Nicorandil Attenuates Ischemia-Reperfusion Injury Via Inhibition of Norepinephrine Release From Cardiac Sympathetic Nerve Terminals

Yasutaka Fukui, MD, Takashi Nozawa, MD, Hiroaki Ihori, MD, Mitsuo Sobajima, MD, Teruo Nakadate, MD, Akira Matsuki, MD, Makoto Nonomura, MD, Nozomu Fujii, MD, Hiroshi Inoue, MD and Koichiro Kinugawa, MD

Summary
A large amount of norepinephrine (NE) released from cardiac sympathetic nerve terminals might accelerate myocardial ischemic injury. Nicorandil (NICO), K<sub>ATP</sub> channel opener, could attenuate cardiac NE release from the sympathetic nerve terminals during ischemia. The present study aimed to investigate the effects of NICO-induced attenuation of cardiac NE release on myocardial ischemia-reperfusion (I/R) injury in rats, by comparison with the effect of cardiac sympathetic denervation on I/R injury.

Cardiac interstitial NE (iNE) concentrations were determined using a microdialysis method. Rats were divided into 3 groups; control, NICO, and denervation groups. Cardiac sympathetic denervation was performed by painting 10% phenol on the left ventricular epicardium 7 days before producing ischemia. The left coronary artery was ligated for 30 minutes and then re-perfused for 120 minutes. NICO (50 μg/kg/minutes) was infused intravenously starting 20 minutes before the coronary occlusion to the end of the ligation.

The infarct size of the left ventricle was smaller in rats treated with NICO than in control rats (20.2 ± 3.0% versus 50.6 ± 14.7%, P < 0.01). Sympathetic denervation also reduced infarct size (28.5 ± 10.4 %, P < 0.01), which was not significantly different from that in the NICO group. At the end of 30-minute ischemia, iNE increased markedly in control rats (0.1 ± 0.1 to 20.6 ± 5.3 × 10<sup>3</sup> pg/mL), whereas the increase was completely inhibited in denervated rats. NICO markedly attenuated the increase (4.9 ± 3.0 × 10<sup>3</sup> pg/mL, P < 0.01) during ischemia.

NICO-induced attenuation of neural NE release during ischemia might, at least in part, contribute to myocardial protection against I/R injury.

Key words: K<sub>ATP</sub> channel opener, Sympathetic denervation, Microdialysis, Rat
denervation on I/R injury in the rat.

Methods

The present study was performed with the approval of the Animal Experiment Committee of the University of Toyama. **Experimental animals:** Male Wistar rats weighing 300-350 g were used for induction of myocardial ischemia and reperfusion, as described previously. Briefly, the animals were divided into 3 groups, i.e., control, NICO, and cardiac sympathetic denervation groups. Rats were anesthetized with sodium pentobarbital (30 mg/kg, ip), and a left thoracotomy was performed to exteriorize the heart. The left coronary artery was ligated 2-3 mm from its origin with a 5-0 Prolene (ETHICON, INC, Somerville, NJ, USA) suture for 30 minutes, and then the ligation was released and reperfusion was confirmed visually. Evaluation of cardiac hemodynamics was performed before coronary occlusion, and 5 and 20 minutes after inducing coronary occlusion. A 2F micromanometer-tipped catheter was inserted into the right carotid artery to measure aortic pressure.

Myocardial infarct size was determined 2 hours after reperfusion. After an overdose of sodium pentobarbital (70 mg/kg), the heart was removed quickly and mounted on a Langendorff apparatus. The left coronary artery was re-ligated at the same position and the heart was perfused with blue dye to stain the perfused myocardium blue, whereas the area at risk remained unstained. The left ventricle (LV) was then sliced into 2-mm-thick sections for incubation in triphenyl tetrazolium chloride for 10 min at 37°C to distinguish stained viable tissue from unstained infarct area. The area at risk and infarct area were quantified using computer-assisted planimetry. NICO was infused intravenously at a rate of 50 μg/kg/min starting from 20 minutes before the onset of coronary ligation to the end of coronary occlusion.

**Cardiac sympathetic denervation:** Regional cardiac denervation was performed 1 week before the transient coronary artery occlusion, as described previously. Briefly, the rats were anesthetized with sodium pentobarbital (30 mg/kg, ip), and a left thoracotomy was performed to exteriorize the heart. A solution of 10% phenol in ethanol was used for the determination of myocardial infarct size, cardiac microdialysis was performed to determine iNE concentrations in LV tissue, as described previously. Rats were anesthetized with sodium pentobarbital (30 mg/kg, ip) followed by continuous, intravenous infusion (3 mg/kg/hour). Body temperature was maintained with a heated pad and lamp. The heart was exposed by midline incision and a microdialysis probe was inserted into the myocardium along the left coronary artery using an attached needle. A linear microdialysis probe was used to minimize tissue damage while providing a secure implantation. Both ends of the dialysis fiber (length 8 mm, outer diameter 0.31 mm, inner diameter 0.2 mm, molecular weight cutoff 50,000, PAN-130SF, Asahi Chemical, Tokyo, Japan) were connected to polyethylene tubes. The dialysis probe was perfused with Ringer’s solution at a rate of 2 μL/minute. A baseline dialysate sample was collected after a 60-minute stabilization period. The left coronary artery was then ligated 2-3 mm from its origin for 30 minutes. Dialysate sampling was performed between 15 and 30 minutes after the start of ligation. Dialysate samples were stored at -80°C for later analyses. After the sampling, the heart was isolated and perfused with Krebs solution. The position of the microdialysis probe was confirmed by determination of the ischemic area using blue dye.

The NE concentration of dialysate was measured by high performance liquid chromatography (HPLC) with electrochemical detection (EC 300, ELICOM, Kyoto, Japan). NE concentrations of dialysate were determined by comparison with the heights of chromatographic peaks corresponding in retention times to those obtained by the standard solution of NE (0.5 ng/mL).

**Statistics:** Data are expressed as means ± SD. Groups were compared using an ANOVA followed by a Bonferroni test to identify differences among groups. A P value of < 0.05 was considered significant.

**Results**

**Hemodynamic data and infarct size:** Before coronary occlusion, neither heart rate nor blood pressure was different among the control, NICO, and phenol groups (Table). During coronary occlusion, both systolic and diastolic blood pressures in the NICO group were not significantly different from those of the control and phenol groups, although there was a trend toward a decrease in systolic blood pressure in rats treated with NICO and phenol.

Examples of images for blue dye and triphenyl tetrazolium chloride stains in each group are shown in Figure 1. The size of the area at risk was 60% of the LV in the control group, and it was not different among the 3 study groups; however, the ratio of infarct area to area at risk

<table>
<thead>
<tr>
<th>Table. Heart Rate and Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>Nicorandil (n = 7)</td>
</tr>
<tr>
<td>Phenol (n = 5)</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>Before ischemia</td>
</tr>
<tr>
<td>HR (bpm)</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
</tr>
<tr>
<td>5 minutes after occlusion</td>
</tr>
<tr>
<td>HR (bpm)</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
</tr>
<tr>
<td>20 minutes after occlusion</td>
</tr>
<tr>
<td>HR (bpm)</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
</tr>
</tbody>
</table>

HR indicates heart rate; sBP, systolic blood pressure; dBP, diastolic blood pressure; n, number of rats; and NS, no significant. Data are means ± SD.
Effects of Nicorandil on cardiac NE release

Figure 1. Images of serial sections for both blue dye (A, area at risk) and triphenyl tetrazolium chloride stains (B, infarct size) in each group. The area at risk (A) and infarct area (B) are unstained.

Figure 2. The area at risk (left panel) and infarct area (right panel) following 30-minute ischemia and 2-hour reperfusion were determined using a Langendorff apparatus with blue dye and triphenyl tetrazolium chloride, respectively. The size of the area at risk is not different among the 3 study groups; however, the ratio of infarct area to area at risk is smaller in the NICO and phenol groups than in the control group. The number of rats is 7 in each group. Data are mean ± SD.

Discussion

The major findings of the present study were as follows. First, iNE concentrations in ischemic tissue were markedly increased during 30-minute coronary occlusion. NICO strongly inhibited the ischemia-induced increase in iNE, while coronary occlusion did not increase iNE in the rats treated with phenol. Second, NICO attenuated myocardial I/R injury and the infarct size decreased to 39% of that of control rats, and sympathetic denervation also reduced the infarct size to 57% of that of control rats, although the severity of myocardial injury was determined only by infarct size, not using biomarkers such as troponin T. These results were consistent with those of previous studies, i.e., NICO has been shown to protect against I/R injury and iNE release during ischemia was attenuated by NICO. Based on the present results together with those of previous studies, we propose that NICO-induced attenuation of cardiac NE release from sympathetic nerve fibers was smaller in the NICO group than in the control group (20.2% versus 50.6%, P < 0.01, Figure 2). The ratio was also smaller in the phenol group (28.5%), but it was not significantly different between the phenol and NICO groups.

Interstitial norepinephrine concentration of LV tissue: Before coronary occlusion, a small peak of NE in dialysate, i.e., iNE, was detected on an HPLC chart, but the peak was markedly increased during coronary occlusion in a control rat (Figure 3). However, the peak during coronary occlusion was markedly attenuated in a rat treated with NICO, and it disappeared in a rat treated with phenol painting.

As shown in Figure 4, NICO did not affect iNE concentrations before coronary occlusion. In control rats, iNE was 87 pg/mL before coronary occlusion, and it increased markedly to 20.6 × 10^3 pg/mL during 30-minute coronary occlusion. NICO significantly attenuated the ischemia-induced increase in iNE (4.1 × 10^3 pg/mL). Concentrations of iNE were measured in 2 rats treated with phenol, because we had previously confirmed that both iNE concentrations and NE contents of LV tissue were quite low in rats treated with phenol. As expected, coronary occlusion did not affect cardiac iNE (26 and 60 pg/mL before and during coronary occlusion, respectively) in the present study, indicating sympathetic denervation in these rats.
Figure 3. Representative examples of NE concentrations in dialysate detected using an HPLC system. The height of the chromatographic peaks corresponding to retention times to those of the directly injected NE standards were determined. A red peak in each panel corresponds to the level of NE. HPLC charts in a control rat before (A) and during coronary occlusion (B), and in nicorandil (C) and phenol rats (D) during coronary occlusion.

Figure 4. NE concentrations in dialysate before (left panel) and during coronary occlusion (right panel). Before coronary occlusion, there are no differences in NE concentrations between control and nicorandil-treated animals. During coronary occlusion, however, NE concentrations increase markedly in controls, but their increase is significantly attenuated with nicorandil. As expected, coronary occlusion does not increase NE concentrations in phenol-treated animals. The number of rats is 6, 6, and 2 in the control, nicorandil, and phenol groups, respectively. Data are mean + SD.

 terminals during ischemia, at least in part, contribute to the reduction of infarct size in I/R hearts.

**Interstitial NE concentrations during ischemia:** Microdialysis has been used to sample the extracellular compartment of organs. The levels of iNE are affected by various factors, including the magnitude of sympathetic nerve drive to each organ, the amount of NE release from the nerve terminals, and neuronal function at nerve terminals. Circulating NE in plasma may also affect iNE levels. During the early phase of myocardial ischemia (less than 10 minutes after the onset of ischemia), the release of NE is exocytotic and depends on the activation of efferent sympathetic neurons. During 10- to 40-minute myocardial ischemia, however, an increase in cardiac iNE is primarily caused by non-exocytotic NE release, which is transported across the axolemma from the cytoplasm to the interstitial space via the neuronal uptake carrier in the reverse direction to that of its normal transport. This release is independent of both central sympathetic activation and extracellular calcium. In the present study, iNE concentrations increased more than 200-fold during 15 to 30 minute-coronary occlusion, compared to the basal level before coronary occlusion, a finding consistent with previous studies. In rats treated with nicorandil, the increase in iNE during ischemia was markedly attenuated and the levels of iNE were reduced to 25% of those in control rats without NICO in the present study. In a previous study, however, the effect of NICO on iNE was completely abolished by a K<sub>ATP</sub> channel blocker, glibenclamide. The existence of K<sub>ATP</sub> channels on nerves has been demonstrated, and, therefore, K<sub>ATP</sub> channels on the sympathetic nerve terminals may act as effector of neural protection by NICO. However, Schömig, et al. reported that ischemia-induced NE release was mediated by starvation of axoplasmic ATP, leading to counterclockwise NE release through uptake-1 carriers on the sympathetic nerve terminals. The precise mechanism of NICO-induced reduction of NE release from the sympathetic nerve terminals remains unclear, but suppression of energy starvation and K<sub>ATP</sub> channels on the
nerve terminals may be involved.

Effect of interstitial NE concentration on ischemia-reperfusion injury: Previous studies have shown that the epicardial application of phenol results in cardiac sympathetic denervation.20,21 Three days following application of phenol, a thin layer of subepicardial myocardium showed coagulation type of necrosis with mild reactive inflammation extending to a depth of about 0.25 mm in areas where there was no epicardial fat, and the underlying myocardium was normal where the epicardium contained a thin layer of fat.22 Moreover, phenol does not affect contraction or electrophysiological characteristics of ventricular muscle.20,21 Thus, the influence of phenol on myocardium might be small, and phenol painting might little affect the infarct size in the present study. In our previous study using the same method as the present study,23 phenol painting depleted the cardiac tissue content of NE to 3% of control animals and 30-minute coronary occlusion did not affect cardiac iNE, a finding compatible with sympathetic denervation.

It is generally accepted that excess levels of NE could lead to myocardial injury.3,4 Previous studies2,3,4 showed beneficial effects of cardiac denervation on infarct size during ischemia. Other studies, however, demonstrated that cardiac denervation had no protective effect on ischemic injury,35 or rather had a detrimental influence.36 These conflicting data may come from differences in the experimental methods used and in the duration of coronary artery occlusion. A prolonged duration of ischemia may mask a beneficial effect of cardiac denervation.27

Several mechanisms to inhibit ischemic injury in denervated hearts have been proposed. A study showed that sympathetic denervation might develop collateral vessels in dog hearts.25 However, rat hearts have relatively poor native collateral networks26 and, therefore, beneficial effects of denervation may not primarily result from increasing collateral flow in the present study. During the 30-minute coronary occlusion of the present study, iNE increased more than 200-fold compared with iNE before coronary occlusion in control rats, but it did not increase in denervated rats. Schömig27 also demonstrated that, during 20-40 minutes of ischemia, the extracellular concentration of NE due to the non-exocytotic release reaches 100-1000 times the normal concentration. Such a concentration of NE is capable of producing myocardial necrosis even in non-ischemic hearts.37 The pathogenesis of catecholamine-induced myocardial injury is beyond the scope of the present study but may be multifactorial. Excess NE might induce intracellular calcium overload, changes in permeability of the sarcolemmal membrane, and increased oxidative stress.38,39 Moreover, in the ischemic myocardium, adrenergic stimulation is aggravated by β-receptor specific sensitization of the β-adrenergic system.40

NICO has both ATP-sensitive KATP channel opener and nitrate properties, and it has a protective effect on myocardial I/R injury in animal models41,42 and humans.43-46 Ishii, et al.47 reported in a randomized study that the addition of intravenous NICO to percutaneous coronary intervention prevented cardiovascular events in patients with acute myocardial infarction, although NICO did not limit infarct size in a multicenter study that was performed using a lower dose of NICO compared to a previous study.48 The cardioprotective effect of NICO may be primarily mediated by selective activation of KATP channels in the mitochondrial inner membrane of myocytes,49 while NICO markedly reduced NE release during ischemia. The complete inhibition of NE release by sympathetic nerve denervation significantly reduced infarct size in reperfused hearts. Taken together, NICO-induced myocardial protection against I/R injury might result, at least in part, from the attenuation of massive NE release during prolonged ischemia.

Limitations: The present study had several limitations. First, the dose of NICO in the present study was determined according to a study, in which NICO was infused at a rate of 50 µg/kg/minute from 20 minutes before coronary occlusion to the end of ischemia in rats and the infarct size after reperfusion decreased to 58% of that of control rats.40 Further works are needed to elucidate the dose-response effect of NICO for protection against I/R injury and inhibition of NE release. Second, during more than 40-minute ischemia, the release of NE might result from structural changes in the membrane of cardiac neurons50 and, therefore, the beneficial effects of NICO on NE release and/or myocardial I/R injury may be attenuated in a heart after a long period of ischemia lasting more than 40 minutes. Unfortunately, circulating NE levels were not evaluated in the present study. Plasma levels of NE may be increased during coronary occlusion, but their increase would be relatively small in the present study because neither heart rate nor blood pressure was significantly affected during coronary occlusion (Table). Third, in the present study, the effect of NICO on myocardial infarct size in denervated hearts was not evaluated. The infarct size of NICO-treated animals might be smaller in denervated hearts than in innervated hearts, but the difference might be small because, during coronary occlusion, NICO markedly reduced the levels of iNE in innervated hearts to 25% of those without NICO. Finally, NICO has both neural protection and direct myocyte protection effects in ischemic hearts. An intervention using a KATP channel blocker might abolish NICO’s effect of direct myocyte protection via the mitochondrial KATP channels, while it might also abolish neural protection51 because there are KATP channels in nerve terminals.52,53 To the best of our knowledge, pharmacological agents that selectively block the activation of mitochondrial KATP channels in myocytes but do not affect KATP channels in nerve terminals have not been developed. A further study using a drug specific to cardiac sympathetic nerves will clearly differentiate NICO-induced myocardial protection through the inhibition of NE release from its direct effect on myocytes.

Conclusion

Clinical trials have shown that NICO exerts beneficial effects as an adjunctive therapy for patients with acute myocardial infarction who receive coronary reperfusion therapy.44,45 NICO could markedly inhibit cardiac iNE concentrations during ischemia, and this neural protection
induced by NICO might, at least in part, contribute to the attenuation of infarct size in I/R injury.

**Disclosures**

**Conflict of Interest:** None.

**References**


Supplemental Files

Supplemental Figure 1, 2
Please see supplemental files:
https://www.jstage.jst.co.jp/article/ihj/58/6/58_16-391/-article/supplement