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Differential activation of mitogen-activated protein kinases in ischemic and nitroglycerin-induced preconditioning

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Abstract Previous studies have shown that the cardioprotective effect of ischemic preconditioning (IPC) can be mimicked pharmacologically with clinically relevant agents, including nitric oxide (NO) donors. However, whether pharmacological preconditioning shares the same molecular mechanism with IPC is not fully elucidated. The present study aimed to determine the activation of mitogen-activated protein kinases (MAPKs) (ERK1/2, p38 MAPK and p46/p54 JNKs) during ischemia and at reperfusion in nitroglycerin-induced preconditioning as compared to IPC and to correlate this with the conferred cardioprotection in anesthetized rabbits. Sixty minutes of intravenous administration of nitroglycerin was capable of inducing both early and late phase preconditioning in anesthetized rabbits, as it was expressed by the reduction of infarct size. Despite the cardioprotective effect conferred by both ischemic and nitroglycerin-induced preconditioning, there was a differential phosphorylation of MAPKs between the studied groups. p38 MAPK was activated early in ischemia in both ischemic and the early nitroglycerin-induced preconditioning while JNKs were markedly increased only after IPC. Furthermore, in these groups, ERK1/2 were activated during reperfusion. A different profile was observed in the late preconditioning induced by nitroglycerin with increased p38 MAPK and ERK1/2 phosphorylation during late ischemia. No activation of JNKs was observed at any time point in this group. It seems that activation of individual MAPK subfamilies depends on the nature of preconditioning stimulus.

Key words MAPK – ischemia – nitroglycerin – preconditioning – rabbit

Introduction

Preconditioning by brief episodes of ischemia/reperfusion exerts a powerful protective influence on the heart [26]. Preconditioning induces both an early and a late (second window) phase of protection and it has become the focus of increasing attention because of the potential pharmacological exploitation to alleviate the consequences of sustained myocardial ischemia in patients [11, 41]. There is compelling evidence that the cardioprotective effects of ischemic preconditioning (IPC) can be mimicked pharmacologically with clinically relevant agents, termed pharmacological preconditioning. Along these lines, exogenous administration of many G protein-coupled receptor agonists, such as

adenosine, opioids, bradykinin, catecholamines, angiotensin II or acetylcholine, as well as K_{ATP} channel openers like diazoxide or nicorandil was found to mimic or to reactivate IPC [15, 20, 24, 38–40]. Recent evidence suggests that a delayed cardioprotective effect indistinguishable from the late phase of IPC can be induced with the administration of nitric oxide (NO) donors in the absence of ischemia, which is consistent with the concept that NO plays a major role in initiating the cardioprotective adaptation [5, 27]. In fact, it has been shown that nitroglycerin, a NO-releasing agent, which is widely used in clinical practice, elicits a delayed cardioprotection against both myocardial stunning and infarction [18, 19, 35].

The signal transduction mechanisms involved in preconditioning have not yet been fully clarified, although mounting evidence indicates that protein kinase C (PKC) plays a key role in the signaling pathways underlying both phases of IPC [33, 42]. K_{ATP} channels and several kinases like tyrosine kinase and mitogen-activated protein kinases (MAPKs) have been also implicated in the signal transduction mechanism. However, the reported data on MAPKs are inconsistent and their role in the mechanism of protection remains elusive [25, 33]. We have previously shown that the phosphorylation of p38 MAPK and p46/p54 JNKs is increased at the time of sustained ischemia in preconditioned hearts, both by short ischemia or by acute mechanical stretch, in anesthetized rabbits although this effect may be dissociated from the protective effect of IPC [21]. Whether the various forms of pharmacological preconditioning share the same molecular mechanism with IPC is not fully elucidated. To date, no data are available with respect to the role of MAPKs in the NO-induced preconditioning. Therefore the current study aimed to determine the activation of MAPKs (ERK1/2, p38 MAPK and p46/p54 JNKs) during ischemia (5 and 20 min) and at reperfusion (20 min) in nitroglycerin-induced preconditioning as compared to IPC and to correlate this with the conferred cardioprotection in anesthetized rabbits.

Materials and methods

Animals

New Zealand White male rabbits weighing between 2.6 and 3.3 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.

Surgical preparation

All the animals were anesthetized by slowly injecting 30 mg/kg sodium thiopentone (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 adjusted to keep blood gases within the normal range. Two polyethylene catheters were inserted; one in the left jugular vein for fluids or anesthetic and one 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 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.

Experimental protocols

The animals were divided in two series of experiments each consisting of five groups named respectively control, IPC, early nitroglycerin preconditioning (ENTG10 and ENTG30) and late nitroglycerin preconditioning (LNTG). For the evaluation of the MAPKs, animals were exposed to either 5 or 20 min ischemia, or 30 min of ischemia followed by 20 min of reperfusion. For the estimation of the infarct size, animals were exposed to 30 min ischemia followed by 180 min of reperfusion. In both series the control groups were not subjected to any additional intervention before the sustained ischemia, the IPC groups were subjected to two cycles of 5-min ischemia of the heart and 10-min reperfusion, the ENTG and LNTG groups were treated with nitroglycerin which was intravenously given into another ear vein for 60 min. In the ENTG10 group the infusion of nitroglycerin was discontinued 10 min before the sustained ischemia and in the ENTG30 group 30 min before ischemia, in order to simulate pharmacologically the early phase of preconditioning. In the LNTG group the infusion of nitroglycerin was discontinued 24 h before the sustained ischemia, in order to simulate the late phase of preconditioning. Nitroglycerin was dissolved in normal saline (50 μg/ml) and was continuously infused via the ear vein at a dose of 2 μg/kg/min as previously described [19]. The protocol is presented schematically in Fig. 1. Mean heart rate and mean blood pressure were measured immediately before sustained ischemia (baseline) at the end of sustained ischemia and at the end of reperfusion.

Animal exclusions

Twelve animals from the first and second series were excluded for technical and hemodynamic reasons. Thus, 95 out of 107 rabbits completed the study.

Risk area and infarct size measurement

The animals were exposed to 30-min regional ischemia of the heart followed by 180-min reperfusion. After the end of reperfusion period hearts were harvested, mounted on a reperfusion apparatus and perfused (50 mmHg) retrogradely via the aorta with normal

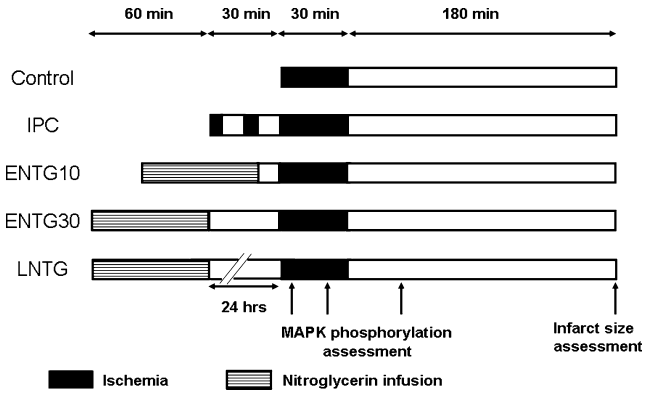

Fig. 1 Experimental protocol for the study of the effect of ischemic and nitroglycerin-induced preconditioning on infarct size and MAPK phosphorylation. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning with 10 min interval between nitroglycerin infusion and ischemia induction; ENTG30: Early nitroglycerin preconditioning with 30-min interval between nitroglycerin infusion and ischemia induction; LNTG: Late nitroglycerin preconditioning

saline (10 ml/min, room temperature) for 2 min. When all residual blood had been removed from the coronary arteries, the coronary ligature was retightened at the same site and 5 ml of Zn–Cd fluorescent particles (1–10 μm diameter, Duke Scientific Corp., Paolo Alto, CA, USA, suspended in saline) were 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 they were sliced into 3 mm thick sections from the apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) in isotonic phosphate buffer solution, pH 7.4 for 20 min at 37°C. TTC reacts with dehydrogenase enzymes and nicotinamide adenine dinucleotide in viable tissue; the infarcted area was defined as the negative staining region. 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 UV 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 and were subsequently scanned with the Adobe Photoshop 6.0 and measured with the Scion Image program. The areas of myocardial tissue at risk and of infarction were automatically transformed into 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 ratio (%I/R) was calculated.

Tissue sample preparation

Five or twenty minutes after the beginning of prolonged ischemia and 20 min after the onset of reperfusion, two small heart samples were rapidly removed, one from the center of the area at risk and another one from the opposite non-ischemic area (postero-inferior wall and part of the right ventricle), and immediately immersed into liquid nitrogen. They were then stored at −80 °C until the subsequent analyses. The tissue samples were homogenized with 3 v/wt of 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, 300 μM phenyl methyl sulfonyl fluoride (PMSF), and 0.5% (v/v) Triton X-100) and extracted on ice for 30 min. The samples were centrifuged (10,000*g*, 5 min, 4 °C) and the supernatants were boiled with 0.33 vol. 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, and 0.2% (w/v) bromophenol blue). Protein concentrations were determined using the Bradford assay (BioRad, Hercules, California 94547, USA).

Immunoblotting

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 & Schuell, Keene N.H. 03431, USA). Non-specific binding sites on the membranes were blocked with 5% (w/v) non-fat milk in TBST (20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 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 ERK1/2, p38 MAPK or JNKs (Cell Signaling, Beverly, MA) according to the manufacturer's instructions. After washing in TBST, the blots were incubated with horseradish peroxidase-linked anti-mouse IgG antibody (DAKO A/S Glostrup, Denmark) for 1 h at room temperature. The blots were washed again in TBST and the bands were detected using ECL (Santa Cruz) with exposure to X-OMAT AR film (Eastman Kodak Company, New York, 14650, USA). Blots were quantified by laser scanning densitometry.

Statistical analysis

Values are expressed as mean ± SEM. Infarct sizes were plotted against risk zone volumes for all groups, and regression lines for groups with interventions were compared with the regression line for the control group by analysis of covariance with Bonferroni's correction for multiple comparisons. Statistical analysis was performed using the statistical package SPSS vr 10.00 (Statistical Package for the Social Sciences) and for all tests, a probability of <0.05 was considered statistically significant. Hemodynamic and 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 significant.

Results

Hemodynamic variables

Mean heart rate and mean blood pressure at baseline, at the end of index ischemia and at the end of reperfusion are shown in Table 1. No significant differences were observed between the groups at various time points.

Infarct size

The effect of various interventions on infarct size is shown in Fig. 2. The infarcted to risk ratio was 47.6 ± 3.4% in the control group and 13.9 ± 1.7% in the IPC group ($P < 0.01$). The infarct size did not differ between the nitroglycerin-treated groups (23.77 ± 4.58% in ENTG10, 34.5 ± 4.1% in ENTG30 and 27.4 ± 2.7% in LNTG). However, the infarct size was significantly smaller in all three nitroglycerin-preconditioned groups compared to the control

Table 1 Hemodynamic variables of the different groups of the study at baseline, the end of sustained ischemia and 120-min reperfusion

	Baseline		Ischemia		Reperfusion	
	HR	MBP	HR	MBP	HR	MBP
Control	280.0 ± 7.9	75.0 ± 3.9	280.7 ± 7.3	67.7 ± 4.1	263.6 ± 7.6	64.1 ± 3.2
IPC	281.6 ± 6.0	75.3 ± 3.9	280.0 ± 8.1	67.5 ± 3.2	259.1 ± 6.9	66.3 ± 3.1
ENTG10	285.8 ± 8.0	77.3 ± 3.8	281.7 ± 6.0	69.0 ± 2.7	254.2 ± 8.8	61.7 ± 3.2
ENTG30	274.4 ± 3.7	73.9 ± 3.4	277.5 ± 4.4	70.2 ± 3.5	258.7 ± 2.3	66.6 ± 3.0
LNTG	285.0 ± 3.8	78.6 ± 2.5	282.5 ± 2.5	74.5 ± 2.5	266.2 ± 5.0	63.2 ± 2.1

HR: Heart rate in beats/min; MBP: Mean blood pressure in mmHg. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning (60-min infusion, started 90 min and ended 30 min before index ischemia); ENTG30: Early nitroglycerin preconditioning (60-min infusion, started 70 min and ended 10 min before index ischemia); LNTG: Late nitroglycerin preconditioning (60-min infusion ended 24 h before index ischemia)

($P < 0.05$ for ENTG30 and $P < 0.01$ for ENTG10 and LNTG) but significantly larger compared to the ischemic preconditioned group ($P < 0.01$ for ENTG30 and $P < 0.05$ for ENTG10 and LNTG). Infarct sizes were plotted against risk zone volumes for all groups (Fig. 3). The regression lines for ischemic preconditioned and nitroglycerin-treated hearts were different from the regression line for control hearts ($P < 0.05$).

Activation of MAPKs

Activation of p38 MAPK, p46/p54 JNKs and ERK1/2, as evidenced by their dual phosphorylation, was determined after 5 and 20 min of prolonged ischemia and after 20 min of the reperfusion following 30-min ischemia, in all groups. For each group, phosphorylation of the kinases was determined in ventricular samples obtained from both the ischemic and non-ischemic regions of the heart. The basal levels of phosphorylation of the kinases in these regions were determined in samples obtained from control hearts that were not subjected to any interventions (Fig. 4). At early stages of ischemia (5 min), a robust increase in the phosphorylation of p38 MAPK was observed in the ischemic preconditioned group (12.69 ± 1.9-fold comparing ischemic to non-ischemic regions) and the early-treated nitroglycerin groups (about 12.01 ± 1.27 in ENTG10 and 12.89 ± 2.25 in ENTG30) as compared with the non-preconditioned control group (Fig. 5). The phosphorylation of the kinase remained increased, although at significantly lower levels, in the IPC and ENTG10 groups after 20 min of prolonged ischemia. On the other hand, phosphorylation of p38 MAPK was not different from the control in the LNTG group determined at 5 min of prolonged ischemia, whereas it was significantly increased after 20 min of ischemia (8.1 ± 2.15-fold comparing ischemic to non-ischemic region). The phosphorylation of p38 MAPK

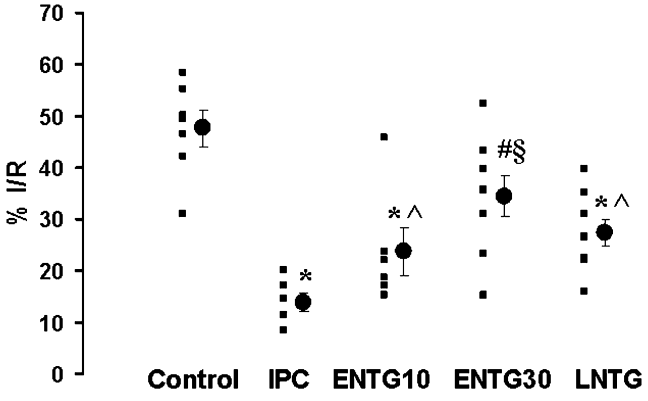

Fig. 2 The effect of various interventions on infarct size (expressed as a percent of risk zone size) in rabbit hearts following 30-min ischemia and 180-min reperfusion. Squares represent individual experiments, circles depict group means with SEM. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning (60-min infusion ending 10 min before index ischemia); ENTG30: Early nitroglycerin preconditioning (60-min infusion ending 30 min before index ischemia); LNTG: Late nitroglycerin preconditioning (60-min infusion ending 24 h before index ischemia). *$P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. control; $^{\S}P < 0.01$ vs. IPC; $^{\wedge}P < 0.05$ vs. IPC

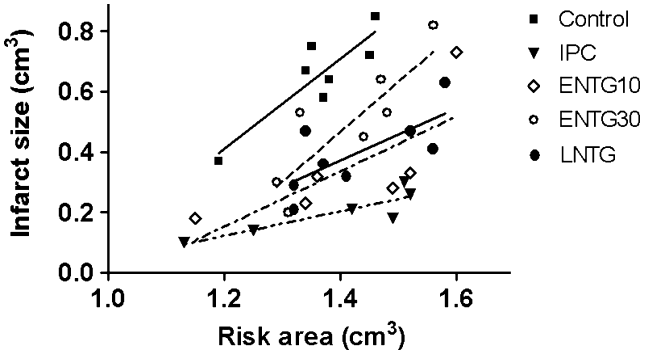

Fig. 3 Infarct size plotted against risk zone for control, ischemic preconditioned and early and late nitroglycerin-treated hearts. Control (*closed squares*); IPC (*closed triangles*): Ischemic preconditioning; ENTG10 (*open diamonds*): Early nitroglycerin preconditioning (60-min infusion ending 10 min before index ischemia); ENTG30 (*open circles*): Early nitroglycerin preconditioning (60-min infusion ending 30 min before index ischemia); LNTG (*closed circles*): Late nitroglycerin preconditioning (60-min infusion ending 24 h before index ischemia). Regression lines for the control hearts are significantly different from that for all the other hearts ($P < 0.05$)

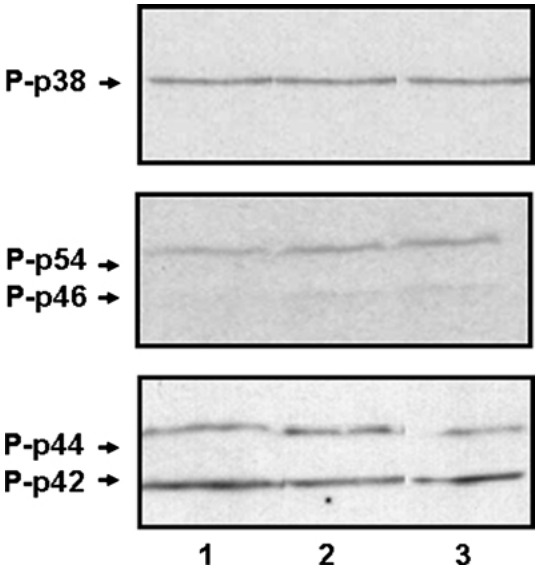

Fig. 4 Phosphorylation levels of p38 MAPK, JNKs and ERK1/2 in samples obtained from the LV (1), the RV (2) and the postero-inferior wall (3) of control hearts not subjected to any interventions. Phosphorylated kinases were detected by immunoblotting. Representative blots are shown. The experiment was repeated three more times with comparable results

did not vary significantly among the groups after 20 min of reperfusion (Fig. 5).

IPC resulted in a significant increase in the phosphorylation level of JNKs at 5 min and 20 min of ischemia (4.57 ± 1.12-fold and 6.79 ± 0.60-fold, respectively) whereas nitroglycerin treatment had no effect (Fig. 6). At reperfusion, increased levels of JNK phosphorylation were observed not only in the IPC group but also in control and the early-treated nitroglycerin groups, ENTG10 and ENTG30. However, phosphorylation of JNKs remained at low levels either during ischemia or at reperfusion in the LNTG group.

The phosphorylation level of ERK1/2 was also determined by immunoblotting during sustained ischemia and reperfusion, in control, ischemic preconditioned and nitroglycerin-treated groups. No change in ERK1/2 phosphorylation between ischemic and non-ischemic regions of the heart was detectable in any of the groups at early ischemia (Fig. 7). Similarly, no change in ERK1/2 phosphorylation was observed in the control, preconditioned or early nitroglycerin-treated groups at 20 min of ischemia. However, a significant increase (about 3-fold, comparing ischemic to non-ischemic regions) in the phosphorylation level of ERK1/2 was observed in the late phase of nitroglycerin-treated hearts. Reperfusion of the hearts resulted in a significant increase in the phosphorylation of ERK1/2 in all groups as compared with the non-preconditioned control at the same time point (Fig. 7).

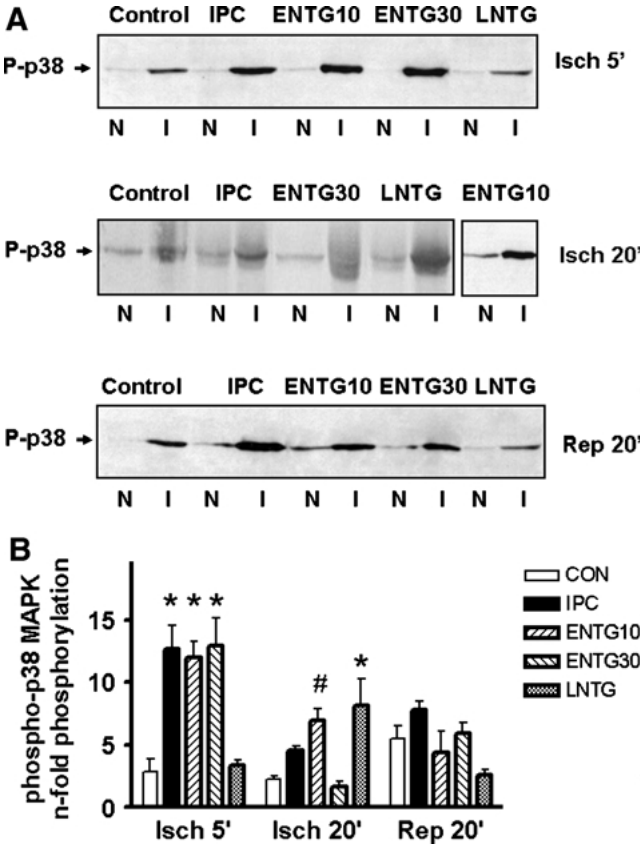

Fig. 5 Analysis of the phosphorylation state of p38 MAPK in the non-ischemic (N) and ischemic (I) regions of the heart after 5 and 20 min of sustained ischemia and 20 min of reperfusion. (A) Phosphorylated p38 MAPK was detected by immunoblotting with an antibody specific to the dually phosphorylated form of the kinase. Representative blots are shown. (B) Blots were quantified by laser densitometry. Each point is the mean ± SEM of four independent observations. $^{*}P < 0.01$ vs. control at the same time point; $^{\#}P < 0.05$ vs. control at the same time point. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning (60-min infusion ending 10 min before index ischemia); ENTG30: Early nitroglycerin preconditioning (60-min infusion ending 30 min before index ischemia); LNTG: Late nitroglycerin preconditioning (60-min infusion ending 24 h before index ischemia)

Discussion

The present study demonstrates that 60 min of intravenous administration of nitroglycerin is capable of inducing both early and late phase preconditioning in anesthetized rabbits, as it is expressed by the reduction of the infarct size. Furthermore, despite the cardioprotective effect conferred by ischemic and pharmacological preconditioning, there is a differential phosphorylation of MAPKs (p38 MAPK, JNKs, ERK1/2) between the studied groups.

The cardioprotective effect of IPC occurs in two phases called early or classic and late or second window of protection. The first is immediate and lasts for a few hours while the delayed phase, which is weaker, starts 24 h after a sub-lethal ischemic insult and lasts 72 h [2,

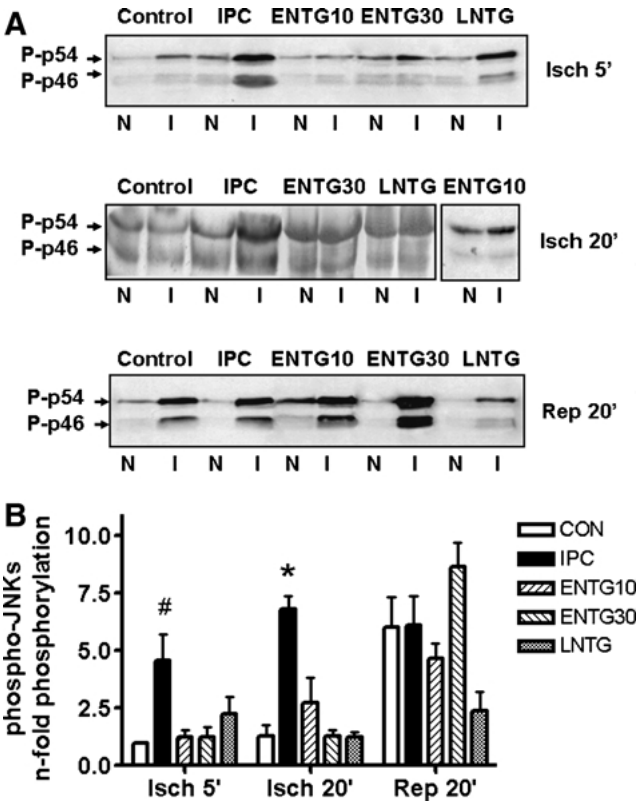

Fig. 6 Analysis of the phosphorylation state of p46/p54 JNKs in the non-ischemic (N) and ischemic (I) regions of the heart after 5 and 20 min of sustained ischemia and 20 min of reperfusion. (A) Phosphorylated JNKs were detected by immunoblotting with an antibody specific to the dually phosphorylated form of the kinases. Representative blots are shown. (B) Blots were quantified by laser densitometry. Each point is the mean ± SEM of four independent observations. $^{*}P < 0.01$ vs. control at the same time point; $^{\#}P < 0.05$ vs. control at the same time point. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning (60-min infusion ending 10 min before index ischemia); ENTG30: Early nitroglycerin preconditioning (60-min infusion ending 30 min before index ischemia); LNTG: Late nitroglycerin preconditioning (60-min infusion ending 24 h before index ischemia)

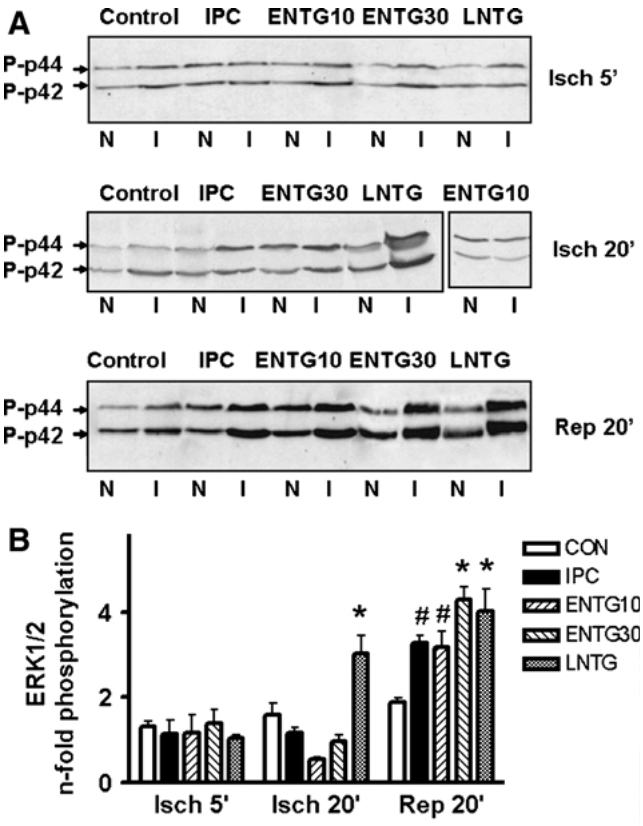

Fig. 7 Analysis of the phosphorylation state of ERK1/2 in the non-ischemic (N) and ischemic (I) regions of the heart after 5 and 20 min of sustained ischemia and 20 min of reperfusion. (A) Phosphorylated ERK1/2 were detected by immunoblotting with an antibody specific to the dually phosphorylated form of the kinases. Representative blots are shown. (B) Blots were quantified by laser densitometry. Each point is the mean±SEM of four independent observations. $^{*}P<0.05$ vs. control at the same time point; $^{\#}P<0.05$ vs. control at the same time point. IPC: Ischemic preconditioning; ENTG10: Early nitroglycerin preconditioning (60-min infusion ending 10 min before index ischemia); ENTG30: Early nitroglycerin preconditioning (60-min infusion ending 30 min before index ischemia); LNTG: Late nitroglycerin preconditioning (60-min infusion ending 24 h before index ischemia)

3]. In recent years, several pharmacological agents have been identified that can substitute for the short ischemic insults but this approach has been met with varying success, probably due to the complex intracellular transduction signaling [33, 42]. Nitroglycerin, which is an NO-releasing drug extensively used in the treatment of coronary artery disease, has been previously tested as a pharmacological analogue of preconditioning [18]. Although endogenous NO is not necessary for ischemia-induced early preconditioning [27, 31], exogenous NO can elicit a preconditioning-like protection [32]. Furthermore, considerable evidence indicates a critical role for NO in the late preconditioning [5]. The results of the present study confirm previous observations that pre-treatment with nitroglycerin induces early and delayed cardioprotection in different species [19, 44]. Moreover, studies in humans have shown that nitroglycerin induces delayed preconditioning and protects the myocardium against ischemic injury in patients undergoing coronary angioplasty; the ability of nitroglycerin to elicit delayed preconditioning is not hampered by the presence of tolerance to the hemodynamic actions of nitrates [19, 23].

The elucidation of the factors involved in the signal transduction pathway of preconditioning has been the subject of intense investigation and although the participation of PKC and K_{ATP} channels [7, 9, 10, 16] is well established, their relevance and the sequence of activation remain controversial. Functional evidence indicates that NO plays a prominent role both in initiating and mediating the protective responses of late preconditioning in the myocardium probably

through the activation of PKC [1, 5, 30]. So, it seems that PKC plays a central role in the signaling of both ischemic and pharmacological preconditioning. However, a complex network of other protein kinases, particularly the stress-related MAPKs, has been increasingly implicated in triggering and mediating cardiac preconditioning although considerable controversy surrounds their precise role [25, 33]. In the present study we sought to determine the activation of MAPKs (p38, p46/p54 JNKs and ERK1/2) in ischemic and nitroglycerin-induced preconditioning and correlate this activation with the degree of protection observed. IPC and nitroglycerin-induced preconditioning exhibit a differential profile of MAPK phosphorylation. p38 MAPK is activated in both ischemic and the early nitroglycerin-induced preconditioning during early ischemia while JNKs are markedly increased during sustained ischemia only after IPC. Furthermore, ERK1/2 are activated at reperfusion in all groups where protection is observed. ERK1/2 activation during ischemia is detected only in the late preconditioning induced by nitroglycerin.

The role of p38 MAPK in acute preconditioning has been extensively studied, although conflicting results have been obtained. We, and others, have previously shown that the phosphorylation and hence activation of p38 MAPK increases in conditions where there is protection of the heart as is the case in ischemic and mechanically induced preconditioning in vivo [21, 41]. In the present study, we showed that the phosphorylation of p38 MAPK also increases in the nitroglycerin-induced preconditioning (Fig. 5). Maximal p38 MAPK activation was comparable in the preconditioned and the early-nitroglycerin groups and it was observed during early ischemia (5 min). However, it appears that there is no correlation between the degree of protection and the level of activation of p38 MAPK (Figs. 2, 4) and this is in accordance with previous studies [4, 21, 37]. It should be mentioned that, in this study, we did not differentiate between p38 MAPK isoforms although there is evidence that p38α and p38β MAPK activities are differentially activated by preconditioning [34]. Increased p38 MAPK phosphorylation was also observed in the late nitroglycerin-treated group (Fig. 5). In this case, however, the maximal activation was shifted to late ischemia. Very few studies exist with respect to the role of p38 MAPK in the late preconditioning. Increased phosphorylation of p38 MAPK was observed 24 h after stimulation of adenosine A1 receptors in the rabbit, mouse and rat hearts suggesting this pathway as a potential distal effector of late preconditioning [8, 43]. In addition, it has been shown [6] that late preconditioning in human atrial myocytes could be prevented by SB203580.

Few reports have investigated the importance of JNKs in cardioprotection. JNKs have been implicated in the signal transduction cascade of IPC in rats [13] and rabbits [21, 29]. In agreement with these studies, the phosphorylation state of JNKs was significantly increased during prolonged ischemia in the preconditioned hearts compared with the controls (Fig. 6). However, this effect was not observed in the early nitroglycerin-treated groups although JNKs phosphorylation at 20-min ischemia in the ENTG10 group tends to be higher than control. The increase in JNKs phosphorylation during ischemia in the preconditioned animals may account for the greater reduction in infarct size observed in these animals vs. animals treated with nitroglycerin. Fryer et al. [13] suggested that activation of JNKs during early reperfusion may be important for cardioprotection. However, the results of the present study do not support this notion since increased JNKs phosphorylation was also observed in control hearts during reperfusion. In addition, we could not detect any significant effect of nitroglycerin-induced preconditioning, in the late phase, on the activation of JNKs (Fig. 6). To our knowledge, there is no evidence that JNKs are activated during the delayed phase of ischemic or pharmacological preconditioning.

The ERK pathway is the best studied from all MAPK subfamilies and it has been implicated in cell survival. A robust increase in ERK1/2 phosphorylation was observed at reperfusion after sustained ischemia in all groups compared to control (Fig. 7), implicating these kinases in the protection. Previous studies have shown that IPC induces a potent activation of ERK1/2 pathway during the reperfusion phase after lethal ischemia and that these kinases are essential for IPC-induced protection [17]. Furthermore, increased ERK1/2 phosphorylation at reperfusion was observed in opioid-induced cardioprotection [14]. Other studies have shown activation of ERK1/2, when determined after the ischemia/reperfusion period of preconditioning [14, 17, 28], which declines during sustained ischemia [14, 17]. We could not detect any change in ERK1/2 phosphorylation during sustained ischemia in the IPC or the early nitroglycerin-treated groups (Fig. 7) and this is in accordance with previous reports [21, 36]. However, a robust activation of both ERK isoforms was detected in the late phase of nitroglycerin-induced preconditioning implicating the kinase in the protection (Fig. 7). In support of this notion, it has been shown that delayed preconditioning induced by adenosine is mediated by both ERK and p38 MAPK activation in the rat heart in vivo [22] whereas inhibition of ERK1/2 by PD098059 blocked delta-opioid agonist delayed preconditioning in in vivo rat myocardium [12].

In conclusion, the results of the present study suggest that nitroglycerin induces early and late preconditioning in anesthetized rabbits. Protection is weaker than the IPC and it is accompanied by a differential degree of phosphorylation of the various MAPKs. Further studies are required to assess the precise role of individual MAPK pathways in nitroglycerin-induced preconditioning.

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