSO4 1.2, CaCl2 1.25, NaHCO3 25 and glucose 11. The solution was equilibrated with a gas mixture of 95% O2 and 5% CO2 and maintained at pH of 7.40–7.42 throughout perfusion. The PO2 value for the solution was more than 600 mmHg when measured by means of a blood gas analyzer (Model 288, Ciba-Corning, East Walpole, U.S.A.). The heart was preloaded with an initial tension of 1.5 g and paced at 300 beats/min by an electronic stimulator (ISEN-3301, Nihon Kohden, Tokyo). Cardiac contractile force was estimated by monitoring isometric tension development generated with the initial resting tension through a hook attached to the apex of the heart by means of a force displacement transducer (TB-612T, Nihonkohden, Tokyo, Japan). After 25 min of equilibration, the hearts were perfused with hypoxic KH buffer for 20 min of hypoxia. The hypoxic KH buffer was previously equilibrated with a gas mixture of 95% N2 and 5% CO2, with replacement of 11 mm glucose to 11 mm Tris–HCl, pH 7.4. The PO2 value of the hypoxic buffer was less than 15 mmHg when monitored with the blood gas analyzer. After 20-min of hypoxic perfusion, the hearts were perfused for 45 min with the normal KH buffer containing 11 mm glucose as used for perfusion in the equilibration period (reoxygenation).

In the first set of experiments, hearts were treated with different, newly synthesized compounds at each concentration.
of 100 µg/min. Syntheses of the compounds were reported previously. In the next set of experiments, we examined the effects of one of the derivatives, 2-[4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]butyl]hexahydro-1H-isoindol-1,3(2H)-dione (ST-6), on hypoxia/reoxygenation injury of the perfused heart. The chemical structure of the agent is shown in Figure 1. In this study, 30 µg/min of ST-6 was used because we found in a preliminary study that the submaximal effect on post-hypoxic contractile function was detected at this dose. For treatment with ST-6, we carried out delayed treatment; that is, the agent was infused into the perfusate from 3 min after the onset of hypoxia to the end of hypoxia (total 17 min during hypoxia).

The perfusate eluted from the heart was collected under ice-cooling during hypoxia and reoxygenation. Purine nucleosides and bases in the perfusate were determined by HPLC as described elsewhere. Creatine kinase (CK) activity in the perfusate was also determined according to the method of Bergmeyer et al.

**Determination of Tissue Ion Content** In the next series of experiments, tissue ion content of the heart during hypoxia was determined to elucidate myocardial ion changes. After hypoxia, the vascular and readily exchangeable spaces were washed through the aortic cannula by infusion of 8 ml of cold 320 mm sucrose/20 mM Tris-HCl, pH 7.4. In brief, the myocardium was dried at 120°C for 48 h. The dried myocardium was digested at 180°C with concentrated HNO₃. The residue was reconstituted with 0.75 N HNO₃ and used for determination of tissue ion concentration by atomic absorption spectrophotometer (AA-680, Shimazu, Kyoto, Japan).

**Statistics** The results are expressed as the means ± S.E.M. Statistical significance was evaluated using two-way analysis of variance followed by Bonferroni’s multiple comparison. A confidence level of more than 95% was considered significant (p<0.05).

**RESULTS**

**Effects of Various Synthetic Agents on Post-hypoxic Recovery of Cardiac Contractile Force (Screening Test)** The effects of various synthetic compounds on post-hypoxic cardiac function of hypoxic/reoxygenated heart were examined. The results are shown in Table 1. Compounds 2, 3, 5, 6, and 7 at each dose of 100 µg/min improved the post-hypoxic recovery of cardiac contractile force appreciably. Among these compounds, compound 6 (ST-6) was most effective in enhancing the post-hypoxic contractile force. Thus, we examined the effects of this agent on hypoxia/reoxygenation injury in detail.

**Effects of ST-6 on Hypoxia/Reoxygenation Injury** Cardiac Function: Typical tracing of hypoxia/reoxygenated hearts in the presence and absence of ST-6 is shown in Fig. 2. Hypoxia/reoxygenation increased the resting tension and recovered very little the post-hypoxic contractile force of the

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Table 1. Chemical Structures of Cyclohexane Dicarboximide Derivatives and Their Effects on Hypoxia/Reoxygenation-Induced Contractile Dysfunction of Isolated Perfused Rat Hearts

<table>
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<tr>
<th>No.</th>
<th>Chemical structure</th>
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All these compounds are the hydrochloride salts. Each value represents % recovery of contractile force for one preparation.
perfused heart. Infusion of 30 μg/min of ST-6 appreciably reversed the contractile force of the heart. Figure 3 shows the time courses of changes in contractile force, resting tension and perfusion pressure of hypoxic/reoxygenated hearts in the absence and presence of ST-6. Baseline values for contractile force of agent-treated and untreated hearts were 4.5 ± 0.3 and 4.6 ± 0.3 g, n = 6, respectively, and those for perfusion pressure, 76.0 ± 7.6 and 78.2 ± 3.8 mmHg, n = 6, respectively. This indicates that there were no significant differences in the baseline values between the agent-treated and untreated groups. Cardiac contractile force immediately declined after the onset of hypoxia. Reoxygenation did not regenerate the contractile force of the untreated heart. Cardiac contractile force of the heart treated with ST-6 was restored when the heart was reoxygenated. Resting tension was increased during hypoxia and gradually recovered toward the baseline level during reoxygenation. Treatment with ST-6 significantly reduced the rise in resting tension induced by hypoxia and reoxygenation (by two-way ANOVA). Perfusion pressure decreased immediately after the onset of hypoxia and thereafter reversed to the baseline level during hypoxia. Perfusion pressure tended to be higher than the baseline value during reoxygenation. Treatment with ST-6 significantly suppressed the reoxygenation-induced rise in perfusion pressure.

CK Release: To elucidate myocardial necrosis of the perfused heart, the release of creatine kinase was measured in the perfusate eluted from hypoxic and reoxygenated heart. As shown in Fig. 4, CK was released during hypoxia to a small degree. This release was inhibited by treatment with 30 μg/ml ST-6. A marked increase in the release of CK from reoxygenated hearts was seen. This increase was greatly attenuated by treatment with ST-6.

ATP Metabolites Released from Hypoxic and Reoxygenated Heart: To elucidate the release of purine nucleosides and bases (ATP metabolites) from hypoxic and reoxygenated heart, we determined the concentrations of ATP metabolites in the perfusate eluted from the hypoxic as well as reoxygenated hearts (Fig. 5). ATP metabolites were released to a minimal degree in normoxic hearts (<0.1 μmol/g wet tissue). The ATP metabolites were markedly increased

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Fig. 2. Tracings of Changes in Perfusion Pressure and Contractile Force of Hypoxic and Reoxygenated Hearts Untreated (Lower Panel) and Treated with 30 μg/min of ST-6 (Upper Panel)

The agent was administered starting 1 min after the onset of hypoxia to the end of hypoxia.

Fig. 3. The Time Courses of Changes in Contractile Force, Resting Tension and Perfusion Pressure of the Hypoxic and Reoxygenated Heart Untreated (○) and Treated with 30 μg/min of ST-6 (●)

Each value represents the mean ± S.E.M. of 6 experiments. Note that some S.E.M. bars are not shown because standard errors were too small to be revealed on the scale of plot. There are significant differences in the 3 parameters between the agent-treated and untreated groups, when evaluated by two-way ANOVA. Asterisks (*) indicate statistical differences between the agent-treated and untreated groups at the end of reoxygenation (p < 0.05).
DISCUSSION

In a previous study, we demonstrated that the class I type antiarrhythmic agents such as quinidine,6 flecaïnide,7 pilscainide,7 mexiletine,8 lidocaine,8 and aprindine,16 enhanced the post-ischemic recovery of cardiac contractile function of the heart in a concentration-dependent manner. This effect was closely related to the suppression of sodium accumulation during ischemia.9 Sodium accumulation of the perfused heart determined in this way may be considered as one of the markers for sodium overload in the heart.15 Furthermore, several investigators have postulated that sodium overload may play a crucial role in the genesis of ischemia/reperfusion injury.1,17–20 Thus, we examined agents that are sodium channel blockers as possible protective drugs against ischemia/reperfusion or hypoxia/reoxygenation injury. In the present study we tested the effects of agents by treating isolated rat hearts from 3 min after the onset of hypoxia to the end of 20-min of hypoxia. To test agents if they have a protective effect against ischemia/reperfusion injury, isolated perfused hearts are commonly used after pretreatment with the agents. In the present study, however, the agent treatment was carried out after the episode of hypoxia.

As described in the Introduction section, some agents of the cyclohexane dicarboximide derivatives as listed in Table 1 showed local anesthetic action, which is believed to be a rough marker of blockade of voltage-gated sodium channels.10 Thus, we tested if these compounds have cardioprotective action against hypoxia/reoxygenation injury. We found that ST-6 was most effective in enhancing post-hypoxic contractile recovery of the hypoxia/reoxygenation heart among these agents. To further examine the effects of the agent, we characterized the properties of this agent in more detail. The decrease in isometric tension development of the hypoxic/reoxygenated heart was partially but significantly reversed by treatment with ST-6. This improvement was associated with reduction in the release of CK from the perfused heart, suggesting prevention of cardiac cell necrosis.5 Purine nucleosides and bases, such as adenosine, inosine and hypoxanthine, are released from hypoxic and reoxygenated hearts or reperfused hearts, which represents a release of ATP metabolites. The release did not occur under normoxic conditions. This is considered to indicate changes in cell membrane permeability of the perfused heart.21 In the present study, we observed a marked increase in the release of ATP metabolites during hypoxia as well as reoxygenation, and a significant attenuation of their release by treatment with ST-6. These results suggest that the agent may reduce the hypoxia-induced increase in cell membrane permeability.

To examine tissue cation changes, we examined the ion content at the end of hypoxia. In a previous study, we showed that hypoxia/reoxygenation and hypoxia per se induced an increase in tissue sodium content of the perfused heart.22,23 This may represent ionic disturbance across cardiac sarcolemma, one of the pathophysiological changes of ischemia/reperfusion injury.22,23 Hypoxia-induced accumulation of sodium ion and loss of potassium ion were attenuated by treatment with ST-6. Prevention of the loss of potassium during ischemia does not appear to play a significant role in the recovery of post-ischemic contractile function.15 In contrast, the agents that have cardioprotective effects are known during hypoxia and reoxygenation. Treatment with ST-6 reduced the increase in the release of ATP metabolites during hypoxia and reoxygenation significantly.

Tissue Ion Content in Hypoxia: To examine the effects of ST-6 on myocardial ion changes, tissue ion content was determined in the heart at the end of hypoxia. The results are shown in Fig. 6. Tissue calcium accumulation was not seen at the end of hypoxia. A marked increase in tissue sodium and a significant decrease in tissue potassium were seen at the end of hypoxia. Treatment with ST-6 reduced sodium accumulation and potassium loss during hypoxia.

![Figure 4. CK Activity in the Perfusate Eluted during Hypoxia (Hypo) and Reoxygenation (Re) from the Hearts Untreated (Un) and Treated with 30 μg/min of ST-6 (ST-6)

Each value represents the mean±S.E.M. of 6 experiments. * Significantly different from the untreated group (p<0.05).

![Figure 5. ATP Metabolite Concentrations in the Perfusate Eluted during Hypoxia (Hypo) and Reoxygenation (Re) from the Hearts Untreated (Un) and Treated with 30 μg/min of ST-6 (ST-6)

Each value represents the mean±S.E.M. of 6 experiments. * Significantly different from the untreated group (p<0.05).

![Figure 6. Tissue Ion Contents of Ca²⁺, Na⁺, and K⁺ of the Hypoxic Hearts Untreated (○) and Treated with 30 μg/min of ST-6 (●)

Each value represents the mean±S.E.M. of 6 experiments. * Significantly different from the untreated group (p<0.05).]
to prevent sodium ion disturbance of the heart, similar to the results in the present study. Thus, ST-6 is a possible agent to exert cardioprotective action of the ischemia/reperfusion or hypoxia/reoxygenation injury by preventing sodium overload during oxygen deficiency.

REFERENCES