Long-term administration of nicorandil abolishes ischemic and pharmacologic preconditioning of the human myocardium: Role of mitochondrial adenosine triphosphate–dependent potassium channels

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Background: Acute administration of mitochondrial adenosine triphosphate–dependent potassium channel openers preconditions the heart, but whether their long-term administration induces a permanent state of protection is unknown. These studies investigate the effect of long-term treatment with the mitochondrial adenosine triphosphate–dependent potassium channel opener nicorandil on the response of the human myocardium to ischemia and preconditioning.

Methods: Right atrial tissue obtained from patients regularly treated with or without nicorandil (mean of 20 mg/d for 18.6 ± 2.5 months) and undergoing cardiac surgery was sliced and equilibrated for 30 minutes and then subjected to 90 minutes of simulated ischemia, followed by 120 minutes of reoxygenation. In study 1 the following groups were studied to investigate the effect of nicorandil on the susceptibility of the myocardium to ischemia and on the protective effect of ischemic and pharmacologic preconditioning: (1) aerobic control; (2) simulated ischemia and reoxygenation alone; (3) ischemic preconditioning with 5 minutes of simulated ischemia and 5 minutes of reoxygenation; and (4) phenylephrine ($0.1 \mu\text{mol/L}$) for 5 minutes and 5 minutes' washout before simulated ischemia and reoxygenation. In study 2 the following groups were studied to investigate the effect of nicorandil on the responsiveness of mitochondrial adenosine triphosphate–dependent potassium channels: (1) aerobic control; (2) simulated ischemia and reoxygenation; (3) ischemic preconditioning; (4) diazoxide ($100 \mu\text{mol/L}$) for 10 minutes before simulated ischemia and reoxygenation, and (5) 5-hydroxydecanoate (1 mmol/L) for 10 minutes before simulated ischemia and reoxygenation. In study 3 the following groups were included to investigate the effect of the long-term administration of nicorandil on the kinase pathway involved in preconditioning: (1) aerobic control; (2) simulated ischemia and reoxygenation alone; (3) ischemic preconditioning; (4) phorbol 12-myristate 13-acetate ($1 \mu\text{mol/L}$), a protein kinase C activator, for 10 minutes before simulated ischemia and reoxygenation; and (5) anisomycin (1 nmol/L), a p38 mitogen-activated protein kinase activator, for 10 minutes before simulated ischemia and reoxygenation. At the end of each protocol, the leakage of creatine kinase (in units per gram wet weight) and the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide into insoluble formazan dye (in millimoles per gram wet weight) were measured.

Results: In study 1 the leakage of creatine kinase and the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide induced by simulated ischemia and reoxygenation were similar in the groups with or without nicorandil (creatin kinase, 3.4 ± 0.1 and 3.5 ± 0.2 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 74.6 ± 3.9 and 67.9 ± 7.3 , respectively; $P > .2$ in each instance). Ischemic preconditioning and pharmacologic preconditioning protected the myocardium from patients without nicorandil (creatin kinase, 2.3 ± 0.1 and 2.4 ± 0.1 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetra-

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lium bromide, 131.4 ± 4.9 and 128.4 ± 5.6 , respectively; $P < 0.001$ vs simulated ischemia and reoxygenation alone in each instance) but not the myocardium from patients receiving nicorandil (creatin kinase, 3.3 ± 0.1 and 3.3 ± 0.2 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 89.7 ± 6.5 and 86.4 ± 5.2 , respectively; $P > .2$ vs simulated ischemia and reoxygenation alone in each instance). In study 2 the administration of diazoxide had identical protection to that of ischemic preconditioning in the myocardium of patients not receiving nicorandil (creatin kinase, 2.1 ± 0.2 and 2.3 ± 0.1 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 141.4 ± 7.4 and 131.4 ± 4.9 , respectively; $P < 0.001$ vs simulated ischemia and reoxygenation alone in each instance) but failed to precondition the myocardium from patients treated with nicorandil (creatin kinase, 3.3 ± 0.2 and 3.4 ± 0.1 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 90.1 ± 7.2 and 86.4 ± 5.2 , respectively; $P > 0.2$ vs simulated ischemia and reoxygenation alone in each instance). In study 3 phorbol 12-myristate 13-acetate or anisomycin given for 10 minutes before simulated ischemia and reoxygenation afforded similar protection to that of ischemic preconditioning in the myocardium from patients with (creatin kinase, 1.5 ± 0.3 and 1.4 ± 0.1 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 147.0 ± 4.9 and 160.0 ± 16.1 , respectively; $P < 0.001$ vs simulated ischemia and reoxygenation alone in each instance) and without nicorandil (creatin kinase, 1.7 ± 0.4 and 1.4 ± 0.2 , respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 160.3 ± 13.6 and 158.3 ± 11.8 , respectively; $P < .001$ vs simulated ischemia and reoxygenation alone in each instance).

Conclusion: The myocardium of patients chronically treated with nicorandil cannot be preconditioned either by ischemia or pharmacologically, and this is because of unresponsive mitochondrial adenosine triphosphate–dependent potassium channels. However, protection can be obtained by protein kinase C and p38 mitogen-activated protein kinase activation, which are downstream of mitochondrial adenosine triphosphate–dependent potassium channels in the signaling transduction pathway of preconditioning.

Nicorandil was first introduced in clinical practice in 1984 as the first in a new class of antianginal drugs. Since then, it has become widely used for the control of angina as part of combination therapy, and more recently, it is being increasingly used as the first-line and sole treatment in both stable^{1,2} and unstable angina.³ Nicorandil is a nicotinamide nitrate ester that has been shown to have an antianginal effect comparable with that of β -blockers and calcium antagonists. It has a bimodal mechanism of action combining 2 vasodilator mechanisms; it increases potassium conductance in the cell membrane, resulting in potassium outflow from the cell and causing membrane hyperpolarization⁴; and it also increases cellular levels of cyclic guanosine monophosphate,⁵ with both actions causing vasorelaxation. The nitrate-like action of nicorandil dilates epicardial coronary arteries,⁶ which

results in an increase in the blood supply to the ischemic region of the myocardium. In addition, nicorandil has been shown to open adenosine triphosphate–dependent potassium (K_{ATP}) channels in ischemic cardiomyocytes,⁷ and there is strong evidence that the mitochondrial, rather than the sarcolemmal, K_{ATP} channels are involved in the protection of ischemic preconditioning,^{8–11} probably by decreasing the mitochondrial membrane potential.^{12,13} Yellon's laboratory has reported that nicorandil can mimic the protection of ischemic preconditioning,¹⁴ which has led to the suggestion that patients receiving nicorandil for the control of angina might be permanently protected.¹⁵

The aims of this study were to investigate the effects of long-term administration of nicorandil on the tolerance of the human myocardium to ischemia and on the protection of ischemic and pharmacologic preconditioning and on its signal transduction mechanism.

Methods

Patient Selection and Experimental Preparation

Experiments were performed on muscle obtained from the right atrial appendage of patients undergoing elective coronary artery surgery in a cell necrosis model characterized in our laboratory and described previously.¹⁶ Patients were excluded if they had large atriums, atrial arrhythmias, poor left ventricular function (ejection fraction <30%), diabetes, and right ventricular failure or were receiving oral hypoglycemic agents, opioid analgesia, or catecholamines. Patients taking nicorandil had their last dose on the morning of the operation 2.5 ± 0.4 hours before harvesting of the atrial appendage. These patients were taking nicorandil for a mean of 18.6 ± 2.5 months at a mean dosage of 20 mg/d. Local ethical committee approval was obtained for the harvesting technique. The specimens were collected in oxygenated N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffered solution at 4°C to 5°C and immediately sectioned and prepared for study. The appendage was mounted onto a ground glass plate with the epicardial surface face down and then sliced freehand (to prevent crushing) with surgical skin graft blades (Shwamm-Morton) to a thickness of between 300 and 500 μm . The muscles (weight, 30-50 mg) were then transferred to conical flasks (25-mL Erlenmeyer flasks, Schott Glaswerk) containing 10 mL of oxygenated buffered solution, and the flasks were placed in a shaking water bath maintained at 37°C. The oxygenation of the incubation medium was maintained by a continuous flow of 95% O₂/5% CO₂ gas mixture to obtain a Po₂ of between 25 and 30 kPa and a Pco₂ of between 6 and 6.5 kPa. The Po₂, Pco₂, and pH in the incubation medium were monitored by means of intermittent analyses of the solution with an automated blood gas analyzer (model 855 Blood Gas System, Chiron Diagnostics), and the pH was kept at between 7.36 and 7.45. For the induction of simulated ischemia, the medium was bubbled with 95% N₂/5% CO₂ (pH 6.80-7.00), and D-glucose was replaced with 2-deoxy glucose.

Assessment of Tissue Injury and Viability

Tissue injury was determined by measuring the leakage of creatine kinase (CK) into the incubation medium during the 120-minute reoxygenation period. This was assayed by using a kinetic UV method on the basis of the formation of NAD (Sigma Catalogue No. 1340-K), and the results were expressed as units per gram wet weight.

Tissue viability was assessed by the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to a blue formazan product at the end of the experimental time. The absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad Laboratories) at 550 nm, and the results were expressed as millimoles per gram wet weight. In this preparation the atrial tissue was not paced, and the force developed was not measured.

Solutions and Drugs

The incubation medium was prepared daily with deionized distilled water and contained the following: NaCl₂, 118 mmol/L; KCl, 4.8 mmol/L; NaHCO₃, 27.2 mmol/L; MgCl₂, 1.2 mmol/L; KH₂PO₄, 1.0 mmol/L; CaCl₂, 1.25 mmol/L; D-glucose, 10 mmol/L; and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 20 mmol/L. During simulated ischemia, D-glucose was sub-

stituted with 2-deoxy glucose (10 mmol/L) to maintain a constant osmolarity. Phenylephrine, prazosin, 5-hydroxydecanoate, and anisomycin were dissolved in deionized distilled water, whereas diazoxide and phorbol 12-myristate 13-acetate (PMA) were dissolved in dimethyl sulfoxide. Anisomycin is an antibiotic that inhibits protein synthesis and has been demonstrated to activate p38 mitogen-activated protein kinase (MAPK), whereas PMA is a phorbol ester that is widely used to activate protein kinase C (PKC). All the drug doses were chosen after extensive preliminary dose-response experiments. All reagents were obtained from Sigma.

Experimental Protocols

All atrial muscles were allowed to equilibrate under aerobic conditions for 30 minutes before 90 minutes of simulated ischemia and 120 minutes of reoxygenation. There were 6 specimens in each group from 6 different patients, with a total of 9 to 13 patients being enrolled in each study.

Study 1: To investigate the effect of long-term administration of nicorandil on myocardial tolerance to ischemia and on protection of ischemic and pharmacologic preconditioning with phenylephrine. Atrial muscles obtained from appendages of patients treated or not treated with nicorandil were randomly assigned to one of the following groups: (1) aerobic control; (3) simulated ischemia and reoxygenation alone; (3) ischemic preconditioning with 5 minutes of simulated ischemia and 5 minutes of reoxygenation; and (4) phenylephrine (0.1 $\mu\text{mol/L}$) for 5 minutes and 5 minutes' washout before simulated ischemia and reoxygenation.

Study 2: To investigate the effect of long-term administration of nicorandil on responsiveness of mitochondrial K_{ATP} channels during preconditioning. Atrial slices obtained from appendages of patients treated or not treated with nicorandil were randomly assigned to one of the following groups: (1) aerobic control; (2) simulated ischemia and reoxygenation alone; (3) ischemic preconditioning with 5 minutes of simulated ischemia and 5 minutes of reoxygenation; (4) diazoxide (100 $\mu\text{mol/L}$), a mitochondrial K_{ATP} channel opener, for 10 minutes before simulated ischemia and reoxygenation; and (5) 5-hydroxydecanoate (1 mmol/L), a mitochondrial K_{ATP} channel blocker, for 10 minutes before simulated ischemia and reoxygenation.

Study 3: To investigate the effect of long-term administration of nicorandil on the kinase pathway involved in preconditioning. Atrial slices obtained from appendages of patients treated or not treated with nicorandil were randomly assigned to one of the following groups: (1) aerobic control; (2) simulated ischemia and reoxygenation alone; (3) ischemic preconditioning with 5 minutes of simulated ischemia and 5 minutes of reoxygenation; (4) PMA (1 $\mu\text{mol/L}$), a PKC activator, for 10 minutes before simulated ischemia and reoxygenation; and (5)

anisomycin (1 nmol/L), a p38MAPK activator, for 10 minutes before simulated ischemia and reoxygenation.

Statistical Analysis

All data are presented as means $\pm$ SEM. Mean values were compared by means of analysis of variance with application of the post hoc Tukey test.

Results

Effect of Long-Term Administration of Nicorandil on Myocardial Tolerance to Ischemia and on Protection of Ischemic and Pharmacologic Preconditioning with Phenylephrine (Study 1)

As shown in Figure 1, the increased CK leakage and decreased MTT reduction induced by ischemia and reoxygenation were similar in the muscles from patients with and without long-term nicorandil treatment. Figure 1 also shows that ischemic and pharmacologic preconditioning with phenylephrine resulted in identical protection in the nicorandil-free group but failed to protect the myocardium in the nicorandil-treated group.

Effect of Long-Term Administration of Nicorandil on Responsiveness of Mitochondrial K_{ATP} Channels During Preconditioning (Study 2)

As expected, Figure 2 shows that the selective opening of mitochondrial K_{ATP} channels with diazoxide resulted in protection (ie, reduced CK leakage and increased MTT reduction) similar to that provided by ischemic preconditioning, with no detrimental effect beyond that of ischemia and reoxygenation alone when the channels were blocked with 5-hydroxydecanoate. In contrast with these results, diazoxide did not protect the myocardium in the nicorandil-treated group.

Effect of Long-Term Administration of Nicorandil on the Kinase Pathway Involved in Preconditioning (Study 3)

The results shown in Figure 3 demonstrate that activation of PKC and p38MAPK resulted in similar protection as that provided by ischemic preconditioning, as shown on the basis of the CK leakage and MTT reduction values in both the nicorandil-free and nicorandil-treated groups.

Discussion

The present studies have demonstrated that the long-term administration of nicorandil, a mitochondrial K_{ATP} channel opener¹⁴ and nitric oxide donor,¹⁷ abolishes the ability of the human myocardium to be protected by ischemic and pharmacologic preconditioning without exacerbating the susceptibility to ischemic injury. In addition, they have shown that the likely cause of the failure to precondition the myocardium of patients receiving nicorandil is the unre-

sponsiveness of the mitochondrial K_{ATP} channels because protection cannot be obtained with diazoxide, a specific mitochondrial K_{ATP} channel opener, but can be elicited by activation of PKC and p38MAPK, which are downstream of mitochondrial K_{ATP} channels in the signaling transduction cascade of preconditioning.¹⁸ These results have important clinical implications and shed light onto the mechanism of protection by preconditioning that are discussed below.

Opening of the mitochondrial K_{ATP} channels has been demonstrated to be an obligatory step in the signal transduction mechanism of preconditioning.^{8,9,11} Thus nicorandil and other mitochondrial K_{ATP} channel openers have been shown to mimic the cardioprotection of ischemic preconditioning when given acutely (ie, immediately before the ischemic insult) in both animal^{7,11,15} and human^{8,9} studies. As a result, and as suggested by Carr and Yellon,¹⁵ one might be tempted to hypothesize that the long-term administration of mitochondrial K_{ATP} channel openers induces a permanent state of protection against ischemic injury. However, the present studies are the first to report that when mitochondrial K_{ATP} channel openers are given on a long-term basis, as occurs clinically, these channels become unresponsive, with loss of the cardioprotection of preconditioning.

Our findings contrast with those reported by Carr and Yellon¹⁵ showing that the long-term administration of nicorandil is actually protective. Their and our studies used human atrial myocardium, and the only difference between the 2 studies is that they assessed recovery of contractile function in their study as opposed to tissue viability in ours, which might offer an explanation as to the differing results. Further fuel is added to the controversy when the same authors observed that the protection of the myocardium of patients receiving long-term nicorandil is in fact abolished by the application of ischemic preconditioning. These results require clarification because it is difficult to understand how 2 protective interventions using identical signal transduction mechanisms can annul each other.

The mechanism by which the long-term administration of nicorandil renders the mitochondrial K_{ATP} channels unresponsive to preconditioning with ischemia and with diazoxide is not completely elucidated by the present studies, but they have shown that the activation of kinases that we have previously shown to be downstream of mitochondrial K_{ATP} channels¹⁸ is unaffected because their activation can still elicit protection. It has been suggested that the generation of free radical species is the link between mitochondrial K_{ATP} channels and the activation of PKC.¹¹ If this is the case, then it might be speculated that a permanent opening state of mitochondrial K_{ATP} channels results in a reduction in the formation of free radicals, a thesis that gains support from the demonstration that nicorandil possesses antioxidant properties.¹⁹

Figure 1. CK leakage into the media: A, during the 120-minute reoxygenation period and MTT reduction by the slices; B, at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the myocardial tolerance to ischemia and on the protection of ischemic and pharmacological preconditioning with phenylephrine (study 1). Data are expressed as means $\pm$ SEM of 6 experiments. * $P < .001$ versus simulated ischemia and reoxygenation alone.

The present studies raise fundamental questions on the clinical utility and safety of nicorandil and other mitochondrial K_{ATP} channel openers for the control of angina symptoms. The permanent opening of the mitochondrial K_{ATP} channels deprive the heart of the intrinsic protective mechanism of preconditioning that might be a risk factor in the presence of ischemic heart disease. Therefore if the bene-

ficial action of nicorandil is solely due to its nitrate effect, then the use of this compound might not be fully justified. However, if, as discussed earlier, the maintenance of mitochondrial K_{ATP} channels in an open state might reduce the generation of free radicals and oxidative stress, then these beneficial effects of nicorandil might counterbalance the loss of preconditioning. Indeed, oxidative stress has been

Figure 2. CK leakage into the media: **A**, during the 120-minute reoxygenation period and MTT reduction by the slices; **B**, at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the responsiveness of mitochondrial K_{ATP} channels during preconditioning (study 2). Data are expressed as means $\pm$ SEM of 6 experiments. * $P < .001$ versus simulated ischemia and reoxygenation alone. 5-HD, 5-Hydroxydecanoate.

suggested as an important mechanism of many disease states, including the inflammatory response in diabetes,²⁰ atherosclerosis,²¹ cardiac hypertrophy,²² and heart failure.²³ It is clear that further experimental and clinical studies are required to fully elucidate the mechanism and the clinical repercussions of nicorandil and similar agents on ischemic heart disease and ischemic syndromes.

It is necessary to mention that our studies were performed in an in vitro preparation that was not electrically stimulated (ie, nonbeating), and it was not possible to obtain functional data. Therefore any extrapolation to the clinical setting should be made with caution. Another potential limitation of our studies was the use of atrial, as opposed to ventricular, myocardial tissue, and again, any extrapolation

Figure 3. CK leakage into the media: **A**, during the 120-minute reoxygenation period and MTT reduction by the slices; **B**, at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the kinase pathway involved in preconditioning (study 3). Data are expressed as means $\pm$ SEM of 6 experiments. * $P < .001$ versus simulated ischemia and reoxygenation alone.

of the conclusions from these studies to the ventricular myocardium should also be made with caution.

Although these studies have demonstrated the unresponsiveness of the mitochondrial K_{ATP} channels as the cause of failure to precondition the myocardium of nicorandil-treated patients, they show that protection can still be obtained by direct activation of PKC or p38MAPK, which are down-

stream of mitochondrial K_{ATP} channels in the signal transduction pathway of ischemic preconditioning. Therefore protection can be elicited by bypassing the steps of the transduction cascade that might be affected by disease states, such as diabetes or heart failure,²⁴ or by medication, such as sulphonylureas,²⁴ and antianginal treatment, such as nicorandil, as shown from the current studies. Undoubtedly

greater understanding of the various elements participating in the signal transduction pathway and identification of agents with selective activity on factors to avoid unwanted side effects will be required before its use might be considered in the clinical setting.

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