Sustained activation of p42/p44 mitogen-activated protein kinase during recovery from simulated ischaemia mediates adaptive cytoprotection in cardiomyocytes

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Delayed cytoprotection (preconditioning) occurs 24 h after sublethal simulated ischaemia and reperfusion (SI/R) in neonatal rat ventricular cardiomyocytes. SI/R was used to investigate the role of activation of mitogen-activated protein kinases (MAPKs), stress-activated protein kinases (SAPKs) and phosphoinositide 3-kinase-dependent protein kinase B (PKB)/Akt in cytoprotection. SI resulted in transient dual (Thr/Tyr) phosphorylation of p42/p44-MAPK and p38-MAPK, weak phosphorylation of p46/p54-SAPK, but no phosphorylation of PKB. 'Reperfusion' caused further transient phosphorylation of p38-MAPK, but sustained phosphorylation of p42/p44-MAPK (lasting 4 h) and of Ser^{473} of PKB (lasting 2 h). Furthermore, SI/R (24 h) induced delayed protection against lethal SI, as determined by an increase in cell viability {bioreduction of MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]} and a decrease in cell injury (release of creatine kinase). Both protection and phosphorylation of p42/p44-MAPK were blocked by the MEK-1/2 (MAPK/Erk kinase-1/2) inhibitor PD98059 (50 μM) when given during SI/R, but not when given during SI alone. The p38-MAPK inhibitor SB203580 (10 μM) blocked the p38-MAPK-dependent phosphorylation of activating transcription factor 2 *in vitro*, and the phosphoinositide 3-kinase inhibitor wortmannin (100 nM) blocked PKB phosphorylation on Ser^{473}. However, neither SB203580 nor wortmannin had any effect on delayed protection. Therefore sustained activation of p42/p44-MAPK during simulated 'reperfusion' following sublethal SI mediates preconditioning in cardiomyocytes independently of transient activation of p38-MAPK or sustained activation of PKB.

Key words: PKB/Akt, p38-MAPK, p42/p44-MAPK, preconditioning, reperfusion.

INTRODUCTION

The signal transduction pathways that mediate cell survival in cardiac myocytes during adaptation to brief sublethal myocardial stresses, such as heat stress, ischaemia, withdrawal of survival factors or metabolic substrates, remain to be defined in detail. A number of studies have indicated that p38-MAPK (mitogen-activated protein kinase) [1] and/or MAPKAPK2 (MAPK-activated protein kinase 2) [2] are transiently activated by cardiac ischaemic 'preconditioning' and that adenosine, an endogenous mediator of ischaemic preconditioning, activates p38-MAPK, JNK1/2 (c-Jun N-terminal kinase 1/2) and p42/p44-MAPK in the perfused rat heart [3]. Furthermore, other G-protein-coupled receptor agonists, such as noradrenaline [4] and endothelin [5], activate these pathways in rat hearts.

In many cell types, the p42/p44-MAPK (Erk1/2, where Erk is extracellular-signal-regulated kinase) cascade appears to mediate cell growth and survival signals. For instance, the small GTP-binding protein Ras is a key mediator of growth-factor-dependent survival [6,7]. Ras signals through Raf-1 to MEK-1/2 (MAPK/Erk kinase-1/2) and p42/p44-MAPK, which promotes survival and inhibits apoptosis [8]. In addition, Erk activation has been shown to protect cardiomyocytes from oxidant stress [9].

We have recently shown that the growth-factor-associated kinase Akt [also known as protein kinase B (PKB)] is also phosphorylated following simulated ischaemia/reperfusion (SI/R) in cardiomyocytes in a phosphoinositide 3-kinase (PI 3-kinase)-dependent manner [10]. PKB has also been suggested to be involved in cell survival pathways [11]. PKB is activated downstream of PI 3-kinase by the phosphoinositide-dependent protein kinases PDK-1 and -2 [12]. PKB in turn phosphorylates a number of downstream targets relevant to cell survival functions, including the pro-apoptotic Bcl-2 family member BAD [13], nuclear factor κB [14] and nitric oxide synthase [15]. Phosphorylation of BAD on Ser^{136} by PKB inhibits is pro-apoptotic function, and so promotes cell survival [16].

The p38-MAPK and JNK [stress-activated protein kinase (SAPK)-1/2] families are activated by a variety of cellular stresses, including heat shock, UV radiation, osmotic shock and pro-inflammatory cytokines, and appear to be involved in a variety of responses, including cell death/survival pathways. In particular, p38-MAPK and JNK signalling appears to be pro-apoptotic in many cell types. For instance, activation of p38-MAPK in response to Erk inhibition in HeLa cells led to caspase activation and apoptosis. This was inhibited by activation of the PI 3-kinase $\rightarrow$ Akt/PKB pathway, either by serum (which was sensitive to inhibitors of PI 3-kinase or Akt) or by expression of active mutants of PI 3-kinase or Akt [17]. However, the role of these pathways in cardiomyocyte cell death, and in particular necrosis rather than apoptosis, is less certain. For instance, activation of p38-MAPK during lethal SI appears to promote cell death in cardiomyocytes [18] and in Langendorff perfused rabbit hearts [19]. In contrast, in a model which involved 'ischaemic pelleting' of isolated adult cardiomyocytes, the pro-

Abbreviations used: SI, simulated ischaemia; SI/R, SI/reperfusion; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPKAPK, MAPK-activated protein kinase; Erk, extracellular-signal-regulated kinase; MEK, MAPK/Erk kinase; PKB, protein kinase B; PI 3-kinase, phosphoinositide 3-kinase; TBST, Tris-buffered saline/Tween-20; TBSTM, TBST/milk; ATF, activating transcription factor.

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tein phosphatase inhibitors calyculin A and fostreicin attenuated osmotic fragility. Protection by the phosphatase inhibitors was concomitant with a potentiation of p38-MAPK dual phosphorylation and downstream phosphorylation of the small heat-shock protein HSP27 [20]. Indeed, MAPKAPK2 (which is activated by p38-MAPK and is probably responsible for HSP27 phosphorylation) has been implicated in the early phase of ischaemic preconditioning [21].

It is clear that the precise roles of activation of the MAPK/SAPK and PI 3-kinase pathways during sublethal ischaemia, during intervening reperfusion and during subsequent severe lethal ischaemia deserve further characterization. Our findings indicate that a delayed, sustained activation of p42/p44-MAPK that occurs during 'reperfusion' following reversible, sublethal SI in isolated neonatal rat cardiomyocytes is essential for the subsequent development of delayed protection, but that protection is independent of both p38-MAPK and PKB activation.

MATERIALS AND METHODS

Materials

Tissue culture products were obtained from Life Technologies Inc. (Paisley, Scotland, U.K.). Antibodies recognizing the dual-phosphorylated (Thr/Tyr) forms of p42/p44-MAPK (Erk1/2) and p38-MAPK, PKB/Akt phosphorylated on either Ser^{473} or Thr^{308} and non-phosphorylated forms of p38-MAPK, p54-SAPK/JNK and PKB/Akt were obtained from New England Biolabs (Hitchin, Herts., U.K.). Phosphospecific antibodies recognizing dual-phosphorylated (Thr/Tyr) p46/p54-SAPK (JNK1/2) were from Promega. Monoclonal antibodies to Erk2 were obtained from Santa Cruz (Insight Biotechnology, Wembley, Middx., U.K.). Rabbit anti-mouse and swine anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from DAKO. SB203580, PD98059 and wortmannin were obtained from Calbiochem, and stock solutions were prepared in DMSO. Prestained molecular-mass markers, enhanced-chemiluminescence blotting reagents, Hybond C nitrocellulose and Hyperfilm were from Amersham International. Premixed acrylamide/bisacrylamide was from National Diagnostics. General laboratory reagents were from Sigma and Merck.

Cell culture

For each experiment, primary cultures of cardiomyocytes were prepared from three litters of between eight and 12 neonatal Sprague–Dawley rats, as described previously [22], and pooled. Briefly, hearts were removed, and ventricles were trisected and dispersed in a series of incubations at 37 °C in nominally calcium-free Hepes-buffered salt solution (ADS buffer) containing pancreatin (0.6 mg/ml; Life Technologies) and type II collagenase (0.5 mg/ml; Worthington Biochemicals). The dispersed cells were pre-plated for 30 min to minimize fibroblast contamination, thus giving cultures which were generally $> 95\%$ cardiomyocytes. Myocytes were plated on to gelatin-coated plates at a final density of 7×10^5 cells per well on 12-well plates for cell viability studies, or of 2×10^6 cells per well on 60 mm dishes for signalling experiments in 4:1 (v/v) Dulbecco's modified Eagle's medium/Medium 199 with Hanks salts (M199) supplemented with 10 % (v/v) horse serum, 5 % (v/v) fetal calf serum and 1 % penicillin/streptomycin (100 units/ml). After 24 h in culture, cells were transferred to low-serum maintenance medium (4:1 Dulbecco's modified Eagle's medium/M199 containing 1 % fetal calf serum and 1 % penicillin/streptomycin) for 24 h prior to experimentation.

SI protocol

Sublethal SI was induced by treating myocytes for 1 h at 37 °C with a modified Krebs buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 4.0 mM Hepes) supplemented with 10 mM 2-deoxyglucose, 20 mM sodium lactate, 1 mM sodium dithionite and 12 mM KCl, pH 6.5, to simulate the extracellular milieu of myocardial ischaemia [22]. Following SI, the buffer was washed off and the cultures were reperfused with maintenance medium. Approx. 24 h after SI/R, myocytes were exposed to lethal SI by incubating them in the same buffer for 3 h at 37 °C. Following 3 h of lethal SI, cells were returned to maintenance medium (0.5 ml) and incubated for a further 3 h, and then the supernatant was collected to assess cell membrane damage by measuring creatine kinase activity. The medium was replaced (1 ml) and cells were incubated for a further 20 h prior to assessment of cell viability using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] staining. In control wells containing cells not exposed to SI/R or lethal SI, the medium was replaced at the same time points to control for loss of survival factors and changes in pH, etc. In addition, some cells were exposed to control buffer (modified Krebs buffer, pH 7.4, containing 20 mM glucose and 1 mM sodium pyruvate) for the SI/R and lethal SI periods of the experiment. This treatment had no effect on cell viability (results not shown).

Alternatively, cardiomyocytes were harvested for protein analysis at the appropriate time points, as described below. To investigate the roles of p42/p44-MAPK and p38-MAPK in adaptation to SI/R, the selective MEK-1/2 and p38-MAPK inhibitors PD98059 and SB203580 respectively were employed. Cardiomyocytes were treated with 10 μM SB203580, 50 μM PD98059 or 100 nM wortmannin for 30 min prior to SI, during the SI period or during SI and for the next 4 h of simulated reperfusion, as appropriate to the experiment. Inhibitors were then washed off and cardiomyocytes were returned to maintenance medium.

Determination of cardiomyocyte viability

Cell injury (membrane damage) following lethal SI was determined by measuring creatine kinase efflux during reperfusion, and cell viability (reductive capacity) was assessed by MTT bioreduction. For the MTT assay, cells were washed with PBS at 37 °C and incubated with 0.5 mg/ml MTT in 0.5 ml of PBS for 20 min at 37 °C. The reaction was stopped and cells were lysed by the addition of an equal volume of 10 % Triton X-100/0.1 M HCl in propan-2-ol, and the absorbance was read at 570 nm. Creatine kinase activity was determined in the myocyte supernatant collected 3 h after lethal SI. Creatine kinase activity was measured spectrophotometrically at 340 nm using a creatine kinase assay kit (Boehringer Mannheim) according to the manufacturer's instructions.

PAGE and Western blot analysis

Following treatment, cardiomyocytes were rinsed with ice-cold PBS and harvested in 200 μl of 2× concentrated SDS/PAGE sample buffer. Proteins were separated on SDS/10 %-polyacrylamide gels and transferred to nitrocellulose membranes. SDS/PAGE and Western blotting were performed using a Mini-Protean II apparatus (Bio-Rad). For MAPK blots, following transfer, membranes were blocked for 2 h with 5 % non-fat milk in Tris-buffered saline (pH 7.6) containing 0.1 % Tween-20 (TBST) and probed overnight at 4 °C with phospho-specific antibodies against p42/p44-MAPK, JNK, p38-MAPK or PKB

at 1:1000 dilution in the same buffer. Membranes were washed three times for 5 min in TBST containing 0.1% non-fat milk (TBSTM) prior to addition of swine anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2500 dilution in TBSTM) for 2 h at room temperature. Membranes were washed three times for 5 min with TBSTM (MAPKs) and the antibody–antigen complexes were visualized by enhanced chemiluminescence. Membranes were exposed to X-ray film (Hyperfilm; Amersham) for between 30 s and 2 min. For signalling experiments, membranes were also probed with antibodies specific for non-phosphorylated p42-MAPK, JNK, p38-MAPK and PKB, to confirm that levels did not change during the experiment. Protein loadings were equalized prior to Western blotting by Coomassie Blue staining and densitometry of gels and by Ponceau S staining of membranes following transfer. Phosphorylation levels were quantified by scanning the films using an Agfa Snapscan 600 and Photolook SA software, and the integrated band density was determined using NIH Image software.

In vitro kinase assay for p38-MAPK

To determine p38-MAPK activity, cells were lysed in lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF) and phosphorylated p38-MAPK was immunoprecipitated using anti-phospho-p38-MAPK agarose-conjugated antibody overnight at 4 °C. Following incubation, immunoprecipitates were washed twice with kinase buffer and the kinase reaction was carried out at 30 °C for 30 min in 40 μl of kinase buffer (25 mM Tris/HCl, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 and 1 mM $MgCl_2$) supplemented with 5 μM activating transcription factor 2 (ATF2) as the exogenous substrate and 10 μM ATP. The reaction was stopped by the addition of 2 μl of 3 × concentrated SDS/PAGE sample buffer, and samples were analysed for ATF2 phosphorylation by Western blotting using an anti-phospho-ATF2 antibody (NEB).

Statistical analysis

Data are expressed as means ± S.E.M. from at least three independent experiments. Each treatment in each expriment was performed in triplicate wells. In each experiment, cells were pooled from three different litters. The data from the three experiments were then pooled ($n = 9$). Statistical analysis was performed by ANOVA followed by the modified least significant difference test using StatView SE software.

RESULTS

Phosphorylation of p38-MAPK and p42/p44-MAPK following SI and reperfusion

To investigate whether MAPKs were activated during the adaptation protocol, cells were harvested at different times during SI or simulated reperfusion following SI (SI/R). Phosphorylation of p38-MAPK, JNK and p42/p44-MAPK (Erk1/2) was determined by Western blotting using phospho-specific antisera. As shown in Figure 1(A), transient phosphorylation of both p42- and p44-MAPK isoforms was observed, with maximal phosphorylation of both isoforms occurring after 10–20 min of SI. Phosphorylation was transient, returning to control levels by the end of SI (60 min), with the p42 isoform appearing to be the predominantly phosphorylated form under basal conditions. Upon reperfusion, a similar increase in phosphorylation of both p42- and p44-MAPKs was detected after 10 min; however, in

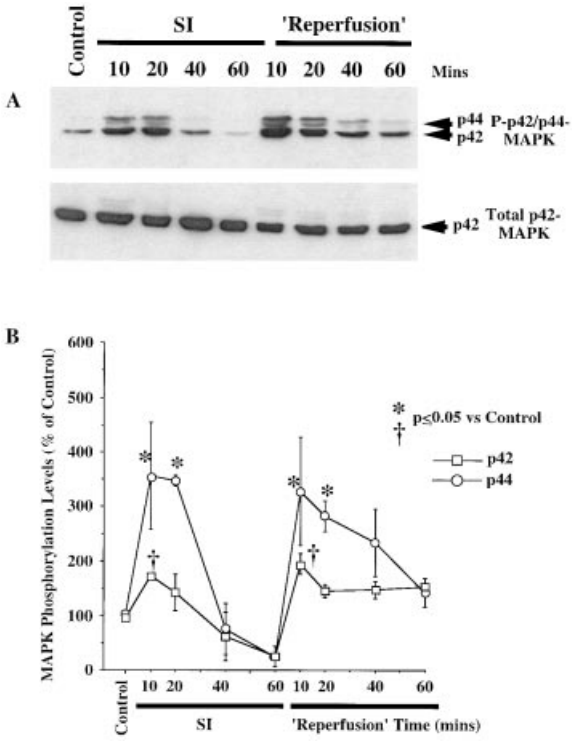

Figure 1 Time course of p42/p44-MAPK phosphorylation during SI and early reperfusion

Cardiomyocytes were treated with SI and harvested for analysis of MAPK phosphorylation at the indicated time points (min) during SI itself or during 'reperfusion' following 1 h of SI. Samples were analysed by Western blotting with antibodies recognizing dual-phosphorylated MAPKs, or non-phosphorylated MAPKs to control for loading. (**A**) Upper panel, phosphorylation of p42/p44-MAPK (Erk1/2); lower panel, total p42-MAPK (Erk2) shown as a control for loading. (**B**) Membranes were probed with antibodies specific for phosphorylated p42/p44-MAPK. Protein loadings were equalized by Coomassie Blue staining and densitometry of gels and by Ponceau S staining of membranes following transfer. Levels of phosphorylation of p42/p44-MAPK were quantified by densitometric analysis and expressed relative to total p42-MAPK as a percentage of control levels. Results are expressed as means ± S.E.M. for three independent experiments ($n = 3$).

contrast with that seen during SI, phosphorylation of both isoforms remained elevated beyond 40–60 min of reperfusion. Total p42-MAPK (Erk2) is shown in the lower panel of Figure 1(A) as a loading control. Figure 1(B) shows quantitative analysis of the p42- and p44-MAPK phosphorylation levels during SI and 'reperfusion'. It can be seen that, during SI, phosphorylation of both p42- and p44-MAPK peaked at 10 min, reaching approx. 172% and 350% respectively of control levels. During the first 60 min of reperfusion, p42- and p44-MAPK phosphorylation again peaked at 10 min, reaching approx. 194% and 330% respectively of control levels.

p46/p54-SAPKs (JNKs) were only very weakly activated at 10 min of SI and after 10–20 min of reperfusion (Figure 2A). The weak JNK dual phosphorylation was not due to a failure in detection, since relatively strong JNK phosphorylation was detected in cardiomyocytes following heat stress (see Figure 2C, centre panel). Phosphorylation of p38-MAPK was detected after 10 min of SI, being maximal after 20 min and returning to control levels by 60 min (Figure 2B). In a similar manner to p42/44-MAPK, p38-MAPK was again activated upon reperfusion, but to a lesser extent, with p38-MAPK phosphorylation peaking after 10 min and returning to below basal levels by

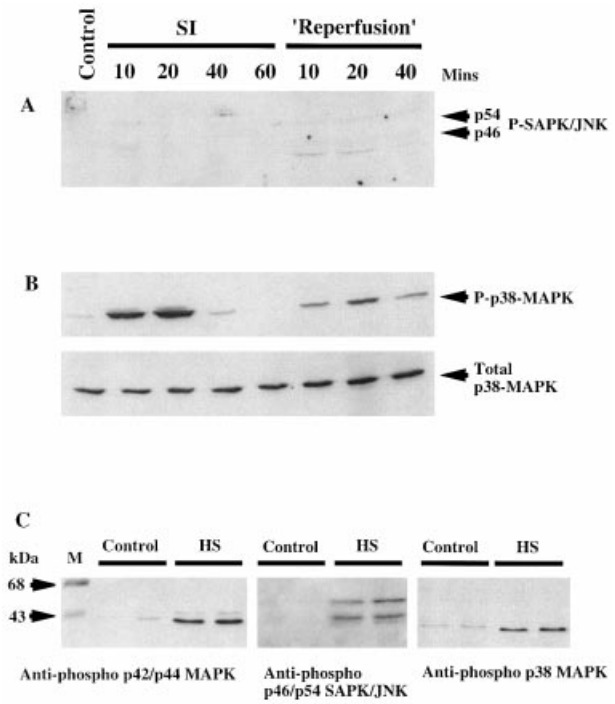

Figure 2 Time course of p46/p54-SAPK and p38-MAPK phosphorylation during SI and early reperfusion

Cardiomyocytes were treated with SI and harvested for analysis of MAPK phosphorylation at the indicated time points (min) during SI itself or during 'reperfusion' following 1 h of SI. Samples were analysed by Western blotting with antibodies recognizing dual-phosphorylated MAPKs, or non-phosphorylated MAPKs to control for loading. (**A**) Phosphorylation of p46/54-SAPK (JNK). (**B**) Upper panel, phosphorylation of p38-MAPK; lower panel, non-phosphorylated p38-MAPK as a control for loading. (**C**) Samples harvested 10 min after heat stress (HS) at 43 °C for 30 min were analysed by Western blotting with antibodies recognizing dual-phosphorylated MAPKs, as indicated, to confirm the efficacy and specificity of the antibodies and the relative molecular masses of the phosphorylated kinases.

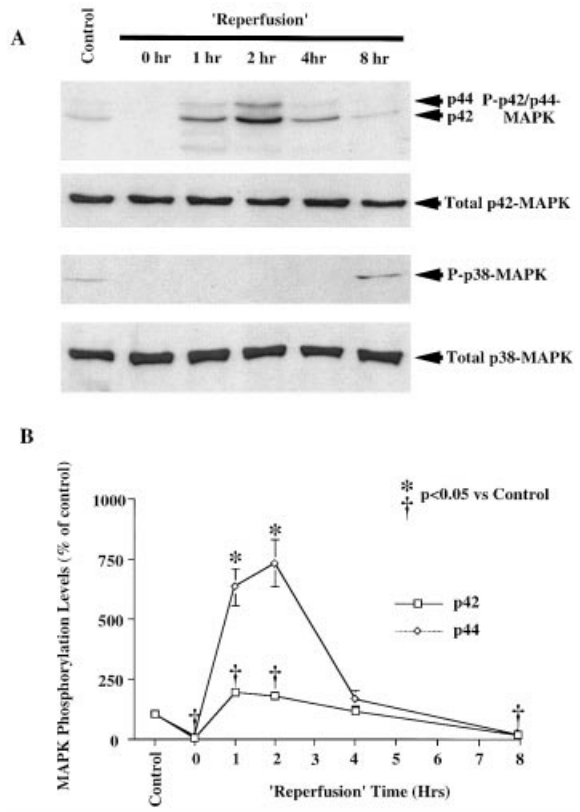

Figure 3 Time course of p42/p44- and p38-MAPK phosphorylation during late reperfusion

Cardiomyocytes were treated with SI for 1 h and harvested for analysis of MAPK phosphorylation at the indicated time points (h) during 'reperfusion'. Samples were analysed by Western blotting with antibodies recognizing dual-phosphorylated p42/p44-MAPKs or p38-MAPK. (**A**) Phosphorylation of p42/p44-MAPK (upper panels) and p38-MAPK (lower panels). Total p42- (Erk2) and p38-MAPKs are shown as loading controls. (**B**) Membranes were probed with antibodies specific for phosphorylated p42-MAPK and p38-MAPK as shown in (**A**). Protein loadings were equalized by Coomassie Blue staining and densitometry of gels and by Ponceau S staining of membranes following transfer. Levels of phosphorylation of p42/p44-MAPK were quantified by densitometric analysis and expressed relative to total p42-MAPK as a percentage of control levels. Results are expressed as means $\pm$ S.E.M. for three independent experiments ($n = 3$).

60 min (Figures 2B and 3A). Blots probed with antibodies against non-phosphorylated p38-MAPK showed similar levels in each sample, and therefore the changes were not due to differences in protein loading (Figure 2B, lower panel). In order to confirm the efficacy and specificity of the antibodies and the relative molecular masses of the different phosphorylated kinases, and to ensure that the lack of p46/p54-SAPK (JNK) phosphorylation was not due to a failure in detection, we analysed samples from heat-stressed cardiomyocytes. Heat stress activated all three kinase pathways (Figure 2C). Heat stress appeared to be more selective for phosphorylation of p42-MAPK, but p46- and p54-SAPKs were phosphorylated equally following heat stress.

Since p42/44-MAPK activity was still elevated at 40 min of reperfusion, the time course of reperfusion was extended to ascertain the exact duration of activation. As can be seen from Figure 3(A), p42/44-MAPK dual phoshorylation remained elevated at 1 h of reperfusion. Furthermore, this phosphorylation increased further and was maximal at 2 h of reperfusion, returning to baseline levels by 8 h of reperfusion. Therefore p42/44-MAPK showed a sustained, biphasic activation during reperfusion following SI, lasting up to 4 h. In contrast, p38-MAPK was phosphorylated transiently on reperfusion and returned to below baseline levels of phosphorylation by 1 h (compare Figures 2B and 3A). Therefore p38-MAPK did not show sustained activation during reperfusion, in contrast with p42/44-MAPK. During de-activation of both p42/44-MAPK and p38-MAPK, the phosphorylation levels were suppressed below the control basal level at the end of SI and prior to reperfusion. p38-MAPK phosphorylation only returned to control basal levels after 8 h of reperfusion. Quantitative analysis of p42-MAPK phosphorylation during late reperfusion (Figure 3B) confirms that it peaked at 1–2 h of reperfusion, reaching approx. 200 % of control levels. p44-MAPK also peaked at 1–2 h of reperfusion, reaching approx. 700 % of control levels.

PKB phosphorylation was determined using antibodies recognizing phospho-Ser^{473} or phospho-Thr^{308}. As shown in Figure 4(A), Ser^{473} phosphorylation occurred after 10 min of reperfusion following SI, peaking at 40 min and beyond. The extended time course shown in Figure 4(B) demonstrates that PKB Ser^{473} phosphorylation peaked between 40 min and 1 h, and decreased towards baseline from 2 to 8 h of reperfusion. Since peak PKB phosphorylation following SI/R was completely abolished by 100 nM wortmannin (Figure 4C), this indicates that it was PI 3-kinase-dependent. Similar results were obtained for Thr^{308} (not shown; see [10]).

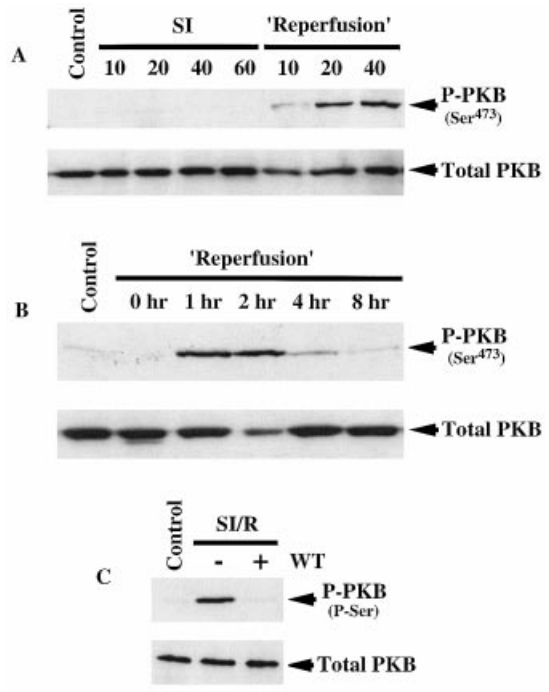

Figure 4 Time course of PKB/Akt phosphorylation during SI and reperfusion

Cardiomyocytes were treated with SI and harvested for analysis of PKB phosphorylation at the indicated time points (min) during SI itself or (min/h) during 'reperfusion' following 1 h of SI. Samples were analysed by Western blotting with antibodies recognizing phosphorylated Ser^{473} of PKB, or non-phosphorylated PKB to control for loading. (**A**) Phosphorylation of PKB Ser^{473} during SI and early reperfusion (upper panel) compared with total (non-phosphorylated) PKB (lower panel). (**B**) Phosphorylation of PKB Ser^{473} during extended reperfusion following 1 h of SI (upper panel) compared with total (non-phosphorylated) PKB (lower panel). (**C**) Cardiomyocytes were pretreated with the PI 3-kinase inhibitor wortmannin (WT; 100 nM) before and during SI and reperfusion. Samples were Western-blotted for phosphorylated PKB Ser^{473} (as for **A** and **B**; upper panel) and compared with total (non-phosphorylated) PKB (lower panel).

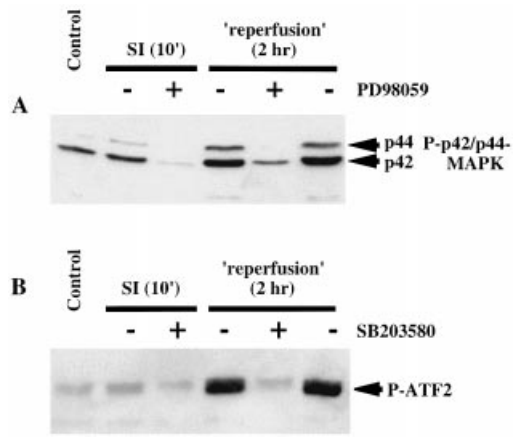

Figure 5 Effects of MAPK inhibitors on phosphorylation

To test the efficacy of inhibitors, SI and reperfusion were carried out in the presence of the inhibitor PD98059 for MAPKK (MEK) (an upstream activator of p42/p44-MAPK) or the p38-MAPK inhibitor SB203580, and harvested at the time points of maximal MAPK activation, i.e. SI at 10 min and reperfusion at 2 h. (**A**) Representative blot probed with anti-phospho-specific p42/p44-MAPK. (**B**) Blot probed with anti-phospho-ATF2 (a downstream substrate of p38-MAPK) following immunoprecipitation of p38-MAPK and *in vitro* phosphorylation of an ATF2 substrate. Sorbitol (hyperosmotic stress) was used as a positive control for activation of p38-MAPK in the pull-down assay (results not shown)

Role of MAPK and PI 3-kinase/PKB activation in adaptive cytoprotection of cardiomyocytes

To determine the role of activation of p42/44-MAPK, p38-MAPK and PI 3-kinase/PKB in delayed protection, SI was carried out in the absence or presence of selective inhibitors of the pathways. PD98059 inhibits the MAPK kinases MEK-1 and -2, upstream activators of p42/44-MAPK, and therefore blocks p42/44-MAPK phosphorylation and activation. This can be readily detected using Western blotting with phospho-specific antibodies against p42/44-MAPK. SB203580 blocks p38-MAPK activity directly, but does not affect the dual phosphorylation of p38-MAPK on the consensus TGY motif by its upstream activator(s) [i.e. MKK (MAPK kinase) 3/6]. Therefore the effect of SB203580 on p38-MAPK was detected using an assay for phosphorylation of recombinant ATF2 *in vitro* following immunoprecipitation of p38-MAPK. The transcription factor ATF2 is also phosphorylated by JNK/p46/p54-SAPK. However, since we detected very little JNK activation by SI or reperfusion (Figure 2A), we assumed that ATF2 phosphorylation was due predominantly to p38-MAPK activity. JNK phosphorylation, as determined by Western blotting, was strongly induced by heat stress (Figure 2C), and therefore the inability to detect phosphorylation following SI and reperfusion was not due to a failure in detection. Figure 5(A) demonstrates that PD98059 was able to block p42/44-MAPK phosphorylation following SI and SI/R. Furthermore, SB203580 blocked ATF2 phosphorylation *in vitro* following SI/R (Figure 5B). SB203580 was able to block ATF2 phosphorylation completely following reperfusion, confirming that ATF2 phosphorylation was due entirely to p38-MAPK activity under these conditions.

To demonstrate delayed cytoprotection in cardiomyocytes using the SI/R protocol, cells were treated with SI for 1 h, followed by 24 h of reperfusion prior to 3 h of lethal SI, and compared with cells receiving lethal SI alone. Figure 6 demonstrates that cardiomyocytes showed a significant increase in survival following lethal SI when preceded by SI/R, as determined by preservation of MTT bioreductive capacity ($P \leqslant 0.001$ compared with lethal SI alone) and a decrease in creatine kinase release ($P \leqslant 0.001$ compared with lethal SI). Neither PD98059 nor SB203580 given before and during SI, but not reperfusion, were able to block protection ($P \leqslant 0.01$ compared with lethal SI). Since we observed that both p42/44-MAPK and p38-MAPK were re-activated during reperfusion following SI, cardiomyocytes were subjected to SI with PD98059 and SB203580 present both during SI and during 4 h of the following reperfusion, i.e. spanning the times at which p42/44-MAPK is maximally activated. As can be seen from Figure 7, in this case PD98059 completely blocked protection induced by SI/R. In contrast, SB203580 alone did not affect protection ($P \leqslant 0.001$ compared with lethal SI). When PD98059 and SB203580 were given together, the development of delayed protection was blocked in a similar manner to that with PD98059 alone. There was no effect of PD98059 or SB203580 on survival in the absence of lethal SI. Since PD98059 blocked the phosphorylation, and therefore activation, of p42/44-MAPK (Figure 5A), these results indicate that activation of p42/44-MAPK during SI/R is essential for the delayed protective effect.

Whereas the PI 3-kinase inhibitor wortmannin (100 nM) completely blocked phosphorylation of Ser^{473} and Thr^{308} of PKB (Figure 4C), wortmannin had no effect on the delayed protection following SI/R when given during SI and reperfusion (Figure 8).

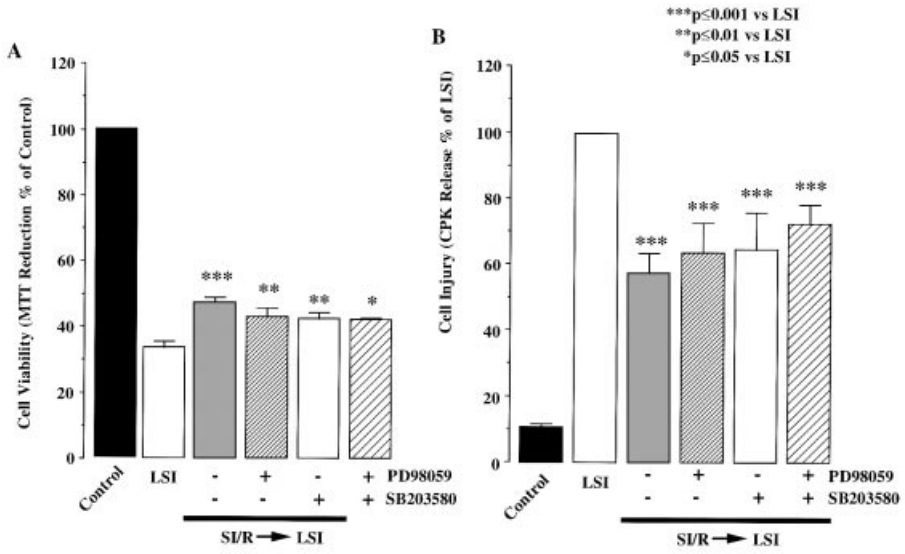

Figure 6 Effect of inhibition of MAPK during SI on cytoprotection

Cardiomyocytes were subjected to 3 h of lethal SI (LSI) either with or without preceding SI/R 24 h earlier. SI was carried out either alone or following pretreatment with PD98059 or SB203580. Inhibitors were present during SI, but not reperfusion. (**A**) After 24 h of reperfusion, cell viability was determined using MTT bioreduction, as described in the Materials and methods section. (**B**) Following lethal SI, cardiomyocytes were 'reperfused' in normal maintenance medium and the supernatants were collected for analysis of creatine kinase (CPK) release (cell injury) after 3 h. Results represent means ± S.E.M. for $n = 9$ (pooled from three separate experiments). Statistical significance of differences compared with lethal SI: $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$.

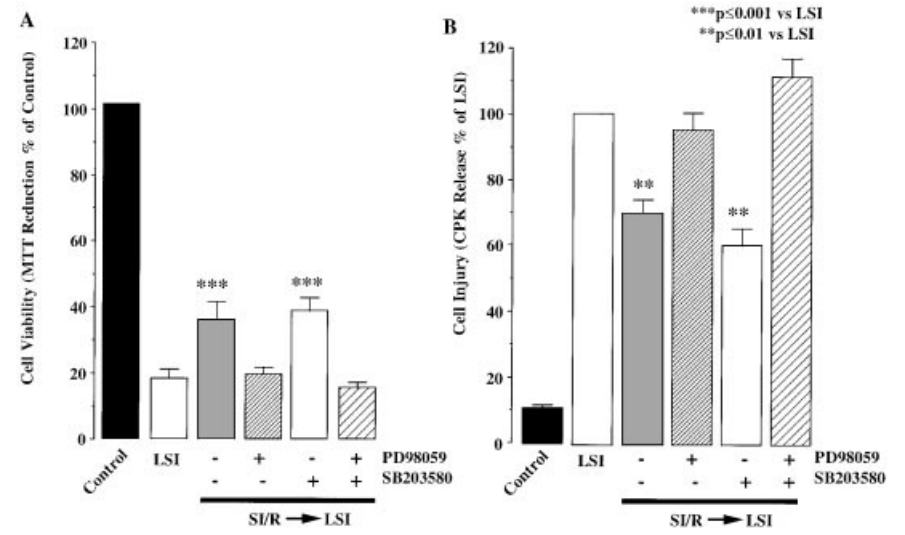

Figure 7 Effects of inhibition of MAPK during SI and reperfusion on cytoprotection

Cardiomyocytes were subjected to 3 h of lethal SI (LSI) either with or without preceding SI 24 h earlier. SI was carried out either alone or following pretreatment with PD98059 or SB203580. Inhibitors were present during SI and for 4 h of the subsequent reperfusion. (**A**) After 24 h of reperfusion following lethal SI, cells were stained with MTT to determine viability. (**B**) Following lethal SI, cardiomyocytes were 'reperfused' in normal maintenance medium and the supernatants were collected for analysis of creatine kinase (CPK) release (cell injury) after 3 h. Results represent means ± S.E.M. for $n = 9$ (pooled from three separate experiments). Statistical significance of differences compared with lethal SI: $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$.

These results indicate that activation of PI 3-kinase/PKB during SI/R does not play a significant role in the delayed protection observed in this model.

DISCUSSION

In a model of reversible, sublethal SI/R in isolated cardiomyocytes, we have demonstrated that p42/44-MAPK, p38-MAPK and PKB are transiently activated both during SI itself and during subsequent 'reperfusion' in normal maintenance medium. Furthermore, during reperfusion a sustained activation of p42/44-MAPK occurs which appears to be essential for the induction of the delayed cytoprotective effect of SI/R. Multiple MAPK/SAPK pathways have been reported to be activated by ischaemia and reperfusion, oxidative stress, anisomycin, ATP depletion, osmotic stress and hypoxia/reoxygenation in the heart

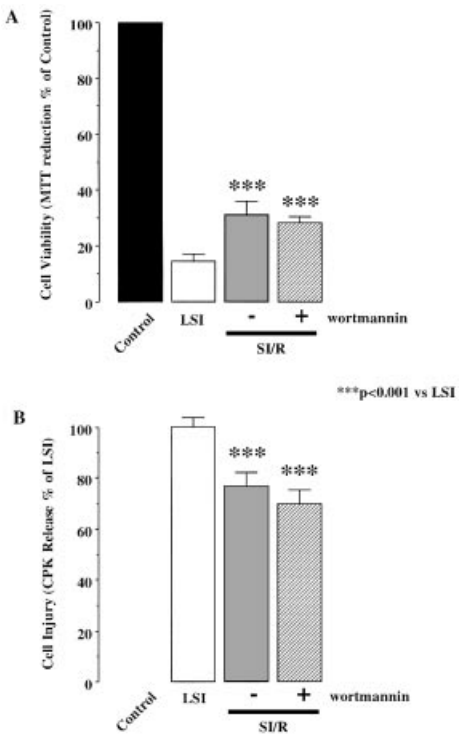

Figure 8 Effect of inhibition of PI 3-kinase/PKB during SI and reperfusion on cytoprotection

Cardiomyocytes were subjected to 3 h of lethal SI (LSI) either with or without preceding SI 24 h earlier. SI was carried out either alone or following pretreatment with the PI 3-kinase inhibitor wortmannin (100 nM). Wortmannin was present during SI and for 4 h of the subsequent reperfusion. (**A**) After 24 h of reperfusion following lethal SI, cells were stained with MTT to determine viability. (**B**) Following lethal SI, cardiomyocytes were 'reperfused' in normal maintenance medium and the supernatants were collected for analysis of creatine kinase (CPK) release (cell injury) after 3 h. Results represent means $\pm$ S.E.M. for $n = 9$ (pooled from three separate experiments). Statistical significance of differences compared with lethal SI: $^{***}P \leqslant 0.001$.

or in cardiomyocytes [23–27]. In the isolated Langendorff perfused rat heart exposed to global ischaemia/reperfusion, both p42/44-MAPK and JNK were reported to be activated during reperfusion [28]. The pattern of MAPK activation described herein was very similar to a model of ATP depletion using cyanide and 2-deoxyglucose in neonatal rat cardiomyocytes, in which strong activation of p42/44-MAPK, but no activation of JNK, was observed [25].

A variety of *in vitro* SI models have been described for the study of preconditioning. These include hypoxia, glucose-free hypoxia/anoxia and glucose-free buffer supplemented with 2-deoxyglucose and sodium lactate. In fact, there are now over 25 published studies using such models (reviewed recently by Marber [29]). Furthermore, oxygen/glucose deprivation (OGD) is well established as a model of neuronal preconditioning [30]. The model of SI used in the present study utilizes glycolytic and respiratory inhibition under acidotic conditions and was the first description of *in vitro* preconditioning in isolated cardiomyocytes [22]. This model has been used subsequently by others to investigate mechanisms of preconditioning [31,32]. Inhibitors of mitochondrial respiration, including cyanide, are unsuitable for preconditioning/cytoprotection studies, due to a lack of, or slow, reversibility (R. J. Heads, unpublished work). In contrast, the oxygen scavenger sodium dithionite is effective due to its rapid depletion of oxygen from the solution and the rapid reversibility of its effects on washout. Sodium dithionite reduces cytochrome a_3 of the mitochondrial respiratory chain [33]. Cytochrome oxidase is thus the target for reversible inhibition of the respiratory chain by dithionite, and reversible mitochondrial inhibition has been postulated as a trigger/mediator of ischaemic preconditioning [34,35]. Furthermore, oxidative stress occurring on washout is likely to be a direct cause of the 'reperfusion'-associated sustained phosphorylation of MAPK (but see Note added in proof).

The biphasic pattern of p38-MAPK activation that we observed following SI/R was similar to that observed in isolated perfused rat hearts, where activation of p38-MAPK and MAPKAPK2 during ischaemia was followed by a second phase of activation during reperfusion [23,36]. The finding that delayed, sustained activation of p42/44-MAPK is essential for the development of delayed cytoprotection is consistent with a model of ischaemic preconditioning described in rabbit hearts *in vivo*. In this model, Ping et al. [37] have shown that both the p42 and p44 isoforms of MAPK are activated by six cycles of 4 min of coronary artery ligation/4 min of reperfusion. Furthermore, activation of p42- and p44-MAPK by MEK in the cytosol was followed by translocation of both isoforms to the nucleus. In a similar report in isolated rat hearts, nuclear translocation of MAPK during ischaemia was again observed [38]. Biphasic activation of p42/p44-MAPK has also been observed following exposure of glomerular mesangial cells to a long-lived NO donor (spermine-NO). The initial rapid activation phase was followed by a sustained activation similar to that described here [39].

The p42/p44-MAPK and PI 3-kinase/Akt pathways may promote cell survival, whereas the p38-MAPK and JNK pathways may promote cell injury under certain conditions. For instance, activation of p38-MAPK and JNK, particularly following down-regulation of p42/p44-MAPK and PI 3-kinase/Akt, promote apoptosis in a number of cell types [17,40]. As such, it has been suggested that activation of different isoforms of p38-MAPK may mediate hypertrophic compared with apoptotic pathways [41]. However, the precise role of different p38-MAPK isoforms in the heart requires further investigation.

A number of recent studies have suggested that activation of p38-MAPK during ischaemia in the heart may be detrimental [18,19], whereas others have suggested that p38-MAPK may mediate early preconditioning [21]. Despite the controversy surrounding the apparently contradictory results with respect to the role of p38-MAPK (see [42]), this paradox can be at least partly explained if elements activated during the preconditioning phase (such as protein kinase C or p38-MAPK itself) then lead to a subsequent inhibition of p38-MAPK activation during ischaemia, as indeed is suggested by our own recent results [43]. This conclusion is also supported by our observation that phosphorylation of p38-MAPK is suppressed below basal levels following preconditioning with SI/R and takes at least 8 h to recover (see Figure 3A). Therefore if the lethal insult is introduced within this 8 h period, i.e. 90 min after SI/R, as is typical in an acute *in vitro* preconditioning protocol [43], p38-MAPK may fail to be activated by the lethal SI, thus resulting in protection. This is also borne out by the fact that the p38-MAPK inhibitor SB203580 protects cardiomyocytes when given immediately before and during lethal SI ([43]; R. J. Heads, unpublished work).

Activation of the PI 3-kinase $\rightarrow$ PKB/Akt pathway appears to promote cell survival in a number of cell types, including cardiomyocytes [44]. Furthermore, we have recently demonstrated that PKB/Akt is activated in a PI 3-kinase-dependent manner following SI/R ([10]; the present study). However,

despite the rapid and strong phosphorylation of PKB during simulated reperfusion, this does not appear to contribute to the delayed cytoprotection observed in this model, as the PI 3-kinase inhibitor wortmannin is unable to block protection. The mode of cell death observed following lethal SI is rapid and predominantly necrotic. It remains possible that activation of PKB/Akt may play a survival function in other SI models in which delayed cell death, or apoptosis, is likely to play a more prominent role, such as following hypoxia, serum withdrawal or glycolytic ATP depletion [45].

Note added in proof (received 9 August 2000)

Since the present paper was accepted, it has been shown that mitochondrial ATP is necessary for reoxygenation-induced ERK activation [46].

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