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Dissociation of Stress-activated Protein Kinase (p38-MAPK and JNKs) Phosphorylation from the Protective Effect of Preconditioning *in vivo*

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E. K. Iliodromitis, C. Gaitanaki, A. Lazou, E. Bofilis, G. K. Karavolias, I. Beis and D. Th. Kremastinos. Dissociation of Stress-activated Protein Kinase (p38-MAPK and JNKs) Phosphorylation from the Protective Effect of Preconditioning *in vivo*. *Journal of Molecular and Cellular Cardiology* (2002) **34**, 1019–1028. The aim of the present study was to examine and compare the role of the stress-activated protein kinases in ischemic and stretch-induced preconditioning. A model of anesthetized rabbits was used, and the preconditioning protocol included one or three cycles of short ischemia/reperfusion, or short mechanical stretch with acute pressure overload without or with the addition of the stretch blocker gadolinium. Infarct size was determined after 2 h reperfusion and p38 MAPK and JNKs phosphorylation was determined after 20 min of prolonged ischemia. Preconditioning stimuli were equally effective in reducing the infarct size (14.2 ± 3.4%, 12.9 ± 3.0%, 15.9 ± 3.3%, $P < 0.01$ *vs* control). The addition of the stretch channel blocker gadolinium abrogated the effect of stretch preconditioning only, without any effect on ischemic preconditioning. Comparing p38-MAPK and p46/p54 JNKs phosphorylation in the ischemic and non-ischemic regions of the heart at the time of sustained ischemia, activation was observed in the ischemic or mechanically preconditioned groups compared with the control. The addition of gadolinium abolished this activation. The above results indicate that the phosphorylation of p38-MAPK and p46/p54 JNKs is increased in preconditioning but this effect can be dissociated from the protective effect of ischemic preconditioning. Activation of the stress-activated protein kinases may be related to the increased contracture, a characteristic of ischemic preconditioning. © 2002 Elsevier Science Ltd. All rights reserved.

Key Words: Stress-activated protein kinases; Ischemia; Stretch; Preconditioning; Rabbit.

Introduction

Short sublethal periods of ischemia render the heart more tolerant to a subsequent prolonged ischemic insult, an effect known as ischemic preconditioning.[1] Preconditioning has been observed in every species studied but the precise intracellular mechanism has not been completely elucidated so far. Most studies have shown that repeated episodes of brief ischemia and reperfusion are equally effective up to a certain number of ischemic bouts and do not yield a cumulative "dose-dependent" effect.[2,3] Preconditioning

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can be activated not only by short ischemic insults but also by other means such as pharmacological agents,[4,5] rapid ventricular pacing,[6] exposure to hypoxia[7] or to myocardial stretch.[8] The initial hypothesis of protection was that binding of several receptors and then activation of protein kinase C isoforms and mitogen-activated protein kinases (MAPKs) open the mitochondrial K_{ATP} channels, which are the final intracellular step for protection.[9,10] Of note, more recent data suggest that mitochondrial K_{ATP} channels may be upstream of the above cascade of events.[11]

Several studies have examined the potential role of MAPKs in preconditioning.[12] In particular, activation of p38-MAPK, which appears to be more sensitive to stress signals,[13] has been studied intensively as a possible mediator in the signaling pathway to the generation of a protective protein during ischemic preconditioning. However, the reported data are inconsistent and the role of MAPKs remains under dispute. Although it has been shown that strectch-induced cardioprotection may share a common mechanism with ischemic preconditioning, acting through PKC,[14] the role of MAPKs has not been examined in this model of preconditioning. The present study was designed to (1) examine the role of the two stress-activated protein kinases, the p38-MAPK and the c-Jun-N-terminal kinases (JNK 1 and 2) in a condition of stretch-induced cardioprotection and (2) compare their activation with that observed after one or three cycles of ischemic preconditioning. MAPK phosphorylation was measured in samples collected from the ischemic and the non-ischemic regions of rabbit hearts in an *in vivo* experimental model. The effect of gadolinium, a blocker of stretch-activated ion channels, on infarct size and the activation of the kinases in both ischemic and mechanical preconditioning was also determined.

Materials and Methods

Surgical preparation

New Zealand White male rabbits weighing between 2.5 and 2.9 kg were used in this study. All animals 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 Institutes of Health (NIH Publication No. 86-23, revised 1985). Sixteen groups of male rabbits were divided in two equal series each consisted of eight groups. All the animals were anaesthetised by slowly injecting 20 mg/kg sodium thiopentone (Pentothal, Abott) into an ear vein. After midline tracheal incision, they were intubated and mechanically ventilated under positive pressure using a respirator for small animals (MD Industries, Mobile, AL, USA) at a rate adjusted as necessary to maintain arterial blood gases and pH within the normal range. Two polyethylene catheters were inserted; one was positioned in the jugular vein for fluid infusion (1 ml of normal saline containing 1000 IU heparin/100 ml was administered every 30 min) and top up anaesthesia and the other in the carotid artery for continuous blood pressure monitoring. A bipolar chest lead was used for electrocardiographic recording. The surface electrocardiogram and intra-arterial pressure were monitored using a Nihon-Koden RM 6000 recorder. The chest was opened via a left thoracotomy in the fourth intercostal space and the beating heart was exposed. After pericardiotomy a 3-0 silk suture on a tapered needle was passed through the myocardium around a prominent branch of the left coronary artery. Regional ischemia was induced by pulling the ends of the thread through a small segment of soft tubing and a clamp thus firmly compressing the coronary artery. Ischemia was verified by visual inspection of a cyanotic region and by ST elevation on the electrocardiogram. Reperfusion was achieved by releasing the snare and was verified by the "blushing" of the ischemic region and by refilling of the artery.

Experimental protocols

Experimental protocols are schematically presented in Figure 1.

The effect of different triggers of preconditioning on infarct size

In the first series of experiments eight groups of animals were subjected to 30 min regional ischemia and 2 hours reperfusion with one of the following interventions: Control group ($n = 7$), no intervention prior to prolonged ischemia; 1PC group ($n = 6$), one cycle of 5 min ischemia and 10 min reperfusion; 3PC group ($n = 6$), three cycles of 5 min ischemia and 10 min reperfusion; aortic clamp group ($n = 6$) which was exposed to mechanical obstruction of the descending thoracic aorta in an attempt to verify whether acute myocardial stretch due to pressure overload can precondition rabbit hearts. Mechanical pressure overload was obtained by placing a silk thread around the descending thoracic aortic wall

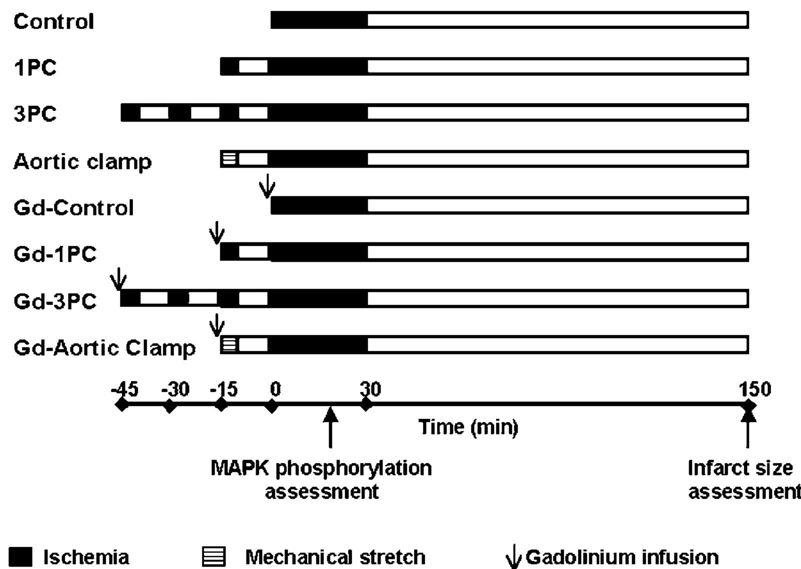

Figure 1 Experimental protocol for the study of the effect of ischemic and mechanical preconditioning without or with gadolinium on infarct size and stress-activated protein kinase phosphorylation. Gd: gadolinium, 1PC: ischemic preconditioning with one cycle of 5 min ischemia/10 min reperfusion, 3PC: ischemic preconditioning with three cycles of 5 min ischemia/10 min reperfusion, Aortic clamp: mechanical preconditioning with one cycle of aortic clamping/10 min reperfusion.

and the ends of the suture were passed through a small piece of soft tubing. Ischemic preconditioning was simulated by mechanical hypertension for 5 min, starting 15 min before sustained ischemia, by pulling the ends of the thread and fixing the soft tubing against the vessel wall by a clamp. Blood pressure recordings were taken periodically from catheters inserted into the femoral artery and left atrium. Normalization of blood pressure measurements was observed immediately after releasing the snare and remained stable for 10 min in order to simulate the reperfusion interval between the short and long ischemia. Four additional groups, respectively called Gd-Control ($n=8$), Gd-1PC ($n=6$), Gd-3PC ($n=6$), Gd-Aortic clamp ($n=7$), were subjected to the same manipulations as in the previous groups but they additionally received a bolus of gadolinium into the jugular vein, at a dose of 40 μmol/kg starting one minute before any intervention on the heart or aorta in order to block stretch activated channels.

The effect of different triggers of preconditioning on stress-activated protein kinases

In the second series of experiments, eight groups of animals ($n=4$/group) were subjected to the same initial manipulations as in the first series of experiments but were destinated for the assessment of p38-MAPK and JNKs phosphorylation. Twenty minutes after the beginning of prolonged ischemia, heart samples were rapidly removed from the ischemic and the non-ischemic left ventricular regions, immediately immersed into liquid nitrogen and were then stored at $-80°C$ until the subsequent analysis.

Animal exclusions

Fifty-four animals from the first series and thirty-two animals from the second series completed the study. Seven rabbits from the first series and four rabbits from the second series were excluded for various reasons.

Risk area and infarct size measurement

After the end of reperfusion period hearts were harvested, mounted on a reperfusion apparatus and perfused (50 mmHg) retrogradely via the aorta with normal saline (15 ml/min, 20°C) 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 ($1-10\,\mu$m 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 quantified by planimetry with the aid of a digitizer interfaced with a computer. The areas of myocardial tissue at risk and 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 as previously described.

Tissue sample preparation

The tissue samples were homogenized with 3 ml/g 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 sulphonyl fluoride (PMSF), 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, 0.2% (w/v) bromophenol blue). Protein concentrations were determined using the Bradford assay (BioRad, Hercules, California 94547, USA).

Immunoblotting analysis

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) nonfat 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 ERKs, p38-MAPK or JNKs (Cell Signaling, Inc., Beverly, MA, USA) 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 (DK-2600 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 ± S.E. Statistical analysis was performed using the CSS-Statistical Software package. One-way ANOVA was used to test for differences in area at risk and infarct size between groups. Hemodynamic and MAPK phosphorylation data were compared by two-way ANOVA. When significant differences were detected, individual mean values were compared by post hoc tests. A probability of $P < 0.05$ was considered significant.

Results

Hemodynamic variables

Heart rate and blood pressure were measured from the carotid artery at baseline, during sustained ischemia and reperfusion. Table 1 summarizes the hemodynamic data; no significant differences were detected between the groups at the various time points. The mean blood pressure of the Ao-Clamp and Gd-Ao-clamp groups was increased significantly in the carotid artery at the time of mechanical pressure overload (150.2 ± 3.8 mmHg and 151.4 ± 8.2 mmHg respectively, $P < 0.001$ *vs* baseline). At the same time the mean left atrial pressure increased from 9.8 ± 0.7 to 28.3 ± 1.0 mmHg and from 8.7 ± 0.9 to 26.1 ± 1.1 mmHg whilst the mean femoral artery pressure decreased from 62.9± 2.8 to 12.5 ± 1.1 mmHg and from 64.7 ± 2.5 to 11.7 ± 0.9 mmHg respectively in the above groups.

Infarct size

No significant differences were detected in risk areas (the areas exposed to sustained ischemia) between groups. In contrast, significant smaller infarct size

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

Group		Baseline	30′ Ischemia	120′ Reperfusion
Control	HR	284.3 ± 6.1	287.1 ± 5.8	260.7 ± 4.9
	BP	73.3 ± 4.0	66.6 ± 3.0	63.8 ± 5.9
1PC	HR	290.8 ± 5.5	288.3 ± 5.6	263.3 ± 5.1
	BP	70.3 ± 4.0	68.7 ± 4.8	67.0 ± 5.1
3PC	HR	283.3 ± 9.1	284.2 ± 9.0	258.3 ± 6.0
	BP	73.2 ± 2.2	68.9 ± 2.8	65.1 ± 2.0
Ao-clamp	HR	281.7 ± 4.0	288.7 ± 4.2	266.7 ± 3.3
	BP	70.5 ± 2.9	63.7 ± 2.8	59.4 ± 2.2
Gd-Control	HR	285.0 ± 6.5	280.6 ± 4.5	260.6 ± 5.1
	BP	74.1 ± 2.9	70.6 ± 2.3	62.7 ± 3.5
Gd-1PC	HR	281.7 ± 9.2	285.8 ± 10.4	260 ± 5.8
	BP	73.0 ± 4.7	66.4 ± 4.9	60.0 ± 2.4
Gd-3PC	HR	280 ± 6.3	288.3 ± 6.0	265 ± 5.6
	BP	72.8 ± 2.1	69.7 ± 1.9	63.0 ± 3.0
Gd-Ao-clamp	HR	284.3 ± 8.1	285.7 ± 5.7	260 ± 3.1
	BP	74.0 ± 3.4	70.0 ± 2.5	58.1 ± 2.0

HR, Heart rate in beats/min; BP, Mean blood pressure in mmHg. Comparisons between groups did not reveal any statistically significant differences.

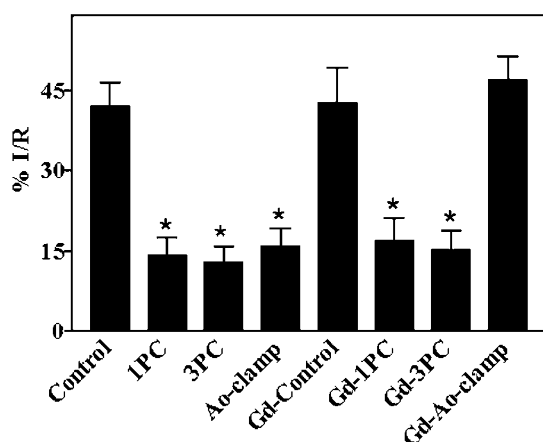

Figure 2 The effect of various interventions on infarct size (expressed as a percent of risk zone size) in rabbit hearts following 30 min ischemia and 120 min reperfusion. Gd: gadolinium, 1PC: ischemic preconditioning with one cycle of 5 min ischemia/10 min reperfusion, 3PC: ischemic preconditioning with three cycles of 5 min ischemia/10 min reperfusion, Ao-clamp: mechanical preconditioning with one cycle of aortic clamping/10 min reperfusion. *$P < 0.01$ *vs* Control, Gd-Control, Gd-Ao clamp.

was found in all preconditioned groups ($P < 0.05$). As shown in Figure 2, the infarcted to risk zone ratio in percent (%I/R), which is the most reliable index of protection, was 42.0 ± 4.4% in control group and 14.2 ± 3.4%, 12.9 ± 3.0%, 15.9 ± 3.3% respectively in groups 1PC, 3PC and Ao-clamp ($P < 0.01$). Gadolinium did not change the infarct size in the control and ischemic preconditioned groups: Gd-Control = 46.2 ± 6.7% (P = NS *vs* Control), Gd-1PC = 16.9 ± 4.2% (P = NS *vs* 1PC) and Gd-3PC = 15.2 ± 3.7% (P = NS *vs* 3PC). In contrast, the protection was negated in Gd-Ao-clamp group (46.9 ± 4.5%, $P < 0.01$ *vs* Ao-clamp).

Activation of stress-activated protein kinases

Activation of p38-MAPK and p46/p54 JNKs, as evidenced by their dual phosphorylation, was determined after 20 min of prolonged 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. As expected, sustained ischemia was associated with an increase in p38-MAPK phosphorylation and hence activation (about 2-fold) in the control group (Fig. 3). However, ischemia resulted in a more pronounced activation of p38-MAPK in ischemic preconditioned hearts (about 4.5-fold in 1PC and 3PC groups, comparing ischemic to non-ischemic regions). Stretch-induced preconditioning also resulted in a significant increase in p38 MAPK phosphorylation in both the ischemic and the non-ischemic regions of the heart as compared with the respective regions in the control group (Fig. 3). The addition of gadolinium, which blunted the beneficial effect of mechanical stretch on infarct size, decreased the activation of p38-MAPK to the control values level. It is of interest that, while gadolinium did not abrogate the effect of ischemic preconditioning on infarct size reduction (Fig. 2), it significantly decreased the phosphorylation of

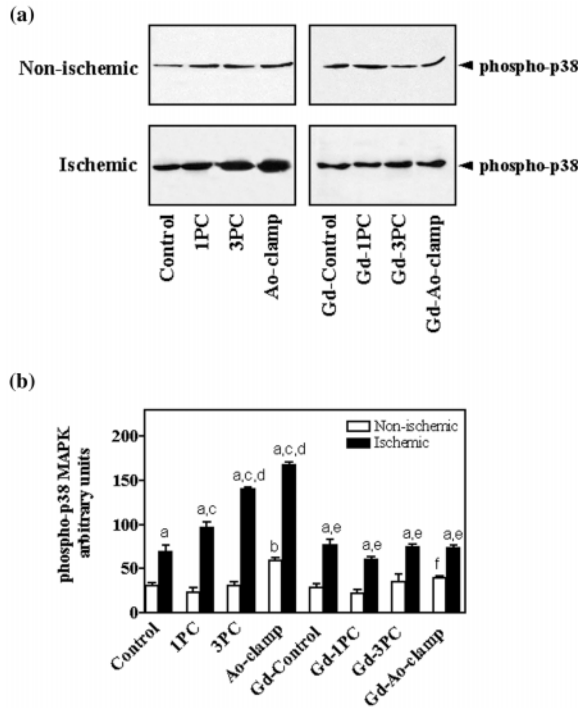

Figure 3 Analysis of the phosphorylation state of p38-MAPK in the non-ischemic and ischemic regions of the heart after 20 min of sustained ischemia. (a) Phosphorylated p38-MAPK was detected by immunoblotting with an antibody specific to the dually phosphorylated form of the kinase. Representative blots are shown. (b) Blots were quantified by laser densitometry. Each point is the mean ± S.E. of four independent observations. $^{a}P < 0.001$ *vs* the respective non-ischemic; $^{b}P < 0.01$ *vs* non-ischemic control; $^{c}P < 0.01$ *vs* ischemic control; $^{d}P < 0.01$ *vs* ischemic 1PC; $^{e}P < 0.001$ *vs* the respective group without the addition of gadolinium; $^{f}P < 0.01$ *vs* non-ischemic Ao-clamp. Gd: gadolinium, 1PC: ischemic preconditioning with one cycle of 5 min ischemia/10 min reperfusion, 3PC: ischemic preconditioning with three cycles of 5 min ischemia/10 min reperfusion, Ao-clamp: mechanical preconditioning with one cycle of aortic clamping/10 min reperfusion.

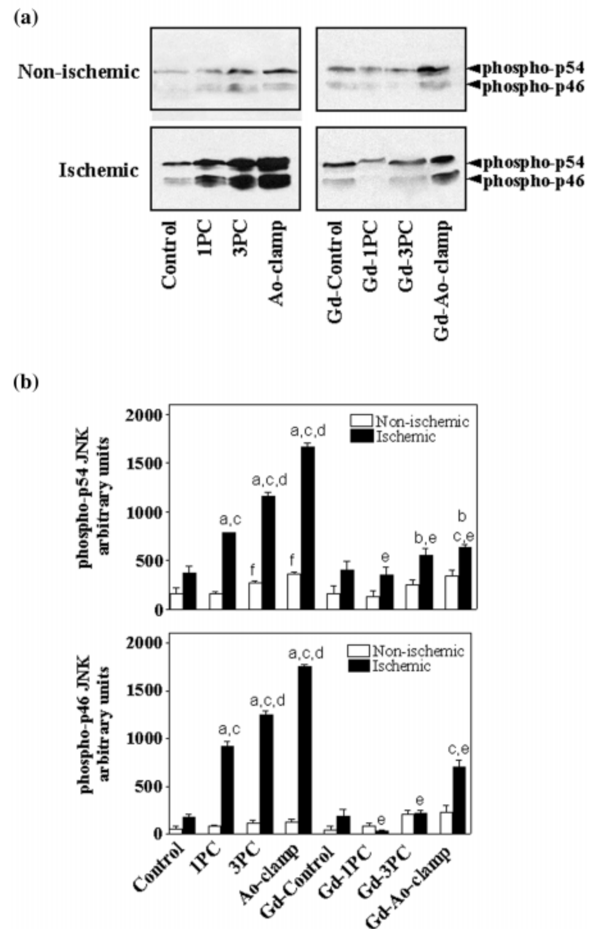

Figure 4 Analysis of the phosphorylation state of p46/p54 JNKs p38-MAPK in the non-ischemic and ischemic regions of the heart after 20 min of sustained ischemia. (a) Phosphorylated JNKs were detected by immunoblotting with an antibody specific to the dually phosphorylated form of the kinases. Representative blot are shown. (b) Blots were quantified by laser densitometry. Each point is the mean ± S.E. of four independent observations. $^{a}P < 0.001$ and $^{b}P < 0.01$ *vs* the respective non-ischemic; $^{c}P < 0.01$ *vs* ischemic control; $^{d}P < 0.01$ *vs* 1PC; $^{e}P < 0.001$ *vs* the respective group without the addition of gadolinium; $^{f}P < 0.05$ *vs* non-ischemic control. Gd: gadolinium, 1PC: ischemic preconditioning with one cycle of 5 min ischemia/10 min reperfusion, 3PC: ischemic preconditioning with three cycles of 5 min ischemia/10 min reperfusion, Ao-clamp: mechanical preconditioning with one cycle of aortic clamping/10 min reperfusion.

p38-MAPK in Gd-1PC and Gd-3PC groups comparable to that observed in Gd-Ao-clamp group (Fig. 3).

Measurement of p46/p54 JNKs phosphorylation in ventricular samples obtained from the ischemic and the non-ischemic regions of hearts revealed that there was a slight but non-significant increase in their phosphorylation in the control group (Fig. 4). A robust increase in the phosphorylation level of both JNK isoforms was observed in preconditioned hearts (7 to 11-fold for p46 and 6 to 7-fold for p54). Interestingly, the activation of JNKs was higher when preconditioning was induced by three ischemic cycles or by mechanical stretch. In addition, phosphorylation of p54 JNK in the non-ischemic samples was higher in 3PC and Ao-Clamp groups compared with the control. As is the case for p38-MAPK, addition of the stretch blocker gadolinium to any of the preconditioned groups decreased the activation of both JNKs to the control values (Fig. 4).

The phosphorylation level of ERK1/2 was also determined by immunoblotting during sustained ischemia in control and preconditioned groups. However, no change in ERK1/2 phosphorylation was detectable either in the control group or in

response to any of the preconditioning stimuli (data not shown).

Discussion

Ischemic preconditioning confers protection to the vulnerable ischemic heart and delays the ensuing necrosis. This phenomenon is receptor mediated and current evidence indicates that several receptors such as adenosine,[15] opioid,[16] bradykinin,[17] stretch[8,18] and others[19,20] are activated and then trigger the cascade of intracellular signaling. Stimulation of these receptors couples through phospholipases C and D which then activate protein kinase C, tyrosine kinases, mitogen-activated protein kinases and finally phosphorylate the mitochondrial K_{ATP} channels which are considered to be the end-effectors of ischemic preconditioning.[9,10] However, more recent data suggest that, in some cases, K_{ATP} channels may be the upstream triggers rather than the end-effectors of ischemic preconditioning.[11,21]

Several studies have elucidated the natural history of preconditioning regarding the length of the short ischemic insult and the following reperfusion interval,[22,23] the number of short ischemic bouts,[22,24] the possibility of reactivation[25,26] and the alternative means of triggering the mechanism of protection. However, controversy exists over the "frequency-dependency" of preconditioning, with some studies suggesting that one cycle is as efficacious as multiple cycles of preconditioning[22,24] whereas others, especially those using arrhythmias as an endpoint, have shown opposite results.[27] Furthermore, preconditioning is a graded phenomenon which depends on the duration of the short ischemia and of the stabilization period.[28,29] In the first step of this study, we repeated the experiments with one or three cycles of short ischemia and reperfusion and we confirmed previous findings that these stimuli are equally effective in protecting the ischemic heart *in vivo* (Fig. 2). In addition, corroborating other studies,[8,14] we showed that myocardial stretch induced by mechanical hypertension is an analogue of ischemic preconditioning conferring similar protection to the heart. The beneficial effect was completely abrogated when gadolinium—the stretch activated ion channel blocker—was given before stretch. On the other hand, administration of gadolinium before the ischemic preconditioning did not interfere with the protective effect (Fig. 2). These results disagree with those of Gysembergh *et al.*[14] who found that gadolinium abolishes the infarct size limiting effect of ischemic preconditioning. The reason for this discrepancy is not clear but it should be noted that the preconditioning protocols used in this study and that of Gysembergh *et al.*[14] are different. First, a shorter reperfusion interval (5 min) in the latter study might allow gadolinium to interfere with the protective mediators at the beginning of sustained ischemia, whereas the longer reperfusion interval (10 min) in this study would abolish such an effect. Second, it has been well recognized that the type of anesthesia can modify the results in cardiovascular experiments; they used xylazine and ketamine whilst we used pentothal. Finally, the areas at risk were defined with different techniques; unisperse blue pigment in that study and fluorescent Zn-Cd particles in our study.

The fact that stretch can induce preconditioning in the absence of short periods of ischemia does not exclude the possibility that these two mechanisms share some of the downstream intracellular pathways. In this regard, both classical ischemic preconditioning and preconditioning provided by mechanosensitive ion channel activation were prevented by staurosporine or polymyxin B which are well recognized protein kinase C inhibitors.[14,30] Thus, in the second step of the present study, we investigated the changes in the activation of MAPKs at the time of sustained ischemia in the control group and in the two models of preconditioning *in vivo*. Activation was assessed by immunoblotting with antibodies specific for the dually phosphorylated form of the kinases. MAPK require phosphorylation of both tyrosine and threonine in order to become activated. However, assessment of MAPK phosphorylation may not always be a fully indicator of its activity and this limitation should be taken into consideration in the interpretation of the data.[31] By comparing the ventricular samples of the ischemic to the non-ischemic regions of the heart we found that there is an activation of p38-MAPK in the control group, which becomes more pronounced in the preconditioned hearts, independently of the way of preconditioning (Fig. 3).

The role of MAPKs in the signal transduction of preconditioning has been addressed in several studies; however, the reported data are not conclusive. Increased activation of p38-MAPK during sustained ischemia was reported in preconditioned isolated rabbit and rat hearts[32–34] and rabbit cardiomyocytes.[35] Furthermore, MAPKAPK2, a downstream substrate of p38-MAPK, was found to be activated in preconditioned rabbit hearts implicating a role for p38-MAPK.[36] On the other hand, several studies have reported that activation of p38-MAPK during ischemia is transient and does not correlate with the preconditioning effect.[37,38] To further complicate the matters, recent studies demonstrated that p38-MAPK activity is attenuated in preconditioned

rabbit[39] and rat hearts[40,41] and this is associated with cardioprotection. Along these lines, it has been shown by several investigators that inhibition of p38-MAPK protects the heart against ischemic damage.[42–44] However, inhibition of p38-MAPK was also shown to completely block the cardioprotective effect of preconditioning.[34,45] Thus, it is still controversial whether the ischemia-induced activation of p38-MAPK is beneficial or deleterious. A plausible explanation for this controversy may be the differential actions of individual isoforms of the p38-MAPK family. These isoforms may have divergent roles with activation of p38α purportedly contributing to myocyte death and p38β potentially contributing to myocyte survival.[46,47]

The JNK family consists of at least 2 isoforms, the 46 kDa JNK1 and the 54 kDa JNK2, both of which are present in the heart. Most investigations have reported that both are strongly activated on reperfusion but are not affected by ischemia alone.[13] By determining the phosphorylation level of JNKs in ventricular samples from the ischemic and the non-ischemic regions of the heart we found that there is a non-significant increase of both p46 and p54 JNKs in the control group (Fig. 4). However, a robust increase in JNKs activation was observed in the preconditioned hearts. Our findings are in agreement with Ping *et al.*[48] who reported a PKC-dependent activation of p46 and p54 JNKs during ischemic preconditioning in conscious rabbits. However, in another study, there was no increase in either non-preconditioned or preconditioned isolated rabbit hearts before or during ischemia.[36] The reason for the divergent results between that study and the present study is unclear but may be related to the different experimental models since they used isolated rabbit hearts whereas we used a model of anaesthetized rabbits.

Hypertension *per se* by mechanical obstruction of the aorta induced a significant stretch on the left ventricle and this stretch activated p38-MAPK and JNKs. Our findings are in accordance with previous studies which have shown that mechanical stretch activates MAP kinases.[49,50] However, this is the first study to show an activation of p38-MAPK and JNKs during sustained ischemia in mechanically preconditioned hearts (Figs 3 and 4). The addition of the stretch channel blocker gadolinium reversed this effect and blocked the protection. Interestingly, gadolinium abolished the increase in the stress-activated kinases phosphorylation when administered in ischemic preconditioned hearts whereas it had no effect on the beneficial effect on infarct size. Thus, mechanical and ischemic preconditioning may not entirely share a common mechanism. Ischemic preconditioning intensifies myocardial contracture and there is a clear dissociation between protection and contracture.[51,52] In mechanical preconditioning, increased stretch is the pivotal stimulus for protection but in ischemic preconditioning the increased contracture or the dyskinetic myocardium may stimulate the stretch activated channels and MAPK activation without any further role. This satisfactorily explains that the stretch channel blocker gadolinium decreases the MAPK activation in all cases but abrogates protection in the mechanical preconditioning only. Ischemically preconditioned hearts would still be protected because of the activation of other receptors.

In conclusion, we showed that one or three cycles of short ischemia and reperfusion or mechanical hypertension induced by aortic clamping, are equally effective in preconditioning the ischemic heart. The protective effect of mechanical hypertension is blunted by the use of the stretch channel blocker gadolinium whilst there is no effect in the outcome of the control or ischemic preconditioned hearts. Increased phosphorylation of the stress-activated protein kinases, p38-MAPK and p46/p54 JNKs, is observed at the time of sustained ischemia in preconditioned hearts, both by short ischemia or by acute mechanical stretch, compared with the control. This activation is abolished when gadolinium is added to either the ischemic or the mechanically preconditioned hearts. Thus, the activation of stress-activated protein kinases can be dissociated from the protective effect of ischemic preconditioning in anesthetized rabbits and it may occur as an epiphenomenon.

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