Hydroxyl radical scavenging effect of nicaraven in myocardial and coronary endothelial preservation and reperfusion injury

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Abstract

Objective: We investigated the efficacy of nicaraven in reducing myocardial as well as coronary endothelial preservation-reperfusion (P/R) injury. Methods: In experiment 1, isolated rat hearts were mounted on a Langendorff (L) apparatus to estimate the baseline cardiac function. Group 1, 8- and 12-h storage in histidine-tryptophan-ketoglutarate (HTK) solution; group 2, 8- and 12-h storage in HTK solution with superoxide dismutase (2.5 × 10^5 U/l) and catalase (2 × 10^5 U/l); group 3, 8- and 12-h storage in HTK solution with nicaraven (10^-3 M). Following storage for 8 and 12 h at 4°C, they were reperfused and post-preservative cardiac function was evaluated. The hearts were then switched back to L-mode and paced at 330 beats/min. Coronary flow (CF) following perfusion with KHB solution containing 5-hydroxytryptamine (5-HT) and nitroglycerin was also measured. In experiment 2, segments of pig coronary artery were suspended in organ chambers and exposed to hydroxyl radicals in the presence or absence of nicaraven. The sensitivity of relaxation response to bradykinin of the prior-exposed rings was measured. Results: The recovery of CF and LV dp/dt following 8 h of storage in group 3 was higher than that in group 1, although there were no significant differences in the other parameters of cardiac functional recovery among the groups. The absolute values of cardiac function following 12 h of storage in groups 1–3 were as follows: 6.6 ± 2.4, 9.1 ± 0.8, 15.6 ± 3.1 ml/min of cardiac output (CO); 1.9 ± 0.9, 2.3 ± 0.4, 6.0 ± 2.3 ml/min of aortic flow (AF); 4.5 ± 1.3, 6.0 ± 0.5, 9.5 ± 0.8 ml/min of CF, respectively. The recovery of CO, AF, CF, SP, MP, and left ventricular (LV) dp/dt were significantly improved in group 3, compared with those in group 1. CF and CO in group 3 were higher than those in group 2. 5-HT caused vasoconstriction in all groups, but the vasoconstriction in group 3 was less than in group 1. Prior exposure to FeSO4/H2O2 produced significant endothelial damage as reflected by the right-ward shift of the dose-response curve of bradykinin-induced endothelium-dependent relaxation. In the presence of nicaraven, the dose-response curve recovered to the control level. Conclusions: Nicaraven may improve coronary endothelial and myocardial function following P/R by its hydroxyl radical scavenging activity.

Keywords: Nicaraven; Free radicals; Preservation-reperfusion injury; Myocardium; Coronary endothelium; Rat; Pig

1. Introduction

Optimal myocardial as well as coronary endothelial preservation remains a major concern in heart transplantation. A growing body of evidence has been produced by direct demonstration of hydroxyl radical (·OH) with electron paramagnetic resonance (EPR), indicating that ·OH plays a pathogenetic role in myocardial stunning [1] and coronary endothelial damage [2]. Myocytes as well as vascular endothelial cells are the source of the intrinsic burst of super-oxide-derived ·OH generation that occurs on reperfusion of an ischaemic heart, and in turn causes myocardial and endothelial injury [1,3].

The oxygen free radicals and their metabolites that may play a part in ischaemia-reperfusion (I/R) injury include the superoxide radical (O2·-), hydrogen peroxide (H2O2), and ·OH. O2·- and H2O2 can be scavenged by naturally

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occurring enzyme systems. However, \(\cdot OH\), which can be formed via a superoxide-driven, iron-catalyzed Fenton reaction, has no known naturally occurring scavenging systems and thus plays a significant role in I/R injury [4,5]. It has been suggested that \(\cdot OH\) is the major culprit as it displays much greater chemical reactivity than \(O_2^-\) or \(H_2O_2\). If this hypothesis is correct, then therapy with oxygen free radical scavengers should be directed toward the inactivation of hydroxyl radicals rather than those of \(O_2^-\) and \(H_2O_2\) [6]. This concept is supported by the observation that myocardial stunning and coronary vascular damage are significantly attenuated by \(\cdot OH\) scavengers [1,2].

A promising candidate which emerged from a synthetic program is nicaraven: 1,2-bis(nicotinamido)-propane, an effective scavenger of \(\cdot OH\). Koide et al. [7] and Asano et al. [8] reported the beneficial effects of nicaraven as \(\cdot OH\) scavenger in the treatment of prolonged cerebral vasospasm after subarachnoid haemorrhage and of ischaemic cerebral oedema, respectively. Therefore, we designed this experiment to evaluate whether nicaraven has the efficacy to reduce myocardial as well as coronary endothelial P/R injury by measuring cardiac and coronary endothelial function in isolated rat hearts. In addition, we developed a model of oxidative stress of pig coronary artery, generating \(\cdot OH\) in vitro by adding FeSO\(_4\) and H\(_2\)O\(_2\) in organ chambers, and evaluated the efficacy of nicaraven as \(\cdot OH\) scavenger.

2. Methods

2.1. Experiment 1

2.1.1. Isolated rat heart preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Male wistar rats (250–350 g) were systemically heparinized (500 IU intraperitoneally), and anaesthetized with sodium pentobarbital (65 mg/kg intraperitoneally). The heart was excised and immediately immersed in Krebs-Henseleit bicarbonate buffer (KHB) solution, consisting of (mmol/l): NaCl (118), KCl (4.7), MgSO\(_4\) (1.2), KH\(_2\)PO\(_4\) (1.2), CaCl\(_2\) (2.5), NaHCO\(_3\) (25.0), and glucose (11.0) at 37°C. Then it was mounted on a Langendorff (L) apparatus (IPW-W, Labo Support, Osaka, Japan) via the aorta, and perfused with KHB solution at a constant pressure of 60 mmHg for 3 min in Langendorff mode (L-mode). KHB solution was filtered (0.22 μm), equilibrated with 95% O\(_2\) and 5% CO\(_2\) and maintained at 37°C. KHB perfusate in this circuit was not recycled. Following cannulation of the left atrium (LA) via the pulmonary vein, the heart was switched to working mode (W-mode) with an LA perfusion pressure of 10 mmHg and an afterload of 60 mmHg. Baseline function was determined following 2 min of W-mode. Measurements were as follows: aortic flow (AF), coronary flow (CF), cardiac output (CO), heart rate (HR), systolic pressure (SP), mean pressure (MP), and rate X pressure product (RPP, HR X max SP). AF and CF were measured by collecting the circulatory fluids from aortic root and coronary sinus, respectively. CO was calculated by adding AF to CF. HR, SP, and MP were recorded simultaneously (RM 6000, NIHON Koden Co., Tokyo, Japan).

2.1.2. Experimental protocol

The experimental protocol is shown in Fig. 1. The hearts were divided into 3 groups (8-h storage, \(n = 5\) hearts/group; 12-h storage, \(n = 6\) hearts/group): group 1, 8- and 12-h storage in HTK solution; group 2, 8- and 12-h storage in HTK solution with 2.5 X 10\(^5\) U/l superoxide dismutase (SOD) and 2 X 10\(^5\) U/l catalase (CAT); group 3, 8- and 12-h storage in HTK solution with 10\(^{-3}\) M nicaraven. The hearts in all groups were arrested by administration of each preservation solution (50 ml/kg body

![Fig. 1. Experimental protocol. (1) Harvesting and preparation. (2,3) Following measurement of baseline cardiac function in W-mode they were arrested and stored for 8 and 12 h. (4) Collection of coronary perfusate for evaluation of lactate, CPK, and troponin-T. (5) Measurement of cardiac function following preservation and reperfusion. (6,8) Measurement of CF perfused with the drug-free KHB solution in L-mode paced in an atrial mode at 330 beats/min. (7,9) Measurement of CF perfused with the KHB solution containing 10\(^{-5}\) M 5-HT and 10\(^{-5}\) M NTG, respectively. W = working mode perfusion; L = Langendorff mode perfusion; CPK = creatine phosphokinase; KHB = Krebs-Henseleit bicarbonate buffer; SOD = superoxide dismutase; CAT = catalase; 5-HT = 5-hydroxytryptamine; NTG = nitroglycerin; CF = coronary flow.](image-url)
weight at 4°C) via the aortic cannula at a pressure of 60 mmHg. They were stored in each preservation solution (30 ml) at 4°C. Following cold storage, they were mounted on a Langendorff apparatus again and reperfused with KHB solution. During reperfusion, the hearts were infused with SOD 1500 U/kg/min and CAT 1500 U/kg/min (2000 U/mg) in group 2 and with nicaraven 1 mg/kg/min in group 3 [9–11]. Then, they were switched to W-mode. The coronary perfusate was collected for 5 min of W-mode reperfusion for evaluation of lactate, CPK, and troponin-T in each group. Lactate, CPK, and troponin-T were determined as previously described [12]. Cardiac functional recovery of the stored heart was measured at the end of 45 min of W-mode reperfusion. LV dp/dt (mmHg/s) was then measured by puncture via the left ventricular apex (pressure transducer, Nihon Kohden Corp., Tokyo, Japan), and the heart was switched back to L-mode and paced in an atrial mode at 330 beats/min (PGM-330, Labo Support, Osaka, Japan).

The hearts were then perfused with the drug-free KHB solution for 20 min for stabilization, during which CF was measured continuously. After stabilization, the hearts were perfused with KHB solution containing 10^{-5} M 5-HT for 15 min and CF was measured for the last 2 min of perfusion. The coronary circulation was washed with the drug-free KHB for the next 20 min to re-establish the basal CF. The hearts were subsequently perfused with KHB solution containing 10^{-5} M nitroglycerin (NTG) for 15 min and CF was measured for the last 2 min of perfusion. A ventricular specimen was weighed immediately, dried at -80°C to constant weight, and reweighed after 24 h. Water content was computed using the following formula: water content (%) = (wet weight - dry weight)/wet weight × 100.

2.2. Experiment 2

2.2.1. Vascular preparation and isometric force measurement

Pig hearts obtained from a slaughter house were immediately drained of blood, within a few minutes of death immersed in ice-cold (4°C) KHB solution, and the left anterior descending (LAD) coronary arteries were dissected. The preparation and method of measuring the mechanical responses of the pig coronary artery were performed according to methods previously reported by Hashimoto et al. [13]. Prior to estimating the relaxation responses to bradykinin a cumulative dose–response curve to prostaglandin F_{2α} was obtained to find the concentration that produces 70% of the maximal contractile response (ED_{70}).

2.2.2. •OH generating system and •OH exposure studies

•OH was generated from Fenton’s reagent by adding 0.28 mM FeSO_{4} plus 0.28 mM H_{2}O_{2} in the KHB solution continuously aerated with 95% O_{2} and 5% CO_{2}. At the plateau phase of contraction induced by PGF_{2α} (2–5 × 10^{-6} M) with the previously determined ED_{50}, the rings were exposed to an •OH generating system for 60 min. At the same time, one parallel group of vessels was incubated in KHB solution without 0.28 mM FeSO_{4} and 0.28 mM H_{2}O_{2}, another parallel group of vessels was incubated in the presence of nicaraven (10^{-4}–10^{-6} M) and •OH generating system and both groups were exposed for 60 min. Nicaraven was added 2 min before the addition of •OH generating system. The rings were serially washed and re-equilibrated for 30 min and after contraction with PGF_{2α} (2–5 × 10^{-6} M), a cumulative dose–response curve of bradykinin-induced relaxation was made. In some experi-

![Fig. 2. A tracing of actual record showing attenuation caused by prior exposure to 0.28 mM H_{2}O_{2} and 0.28 mM FeSO_{4} on bradykinin-induced relaxation of a pig coronary artery. After being contracted by 80 mM KCl, prostaglandin F_{2α} (PGF_{2α}) was cumulatively added to find the concentration that produces 70% of the maximum contractile response (ED_{70}). Ring segments precontracted with PGF_{2α} (ED_{50}; 2.5 × 10^{-6} M) were exposed to H_{2}O_{2}/FeSO_{4} with and without nicaraven for 60 min, were washed out by Krebs-Henseleit bicarbonate buffer solution and bradykinin was cumulatively added to the ring segments precontracted with PGF_{2α} (ED_{70}). Nicaraven (10^{-4} M, 10^{-5} M, and 10^{-6} M) was added 2 min before the addition of H_{2}O_{2}/FeSO_{4}.](image-url)
Table 1
Cardiac functional recovery following preservation and reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>AF (%)</th>
<th>CF (%)</th>
<th>CO (%)</th>
<th>SP (%)</th>
<th>MP (%)</th>
<th>RPP (%)</th>
<th>LV dp/dt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (n = 8)</td>
<td>3.9 ± 2.3</td>
<td>29.7 ± 9.2</td>
<td>11.7 ± 3.9</td>
<td>37.9 ± 8.7</td>
<td>52.9 ± 10.5</td>
<td>28.1 ± 6.4</td>
<td>23.6 ± 5.2</td>
</tr>
<tr>
<td>2. (n = 8)</td>
<td>5.1 ± 0.9</td>
<td>43.0 ± 3.1</td>
<td>15.0 ± 1.4</td>
<td>53.4 ± 3.7</td>
<td>75.9 ± 3.9</td>
<td>40.8 ± 8.0</td>
<td>32.6 ± 4.5</td>
</tr>
<tr>
<td>3. (n = 8)</td>
<td>13.0 ± 5.4</td>
<td>62.8 ± 5.6</td>
<td>25.6 ± 5.1</td>
<td>61.0 ± 3.9</td>
<td>80.6 ± 2.8</td>
<td>42.7 ± 6.0</td>
<td>42.7 ± 3.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± s.e. The haemodynamics of the unstored control hearts were: AF, 46.4 ± 2.9 ml/min; CF, 15.4 ± 1.1 ml/min; CO, 61.3 ± 3.4 ml/min; HR, 239 ± 15 beats/min; SP, 112.3 ± 4.6 mmHg; MP, 55.3 ± 0.6 mmHg; RPP, 25634 ± 421; LV dp/dt, 2350 ± 140. * P < 0.05 vs Group 1. ** P < 0.05 vs Group 2. Group 1 = control group; Group 2 = SOD + CAT group; Group 3 = nicaraven group. SOD = superoxide dismutase 2.5 X 10^5 U/l; CAT, catalase 2.0 X 10^5 U/l; nicaraven 10^-3 M. AF = aortic flow; CF = coronary flow; CO = cardiac output; SP = systolic pressure; MP = aortic mean pressure; RPP = rate X pressure product; LV dp/dt = left ventricular maximum dp/dt.

2.3. Chemicals and reagents

Nicaraven was obtained from Chuogai Pharmaceutical Co. Ltd (Tokyo, Japan). 5-HT and NTG was purchased from Wako Pure Chemical Industries, Ltd (Japan) and Nihon Kayaku Co., Ltd (Japan), respectively. Prostaglandin E2a, tromethamin (PGF2a) was from Cayman Chemical Company (Ann Arbor, MI, USA). Bradykinin, SOD, and CAT were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Statistical analysis

Data are expressed as mean ± s.e.m. In all experiments n refers to the number of animals. Statistical evaluation of the data was performed by using one-way ANOVA and Scheffe's F-test in experiment 1, and Student's paired t-test in experiment 2. Values were considered to be statistically significant when P was less than 0.05.

3. Results

3.1. Experiment 1

3.1.1. Baseline cardiac functions

The mean values of baseline cardiac function (n = 33), measured prior to preservation, were as follows: 45.7 ± 3.4 ml/min of AF, 14.9 ± 1.3 ml/min of CF, 60.2 ± 3.9 ml/min of CO, 245 ± 17 beats/min of HR, 110.3 ± 5.2 mmHg of SP, 55.0 ± 1.0 mmHg of MP, 25.2 ± 0.5 of RPP(X 10^3), and 2350 ± 140 mmHg/s of LV dp/dt.

Table 2
Coronary flow (ml/min)

<table>
<thead>
<tr>
<th>Group</th>
<th>KHB (20 min)</th>
<th>5-HT (15 min)</th>
<th>KHB (20 min)</th>
<th>NTG (15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>5.1 ± 0.6</td>
<td>4.1 ± 0.5 *</td>
<td>4.8 ± 0.5</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>2. SOD + CAT</td>
<td>5.5 ± 0.4</td>
<td>4.6 ± 0.4 *</td>
<td>5.3 ± 0.3</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>3. Nicaraven</td>
<td>5.8 ± 0.4</td>
<td>5.4 ± 0.3 *</td>
<td>5.6 ± 0.3</td>
<td>5.8 ± 0.4</td>
</tr>
</tbody>
</table>

n = 8 hearts per group. Data are expressed as mean ± s.e. SOD = superoxide dismutase 2.5 X 10^5 U/l; CAT = catalase 2.0 X 10^5 U/l; nicaraven 10^-3 M. KHB = Krebs-Henseleit bicarbonate buffer; 5-HT = 5-hydroxytryptamine; NTG = nitroglycerin.

3.1.2. Cardiac functional recovery

The absolute values of cardiac functional recovery following 8 h of storage in groups 1–3 were as follows: 34.5 ± 3.6, 32.7 ± 5.2, 35.1 ± 4.1 ml/min of AF; 10.5 ± 13.3, 13.3 ± 0.7, 14.4 ± 0.5 ml/min of CF; 43.4 ± 3.7, 45.7 ± 4.6, 48.1 ± 3.3 ml/min of CO; 209 ± 13, 196 ± 8, 214 ± 12 beats/min of HR: 20.1 ± 1.4, 19.2 ± 1.8, 22.2 ± 1.7 of RPP(X 10^3); 1690 ± 120, 1870 ± 140, 2210 ± 10 mmHg/s of LV dp/dt. Recovery of CF and LV dp/dt in group 3 were higher than that in group 1 (P < 0.05). There were no significant differences in the other parameters of cardiac functional recovery among the groups.

Table 1 shows the recovery of haemodynamic data on the heart for 12 h of storage. Recovery of CF, CO, SP, MP, and RPP in group 3 was significantly greater than that in group 1. Recovery of CF in the nicaraven group was also superior to that in group 2 (P < 0.05). Recovery of CO in group 3 showed a tendency to increase, compared with that in group 2 (P = 0.07).

There were no significant differences in CF perfused with 5-HT and NTG following 8 h of storage among the groups (data are not shown). Table 2 shows the effects of 5-HT and NTG on post-preservative CF following 12 h of storage. CF perfused with 5-HT was higher in group 3 than in group 1 (P < 0.05). There were no significant differences in NTG-induced CF among the groups.

3.1.3. CPK, lactate, and troponin-T leakage

Following 8 h of storage, the values of CPK, lactate, and troponin-T leakage in groups 1–3 were as follows: 0.06 ± 0.02, 0.10 ± 0.03, and 0.04 ± 0.01 IU/min/g dry.
heart weight of CPK; 6.8 ± 1.2, 7.4 ± 1.9, and 6.5 ± 2.1 mg/min/g dry heart weight of lactate; 13.4 ± 2.1, 10.7 ± 4.0, and 11.0 ± 2.0 ng/min/g dry heart weight of troponin-T, respectively. There were no significant differences in CPK, troponin-T, and lactate leakage among the 3 groups. Following 12 h of storage, there also were no significant differences in CPK, troponin-T, and lactate leakage among the groups (data are not shown).

3.1.4. Water content

The tissue water content following 8 h of storage in groups 1–3 was 79.5 ± 1.2, 78.2 ± 2.1, and 79.8 ± 1.7%, respectively. There were no statistically significant differences among the groups. There also were no significant differences in tissue water content following 12 h of storage among the groups (data are not shown).

3.2. Experiment 2

3.2.1. Effects of prior exposure to \( \cdot \)OH and \( \cdot \)OH in the presence of nicaraven on bradykinin-induced relaxation

As shown in Fig. 3, bradykinin dose-dependently relaxed pig coronary arteries precontracted with PGF\(_{2\alpha}\) (2–5 \( \times \) \( 10^{-6} \) M) which was previously determined ED\(_{70}\). Prior exposure of the arteries to H\(_2\)O\(_2\)/FeSO\(_4\) significantly shifted the dose–response curve of bradykinin-induced relaxation to the right. The maximum relaxation response to bradykinin, however, was not changed by prior exposure of these reagents. The bradykinin did not elicit relaxation in a rubbed artery (data are shown). The plateau phase of contraction induced by PGF\(_{2\alpha}\) was not affected in the presence of nicaraven (10\(^{-4}\)–10\(^{-6}\) M).

When nicaraven at both 10\(^{-4}\) and 10\(^{-5}\) M was added to the arteries with H\(_2\)O\(_2\)/FeSO\(_4\) in advance, the rightward shift of the dose–response curve of bradykinin returned to the control levels. On the other hand, nicaraven at 10\(^{-6}\) M did not exhibit the preventing effect of the drug on bradykinin-induced relaxation of the arteries.

4. Discussion

Stunned myocardium, endothelial damage, and no-reflow involve the production of oxygen-derived free radicals and have recently become the focus of considerable interest. During myocardial ischaemia, xanthine dehydrogenase, which appears to be located in the endothelial cells and myocytes, is converted to xanthine oxidase, an enzyme that produces O\(_2^\cdot\), H\(_2\)O\(_2\), and uric acid from hypoxanthine and molecular oxygen [14]. At the same time, ischaemia is associated with rapid catabolism of ATP and accumulation of hypoxanthine, one of the two substrates for xanthine oxidase. The other substrate (molecular oxygen) becomes

Fig. 3. Effects of prior exposure to H\(_2\)O\(_2\)/FeSO\(_4\) with and without nicaraven on the dose–response curves of bradykinin-induced relaxation in pig coronary arteries. Hydroxyl radical (-OH) was generated from Fenton's reaction by adding 0.28 mM H\(_2\)O\(_2\) and 0.28 mM FeSO\(_4\) in the Krebs-Henseleit bicarbonate buffer solution. Arteries were precontracted with prostaglandin F\(_{2\alpha}\) (ED\(_{70}\): 2–5 \( \times \) \( 10^{-6} \) M). Relaxation was expressed as a percentage of response to the prostaglandin F\(_{2\alpha}\). Nicaraven at 10\(^{-4}\) M (A), 10\(^{-5}\) M (B), and 10\(^{-6}\) M (C) was added 2 min before the addition of H\(_2\)O\(_2\)/FeSO\(_4\). Each point represents mean ± s.e.m. * and # indicate a significant difference between \( \cdot \)OH and control and between \( \cdot \)OH and nicaraven plus \( \cdot \)OH at \( P < 0.05 \), respectively. ED\(_{70}\) = 70% effective dose.
oxidation of hypoxanthine and xanthine proceeds rapidly, available at reperfusion, suddenly and in excess, and the peroxides which may overcome the endogenous antioxidant system [15]. Both ischaemia and O$_2^-$ lead to iron release [5,6]. Therefore, conditions are created in which highly reactive •OH radicals are formed in the ‘oxygen paradox’ [16]. •OH probably exerts its damaging effects by disrupting membrane lipids or membrane-bound proteins which may lead to reperfusion injuries such as myocardial stunning, vascular damage and no-reflow, and arrhythmias [11,17].

Sekili et al. [1] provided direct evidence with EPR that •OH plays an important role in the pathogenesis of myocardial stunning, which can be markedly attenuated without subsequent adverse effects by an •OH scavenger. In spin-trapping experiments designed to measure free-radical concentration, nicaraven was shown to scavenge •OH in a dose-dependent manner [18]. Recently, the spin trapping and EPR observation methods have shown that nicaraven may have strong hydroxyl radical scavenging effects [19]. Therefore, in this study, we investigated the protective effects of nicaraven, a new •OH scavenger, in myocardial and coronary endothelial preservation and reperfusion injury of rat heart. We found better recovery of cardiac function in the nicaraven-treated group than in the non-treated control and SOD/CAT-treated groups. Our results suggest that nicaraven may reduce myocardial P/R injury more effectively than combined treatment with SOD/CAT. SOD and CAT metabolize O$_2^-$ and H$_2$O$_2$, respectively, to benign metabolites. It has been reported that SOD has an extremely short half-life, which may limit its efficacy since considerably more time may be required until an effective tissue concentration is reached [20]. It might be partly attributable to the fact that, in this study, recovery of cardiac function in the SOD/CAT-treated group did not improve as much as that in the non-treated control group. Mak et al. [21] have reported that scavengers such as SOD and CAT may fail to scavenge free radicals when they are generated within the membrane. Nicaraven, which is not only hydrophilic but also lipophilic, might be able to contact and/or permeate the cell membrane and scavenge hydroxyl radicals formed at the cell membrane [7,11,19,22]. This may explain the improvement in cardiac function in the nicaraven-treated group in this study.

The importance of coronary artery endothelium in regulating coronary blood flow and cardiac function has been well established [23,24]. The first target of oxygen-derived free radicals, generated in several pathological processes, is the vascular system, especially the endothelium [14,25]. Vascular damage is one of the major consequences of reperfusion-associated events. No-reflow is a specific type of vascular damage caused by reperfusion [17]. In the isolated rat heart, 5-HT may cause release of endothelium-derived relaxant factor (EDRF). But when the release of EDRF is blocked or the endothelium is damaged, the vasoconstrictive effect of 5-HT on the coronary vasculature is unmasked [26]. Studies of myocardial ischaemia and reperfusion have shown marked alternations in endothelium-dependent relaxation of the coronary vasculature [27]. •OH has been shown to be an important cause of endothelial cell damage and dysfunction [2]. In this study, 5-HT caused a decrease in CF, from which it can be postulated that the coronary endothelium of rat heart was damaged during P/R. Examination of cardiac function in the working mode revealed significantly better recovery of CF in the nicaraven-treated group than in the non-treated control and SOD/CAT-treated groups. Ohta et al. [22] reported that nicaraven may attenuate cerebral vasospasm by restraining abnormal lipid peroxidation initiated in subarachnoid haemorrhage. Nicaraven may exert a protective effect on the coronary endothelium and led to an attenuation of the decrease in CF following P/R similarly in isolated rat hearts. In our study, there were no significant differences in NTG-induced CF among the groups. NTG relaxes vascular smooth muscle through an endothelium-independent pathway; this action of NTG in coronary vascular smooth muscle might have been preserved even after the endothelial dysfunction.

The coronary artery is of particular interest, in this respect, because both the formation of oxygen-derived free radicals and alterations in local blood flow occur simultaneously. Therefore, to search for the endothelial protective effect and to clarify the precise mechanism of action of nicaraven, we developed an in vitro model of oxidative stress of pig coronary artery by generating •OH (H$_2$O$_2$/FeSO$_4$) in organ chambers. The vasodilator, bradykinin, that mediates the release of EDRF was given after the •OH generating system had been removed from the organ chambers. In the present experiments, prior exposure of pig coronary arteries to H$_2$O$_2$/FeSO$_4$ damaged the endothelial function, as depicted by the decreased sensitivity of the artery to bradykinin. The decreased sensitivity was attenuated in the presence of ($10^{-4}$ and $10^{-5}$ M) nicaraven. In this study, however, SOD did not show a protective effect on the coronary endothelium after exposure to •OH (data not shown). SOD metabolizes superoxide radicals to benign metabolites, whereas nicaraven directly scavenges •OH [7,8,17]. Our results suggest that nicaraven might exert a protective effect on the coronary endothelium by its •OH scavenging activity.

We have previously reported that HTK solution is superior to University of Wisconsin (UW) solution for cardiac preservation following 8-h storage [12]. Therefore, in this study, we used HTK solution as a basal preservation solution and examined cardiac function following preservation and reperfusion. In our isolated rat heart model, neutrophils and platelets were absent from the perfusion solution, but other sources of free radical production such as mitochondria, catecholamine oxidation, arachidonic acid metabolism, and xanthine oxidase systems could have produced free radicals [28]. Furthermore, we used pig
coronary arteries in experiment 2, because of their anatomical and pharmacological similarities to human arteries and because they were easy to isolate for this experiment. In our study, there were no significant differences in CPK, troponin-T, and lactate leakage among the 3 groups. A more appropriate collection time may be during the initial period of Langendorff reperfusion when the peak release of these occurs.

In conclusion, nicaraven has protective effects on both myocardium and endothelium during preservation and reperfusion due to its hydroxyl radical scavenging activity. Therefore, combined treatment with nicaraven during both preservation and reperfusion may lead to a safe extension of the heart preservation time and improve morbidity and mortality rates for patients undergoing cardiac transplantation.

Acknowledgements

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