Noricandil induces late preconditioning against myocardial infarction in conscious rabbits

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Tang, Xian-Liang, Yu-Ting Xuan, Yanqing Zhu, Gregg Shirk, and Roberto Bolli. Nicorandil induces late preconditioning against myocardial infarction in conscious rabbits. Am J Physiol Heart Circ Physiol 286: H1273–H1280, 2004. First published December 18, 2003; 10.1152/ajpheart.01055.2003.—Nicorandil has been shown to induce an infarct-limiting effect similar to that induced by the early phase of ischemic preconditioning (PC). The goals of this study were to determine whether nicorandil induces a delayed cardioprotection that is analogous to the late phase of ischemic PC and, if so, whether nicorandil-induced late PC is associated with upregulation of cardioprotective proteins. Chronically instrumented, conscious rabbits received vehicle (intravenous normal saline; control group, n = 10), nicorandil (100 μg/kg bolus + 30 μg·kg⁻¹·min⁻¹ iv for 60 min; nicorandil group, n = 10), or ischemic PC (6 cycles of 4-min coronary occlusion/4-min reperfusion; PC group, n = 8). Twenty-four hours later, rabbits underwent a 30-min coronary occlusion, followed by 3 days of reperfusion. Myocardial infarct size was significantly reduced in rabbits pretreated with nicorandil (27.5 ± 5.3% of the risk region) or with ischemia (30.3 ± 4.2%) versus controls (59.1 ± 4.7%, P < 0.05 vs. both). Furthermore, the expression of cyclooxygenase-2 (COX-2) and Bcl-2 was significantly elevated (+38% and +126%, respectively; P < 0.05) in myocardium of rabbits given nicorandil 24 h earlier versus controls. We conclude that nicorandil induces delayed cardioprotection against myocardial infarction similar to that afforded by the late phase of ischemic PC, possibly by upregulating COX-2 and Bcl-2.

Nicorandil is a hybrid agent with ATP-sensitive K⁺ (KATP) channel opener-like and nitrate-like properties (40, 61) and has been shown to have a protective effect in several experimental animal models of myocardial ischemia-reperfusion (1, 15, 16, 21, 24, 30, 31, 36, 37, 45, 46, 57, 69). Clinical studies (23, 34, 43, 54, 60) have shown that nicorandil attenuates ECG changes during coronary occlusion and that it improves the functional recovery of the reperfused myocardium in patients with acute myocardial infarction. More recently, the Impact of Nicorandil in Angina study (double-blind placebo-controlled trial of >5,000 patients) demonstrated that nicorandil improves the prognosis in stable angina pectoris (22). In addition to its direct beneficial effect on hemodynamics, nicorandil has also been shown to induce an early preconditioning (PC)-like cardioprotective effect (34, 35, 53), to lower the threshold of ischemic PC (38), and to potentiate the infarct size-limiting effect of the early phase of ischemic PC (39). Whether nicorandil also induces a delayed cardioprotection similar to the late phase of ischemic PC, however, remains unknown. Because of the sustained duration of late PC (4), elucidation of this issue may have considerable importance to explain the salubrious actions of nicorandil in the clinical arena (22).

In addition, because the late phase of ischemic PC provides sustained cardioprotection (4), exploitation of late PC is of clinical significance. Recent studies in various experimental animal preparations have demonstrated that a delayed cardioprotective effect indistinguishable from the late phase of ischemic PC can be elicited by a variety of pharmacological manipulations including nitric oxide donors (2, 20, 47, 65, 67), adenosine receptor agonists (3, 62), opioid receptor agonists (11, 12, 27, 44), reactive oxygen species (69), endotoxin derivatives (70, 72), and the KATP channel opener diazoxide (41, 66). However, many of these interventions are not clinically applicable or have significant side effects. Because nicorandil is already used in patients with coronary artery disease and is well tolerated, determining whether it induces a late PC-like cardioprotection is clinically important. Because previous studies have shown that late PC is mediated by upregulation of cyclooxygenase-2 (COX-2) (27, 58, 59) and Bcl-2 (51), we also tested whether these cardioprotective proteins are involved in the late PC-like effect of nicorandil.

Accordingly, the goals of the present study were to determine whether pretreatment with nicorandil, in the absence of ischemia, can reproduce the protective effect of the late phase of ischemic PC against myocardial infarction and to determine whether nicorandil-induced delayed cardioprotection is associated with expression of COX-2 and Bcl-2, which has previously been shown to increase 24 h after ischemic PC (51, 59). All studies were performed in our well-established conscious rabbit model (51, 59). The choice of a conscious model was dictated by the preclinical nature of this study and by our motivation to rigorously test the cardioprotective actions of nicorandil under conditions that are as physiological as possible. Open-chest animal preparations are associated with several factors (such as anesthesia, surgical trauma, fluctuations in temperature, elevated catecholamine levels, abnormal hemodynamics, and exaggerated formation of reactive oxygen species) that may interfere with myocardial infarct size (9, 56) and/or with ischemic PC (18, 55).

MATERIALS AND METHODS

The experimental procedures and protocols used in this study were reviewed and approved by the Animal Care and Use Committee of the University of Louisville, School of Medicine, and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996).

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Experimental Protocols

Infarct size studies. Chronically instrumented New Zealand White male rabbits (2.0–2.5 kg wt; age 3 to 4 mo) were prepared as described previously (5, 6, 25, 26, 47, 49, 50, 63–65, 71). Briefly, rabbits were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal in the center of the region to be rendered ischemic, and bipolar ECG leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for 3 days to aspirate air and fluid postoperatively. Gentamicin was administered before surgery and on the first and second postoperative days (5 mg/kg im each day).

After a minimum of 10 days postsurgical recovery, rabbits were studied in the conscious state. An intravenous infusion line was established by cannulating the marginal ear vein with a 24-gauge angiocatheter. Rabbits were divided into three groups and given one of the following treatments: vehicle (intravenous normal saline; control group), nicorandil (100 μg/kg bolus injection, followed by 30 μg·kg⁻¹·min⁻¹ infusion for 60 min iv; nicorandil group), or ischemic PC (a sequence of six 4-min coronary occlusion/4-min reperfusion cycles; PC group) (Fig. 1). Nicorandil (Chugai Pharmaceutical; Tokyo, Japan) was dissolved in normal saline; the total volume infused over 60 min was 6 ml/kg. To confirm the absence of hemodynamic changes, arterial pressure and heart rate were monitored in the nicorandil group. Arterial pressure was measured by cannulating the ear dorsal artery with a 22-gauge angiocatheter, as previously described (5). Ischemia-reperfusion in the PC group was produced by inflating and deflating the balloon occluder (5). The performance of successful coronary occlusions was verified by observing the development of ST-segment elevation and the changes in the QRS complex on the ECG and the appearance of paradoxical systolic wall thinning on the ultrasonic crystal recordings. No sedative or antiarrhythmic agent was given at this time. Twenty-four hours later, all rabbits were subjected to a 30-min coronary artery occlusion, followed by 3 days of reperfusion. Diazepam was administered 20 min before the 30 min occlusion (4 mg/kg ip) to relieve the stress caused by ischemia.

At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with triphenyl tetrazolium chloride and phthalo blue dye (26, 50, 63, 65). Infarct size was calculated by using computerized planimetry (26, 47, 50, 59, 63, 65, 71).

Western Blot Analysis

Two additional groups of rabbits were studied to determine whether nicorandil induces upregulation of COX-2 and Bcl-2. Rabbits received vehicle or nicorandil (same dose) (Fig. 1). Twenty-four hours later, rabbits were euthanized and myocardial samples were rapidly removed from the left ventricular (LV) wall and frozen in liquid N₂. Tissue samples were homogenized in buffer A composed of 25 mM Tris·HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 25 μg/ml leupeptin, 1 mM DTT, 25 mM NaF, and 1 mM Na₃VO₄ and centrifuged at 14,000 g for 12 min at 4°C, and the resulting supernatants were collected as cytosolic fractions (48). The pellets were incubated in a lysis buffer (buffer A + 1% Triton X-100) for 2 h and centrifuged 14,000 g for 15 min at 4°C, and the resulting supernatants were collected as membranous fractions (48). Protein expression was assessed by standard SDS-PAGE immunoblotting techniques (48, 49, 71). Gel transfer efficiency was recorded carefully by making photocopies of membranes dyed with reversible Ponceau staining (48, 49); gel retention was determined by Coomassie blue staining (48, 49).
Mouse monoclonal (C-33) anti-rat COX-2 antibodies (Transduction Laboratories; Lexington, KY) were used for the assay of COX-2 protein, and mouse monoclonal (C-2) anti-human/rat Bcl-2 antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) were used for the assays of Bcl-2 protein. Protein signals and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated by an image scanning densitometer, and each protein signal was normalized to its corresponding Ponceau stain signal (48, 49). In all samples, the content of each protein was expressed as a percentage of the corresponding protein in vehicle control.

Statistical Analysis

Data are reported as means ± SE. Hemodynamic variables were analyzed by a one-way ANOVA, followed by Student’s t-tests with Bonferroni’s correction. Infarct size and protein expression were analyzed by a one-way ANOVA, followed by unpaired Student’s t-tests with Bonferroni’s correction. The relationship between infarct size and risk region size was compared among groups with an analysis of covariance (ANCOVA) using the size of the risk region as the covariate. The correlation between infarct size and risk region size was assessed by linear regression analysis using the least-squares method. All statistical analyses were performed using SPSS for Windows version 8.0 and SigmaStat for Windows version 2.0. A P value < 0.05 was considered significant.

RESULTS

A total of 55 rabbits was used in this study (5 for the pilot studies, 30 for the studies of myocardial infarction, and 20 for the studies of protein expression). Two rabbits in the PC group were excluded due to failure of the occluder balloon.

Pilot Studies

Pilot studies were conducted in five rabbits to determine the dose of nicorandil that would not cause significant hemodynamic alterations. The concern was that hemodynamic perturbations caused by this agent (e.g., a drop in blood pressure or an increase in heart rate) could contribute nonspecifically to induce a late PC effect unrelated to the direct actions of nicorandil on the heart. Three rabbits received a 100 μg/kg iv bolus injection of nicorandil, followed by a 50 μg·kg⁻¹·min⁻¹ infusion for 60 min. This dose of nicorandil caused a significant increase (+25% at the end of the infusion) in heart rate and a mild drop (−11% at 40 min into the infusion) of arterial pressure (Fig. 2). Because the bolus dose did not cause significant changes in heart rate and arterial pressure, in two additional rabbits we reduced only the infusion rate to 30 μg·kg⁻¹·min⁻¹ for 60 min; we found no significant changes in heart rate and arterial pressure with this lower dose (data not shown). On the basis of these pilot studies, we selected this lower infusion rate of 30 μg·kg⁻¹·min⁻¹ of nicorandil for the present studies.

Hemodynamic Variables

Heart rate and mean arterial pressure remained stable in rabbits receiving nicorandil (Fig. 3). There were no significant differences in heart rate among the three groups at baseline, after diazepam injection (immediately before occlusion), during the 30-min coronary occlusion, or during the 72-h reperfusion period (data not shown).

Region at Risk and Infarct Size

There were no significant differences among the three groups with respect to the weight of the region at risk: 0.80 ± 0.12 g (17.4 ± 2.3% of LV weight), 0.90 ± 0.11 g (20.6 ± 1.9% of LV weight), and 0.81 ± 0.07 g (18.03 ± 1.4% of LV weight) in the control, nicorandil, and PC groups, respectively.

Fig. 2. Pilot studies of arterial pressure (A) and heart rate (B) in three rabbits receiving an intravenous nicorandil infusion at 100 μg/kg bolus plus 50 μg·kg⁻¹·min⁻¹ for 60 min. Values are means ± SE.
The average infarct size was 54% smaller in the nicorandil-pretreated rabbits compared with control (27.5 ± 5.3% vs. 59.1 ± 4.7% of the region at risk, P < 0.05) (Fig. 4), indicating that nicorandil elicited delayed cardioprotection 24 h later. The infarct size in the nicorandil group was similar to that observed in the rabbits preconditioned with ischemia (PC group, 30.3 ± 4.2% of the region at risk) (Fig. 4), indicating that the protection induced by nicorandil was equivalent to that induced by ischemic PC.

In all three groups, the size of the infarction was positively and linearly related to the size of the region at risk (r = 0.93, 0.63, and 0.78, respectively, in control, nicorandil, and PC group). The regression line was shifted downward in the nicorandil and PC groups compared with the control group (P < 0.05 by ANCOVA) (Fig. 5), indicating that for any given size of the region at risk, the resulting infarct size was reduced either by pretreatment with nicorandil or by ischemic PC.

**Western Blot Analysis**

**Expression of COX-2 protein.** A representative Western blot from four control and four nicorandil-pretreated rabbits is illustrated in Fig. 6A. Similar to our previous findings (27, 59), a detectable COX-2 signal was noted in the myocardium of both control and nicorandil-pretreated rabbits. In contrast, no detectable COX-2 protein was observed in the myocardium of rabbits preconditioned with ischemia (PC group).

![Fig. 5. Relationship between size of the region at risk and size of myocardial infarction. The figure illustrates both individual values and regression lines obtained by linear regression analysis for the control group (n = 10), the nicorandil group (n = 10), and the ischemic PC group (n = 8). In all groups, infarct size was positively and linearly related to risk region size. The linear regression equations were as follows: control group, y = 0.72x + 0.08 (r = 0.93); nicorandil group, y = 0.38x - 0.09 (r = 0.63); and ischemic PC group, y = 0.54x - 0.18 (r = 0.78). The regression line was shifted downward in the nicorandil and PC groups compared with the control group (both P < 0.05 by ANCOVA), indicating that for any given risk region size, infarct size was smaller in rabbits pretreated with nicorandil or ischemic PC compared with control rabbits.](http://ajpheart.physiology.org/)

![Fig. 3. Arterial pressure (A) and heart rate (B) in 10 rabbits in the nicorandil group given intravenous nicorandil at 100 μg/kg bolus plus 30 μg·kg⁻¹·min⁻¹ for 60 min. Data are means ± SE.](http://ajpheart.physiology.org/)
control rabbits. Densitometric measurements of COX-2 immunoreactivity showed that the expression of COX-2 protein increased by 38% versus control, $P < 0.05$ (Fig. 6B), in myocardium of rabbits given nicorandil 24 h earlier.

Expression of Bcl-2 protein. A representative Western blot from four control and four nicorandil-pretreated rabbits is illustrated in Fig. 7A. A weak Bcl-2 signal was detected in the myocardium of control rabbits. When rabbits were given nicorandil 24 h earlier, Bcl-2 expression increased markedly (+126 ± 7% vs. control, $P < 0.05$) (Fig. 7B).

**DISCUSSION**

The ultimate goal of studying PC is to exploit this phenomenon for the protection of the ischemic myocardium in patients with coronary artery disease. Although a variety of pharmacological interventions, including nitric oxide donors (2, 20, 47, 65, 67), adenosine receptor agonists (3, 62), opioid receptor agonists (11, 12, 27, 44), reactive oxygen species (68), endotoxin derivatives (70, 72), and the KATP channel opener diazoxide (41, 66) have been demonstrated to induce a late PC-like cardioprotection in experimental models, many of these agents are not clinically applicable or have significant side effects. Therefore, it is important to develop PC-mimetic therapies that are clinically relevant (20). In this connection, nitroglycerin has been shown to induce a late PC effect in conscious rabbits (2, 20) and in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) (19, 32). In the present study, we demonstrate in conscious rabbits that administration of nicorandil, another clinically relevant agent, elicits a delayed cardioprotective effect that is manifested by a reduction in infarct size 24 h later and that is equivalent to that observed after ischemic PC. The ability of nicorandil to induce late PC cannot be ascribed to myocardial ischemia secondary to a decrease in arterial pressure or an increase in heart rate, because the dose of nicorandil used did not produce appreciable hemodynamic changes; accordingly, the late PC effect must have been the result of a direct action of nicorandil on the heart. Furthermore, this study demonstrates that nicorandil-induced late PC is associated with upregulation of COX-2 and Bcl-2.

Nicorandil is a hybrid compound that consists of an $N$-(2-hydroxyethyl)nicotinamide vitamin group and an organic nitrate moiety and therefore is thought to have dual actions, a nitrate-like action and opening of $K_{ATP}$ channels (33). Like nitrates, nicorandil activates cytoplasmic guanylate cyclase leading to an increase in cellular levels of cGMP and a reduction in cytosolic calcium and thus to a relaxation of vascular smooth muscle (10). As a $K_{ATP}$ channel opener, nicorandil increases efflux of potassium ions from the cell, leading to a more negative resting membrane potential (hyperpolarization), and also shortens the action potential duration. This inhibits calcium influx, causing a reduction in intracellular calcium leading to relaxation of vascular smooth muscle and vasodilatation (indirect calcium channel blocking effect) (28, 29). Through its dual actions, nicorandil reduces both preload (nitrate-like effect) and afterload ($K_{ATP}$ channel opener effect), and improves coronary blood flow (14).

Clinical studies (22, 23, 54, 60) have shown that nicorandil improves functional and clinical outcomes in patients with acute myocardial infarction, but the mechanism for these effects is not known.
salubrious actions is unclear. Nicorandil reduces myocardial infarct size in various animal models when given before or during coronary occlusion (7, 8, 13, 21, 36, 42, 69); this infarct size-limiting effect seems independent of hemodynamic alterations (8, 36). Besides its direct action in alleviating ischemia-reperfusion injury, nicorandil has been suggested to induce an early PC-like cardioprotection in patients (34, 35, 53). In these studies, pretreatment of patients undergoing PTCA with a 1-min intravenous infusion of nicorandil 5 min before the initial balloon inflation significantly reduced ST elevation (34, 35, 53) and troponin T release (53) compared with control patients. However, because in these studies nicorandil was given just 5 min before ischemia (34, 35, 53), it is difficult to make a distinction between the cardioprotection resulting from its direct actions and that afforded by initiation of PC. In contrast, the infarct-sparing effect observed 24 h after nicorandil pretreatment in our study must be the result of late PC and cannot be ascribed to a direct drug action because a single dose of nicorandil is eliminated from plasma almost entirely within 8 h (33). The mechanism whereby nicorandil triggers the development of late PC is unclear. Our previous studies (2, 17, 20) have demonstrated that late PC can be elicited by NO donors such as nitrates, whereas others (41, 66) have shown that it can be elicited by the K\textsubscript{ATP} channel opener diazoxide. Thus, in principle, either the nitrate-like or the K\textsubscript{ATP} channel activating properties of nicorandil, or both, could be responsible for the development of late PC against myocardial infarction.

The finding that nicorandil pretreatment upregulates the expression of COX-2 (Fig. 6) and Bcl-2 (Fig. 7) in the myocardium has potentially important implications for our understanding of the mechanism responsible for mediating nicorandil-induced delayed cardioprotection. In previous studies in rabbits, expression of COX-2 was shown to be upregulated 24 h after ischemic PC (59) and after pretreatment with the opioid receptor agonist BW-373U86 (27). Inhibition of COX-2 activity abrogated the cardioprotective effect of late PC induced by ischemia (59) and by the opioid receptor agonist (27). In addition, expression of Bcl-2 has been shown to be enhanced 24 h after ischemic PC in rat hearts (51). In view of these studies (27, 51, 59), it is plausible that nicorandil-induced delayed cardioprotection may be mediated by upregulation of COX-2 and/or Bcl-2. Establishing a cause-and-effect relationship between the induction of these proteins and nicorandil-induced cardioprotection will require further investigation.

In summary, the present study demonstrates the PC-mimetic properties of another clinically relevant agent (besides nitroglycerin), i.e., nicorandil. Specifically, using a conscious animal model devoid of the problems associated with anesthetized models (27, 51, 59), we found that administration of nicorandil reduced myocardial infarct size 24 h later. This delayed cardioprotection was associated with enhanced expression of myocardial COX-2 and Bcl-2. The ability of nicorandil to elicit a sustained protected phenotype may explain, at least in part, the salubrious effects of the drug in clinical trials (22, 23, 33, 34, 43, 52, 54, 73). Because nicorandil has been used clinically to treat patients with coronary artery disease and is well tolerated (43), the present results have potential clinical implications. The notion that in addition to its immediate anti-ischemic effects, nicorandil can also trigger a sustained phenotypic change that renders the heart resistant to infarction at a distance of 24 h suggests novel applications of this drug for protecting the ischemic myocardium in patients. If nicorandil exerts late PC-mimetic actions in humans, a protracted or even chronic PC state could be implemented by administering nicorandil on a regular basis. Thus the present results provide a rationale for studies aimed at investigating the usefulness of nicorandil as a prophylactic treatment against infarction in patients with coronary artery disease.

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