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Oral nicorandil recaptures the waned protection from preconditioning *in vivo*

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1 Protection from preconditioning (PC) wanes and is eventually lost when multiple bouts of short ischemia or a prolonged reperfusion interval precedes the following sustained ischemia. The activation of mitochondrial K_{ATP} channels plays a pivotal role in the intracellular signaling of PC. We tested whether the K_{ATP} channel opener nicorandil (nic) preserves the given protection from PC in conditions where this benefit decays and is lost.

2 Eight groups of rabbits were divided into two equal series of experiments, one without nic (placebo) and one with nic treatment. Nic was given orally for 5 consecutive days in a dose of $5\,mg\,kg^{-1}\,d^{-1}$. In a second step, four additional groups were treated with nic plus the K_{ATP} channel blocker 5HD and 1 additional control group with nitroglycerin only. All the animals were anesthetized and then subjected to 30 min of myocardial ischemia and 2 h of reperfusion with one of the following interventions before the sustained ischemia: *Control* groups to no intervention; *3PC* groups to three cycles of 5-min ischemia–10-min reperfusion; *8PC* groups to eight cycles of 5-min ischemia – 10-min reperfusion; and *3PC90* groups to the same interventions as the *3PC* groups but with a prolonged (90 min) intervening reperfusion interval before the sustained ischemia. The infarcted and the risk areas were expressed in percent.

3 There was no significant change in infarct size between the placebo, the nic and the 5HD-nic in the *control* groups (41.5 ± 4.7, 43.9 ± 7.1 and $48.7\pm6.4\%$) and *3PC* groups (10.3 ± 3.4, 12.2 ± 3.9 and $12.6\pm4.5\%$). However, there was a significant decrease after nic treatment in groups *8PC* ($47.7\pm8.8\%$ *vs* $13.0\pm2.6\%$, $P<0.01$) and *3PC90* ($37.3\pm6.0\%$ *vs* $14.2\pm2.4\%$, $P<0.01$), which was abrogated (38.2 ± 4.7 and $42.7\pm4.4\%$, respectively, for *8PC* and *3PC90* groups). Nitroglycerin had no effect on infarct size ($39.1\pm3.1\%$, $P=$NS *vs* other controls).

4 Oral treatment with nic recaptures the waned protection of PC, both after repetitive bouts of short ischemia or after a prolonged reperfusion interval, preserving the initially obtained benefit. Nic by itself is insufficient to initiate PC *in vivo*.
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Keywords: Preconditioning; nicorandil; ischemia reperfusion; protection

Abbreviations: I, infarcted area; nic, nicorandil; NTG, nitroglycerin; R, risk area; PC, preconditioning; TTC, tetrazolium chloride; u.v., ultraviolet; 5HD, 5-hydroxydecanoate

Introduction

New therapeutic strategies, based on the knowledge of the intracellular signaling pathways of preconditioning, have emerged in order to confer pharmaceutical protection to the ischemic heart. So far, several experimental and clinical studies have used various substances, either with preconditioning (Liu *et al*., 1991; Leesar *et al*., 1997; Cohen *et al*., 2001) or with antipreconditioning effect (Tomai *et al*., 1996, 1997; Iliodromitis *et al*., 1998), in an attempt to preserve or abolish, respectively, any benefit obtained from preconditioning. Opening of the mitochondrial K_{ATP} channels appears to play a central role in the protective mechanism of preconditioning either downstream (Gross & Fryer, 2000; Cohen *et al*., 2001) or upstream (Garlid *et al*., 1997; Pain *et al*., 2000) in the signal transduction pathways. Nicorandil, a selective mitochondrial K_{ATP} channel opener, has been extensively used in many experimental studies and in a multicenter clinical trial with very promising results (Patel *et al*., 1999; Geshi *et al*., 1999; Sato *et al*., 2000).

We and other teams have previously shown that the protection from preconditioning wanes is eventually lost when multiple cycles of ischemic bouts precede the sustained ischemia (Cohen *et al*., 1994; Iliodromitis *et al*., 1997) or when the intervening reperfusion interval between the short and long ischemia is very prolonged (Li & Kloner, 1994; Iliodromitis *et al*., 1996). The aim of the present study is to clarify the effect of nicorandil on infarct size after sustained ischemia in anesthetized rabbits without preconditioning and in various preconditioning modes.

Methods

New Zealand white male rabbits weighing between 2.3 and 3.3 kg were used in this study and received proper care in

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compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institute of Health.

Study design

First step Eight groups of male rabbits were divided into two equal series of experiments consisting of four respective groups. In the first series, placebo tablets were given orally for 5 days every 12 h. In the second series of experiments, the K_{ATP} channel opener nicorandil (Ikorel, Aventis) was given orally for 5 consecutive days in a dose of $5\,mg\,kg^{-1}$ per day divided into two equal doses every 12 h, one in the morning and the other late in the evening. Tablets were crushed, diluted in dextrose 5% fluid, then given orally to the animals through a 5-ml syringe. The last dose of nicorandil or placebo was given 2–3 h before surgical intervention and both tablets had the same color and size. All the animals were subjected to 30-min regional ischemia and 2-h reperfusion. In both series, *Control* groups were not subjected to any intervention apart from the sustained ischemia and reperfusion; all the other groups were named according to preconditioning cycles and were subjected to the following interventions: *3PC* groups subjected to three cycles of 5-min ischemia and 10-min reperfusion, *8PC* groups to eight cycles of 5-min ischemia and 10-min reperfusion, and *3PC90* groups to the same intervention as the *3PC* groups but with a prolonged (90 min) intervening reperfusion interval between the last preconditioning stimulus and the beginning of the sustained ischemia.

Second step In the second step of the study, four groups of male rabbits were treated with nicorandil as previously described and were exposed to the same protocol as the first step but with the addition of the mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (5HD). Similar to the first step of the protocol, these groups are called *Control, 3PC, 8PC* and *3PC90*. 5HD (Sigma, 5-hydroxydecanoic acid sodium salt) was dissolved in normal saline ($10\,mg\,ml^{-1}$) and was given bolus into the jugular vein 10 min before sustained ischemia at a dose of $10\,mg\,kg^{-1}$ (Tanhehco *et al*., 2000). Finally, an additional group, called *NTG-Control,* was treated with nitroglycerin in order to compare the net effect of nitrates on infarct size since nicorandil is not only a K_{ATP} channel opener but it also has a nitrate component. Nitroglycerin was dissolved in normal saline ($50\,\mu g\,ml^{-1}$) and was continuously infused *via* the jugular vein for 90 min, starting 60 min before the long ischemia and for the whole period of the coronary occlusion at a dose of up to $2\,\mu g\,kg^{-1}\,min^{-1}$ as previously described (Hill *et al*., 2001). The infusion rate was properly adjusted when the systolic blood pressure decreased to 80 mmHg.

We administered nitroglycerin acutely and not over a prolonged period of time as the drug is known to have a high gastrointestinal clearance and a low oral bioavailability in rabbits (Tam *et al*., 1988). Furthermore, we did not want to risk a missed inappropriate drop in blood pressure or any further hemodynamic compromise during a long-term oral administration of nitroglycerin. The protocol is presented in Figure 1.

Surgical preparation

All the animals were anesthetized by slowly injecting $30\,mg\,kg^{-1}$ of sodium thiopeptone (Pentothal, Abbott) into an ear vein, intubated through a midline tracheal incision and mechanically ventilated with a positive pressure respirator for small animals (MD Industries, Mobile, AL, USA) at a rate that adjusted them to keep blood gases within the normal range. Two polyethylene catheters were inserted; one in the left jugular vein for fluids or top-up anesthesia and another in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multi-channel recorder (Nihon-Koden, Model 6000, Japan). A bipolar chest lead was used for continuous electrocardiographic monitoring. The chest was opened via a left thoracotomy in the fourth intercostal space and after pericardiotomy the beating heart was exposed. A 3-0 silk thread was passed through the myocardium around a prominent branch of the left coronary artery. Ischemia was induced by pulling the ends of the suture through a small segment of a soft tube, which was firmly attached against the artery with a clamp. The successful induction of ischemia was verified by ST segment elevation on the electrocardiogram and by visual inspection (cyanosis) of the heart. Reperfusion was achieved by releasing the clamp and was verified by refilling of the artery.

Hemodynamics

Heart rate and blood pressure were continuously monitored and measured at baseline, in the middle of sustained ischemia and at the end of long reperfusion.

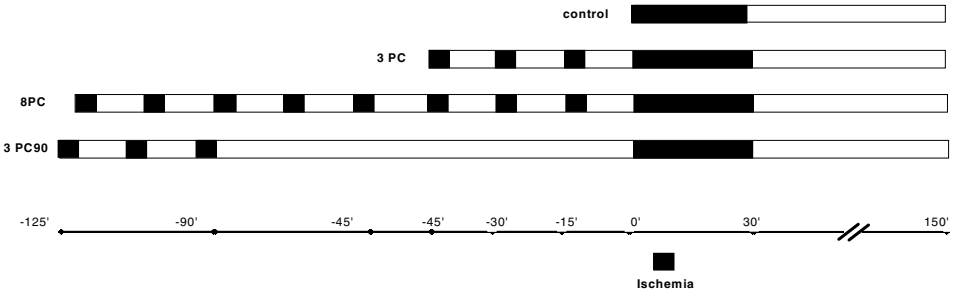

Figure 1 Diagrammatic representation of the experimental protocol used to evaluate the effect of placebo, nicorandil and 5HD-nicorandil on infarct size, in different modes of preconditioning of the rabbit heart *in vivo*.

Risk area and infarct size

After 2 h of reperfusion the hearts were harvested, the coronary ligature was retightened at the same site and 5 ml of Zn–Cd fluorescent particles was infused over 5 min for the delineation of the normally perfused tissue from the risk zone. Hearts were then frozen at −20°C and 24 h later were sliced into 3-mm-thick sections from apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) in an isotonic phosphate buffer solution, at a pH of 7.4 for 20 min at 37°C. The heart slices were immersed in 10% formaldehyde solution for 24 h to delineate the infarcted areas more clearly. For examination, the slices were pressed between glass plates; to identify the borders between the risk zone and the normal area, slices were examined under u.v. light (wavelength 366 nm). The infarcted, the risk and the normal areas were traced onto an acetate sheet that had been placed over the top glass plate. The tracings were then photographically enlarged and quantified by planimetry with the aid of a computer-interfaced scanner. The areas of myocardial tissue at risk and tissue of infarction were automatically transformed in volumes by multiplying the corresponding areas by thickness (3 mm). Infarct and risk area volumes were expressed in cm^3 and the percent of infarct to risk area (%I/R) calculated.

Statistical analysis

Values are expressed as mean ± s.e.m. Differences in infarct to risk ratio in percent were assessed using one-way analysis of variance (ANOVA). *Post hoc* analysis with the least significant difference test was used for the statistical comparisons. A calculated P value of less than 0.05 was considered to be statistically significant.

Results

Animals and exclusions

First step One rabbit from the first series and one from the second died prior to surgery while on the treatment with placebo and nicorandil, respectively. Three animals from the first series and four from the second died from various reasons such as hypoxia at the time of surgical manipulations (one animal), hemorrhage at the time of ischemia (one animal), progressive hypotension at the time of reperfusion (two animals) and intractable ventricular fibrillation at the time of ischemia and reperfusion (three animals). Therefore, 53 rabbits completed the first step of the study.

Second step Three rabbits died from various reasons, such as hemorrhage at the time of surgical manipulations (one animal), intractable ventricular fibrillation at the time of ischemia (one animal) and severe hypoxia because of intratracheal hemorrhage at the time of incision and intubation. Therefore, 34 animals completed the second step of the study.

Hemodynamic variables

Baseline characteristics for all the study groups and hemodynamic variables at the various points of time were not different between the groups that received placebo, nicorandil, combined nicorandil plus 5HD or nitroglycerin. These findings are presented in Table 1.

Infarct size

Table 2 details the means ± 1 s.e.m. values of the infarct size and risk zone size for the various study groups and Figure 2 their mean infarct to risk zone ratio in percent (%I/R). The control group that was subjected to extended ischemia reperfusion without nicorandil treatment developed infarcts that represented 41.5±4.7% of the risk zone *vs* 43.9±7.0% in the control group with nicorandil treatment, P = NS. The addition of 5HD did not alter the infarct size (48.7±6.4%). The *3PC* group developed an infarct size of 10.3±3.4% without nicorandil and 12.2±3.9% with nicorandil treatment, P = NS, and again the addition of 5HD had no effect on infarct size (12.6±4.5%). The *8PC* and the *3PC90* groups without nicorandil developed an infarct size of 47.7±8.8% and 37.3±6.0%, respectively, which decreased significantly with nicorandil treatment (13.0±2.6% and 14.2±2.4%, $P<0.01$ *vs* respective placebo groups), but was abrogated

Table 1 Hemodynamic variables of the various studied groups at baseline, on the 15th minute of sustained ischemia and on the 120th minute of reperfusion

	Baseline		*Ischemia*		*Reperfusion*	
	HR	*BP*	*HR*	*BP*	*HR*	*BP*
Placebo control	300.0±9.8	82.3±4.1	313.6±9.7	76.8±4.1	287.3±7.1	66.3±2.9
Nicorandil control	288.0±5.6	78.3±4.2	295.2±6.5	72.6±3.1	279.4±10.4	68.5±3.7
5HD-Nicorandil control	280.8±7.3	78.9±3.6	286.6±5.1	75.5±3.2	262.5±6.1	69.3±3.2
Placebo 3 PC	275.7±11.2	81.4±3.8	284.9±10.6	75.7±4.2	270.2±9.4	65.7±3.5
Nicorandil 3 PC	302.8±8.9	80.8±3.6	309.5±10.5	78.5±3.8	276.2±12.3	69.2±4.4
5HD-Nicorandil 3PC	293.3±8.0	80.1±5.1	292.5±4.7	75.5±3.4	265.0±7.7	68.0±2.9
Placebo 8 PC	281.2±4.7	78.9±3.4	289.9±7.9	74.5±3.1	269.7±7.8	65.5±2.6
Nicorandil 8 PC	279.0±5.1	79.5±3.9	288.6±8.9	74.7±2.3	263.2±7.4	70.4±3.5
5HD-Nicorandil 8PC	287.8±6.7	82.5±3.1	288.5±4.9	75.1±2.6	267.1±5.7	65.0±1.8
Placebo 3 PC 90	288.7±7.2	81.2±4.1	297.6±6.9	72.2±4.4	261.1±9.1	63.6±3.9
Nicorandil 3 PC 90	285.6±9.6	80.7±3.1	289.9±9.6	72.4±4.2	259.3±6.8	61.9±3.6
5HD-Nicorandil 3PC 90	280.0±5.6	77.1±3.5	283.1±4.1	72.7±3.1	256.2±5.3	63.7±2.4
NTG control	285.7±5.7	70.6±3.2	289.2±5.5	65.8±2.3	268.5±5.0	60.8±2.1

HR: heart rate in beats per minute, BP: mean blood pressure in mmHg. In NTG control group the baseline values are 5 min before sustained ischemia while on nitroglycerin treatment.

Table 2 Mean values ±1 s.e.m. expressed in cm^3 of the areas of infarct and risk in the various study groups

	N	*Infarct size*	*Risk area*
Placebo control	**7**	0.56±0.0.9	1.28±0.11
Nicorandil control	7	0.58±0.10	1.32±006
5HD-Nicorandil control	6	0.68±0.11	1.36±0.12
Placebo 3 PC	6	0.17±0.06	1.59±0.08
Nicorandil 3 PC	7	0.17±0.05	1.52±0.11
5HD-Nicorandil 3PC	6	0.19±0.07	1.48±0.08
Placebo 8 PC	7	0.63±0.10	1.35±0.08
Nicorandil 8 PC	6	0.18±0.04	1.31±0.09
5HD-Nicorandil 8PC	7	0.52±0.09	1.33±0.13
Placebo 3 PC 90	6	0.54±0.11	1.41±0.11
Nicorandil 3 PC 90	7	0.20±0.04	1.35±0.10
5HD-Nicorandil 3PC 90	8	0.57±0.07	1.34±0.12
NTG control	7	0.59±0.07	1.53±0.19

with the addition of 5HD (38.2±4.7 and 42.7±4.4%, respectively, $P<0.01$ *vs* nicorandil). Finally, the nitroglycerin-treated NTG-*Control* group developed an infarct size of 39.1±3.1%, which was not statistically significant in comparison with the other controls.

Discussion

Our study demonstrates that the oral treatment with the K_{ATP} channel opener nicorandil is effective in limiting the infarct size in conditions where protection from preconditioning has been lost. Nicorandil alone, with no previous preconditioning stimulus, appears to be insufficient in protecting the ischemic heart.

After the seminal definition of preconditioning, a large number of studies described the natural history of this phenomenon. It has been shown that protection wanes and is eventually lost when multiple cycles of preconditioning are applied before the sustained ischemia (Cohen *et al*., 1994; Iliodromitis *et al*., 1997) or when the time interval between short and long ischemia is very prolonged (Van Winkle *et al*., 1991; Miura *et al*., 1992).

The lost protection of preconditioning can be recaptured when an additional brief ischemic stimulus is given shortly before the sustained ischemia (Cohen *et al*., 1994; Li and Kloner, 1994; Iliodromitis *et al*., 1996). Thus, the effectiveness of preconditioning in limiting the infarct size depends on the number of short cycles of ischemia and on the length of the intervening interval between the short and long ischemic period.

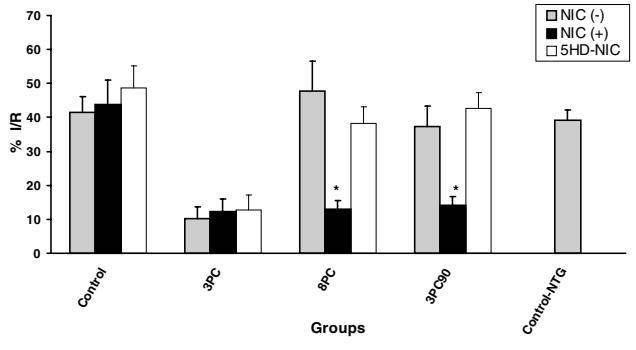

Figure 2 The effect of placebo, nicorandil and 5HD-nicorandil on infarct size, expressed as a percentage of risk zone size, after various modes of preconditioning. This benefit is abolished with the addition of the K_{ATP} channel blocker 5-hydroxydecanoate. *$P<0.01$ *vs* placebo-treated and 5HD-nicorandil groups. Nitroglycerin does not reduce the infarct size in the control group.

Among the various ligands that stimulate the cardiomyocyte membranes, adenosine (Liu *et al*., 1991; Tsuchida *et al*., 1992), bradykinin (Goto *et al*., 1995), opioids (Schulz *et al*., 1995) and many others (Wang *et al*., 1996; Cohen *et al*., 2001) initiate one or more signal transduction pathways. Therefore, several triggers and mediators are involved in the mechanism of preconditioning, probably by opening the mitochondrial K_{ATP} channels, which appear to be the end effectors for the protection (Ockaili *et al*., 1999; Wang *et al*., 2001). However, more recent evidence suggests that direct activation of the mitochondrial K_{ATP} channels may put the heart in a preconditioned state (Liu *et al*., 1998; Pain *et al*., 2000). Thus, K_{ATP} channels may act as triggers, mediators, or end effectors.

Mitochondrial K_{ATP} channel openers such as nicorandil (Sato *et al*., 2000), diazoxide (Garlid *et al*., 1997; Liu *et al*., 1998) and pinacidil (Critz *et al*., 1997) have been used as preconditioning triggers. In the present study, we used the selective agent nicorandil and found that it is capable of recapturing the protection from preconditioning in conditions where it had waned or was lost. Oral treatment with nicorandil, without any previous short ischemic insult, appears to be insufficient in triggering the protective mechanism of preconditioning and limiting the infarct size. Furthermore, a brief preceding ischemia of eight cycles or more, or an extended duration of reperfusion, which would have been ineffective appear to become effective when combined with oral nicorandil. Thus, we can assume that subthreshold stimuli or insufficient mediators emerging from the short ischemic bouts or by nicorandil itself should be combined in order to reinstate the lost protection from preconditioning. Our findings are in accordance with Mizumura *et al*. (1997), who found that nicorandil was ineffective without any short ischemia in a canine model but was effective when added to a subthreshold short ischemic insult. Similarly, Critz *et al*. (1997) found that nicorandil alone does not protect the rabbit myocytes, and Minatoguchi *et al*. (2001) observed that nicorandil alone is rather ineffective in limiting the infarct size, while Imagawa *et al*. (1998) reported that the intravenously given nicorandil shows an intermediate protective efficacy between the control and the preconditioned groups. It should be noted that in these studies nicorandil was administered intravenously. To our knowledge, ours is the first study of preconditioning in which nicorandil was given orally.

The addition of the K_{ATP} channel blocker 5HD abrogated the beneficial effect of nicorandil, meaning that the opening of the K_{ATP} channels, either as trigger or as mediator, plays a significant role in conditions of ischemic preconditioning, where the protection waned or had been lost. However, we did not observe any change in the infarct size in the condition where the 5HD was added to the group treated with nicorandil and subjected to classic ischemic preconditioning. This may happen because ischemic preconditioning is a sufficient stimulus for the mechanism of protection, and follows alternative pathways for intracellular signaling, *via* the phosphorylation of an unknown end effector (Oldenburg *et al*., 2002) and without using K_{ATP} channels (Garcia-Dorado

et al., 2002; Oldenburg *et al*., 2002). Another possibility is that although the 5HD is sufficient to block the opening of the K_{ATP} channels from previous nicorandil treatment, it is insufficient to block any additional opening of the K_{ATP} channels from the ischemic preconditioning. Interestingly, although we agree with Imagawa from Yellon's group that the addition of nicorandil does not increase the infarct size in the preconditioned rabbits and the 5HD infusion has no effect in control animals but blunts the protective effect of nicorandil, Carr & Yellon (1997) reported that the exposure to nicorandil abolishes the functional recovery of preconditioned human atrial trabeculae. These divergent results may be explained because of the differences in the surrogate end points (functional recovery *vs* infarct size), in the chosen species (human tissue *vs* rabbits), and in the mode of perfusion (Tyrode's solution *vs* circulating blood *in vivo*).

Although blood nicorandil levels were not measured, the total daily dose was more than sufficient compared to doses administered in previous trials on humans (Patel *et al*., 1999; The IONA Study Group, 2002). Orally administered nicorandil is rapidly absorbed with peak plasma concentration occurring 0.5–1 h after administration and causes a steady-state plasma level within 4 days after the first dose; the apparent volume of distribution is equal between a 20 mg oral or a 5 mg i.v. dose (Markham *et al*., 2000). Imagawa's (1998) study describes an intravenous dosage of $100\,\mu g\,kg^{-1}$ bolus administration, then $10\,\mu g\,kg^{-1}\,min^{-1}$ for 60 min, giving a total dose of $700\,\mu g\,kg^{-1}\,min^{-1}$, or approximately 2 mg per animal. In our protocol, we administered nicorandil orally at $5\,mg\,kg^{-1}$, therefore, the total daily dose was approximately 15 mg per animal. Thus, the total oral dose in our protocol was higher than the equivalent iv dose used in Imagawa's study.

One of the clinical equivalents of preconditioning is unstable angina. Transferring the acquired experience from experimental conditions to clinical practice, we can assume that some patients with preinfarct angina are protected from preconditioning but others may not be because of inappropriate time intervals or because of the inappropriate frequency of the ischemic episodes. This may explain the divergent results that emerged from various clinical studies in the preinfarct angina era.

In the CESAR 2 multicenter clinical trial (Patel *et al*., 1999), patients with unstable angina were treated with conventional therapy and with oral nicorandil or placebo for 5 consecutive days. Using ST changes as a surrogate end point of protection, the researchers found that patients taking nicorandil had less ischemic changes in 24-h Holter monitoring. Our experimental results agree with this study, because we observed that the addition of nicorandil to short ischemic insults confers protection while the addition of a placebo does not. We also agree with Matsubara *et al*. (2000), who found that the addition of nicorandil to an ineffective stimulus for preconditioning is capable of rendering it effective, and Lee *et al*. (2002), who recently proved in elderly patients that impaired preconditioning is reversed by intravenous nicorandil.

Nicorandil has a nitrate component and therefore it should be assumed that the conferred protection may be related to its action as nitroglycerin. Although there is no strong evidence to support the hypothesis that nitrates decrease the infarct size, this possibility can be excluded as we did not observe any benefit either in the nicorandil- or in the nitroglycerin-treated control groups.

Limitation

In our study, reperfusion was limited to 2 h. This might be seen as a deficiency in our method, as the observed protection against infarct development may represent a delay in progression to cell death rather than prevention of irreversible tissue injury. However, our model was similar to others in which the infarct size was assessed after a rather short reperfusion time following the ischemic period, and not a reperfusion period of several days' duration.

In conclusion, oral treatment with nicorandil recaptures the waned protection of preconditioning, both after repetitive bouts of short ischemia or after a prolonged reperfusion interval, preserving the initially obtained benefit. Nicorandil by itself is not sufficient to initiate preconditioning *in vivo*.

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