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p38-MAPK is involved in restoration of the lost protection of preconditioning by nicorandil in vivo

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Abstract

Nicorandil, a selective mitochondrial K_{ATP} channel opener, reinstates the waned protection after multiple cycles of preconditioning. In this study, we determined the signal transduction activated in heart after 3 or 8 cycles of preconditioning and prolonged ischemia in rabbits treated with placebo or nicorandil. In a first series (eight groups) we evaluated the (%) infarct to risk ratio after 30 min ischemia/3 h reperfusion and in a second series (six groups), we assessed the intracellular levels of cyclic GMP (c-GMP), protein kinase C (PKC) activity and p38-mitogen activated protein kinase (p38-MAPK) phosphorylation from heart samples taken during the long ischemia. Cardioprotection by 3 cycles of preconditioning (11.7±3.8% vs 45.9±5.2% in the control, $P<0.001$) was lost after 8 cycles (43.9±5.1%, P=NS vs control). Nicorandil restored it to the levels of classic preconditioning (13.7±2.4% vs 40.8±3.5% in respective controls, $P<0.001$). This was reversed by the p38-MAPK inhibitor SB203580 (48.8±5.1%) which had no protective effect in the control group (44.6±5.8%). In the placebo-treated rabbits, intracellular c-GMP and PKC were increased only in the group subjected to 3 cycles of preconditioning. Despite that nicorandil equalizes the intracellular levels of c-GMP, PKC and activated p38-MAPK at the long ischemia, specific alterations of p38-MAPK phosphorylation differentiate the protected groups. Our data delineate the signal transduction mechanism mediating the beneficial effect of nicorandil and imply that the recapture of the lost protection is due to a dynamic process of the intracellular mediators accompanied by an increase in p38-MAPK phosphorylation and not to an instantaneous event.

Keywords: Nicorandil; Ischemia; Preconditioning; p38-MAPK; Cardioprotection

1. Introduction

Murry et al. (1986) found that brief sub-lethal periods of ischemia prior to a subsequent prolonged ischemic insult render the heart more tolerant against infarction, an effect known as ischemic preconditioning. Long before studies on preconditioning, Noma (1983) was the first to report the existence of K_{ATP} channels in the myocardium. Since then, it has been established that opening of the mitochondrial (Ma et al., 1999) rather than sarcolemmal (Fryer et al., 2000) K_{ATP} channels causes a transient generation of reactive oxygen species, which in turn trigger downstream cascades that may confer protection against infarction (Pain et al., 2000). Thus, there is accumulating evidence showing that reactive oxygen species have an important physiological role as intracellular messengers. Furthermore, several ligands like adenosine, opioids and bradykinin (Cohen et al., 2001; Krieg et al., 2005; Oldenburg et al., 2004) acting before long ischemia may trigger preconditioning while several intracellular agents such as PKC and Mitogen-Activated Protein Kinases (MAPKs) (Michel et al., 2001; Mitchell et al., 1995; Miura et al., 1998; Schulz et al., 2001) are the acting mediators at the time of sustained ischemia. Furthermore, levels of circulating c-GMP are increased from the beginning of sustained ischemia after an effective preconditioning stimulus (Iliodromitis et al., 1996) while intracellular levels of c-GMP are increased at the 20th min of ischemia (Andreadou et al., 2006) and 24 h after preconditioning (Kodani et al., 2002). On the other hand, nicorandil is a selective mitochondrial K_{ATP} channel opener that

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has been tested in numerous experimental studies as well as in multicenter clinical trials with promising results (IONA study group 2002; Krumenacher and Roland, 1992; Ota et al., 2006; Patel et al., 1999; Sato et al., 2000b). We have previously shown that nicorandil recaptures the waned protection of preconditioning after multiple cycles in a rabbit model in vivo (Iliodromitis et al., 2003) but no data are available regarding the signal transduction cascades mediating this effect, even though research has progressed in the area of late preconditioning and postconditioning.

In the present study we thus endeavored to assess the intracellular levels of c-GMP, PKC activity as well as the activation profile of p38-MAPK at the time of prolonged ischemia following two modes of preconditioning: an effective classic one and an ineffective with multiple cycles of brief ischemia/reperfusion periods, without or with oral nicorandil treatment. Our results disclose and elucidate p38-MAPK vital role in the signal transduction regulatory mechanism mediating restoration of the waned protection after 8 cycles of preconditioning conferred by nicorandil.

2. Materials and methods

2.1. Animals

New Zealand White male rabbits weighing between 2.5 and 3.1 kg were used in this study and received proper care in 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.

2.2. Experimental protocols

Fourteen groups of male rabbits were divided into two respective series of experiments consisting of eight and six groups, respectively. In both series, placebo or nicorandil tablets were given orally for 5 consecutive days in a dose of 5 mg/kg/day every 12 h, one in the morning and one late in the evening. Tablets were crashed, diluted in dextrose 5% fluid and then given orally to the animals through a 5-ml syringe. The last dose was given 2 h before surgical intervention. The first series was used for the evaluation of the infarct size as the percent of infarct to risk area ratio and the second one for the assessment of levels of several intracellular mediators at the time of long ischemia.

2.3. Surgical preparation

All animals were anesthetized by slowly injecting 30 mg/kg of sodium thiopentone (Pentothal, Abbot) 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 adjusted to keep blood gases within the normal range. Two polyethylene catheters were inserted in the left jugular vein for fluids, drug administration or additional infusion of anesthesia and in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multichannel recorder (Nihon-Koden, Model 6000, Japan). A bipolar chest lead was used for continuous electrocardiographic recording. 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.

2.4. First series protocol

All animals were subjected to a 30 min regional ischemia of the heart and a 3 h reperfusion with the following interventions before long ischemia: Control groups were treated with either placebo ($n=8$) or nicorandil ($n=8$) without any other intervention apart from the prolonged ischemia and reperfusion; all other groups were subjected to either 3 or 8 cycles of preconditioning after treatment with placebo or nicorandil tablets. In particular, animals in the classic preconditioning groups ($n=7$ for either placebo or nicorandil) were subjected to 3 cycles of 5 min ischemia and 10 min reperfusion and animals in the multiple preconditioning groups ($n=7$ for placebo and $n=8$ for nicorandil) were subjected to 8 cycles of 5 min ischemia and 10 min reperfusion prior to long ischemia.

Given that the role of p38-MAPK in the mechanism of myocardial protection remains controversial, we investigated its role in our experimental setting using 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), a potent inhibitor of this protein kinase activity. SB203580 (Alexis Biochemicals, Lausen, Switzerland) was dissolved in DMSO (0.5 mg/50 μl) and the solution was further diluted in 1 ml of 0.9% (w/v) saline. The drug was given (0.16 mg/kg) as an intravenous bolus before the onset of the 8 cycles of preconditioning ($n=5$) as well as in a respective control group ($n=5$). The dose at which it was given was more than 10-fold less than what is routinely applied so as to ensure maximal specificity and avoid any cross-effects with other kinases reported to be also inhibited by high concentrations of SB203580 (e.g. JNKs).

After 3 h of reperfusion, the hearts were harvested, mounted on a reperfusion apparatus and perfused retrogradely (50 mmHg) for 2 min via the aorta with normal saline (15 ml/min, 20 °C). When all residual blood had been removed from the coronary arteries, the coronary ligature was retightened at the same site and 5 ml of green fluorescent micro spheres (2–9 μm diameter, Duke Scientific Corp., Palo Alto, CA, USA), suspended in saline, were infused over 5 min for the delineation of the normally perfused tissue from the risk area. Hearts were then frozen at −20 °C and 24 h later, sliced into 3 mm-thick sections from the apex to base. The slices were incubated in 1% (w/v) triphenyl tetrazolium chloride (TTC) in isotonic phosphate buffer solution, pH 7.4 for 20 min at 37 °C. TTC reacts with dehydrogenases and NAD in viable tissue; the infarcted area was defined as the

negative stained region. Heart slices were immersed in 10% (v/v) formaldehyde solution for 24 h to delineate the infarcted areas more clearly. Subsequently, the slices were pressed between glass plates and in order to identify the borders between the risk zone and the normal area, they were examined under ultraviolet light (wavelength 366 nm). The infarcted, the risk and the normal areas were traced onto an acetate sheet, which had been placed over the top glass plate. The tracings were then photographically enlarged (Adobe Photoshop 6.0) and quantified by planimetry with the aid of the Scion Image program interfaced with a computer. The areas of myocardial tissue at risk and of infarction were automatically transformed into volumes by multiplying the corresponding areas by the slice thickness (3 mm).

Infarct and risk area volumes were expressed in cm^3 and the percent of infarct to risk area ratio calculated as previously described (Iliodromitis et al., 1997).

2.5. Second series protocol

Animals were exposed to a 20 min regional ischemia of the heart with the same additional interventions prior to long ischemia as in the first series of experiments. After the end of the ischemic period, heart samples were cut by a biopsy scissor distal to the silk thread into the center of the area at risk which appears to be blue by visual inspection, rapidly removed and immediately immersed into liquid nitrogen. They were then stored at −80 °C until the subsequent analyses. For the detection of p38-MAPK phosphorylation levels, samples were also taken from the non-ischemic region of the respective hearts. Tissue samples were homogenized with 3 ml/g extraction buffer [20 mM Tris–HCl pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 10 mM benzamidine, 200 μM leupeptin, 120 μM pepstatin A, 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane (E64), 300 μM phenyl methyl sulphonyl fluoride (PMSF), 0.5% (v/v) Triton X-100] and extracted on ice for 15 min. Samples were centrifuged (10,000 g, 5 min, 4 °C) and the supernatants were boiled with 0.33 volumes of SDS/PAGE sample buffer [0.33 M Tris/HCl pH 6.8, 10% (w/v) SDS, 13%(v/v) glycerol, 20%(v/v) 2-mercaptoethanol, 0.2%(w/v) bromophenol blue]. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, California 94547, USA).

The various experimental protocols are presented schematically in Fig. 1.

2.6. c-GMP measurement

Heart powders were homogenized with 5 ml/g absolute ethanol and extracted on ice for 10 min. Samples were centrifuged (10,000 g, 5 min, 4 °C) and supernatants were used for c-GMP determination by radioimmunoassay using the [^{3}H]c-GMP assay Biotrak system (TRK 500) according to the manufacturer's protocol (Amersham Biosciences, Buckinghamshire, HP7 9NA, England). c-GMP concentration was normalized to tissue weight (The number of independent experiments in each group was at least four).

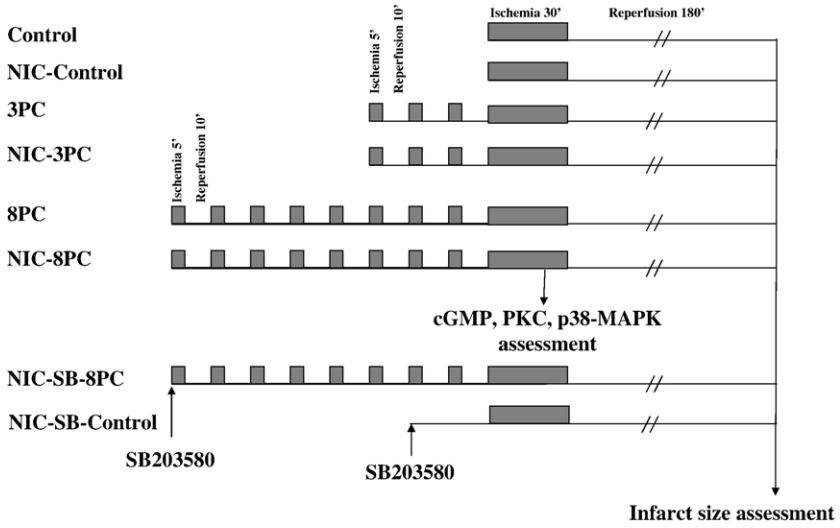

Fig. 1. Experimental protocols. Control: oral treatment with placebo tablets for 5 consecutive days before subjecting the hearts to 30 min sustained ischemic period, NIC-Control: oral treatment with nicorandil for 5 consecutive days before subjecting the hearts to 30 min sustained ischemia. 3PC: oral treatment with placebo tablets for 5 consecutive days before subjecting the hearts to 3 cycles of preconditioning i.e. : (5 min ischemia+10 min reperfusion)×3. NIC-3PC: oral treatment with nicorandil for 5 consecutive days before subjecting the hearts to 3 cycles of preconditioning [(5 min ischemia+10 min reperfusion)×3] followed by 30 min sustained ischemia. 8PC: oral treatment with placebo tablets for 5 consecutive days before subjecting the hearts to 8 cycles of preconditioning [(5 min ischemia+10 min reperfusion)×8] followed by 30 min sustained ischemia. NIC-8PC: oral treatment with nicorandil before subjecting the hearts to 8 cycles of preconditioning [(5 min ischemia+10 min reperfusion)×8] followed by 30 min sustained ischemia. NIC-SB-Control: oral treatment with nicorandil before subjecting the hearts to administration of a bolus injection of SB203580 and subsequently to 30 min sustained ischemia. NIC-SB-8PC: oral treatment with nicorandil before subjecting the hearts to administration of a bolus injection of SB203580 and subsequently to 8 cycles of preconditioning [(5 min ischemia+10 min reperfusion) X 8] followed by 30 min sustained ischemia. c-GMP levels, PKC activity and p38-MAPK phosphorylation assessment was performed in samples taken at the 20th min of sustained ischemia, while infarct size assessment was performed after 180 min of reperfusion following the 30 min sustained ischemia. NIC: nicorandil, PC: preconditioning, SB: SB203580.

2.7. Determination of PKC activity

PKC enzyme activity was determined with an Upstate assay kit (#17-139, Lake Placid, NY), which measures the phosphotransferase activity of PKC in crude tissue lysates. All assays were performed in the presence of an inhibitor cocktail, blocking the activity of other serine/threonine kinases such as PKA (c-AMP — dependent protein kinase) and calmodulin-dependent kinases. Suitable blanks were also performed so as to correct for any non-specific binding of [γ-^{32}P] ATP and its breakdown products to the phosphocellulose paper. Samples were assayed for PKC activity using the kit mentioned above according to the manufacturer's protocol. (The number of independent experiments in each group was at least four).

2.8. Immunoblotting for phosphorylated p38-MAPK

Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Keene N.H. 03431, USA). Non-specific binding sites on the membranes were blocked with 5% (w/v) nonfat milk in buffer A consisting of 20 mM Tris–HCl pH 7.5, 137 mM NaCl and 0.1% (v/v) Tween 20 for 30 min at room temperature. Subsequently, the membranes were incubated with a mouse monoclonal antibody recognizing the dually phosphorylated forms of p38-MAPK (Cell Signaling, Inc., Beverly, MA) according to the manufacturer's instructions. After washing in buffer A, blots were incubated with horseradish peroxidase-linked anti-mouse IgG antibody (DAKO A/S DK-2600 Glostrup, Denmark) for 1 h at room temperature. The blots were washed again in buffer A and bands were detected using an enhanced chemiluminescence reagent kit (Amersham Biosciences, Uppsala, Sweden) with exposure to Fuji photo film GmbH (Dusseldorf, Germany). Blots were quantified by laser scanning densitometry. (The number of independent experiments in each group was at least four).

2.9. Statistical analysis

Values are expressed as mean±S.E.M. c-GMP levels, PKC activity values and p38-MAPK phosphorylation data were compared by two-way ANOVA. When significant differences were detected, individual mean values were compared by Tukey test. A probability of $P<0.05$ was considered statistically significant.

Table 1
Mean values±S.E.M. expressed in cm^3 of the areas of infarct size and risk area in the various study groups

Group	N	Infarct size	Risk area
Control	8	0.68±0.09	1.63±0.39
3 cycles of preconditioning	7	0.17±0.06*	1.51±0.07
8 cycles of preconditioning	7	0.81±0.09	1.86±0.15
Nicorandil–control	8	0.59±0.08	1.42±0.15
Nicorandil–3 cycles of preconditioning	7	0.17±0.06*	1.59±0.06
Nicorandil–8 cycles of preconditioning	8	0.24±0.05*	1.72±0.07
Nicorandil–SB203580–control	5	0.76±0.11	1.72±0.12
Nicorandil–SB203580–8 cycles of preconditioning	5	0.79±0.09	1.65±0.017

* P<0.001 vs. control group.

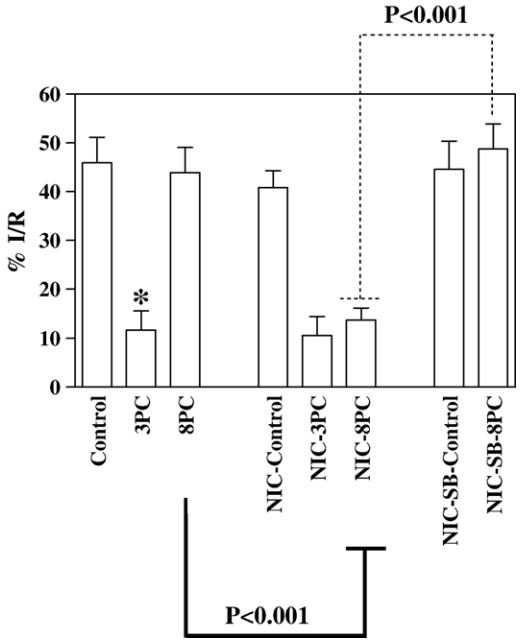

Fig. 2. The effect of placebo and nicorandil on infarct size (expressed as a percent of risk area size) in rabbit hearts, after different modes of preconditioning. For details of experimental protocols see legend of Fig. 1. Data is presented as means±S.E.M. for at least five independent experiments. *$P<0.001$, compared to placebo-treated control group. NIC: nicorandil, PC: preconditioning, SB: SB203580.

3. Results

3.1. Infarct size and hemodynamic variables

The effect of various interventions on infarct size and risk area is shown in Table 1 and in Fig. 2. The infarcted to risk area ratio was 45.9±5.2% in the placebo-treated control group, 11.7±3.8% in the group of the hearts subjected to 3 cycles of preconditioning ($P<0.001$) and 43.9±5.1% in the group of the hearts subjected to

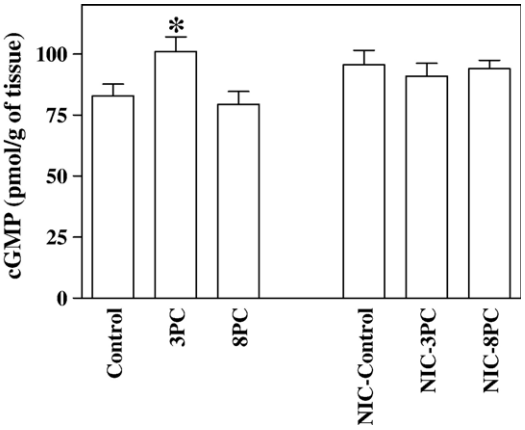

Fig. 3. c-GMP levels (pmol/g of tissue) from the ischemic region of hearts subjected to the various experimental protocols mentioned in Fig. 1. Data is presented as means±S.E.M. for at least four independent experiments. *$P<0.05$, compared to placebo-treated control group. NIC: nicorandil, PC: preconditioning.

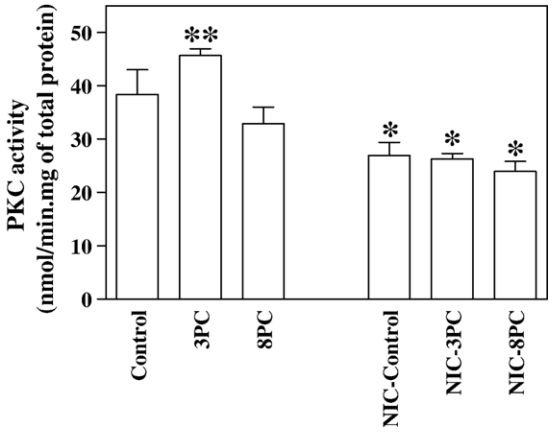

Fig. 4. Activity of cardiac PKC (nmol/min mg of total protein) in whole extracts from the ischemic region of hearts subjected to the various experimental protocols mentioned in Fig. 1. PKC activity was assayed in the presence of Ca^{2+} and phospholipid as described in the Materials and methods section. Data is presented as means±S.E.M. for at least four independent experiments. *$P<0.05$, compared to placebo-treated respective group values; **$P<0.05$, compared to placebo-treated control group. NIC: nicorandil, PC: preconditioning.

8 cycles of preconditioning (P=NS vs control). In the group of animals treated with nicorandil, 8 cycles of preconditioning resulted in a considerable reduction of the infracted to risk area ratio (13.7±2.4%, $P<0.001$ vs placebo-treated control with 8 cycles of preconditioning group), while no notable change was observed in the groups of animals treated with nicorandil followed by either no intervention (40.8±3.5%) or 3 cycles of preconditioning (10.6±3.7%), compared to the placebo-treated control and 3 cycles of preconditioning groups, respectively. Therefore, our results indicate that 3 cycles of preconditioning protect the heart against the 30 min following ischemia/3 h reperfusion, with this protection being lost after 8 cycles of preconditioning. However, nicorandil restores the observed loss of protection after 8 cycles of preconditioning (Fig. 2). SB203580 negates this benefit that nicorandil confers after 8 cycles of preconditioning, with no additional effect in the nicorandil treated control group (48.8±5.1% and 44.6±5.8% respectively, P=NS vs control) (Table 1 last two lines and Fig. 2 last two bars). Means±S.E.M. values of the (%) infarct to risk area ratio are detailed in Fig. 2. Mean heart rate and mean blood pressure at baseline and at the end of sustained ischemia were monitored with no significant differences observed between the groups at various time points (data not shown).

3.2. c-GMP levels

Cyclic-GMP concentration was significantly greater in the protected by 3 cycles of preconditioning group compared to the non-protected placebo-treated control and 8 cycles of preconditioning groups ($P<0.05$) (Fig. 3). All groups treated with nicorandil exhibit similar intracellular levels of c-GMP at the time of long ischemia (Fig. 3, three last bars).

3.3. PKC activity

In accordance with the widely accepted role of PKC as a mediator in cardioprotection induced by preconditioning, higher

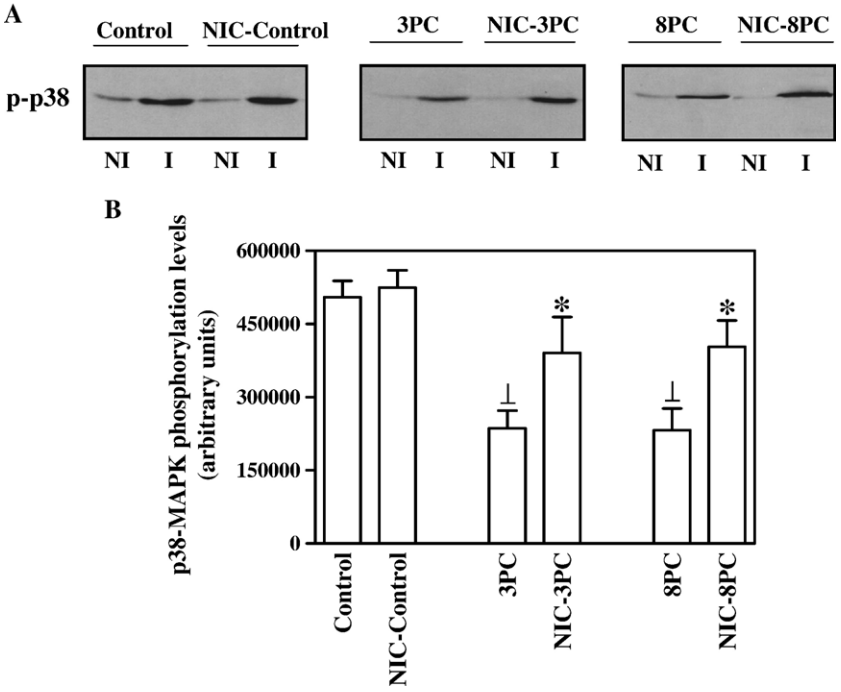

Fig. 5. Phosphorylation profile of p38-MAPK in the various experimental groups studied. Heart tissue extracts (100 μg) of the non-ischemic (NI) and ischemic (I) regions, were subjected to SDS-PAGE and immunoblotted with an appropriate antibody for the phosphorylated p38-MAPK (Thr180/Tyr182). Bands were quantified by laser scanning densitometry. Blots (top panels) shown are representative of at least four independent experiments. Graph bars represent densitometric data of p38-MAPK phosphorylation levels in samples from the ischemic region of the heart. Results are means±S.E.M. for at least four independent experiments. *$P<0.01$, compared to placebo-treated respective group values; ⊥ $P<0.001$, compared to placebo-treated control group. NIC: nicorandil, PC: preconditioning.

levels of this kinase activity were measured in samples from the protected by 3 cycles of preconditioning group vs the non-protected placebo-treated control and 8 cycles of preconditioning groups ($P<0.05$, Fig. 4). Nicorandil significantly decreased PKC activity in all protected groups ($P<0.05$ vs the respective placebo-treated groups) which showed similar intracellular activity levels of this kinase at the time of long ischemia. Of note, nicorandil also decreased PKC activity in the non-protected placebo-treated control group (Fig. 4).

3.4. Activation of p38-MAPK

Ischemic preconditioning with 3 or 8 cycles of brief ischemia/reperfusion causes an equal reduction by ~53.6±5.7% of p38-MAPK phosphorylation (thus activation) levels ($P<0.001$ vs control value). Nicorandil significantly enhanced p38-MAPK phosphorylation levels in both protected by 3 or 8 cycles of preconditioning groups (by ~90±2.4%, $P<0.01$). The kinase phosphorylation levels remained unchanged in the control groups treated with either placebo or nicorandil (Fig. 5B, first two bars). As shown in Fig. 5, p38-MAPK phosphorylation was barely detectable in respective samples from the non-ischemic region of all the hearts examined.

4. Discussion

The present study investigates the changes in signal transduction pathways involved in restoration of lost benefit after multiple cycles of preconditioning in vivo by nicorandil. In comparison to the non-protected placebo-treated control and multiple cycles of preconditioning groups, the classic preconditioning increases the intracellular levels of c-GMP and PKC activity at the time of long ischemia. Furthermore, recapture of the lost protection by oral nicorandil treatment is accompanied by significant intracellular alterations: no change in c-GMP levels, a decrease in PKC activity but also a simultaneous increase in p38-MAPK phosphorylation (thus activation) levels. Despite that nicorandil equalizes the intracellular levels of c-GMP, PKC activity and p38-MAPK phosphorylation levels at the time of long ischemia, specific alterations of p38-MAPK activation differentiate the protected groups. This finding implies that the recovery of the lost protection is due to a dynamic process rather than an instantaneous event.

Developing successful interventions for cardioprotection by establishing safe and potent therapeutic strategies applicable in the clinical setting constitutes a challenge. Cardioprotective utility of K_{ATP} channel openers seems advantageous over ischemic preconditioning, since the drugs can be given and metabolized in a dosage that can be precisely adjusted. Several studies so far have shown that the protection induced by preconditioning depends on the natural history of this phenomenon and is lost when inappropriate stimuli or time intervals precede the potentially lethal ischemic insult (Cohen et al., 1994; Iliodromitis et al., 1997, 2003). Corroborating the aforementioned studies, we observed that a preceding brief ischemic period consisting of three cycles protects the heart unlike one of eight cycles (Iliodromitis et al., 1997). Quite interestingly however, eight cycles of brief ischemia combined with oral nicorandil treatment lead to the recovery of cardioprotection against subsequent sustained ischemia (Iliodromitis et al., 2003). Therefore, while nicorandil preserves protection from classic ischemic preconditioning, in conditions where the latter is lost, i.e. after repetitive multiple cycles of ischemia/reperfusion, it appears to be insufficient to promote alone a preconditioning-like cardioprotective effect, a result that is in accordance with previous reports (O'Rourke, 2004). Nicorandil being a promising alternative that functions via opening of the mitochondrial K_{ATP} channels, it appears essential to characterize the signal transduction cascades involved in the case of nicorandil-conferred recovery of protection. In the present study we evaluated some of the most important mediators of the intracellular signal transduction pathways during a long ischemic insult in vivo and correlated our findings with the infarct size measured. Similar to previous studies of our group (Andreadou et al., 2006; Iliodromitis et al., 2002, 2006) samples for these evaluations were taken at the 20th min of prolonged ischemia, because this time period has been proven to be sufficient to cause irreversible cell damage.

It has been previously shown that circulating c-GMP levels are increased at the beginning of prolonged ischemia after a proper preconditioning stimulus (Iliodromitis et al., 1996). c-GMP is involved in the mechanism of preconditioning (Oldenburg et al., 2004) and its intracellular levels are increased in preconditioned hearts during long ischemia (Andreadou et al., 2006) or 24 h later (Kodani et al., 2002) indicating that it is required for the protection independently of its effect as a trigger. In agreement with this, in the present study we found that c-GMP levels are increased in the classically preconditioned group with three cycles of 5 min ischemia/10 min reperfusion and are not affected by the administration of nicorandil. This is in accordance with studies reporting c-GMP-dependent protein kinase G (PKG) "localization" upstream of the mitochondrial K_{ATP} channels inducing the opening of the latter when activated (Horinaka et al., 2006; Krieg et al., 2005). Lochner et al. (2000) using rat hearts in vitro, demonstrated that intracellular c-GMP levels are increased during the short ischemic insults and decreased during the following reperfusion period; other studies have also shown that c-GMP is operative during the trigger phase of both classic and delayed preconditioning (Cohen et al., 2006; Kodani et al., 2002; Szilvassy et al., 1994). Minamiyama et al. (2007) showed that nicorandil promoted an increase in c-GMP levels in rat liver, aorta and human coronary smooth muscle cells in vitro in a nitric oxide-dependent fashion. In the present in vivo study, we did not observe a similar effect on heart muscle. This discrepancy may be related to the experimental conditions, to the route and the dose of nicorandil administration, to tissue as well as species differences. Thus, additional samples taken at earlier or later stages of ischemia would be useful tools in evaluating c-GMP levels at various time points of the ischemic and reperfusion periods; however, this would be beyond the scope of our present study.

Corroborating studies implicating PKC as a significant mediator of preconditioning (Armstrong et al., 1994; Liu et al., 1994; Saurin et al., 2002), we also found that PKC activity is elevated in the classic preconditioned heart group. Of note, nicorandil, which is known to stimulate a transient generation of reactive oxygen species, failed to enhance the assayed PKC activity. The mechanism of reactive oxygen species-dependent cardioprotection has recently been the focus of intensive studies with rather controversial results (Carroll et al., 2001; Gopalachrishna and Anderson, 1989; Oldenburg et al., 2002; Tritto et al., 1997). Those groups that support stimulation of PKC activity by reactive oxygen species attribute the latter to a modification of the kinase regulatory domain (Cohen et al., 1996) or to activation by phospholipases C and D (Harada et al., 2004). Overall, the observed dissociation between the infarct size and PKC activity in the nicorandil treated group after multiple cycles of preconditioning, may be attributed to the fact that nicorandil increases the levels of reactive oxygen species generated by mitochondria which in fact subsequently mediate protection via at least one tyrosine kinase (Baines et al., 1998) with a p38-MAPK potential key role still remaining under dispute (Mackay and Mochly-Rosen, 1999; Mocanu et al., 2000).

Indeed, the role of MAPKs in the signal transduction of preconditioning has been addressed in several studies but the reported data are not conclusive (Hausenloy et al., 2005; Iliodromitis et al., 2002; Nakano et al., 2000a; Sato et al., 2000a; Yue et al., 2002). Looking into intracellular pathways, Cohen et al. (2001) focused on pharmacological preconditioning and found p38-MAPK to participate in various agonists of G_i-coupled receptors-induced signaling cascades. Given this finding, we decided to investigate the possible role of p38-MAPK in the mechanism mediating recovery of protection in the treated with nicorandil group which was subjected to multiple cycles of preconditioning. Nicorandil was found to induce a significant increase in p38-MAPK phosphorylation levels in the protected groups of both, classic and multiple preconditioning, conferring no change in the control group. What is more, in the presence of SB203580, a selective p38-MAPK inhibitor, the beneficial effect of nicorandil in the group subjected to 8 cycles of preconditioning was ablated. The concentration at which this inhibitor was used in our experiments (more than 10-fold less than what is routinely applied) excludes any possibility of an inhibitory side effect on other protein kinases such as JNKs (Zhang et al., 2000). The above result is indicative of the pivotal role of p38-MAPK in the recaptured protection by nicorandil after 8 cycles of preconditioning. However, one must point out that elevated p38-MAPK phosphorylation levels were not sufficient to confer protection in the group subjected to 8 cycles of preconditioning, suggesting that other factors also modulate the equilibrium between survival and cell injury in this case.

Interestingly, nicorandil also induced a significant increase in p38-MAPK phosphorylation levels in the classically preconditioned group, but did not confer any additional cardioprotection as exemplified by the infarct to risk area ratio measured. Nevertheless, previous studies have shown SB203580, a p38-MAPK selective inhibitor, to negate the cardioprotection afforded by classic preconditioning in rabbits (Nakano et al., 2000b) as well as in pigs (Schulz et al., 2002). Thus, further studies investigating p38-MAPK phosphorylation profile in different modes of ischemic preconditioning in the absence or presence of nicorandil and/or SB203580 could confer additional evidence elucidating the precise role of this signaling pathway in cardioprotection during long ischemia and/or reperfusion.

In conclusion, classic preconditioning in vivo results in increased intracellular c-GMP and PKC activity levels at the time of long ischemia, conversely to the non-protected groups. Nicorandil appears to modify intracellular signaling mechanisms by equalizing the intracellular differences of c-GMP, PKC activity and p38-MAPK phosphorylation levels. What is more, nicorandil stimulates p38-MAPK phosphorylation and leads to the recapture of protection via a dynamic intracellular process. Our results provide novel evidence regarding the signaling pathways involved in vivo and at the same time underline the necessity of careful interpretation of experimental results. In particular, assessment of the instantaneous levels of several mediators should be interpreted with caution as all the changes that precede should also be taken into account. Given the prominent role of nicorandil as a promising candidate for adjunctive treatment in acute myocardial infarction, further studies are required to fully decipher the diversity and multiplicity of interactions between the signaling cascades mediating the regulation of its mode of function. These novel data contribute to our understanding of the mechanisms of triggering and recapturing the protection of the ischemic heart in vivo.

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References

Andreadou, I., Iliodromitis, E.K., Tsovolas, K., Aggeli, I.K., Zoga, A., Gaitanaki, C., Paraskevaidis, I.A., Markantonis, S.L., Beis, I., Kremastinos, D.T., 2006. Acute administration of vitamin E triggers preconditioning via K (ATP) channels and cyclic-GMP without inhibiting lipid peroxidation. Free Radic. Biol. Med. 41, 1092–1099.

Armstrong, S., Downey, J.M., Ganote, C.E., 1994. Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. Cardiovasc. Res. 28, 72–77.

Baines, C.P., Wang, L., Cohen, M.V., Downey, J.M., 1998. Protein tyrosine kinase is downstream of protein kinase C for ischemic preconditioning's anti-infarct effect in the rabbit heart. J. Mol. Cell. Cardiol. 30, 383–392.

Carroll, R., Gant, V., Yellon, D.M., 2001. Mitochondrial K_{ATP} channel opening a human atrial-derived cell line by a mechanism involving free radical generation. Cardiovasc. Res. 51, 691–700.

Cohen, M.V., Yang, X.M., Downey, J.M., 1994. Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. Circ. Res. 74, 998–1004.

Cohen, M.V., Liu, Y., Liu, G.S., Wang, P., Weinbrenner, C., Cordis, G.A., Das, D.K., Downey, J.M., 1996. Phospholipase D plays a role in ischemic preconditioning in rabbit heart. Circulation 94, 1713–1718.

Cohen, M.V., Yang, X.M., Liu, G.S., Heusch, G., Downey, J.M., 2001. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine

trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels. Circ. Res. 89, 273–278.
Cohen, M.V., Yang, X.M., Downey, J.M., 2006. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc. Res. 70, 231–239.
Fryer, R.M., Eells, J.T., Hsu, A.K., Henry, M.M., Gross, G.J., 2000. Ischemic preconditioning in rats: role of mitochondrial K_{ATP} channel in preservation of mitochondrial function. Am. J. Physiol, Heart Circ. Physiol. 278, H305–H312.
Gopalachrishna, R., Anderson, W.B., 1989. Calcium and phospholipids independent activation of protein kinase C by selective oxidative modification of the regulatory domain. Proc. Natl. Acad. Sci. 86, 6758–6762.
Harada, N., Miura, T., Dairaku, Y., Kametani, R., Shibuya, M., Wang, R., Kawamura, A., Matsuzaki, M., 2004. NO donor-activated PKC delta plays a pivotal role in ischemic myocardial protection through accelerated opening of K-ATP mitochondrial channels. J. Cardiovasc. Pharmacol. 44, 35–41.
Hausenloy, D.J., Tsang, A., Mocanu, M., Yellon, D.M., 2005. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am. J. Physiol, Heart Circ. Physiol. 288, H971–H976.
Horinaka, S., Kobayashi, N., Yagi, H., Mori, Y., Matsuoka, H., 2006. Nicorandil but not ISDN upregulates endothelial nitric oxide synthase expression, preventing left ventricular remodeling and degradation of cardiac function in Dahl salt-sensitive hypertensive rats with congestive heart failure. J. Cardiovasc. Pharmacol. 47, 629–635.
Iliodromitis, E.K., Papadopoulos, C.C., Markianos, M., Paraskevaidis, I.A., Kyriakides, Z.S., Kremastinos, D.T., 1996. Alterations in circulating cyclic guanosine monophosphate (c-GMP) during short and long ischemia in preconditioning. Basic Res. Cardiol. 91, 234–239.
Iliodromitis, E.K., Kremastinos, D.T., Katritsis, D.G., Papadopoulos, C.C., Hearse, D.J., 1997. Multiple cycles of preconditioning cause loss of protection in open-chest rabbits. J. Mol. Cell. Cardiol. 29, 915–920.
Iliodromitis, E.K., Gaitanaki, C., Lazou, A., Bofolis, E., Karavolias, G.K., Beis, I., Kremastinos, D., 2002. Dissociation of stress-activated protein kinase (p38-MAPK and JNKs) phosphorylation from the protective effect of preconditioning in vivo. J. Mol. Cell. Cardiol. 34, 1019–1028.
Iliodromitis, E.K., Cokkinos, P., Zoga, A., Steliou, A., Vrettou, A.R., Kremastinos, D.T., 2003. Oral nicorandil recaptures the waned protection from preconditioning in vivo. Br. J. Pharmacol. 138, 1101–1106.
Iliodromitis, E.K., Gaitanaki, C., Lazou, A., Aggeli, I.K., Bofilis, E., Zoga, A., Beis, I., Kremastinos, D.T., 2006. Differential activation of mitogen activated protein kinases to ischemic and nitroglycerin-induced preconditioning. Basic Res. Cardiol. 101, 327–335.
Kodani, E., Xuan, Y.T., Takano, H., Shinmura, K., Tang, X.L., Bolli, R., 2002. Role of cyclic guanosine monophosphate in late preconditioning in conscious rabbits. Circulation 105, 3046–3052.
Krieg, T., Phillipp, S., Cui, L., Dostmann, W.R., Downey, J.M., Cohen, M.V., 2005. Peptide blockers of PKG inhibit ROS generation by acetylcholine and bradykinin in cardiomyocytes but fail to block protection in the whole heart. Am. J. Physiol, Heart Circ. Physiol. 288, H1976–H1981.
Krumenacher, M., Roland, E., 1992. Clinical profile of nicorandil: an overview of its hemodynamic properties and therapeutic efficacy. J. Cardiovasc. Pharmacol. 20, S93–S102.
Liu, Y., Ytrehus, K., Downey, J.M., 1994. Evidence that translocation of protein kinase C is a key event during ischemic preconditioning of rabbit myocardium. J. Mol. Cell. Cardiol. 26, 661–668.
Lochner, A., Marais, E., Genade, E., Moolman, J.A., 2000. Nitric oxide: a trigger for classic preconditioning? Am. J. Physiol, Heart Circ. Physiol. 279, H2752–H2765.
Ma, X.L., Kumar, S., Gao, F., Louden, C.S., Lopez, B.L., Christopher, T.A., Wang, C., Lee, J.C., Feuerstein, G.Z., Yue, T.L., 1999. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. Circulation 99, 1685–1691.
Mackay, K., Mochly-Rosen, D., 1999. An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. J. Biol. Chem. 274, 6272–6279.
Michel, M.C., Li, Y., Heuch, G., 2001. Mitogen-activated protein kinases in the heart. Naunyn-Schmiedebergs Arch. Pharmakol. 363, 245–266.
Minamiyama, Y., Takemura, S., Hai, S., Suehori, S., Okada, S., Funae, Y., 2007. Nicorandil elevates tissue cGMP levels in a nitric-oxide-independent manner. J. Pharmacol. Sci. 103, 33–39.
Mitchell, M.B., Meng, X., Ao, L., Brown, J.M., Harken, A.H., Banerjiee, A., 1995. Preconditioning of isolated rat heart is mediated by protein kinase C. Circ. Res. 76, 73–81.
Miura, T., Miura, T., Kawamura, S., Goto, M., Sakamoto, J., Tsuchida, A., 1998. Effect of protein kinase C inhibitors on cardioprotection by ischemic preconditioning depends on the number of preconditioning episodes. Cardiovasc. Res. 37, 700–709.
Mocanu, M.M., Baxter, G.F., Yue, Y., Critz, S.D., Yellon, D.M., 2000. The p38-MAPK inhibitor, SB203580, abrogates ischemic preconditioning in rat heart but timimg of administration is critical. Basic Res. Cardiol. 95, 472–478.
Murry, C.E., Jennings, R.B., Reimer, K.A., 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74, 1124–1136.
Nakano, A., Baines, C.P., Kim, S.O., Pelech, S.L., Downey, J.M., Cohen, M.V., Critz, S.D., 2000a. Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. Circ. Res. 86, 144–151.
Nakano, A., Cohen, M.V., Critz, S., Downey, J.M., 2000b. SB203580, an inhibitor of p38 MAPK, abolishes infarct-limiting effect of ischemic preconditioning in isolated rabbit hearts. Basic Res. Cardiol. 95, 466–471.
Noma, A., 1983. ATP-regulated K^+ channels in cardiac muscle. Nature 305, 147–148.
Oldenburg, O., Qin, Q., Krieg, T., Yang, X.M., Phillipp, S., Critz, S.D., Cohen, M.V., Downey, J.M., 2004. Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoK_{ATP} channel opening and leads to cardioprotection. Am. J. Physiol, Heart Circ. Physiol. 286, H468–H476.
Oldenburg, O., Qin, Q., Sharma, A.R., Cohen, M.V., Downey, J.M., Benoit, J.N., 2002. Acetylcholine leads to free radical production dependent on K_{ATP} channels, Gi proteins, phosphatidylinositol 3-, and tyrosine kinases. Cardiovasc. Res. 55, 544–552.
O'Rourke, B., 2004. Evidence for mitochondrial K^+ channels and their role in cardioprotection. Circ. Res. 94, 420–432.
Ota, S., Nishikawa, H., Takeuchi, M., Nakajima, K., Nakamura, T., Okamoto, S., Setsuda, M., Makino, K., Yamakado, T., Nakano, T., 2006. Impact of nicorandil to prevent reperfusion injury in patients with acute myocardial infarction: Sigmart Multicenter Angioplasty Revascularization Trial (SMART). Circ. J. 70, 1099–1104.
Pain, T., Yang, X., Critz, S.D., Yue, Y., Nakano, A., Liu, G.S., Heusch, G., Cohen, M.V., Downey, J.M., 2000. Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. Circ. Res. 87, 460–466.
Patel, D.J., Pucel, H.J., Fox, K.M., 1999. Cardioprotection by opening of the K_{ATP} channel in unstable angina. Is this a clinical manifestation of myocardial preconditioning? Results of a randomized study with nicorandil. Eur. Heart J. 20, 51–57.
Sato, M., Cordis, G.A., Maulik, N., Das, D.K., 2000a. SAPKs regulation of ischemic preconditioning. Am. J. Physiol, Heart Circ. Physiol. 279, H901–H907.
Sato, T., Sasaki, N., O'Rourke, B., Marban, E., 2000b. Nicorandil, a potent cardioprotective agent, acts by opening mitochondrial ATP-dependent potassium channels. J. Am. Coll. Cardiol. 35, 514–518.
Saurin, A.T., Pennington, D.J., Raat, N.J.H., Latchman, D.S., Owen, M.J., Marber, M.S., 2002. Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. Cardiovasc. Res. 55, 672–680.
Schulz, R., Cohen, M.V., Behrens, M., Downey, J.M., Heusch, G., 2001. Signal transduction of ischemic preconditioning. Cardiovasc. Res. 52, 181–198.
Schulz, R., Belosjorow, S., Gres, P., Jansen, J., Michel, M.C., Heusch, G., 2002. p38 MAP kinase is a mediator of ischemic preconditioning in pigs. Cardiovasc. Res. 55, 690–700.
Szilvassy, Z., Ferdinandy, P., Bor, P., Jakab, I., Szilvassy, J., Nagy, I., Lonovics, J., Koltai, M., 1994. Loss of preconditioning in rabbits with vascular tolerance to nitroglycerin. Br. J. Pharmacol. 112, 999–1001.
The IONA study group, 2002. Effect of nicorandil on coronary events in patients with stable angina: the impact of nicorandil in angina (IONA) randomized trial. Lancet 359, 1269–1275.

Tritto, I., D'Andrea, D., Eramo, N., Sconamiglio, A., De Simone, C., Violante, A., Esposito, A., Chiariello, M., Ambrosio, G., 1997. Oxygen radicals can induce preconditioning in rabbit hearts. Circ. Res. 80, 743–748.

Yue, Y., Qin, Q., Cohen, M., Downey, J.M., Critz, S.D., 2002. The relative order of mK_{ATP} channels, free radicals and p38 MAPK in preconditioning's protective pathway in rat heart. Cardiovasc. Res. 55, 681–689.

Zhang, J., Gao, J.X., Salojin, K., Shao, Q., Grattan, M., Meagher, C., Laird, D.W., Delovitch, T.L., 2000. Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. J. Exp. Med. 191, 1017–1030.