ELSEVIER

Cardiovascular Research 63 (2004) 305–312

Cardiovascular Research

www.elsevier.com/locate/cardiores

Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning

Derek J. Hausenloy, Mihaela M. Mocanu, Derek M. Yellon*

The Hatter Institute and Centre for Cardiology, University College London Hospital, and Medical School, Grafton Way, London WC1E 6DB, UK

Received 8 December 2003; received in revised form 9 April 2004; accepted 14 April 2004
Available online 14 May 2004
Time for primary review 7 days

Abstract

Objectives: Recruitment of the survival kinase cascades, PI3K–Akt and Raf–MEK1/2–Erk1/2, at the time of reperfusion, following a lethal ischemic insult, may mediate the protection associated with ischemic preconditioning (IPC). The exact interplay between these two kinase cascades in mediating this effect is not clear. We examine the 'cross-talk' between these kinase cascades in their contribution to IPC-induced protection. **Methods and results:** In isolated perfused rat hearts subjected to 35 min of lethal ischemia ± ischemic preconditioning, the phosphorylation states of Akt, Erk1/2, p70S6K were determined after 15 min of reperfusion, and infarct size was measured after 120 min of reperfusion. IPC induced a threefold increase in Akt, Erk1/2, and p70S6K phosphorylation, at reperfusion. We found that inhibiting the PI3K–Akt (using LY294008) at reperfusion induced the phosphorylation of Erk1/2–p70S6K, and conversely, that inhibiting the MEK1/2–Erk1/2 pathway (using PD 98059) at reperfusion, induced the phosphorylation of Akt, suggesting 'cross-talk' between the two kinase pathways. However, this effect was not accompanied by a reduction in infarct size (43.1 ± 7.2% with LY 294008 and 57.7 ± 7.0% with PD 98059 vs. 46.3 ± 5.8% in control; P = NS), suggesting that both the kinase cascades may need to be activated to mediate IPC-induced protection. IPC reduced the infarct–risk volume ratio to 17.8 ± 2.3% from 46.3 ± 5.8% in control ($P < 0.01$). Inhibiting p70S6K, a kinase situated downstream of both PI3K and Erk1/2, using rapamycin, abolished IPC-induced protection (46.0 ± 7.7% with IPC + RAPA vs. 17.8 ± 2.3% with IPC; $P < 0.01$). **Conclusions:** We report that, the survival kinase cascades PI3K–Akt and MEK1/2–Erk1/2, which are recruited at the time of reperfusion in response to ischemic preconditioning, exhibit 'cross-talk' such that inhibiting one cascade activates the other and vice versa. Furthermore, at the time of reperfusion, these kinase cascades mediate IPC-induced protection, by acting in concert via p70S6K.

Keywords: Reperfusion injury; Ischemic preconditioning; Survival kinases; Cross-talk

1. Introduction

The phosphatidylinositol 3-OH kinase (PI3K)–Akt [1,2], and the Raf–mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (Raf–MEK1/2–Erk1/2) cascades [3], are activated in response to ischemia reperfusion injury, where they mediate protection through the recruitment of downstream antiapoptotic pathways of cellular survival. These include the phosphorylation and inactivation of proapoptotic proteins such as BAD [4], Bax [5,6], Bim [6], and caspases [7,8], and the phosphorylation and activation of endothelial nitric oxide synthase (eNOS) [9], p70S6 kinase [10,11], and protein kinase C (PKC) [12].

We and others have previously demonstrated that pharmacologically activating these kinase cascades at the time of reperfusion, by administering growth factors or other agents during the first few min of reperfusion, confers cardioprotection by limiting both the apoptotic and necrotic components of cell death [13,14]. Recruitment of these kinase cascades also occurs in the setting of ischemic preconditioning (IPC) [15,16], the phenomenon in which transient episodes of ischemia/reperfusion render the myocardium resistant to a subsequent episode of lethal ischemia [17]. In this scenario, the kinase cascades are activated in the

* Corresponding author. Tel.: +44-207-380-9776; fax: +44-207-388-5095.
E-mail address: hatter-institute@ucl.ac.uk (D.M. Yellon).

0008-6363/$ - see front matter © 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2004.04.011

preconditioning phase and they relay the preconditioning signal from the cell membrane to mediators situated further downstream on the signalling pathway [15,16].

Furthermore, we have recently demonstrated that the activation of these kinase cascades at the time of reperfusion may mediate IPC-induced protection, suggesting an additional role for these kinases in the setting of myocardial preconditioning [18]. In that study, IPC resulted in the activation, at the time of reperfusion, of both the PI3K–Akt and the Raf–MEK1/2–Erk1/2 cascades. Interestingly, inhibiting the activation of either Akt or Erk1/2 at the time of reperfusion abolished protection, suggesting that IPC-induced protection may require the activation of both the kinase cascades [18].

The exact interplay that exists between the PI3K–Akt and Raf–MEK1/2–Erk1/2 kinase cascades in mediating their protective effect is unclear, and their interaction at the time of reperfusion has not been previously examined. In various immortal cell lines, these kinase cascades have been demonstrated to exhibit a variety of complex, sometimes contradictory, interactions which enable mutual amplification or inhibition of the signal. For example: (i) the G-protein Ras can stimulate both the PI3K–Akt and Raf–MEK1/2–Erk1/2 kinase cascades [19]; (ii) it has been demonstrated that the PI3K–Akt cascade can both inhibit [20,21] and facilitate [22–24] the Raf–MEK1/2–Erk1/2 kinase cascade; (iii) the Raf–MEK1/2–Erk1/2 pathway can activate the PI3K–Akt–p70S6K cascade [25,26]; and (iv) signalling through these kinases cascades may converge on a distal target, such as the proapoptotic protein, BAD [25,26].

However, the cross-talk between these kinase cascades has not been investigated in the setting of myocardial ischemia-reperfusion. Therefore, the aim of the present study is to examine the interactions that exist between the PI3K–Akt and Raf–MEK1/2–Erk1/2 kinase cascades, at the time of reperfusion, in their contribution to IPC-induced protection. Furthermore, we examine the role of p70S6K, at the time of reperfusion, as a possible point of convergence for these kinase cascades in IPC-induced protection.

2. Methods

2.1. Animals

A total of 72 male Sprague–Dawley rats (300 ± 50 g body weight) were used. All animals were obtained from Charles River UK, (Margate, UK), and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationery Office, London, UK). The investigation conforms with the Guide for the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Materials

Rapamycin (Tocris), LY 294008 (Tocris), and PD 98059 (Tocris) were dissolved in dimethyl sulphoxide (DMSO) and added to the Krebs–Henseleit buffer such that the final DMSO concentration was less than 0.02%. All other reagents were of standard analytical grade.

2.3. Western blot analysis

Hearts excised from male Sprague–Dawley rats were Langendorff-perfused with Krebs–Henseleit buffer, according to the method outlined in a previous study [29], and were subjected to 35 min regional ischemia followed by 15 min of reperfusion, after which, samples taken from the region-at-risk were snap-frozen in liquid nitrogen for subsequent Western blot analysis for Akt, Erk1/2, and p70S6K phosphorylation ($N = 6$ per group). Isolated rat hearts were randomly assigned to treatment groups 1–4 (as outlined in Fig. 1).

For each extracted protein sample, the tissue was homogenised on ice in 250 μl of suspension buffer, which comprised (in mmol/l): NaCl 100, TRIS 10 (pH 7.6), EDTA 1 (pH 8.0), sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2, phenyl methyl sulphonyl fluoride (PMSF) 0.1 μg/ml, and 1 μg/ml each of aprotonin, leupeptin, trypsin inhibitor, and protease inhibitor. The homogenised samples were then centrifuged at 10,000 rpm for 10 min at 4 °C and the pellet discarded. The protein content of the supernatant from each protein sample was determined using the Bicinchoninic acid-based (BCA™) protein assay reagent system (Pierce, Rockford, USA). The protein sample was then diluted in 2 × sample buffer, comprising (in mmol/l): Tris 100 (pH 6.8), dithiothreitol (DTT) 200 mM, sodium dodecylsulphate (SDS) 2%, bromophenol blue 0.2%, and glycerol 20%, and subsequently heated for 10 min at 100 °C, and then stored at −70 °C for later analysis.

Western blot analysis was performed as previously described [16]. Briefly, for each sample, 30 μg of protein/lane was loaded onto a 12.5% SDS–PAGE gel and subsequently transferred onto a Hybond ECL nitrocellulose membrane (Amersham, UK). Adequate transfer of proteins was confirmed by Coomassie blue staining of the gel and Ponceau Red staining (Sigma) of the membrane. Equal protein loading was confirmed by probing for β-actin.

The membranes were probed with polyclonal antibodies (1:1000 dilution) for phospho–Erk1/2 (Thr202/Thr204), phospho–Akt (Ser473), phospho–p70S6 kinase (Thr389) and phospho–p70S6 kinase (Thr421/Ser424), and were used in accordance with the manufacturer's instructions (Cell Signalling, Hitchin, Kent). The membranes were subsequently probed with horse radish peroxidase-conjugated antirabbit antibody (1:2000). Proteins were detected using enhanced chemiluminescence ECL Western blotting detection reagent and bands were visualized by exposure to

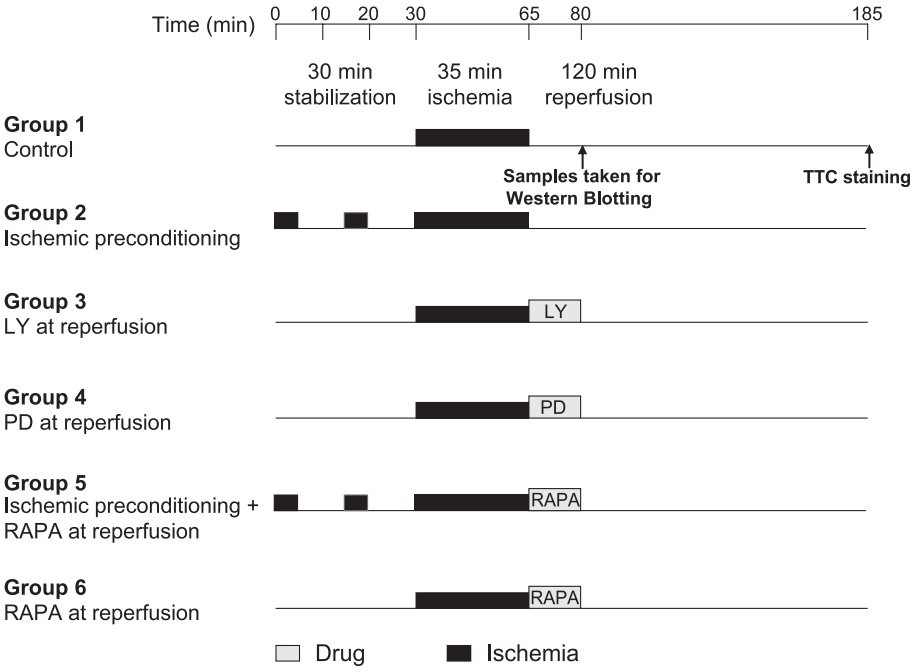

Fig. 1. Treatment protocols for isolated perfused rat heart studies (TTC-triphenyltetrazolium–chloride. LY-LY 294002, PD-PD 98059).

photographic film. The developed films were scanned, and the relative densitometry was assessed using the National Institutes of Health (NIH) Shareware program, NIH Image 1.63.

2.4. Langendorff-perfused rat heart studies

Hearts excised from male Sprague–Dawley rats were Langendorff-perfused with Krebs–Henseleit buffer and subjected to 35 min regional ischemia followed by 120 min of reperfusion, after which, the infarct–risk volume ratio was determined by triphenyltetrazolium–chloride staining.

Isolated rat hearts were randomly assigned to one of the following treatment groups ($n = 6$ per group; see Fig. 1):

(1) Control hearts received either 0.02% DMSO or Krebs–Henseleit buffer alone for the first 15 min of reperfusion;
(2) IPC hearts were treated with two 5-min periods of global ischemia with an intervening 10-min period of reperfusion prior to the lethal ischemic insult;
(3) Hearts were given LY 294008 (LY, 15 μmol/l) alone for the first 15 min of reperfusion. At this concentration, LY 294008 has been shown to act as a specific inhibitor of PI3K [30] and has been demonstrated to inhibit Akt phosphorylation in the isolated perfused rat heart [16];
(4) Hearts were given PD 98059 (PD, 10 μmol/l) alone for the first 15 min of reperfusion. This concentration of PD 98059 has been demonstrated to inhibit Erk1/2 phosphorylation in the isolated perfused rat heart [16];
(5) IPC hearts were given rapamycin (RAPA, 0.5 nmol/l) for the first 15 min of reperfusion. This concentration of rapamycin has been shown to inhibit p70S6K phosphorylation in the isolated perfused rat heart [31];
(6) Hearts were given rapamycin alone for the first 15 min of reperfusion.

2.5. Statistical analysis

All values are expressed as mean ± S.E.M. Data were analyzed using one-way analysis of variance and the Fisher's protected least significant difference test for multiple comparisons. A $P < 0.05$ was considered significant.

3. Results

3.1. Ischemic preconditioning induces Akt–p70S6K and Erk1/2–p70S6K phosphorylation at the time of reperfusion

In hearts subjected to IPC, there was a threefold increase in Akt phosphorylation at the time of reperfusion (relative densitometry in arbitrary units [a.u.]: 891 ± 99 with IPC vs. 281 ± 58 a.u. in control; $P < 0.001$; Fig. 2a), as well as an eightfold increase in phosphorylation of p70S6K at Thr389, the site phosphorylated by Akt–mTOR [10,11] (1343 ± 259 a.u. with IPC vs. 163 ± 41 a.u. in control; $P < 0.001$; Fig. 2b). The presence of LY 294008 for the first 15 min of reperfusion abolished the IPC-induced phosphorylation of Akt (1343 ± 259 a.u. with IPC vs. 226 ± 28 a.u. in IPC + LY; $P < 0.001$; Fig. 2a), confirming the inhibitory effect of LY 294008 on PI3K.

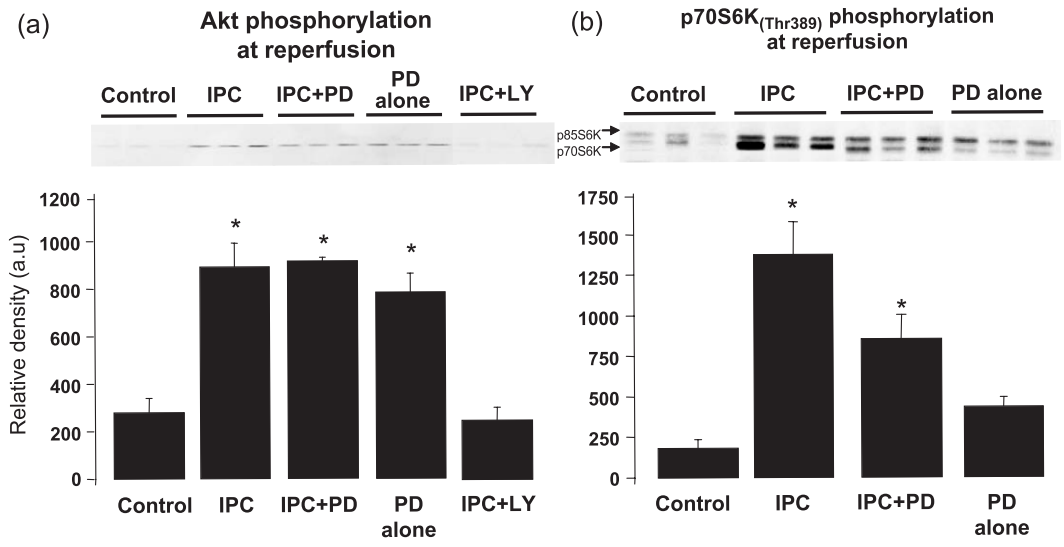

Fig. 2. Representative Western blots and relative densitometry showing that IPC results in the activation of (a) Akt and (b) p70S6K at Thr389 (the site phosphorylated by Akt–mTOR) at the time of reperfusion. The lower bands of the Western blot shown in (b) represent p70S6K and the upper bands depict p85S6K. Inhibiting MEK1/2, using PD 98059 (PD), at the time of reperfusion, results in the phosphorylation of (a) Akt and (b) p70S6K ($N=6$ per group. *: $P<0.05$ compared to control).

IPC also induced the phosphorylation of Erk1/2 (1122 ± 79 a.u. with IPC vs. 334 ± 67 a.u. in control; $P<0.001$; Fig. 3a), and p70S6K at Thr421/Ser424, the sites phosphorylated by Erk1/2 [10,11] (1906 ± 110 a.u. with IPC vs. 450 ± 34 a.u. in control; $P<0.01$; Fig. 3b), at the time of reperfusion. The presence PD 98059 for the first 15 min of reperfusion abolished the IPC-induced phosphorylation of Erk1/2 (1906 ± 110 a.u. with IPC vs. 446 ± 117 a.u. in IPC + PD; $P<0.001$; Fig. 3a), confirming the inhibitory effect of PD 98059 on MEK1/2.

3.2. 'Cross-talk' between the PI3K–Akt and MEK1/2–Erk1/2 kinase cascades at the time of reperfusion

Inhibiting MEK1/2 in control hearts, during the reperfusion phase, using PD 98059 alone, resulted in the phosphorylation of Akt (785 ± 78 a.u. with PD alone vs. 281 ± 58 a.u. in control; $P<0.01$; Fig. 2a) and a nonsignificant increase in phosphorylation of the downstream p70S6K at Thr389 (356 ± 52 a.u. with PD alone vs. 163 ± 41 a.u. in control; $P=$NS; Fig. 2b). Inhibiting

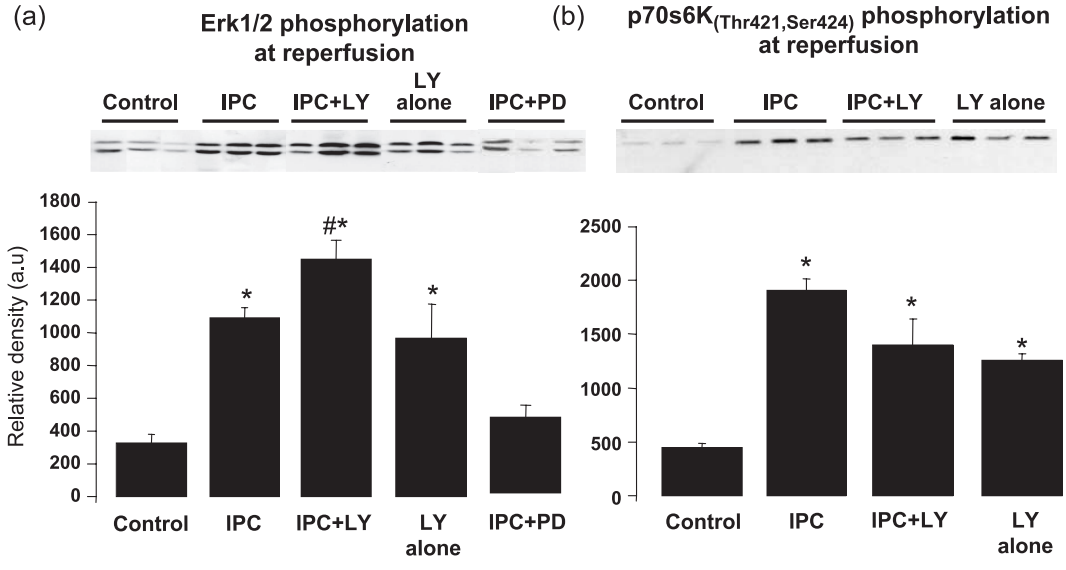

Fig. 3. Representative Western blots and relative densitometry showing that IPC results in the activation of (a) Erk1/2 and (b) p70S6K at Thr421/Ser424 (the sites phosphorylated by Erk1/2) at the time of reperfusion. Inhibiting PI3K, using LY 294008 (LY), at the time of reperfusion, results in the phosphorylation of (a) Erk1/2 and (b) p70S6K ($N=6$ per group. *: $P<0.05$ compared to control. #: $P<0.05$ compared to IPC treatment).

MEK1/2 at reperfusion in IPC-treated hearts did not increase Akt phosphorylation above that observed in hearts that had only received IPC treatment (914 ± 18 a.u. in IPC + PD vs. 891 ± 99 a.u. in IPC; P = NS; Fig. 2a).

Conversely, inhibiting PI3K in control hearts, during the reperfusion phase, using LY 294002, resulted in the phosphorylation of Erk1/2 (913 ± 225 a.u. with LY alone vs. 334 ± 67 a.u. in control; $P < 0.01$; Fig. 3a) and the downstream p70S6K at Thr421, Ser424 (1260 ± 60 a.u. with LY alone vs. 450 ± 34 a.u. in control; $P < 0.01$; Fig. 3b). Interestingly, in this case, the inhibition of PI3K at the time of reperfusion resulted in the activation of Erk1/2 over and above that induced by IPC alone (1442 ± 134 a.u. in IPC + LY vs. 1122 ± 79 a.u. in IPC; $P < 0.001$; Fig. 3a).

3.3. The 'cross-talk' demonstrated between the PI3K–Akt and MEK1/2–Erk1/2 kinase cascades is not associated with protection

In order to determine whether the PD 98059-induced phosphorylation of Akt and the LY 294008-induced phosphorylation of Erk1/2 are associated with protection at the time of reperfusion, we examined the effect of these kinases on infarct size. Inhibiting PI3K, using LY 294002 for the first 15 min of reperfusion in control hearts, did not influence infarct size (43.1 ± 7.2% with LY vs. 46.3 ± 5.8% in control; P = NS; Fig. 4a), suggesting that the LY 294002-induced Erk1/2 phosphorylation (as seen in Fig. 3a) is not sufficient in itself to induce protection. Similarly, inhibiting MEK1/2, using PD 98059 for the first 15 min of reperfusion in control hearts did not influence infarct size (57.7 ± 7.0% in PD vs. 46.3 ± 5.8% in control; P = NS; Fig. 4a), suggesting that the PD 98059-induced Akt phosphorylation (as seen in Fig. 3b) is also not sufficient in itself to induce protection.

3.4. Inhibiting p70S6K phosphorylation at the time of reperfusion abrogates the IPC-mediated reduction in infarct size

IPC reduced infarct size to 17.8 ± 2.3% from 46.3 ± 5.8% in control ($P < 0.01$; Fig. 4). The presence of rapamycin (the p70S6K inhibitor) for the first 15 min of reperfusion abrogated the IPC-induced reduction in infarct size (46.0 ± 7.7% with IPC + RAPA vs. 17.8 ± 2.3% with IPC; $P < 0.01$; Fig. 4b), suggesting that the activation of p70S6K may be a mediator of IPC-induced protection. Rapamycin given alone at time of reperfusion to control hearts did not influence infarct size (36.0 ± 5.4% with RAPA vs. 46.3 ± 5.8% in control; P = NS; Fig. 4b). DMSO vehicle (0.02%) did not influence either infarct size or kinase phosphorylation.

4. Discussion

In the present study, we demonstrate that the PI3K–Akt and Raf–MEK1/2–Erk1/2 kinase cascades, which are activated at the time of reperfusion in response to ischemic preconditioning, exhibit 'cross-talk'. We found that, inhibiting PI3K, using LY 294002, at the time of reperfusion resulted in the activation of the MEK1/2–Erk1/2–p70S6K kinase cascade, and that the inhibition of MEK1/2 with PD 98059 at the time of reperfusion resulted in the activation of

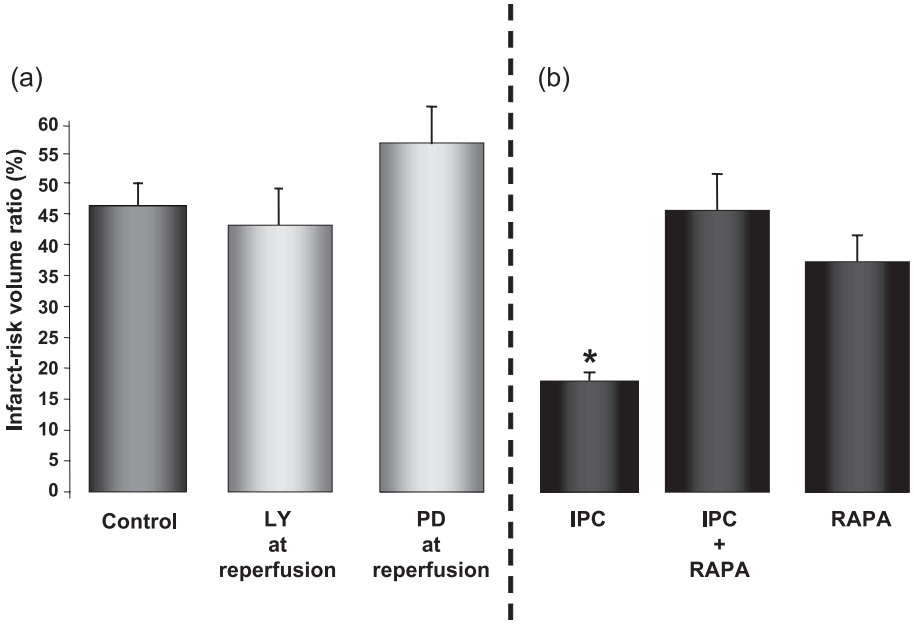

Fig. 4. Inhibiting PI3K, MEK1/2 or p70S6K using LY 294008 (LY), PD 98059 (PD), or rapamycin (RAPA), respectively, at the time of reperfusion, in control hearts does not influence infarct size. However, inhibiting p70S6K, using rapamycin at the time of reperfusion, abolishes the ischemic preconditioning (IPC)-mediated reduction in infarct–risk volume ratio (N = 6 per group. *: $P < 0.01$ compared to control).

the PI3K–Akt kinase cascade but not p70S6K. Interestingly, this 'cross-talk' between the kinase cascades was not associated with protection, in terms of a reduction in infarct size, which suggests that the PI3K–Akt phosphorylation induced by PD 98059 (the MEK1/2 inhibitor) and the MEK1/2–Erk1/2–p70S6K phosphorylation induced by LY 294008 (the PI3K inhibitor) were insufficient in themselves to induce protection, and indicates that both the kinase cascades may be required to be activated at the time of reperfusion to mediate IPC-induced protection.

Our previous study had demonstrated that inhibiting either the Akt or the Erk1/2 activation that occurred at the time of reperfusion, using LY 294008 or PD 98059, respectively, completely abrogated IPC-induced protection, also indicating the requirement for both kinase cascades to mediate protection [18]. In the present study, we tested this proposal by investigating p70S6K, which, because it is downstream of both the PI3K–Akt and MEK1/2–Erk1/2 cascades, may act as a point of convergence for the signal relayed through these kinase cascades. In the present study, we demonstrated that IPC results in the activation of p70S6K at phosphorylation sites specific for Akt (at Thr 389) and Erk1/2 (at Thr421, Ser424), at the time of reperfusion [10,11]. We found that the presence of the known p70S6K inhibitor, rapamycin, at the time of reperfusion completely abrogated IPC-induced protection, suggesting that p70S6K may act as the point of convergence for these kinase cascades. However, without including data actually demonstrating rapamycin-mediated inhibition of the IPC-induced phosphorylation of p70S6K, we cannot categorically state that p70S6K is implicated in IPC-induced protection, and we cannot exclude the possibility of rapamycin blocking IPC-induced protection by a mechanism other that p70S6K inhibition. We speculate that the activation of p70S6K requires the phosphorylation at both Thr 389 (the site phosphorylated by Akt–mTOR) and Thr421, Ser424 (the sites phosphorylated by Erk1/2), which would necessitate the activation of both the PI3K–Akt and MEK1/2–Erk1/2 pathways to mediate IPC-induced protection.

Previous studies have demonstrated that signalling through these kinase cascades can converge on another distal target, the proapoptotic protein, BAD, the phosphorylation and inactivation of which would mediate cellular survival by an antiapoptotic mechanism [4,27,28]. These kinase cascades may also phosphorylate and inactivate BAD via the recruitment of p70S6K, providing a role for the latter in cellular protection [32]. In this regard, we have previously demonstrated that the protection induced by insulin at the time of reperfusion is mediated by activation of the Akt–p70S6K–BAD pathway [31].

The phenomenon of 'cross-talk' observed between the kinase cascades, in which the inhibition of one kinase cascade results in the activation of the other and vice versa, has been observed in other organ tissue, including the lens [33] and neuronal cells [23]. In previous studies, using immortal cell lines, it has been demonstrated that Akt inhibits the Raf–MEK1/2–Erk1/2 kinase cascade by phosphorylation and inactivation of Raf at Ser^{259} [20,21], and this inhibitory pathway may be recruited at different stages of cell development [20], or vary according to the concentration and type of ligand exposure [24]. Therefore, the inhibition of the PI3K–Akt pathway may activate the Raf–MEK1/2–Erk1/2 cascade, providing a form of 'compensatory regulation'. This would explain why the inhibition of PI3K at the time of reperfusion induces the activation of MEK1/2–Erk1/2 as the inhibitory pathway from Akt to Raf–MEK1/2–Erk1/2 is removed (see Fig. 5).

Whether the corollary occurs, in which the activation of Akt occurring in response to the inhibition of the Raf–MEK1/2–Erk1/2 cascade is the result of the removal of an inhibitory pathway from Erk1/2 on the PI3K–Akt pathway, is unclear at present but has been suggested in a previous study [33]. We did not find any evidence to suggest that the PI3K–Akt cascade facilitates the Raf–MEK1/2–Erk1/2 kinase cascade as has been shown in previous studies [22–24]. Nor did we find evidence that MEK1 activation activates the PI3K–Akt–p70S6K pathway as been suggested by several studies [25,26]. The disparity in findings may rest with the different experimental models used and vary with the experimental conditions [22].

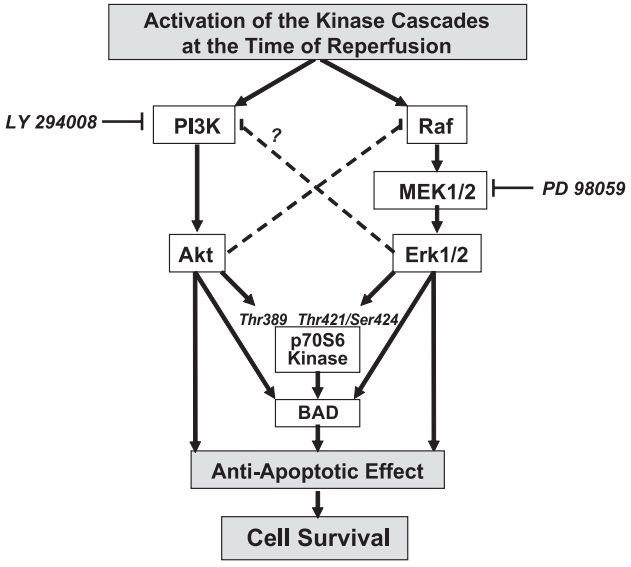

Fig. 5. Hypothetical scheme outlining the 'cross-talk' which occurs between the PI3K–Akt–p70S6K and MEK1/2–Erk1/2–p70S6K cascades, at the time of reperfusion, in response to ischemic preconditioning. The existence of inhibitory pathways between the kinase cascades may explain why the inhibition of one cascade using either LY 294008 or PD 98059 results in the activation of the other and vice versa. Convergence on the downstream kinase p70S6K and the proapoptotic factor, BAD, may explain the requirement for both kinase cascades to be activated to mediate IPC-induced protection.

The hypothetical scheme in Fig. 5 demonstrates that, at the time of reperfusion, both the PI3K–Akt–p70S6K and MEK1/2–Erk1/2–p70S6K cascades are activated in response to ischemic preconditioning. The existence of inhibitory pathways between the kinase cascades may explain why the inhibition of one cascade using either LY294008 or PD98059 results in the activation of the other and vice versa. Convergence on the downstream kinase p70S6K and the proapoptotic factor, BAD, may explain the requirement for both kinase cascades to be activated to mediate IPC-induced protection.

Interestingly, the 'cross-talk' observed between the two kinase cascades did not appear to be equal in that inhibiting MEK1/2 at the time of reperfusion in control hearts resulted in the phosphorylation of Akt but not p70S6K (at Thr389). Furthermore, inhibiting MEK1/2 at the time of reperfusion in IPC-treated hearts did not result in the phosphorylation of either Akt or p70S6K (at Thr389) to a greater level than that observed in hearts undergoing IPC-alone. In direct contrast, we found that inhibiting PI3K at the time of reperfusion in control hearts resulted in the phosphorylation of Erk1/2 and its downstream p70S6K (at Thr421/Ser424). Furthermore, inhibiting PI3K at the time of reperfusion in IPC-treated hearts resulted in the phosphorylation of Erk1/2 but not p70S6K (at Thr421/Ser424) to a greater level than that observed in hearts undergoing IPC-alone. These findings would suggest that the cross-talk between the two kinase cascades is not balanced and that in the scenario of cellular survival, the PI3K–Akt cascade is the more dominant cascade, with the MEK1/2–Erk1/2 cascade playing a more significant role in mediating growth and hypertrophy.

This study relies on the specificity of the pharmacological inhibitors used, and as such, the concentrations employed in the study were taken from previous studies [16]. More importantly, the concentrations used were well within the concentration ranges required to inhibit these kinases specifically [30,34]. However, we cannot categorically exclude the effects of these drugs on other kinase pathways, although we consider this unlikely at the concentrations used.

In conclusion, we report that the activation of the PI3K–Akt and MEK1/2–Erk1/2 kinase cascades that occurs at the time of reperfusion, in response to ischemic preconditioning, occurs in parallel, and that both kinase cascades are required to act in concert to mediate IPC-induced protection. The downstream kinase, p70S6K, may be a point of convergence for the PI3K–Akt and MEK1/2–Erk1/2 kinase cascades in mediating IPC-induced protection, although further studies are required to confirm this. These two pathways appear to interact in such a way that inhibiting one kinase cascade upregulates the activity of the other pathway, thereby acting as a compensatory safeguard, ensuring the signal for cellular protection is executed. Further work is needed to explore the intricate interactions between these two kinase cascades at the time of reperfusion, in the setting of IPC-induced protection. Therefore, pharmacological manipulation of these survival kinases, at the time of reperfusion, which attenuate lethal reperfusion injury, may provide novel adjuncts to thrombolytic therapy in the treatment of an acute myocardial infarction.

Acknowledgements

Dr. Derek Hausenloy is supported by a British Heart Foundation Clinical PhD Studentship (FS/02017).

References

[1] Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation 2001;104(3):330–5.
[2] Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation 2000;101(6):660–7.
[3] Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, et al. Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res 2000;86(6):692–9.
[4] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91(2):231–41.
[5] Tsuruta F, Masuyama N, Gotoh Y. The phosphatidylinositol 3-kinase (PI3K)–Akt pathway suppresses Bax translocation to mitochondria. J Biol Chem 2002;277(16):14040–7.
[6] Weston CR, Balmanno K, Chalmers C, Hadfield K, Molton SA, Ley R, et al. Activation of ERK1/2 by deltaRaf–1:ER* represses Bim expression independently of the JNK or PI3K pathways. Oncogene 2003;22(9):1281–93.
[7] Erhardt P, Schremser EJ, Cooper GM. B-Raf inhibits programmed cell death downstream of cytochrome *c* release from mitochondria by activating the MEK/Erk pathway. Mol Cell Biol 1999;19(8):5308–15.
[8] Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;282(5392):1318–21.
[9] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399(6736):601–5.
[10] Chung J, Grammer TC, Lemon KP, Kazlauskas A, Blenis J. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. Nature 1994;370(6484):71–5.
[11] Lehman JA, Gomez-Cambronero J. Molecular crosstalk between p70S6k and MAPK cell signaling pathways. Biochem Biophys Res Commun 2002;293(1):463–9.
[12] Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science 1998;281(5385):2042–5.
[13] Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. Cardiovasc Res 2004;61(3):448–60.
[14] Yang XM, Krieg T, Cui L, Downey JM, Cohen MV. NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK, and NO. J Mol Cell Cardiol 2004;36(3):411–21.
[15] Tong H, Chen W, Steenbergen C, Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. Circ Res 2000;87(4):309–15.

[16] Mocanu MM, Bell RM, Yellon DM. PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by ischemic preconditioning. J Mol Cell Cardiol 2002;34(6):661–8.
[17] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74(5):1124–36.
[18] Hausenloy DJ, Mocanu MM, Yellon DM. Activation of the pro-survival kinase cascades (PI3 kinase–Akt–p70S6K kinase and ERK 1/2–p70S6K kinase) at reperfusion are essential for preconditioning-induced protection. Circulation 2003;108:I–288 (Abstract).
[19] Schwartz MA, Baron V. Interactions between mitogenic stimuli, or, a thousand and one connections. Curr Opin Cell Biol 1999;11(2):197–202.
[20] Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, et al. Differentiation stage-specific inhibition of the Raf–MEK–ERK pathway by Akt. Science 1999;286(5445):1738–41.
[21] Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 1999;286(5445):1741–4.
[22] Duckworth BC, Cantley LC. Conditional inhibition of the mitogen-activated protein kinase cascade by Wortmannin. Dependence on signal strength. J Biol Chem 1997;272(44):27665–70.
[23] Van Der Heide LP, Hoekman MF, Biessels GJ, Gispen WH. Insulin inhibits extracellular regulated kinase 1/2 phosphorylation in a phosphatidylinositol 3-kinase (PI3) kinase-dependent manner in Neuro2a cells. J Neurochem 2003;86(1):86–91.
[24] Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M. Regulation of Raf–Akt cross-talk. J Biol Chem 2002;277(34):31099–106.
[25] von Gise A, Lorenz P, Wellbrock C, Hemmings B, Berberich-Siebelt F, Rapp UR, et al. Apoptosis suppression by Raf–1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. Mol Cell Biol 2001;21(7):2324–36.
[26] Blalock WL, Navolanic PM, Steelman LS, Shelton JG, Moye PW, Lee JT, et al. Requirement for the PI3K/Akt pathway in MEK1-mediated growth and prevention of apoptosis: identification of an Achilles heel in leukemia. Leukemia 2003;17(6):1058–67.
[27] Hayakawa J, Ohmichi M, Kurachi H, Kanda Y, Hisamoto K, Nishio Y, et al. Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin. Cancer Res 2000;60(21):5988–94.
[28] Scheid MP, Duronio V. Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/Akt: involvement of MEK upstream of Bad phosphorylation. Proc Natl Acad Sci U S A 1998;95(13):7439–44.
[29] Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc Res 2002;55(3):534–43.
[30] Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). J Biol Chem 1994;269(7):5241–8.
[31] Jonassen AK, Sack MN, Mjos OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ Res 2001;89(12):1191–8.
[32] Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci U S A 2001;98(17):9666–70.
[33] Steven ZD, Lou MF. Studies of the mitogen-activated protein kinases and phosphatidylinositol-3 kinase in the lens: 2. The intercommunications. Exp Eye Res 2002;75(2):177–92.
[34] Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 1995;92(17):7686–9.