

## Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart

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### Summary

We investigated the activation of three subfamilies of MAPKs (ERK, JNKs and p38-MAPK) by oxidative stress in the isolated perfused amphibian heart. Activation of p43-ERK by  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  was maximally observed within 5 min, remained elevated for 30 min and was comparable with the effect of  $1 \mu\text{mol l}^{-1}$  PMA. p43-ERK activation by  $\text{H}_2\text{O}_2$  was inhibited by PD98059 but not by SB203580. The p46 and p52 species of JNKs were maximally activated by 2.5- and 2.1-fold, respectively, by  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  within 2 min. JNK activation was still detectable after 15 min, reaching control values at 30 min of treatment. p38-MAPK was maximally activated by 9.75-fold by  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  after 2 min and this activation progressively declined thereafter, reaching control values within 45 min of treatment. The observed dose-dependent profile of p38-MAPK activation by  $\text{H}_2\text{O}_2$  revealed that  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  induced maximal phosphorylation, whereas  $100\text{--}300 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  induced considerable activation of the kinase. Our studies also

showed that the phosphorylation of MAPKAPK2 by  $\text{H}_2\text{O}_2$  followed a parallel time-dependent pattern and that SB203580 abolished this phosphorylation. Furthermore, our experiments clearly showed that  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  induced a strong, specific phosphorylation of HSP27. Our immunohistochemical studies showed that immune complexes of phosphorylated forms of both p38-MAPK and HSP27 were strongly enhanced by  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  in the perinuclear region as well as dispersedly in the cytoplasm of ventricular cells and that SB203580 abolished this phosphorylation. These data indicate that oxidative stress is a powerful activator of all three MAPK subfamilies in the amphibian heart. Stimulation of p38-MAPK and the consequent phosphorylation of HSP27 may be important in cardioprotection under such conditions.

Key words: oxidative stress, amphibian heart, MAPK, p38-MAPK, HSP27, signal transduction, *Rana ridibunda*.

### Introduction

It is well established that toxic reactive oxygen species (ROS) are overproduced in the mammalian heart following hypoxia and reoxygenation *in vivo* or ischaemia and reperfusion *in vitro*, causing oxidation of various cellular components, including proteins, DNA and membrane lipids (Shlafer et al., 1987; Kehrer, 1993; Halliwell, 1994; Kramer et al., 1994; Wang et al., 1998a). The electron carriers of the mitochondrial respiratory chain are reduced during ischaemia, whereas immediate reoxidation of these carriers occurs upon reinitiation of the flow of oxygenated perfusate, leading to oxyradical overproduction (Halliwell, 1994). Moreover, post-ischaemic peroxidation of endoplasmic reticulum causes an increase in cytoplasmic calcium levels that can lead to uncontrolled activation of phospholipases and proteases (Halliwell, 1994). In the mammalian heart undergoing reoxygenation or reperfusion, these free oxyradical-induced events can lead to either severe cell damage, apoptosis and organ failure (Turner et al., 1998; Bolli and Marban, 1999; Mizukami et al., 2001) or to survival (Wang et al., 1998b; Dougherty et al., 2002).

Although the above-described mechanisms leading to oxidative stress are common in all air-breathing organisms, there are some distinguishable differences between higher and lower vertebrates and/or invertebrates. Mammals are designed to function under high oxygen pressure and therefore show a limited capacity for maintaining cellular homeostasis without oxygen and a limited ability to deal with oxidative stress. On the other hand, many lower vertebrate and invertebrate species deal naturally with wide variations and rapid changes in oxygen availability and include some species that have a well-developed capacity to sustain prolonged periods of complete anoxia (Driedzic and Gesser, 1994). Previous studies conducted in organs of anoxia-tolerant species have shown that particular adaptations, including the induction or overexpression of various antioxidant systems, exist in these organisms to allow them to deal effectively with a rapid transition from anoxia back to normoxia, minimising stress or injury resulting from a burst of ROS generation (Hermes-Lima and Storey, 1996;

Venditti et al., 1999; Hermes-Lima et al., 2001; Lushchak et al., 2001; Pritchard, 2002).

Various reports have documented the involvement of the mitogen-activated protein kinase (MAPK) signalling pathways in redox-stressed cells and tissues, including mammalian cardiac myocytes and intact myocardium (reviewed in Sugden and Clerk, 1998; Franklin and McCubrey, 2000). However, the factors that modulate these signalling pathways have not been described fully in any system studied to date.

MAPKs are members of a major intracellular signal transduction pathway that has been demonstrated to play an important role in various physiological processes (Widmann et al., 1999; Kyriakis and Avruch, 2001; Pearson et al., 2001). Three subfamilies of these serine/threonine kinases have been clearly identified in mammals: the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38-MAPKs. The third subfamily, p38-MAPK, is activated by various forms of environmental stress, including hyperosmolarity and heat shock (for a review, see Bogoyevitch, 2000; Kyriakis and Avruch, 2001). The respective MAPK subfamilies in the amphibian heart have been recently characterised in our laboratory (Aggeli et al., 2001a,b, 2002a,b). In the isolated perfused *Rana ridibunda* heart, the one isoform of ERK (p43) detected was activated by phorbol esters [ $1 \mu\text{mol l}^{-1}$  4 $\beta$ -phorbol 12-myristate 13-acetate (PMA)] and mechanical overload. The two isoforms of JNKs identified (p46-JNK1 and p52-JNK2) were found to be phosphorylated in response to  $0.5 \text{ mol l}^{-1}$  sorbitol, mechanical overload and re-oxygenation following anoxia. p38-MAPK was also stimulated by mechanical overload but was most potently activated by hyperosmotic and thermal stresses.

Activated MAPKs are characterised by their localisation in both the cytoplasm and the nucleus, where they interact with their substrates (Bogoyevitch, 2000; Aggeli et al., 2001b). A variety of substrates for the MAPKs has 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 p38-MAPK substrates is MAPK-activated protein kinase 2 (MAPKAPK2), which phosphorylates the small heat shock protein HSP27 (Stokoe et al., 1992; Rouse et al., 1994). In several cell types, phosphorylation of HSP27 is associated with stabilisation of the actin cytoskeleton, protecting cells against damage (Lavoie et al., 1995; Guay et al., 1997; Concannon et al., 2003).

Here, we have investigated the effect of oxidative stress (as exemplified by  $\text{H}_2\text{O}_2$ ) on the phosphorylation levels of the three MAPK subfamilies as well as MAPKAPK2 and HSP27 in the isolated perfused amphibian heart. We have also studied the immunolocalisation pattern of phosphorylated p38-MAPK and HSP27 induced by oxidative stress in the absence or presence of the selective p38-MAPK inhibitor SB203580. Overall, our results provide evidence that, despite the fundamental structural and functional differences between the mammalian and amphibian heart, common MAPK signal transduction pathways are involved in responses such as oxidative stress.

## Materials and methods

### Materials

Most biochemicals used were purchased from Sigma Chemical Co. (St Louis, MO, USA). The enhanced chemiluminescence kit was from Amersham International (Uppsala, Sweden) and the alkaline phosphatase Kwik kit was from Lipshaw (Pittsburgh, PA, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45  $\mu\text{m}$ ) was obtained from Schleicher & Schuell (Keene, NH, USA). MAPK inhibitors PD98059 and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA), and stock solutions ( $10 \text{ mmol l}^{-1}$ ) were prepared in dimethyl sulphoxide (DMSO).

Rabbit polyclonal antibodies specific for the total and dually phosphorylated ERKs (#9102 and #9101, respectively), JNKs (#9252 and #9251, respectively) and p38-MAPK (#9212 and #9211, respectively), as well as for the total and phosphorylated (Thr334) MAPKAPK2 (#3042 and #3041, respectively) and phosphorylated (Ser82) HSP27 (#2401) were purchased from Cell Signalling (Beverly, MA, USA). A mouse monoclonal antibody specific for the total HSP27 (#2402) was also obtained from Cell Signalling. Pre-stained molecular mass markers were from New England Biolabs. Biotinylated anti-rabbit and anti-mouse antibodies were from DAKO A/S (Glostrup, Denmark). X-OMAT AR 13 cm $\times$ 18 cm and Elite chrome 100 films were purchased from Eastman Kodak Company (New York, NY, USA).

### Animals

Frogs (*Rana ridibunda* Pallas) weighing 120–150 g were caught in the vicinity of Thessaloniki, Greece and supplied by a local dealer. They were kept in containers in freshwater and their care met the standards of Good Laboratory Practice.

### Heart perfusions

Frogs were anaesthetised by immersion in 0.01% (w/v) MS222 and sacrificed by decapitation. The hearts were excised and mounted onto the aortic cannula of a conventional Langendorff perfusion system. Perfusions were performed in a non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with bicarbonate-buffered saline ( $23.8 \text{ mmol l}^{-1}$   $\text{NaHCO}_3$ ,  $103 \text{ mmol l}^{-1}$   $\text{NaCl}$ ,  $1.8 \text{ mmol l}^{-1}$   $\text{CaCl}_2$ ,  $2.5 \text{ mmol l}^{-1}$   $\text{KCl}$ ,  $1.8 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ ,  $0.6 \text{ mmol l}^{-1}$   $\text{NaH}_2\text{PO}_4$ , pH 7.4 at  $25^\circ\text{C}$ ) supplemented with  $10 \text{ mmol l}^{-1}$  glucose and equilibrated with 95%  $\text{O}_2$ :5%  $\text{CO}_2$ . The temperature of the hearts and perfusates was maintained at  $25^\circ\text{C}$  using a water-jacketed apparatus. All hearts were equilibrated for 15 min under these conditions. At the end of the equilibration period, hearts were perfused with  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for periods of time ranging from 30 s to 60 min. In another set of experiments, hearts were perfused with different concentrations of  $\text{H}_2\text{O}_2$  (3–1000  $\mu\text{mol l}^{-1}$ ) for 2 min or 5 min (the time point of the respective maximal MAPK activation) after the equilibration period. In addition, hearts perfused with either  $1 \mu\text{mol l}^{-1}$  PMA for 10 min or  $0.5 \text{ mol l}^{-1}$  sorbitol for 15 min after the equilibration period

were used as positive controls. Perfusions were also conducted in the presence of  $1 \mu\text{mol l}^{-1}$  SB203580 or  $25 \mu\text{mol l}^{-1}$  PD98059 during both the equilibration period and the perfusion with either  $30 \mu\text{mol l}^{-1}$  or  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min or 5 min.

At the end of the perfusions, atria were removed, and the ventricles, after being immersed in liquid  $\text{N}_2$ , were pulverised under liquid  $\text{N}_2$ . Powders were stored at  $-80^\circ\text{C}$ .

#### *Tissue extractions*

Heart powders were homogenised with  $3 \text{ ml g}^{-1}$  of buffer [ $20 \text{ mmol l}^{-1}$  Tris-HCl, pH 7.5,  $20 \text{ mmol l}^{-1}$   $\beta$ -glycerophosphate,  $20 \text{ mmol l}^{-1}$  NaF,  $2 \text{ mmol l}^{-1}$  EDTA,  $0.2 \text{ mmol l}^{-1}$   $\text{Na}_3\text{VO}_4$ ,  $5 \text{ mmol l}^{-1}$  dithiothreitol (DTT),  $10 \text{ mmol l}^{-1}$  benzamide,  $200 \mu\text{mol l}^{-1}$  leupeptin,  $120 \mu\text{mol l}^{-1}$  pepstatin A,  $10 \mu\text{mol l}^{-1}$  trans-epoxy-succinyl-L-leucylamido(4-guanidino)butane,  $300 \mu\text{mol l}^{-1}$  phenyl methyl sulphonyl fluoride (PMSF), 0.5% (v/v) Triton X-100] and extracted on ice for 30 min. The samples were centrifuged ( $10\,000 \text{ g}$ , 5 min,  $4^\circ\text{C}$ ) and the supernatants boiled with 0.33 volumes of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [ $0.33 \text{ mol l}^{-1}$  Tris-HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) Bromophenol Blue]. Protein concentrations were determined using the BioRad Bradford assay.

#### *SDS–PAGE and immunoblot analysis*

Proteins were separated by SDS–PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bis-acrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes ( $0.45 \mu\text{m}$ ). Membranes were then incubated in TBS-T [ $20 \text{ mmol l}^{-1}$  Tris-HCl, pH 7.5,  $137 \text{ mmol l}^{-1}$  NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) bovine serum albumin (BSA) for 30 min at room temperature. Subsequently, the membranes were incubated with the appropriate antibody according to the manufacturer's instructions. After washing in TBS-T ( $4 \times 5 \text{ min}$ ), the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies [1:5000 dilution in TBS-T containing 1% (w/v) BSA; 1 h at room temperature]. The blots were washed again in TBS-T ( $4 \times 5 \text{ min}$ ), and the bands were detected using the enhanced chemiluminescence (ECL) reaction with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

#### *Immunolocalisation of phospho-p38-MAPK and phospho-HSP27*

At the end of the perfusions, atria were removed and ventricles were immersed in isopentane pre-cooled in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ . Tissues were sectioned with a cryostat at a thickness of  $5\text{--}6 \mu\text{m}$ , fixed with ice-cold acetone (10 min at room temperature) and stored at  $-30^\circ\text{C}$  until use. Tissue sections were washed in TBS-T [containing 0.1% (v/v) Tween 20], and non-specific binding sites were blocked with 3% (w/v) BSA in TBS-T (1 h at room temperature). Specimens were incubated with primary antibodies specific for phospho-p38-

MAPK and phospho-HSP27, diluted in 3% (w/v) BSA in TBS-T (overnight at  $4^\circ\text{C}$ ), according to the method previously described (Aggeli et al., 2002a). All sections were immunostained by the alkaline phosphatase method using a Kwik kit according to the manufacturer's instructions. The alkaline phosphatase label was visualised by exposing the sections to Fast Red chromogen, and nuclei were counterstained with haematoxylin. Slides were mounted, examined with a Zeiss Axioplan microscope and photographed with a Kodak Elite chrome 100 film.

#### *Statistical evaluations*

All data are presented as means  $\pm$  S.E.M. Comparisons between control and treatments were performed using the unpaired Student's *t*-test. A value of  $P < 0.05$  was considered to be statistically significant. MAPK activation in 'control' hearts was set at 1, and the stimulated MAPK activation in treated hearts was expressed as '-fold' activation over control hearts.

### **Results**

Activation of the 43 kDa ERK previously detected and characterised in the perfused amphibian heart (Aggeli et al., 2001a) by  $\text{H}_2\text{O}_2$  was studied by immunoblot analysis using a rabbit polyclonal antibody specific for the dually phosphorylated (activated) forms of the kinases (namely ERK1 and ERK2). Examination of the time course of the kinase phosphorylation by  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  revealed that the p43-ERK was apparently phosphorylated within 30 s of treatment (approximately  $2.3 \pm 0.1$ -fold relative to control values;  $P < 0.05$ ,  $N=3$ ). Maximal phosphorylation was attained within 5 min of treatment ( $2.8 \pm 0.2$ -fold relative to control values;  $P < 0.05$ ,  $N=3$ ) while the phosphorylation levels remained elevated above basal for 30 min (Fig. 1A,B). As a positive control, extract from hearts perfused with  $1 \mu\text{mol l}^{-1}$  PMA for 10 min was used (Fig. 1A, top panel). The dose-dependent activation of p43-ERK by  $\text{H}_2\text{O}_2$  was also examined. There was a strong activation of the kinase by  $3\text{--}1000 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  (Fig. 1C,D), a result quite different from those described for neonatal rat cardiomyocytes (Clerk et al., 1998a) and rat perfused heart (Clerk et al., 1998b). The mechanism of ERK activation by  $\text{H}_2\text{O}_2$  was investigated using selective inhibitors. PD98059 ( $25 \mu\text{mol l}^{-1}$ ), which selectively inhibits activation of MEK1 [MAPK (or ERK) kinase 1], almost entirely inhibited the ERK activation by  $\text{H}_2\text{O}_2$  ( $30 \mu\text{mol l}^{-1}$ , 5 min), whereas SB203580 ( $1 \mu\text{mol l}^{-1}$ ), which selectively inhibits the p38-MAPK pathway, had no effect on this phosphorylation (Fig. 2A,B). Equivalent protein loading was confirmed by probing identical samples with an antibody recognising total ERK levels (bottom panels of Figs 1A, 2A).

Activation of JNKs by  $\text{H}_2\text{O}_2$  was studied using specific antibodies for the dually phosphorylated forms of these kinases. The 46 kDa (p46-JNK1) and 52 kDa (p52-JNK2) JNKs were rapidly activated by  $100 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  (Fig. 3A). Activation was apparent within 1 min and was maximal at approximately 2 min. p46-JNK1 was activated by

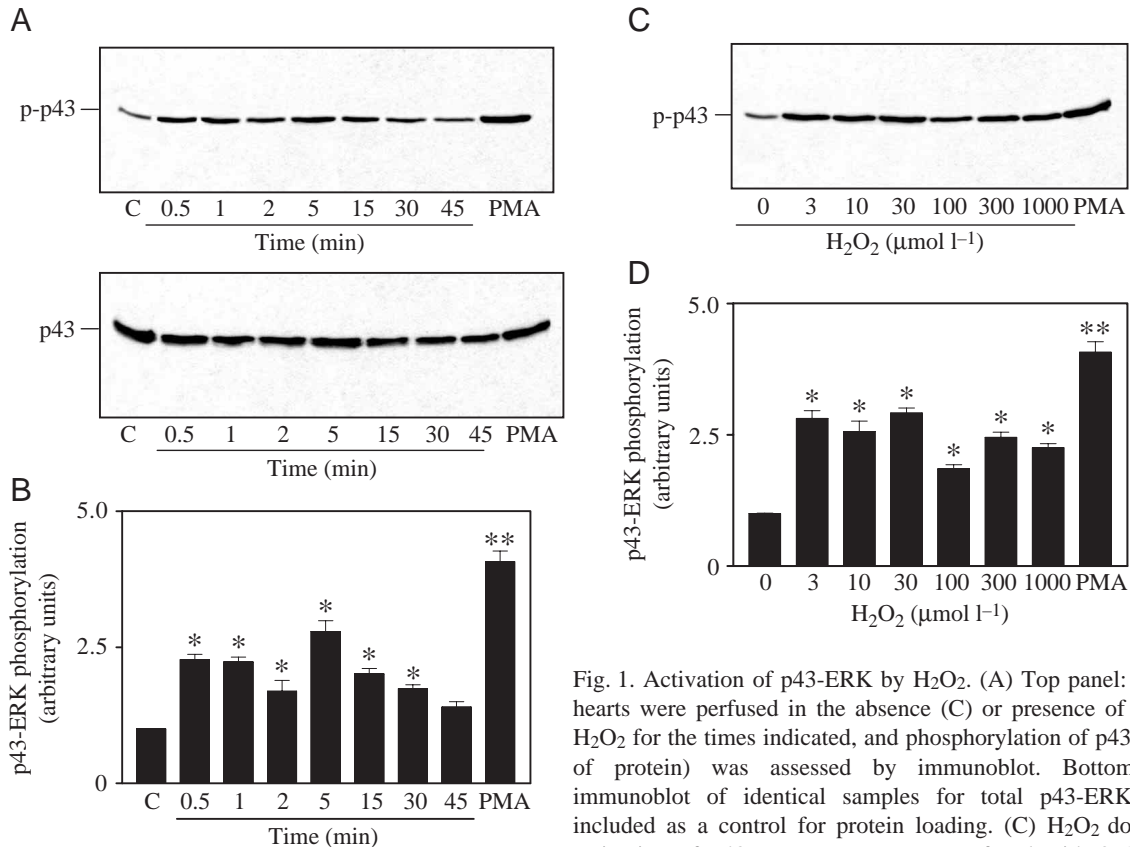


Fig. 1. Activation of p43-ERK by H<sub>2</sub>O<sub>2</sub>. (A) Top panel: *R. ridibunda* hearts were perfused in the absence (C) or presence of 100 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> for the times indicated, and phosphorylation of p43-ERK (50 μg of protein) was assessed by immunoblot. Bottom panel: an immunoblot of identical samples for total p43-ERK levels was included as a control for protein loading. (C) H<sub>2</sub>O<sub>2</sub> dose-dependent activation of p43-ERK. Hearts were perfused with 3–1000 μmol l<sup>-1</sup>

H<sub>2</sub>O<sub>2</sub> for 5 min. As positive controls, extracts from hearts perfused with 1 μmol l<sup>-1</sup> PMA for 10 min were included. Blots are representative of three independent experiments. (B,D) Densitometric analysis of phospho-p43-ERK bands by laser scanning. Results are means ± S.E.M. for three independent experiments performed with similar findings. \**P*<0.05, \*\**P*<0.01 vs control value.

approximately 2.5±0.01-fold (*P*<0.001, *N*=3) and p52-JNK2 by approximately 2.2±0.01-fold (*P*<0.001, *N*=3) relative to control values. Phosphorylation levels showed a progressive decline that reached control values after a period of 30 min. The blot presented in Fig. 3B shows that there were no changes in the total cellular pool of JNKs and therefore provides a control for protein loading under these conditions.

p38-MAPK is activated by dual phosphorylation of Thr and Tyr residues within a Thr-Gly-Tyr motif. Activation of the kinase by H<sub>2</sub>O<sub>2</sub> was therefore studied by immunoblot using a specific antibody that recognises the dually phosphorylated kinase in extracts from amphibian hearts perfused with 100 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> for increasing time periods varying from 30 s to 60 min. The results of this study showed that the phosphorylation of p38-MAPK was apparent within 30 s, maximal at 2 min (approximately 9.75±0.75-fold relative to control values; *P*<0.001, *N*=4) and progressively declined thereafter, reaching control values within 45 min of treatment (Fig. 4A,B). Interestingly, a second band corresponding to 39 kDa was also detected at 2 min of treatment with H<sub>2</sub>O<sub>2</sub>, a result quite similar to that induced by either 0.5 mol l<sup>-1</sup> sorbitol (Aggeli et al., 2001a) or mechanical overload (Aggeli et al., 2001b) in the perfused amphibian heart. Equivalent protein loading was confirmed by probing identical samples with an

antibody recognising the total p38-MAPK levels (Fig. 4A, bottom panel). We also examined the dose-dependent activation of p38-MAPK by H<sub>2</sub>O<sub>2</sub>. As can be seen in Fig. 4C,D, 3 μmol l<sup>-1</sup> of this agent induced strong activation of the kinase (3.40±0.77-fold relative to control value; *P*<0.01, *N*=4) whereas 30 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> induced maximal activation of the kinase (9.94±0.76-fold relative to control values; *P*<0.001, *N*=4). Lower phosphorylation levels were elicited by concentrations of H<sub>2</sub>O<sub>2</sub> of 300 μmol l<sup>-1</sup> (approximately 5.42±1.05-fold relative to control values; *P*<0.01, *N*=4) or 1 mmol l<sup>-1</sup> (4.33±1.34-fold relative to control values; *P*<0.01, *N*=4) (Fig. 4C,D). The maximal phosphorylation of p38-MAPK induced by oxidative stress was comparable to that induced by 0.5 mol l<sup>-1</sup> sorbitol (9.18±1.00-fold relative to control values; *P*<0.001, *N*=4; Fig. 4C,D). SB203580 (1 μmol l<sup>-1</sup>) abolished the phosphorylation of p38-MAPK induced by 100 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> (Fig. 5A,B). As a positive control, extract from heart perfused with 0.5 mol l<sup>-1</sup> sorbitol was used (Fig. 5A,B). The bottom panel in Fig. 5A shows that there were no changes in the total cellular pool of p38-MAPK and therefore provides a control for protein loading under these conditions.

It is well established that MAPKAPK2 is phosphorylated and activated by p38-MAPK, and we therefore studied its

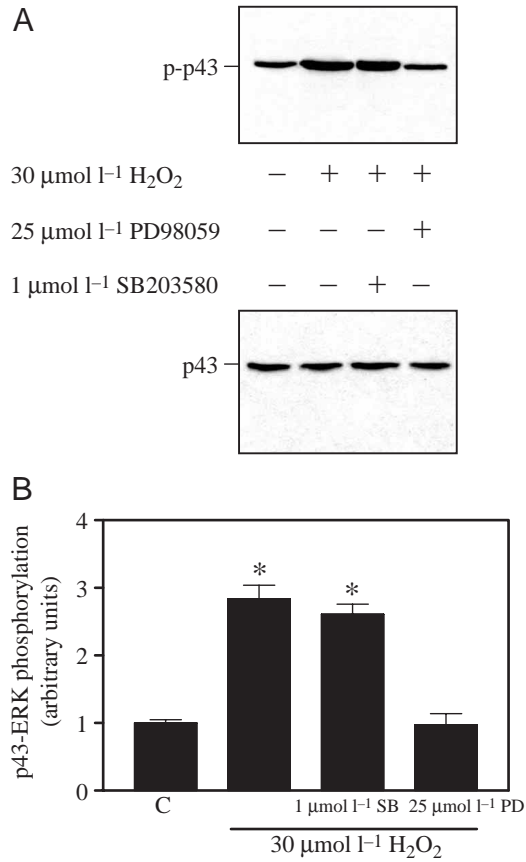


Fig. 2. Effect of selective inhibitors on the p43-ERK phosphorylation induced by oxidative stress. Protein (50  $\mu\text{g}$ ) from hearts perfused with 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 5 min in the absence or presence of 25  $\mu\text{mol l}^{-1}$  PD98059 or 1  $\mu\text{mol l}^{-1}$  SB203580 was assessed by immunoblot using phosphospecific anti-ERK (A, top panel) or total (phosphorylation state independent) anti-ERK antibody (A, bottom panel). Blots are representative of three independent experiments. (B) Densitometric analysis of phospho-p43-ERK bands by laser scanning. Results are means  $\pm$  S.E.M. for three independent experiments. \* $P$ <0.05 vs control value.

phosphorylation state in the amphibian heart perfused with 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  induced a strong phosphorylation of the kinase (Fig. 6A,B). In particular, the time course of the MAPKAPK2 phosphorylation by 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  showed that the kinase phosphorylation was apparent within 1 min, maximised within 5 min (2.57 $\pm$ 0.10-fold relative to control values;  $P$ <0.01,  $N$ =3) and reached control values within 30 min of treatment. Furthermore, the p38-MAPK selective inhibitor SB203580 (1  $\mu\text{mol l}^{-1}$ ) completely inhibited the MAPKAPK2 activation induced by 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$ . As a positive control, extract from heart perfused with 0.5 mol  $\text{l}^{-1}$  sorbitol for 15 min was used. The bottom panel in Fig. 6A shows that equivalent protein was loaded.

HSP27 is phosphorylated at up to three sites (Ser15, Ser78 and Ser82) by MAPKAPK2 and the related kinase MAPKAPK3. The phosphorylation state of HSP27 was assessed by immunoblot using a rabbit polyclonal antibody

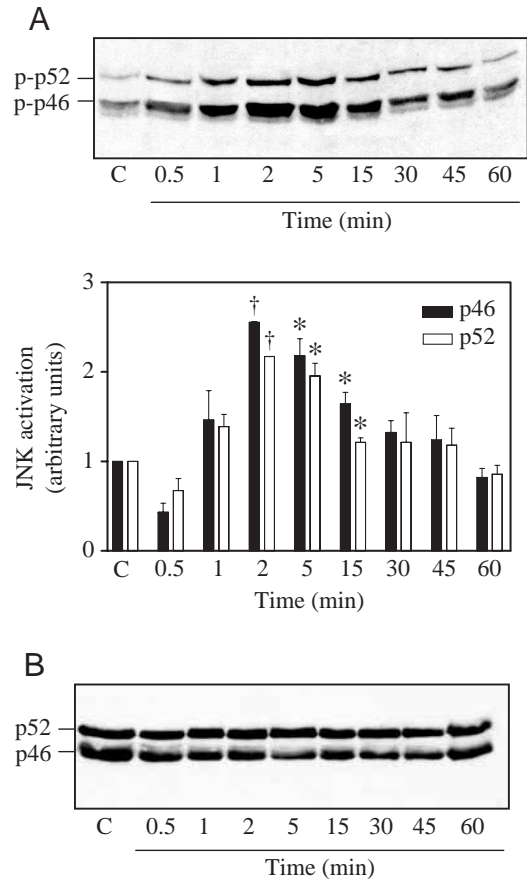


Fig. 3. Time course of JNKs activation by 100  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$ . Protein (100  $\mu\text{g}$ ) from hearts perfused in the absence (C) or presence of 100  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for the times indicated was assessed by immunoblot using phosphospecific anti-JNKs antibody (A, top panel) or total anti-JNKs antibody (B). Blots are representative of three independent experiments. The bottom panel in A shows quantification of JNK1 and JNK2 bands by laser scanning densitometry. Results are means  $\pm$  S.E.M. for three independent experiments. \* $P$ <0.05, † $P$ <0.001 vs control value.

that detects the phosphorylated HSP27 at Ser82 (#2401). As can be seen in Fig. 7A,B, 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  induced a strong phosphorylation of this protein (5.34 $\pm$ 0.25-fold relative to control value;  $P$ <0.001,  $N$ =3). Moreover, the p38-MAPK selective inhibitor SB203580 (1  $\mu\text{mol l}^{-1}$ ) abolished the HSP27 phosphorylation induced by oxidative stress, whereas the ERK pathway selective inhibitor PD98059 at a concentration of 25  $\mu\text{mol l}^{-1}$  had no significant inhibitory effect. The bottom panel in Fig. 7A shows that there were no changes in the total cellular pool of HSP27 and therefore provides a control for protein loading under these conditions. All the above results support the conclusion that oxidative stress induced by  $\text{H}_2\text{O}_2$  leads to the activation of a p38-MAPK-dependent signalling pathway and are consistent with a p38-MAPK $\rightarrow$ MAPKAPK2/3 $\rightarrow$ HSP27 phosphorylation cascade.

In cryosections from hearts perfused with 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min in the absence or presence of 1  $\mu\text{mol l}^{-1}$  SB203580,

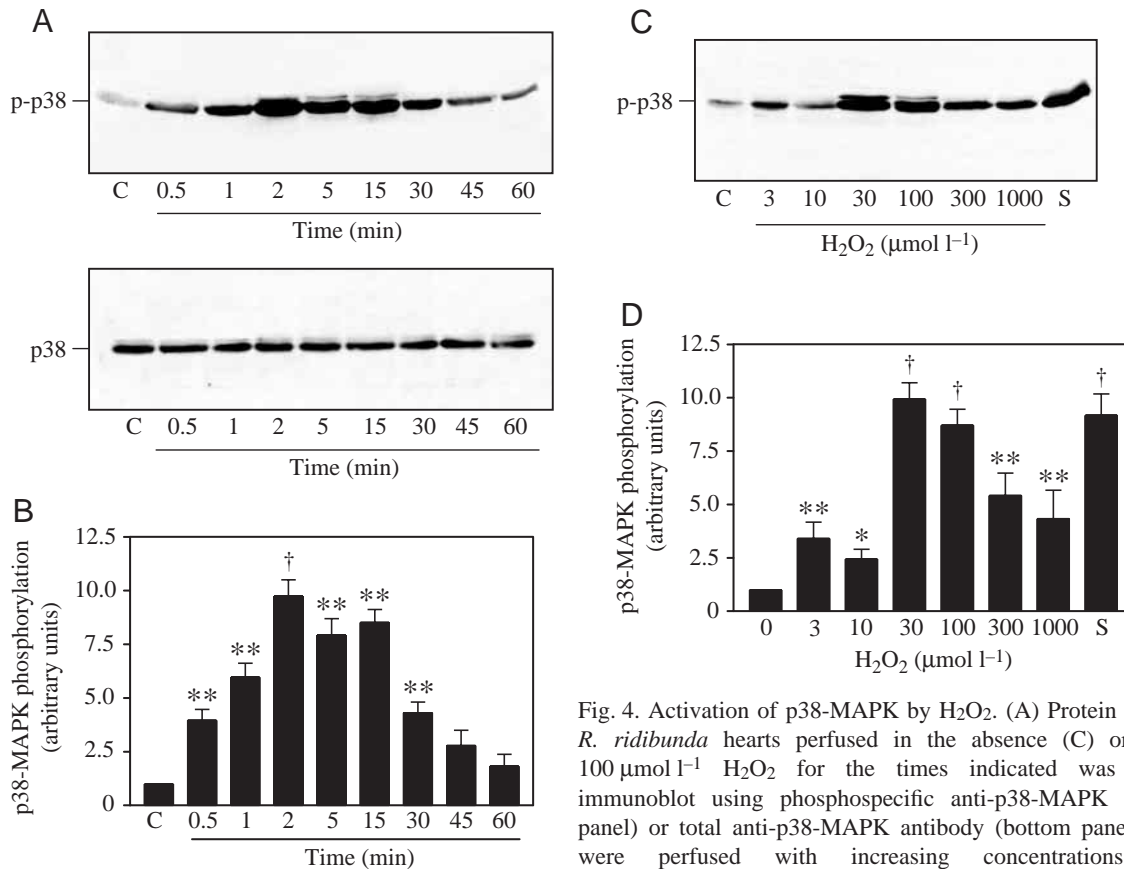


Fig. 4. Activation of p38-MAPK by H<sub>2</sub>O<sub>2</sub>. (A) Protein (100 μg) from *R. ridibunda* hearts perfused in the absence (C) or presence of 100 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> for the times indicated was assessed by immunoblot using phosphospecific anti-p38-MAPK antibody (top panel) or total anti-p38-MAPK antibody (bottom panel). (C) Hearts were perfused with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (3–1000 μmol l<sup>-1</sup>) for 2 min. As positive controls, extracts from hearts perfused with 0.5 mol l<sup>-1</sup> sorbitol (S) for 15 min were included. Blots are representative of four independent experiments. (B,D) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for four independent experiments. \**P*<0.05, \*\**P*<0.01, †*P*<0.001 vs control value.

we tried to immunolocalise the phosphorylated forms of p38-MAPK and HSP27. Neither in control hearts (Figs 8A, 9A) nor in specimens incubated either with the secondary antibody or the chromogen alone was any immunoreactivity detected (data not shown). In specimens from hearts perfused with 30 μmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> for 2 min, strong immunoreactivity staining for phosphorylated p38-MAPK was observed within the cytoplasm as well as in the perinuclear region (Fig. 8B). In particular, perinuclear clustering of deposits indicating phospho-p38-MAPK immunoproducts was more intense in specimens from hearts subjected to either oxidative stress (Fig. 8B) or 0.5 mol l<sup>-1</sup> sorbitol treatment (Fig. 8C). This immunoreactivity pattern disappeared when the hearts were perfused with H<sub>2</sub>O<sub>2</sub> in the presence of the selective p38-MAPK inhibitor SB203580 (Fig. 8D). In respective sections, the anti-phospho-HSP27 antibody produced a discrete pattern of phospho-HSP27 immunoreactivity staining. Observed phospho-HSP27-immunoproducts were localised in the perinuclear region but were also widely dispersed in the cytoplasm (Fig. 9B,C). Overall, our results reveal that the selective p38-MAPK inhibitor SB203580 (1 μmol l<sup>-1</sup>) abolished this HSP27 phosphorylation by oxidative stress (Figs 8D and 9D for phospho-p38-MAPK and phospho-

HSP27, respectively), which is consistent with our results obtained using biochemical approaches.

### Discussion

MAPKs are key participants in signal transduction pathways activated by mitogenic stimuli, environmental stress and inflammatory agents. In mammals, the stress-activated kinase members JNKs and p38-MAPK are activated by ROS generated in response to hypoxia or anoxia as well as during reoxygenation following anoxia, mechanical overload, angiotensin II and exogenous H<sub>2</sub>O<sub>2</sub> (Huot et al., 1997; Seko et al., 1997; Clerk et al., 1998a,b; Turner et al., 1998; Dougherty et al., 2002; Kulisz et al., 2002; Cicconi et al., 2003). However, the precise mechanisms underlying this response are unknown.

We previously reported that neither anoxia nor anoxia/reoxygenation induces p38-MAPK phosphorylation, whereas anoxia/reoxygenation leads to a strong phosphorylation of JNKs (p46-JNK1 and p52-JNK2, respectively) in the perfused amphibian heart (Aggeli et al., 2001a). The present study therefore sought to investigate whether oxidative stress induced by exogenous H<sub>2</sub>O<sub>2</sub> causes phosphorylation (hence

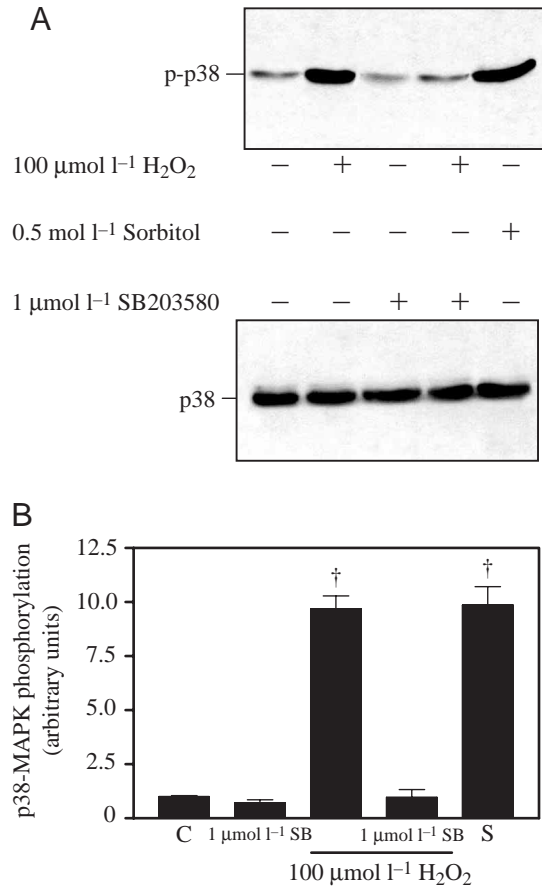


Fig. 5. Effect of the selective inhibitor SB203580 on the p38-MAPK phosphorylation induced by oxidative stress. Protein (100  $\mu\text{g}$ ) from hearts perfused with 100  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min in the absence or presence of 1  $\mu\text{mol l}^{-1}$  SB203580 was assessed by immunoblot using phosphospecific anti-p38-MAPK (A, top panel) or total anti-p38-MAPK antibody (A, bottom panel). As a positive control, extract from hearts perfused with 0.5 mol  $\text{l}^{-1}$  sorbitol was included. Blots are representative of three independent experiments. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means  $\pm$  S.E.M. for three independent experiments.  $\dagger P < 0.001$  vs control value.

activation) of the three well-established MAPK superfamily members in the perfused amphibian heart as well as the possible mechanisms involved in these responses.

Using antibodies that can distinguish between phosphorylated and unphosphorylated forms of these kinases, we detected the activation of the p43-ERK and JNKs with a different time-dependent profile. In particular, p43-ERK was found to be activated by  $\text{H}_2\text{O}_2$  from as early as 30 s and this phosphorylation was sustained for as long as 30 min of treatment (Fig. 1). Similar time-dependent phosphorylation of the kinase was previously detected by mechanical overload (Aggeli et al., 2001b) and by the  $\alpha_1$ -adrenergic agonist phenylephrine (Aggeli et al., 2002b) in the amphibian heart. Furthermore, p43-ERK was activated by  $\text{H}_2\text{O}_2$  in a dose-independent pattern, a result that is different from those previously reported for either neonatal cardiac

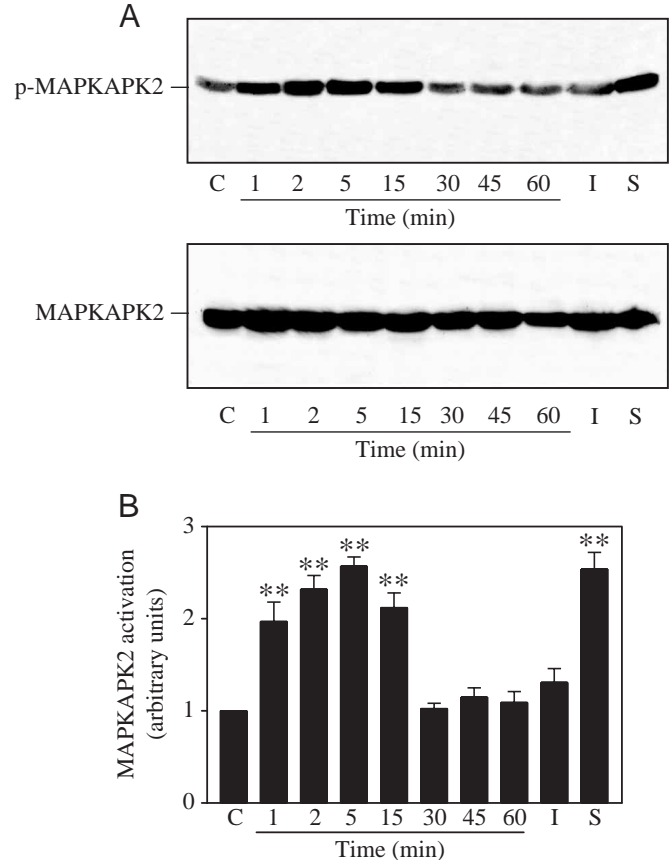


Fig. 6. Phosphorylation of MAPKAPK2 by 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$ . (A) Protein (100  $\mu\text{g}$ ) from control hearts (C), hearts perfused with 0.5 mol  $\text{l}^{-1}$  sorbitol for 15 min (S), hearts perfused with 30  $\mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  in the absence of inhibitor for the times indicated or the presence of 1  $\mu\text{mol l}^{-1}$  SB203580 for 2 min (I) was assessed by immunoblot using phosphospecific (top panel) or total (bottom panel) anti-MAPKAPK2 antibodies. Blots shown are representative of three independent experiments. (B) Densitometric analysis of phospho-MAPKAPK2 bands by laser scanning. Results are means  $\pm$  S.E.M. for three independent experiments.  $**P < 0.01$  vs control value.

myocytes (Clerk et al., 1998a) or perfused rat heart (Clerk et al., 1998b). However, our results are consistent with reports that describe a common, widespread response of ERKs to various stimuli (Steinberg, 2000).

On the other hand, phosphorylation of JNKs by  $\text{H}_2\text{O}_2$  was detected from as early as 1 min of treatment, with its maximal value attained within 2 min and showing a progressive decline thereafter, reaching control values within 30 min of treatment (Fig. 3). A similar time-dependent profile of JNK activation by oxidative stress has also been reported for rat neonatal cardiac myocytes (Clerk et al., 1998a; Dougherty et al., 2002) and adult rat heart (Clerk et al., 1998b). However, the activation of JNKs detected in the present study was low compared with their response to various other stressful stimuli such as hyperosmotic stress, anoxia/reoxygenation or mechanical overload previously examined in this experimental model (Aggeli et al., 2001a,b).

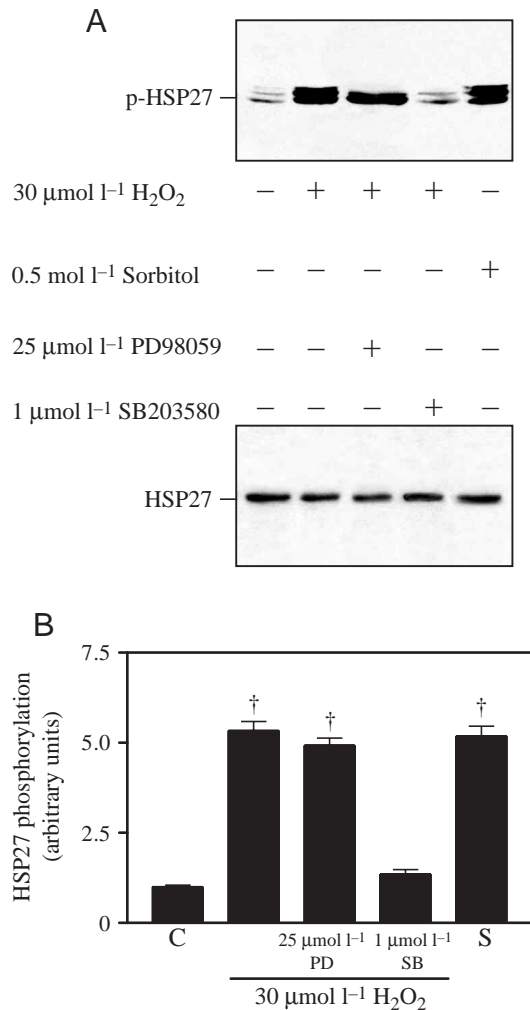


Fig. 7. Phosphorylation of HSP27 by  $\text{H}_2\text{O}_2$ . Protein (100  $\mu\text{g}$ ) from hearts perfused with  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min in the absence or presence of  $25 \mu\text{mol l}^{-1}$  PD98059 or  $1 \mu\text{mol l}^{-1}$  SB203580 was assessed by immunoblot for phosphospecific anti-HSP27 antibody (A, top panel) or total anti-HSP antibody (A, bottom panel). As a positive control, extract from hearts perfused with  $0.5 \text{ mol l}^{-1}$  sorbitol was included. Blots are representative of three independent experiments. (B) Densitometric analysis of phospho-HSP27 bands by laser scanning. Results are means  $\pm$  S.E.M. for three independent experiments.  $\dagger P < 0.001$  vs control value. C, control; S, sorbitol.

Our studies on the stimulation of the principal stress-activated p38-MAPK by  $\text{H}_2\text{O}_2$  showed that this kinase is activated in a time- and dose-dependent manner. The immediate phosphorylation of the kinase by  $\text{H}_2\text{O}_2$  (within the first 30 s of treatment) indicates the importance of an early response of the amphibian ventricular cells to oxidative stress. The immediate response of the p38-MAPK cascade to  $\text{H}_2\text{O}_2$  has been also reported for rat neonatal myocytes (Clerk et al., 1998a). The maximal phosphorylation of the kinase was detected within 2 min of  $\text{H}_2\text{O}_2$  treatment, while a progressive decline of the kinase activation levels was observed thereafter (Fig. 4). Stimulation of the p38-MAPK phosphorylation was

completely inhibited by its selective inhibitor SB203580 at a concentration of  $1 \mu\text{mol l}^{-1}$  (Fig. 5). This transient activation of JNKs and p38-MAPK by oxidative stress may be regulated by either dephosphorylation *via* the respective phosphatases, scaffolding proteins or feedback mechanisms (Kyriakis and Avruch, 2001; Pearson et al., 2001).

To confirm that phosphorylation of p38-MAPK represents its activation, we also tried to examine the phosphorylation state of its specific substrate MAPKAPK2, utilising antibodies that have recently become available and that specifically detect either the phosphorylated (activated) or the total levels of the kinase. The results of these studies clearly showed that  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  induced a strong phosphorylation of MAPKAPK2 similar to the p38-MAPK activation time-dependent profile and that  $1 \mu\text{mol l}^{-1}$  SB203580 abolished this response.

Using antibodies specific to the phosphorylated form of the small heat shock protein HSP27, we also showed that oxidative stress induced a strong phosphorylation of this protein. Overall, using these specific antibodies, we showed a direct pathway from p38-MAPK  $\rightarrow$  MAPKAPK2  $\rightarrow$  HSP27, since the selective p38-MAPK inhibitor SB203580 abolished the oxidative stress-induced phosphorylation of both MAPKAPK2 and HSP27 (Figs 6 and 7 for MAPKAPK2 and HSP27, respectively). Our results are consistent with previous reports describing the possible mechanisms involved in the protective responses of the mammalian heart against oxidative stress (Clerk et al., 1998a,b; Wang et al., 1998b).

Furthermore, the immunohistochemical studies we performed revealed that oxidative stress induced a strong phosphorylation of both p38-MAPK and HSP27 in the ventricular cells of the amphibian heart (Figs 8 and 9 for p38-MAPK and HSP27, respectively). The localisation pattern observed indicates that this signalling pathway was activated by  $\text{H}_2\text{O}_2$ , which was administered exogenously. Previous studies have established that the expression of the cytoplasmic small HSPs is regulated at either the transcriptional level (Frohli et al., 1993) or by post-transcriptional modifications such as phosphorylation, deamination and acylation (Lavoie et al., 1995). The phosphorylation of HSP27 is catalysed by MAPKAPK2 in a stress-dependent manner (Landry et al., 1992; Knauf et al., 1994). Although the influence of HSP27 phosphorylation on cellular stress responses is still controversial and not clearly defined, it is well established that this protein has a wide variety of different and seemingly unrelated functions ranging from a molecular chaperone to a mediator of thermoresistance and chemoresistance. It also inhibits actin polymerisation, regulates apoptosis and protects ribonucleic acids (Sakamoto et al., 2000; Geum et al., 2002; Concannon et al., 2003).

The roles of the different MAPK subfamilies in vertebrate heart function are still under investigation. In many differentiated cells, ERK activation is associated with cell survival (Xia et al., 1995), whereas the stress-activated JNKs and p38-MAPK may promote apoptosis (Xia et al., 1995; Park et al., 1997; Turner et al., 1998). In the amphibian heart, however, p38-MAPK



Fig. 8. Immunohistochemical localisation of phosphorylated p38-MAPK in the ventricle of isolated amphibian heart perfused with  $\text{H}_2\text{O}_2$ . Hearts were perfused (A) under normal conditions, with  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min in (B) the absence or (D) the presence of  $1 \mu\text{mol l}^{-1}$  SB203580 or (C) with  $0.5 \text{ mol l}^{-1}$  sorbitol for 15 min. Cryosections were incubated with phosphospecific anti-p38-MAPK antibody (1:200 dilution) and processed as described in Materials and methods. Representative photographs from three independent experiments are shown. Immunolocalisation deposits are visualised with Fast Red chromogen. Scale bar,  $20 \mu\text{m}$ .

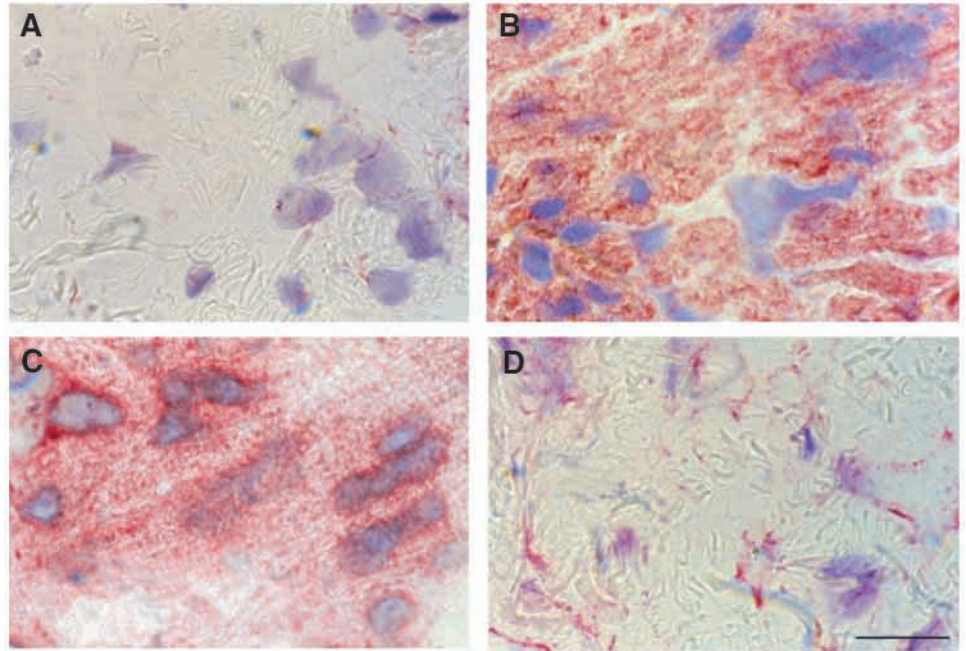
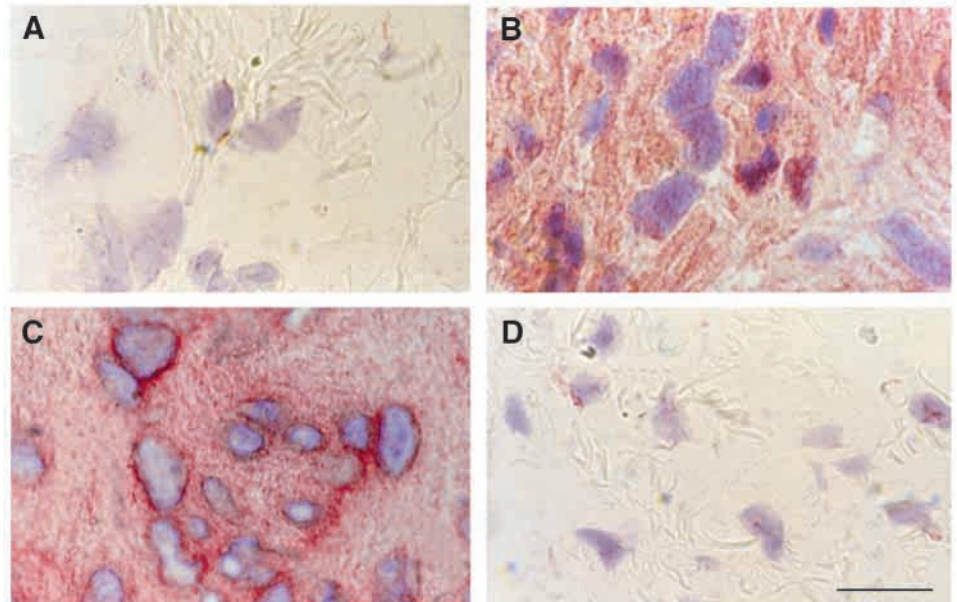


Fig. 9. Immunohistochemical localisation of phosphorylated HSP27 in the ventricle of isolated amphibian heart perfused with  $\text{H}_2\text{O}_2$ . Hearts were perfused (A) under normal conditions, with  $30 \mu\text{mol l}^{-1}$   $\text{H}_2\text{O}_2$  for 2 min in (B) the absence or (D) the presence of  $1 \mu\text{mol l}^{-1}$  SB203580 or (C) with  $0.5 \text{ mol l}^{-1}$  sorbitol for 15 min. Cryosections were incubated with phosphospecific anti-HSP27 antibody (1:200 dilution) and processed as described in Materials and methods. Representative photographs from three independent experiments are shown. Scale bar,  $20 \mu\text{m}$ .



stimulation of MAPKAPK2 and HSP27 phosphorylation is potentially cytoprotective. Both we and others have previously reported that anoxia or anoxia/reoxygenation does not stimulate the p38-MAPK signalling pathway in the perfused *R. ridibunda* heart or in tissues from the freeze-tolerant wood frog *Rana sylvatica* (Greenway and Storey, 2000; Aggeli et al., 2001a). Anoxia or hypoxia tolerance is a common feature of animals that can often face and sustain these environmental conditions *in vivo*. It has also been reported that in various tissues of such tolerant animals the levels of antioxidants are higher than in respective mammalian tissues, a feature representative of the different adaptive mechanisms existing between mammals and lower

vertebrates (Hermes-Lima et al., 2001). In particular, amphibians, as ectotherms, can face tremendous variations *in vivo* in both body temperature and body water content, while their life style is consistent with a wide annual range of activity that obviously modulates their metabolic demands according to the status of their environment. These changes in metabolic demands require compensatory changes in cardiac output. Therefore, environmental stresses leading to cardiac mechanical overload, either directly or indirectly (i.e. thermal or hyperosmotic stresses), could be strong modulators of metabolic ROS overproduction, thus inducing oxidative stress in the amphibian heart.

In summary, we have shown that H<sub>2</sub>O<sub>2</sub>, a ROS that can be produced in the amphibian heart under various conditions of environmental stress, activates all three MAPK subfamilies (ERK, JNKs and p38-MAPK). Furthermore, activation of p38-MAPK stimulates phosphorylation of MAPKAP2, which in turn phosphorylates the HSP27, possibly leading to cell survival. In addition, the immunohistochemical studies we performed provide evidence that under oxidative stress the presence of phosphorylated forms of p38-MAPK and HSP27 is enhanced. All these findings taken together could indicate a possible involvement of p38-MAPK and HSP27 in preservation of amphibian heart homeostasis under similar situations *in vivo*.

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