Stimulation of multiple mitogen-activated protein kinase sub-families by oxidative stress and phosphorylation of the small heat shock protein, HSP25/27, in neonatal ventricular myocytes

Angela CLERK^{*†}, Ashour MICHAEL^{*} and Peter H. SUGDEN^{*1}

^{*}NHLI Division (Cardiac Medicine), Royal Brompton Campus, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, U.K., and [†]Division of Biomedical Sciences, Charing Cross Campus, Imperial College School of Medicine, Fulham Palace Road, London W6 8RF, U.K.

We investigated the activation of three subfamilies of mitogen-activated protein kinases (MAPKs), namely the stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs), the extracellularly responsive kinases (ERKs) and p38-MAPK, by oxidative stress as exemplified by H₂O₂ in primary cultures of neonatal rat ventricular myocytes. The 46 and 54 kDa species of SAPKs/JNKs were activated 5- and 10-fold, respectively, by 0.1 mM H₂O₂ (the maximally effective concentration). Maximal activation occurred at 15–30 min, but was still detectable after 2 h. Both ERK1 and ERK2 were activated 16-fold by 0.1 mM H₂O₂ with a similar time course to the SAPKs/JNKs, and this was comparable with their activation by 1 μ M PMA, the most powerful activator of ERKs that we have so far identified in these cells. The activation of ERKs by H₂O₂ was inhibited by PD98059, which inhibits the activation of MAPK (or ERK) kinases, and by the protein kinase C (PKC) inhibitor, GF109203X. ERK activation was also inhibited by down-regulation of PMA-sensitive PKC isoforms. p38-MAPK was activated by 0.1 mM H₂O₂ as shown by an increase in its phosphorylation.

However, maximal phosphorylation (activation) was more rapid (< 5 min) than for the SAPKs/JNKs or the ERKs. We studied the downstream consequences of p38-MAPK activation by examining activation of MAPK-activated protein kinase 2 (MAPKAPK2) and phosphorylation of the MAPKAPK2 substrate, the small heat shock protein HSP25/27. As with p38-MAPK, MAPKAPK2 was rapidly activated (maximal within 5 min) by 0.1 mM H₂O₂. This activation was abolished by 10 μ M SB203580, a selective inhibitor of certain p38-MAPK isoforms. The phosphorylation of HSP25/27 rapidly followed activation of MAPKAPK2 and was also inhibited by SB203580. Phosphorylation of HSP25/27 was associated with a decrease in its aggregation state. These data indicate that oxidative stress is a powerful activator of all three MAPK subfamilies in neonatal rat ventricular myocytes. Activation of all three MAPKs has been associated with the development of the hypertrophic phenotype. However, stimulation of p38-MAPK and the consequent phosphorylation of HSP25/27 may also be important in cardioprotection.

INTRODUCTION

The importance of reactive oxygen species (ROS) and free radicals in pathological events is becoming increasingly apparent. Exposure of cells to extracellular ROS initiates a variety of intracellular signalling pathways (reviewed in [1]), which may lead to cell death through apoptosis [2]. ROS and free radicals may also be generated intracellularly in response to numerous stimuli [2]. Such events may be particularly relevant to cardiac pathophysiology because the heart is subjected to significant oxidative stress during ischaemia and ischaemia/reperfusion (reviewed in [3–5]). Furthermore, ROS can mimic the effects of ischaemic preconditioning, implying the existence of signalling pathways that are shared with ischaemia [6].

There are at least three subfamilies of the mitogen-activated protein kinase (MAPK) superfamily (reviewed in [7–9]). These are the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs), which are also known as the stress-activated protein kinases (SAPKs), and the p38-MAPKs. The ERKs are involved in anabolic processes (cell growth, division

and differentiation). In contrast, the SAPKs/JNKs and the p38-MAPKs appear to be primarily involved in the response of cells to cytotoxic agents and interventions. Although activation of SAPKs/JNKs and p38-MAPKs has been implicated in apoptosis in a variety of cells [10–14], including ventricular myocytes [15], whether they promote survival or are involved in cell necrosis/apoptosis remains to be clearly established. A variety of substrates for the MAPKs have been identified including several transcription factors and other protein kinases, and these phosphorylations are probably responsible for the ultimate cellular effects of MAPK activation. One of the substrates for p38-MAPK is MAPK-activated protein kinase 2 (MAPKAPK2), which phosphorylates the small heat-shock protein, HSP25/27 [16,17]. In Chinese hamster ovary cells and endothelial cells, phosphorylation of HSP25/27 is associated with stabilization of the actin cytoskeleton, protecting cells against damage [18,19].

All three subfamilies of the MAPKs can be activated in the heart. In cultured neonatal ventricular myocytes, the ERKs are strongly activated by G-protein-coupled receptor (GPCR) agonists, such as endothelin-1 (ET-1) and α_1 -adrenergic agents, and

Abbreviations used: ERK, extracellularly responsive kinase; ET-1, endothelin-1; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; HSP, heat-shock protein; IEF, isoelectric focusing; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MBP, myelin basic protein; MEK, MAPK (or ERK) kinase; PKC, protein kinase C; PKI, cAMP-dependent protein kinase inhibitory peptide; ROS, reactive oxygen species; SAPK, stress-activated protein kinase.

¹To whom correspondence should be addressed (e-mail p.sugden@ic.ac.uk).

by phorbol esters (e.g. PMA) [20–22]. ERKs are also activated, though to a lesser degree, by certain cellular stresses (e.g. hyperosmotic shock) [23]. The SAPKs/JNKs are strongly activated by cellular stresses including hyperosmotic shock and protein-synthesis inhibitors [23]. ET-1 and α_1 -adrenergic agents activate the SAPKs/JNKs, though to a lesser extent [23,24]. In perfused adult rat hearts, the ERKs are activated by GPCR agonists and PMA [25,26], and the SAPKs/JNKs are activated by ischaemia/reperfusion [27–29] or the GPCR agonist, phenylephrine [26]. Activation of p38-MAPK has been demonstrated in adult perfused hearts subjected to ischaemia and ischaemia/reperfusion [28,29] or phenylephrine [26]. Activation of SAPKs/JNKs and p38-MAPK by ischaemia/reperfusion is reduced by antioxidants, implying participation of ROS [30]. Here, we have examined the effects of oxidative stress (as exemplified by H_2O_2) on the activities of the different MAPK subfamilies and MAPKAPK2, and the phosphorylation of HSP25/27.

EXPERIMENTAL

Materials

Tissue culture materials were from previously described sources [22]. Rabbit polyclonal antibodies to JNK1 (sc-474) and HSP25/27 (sc-1049) were from Santa Cruz Biotechnology Inc. and supplied by Autogen Bioclear (Devizes, Wilts., U.K.). Antibodies to the dually phosphorylated form of p38-MAPK were from New England Biolabs (U.K.) Ltd. (Hitchin, Herts., U.K.). The recombinant human c-Jun transactivation domain (residues 1–135) was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* and was purified by glutathione-Sepharose (Pharmacia, St. Albans, Herts., U.K.) chromatography. cAMP-dependent protein kinase inhibitory peptide (PKI; amino acid sequence TTYADFIASGRTGRRNA-IHD) was from Bachem (Saffron Walden, Essex, U.K.). The KKLNRTLSVA MAPKAPK2 peptide substrate [17,31] was synthesized by Severn Biotech (Kidderminster, Worcs., U.K.). PD98059, GF109203X, SB203580 and Ro318220 were obtained from Calbiochem (Beeston, Notts., U.K.) and stock solutions (10–50 mM) prepared in DMSO. [γ - 32 P]ATP was from NEN Life Science Products (Brussels, Belgium). Prestained molecular-mass markers, biotinylated anti-rabbit IgG, enhanced-chemiluminescence blotting reagents and Hyperfilm MP were from Amersham International (Amersham, Bucks., U.K.). SDS/PAGE and isoelectric focusing (IEF) reagents were from Bio-Rad (Hemel Hempstead, Herts., U.K.). Nitrocellulose (Schleicher & Schuell, 0.45 μ m) was supplied by Anderman & Co. (Kingston-upon-Thames, Surrey, U.K.). Protein A-Sepharose, microcystin LR, myelin basic protein (MBP), H_2O_2 and other biochemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.). General laboratory reagents were from Sigma or Merck (Lutterworth, Leics., U.K.).

Primary culture of ventricular myocytes and sample preparation

Myocytes were prepared from the ventricles of neonatal Sprague-Dawley rat hearts by an adaptation of the method of Iwaki et al. [32] as described previously [22]. The cells were pre-plated to remove fibroblasts and then plated (1.4×10^3 cells/mm²) on to 60 mm or 35 mm gelatin-coated dishes in Dulbecco's modified Eagle's medium/Medium 199 (4:1, v/v) containing 10% (v/v) horse serum, 5% (v/v) fetal calf serum and 100 units/ml each of penicillin and streptomycin. After 18 h, the cells were confluent and beating. The cells were incubated for 24 h in 4 ml of serum-free medium and then exposed to H_2O_2 (added directly to the

medium while mixing), 1 μ M PMA or 0.5 M sorbitol. In some experiments, cells were pretreated with 1 μ M PMA for 24 h. For inhibitor studies, the cells were preincubated with inhibitor for 15 min prior to stimulation with agonist + inhibitor. For in-gel assays, FPLC and phospho-p38-MAPK immunoblot analysis, cells in 60 mm dishes were washed in ice-cold Ca^{2+} /Mg²⁺-free Dulbecco's PBS and scraped into 150 μ l of extraction buffer A [20 mM β -glycerophosphate (pH 7.5)/5 mM dithiothreitol/50 mM NaF/2 μ M microcystin/2 mM EDTA/0.2 mM Na_3VO_4 /10 mM benzamide/0.2 mM leupeptin/10 μ M trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane/0.3 mM PMSF]. The samples were centrifuged (10000 g, 5 min, 4 °C) and the supernatants (cell extracts) boiled with 0.33 vol. of SDS sample buffer [330 mM Tris/HCl (pH 6.8)/10% (w/v) SDS/13% (v/v) glycerol/133 mM dithiothreitol/0.2 mg/ml Bromophenol Blue]. Protein concentrations were determined using the Bradford method [33].

FPLC of ERKs and MAPKAPK2

Myocytes (four 60 mm dishes) were scraped into extraction buffer A (150 μ l/60 mm dish) and homogenized. The samples were centrifuged (10000 g, 5 min, 4 °C) and the supernatants were recentrifuged. Proteins (0.5 ml of extract) were separated by FPLC. ERKs were separated on a Mono Q HR5/5 column equilibrated with 50 mM Tris/HCl (pH 7.3)/2 mM EDTA/2 mM EGTA/0.1% (v/v) 2-mercaptoethanol/5% (v/v) glycerol/0.03% (v/v) Brij-35/0.3 mM Na_3VO_4 /1 mM benzamide/4 μ g/ml leupeptin. Following a 5 ml isocratic wash, ERKs were eluted using a linear NaCl gradient (20 ml, 0–0.33 M NaCl) at a flow rate of 1 ml/min with collection of 0.5 ml fractions. They were assayed by the incorporation of 32 P from [γ - 32 P]ATP into MBP by the direct method as described previously [20,21,23]. Samples of fractions were also taken for in-gel kinase assays and were boiled with 0.33 vol of SDS sample buffer.

For MAPKAPK2, myocyte extracts (0.5 ml) were applied to a Mono S HR5/5 column equilibrated with 25 mM β -glycerophosphate (pH 7.3)/2 mM EDTA/5% (v/v) glycerol/0.1% (v/v) Triton X-100/0.3 mM PMSF/0.5 mM dithiothreitol. Following a 5 ml isocratic wash, MAPKAPK2 was eluted using a linear NaCl gradient (15 ml, 0–0.3 M NaCl) at a flow rate of 1 ml/min with collection of 1 ml fractions. MAPKAPK2 was assayed by the incorporation of 32 P from [γ - 32 P]ATP into KKLNRTLSVA peptide substrate [17,31] as described previously [28], with an incubation time of 30 min. Total MAPKAPK2 activity was determined by integration of the FPLC peaks.

JNK1 immunoprecipitation

Myocytes (60 mm dishes) were scraped into 150 μ l/dish immunoprecipitation buffer [10 mM Tris/HCl (pH 7.4)/5 mM EDTA/50 mM NaF/50 mM NaCl/2 mM Na_3VO_4 /0.1% (w/v) fatty acid-free BSA/20 μ g/ml aprotinin] containing 20 mM n-octyl β -D-glucopyranoside and 1% (v/v) Triton X100, and were homogenized. The samples were extracted on ice (10 min) and centrifuged (10000 g, 5 min, 4 °C). The supernatants (150 μ l) were incubated with 4 μ l (0.4 μ g) of JNK1 antibody with mixing (2 h, 4 °C). Protein A-Sepharose was added (20 μ l of a 50% slurry in immunoprecipitation buffer) and the samples mixed (1 h, 4 °C). The samples were centrifuged (10000 g, 1 min, 4 °C), the supernatants were removed and boiled with 0.33 vol. SDS sample buffer. The pellets were washed in immunoprecipitation buffer (3×150 μ l), resuspended in 140 μ l of immunoprecipitation buffer and boiled with 50 μ l of SDS sample buffer.

In-gel protein kinase assays

MAPKs were assayed by the in-gel kinase method [34]. Proteins (50 μ g for myocyte extracts; 30 μ l for FPLC fractions and JNK1 immunoprecipitates) were separated on 10% (w/v) SDS-polyacrylamide gels [6% (w/v) stacking gel], which were formed in the presence of 0.5 mg/ml MBP for the assay of ERKs, or of 0.5 mg/ml GST-c-Jun(1–135) for the assay of SAPKs/JNKs. The gels were washed and processed as described previously [35].

Phospho-p38-MAPK immunoblot analysis

Proteins (50 μ g) were separated by SDS/PAGE on 10% (w/v) polyacrylamide gels and transferred electrophoretically to nitrocellulose [36]. The blots were probed with phospho-p38-MAPK antibody according to the manufacturer's instructions. The bands were detected using enhanced chemiluminescence (ECL) with exposure to Hyperfilm MP.

IEF and immunoblotting for HSP25/27

Myocytes (35 mm dishes) were scraped into 100 μ l/dish of extraction buffer B [12.5 mM Tris/HCl (pH 7.5)/2.5 mM EGTA/1 mM EDTA/100 mM NaF/5 mM dithiothreitol/0.3 mM PMSF/0.12 mM pepstatin A/0.2 mM leupeptin/10 μ M trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane] and centrifuged (10000 *g*, 5 min, 4 °C). Supernatants were mixed with an equal volume of 8 M urea/4% (w/v) Bio-lyte 3-10/1% (v/v) Triton X-100. Samples (2 μ l, 2–3 μ g of protein) were separated by IEF on 5% polyacrylamide gels containing 4 M urea, 0.2% (w/v) CHAPS, 5% (v/v) glycerol, 2% (w/v) Bio-lyte 3-10 at 100 V (15 min), then 200 V (15 min) and finally 450 V (1 h). Proteins were transferred to nitrocellulose and probed with HSP25/27 antibodies (1/100). Blots were processed as described previously [36]. Bands were quantified by laser-scanning densitometry.

Separation of HSP25/27 aggregates by gel-filtration FPLC

Myocytes (60 mm dishes) were scraped into 150 μ l/dish extraction buffer A, centrifuged (10000 *g*, 5 min, 4 °C) and the supernatants were recentrifuged. Supernatants (0.5 ml) were applied to a Superose 12 HR 10/30 column equilibrated with 10 mM Tris/HCl (pH 7.4)/1 mM EDTA/10 mM NaF/1 mM dithiothreitol/0.1 mM PMSF/10% (v/v) glycerol. Proteins were separated at a flow rate of 0.25 ml/min, and 1 ml fractions were collected. Samples from each fraction were boiled with 0.33 vol. SDS sample buffer and immunoblotted for HSP25/27 as described above.

RESULTS

Activation of SAPK/JNK subfamily of the MAPKs

Activation of SAPK/JNKs by H_2O_2 was studied using in-gel kinase assays with GST-c-Jun(1–135) as a substrate. The 46 kDa and 54 kDa SAPK/JNKs were rapidly activated by 0.1 mM H_2O_2 (Figure 1). Activation was apparent within 5 min and was maximal at approx. 15–30 min (Figures 1A and 1B). p46 SAPK/JNK was activated approx. 5-fold and p54 SAPK/JNK was activated approx. 10-fold (Figure 1B). p46 and p54 SAPK/JNK activities remained above basal levels for at least 2 h (Figures 1A and 1B). To confirm the identities of the SAPKs/JNKs activated by H_2O_2 , antibodies to human JNK1, which cross-react with the rat homologue, were used to immunoprecipitate the JNK1 gene products. This antibody immunoprecipitated almost all of the p46 SAPK/JNK activity and approx. 50% of the p54 SAPK/JNK

Figure 1 Activation of SAPKs/JNKs by H_2O_2

(A) Time course of SAPK/JNK activation. Myocytes were exposed to 0.1 mM H_2O_2 for the times indicated and SAPKs/JNKs were assayed by the in-gel kinase method using GST-c-Jun(1–135) as substrate. Results are representative of three independent experiments. (B) Quantification of in-gel kinase assays of p46 SAPK/JNK (●) and p54 SAPK/JNK (▲) by laser-scanning densitometry. Results are means $\pm$ S.E.M. for three independent experiments. (C) Immunoprecipitation of SAPKs/JNKs with antibodies to JNK1 and subsequent in-gel kinase assay. Myocytes were untreated (CON) or exposed to 0.1 mM H_2O_2 for 30 min. SN, supernatant remaining after immunoprecipitation; IP, immunoprecipitate. The experiment was repeated with similar results. (D) Concentration dependence of SAPK/JNK activation by H_2O_2 . Myocytes were exposed to H_2O_2 for 30 min and SAPKs/JNKs were assayed by the in-gel kinase method. Results are representative of three independent experiments.

JNK activity (Figure 1C). Western blots of the supernatants and immunoprecipitated proteins confirmed that there was quantitative immunoprecipitation of p46 JNK1 (results not shown). The dose-dependent activation of SAPKs/JNKs by H_2O_2 was also studied. There was very little (if any) activation of SAPKs/JNKs by 0.03 mM H_2O_2 at 30 min, but maximal activation was obtained with 0.1 mM H_2O_2 . A similar very steep concentration dependence has been seen in perfused hearts [30] and cultured astrocytes [37]. Lower levels of activity were elicited by concentrations of H_2O_2 of > 0.1–1 mM (Figure 1D).

Activation of the ERK subfamily of MAPKs

Activation of 44 kDa and 42 kDa ERKs (ERK1 and ERK2, respectively) was determined using in-gel kinase assays with MBP as a substrate. As with the SAPKs/JNKs, activation of ERK1 and ERK2 was apparent from 5 min stimulation with

Figure 2 Activation of ERKs by H₂O₂

(A) Time course of ERK activation. Myocytes were exposed to 0.1 mM H₂O₂ for the times indicated and ERKs were assayed by the in-gel kinase method using MBP as substrate. Results are representative of three independent experiments. (B) Quantification of in-gel kinase assays of ERK1 (▲, dashed line) and ERK2 (●, solid line) by laser-scanning densitometry. Results are means ± S.E.M. for three independent experiments. (C) Concentration dependence of ERK activation by H₂O₂. Myocytes were exposed to H₂O₂ for 30 min and ERKs were assayed by the in-gel kinase method. Results are representative of three independent experiments.

0.1 mM H₂O₂ (Figures 2A and 2B) and was maximal (approx. 16-fold stimulation) at approx. 30 min. These activities declined to basal levels by approx. 2 h (Figures 2A and 2B). As with SAPKs/JNKs, there was very little (if any) activation of either ERK1 or ERK2 when cells were stimulated with 0.03 mM H₂O₂ (30 min) and maximal activation was achieved with 0.1 mM H₂O₂ (Figure 2C). A similar very steep concentration dependence has been seen in perfused hearts [30] and cultured astrocytes [37]. Lower levels of activation were seen at higher concentrations of H₂O₂.

Other protein kinases including SAPKs/JNKs and p38-MAPK may also phosphorylate MBP in in-gel assays. To confirm that the bands seen on these gels after H₂O₂ stimulation represented ERK1 and ERK2, the kinases were separated by Mono Q FPLC and the activation compared with that by 1 μM PMA, the most potent stimulator of ERKs yet identified in ventricular myocytes [20–22]. Two peaks of activity were detected after stimulation with H₂O₂ (0.1 mM, 30 min) eluting at approx. 0.21 M NaCl (fraction 36) and 0.25 M NaCl (fraction 41) (Figure 3A). PMA (1 μM, 5 min) also stimulated these two peaks of activity (Figure 3A). In-gel assay of fractions 36 and 41 detected ERK2 in fraction 36 and ERK1 in fraction 41 (Figure 3B), consistent with previous studies [21]. No activity was detected in unstimulated cells (Figures 3A and 3B). These experiments confirm that H₂O₂ stimulates strongly ERK1 and ERK2 and this activation is comparable to that obtained with PMA.

Figure 3 Separation of ERKs by FPLC

(A) Myocytes were exposed to 0.1 mM H₂O₂ for 30 min (●, solid line) or 1 μM PMA for 5 min (▲, dashed line), or were left untreated (○, dotted line). ERKs were separated by Mono Q FPLC and assayed by the direct method with MBP as substrate. The NaCl gradient is shown by the dotted line. (B) Fractions showing MBP kinase activity were assayed by the in-gel method with MBP as substrate. ERK1 and ERK2 were identified on the basis of molecular mass (44 kDa and 42 kDa respectively). Results are representative of three independent experiments. CON, untreated.

Figure 4 Inhibition of H₂O₂ stimulation of ERKs

Myocytes were preincubated with 10 μM GF109203X (20 min), 50 μM PD98059 (20 min) or 1 μM PMA (24 h) and exposed to 0.1 mM H₂O₂ (30 min). ERKs were assayed by the in-gel kinase method with MBP as substrate. The experiment was repeated with similar results.

The mechanism of ERK activation by H₂O₂ was investigated using selective inhibitors. PD98059 (50 μM), which selectively inhibits activation of MEK [MAPK (or ERK) kinase] 1 and, to a lesser degree, MEK2 [38,39], almost entirely inhibited the activation of ERKs by H₂O₂ (0.1 mM, 30 min) (Figure 4). Since the upstream activation of MEK1/2 is necessary and specific for ERK activation [40], this provides further evidence that the kinases stimulated by H₂O₂ and detected on MBP in-gel assays were ERKs. Inhibition of protein kinase C (PKC) with the selective inhibitor GF109203X (10 μM) [41] also suppressed ERK activation by H₂O₂ (Figure 4), suggesting that stimulation of ERKs is PKC-dependent. Similar results were obtained with 10 μM Ro318220, another PKC inhibitor [42] (results not shown). The response was also inhibited in myocytes pretreated with

Figure 5 Stimulation of p38-MAPK phosphorylation by H_2O_2

Myocytes were exposed to H_2O_2 (0.1 mM) for the times indicated. p38-MAPK phosphorylation was assessed by immunoblot analysis with an antibody that recognizes dually phosphorylated p38-MAPK. The experiment was repeated with similar results.

PMA (1 μ M, 24 h), a manoeuvre which down-regulates the classical and novel isoforms of PKC [43], providing further evidence that activation of the ERKs by H_2O_2 was PKC-dependent.

Phosphorylation of p38-MAPK

p38-MAPK is activated by dual phosphorylation of Thr and Tyr residues within a Thr-Gly-Tyr motif [44]. Activation of p38-MAPK by H_2O_2 was therefore studied by immunoblot analysis using an antibody specific for the dually phosphorylated (activated) form of p38-MAPK. No phosphorylated p38-MAPK was detected in untreated cells (Figure 5). A large increase in phosphorylated p38-MAPK was detected after 5 min of stimulation with 0.1 mM H_2O_2 (Figure 5). The amount of phosphorylated p38-MAPK declined over 15–30 min, but remained detectable over this period. p38-MAPK is thus more rapidly activated by H_2O_2 than the other MAPKs (ERKs and SAPK/JNKs).

Activation of MAPKAPK2

MAPKAPK2 is phosphorylated and activated by p38-MAPK [17], and we studied its activation in myocytes exposed to H_2O_2 . MAPKAPK2 was purified partially by Mono S FPLC and assayed with a peptide substrate. H_2O_2 (0.1 mM, 5 min) stimulated a single peak of activity eluting at approx. 0.19 M NaCl (Figure 6A). This activation was essentially completely inhibited by 10 μ M SB203580, a selective inhibitor of the p38-MAPK and p38-MAPK β isoforms [45–48] (Figure 6A). Minimal activity was detected in untreated cells (Figure 6A). At 5 min, MAPKAPK2 was stimulated approx. 8-fold by 0.1 mM H_2O_2 (Figure 6B). Although this activity declined subsequently, at 30 min it was still elevated approx. 4-fold, relative to control levels (Figure 6B). The time courses of MAPKAPK2 activation and inhibition by SB203580 are entirely consistent with phosphorylation and activation of MAPKAPK2 by p38-MAPK.

Phosphorylation of HSP25/27

HSP25/27 is phosphorylated on up to three sites (Ser-15, Ser-78 and Ser-82 [49,50]) by MAPKAPK2 and the related kinase, MAPKAPK3 [16,17,51]. The phosphorylation state of HSP25/27 was assessed by IEF in urea/polyacrylamide gels and subsequent immunoblotting. This method depends on the decrease in the isoelectric point induced by the introduction of negatively charged phosphate groups into HSP25/27. Up to three forms of HSP25/27 were detected using this method (Figure 7A). The most positively charged form is referred to as band 1 and the most negatively charged form (i.e. the most phosphorylated form) as band 3. Band 2 presumably represents an intermediate phosphorylation state. In unstimulated cells, HSP25/27 was detected primarily as bands 1 and 2 (approx. 60 % and 35 % of

Figure 6 Activation of MAPKAPK2 by H_2O_2

(A) FPLC of MAPKAPK2. Myocytes were exposed to 0.1 mM H_2O_2 for 5 min in the absence (●, solid line) or presence (▲, solid line) of 10 μ M SB203580 and MAPKAPK2 was partially purified by Mono S FPLC. Fractions were assayed for MAPKAPK2 activity using a peptide substrate. Activity in untreated myocytes is also shown (○, dashed line). The NaCl gradient is shown by the dotted line. (B) Myocytes were exposed to 0.1 mM H_2O_2 for the times indicated and MAPKAPK2 assayed after FPLC. The area under the MAPKAPK2 peaks was integrated. The experiment was repeated with similar results.

total, respectively) (Figures 7A and 7B). In ventricular myocytes exposed to hyperosmotic shock (0.5 M sorbitol, 30 min), a severe stress which induces very significant increase in phosphorylation of p38-MAPK(s) in neonatal myocytes (10-fold, results not shown), HSP25/27 was detected principally as bands 2 and 3 (Figure 7A). This change was completely inhibited by SB203580 (10 μ M in DMSO), whereas DMSO alone had no effect on HSP25/27 phosphorylation. These data indicate that hyperosmotic shock stimulates the phosphorylation of HSP25/27 and is consistent with a mechanism involving p38-MAPK activation of MAPKAPK2, which phosphorylates HSP25/27. In myocytes exposed to H_2O_2 (0.1 mM), there was little change in HSP25/27 phosphorylation at 2 min, but after 5 min, increased proportions of bands 2 and 3 were apparent (Figures 7A and 7B). After myocytes were exposed to H_2O_2 for 20 min, as with hyperosmotic shock, HSP25/27 was detected primarily as bands 2 and 3 (approx. 40 % and 43 % of total, respectively). These data are consistent with increasing phosphorylation of HSP25/27 in response to H_2O_2 , with maximal phosphorylation occurring after approx. 20 min stimulation with 0.1 mM H_2O_2 . Increased phosphorylation remained detectable for up to 60 min. There was no significant change in the total immunoreactivity of HSP25/27 over this period (Figure 7B). As with hyperosmotic shock, the change in HSP25/27 phosphorylation induced by H_2O_2 was completely inhibited by SB203580 (10 μ M in DMSO), whereas

A

B

Figure 7 Stimulation of HSP25/27 phosphorylation by H₂O₂

(A) Myocytes were exposed to 0.1 mM H₂O₂ or 0.5 M sorbitol for the times indicated in the absence or presence of 10 μ M SB203580 or 0.1% (v/v) DMSO (the solvent for SB203580). Phosphorylated forms of HSP25/27 were separated by IEF and identified by immunoblotting. The most positively charged isoform (band 1) migrated closest to the cathode. The most negatively charged isoform (band 3) migrated to the anode. The results are representative of three independent experiments. (B) Quantification of HSP25/27 bands by laser-scanning densitometry. Results are means $\pm$ S.E.M. for three independent experiments.

DMSO alone had no effect on the ratio of the different forms of HSP25/27 in either untreated cells or cells exposed to H₂O₂ (Figure 7A). These data are consistent with a p38-MAPK $\rightarrow$ MAPKAPK2/3 pathway of HSP25/27 phosphorylation.

Aggregation state of HSP25/27

In unstimulated cells, HSP25/27 exists as high-molecular-mass aggregates [18]. Phosphorylation of HSP25/27 by MAPKAPK2 causes these aggregates to dissociate [18]. We investigated the aggregation state of HSP25/27 in neonatal myocytes exposed to hyperosmotic shock or H₂O₂, to determine the effects of phosphorylation on HSP25/27 aggregation in these cells. Proteins were separated by gel-filtration FPLC using a Superose 12 column and fractions were immunoblotted for HSP25/27. In unstimulated myocytes, HSP25/27 aggregates eluted close to the void volume in fractions 7–11, the majority eluting in fractions 8 and 9 (Figures 8A and 8C, top panels). In myocytes exposed to sorbitol (0.5 M, 30 min), HSP25/27 was detected in fractions 7–13, with approximately equal distribution between fractions 8 and 11 (Figure 8A, lower panel). The phosphorylation state of HSP25/27 in these fractions was assessed by IEF. HSP25/27 in fraction 8 from unstimulated cells was detected as bands 1 and 2 (Figure 8B). In contrast, in fraction 11 from cells exposed to hyperosmotic shock, HSP25/27 was detected as bands 2 and 3 (Figure 8B). These data indicate that phosphorylation of HSP25/27 in neonatal myocytes is associated with a decrease in its aggregation state.

We examined the aggregation state of HSP25/27 in myocytes exposed to H₂O₂ (0.1 mM, 20 min). Under control conditions, HSP25/27 was detected principally in fractions 8 and 9, (Figure 8C, upper panel). Although a significant proportion remains in

fractions 8 and 9 in myocytes exposed to H₂O₂, the presence of HSP25/27 in fractions 10–12 is clearly increased (Figure 8C, lower panel). This indicates that, like sorbitol (Figure 8A), H₂O₂ induces a shift of HSP25/27 to lower relative-molecular-mass aggregates.

DISCUSSION

Exposure of the heart or myocytes to ischaemia and ischaemia/reperfusion results in a significant increase in ROS (reviewed in [3–5]) and, in particular, H₂O₂ [52,53]. In cultured ventricular myocytes, H₂O₂ induces apoptosis as shown by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, DNA laddering and CPP32 activation [54]. In endothelial cells, H₂O₂ induces an HSP25/27-dependent formation of actin stress fibres [55]. This is probably mediated by phosphorylation of HSP25/27 through p38-MAPK and MAPKAPK2/3 [55]. In other cells, H₂O₂ has been reported to activate a combination of the three MAPK subfamilies [56]. Because of its potential importance in the heart, we have therefore examined the effects of H₂O₂ on MAPK activities in primary cultures of ventricular myocytes. We have particularly focused on the p38-MAPK $\rightarrow$ MAPKAPK2 $\rightarrow$ HSP25/27 pathway, which may be important in cytoprotection. All three MAPK subfamilies were activated significantly by H₂O₂ in ventricular myocytes (Figures 1, 2 and 5), and activated p38-MAPK induced the activation of MAPKAPK2 and stimulated phosphorylation of HSP25/27 (Figures 7 and 8).

Activation of the ERK cascade and SAPKs/JNKs by H₂O₂

Consistent with findings in other cell types [56], there was strong activation of ERKs in ventricular myocytes (Figure 2; approx.

Figure 8 Disaggregation of HSP25/27 induced by hyperosmotic shock or H₂O₂

(A) Myocytes were unstimulated (upper panel) or exposed to 0.5 M sorbitol (lower panel). HSP25/27 was separated by Superose 12 gel-filtration FPLC and detected by immunoblotting of fractions 6–14. (B) Phosphorylated forms of HSP25/27 in FPLC fractions from unstimulated cells (fraction 8, lanes 1 and 3) or sorbitol-treated cells (fraction 11, lanes 2 and 4) were separated by IEF and identified by immunoblotting. The most positively charged isoform (band 1) migrated closest to the cathode. The most negatively charged isoform (band 3) migrated to the anode. (C) Myocytes were unstimulated (upper panel) or exposed to 0.1 mM H₂O₂ (lower panel). HSP25/27 was separated by gel-filtration FPLC and detected by immunoblotting of fractions 6–14. Results are representative of three independent experiments.

16-fold relative to unstimulated cells). This was comparable with that seen after stimulation with the phorbol ester, PMA (Figure 3). This contrasts with our studies in adult perfused hearts, in which H₂O₂ did not stimulate significant activation of ERKs [30]. A factor contributing to this discrepancy may be the massive down-regulation of ERKs that occurs during development [25]. Activation of ERKs by H₂O₂ was inhibited by the selective MEK1/2 inhibitor, PD98059 (Figure 4), consistent with activation through the well-characterized ERK cascade. ERK activation also appeared to be PKC-dependent since it was attenuated by the selective inhibitor, GF109203X, and by chronic pretreatment (24 h) of the cells with PMA (Figure 4). The effect of PMA is to down-regulate the classical and novel isoforms of PKC which, in the heart, are principally cPKC α , nPKC δ and nPKC ϵ [43]. However, H₂O₂ did not translocate these isoforms from the soluble to the particulate fraction of the myocytes, an event generally associated with cPKC/nPKC activation [57–59], nor was there any stimulation of phosphatidyl [³H]inositol hydrolysis, which generates diacylglycerol, the accepted physiological activator of these PKCs (results not shown). These data are consistent with studies in other cells [60], and it has recently

been proposed that H₂O₂ may activate PKC isoforms by tyrosine phosphorylation and independently of any lipid cofactors [61].

The SAPKs/JNKs were also activated strongly in ventricular myocytes by H₂O₂ (Figure 1). Immunoprecipitation with antibodies selective for JNK1 gene products indicated that, as in other studies [23], these were the principal SAPK/JNK isoforms activated in these cells. The time course for the activation of SAPKs/JNKs (Figures 1A and 1B) and ERKs (Figures 2A and 2B) were similar, with maximal activation at 15–30 min, after which the activities of both these groups of MAPKs declined. The concentration dependency of H₂O₂ activation of SAPKs/JNKs and ERKs was also similar, with no activation detectable at concentrations below 0.03 mM, maximal activation at 0.1 mM and reduced activities at higher concentrations (Figures 1D and 2C). These data indicate that there may be a threshold, which must be attained before H₂O₂ can trigger the activation of these MAPKs.

Phosphorylation and activation of p38-MAPK by H₂O₂

Unlike in the perfused adult rat heart [26,28], we (and others) have not yet demonstrated successful activation of p38-MAPK in neonatal ventricular myocytes using in-gel kinase assays with GST-MAPKAPK2(40–400) as substrate. However, antibodies have recently become available that detect the dually phosphorylated (activated) form of p38-MAPKs. In this study, we have shown that p38-MAPK is phosphorylated in ventricular myocytes exposed to H₂O₂ (Figure 5). These results are consistent with another study which has been published recently, also showing phosphorylation of p38-MAPK [54]. H₂O₂ stimulation of p38-MAPK phosphorylation in ventricular myocytes was very rapid, with maximal stimulation within 5 min, after which the phosphorylation declined (Figure 5). This contrasts with the stimulation of SAPKs/JNKs and ERKs by H₂O₂ which was maximal at 15–30 min (Figures 1A, 1B, 2A and 2B). To confirm that the phosphorylation of p38-MAPK represented its activation, we also assayed the activity of MAPKAPK2, a MAPK substrate that is phosphorylated selectively by p38-MAPK [17]. MAPKAPK2 was activated in ventricular myocytes exposed to H₂O₂ and this activation was inhibited by SB203580, a selective inhibitor for p38-MAPK (Figure 6A). The time course of MAPKAPK2 activation closely paralleled that of p38-MAPK phosphorylation, with maximal activation at 5 min, after which the activity declined. These data indicate that H₂O₂ did indeed activate p38-MAPK.

Our difficulty in demonstrating clearly activation of p38-MAPK (i.e. ability to phosphorylate GST-MAPKAPK2 in in-gel kinase assays) in neonatal ventricular myocytes may be attributable to the very high ERK activities in the neonatal myocytes, which are significantly down-regulated in the adult heart [25]. Since ERKs can phosphorylate MAPKAPK2 in *in vitro* assays (including in-gel assays), and were in fact proposed originally as the MAPKs responsible for the phosphorylation and activation of MAPKAPK2 [31], activation of ERKs in ventricular myocytes results in the appearance of the 42 kDa ERK2 and 44 kDa ERK1 bands on an in-gel assay. It is not possible to distinguish easily between 42 kDa ERK2 and 38 kDa p38-MAPK on the mini-gels used. Larger gels would require large amounts of recombinant GST-MAPKAPK2(40–400). The situation is further complicated by the instability of activated p38-MAPK in extracts from neonatal ventricular myocytes, resulting possibly from high levels of protein phosphatase activity in these cells. This can be reduced by the inclusion of microcystin in the homogenization buffer. However, activity is still lost

during the purification of p38-MAPKs, either by immunoprecipitation or on Mono Q FPLC (results not shown).

Mechanisms of activation of MAPKs by H_2O_2

Net activation of MAPKs could result from the stimulation of MAPK kinases or the inhibition of the protein phosphatases that act on MAPKs or other phosphorylatable components of the MAPK signalling cascades. Like the inhibition by arsenite, the inhibition of protein phosphatases may be brought about by modification of essential thiol groups. Thus arsenite activates SAPKs/JNKs by inhibiting SAPK/JNK phosphatases [62]. The insulinomimetic action of H_2O_2 has also been attributed to the inhibition of the protein tyrosine phosphatase(s) that act on the insulin receptor [63]. A similar scheme has been suggested for the H_2O_2 -induced phosphorylation of the epidermal growth factor receptor [64,65]. Here, H_2O_2 may be an important physiological intracellular signalling molecule in its own right [65].

We did not attempt to define the point of action of H_2O_2 in any detail. Activation of ERKs by H_2O_2 was inhibited by PD98059 (Figure 4), a selective inhibitor of MEK activation [38]. Whereas this could be interpreted as indicating that activation of ERKs by H_2O_2 results from activation of the ERK cascade rather than protein phosphatase inhibition, this is not necessarily the case. Other signalling proteins lying upstream from MEK1/2 are regulated by phosphorylation/dephosphorylation mechanisms (e.g. c-Raf [66]). Inhibition of a hypothetical 'c-Raf phosphatase' would still result in PD98059-sensitive activation of ERKs. This comment is equally applicable to the SAPK/JNK and p38-MAPK cascades, and assay of the phosphorylation or activation of the respective MAPK kinases (MKK4/7 and MKK3/6 [9]) would not be definitive in determining the point of action of H_2O_2 .

Phosphorylation of HSP25/27

In certain cell types, phosphorylation of HSP25/27 is associated with stabilization of the actin cytoskeleton to protect it from damage induced by, amongst other agents, H_2O_2 [18,19]. Since MAPKAPK2 (and the related kinase, MAPKAPK3) phosphorylates HSP25/27 [16,17,51], and since this kinase is activated in myocytes by cellular stresses [e.g. H_2O_2 (Figure 5) or hyperosmotic shock with 0.5 M sorbitol (results not shown)], we examined the phosphorylation state of HSP25/27 in cells exposed to these stresses. HSP25/27 was detected readily in extracts from neonatal ventricular myocytes and 2–3 μ g of total protein extract was sufficient for Western blotting and IEF. The phosphorylation state of HSP25/27 was assessed by IEF (Figure 7). Using this method, three forms of HSP25/27 were detected, representing different phosphorylation states (Figure 7A). HSP25/27 is phosphorylated on up to three sites (Ser-15, Ser-78 and Ser-82 [49,50]) by MAPKAPK2 and MAPKAPK3, which should allow for up to four different forms. The relationship between the different phosphorylation sites and the three HSP25/27 bands detected by IEF was not determined. In unstimulated cells, the two most positively charged forms (least phosphorylated), bands 1 and 2, were detected, whereas in cells subjected to hyperosmotic shock, the two most negatively charged forms, bands 2 and 3, were detected (Figure 7), indicating an increase in phosphorylation state of HSP25/27. As in other cells [55], H_2O_2 induced a similar increase in the phosphorylation state of HSP25/27 and the time course was consistent with phosphorylation by MAPKAPK2 (i.e. phosphorylation was increased from 5 min and maximal at 20 min). Consistent with a p38-MAPK $\rightarrow$ MAPKAPK2 $\rightarrow$ HSP25/27 pathway, SB203580 inhibited the change in HSP25/27 phosphorylation induced by either hyperosmotic shock or H_2O_2 .

In unstimulated cells, unphosphorylated HSP25/27 forms high-molecular-mass aggregates, which inhibit actin polymerization [67,68]. Phosphorylation induces these aggregates to dissociate, the phosphorylation and disaggregation allowing HSP25/27 to stabilize the actin cytoskeleton [19,55]. In ventricular myocytes, we also found that hyperosmotic stress and H_2O_2 stimulated HSP25/27 to disaggregate. Using gel-filtration FPLC followed by immunoblotting of the fractions, a clear shift in the apparent relative molecular mass of the high-molecular-mass aggregates to lower-molecular-mass fractions could be seen (Figures 8A and 8C). This shift was associated with an increase in the phosphorylation state of HSP25/27 (Figure 8B). These data indicate that HSP25/27 is phosphorylated in ventricular myocytes through a p38-MAPK pathway, and suggest that, as in other cells, this may be an important protective mechanism.

Role of the MAPK subfamilies

The roles of the different MAPK subfamilies in myocyte function, or that of any other cell type, are still under investigation. In many cells, ERK activation is associated with cell survival [10], whereas SAPKs/JNKs and p38-MAPK may promote apoptosis [10–14]. However, p38-MAPK stimulation of MAPKAPK2 and HSP25/27 phosphorylation is potentially cytoprotective. In the ventricular myocyte, all three MAPK subfamilies have been linked to the development of the hypertrophic phenotype, particularly that induced by phenylephrine [24,69–75], and the ERKs have also been associated with myocyte survival [54,76]. Although they may be hypertrophic [73,74], the p38-MAPKs, especially p38-MAPK(α), may also be apoptotic [74]. The data from this study show that all three subfamilies can be activated strongly in ventricular myocytes in response to a single stimulus. It is therefore probable that the overall response will depend on the balance between the different MAPK activities and it is not yet clear whether ROS are hypertrophic, apoptotic or anoxic.

In summary, we have shown that H_2O_2 , a ROS which is produced in the heart during ischaemia and ischaemia/reperfusion, when it may participate in activating SAPKs/JNKs and p38-MAPK [24], activates all three MAPK subfamilies (ERKs, SAPKs/JNKs and p38-MAPK) in primary cultures of neonatal rat ventricular myocytes. Furthermore, activation of p38-MAPK stimulates MAPKAPK2 activity and increases the phosphorylation of HSP25/27, inducing its disaggregation. This particular signalling pathway may have an important cytoprotective function in the heart, which will be further investigated.

This work was supported by the British Heart Foundation and the Clinical Research Committee of the Royal Brompton Hospital.

REFERENCES

- Lander, H. M. (1997) *FASEB J.* **11**, 118–124
- Slater, A. F. G., Nobel, C. S. I. and Orrenius, S. (1995) *Biochim. Biophys. Acta* **1271**, 59–62
- Jennings, R. B. and Reimer, K. A. (1991) *Annu. Rev. Med.* **42**, 225–246
- Ferrari, R., Ceconi, C., Curello, C., Alfieri, O. and Visioli, O. (1993) *Eur. Heart J.* **14**, Suppl. G, 25–30
- Jennings, R. B., Steenbergen, Jr., C. and Reimer, K. A. (1995) *Monogr. Pathol.* **37**, 47–80
- Sun, J.-Z., Tang, X.-L., Park, S.-W., Qiu, Y., Turrens, J. F. and Bolli, R. (1996) *J. Clin. Invest.* **97**, 562–576
- Seeger, R. and Krebs, E. G. (1995) *FASEB J.* **9**, 726–735
- Kyriakis, J. M. and Avruch, J. (1996) *J. Biol. Chem.* **271**, 24313–24316
- Cohen, P. (1997) *Trends Cell Biol.* **7**, 353–361
- Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) *Science* **270**, 1326–1331
- Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Gleavy, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I. and Johnson, G. L. (1996) *J. Biol. Chem.* **271**, 3229–3237

- 12 Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F. and Gulbins, E. (1997) *J. Biol. Chem.* **272**, 22173–22181
- 13 Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K. and Gotoh, Y. (1997) *Science* **275**, 90–94
- 14 Park, J., Kim, I., Oh, Y. J., Lee, K., Han, P.-L. and Choi, E.-J. (1997) *J. Biol. Chem.* **272**, 16725–16728
- 15 Yue, T.-L., Ma, X.-L., Wang, X., Romanic, A. M., Liu, G., Loudon, C., Gu, J.-L., Kumar, S., Poste, G., Ruffolo, Jr., R. R. and Feuerstein, G. Z. (1998) *Circ. Res.* **82**, 166–174
- 16 Stokoe, D., Engel, K., Campbell, D. G., Cohen, P. and Gaestel, M. (1992) *FEBS Lett.* **313**, 307–313
- 17 Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994) *Cell* **78**, 1027–1037
- 18 Lavoie, J. N., Lambert, H., Hickey, E., Weber, L. A. and Landry, J. (1995) *Mol. Cell Biol.* **15**, 505–516
- 19 Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J. and Landry, J. (1997) *J. Cell Sci.* **110**, 357–368
- 20 Bogoyevitch, M. A., Glennon, P. E. and Sugden, P. H. (1993) *FEBS Lett.* **317**, 271–275
- 21 Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 1110–1119
- 22 Bogoyevitch, M. A., Clerk, A. and Sugden, P. H. (1995) *Biochem. J.* **309**, 437–443
- 23 Bogoyevitch, M. A., Ketterman, A. J. and Sugden, P. H. (1995) *J. Biol. Chem.* **270**, 29710–29717
- 24 Ramirez, M. T., Sah, V. P., Zhao, X.-L., Hunter, J. J., Chien, K. R. and Brown, J. H. (1997) *J. Biol. Chem.* **272**, 14057–14061
- 25 Lazou, A., Bogoyevitch, M. A., Clerk, A., Fuller, S. J., Marshall, C. J. and Sugden, P. H. (1994) *Circ. Res.* **75**, 932–941
- 26 Lazou, A., Sugden, P. H. and Clerk, A. (1998) *Biochem. J.* **332**, 459–465
- 27 Knight, R. J. and Buxton, D. B. (1996) *Biochem. Biophys. Res. Commun.* **218**, 83–88
- 28 Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996) *Circ. Res.* **79**, 162–173
- 29 Yin, T., Sandhu, G., Wolfgang, C. D., Burrier, A., Webb, R. L., Rigel, D. F., Hai, T. and Whelan, J. (1997) *J. Biol. Chem.* **272**, 19943–19950
- 30 Clerk, A., Fuller, S. J., Michael, A. and Sugden, P. H. (1998) *J. Biol. Chem.* **273**, 7228–7234
- 31 Stokoe, D., Campbell, D. G., Nakielnny, S., Hidaka, H., Leivers, S. J., Marshall, C. and Cohen, P. (1992) *EMBO J.* **11**, 3985–3994
- 32 Iwaki, K., Sukhatme, V. P., Shubaita, H. E. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 13809–13817
- 33 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 34 Kameshita, I. and Fujisawa, H. (1989) *Anal. Biochem.* **183**, 139–143
- 35 Clerk, A. and Sugden, P. H. (1997) *Biochem. J.* **325**, 801–810
- 36 Bogoyevitch, M. A., Parker, P. J. and Sugden, P. H. (1993) *Circ. Res.* **72**, 757–767
- 37 Tournier, C., Thomas, G., Pierre, J., Jacquemin, C., Pierre, M. and Saunier, B. (1997) *Eur. J. Biochem.* **244**, 587–595
- 38 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489–27494
- 39 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686–7689
- 40 Crews, C. M., Alessandrini, A. and Erikson, R. L. (1992) *Science* **258**, 478–480
- 41 Toullec, D., Piannetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J. (1991) *J. Biol. Chem.* **266**, 15771–15781
- 42 Davis, P. D., Elliott, L. H., Harris, W., Hill, C. H., Hurst, S. A., Keech, E., Kumar, M. K., Lawton, G., Nixon, J. S. and Wilkinson, S. E. (1992) *J. Med. Chem.* **35**, 994–1001
- 43 Clerk, A., Bogoyevitch, M. A., Fuller, S. J., Lazou, A., Parker, P. J. and Sugden, P. H. (1995) *Am. J. Physiol.* **269**, H1087–H1097
- 44 Lee, J. C. and Young, P. R. (1996) *J. Leukocyte Biol.* **59**, 152–157
- 45 Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, R. J., Landvatter, S. W. et al. (1994) *Nature (London)* **372**, 739–746
- 46 Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) *FEBS Lett.* **364**, 229–233
- 47 Goedert, M., Cuenda, A., Craxton, M., Jakes, R. and Cohen, P. (1997) *EMBO J.* **16**, 3563–3571
- 48 Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C. and Young, P. R. (1997) *Biochem. Biophys. Res. Commun.* **235**, 533–538
- 49 Gaestel, M., Schröder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V. A. and Bielka, H. (1991) *J. Biol. Chem.* **266**, 14721–14724
- 50 Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A. and Anderson, C. W. (1992) *J. Biol. Chem.* **267**, 794–803
- 51 Clifton, A. D., Young, P. R. and Cohen, P. (1996) *FEBS Lett.* **392**, 209–214
- 52 Slezak, J., Tribulova, N., Pristacova, J., Uhrík, B., Thomas, T., Khaper, N., Kaul, N. and Singal, P. K. (1995) *Am. J. Pathol.* **147**, 772–781
- 53 Vanden Hoek, T. L., Li, C., Shao, Z., Schumacker, P. T. and Becker, L. B. (1997) *J. Mol. Cell. Cardiol.* **29**, 2571–2583
- 54 Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y. and Yazaki, Y. (1997) *J. Clin. Invest.* **100**, 1813–1821
- 55 Huot, J., Houle, F., Marceau, F. and Landry, J. (1997) *Circ. Res.* **80**, 383–392
- 56 Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N. J. (1996) *J. Biol. Chem.* **271**, 4138–4142
- 57 Kraft, A. S., Anderson, W. B., Cooper, H. L. and Sando, J. J. (1982) *J. Biol. Chem.* **257**, 13193–13196
- 58 Kraft, A. S. and Anderson, W. B. (1983) *Nature (London)* **301**, 621–623
- 59 Clerk, A., Bogoyevitch, M. A., Andersson, M. B. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 32848–32857
- 60 Whisler, R. L., Goyette, M. A., Grants, I. S. and Newhouse, Y. G. (1995) *Arch. Biochem. Biophys.* **319**, 23–35
- 61 Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U. and Nishizuka, Y. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11233–11237
- 62 Cavigelli, M., Li, W. W., Lin, A., Su, B., Yoshioka, K. and Karin, M. (1996) *EMBO J.* **15**, 6269–6279
- 63 Heffetz, D., Bushkin, I., Dror, R. and Zick, Y. (1990) *J. Biol. Chem.* **265**, 2896–2902
- 64 Knebel, A., Rahmsdorf, H. J., Ullrich, A. and Herrlich, P. (1996) *EMBO J.* **15**, 5314–5325
- 65 Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B. and Rhee, S. G. (1997) *J. Biol. Chem.* **272**, 217–221
- 66 Marais, R., Light, Y., Paterson, H. F., Mason, C. S. and Marshall, C. J. (1997) *J. Biol. Chem.* **272**, 4378–4383
- 67 Miron, T., Vancompernelle, K., Vandekerckhove, J., Wilchek, M. and Geiger, B. (1991) *J. Cell Biol.* **114**, 255–261
- 68 Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J. and Lutsch, G. (1994) *J. Biol. Chem.* **269**, 20780–20784
- 69 Thorburn, J., Frost, J. A. and Thorburn, A. (1994) *J. Cell Biol.* **126**, 1565–1572
- 70 Thorburn, J., McMahon, M. and Thorburn, A. (1994) *J. Biol. Chem.* **269**, 30580–30586
- 71 Gillespie-Brown, J., Fuller, S. J., Bogoyevitch, M. A., Cowley, S. and Sugden, P. H. (1995) *J. Biol. Chem.* **270**, 28092–28096
- 72 Glennon, P. E., Kaddoura, S., Sale, E. M., Sale, G. J., Fuller, S. J. and Sugden, P. H. (1996) *Circ. Res.* **78**, 954–961
- 73 Zechner, D., Thuerauf, D. J., Hanford, D. S., McDonough, P. M. and Glembofski, C. C. (1997) *J. Cell Biol.* **139**, 115–127
- 74 Wang, Y., Huang, S., Sah, V. P., Ross, J., Brown, J. H., Han, J. and Chien, K. R. (1998) *J. Biol. Chem.* **273**, 2161–2168
- 75 Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J. and Chien, K. R. (1998) *J. Biol. Chem.* **273**, 5423–5426
- 76 Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J. H. and Chien, K. R. (1997) *J. Biol. Chem.* **272**, 5783–5791