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Nicorandil preserves mitochondrial function during ischemia in perfused rat heart

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Abstract

A possible mechanism for the action of nicorandil on the improvement of energy metabolism of ischemic/reperfused hearts was examined. Perfused rat hearts were subjected to 35-min ischemia/60-min reperfusion. The heart was treated with nicorandil at concentrations of 10 to 100 μM for the last 30-min of pre-ischemia. Nicorandil preserved the mitochondrial oxygen consumption rate during ischemia and attenuated the decrease in mitochondrial function during reperfusion in association with the enhanced post-ischemic recovery of the left ventricular developed pressure. To assess the direct effect on mitochondria, myocardial saponin-skinned bundles were incubated under hypoxic conditions in vitro. Hypoxia-induced decrease in the mitochondrial oxygen consumption rate was attenuated by treatment of the bundles with 100 μM nicorandil. This attenuation was abolished by the combined treatment with the K_{ATP} channel blocker, 5-hydroxydecanoate (5-HD). These results suggest that nicorandil is capable of attenuating ischemia/reperfusion injury of isolated perfused hearts through preservation of mitochondrial function during ischemia.

Keywords: High-energy phosphate; Ischemia/reperfusion injury; K_{ATP} channel opener; Mitochondrion; Mitochondrial K_{ATP} channel; Nicorandil

1. Introduction

Nicorandil, *N*-(2-hydroxyethyl)-nicotinamide nitrate ester, has a combined chemical structure of an organic nitrate and a nicotinamide and is an efficacious drug for treatment of ischemic heart disease. Recently, the clinical study by The IONA Study Group (2001) has shown that treatment of patients with angina pectoris with nicorandil reduced the incidence of cardiovascular events. This drug relaxes vascular smooth muscle by stimulating soluble guanylate cyclase leading to increased cGMP levels (Endoh and Taira, 1983; Holzmann, 1983; Meisheri et al., 1991) and also opening of ATP-sensitive K^+ (K_{ATP}) channels to hyperpolarize the plasma membrane (Furukawa et al., 1981; Kukovetz et al., 1991; Holzmann et al., 1992). A number of investigations have shown that nicorandil is not only a vasodilator but also a protector of the ischemic injury in animals and humans (Abiko et al., 1984; Grover et al., 1990; Lamping and Gross, 1985), probably due to its K_{ATP} channel-opening property. Recently, Garlid et al. (1997) suggested that K_{ATP} channel openers protected the ischemic heart in a manner parallel to the degree of the interaction with mitochondrial K_{ATP} channels. In addition, Sakai et al. (1999) described that nicorandil and/or its metabolite are distributed in the mitochondria of the heart after oral administration to rats. Thus, it is possible that nicorandil may protect the ischemic heart through the opening of mitochondrial K_{ATP} channels.

It has been shown that K_{ATP} channels present on the mitochondrial inner membrane regulate mitochondrial volume and energetics (Inoue et al., 1991; Paucek et al., 1992; Garlid et al., 1996; Holmuhamedov et al., 1998). Regulation of volume changes plays an important role in the metabolic control in mitochondria (Halestrap, 1989). Thus, the effects of K_{ATP} channel openers on the mitochondria may be linked indirectly to improvement of cardiac energy production. However, the mechanism by which opening of mitochondrial K_{ATP} channels may lead to cardioprotection remains unclear.

In the present study, we sought to determine whether or not nicorandil may protect cardiac mitochondrial function against ischemia/reperfusion injury through its action on the energy-producing ability of mitochondria.

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2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 230–290 g, were used in the present study. The animals were conditioned at 23 ± 1 °C with a constant humidity of 55 ± 5%, a cycle of 12-h light and 12-h darkness, and were given free access to food and tap water according to the *Guide for the Care and Use of Laboratory Animals* as promulgated by the National Research Council. The protocol of this study was approved by *The Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Science*.

2.2. Agents

Nicorandil, *N*-(2-hydroxyethyl)-nicotinamide nitrate ester, was kindly provided by Chugai Organic Chemistry Laboratory. Sodium 5-hydroxydecanoate (5-HD) was purchased from Sigma (St. Louis, MO).

2.3. Perfusion of hearts

The rats were anaesthetized with diethyl ether. The hearts were rapidly isolated, transferred to a Langendorff apparatus, and perfused at 37 °C with a constant flow rate (9 ml/min) of Krebs–Henseleit bicarbonate buffer of the following composition (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.25; $NaHCO_3$, 25; and glucose, 11. The perfusion buffer was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 to pH 7.4. A latex balloon, with an uninflated diameter of 3.7 mm and connected to a pressure transducer (TP-200, Nihonkohden, Tokyo, Japan), was inserted into the left ventricular cavity through the mitral opening and secured by ligation. A pressure 5-mm Hg of the initial left ventricular end-diastolic pressure was loaded onto the perfused heart. Left ventricular developed pressure, a convenient marker of cardiac contractile function, was monitored by a pressure transducer (TP-200T, Nihonkohden) connected to a carrier amplifier (AP-621G, Nihonkohden) throughout the experiment. Perfusion pressure was monitored through a branch of an aortic cannula by means of an electronic manometer (TP-400, Nihonkohden) connected to a carrier amplifier (AP-621G, Nihonkohden) throughout the experiment. Heart rate was measured by means of a heart rate counter (AT-601G, Nihonkohden). Hemodynamic parameters were recorded on a thermal pen recorder (WT-645G, Nihonkohden).

After a 15-min equilibration, the heart was paced at 300 beats/min with an electrical stimulator via two silver electrodes directly attached to the heart and an additional 45-min perfusion was carried out, and then the perfusion was stopped (ischemia). The heart was immediately submerged in an organ bath filled with the Krebs–Henseleit bicarbonate buffer in which the 11 mM glucose had been replaced with 11 mM Tris–HCl. This buffer had been previously equilibrated with a gas mixture of 95% N_2 and 5% CO_2 to pH 7.4 and maintained at 37 °C during the experiment to avoid hypothermia-induced cardioprotection. After 35 min of ischemia, the buffer in the organ bath was drained, and the hearts were reperfused for 60 min at 37 °C with the Krebs–Henseleit bicarbonate buffer equilibrated with a gas mixture of 95% O_2 and 5% CO_2. The hearts were paced throughout the experiment except for the first 15 min of reperfusion, to prevent contractile irregularities, which might sometimes occur during this period.

For the purpose of comparison, hearts were perfused for 95 min under normoxic conditions, the time equal to that for ischemia plus reperfusion (normoxic group).

Treatment of the perfused hearts with different concentrations of nicorandil (10, 30 or 100 μM) was carried out by perfusing the hearts with the agent in Krebs–Henseleit bicarbonate buffer for the last 30 min of pre-ischemia. Nicorandil was dissolved and diluted in the Krebs–Henseleit buffer.

2.4. Examination of perfusate

The perfusate eluted from the heart was collected to determine creatine kinase activity according to the method of Bergmeyer et al. (1970). The perfusate was also used for determination of purine nucleosides and bases (ATP metabolites) such as adenosine, inosine, and hypoxanthine by the high-performance liquid chromatograph (HPLC) method described previously (Takeo et al., 1988).

2.5. Determination of myocardial energy metabolites

After appropriate sequences of perfusion, the hearts were freeze-clamped with aluminum tongs pre-cooled with liquid nitrogen. The frozen ventricle was pulverized and mixed with 0.3 M $HClO_4$ and 0.25 mM EDTA under liquid nitrogen cooling. The extract was centrifuged at $8000 \times g$ for 15 min at 4 °C, and the resulting supernatant was sampled for determination of myocardial ATP, ADP, AMP, and creatine phosphate by the HPLC method described previously (Takeo et al., 1996). Myocardial creatine phosphate was converted to ATP by the enzymatic method of Lowry and Passonneau (1972). Energy charge was calculated from the formula $(ATP + 0.5 \times ADP)/(ATP + ADP + AMP)$.

2.6. Mitochondrial oxygen consumption rate

The mitochondrial oxygen consumption rate was determined by the method of Sanbe et al. (1993), which is a modification of the method of Saks et al. (1989). After appropriate sequences of perfusion, the untreated and nicorandil-treated hearts were quickly removed from the perfusion apparatus. Myocardial bundles, 0.3 to 0.4 mm in diameter and 3 to 4 mm in length, were prepared from the left ventricular free wall by use of a McIlwain Tissue

Chopper (Mickle Lab. Engineering, NY) and transferred into relaxing medium A of the following composition (mM): EGTA, 10; $MgSO_4$, 3; taurine, 20; dithiothreitol, 0.5; imidazole, 20; potassium 2-(*N*-morpholino)-ethanesulfonate, 160; ATP, 5; creatine phosphate, 15 (pH 7.0). The bundles were incubated for 20 min in 1 ml of medium A containing 75 μg/ml saponin. After incubation, the bundles (skinned bundles) were washed for 10 min in fresh medium B (medium A without ATP and creatine phosphate but supplemented with 0.5% bovine serum albumin) to remove the saponin. All procedures were carried out at 4 °C. The oxygen consumption rate of skinned bundles was determined by means of a Clark-type electrode connected to an Oxygraph (Central Kagaku, Tokyo) containing skinned bundles in 1.0 ml of medium B at 30 °C. The basal oxygen consumption rate was measured following the addition of 5 mM glutamate, 3 mM malate, and 3 mM KH_2PO_4. Maximal oxygen consumption rate was measured after further addition of 1 mM ADP and 7.5 mM creatine. The velocity of ADP-stimulated oxygen consumption rate of skinned bundles was taken as the difference between maximal and basal glutamate/malate-stimulated oxygen consumption rates. After determination of the oxygen consumption rate, the skinned bundles were solubilized with 0.5 ml of 2 N NaOH for 30 min at 60 °C, and then the protein concentration was determined according to the method of Lowry et al. (1951). The mitochondrial oxygen consumption rate was expressed as nano-atoms of oxygen consumed per min per mg protein.

2.7. Release of cytochrome c from mitochondria of the perfused heart after ischemia

At the end of ischemia, untreated and agent-treated hearts were quickly removed from the perfusion apparatus. Tissue was mildly homogenized in an ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM dithiothreitol, 2 μg/ml leupeptin, 2.5 μg/ml aprotonin, and 0.5 mM phenylmethylsulfonyl fluoride (pH7.4). The homogenate was centrifuged at $900 \times g$ for 10 min at 2 °C. The supernatant was aspirated and centrifuged at $8000 \times g$ for 30 min at 2 °C. The resulting supernatant was centrifuged at $100{,}000 \times g$ for 30 min at 2 °C to remove any mitochondrial contamination. The cytosolic fraction was denatured in Laemmli buffer (Tris–HCl 250 mM, sodium dodesylsulfate (SDS) 4%, glycerol 10%, bromophenol blue 0.006%, β-mercaptoethanol 2%, pH 6.8) at 100 °C and fractionated by SDS electrophoresis on a 15% polyacrylamide gel. The fractionated proteins were then transferred onto a nitrocellulose filter, which was probed with a monoclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated donkey anti-mouse or rabbit immunoglobulin G (Amersham Pharmacia Biotech, Buckinghamshire, UK). Bound antibody was detected by the chemiluminescence method using ECL^R reagents (Amersham Pharmacia Biotech). The concentration of cytochrome *c* in the sample was estimated by the densitometric method by comparing with the bands of the samples with the reference obtained with the standard solution of horse heart cytochrome *c* (1.25 ng).

2.8. Hypoxic incubation of skinned bundles

To determine whether nicorandil directly affects mitochondrial function or not, we prepared saponin-skinned bundles of the left ventricular free wall from non-perfused hearts. The bundles were placed into 2 ml of medium B in a tightly sealed chamber at 30 °C for 30 min. The hypoxic condition was induced by saturation of the chamber with 100% N_2 at the flow rate of 20 ml/min as described previously (Tanonaka et al., 1999). After a 30-min hypoxic or normoxic incubation, the skinned bundles were quickly transferred to the glass cell, and then their oxygen consumption rates were determined as described above. Nicorandil was added to the incubation medium in the chamber before addition of the skinned bundles. A combination of nicorandil + 5-HD was also examined in the same manner. 5-HD was dissolved in dimethylsulfoxide (DMSO) and diluted in medium B. The final concentration of DMSO in the medium B was 0.1%. We previously confirmed that this concentration of DMSO had no effect on oxygen consumption rates under hypoxic or normoxic conditions at least for 120 min.

2.9. Statistics

The results were expressed as the means ± S.E.M. The statistical significance of differences in left ventricular developed pressure, level of left ventricular end-diastolic pressure, and perfusion pressure at the end of 35-min ischemia and at the end of reperfusion between the hearts treated and untreated with nicorandil was evaluated by analysis of variance (ANOVA), followed by Bonferroni's or Dunnett's multiple comparison. Unpaired Student's *t*-test was performed for comparison between two groups. Differences with a probability of less than 5% were considered to be statistically significant ($P < 0.05$).

3. Results

3.1. Cardiac function of perfused hearts

The time course of changes in left ventricular developed pressure of ischemic/reperfused hearts treated with 10, 30, or 100 μM nicorandil is shown in Fig. 1. Changes in left ventricular developed pressure were expressed as percentages of the initial values.

Baseline (initial) values for left ventricular developed pressure ranged from 79.0 ± 6.0 to 89.4 ± 14.0 mm Hg ($n = 5$ for each group). Nicorandil at 10, 30 or 100 μM decreased left ventricular developed pressure at the end of the agent-treatment; 91 ± 2%, 91 ± 6% or 81 ± 5% of initial

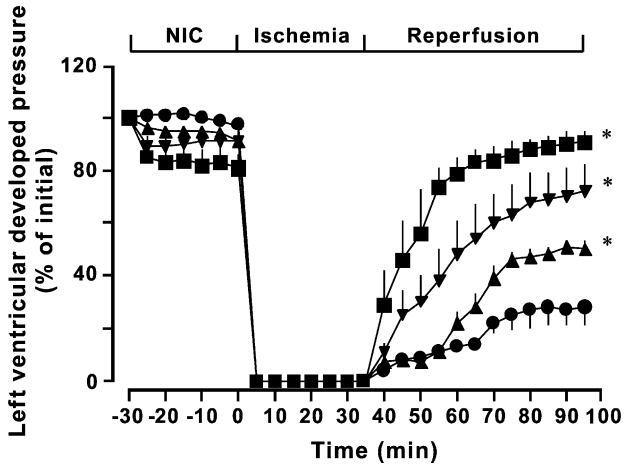

Fig. 1. The time course of changes in left ventricular developed pressure of the ischemic/reperfused untreated heart (●) and the heart treated with 10 (▲), 30 (▼), or 100 μM nicorandil (■). Each value represents the mean ± S.E.M. of five experiments. Treatment with nicorandil was conducted for the last 30 min of pre-ischemia. * Significantly different from the corresponding untreated ischemic/reperfused group ($P < 0.05$).

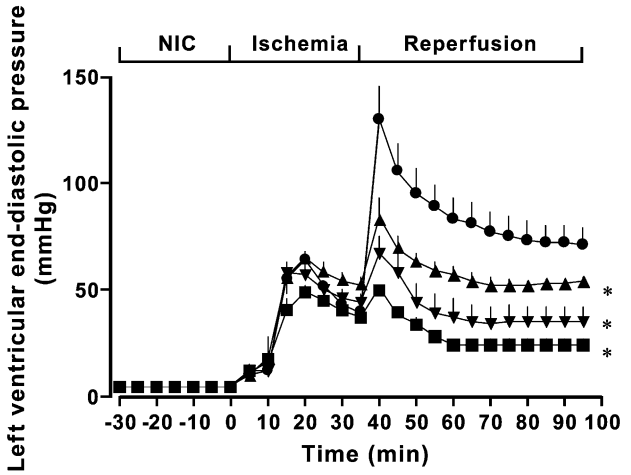

Fig. 2. The time course of changes in level of left ventricular end-diastolic pressure of the ischemic/reperfused untreated heart (●) and the heart treated with 10 (▲), 30 (▼), or 100 μM nicorandil (■). Each value represents the mean ± S.E.M. of five experiments. Treatment with nicorandil was conducted for the last 30 min of pre-ischemia. * Significantly different from the corresponding untreated ischemic/reperfused group ($P < 0.05$).

value ($n = 5$ each), respectively. After the onset of ischemia, left ventricular developed pressure declined to zero within 2.5 min, and it remained at that value during ischemia. The left ventricular developed pressure of the heart recovered to approximately 28 ± 7% of the pre-ischemic value by the end of 60-min reperfusion period. In contrast, left ventricular developed pressures of the hearts treated with 10, 30, or 100 μM nicorandil were significantly recovered to 50 ± 3%, 72 ± 10%, and 91 ± 4%, respectively, of the pre-ischemic value at the end of reperfusion. The level of left ventricular end-diastolic pressure of the untreated heart began to rise at 5 min after the onset of ischemia and reached its peak level approximately 20 min of ischemia (Fig. 2). It was further increased upon reperfusion; the maximum level having been reached at 5 min after the onset of reperfusion. Although the left ventricular end-diastolic pressure gradually declined during reperfusion, this high level of left ventricular end-diastolic pressure was sustained throughout reperfusion. In contrast, treatment with various concentrations of nicorandil attenuated the rise in level of left ventricular end-diastolic pressure during reperfusion in a concentration-dependent manner, but not during ischemia. When perfused hearts were treated with 100 μM nicorandil only during reperfusion, the recovery of left ventricular developed pressure was not enhanced at the end of the reperfusion period (28 ± 7% recovery for untreated heart versus 30 ± 6% recovery for 100 μM nicorandil-treated heart, $n = 5$ each).

Fig. 3 shows the time course of changes in perfusion pressure of ischemic/reperfused hearts. Baseline (initial) values for perfusion pressure ranged from 87.2 ± 5.1 to 97.6 ± 5.0 mm Hg ($n = 5$ for each group). Nicorandil at 10, 30, or 100 μM decreased perfusion pressure at the end of the agent-treatment; 62 ± 1%, 68 ± 8%, or 63 ± 5% of initial value ($n = 5$ each), respectively. No significant relationship between perfusion pressure at the end of pre-ischemia and post-ischemic recovery of left ventricular developed pressure could be seen in hearts treated with 10, 30, or 100 μM nicorandil ($r = 0.253$, $n = 18$, $P > 0.05$).

Since treatment with 100 μM nicorandil resulted in the maximum recovery of left ventricular developed pressure and level of left ventricular end-diastolic pressure, we employed 100 μM nicorandil in the subsequent experiments to further characterize the pharmacological properties of the agent.

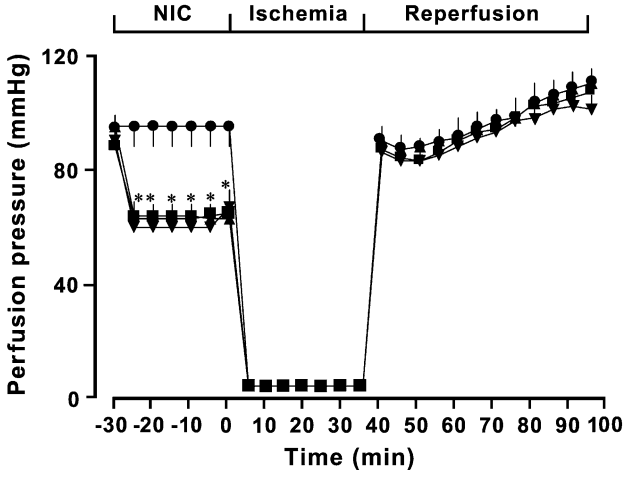

Fig. 3. The time course of changes in perfusion pressure of the ischemic/reperfused untreated heart (●) and the heart treated with 10 (▲), 30 (▼), or 100 μM nicorandil (■). Each value represents the mean ± S.E.M. of five experiments. Treatment with nicorandil was conducted for the last 30 min of pre-ischemia. * Significantly different from the corresponding untreated ischemic/reperfused group ($P < 0.05$).

Table 1
Creatine kinase activity and the amount of purines and bases (ATP metabolites) in the perfusate eluted from ischemic/reperfused or normoxic hearts untreated or treated with 100 μM nicorandil prior to ischemia

	Normoxia	Ischemia/reperfusion
Creatine kinase activity (nmol NADPH/min/g wet tissue)		
None	0.7 ± 0.2	91.5 ± 6.1[a]
Nicorandil	0.6 ± 0.2	13.2 ± 2.6[a,b]
ATP metabolites (μmol/g wet tissue)		
None	0.08 ± 0.02	1.94 ± 0.12[a]
Nicorandil	0.08 ± 0.02	1.29 ± 0.09[a,b]

Each value represents the mean ± S.E.M. of five experiments.
[a] Significantly different from the corresponding normoxic group ($P < 0.05$).
[b] Significantly different from the corresponding untreated ischemic/reperfused group ($P < 0.05$).

3.2. Release of creatine kinase and ATP metabolites in the perfusate

To determine the amounts of creatine kinase and ATP metabolites released from perfused hearts, we collected the perfusate of the heart (Table 1). During the 30-min period of pre-ischemic perfusion, creatine kinase activity in the perfusate was less than 1 nmol NADPH/min/g wet tissue regardless of the presence or absence of nicorandil ($n = 5$ each). Creatine kinase activity in the perfusate of the heart perfused for 95 min under normoxic conditions was less than 1 nmol NADPH/min/g wet tissue regardless of treatment with or without 100 μM nicorandil. Creatine kinase activity in the perfusate from the untreated heart markedly increased during reperfusion, and treatment with nicorandil attenuated this increase.

The amount of ATP metabolites released is shown in Table 1. The ATP metabolites were minimally released from the normoxic heart as well as the nicorandil-treated, normoxic heart. Ischemia/reperfusion induced a marked release of the ATP metabolites. Treatment with nicorandil attenuated this increase significantly.

3.3. Myocardial energy metabolites

Myocardial energy metabolites such as ATP, ADP, AMP and creatine phosphate were determined in the heart treated with nicorandil to examine the myocardial energy profile (Table 2). Myocardial ATP and creatine phosphate contents at the end of the pre-ischemia were 26.61 ± 0.64 and 35.64 ± 1.09 μmol/g dry tissue, respectively ($n = 5$). The metabolite contents of the myocardium at 95 min of normoxia were similar to those at the end of pre-ischemia (at 0 min). Myocardial ATP and creatine phosphate contents at the end of the ischemia were approximately 3% and 10% of the pre-ischemic values, respectively. Reperfusion of the ischemic heart resulted in little restoration of myocardial ATP and creatine phosphate contents (to approximately 15% and 25% of the pre-ischemic values, respectively). Treatment with 100 μM nicorandil for the last 30 min of the pre-ischemia did not alter the pre-ischemic value of these metabolites, nor did it prevent the decreases in myocardial ATP and creatine phosphate during ischemia. During reperfusion, however, myocardial ATP and creatine phosphate contents were restored to approximately 50% and 99% of their pre-ischemic values, respectively, by treatment with 100 μM nicorandil ($n = 5$). The energy charge significantly decreased after ischemia and recovered during reperfusion in both untreated and nicorandil-treated hearts; a larger

Table 2
Effects of nicorandil (100 μM) on high-energy phosphate content and energy charge of perfused hearts

		ATP	ADP	AMP	Creatine phosphate	Energy charge
At 0 min (at the end of pre-ischemia period)						
Un		26.61 ± 0.64	6.92 ± 0.84	0.81 ± 0.05	35.64 ± 1.09	0.88 ± 0.01
Nicorandil		24.56 ± 1.94	6.35 ± 0.77	0.95 ± 0.12	36.97 ± 2.22	0.87 ± 0.01
At 35 min						
Un	Normoxia	24.90 ± 0.71	5.32 ± 0.42	0.38 ± 0.12	36.22 ± 0.90	0.90 ± 0.01
	Ischemia	0.82 ± 0.20[a]	2.24 ± 0.29[a]	20.02 ± 1.49[a]	3.67 ± 0.69[a]	0.09 ± 0.02[a]
Nicorandil	Normoxia	25.78 ± 0.79	7.45 ± 0.67[b]	0.80 ± 0.11	37.00 ± 1.99	0.87 ± 0.01
	Ischemia	0.77 ± 0.15[a]	2.22 ± 0.09[a]	17.67 ± 0.86[a]	2.71 ± 0.45[a]	0.09 ± 0.01[a]
At 95 min						
Un	Normoxia	24.91 ± 1.03	5.34 ± 0.48	0.29 ± 0.15	35.35 ± 1.45	0.90 ± 0.01
	Ischemia	3.84 ± 0.56[a]	2.15 ± 0.38[a]	2.05 ± 0.49[a]	8.86 ± 1.39[a]	0.61 ± 0.05[a]
Nicorandil	Normoxia	24.83 ± 0.89	6.10 ± 0.29	0.72 ± 0.23	35.48 ± 0.95	0.88 ± 0.01
	Ischemia	12.17 ± 0.61[a,b]	4.09 ± 0.25[a,b]	0.72 ± 0.23[a,b]	35.12 ± 2.75[a,b]	0.84 ± 0.01[a,b]

The values of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and creatine phosphate were expressed as μmol per g dry tissue weight. Energy charge was calculated from the formula (ATP + 0.5 × ADP)/(ATP + ADP + AMP). Each value represents as the mean ± S.E.M. of five experiments.
[a] Significantly different from the normoxic group ($P < 0.05$).
[b] Significantly different from the untreated, ischemic group ($P < 0.05$).

recovery of the energy charge was seen in the nicorandil-treated heart. The energy charge of the nicorandil-treated group returned toward that of normoxic group after 60 min of reperfusion, as shown in Table 2.

3.4. Mitochondrial oxygen consumption rate of perfused hearts

The ADP-stimulated mitochondrial oxygen consumption rate of the left ventricular skinned bundles of the nicorandil-treated heart was determined (Fig. 4). The oxygen consumption rate for pre-ischemic hearts was 62.5 ± 1.4 nano-atom O/min/mg protein ($n = 5$). There were no significant differences in the oxygen consumption rate of perfused hearts under normoxic conditions regardless of treatment with or without nicorandil. The oxygen consumption rate of the untreated heart under ischemic conditions was significantly lower than that of the normoxic heart (approximately 30% of the value for the normoxic heart, $n = 5$, the left panel in Fig. 4). A further decline in the oxygen consumption rate was observed upon reperfusion (approximately 23% of the value for the normoxic heart, $n = 5$, the right panel in Fig. 4). In contrast, treatment with 100 μM nicorandil preserved the oxygen consumption rate at the ends of both ischemia and reperfusion (approximately 103% and 89% of the value for the normoxic heart, respectively, $n = 5$ each).

3.5. Release of cytochrome c from the mitochondria of isolated perfused heart

To test whether or not cytochrome *c* might have been released after ischemia from the mitochondria in the perfused heart, we prepared the cytosolic fraction from the perfused heart after 35-min ischemia and used it for Western-blot analysis of cytochrome *c* ($n = 5$ each). Fig. 5 shows that the amount of cytochrome *c* released under normoxic conditions was very low. A marked increase in cytochrome *c* in the cytosolic fraction was seen at the end of ischemia. The increase in the release of cytochrome *c* was substantially inhibited by treatment with 100 μM nicorandil.

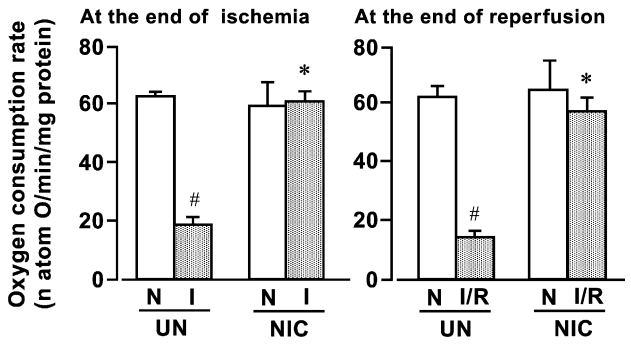

Fig. 4. Mitochondrial oxygen consumption rate of the left ventricular skinned bundles prepared at the ends of ischemia (I; the left panel) and reperfusion (I/R; the right panel) from hearts untreated (UN) or pretreated with 100 μM nicorandil (NIC). The open columns (N) represent the values for normoxic group. Each value represents the mean ± S.E.M. ($n = 5$). * Significantly different from the corresponding untreated ischemic or ischemic/reperfused hearts group ($P < 0.05$). # Significantly different from the corresponding normoxic group ($P < 0.05$).

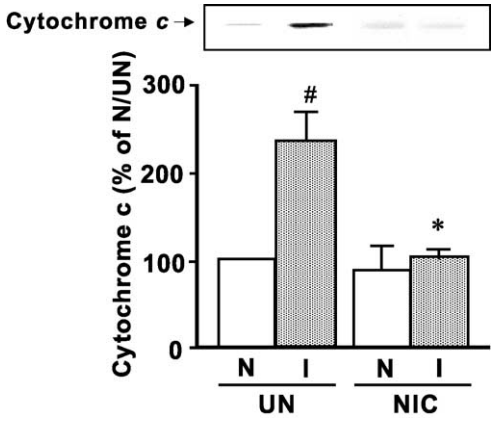

Fig. 5. Effects of treatment with nicorandil on the release of cytochrome *c* from the mitochondria into the cytosol in the perfused heart during ischemia. The perfused heart was subjected to 35-min ischemia without (UN) or with pre-treatment with 100 μM nicorandil (NIC), and the heart was then removed from the apparatus. After mild homogenization of the myocardium, cytosolic fraction was obtained. Cytochrome *c* in the cytosolic fraction was detected by the method as described in the Materials and methods. The upper panel indicates the results of Western blotting of cytochrome *c*. The open column (N) in the lower panel represents the value for normoxic hearts and the close column (I), the value for ischemic hearts. The data are expressed as percentages of the normoxic level (100%). Values represent the means ± S.E.M. of six experiments. # Significantly different from normoxia group ($P < 0.05$). * Significantly different from untreated group ($P < 0.05$).

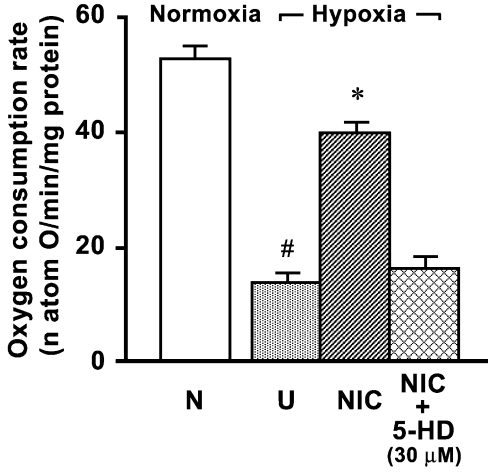

Fig. 6. Effects of 5-hydroxydecanoate (5-HD; cross-striped column) on the mitochondrial oxygen consumption rate of myocardial skinned bundles subjected to 30-min hypoxia in the absence (U) or presence (NIC) of 100 μM nicorandil. The open column (N) represents the value for normoxic group. Each value represents the mean ± S.E.M. of five experiments. # Significantly different from the normoxic group ($P < 0.05$). * Significantly different from the hypoxia untreated group ($P < 0.05$).

3.6. Mitochondrial oxygen consumption rate under hypoxic conditions

To determine whether or not nicorandil may protect the mitochondrial oxygen consumption capacity from hypoxic injury, we prepared skinned bundles from the left ventricular free wall of normal rats and incubated them under hypoxic conditions (Fig. 6). At first, to determine the experimental conditions of hypoxic incubation of skinned bundles, we measured the mitochondrial oxygen consumption rate after 15 to 120 min of hypoxia. The rate was reduced in a time-dependent manner between 0 and 30 min of hypoxia and thereafter the reduction of the rate was stabilized (data not shown). Thus, we employed 30-min hypoxia in subsequent experiments. After the 30-min hypoxic incubation, the mitochondrial oxygen consumption rate was decreased to approximately 26% of the value for the normoxic skinned bundles ($n = 5$). 5-HD itself did not affect the oxygen consumption rate of the both normoxic and hypoxic groups. In contrast, pre-treatment of 100 μM nicorandil prevented the decrease in the oxygen consumption rate after the 30-min hypoxic incubation. The preservation of mitochondrial oxygen consumption capacity by treatment with 100 μM nicorandil was significantly abolished by the combined treatment with 30 μM 5HD ($n = 5$) (Fig. 6).

4. Discussion

In the present study, we observed that treatment with nicorandil during pre-ischemia markedly enhanced the post-ischemic contractile recovery of ischemic/reperfused hearts. The improvement was associated with restoration of myocardial high-energy phosphates and attenuation of the release of creatine kinase. It is well recognized that cardiac contraction basically requires myocardial high-energy phosphates (Katz, 1977). Thus, appreciable levels of high-energy phosphates may be substantially beneficial for the recovery of myocardial contractility of the nicorandil-treated, reperfused heart.

The attenuation of the release of creatine kinase suggests that an ischemia-induced increase in the membrane permeability of macromolecules such as creatine kinase protein across cell membranes, probably due to the genesis of cardiac cell necrosis, was suppressed by treatment with nicorandil. The myocardial creatine phosphate content of the heart treated with nicorandil was restored to 99% of the pre-ischemic value during reperfusion whereas the restoration of ATP of the reperfused heart was relatively moderate. This may be due to the loss of ATP metabolites from the reperfused heart, since ATP metabolites, such as adenosine and inosine, are substrates for the salvage synthesis of ATP in hearts. These results suggest that the ability to produce energy in mitochondria may be appreciably preserved in the nicorandil-treated, ischemic heart.

Recently, it has been shown that a K_{ATP} channel, in addition to its presence in the cell membrane, is present in the mitochondrial inner membrane, where the channels regulate mitochondrial electron transport, volume, and energetics (Inoue et al., 1991; Paucek et al., 1992; Garlid et al., 1996; Holmuhamedov et al., 1998). Several investigators have suggested that mitochondrial K_{ATP} channels may be involved in cardioprotection against ischemia/reperfusion injury (Jovanovic et al., 1998; Liu et al., 1998). Thus, we determined whether or not nicorandil may protect the cardiac mitochondrial function against ischemia/reperfusion injury. In the present study, we used a method to determine mitochondrial functional capacity in the skinned bundles (Saks et al., 1989). Basically, the experimental conditions for measurement of mitochondrial oxygen consumption rate are the same as those for that of mitochondrial oxidative phosphorylation activity (Saks et al., 1989). Thus, this method is efficient to study the quantitative relationship between parameters of mitochondrial respiration and cardiac function in pathophysiological preparations. The 35-min ischemia decreased ADP-stimulated oxygen consumption rate at the end of ischemia as well as at the end of reperfusion. The decreased oxygen consumption rate was not observed in the presence of nicorandil for the last 30-min of pre-ischemia. In addition, cytochrome *c* was detected in the cytosolic fraction of perfused heart. The release of cytochrome *c* from mitochondria was not detected in the presence of nicorandil. It is likely that the decrease in the oxygen consumption rate is attributable to the loss of cytochrome *c* since cytochrome *c* is a critical component of the mitochondrial respiratory chain.

From these observations concerning the mitochondrial activity, nicorandil is capable of preventing mitochondrial functional capacity during ischemia. In a preliminary study, when perfused hearts were treated with nicorandil only during reperfusion, the recovery of left ventricular developed pressure was not enhanced. In addition, it was at the end of ischemia that the mitochondrial oxygen consumption rate of the untreated heart was decreased. These findings positively indicate that the mitochondrial oxygen consumption capacity of the heart had already been impaired under ischemic conditions prior to reperfusion injury and suggest that the presence of nicorandil during the ischemic period is necessary to elicit the improvement in the recovery of cardiac function during reperfusion. If so, ischemia/reperfusion-induced damage to cardiac function and metabolism, at least in part, might be caused by the impairment in mitochondrial function during the ischemic period, but not during reperfusion.

Although we observed that treatment with nicorandil attenuated the ischemia-induced decrease in the mitochondrial activity, it remained to be determined whether nicorandil may directly affect the mitochondria or not. This possibility was addressed in another set of experiments. Myocardial skinned bundles were prepared from the left ventricular free wall of normal rats and then exposed to 30-

min hypoxia in the presence and absence of nicorandil. Incubation of the skinned bundles under hypoxic conditions resulted in a decrease in mitochondrial oxygen consumption rate, which was attenuated by nicorandil in a concentration-dependent manner. This effect of nicorandil was abolished by the presence of a K_{ATP} channel blocker, 5-HD. The 5-HD is believed to affect the mitochondrial K_{ATP} channels rather than sarcolemmal K_{ATP} channels (McCullough et al., 1991; Garlid et al., 1997). The above results suggest that nicorandil preserves mitochondrial oxygen consumption rate under hypoxic conditions, probably through opening of mitochondrial K_{ATP} channels.

It remains unclear how the opening of mitochondrial K_{ATP} channels during the ischemic period might protect against the following reperfusion damage. During the steady state, respiration is balanced by K^+ influx into the mitochondria through the K^+/H^+ anti-porter. One possibility is that the opening of mitochondrial K_{ATP} channels may cause a net influx of K^+ into mitochondria and partially dissipate the mitochondrial membrane potential (McCullough et al., 1991; Garlid, 1996). This may be beneficial against ischemia-induced mitochondrial dysfunction, because partial dissipation of the electrical gradient decreases the paradoxical Ca^{2+} influx into the mitochondria (Liu et al., 1998; Holmuhamedov et al., 1999). Several studies using isolated mitochondria showed an attenuation of Ca^{2+} overload- or reoxygenation-induced decrease in the mitochondrial activity by diazoxide or nicorandil (Holmuhamedov et al., 2001; Ozcan et al., 2002). Thus, we cannot rule out the possibility that K_{ATP} channels might prevent reperfusion-induced damage of ischemia/reperfusion injury in perfused hearts.

Nicorandil is a drug with the chemical structure of organic nitrate. Shinbo and Iijima (1997) investigated whether nitric oxide modulated the K_{ATP} current in a single ventricular cell isolated from guinea pig hearts, and reported that nitric oxide potentiated the action of K_{ATP} channels. This finding leads to the possibility that through K_{ATP} channel activation via nitric oxide formation, nicorandil may synergistically improve the mitochondrial function, especially under ischemic conditions, and augment its cardioprotective effect.

A decrease in perfusion pressure during pre-ischemic period was seen in the heart treated with nicorandil, but no significant vasodilation could not seen during reperfusion. Since this agent was shown to dilate vascular smooth muscle (Endoh and Taira, 1983; Holzmann, 1983; Meisheri et al., 1991), coronary vasodilation might lead to beneficial recovery of post-ischemic cardiac function. To determine this possibility, we examined the relationship between perfusion pressure at the end of pre-ischemia and recovery of left ventricular developed pressure at the end of reperfusion in hearts treated with 10, 30, and 100 μM nicorandil. There was no significant relationship between these two parameters. Furthermore, nicorandil at the concentration of 10 μM appears to maximally dilate coronary artery of the perfused heart since higher concentrations (30 and 100 μM) of the agent did not elicit larger decreases in perfusion pressure of the pre-ischemic heart. In contrast, the higher concentrations of the agent elicited better post-ischemic recovery of cardiac contractile force, suggesting that it is unlikely that vasodilation is largely contribute to the cardioprotection in the present study.

Several investigators showed that K_{ATP} channel openers such as cromakalim and pinacidil preserved myocardial ATP of the perfused heart or the perfused right ventricular free wall during ischemia, that is, energy sparing effect (Grover et al., 1991; McPherson et al., 1993). Fukuda et al. (2001) also proposed the energy sparing effect of nicorandil at high concentrations (300 and 900 μM) in the perfused heart. In contrast, our findings did not support the energy sparing effect under the present experimental conditions (Table 2). The discrepancy may be due to differences in agents and concentrations employed or preparations examined.

In conclusion, the present study has shown that nicorandil is capable of protecting the myocardium against ischemia/reperfusion injury and enhancing the recovery of post-ischemic myocardial contractile function in association with restoration of myocardial high-energy phosphates. The mechanism underlying this cardioprotective effect of nicorandil may be attributed to preservation of mitochondrial function during ischemic period, probably via opening of mitochondrial K_{ATP} channels. The major findings in the present study are that the mitochondrial oxygen consumption capacity of the heart had already decreased at the end of ischemia, prior to reperfusion injury, and that nicorandil protected the mitochondrial oxygen consumption capacity from this ischemic injury.

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