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# Differential roles of p38-MAPK and JNKs in mediating early protection or apoptosis in the hyperthermic perfused amphibian heart

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## SUMMARY

**In the present study the activation of p38 mitogen-activated protein kinase (p38-MAPK) and c-Jun N-terminal kinases (JNKs) by hyperthermia was investigated in the isolated perfused *Rana ridibunda* heart. Hyperthermia (42°C) was found to profoundly stimulate p38-MAPK phosphorylation within 0.5 h, with maximal values being attained at 1 h [4.503(±0.577)-fold relative to control, $P<0.01$]. JNKs were also activated under these conditions in a sustained manner for at least 4 h [2.641(±0.217)-fold relative to control, $P<0.01$]. Regarding their substrates, heat shock protein 27 (Hsp27) was maximally phosphorylated at 1 h [2.261(±0.327)-fold relative to control, $P<0.01$] and c-Jun at a later phase [3 h: 5.367(±0.081)-fold relative to control, $P<0.001$]. Hyperthermia-induced p38-MAPK activation was found to be dependent on the $Na^+/H^+$ exchanger 1 (NHE1) and was also suppressed by catalase (Cat) and superoxide dismutase (SOD), implicating the generation of reactive oxygen species (ROS). ROS were also implicated in the activation of JNKs by hyperthermia, with the $Na^+/K^+$-ATPase acting as a mediator of this effect at an early stage and the NHE1 getting involved at a later time point. Finally, JNKs were found to be the principal mediators of the apoptosis induced under hyperthermic conditions, as their inhibition abolished poly(ADP-ribose) polymerase (PARP) cleavage after 4 h at 42°C. Overall, to our knowledge, this study highlights for the first time the variable mediators implicated in the transduction of the hyperthermic signal in the isolated perfused heart of an ectotherm and deciphers a potential salutary effect of p38-MAPK as well as the fundamental role of JNKs in the induced apoptosis.**

Key words: hyperthermia, frog heart, p38-MAPK, JNKs, PARP, apoptosis, protection

## INTRODUCTION

In the light of the extensive changes observed in the earth's climate in our era and their subsequent effects on the temperature regime of various ecosystems, studying the mechanisms regulating the response to hyperthermia appears to be of considerable interest. In particular, ectothermic vertebrates, including amphibians, can experience large and rapid fluctuations of their body temperature in the process of adapting to the temperature fluctuations occurring in their physical environment (Driedzic and Gesser, 1994). What is more, temperature functions as a major determinant of the kinetics of biochemical pathways affecting cellular homeostasis. Thus, with the cardiac muscle being thermally sensitive as far as its electrophysiological behavior is concerned, the amphibian heart constitutes an excellent candidate experimental model for any relative studies. Therefore, we made an attempt to elucidate the signaling mechanisms triggered by hyperthermia in the perfused *Rana ridibunda* heart, as well as to decipher the effectors involved in the transduction of this signal mediating the terminal response in these organisms.

Mitogen-activated protein kinases (MAPKs) constitute a highly conserved superfamily with a fundamental role in signal transduction of cellular stress stimuli (Bogoyevitch, 2000). The three best-characterized members are the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 reactivating kinase (p38-MAPK) (Kyriakis and Avruch, 2001; Pearson et al., 2001). Our research group has previously identified the respective MAPKs in the amphibian heart (Aggeli et al., 2001) as well as several of their target substrates in both the cytoplasm and the nucleus, which have been shown to transduce a variety of molecular signals similarly to mammals (Bogoyevitch, 2000). JNKs along with p38-MAPK are widely known as stress-activated protein kinases (SAPKs), being activated mainly by stressful conditions (Tibbles and Woodgett, 1999) and eliciting a cascade of interactions related to cellular survival or apoptosis. Thus, it was of interest to examine their activation pattern in the perfused amphibian heart under conditions of heat shock. The effect of antioxidant enzymes (superoxide dismutase scavenging $O_2^-$ and catalase depleting $H_2O_2$) along with specific channel pump inhibitors (cariporide inhibiting the $Na^+/H^+$ exchanger and ouabain suppressing the $Na^+/K^+$-ATPase) was also examined to help elucidate the effectors involved in the observed responses, given the implications of heat stress on membrane ion permeability (e.g. $Na^+$, $H^+$) (Sonna et al., 2002).

Cell fate has been ascribed to the dynamic balance between these signaling networks. JNKs have been proposed to stimulate apoptosis as a response to UV radiation, hyperosmolarity, ischemia–reperfusion, heat shock, or oxidative stress (Westwick et al., 1995; Chen et al., 1996; Cuvillier et al., 1996; Zanke et al., 1996). However, there is also evidence of JNKs promoting survival under adverse conditions (Dougherty et al., 2002). Thus, the role of JNKs appears controversial, as is also the case for p38-MAPK, because it has been shown to elicit cell survival (Liu et al., 2001; Park et al., 2002) but also to occasionally enhance programmed cell death (Porras et al., 2004). Overall, the final effect conferred in each case appears to depend on the nature of the stress stimulus encountered and its duration, as well as the type of experimental setting used. c-Jun activation by JNKs has been reported to exert a leading role

in transmitting and converting stress stimuli into apoptotic signaling (Bossy-Wetzel et al., 1997; Faris et al., 1998), while activation of the p38-MAPK→MAPKAPK2 (MAPK-activated protein kinase 2)→Hsp27 pathway may be cytoprotective (Krajewski et al., 1999; Gaitanaki et al., 2003). Nevertheless, accepting this 'scheme' without substantial evidence would be an oversimplification given the controversy surrounding the actual biological impact of SAPKs.

Several reports have pointed to reactive oxygen species (ROS) generation by heat, leading to oxidative damage to DNA (Eigner et al., 1961; Greer and Zamenhof, 1962; Lindahl, 1993). Given that heat shock disturbs the intracellular redox equilibrium, the resulting oxidative stress would be expected to contribute to the apoptotic impact observed (Privalle and Fridovitch, 1987; Flanagan et al., 1998). Apoptosis may be triggered by variable stimuli and is executed by caspases that can be activated by signal transduction pathways associated with stimulation of death receptors (extrinsic pathway) or mitochondrial stress (intrinsic pathway) leading to the release of cytochrome *c* and stimulation of downstream effector caspases (Fumarola and Guidotti, 2004). Proteolytic processing of poly(ADP-ribose) polymerase (PARP) constitutes a classical hallmark of apoptosis as this family of enzymes, demonstrating poly(ADP-ribosyl)ation activity, participate in various biological functions including DNA repair, genomic stability and apoptosis (Burkle, 2005).

Collectively, our results highlight for the first time the signal transduction mechanisms involved in the phosphorylation and thus activation of p38-MAPK and JNKs by hyperthermia in the isolated perfused *Rana ridibunda* heart. In particular, oxidative stress conditions were shown to be generated by perfusion at 42°C, while our findings also underscore the involvement of two different sodium pumps in the regulation of the response stimulated: the $Na^+/H^+$ exchanger initially and the $Na^+/K^+$-ATPase at a later stage. Furthermore, while we have observed the p38-MAPK cascade to possibly exert a cytoprotective effect under these interventions as an early cellular response, JNKs were found to exert a critical role in the apoptosis that occurs subsequently in a caspase 3-independent manner. Further studies are required, however, to decipher and fully elucidate the identity of the complex signaling mechanisms regulating the response of cardiac muscle of ectotherms to heat shock, and to reveal the unique features of the physiology of these organisms and their exquisite ability to adapt to the variations characterizing their physical environment.

## MATERIALS AND METHODS

### Materials

Catalase (C-30) and superoxide dismutase (SOD; S-2515) were obtained from Sigma Chemical Co. (St Louis, MO, USA). HOE642 (a $Na^+/H^+$-exchanger inhibitor) and ouabain O3125 (a $Na^+/K^+$-ATPase inhibitor) were from Sanofi-Aventis (Frankfurt, Germany) and Sigma Chemical Co. (St Louis, MO, USA), respectively. JNK-inhibitors SP600128 and AS601245 were obtained from Calbiochem (EMD Chemicals Inc., Darmstadt, Germany). Stock solutions of SP600128 (10 mmol $l^{-1}$), AS601245 (10 mmol $l^{-1}$) and HOE642 (50 mmol $l^{-1}$) were prepared in DMSO, whereas ouabain, catalase and SOD were diluted directly in the perfusion buffer.

Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Pre-stained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH, USA). Rabbit polyclonal antibodies specific for dually phosporylated p38-MAPK (no. 9211) and JNKs (no. 9251), and phosphorylated c-Jun (no. 9261) and Hsp27 (no. 2401) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody specific for caspase 3, detecting the endogenous levels of full-length and large active fragments of caspase 3 (no. 9662), was from Cell Signaling Technology. The antibody that detects the endogenous levels of full-length and inactive fragments of PARP resulting from cleavage (no. 9542) was also purchased from Cell Signaling Technology. The rabbit polyclonal antibody specific for actin (A2103) was from Sigma Chemical Co. Secondary antibody conjugated with horse radish peroxidase (HRP) was from DAKO A/S (Glostrup, Denmark). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala, Sweden) while Super RX film was purchased from Fuji photo film GmbH (Dusseldorf, Germany). Most general laboratory reagents used were purchased from Sigma Chemical Co.

### Animals

Frogs (*Rana ridibunda* Pallas 1771) weighing 120–150 g were caught in the vicinity of Thessaloniki, Greece, and were supplied by a local dealer. They were kept in containers in fresh water and received humane care in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the Greek Government (160/1991) based on EC regulations (86/609). They were used 1 week after arrival.

### Heart perfusions

Frogs were anesthetized by immersion in 0.01% (w/v) MS222 and killed by decapitation. The hearts were excised and mounted onto an aortic cannula. Perfusions were performed in a Langendorff perfusion system at a pressure of 4.5 kPa (31.5 mmHg) with a bicarbonate-buffered saline [23.8 mmol $l^{-1}$ $NaHCO_3$, 103 mmol $l^{-1}$ NaCl, 1.8 mmol $l^{-1}$ $CaCl_2$, 2.5 mmol $l^{-1}$ KCl, 1.8 mmol $l^{-1}$ $MgCl_2$, 0.6 mmol $l^{-1}$ $NaH_2PO_4$, pH 7.4 at 25°C] supplemented with 10 mmol $l^{-1}$ glucose and equilibrated with 95% $O_2$:5% $CO_2$. Heart and perfusate temperature were maintained at 25°C using apparatus with a water jacket. All hearts were equilibrated for 0.5 h under these conditions. Following the equilibration period, hearts were perfused at 42°C for periods of time ranging from 0.5 to 6 h. In addition, after the 0.5 h equilibration period, hearts were also perfused at 42°C for 1 or 4 h in the presence of ouabain (100 μmol $l^{-1}$), HOE642 (5 μmol $l^{-1}$), SOD (30 U $ml^{-1}$) or catalase (150 U $ml^{-1}$). Perfusions were also performed in the presence of SP600128 (10 μmol $l^{-1}$) or AS601245 (1 μmol $l^{-1}$) at 42°C for 4 h. When inhibitors (ouabain, HOE642, SP600128 or A601245) were used, they were also present during the equilibration period. Control hearts were also perfused with the inhibitors, catalase or SOD alone. In addition to this, depending on the duration of each experimental protocol, the respective control hearts were assayed. In particular, to ensure that the observed effects were a direct consequence of hyperthermia, samples from hearts perfused at 25°C for increasing time periods ranging from 0.5 to 6 h were also processed. Details of the control used in each case are outlined in the figure legends. In parallel, the electrical and mechanical activity of the hearts were monitored using an appropriate setting. In particular, contractile activity was measured by means of a force displacement transducer (Grass FT03C; Grass Instruments, Quincy, MA, USA), which was connected to the apex of the heart, while continuous electrocardiogram (ECG) measurements of intracardiac activity were performed as previously described (Gaitanaki et al., 2002). Readings were recorded using a HAMEG oscilloscope and respective software (HAMEG Instruments, D-63533, Mainhausen, Germany). Additionally, samples of the effluent perfusate were collected at corresponding time intervals; protein content was estimated by the Bradford method while pH was also monitored. At the end of the

perfusions, atria were removed and ventricles, after being immersed in liquid $N_2$, were pulverized and the powder stored at –80°C.

### Preparation of cytoplasmic and nuclear extracts

Heart powders were homogenized with 3 ml $g^{-1}$ buffer A [10 mmol $l^{-1}$ Hepes, pH 7.9, 10 mmol $l^{-1}$ KCl, 0.1 mmol $l^{-1}$ EGTA, 0.1 mmol $l^{-1}$ EDTA, 1.5 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ NaF, 1 mmol $l^{-1}$ $Na_3VO_4$, 10 mmol $l^{-1}$ β-glycerophosphate, 1 mmol $l^{-1}$ dithiothreitol (DTT), 0.5 mmol $l^{-1}$ phenylmethylsulphonyl fluoride (PMSF), 2 μg $ml^{-1}$ leupeptin, 4 μg $ml^{-1}$ aprotinin]. Following homogenization with a micropestle, and after extraction on ice for 30 min, samples were centrifuged (2700 ***g***, 10 min, 4°C). Supernatants containing the cytoplasmic protein fraction were collected. The remaining pellets were resuspended in 400 μl buffer A and 31 μl of 10% (v/v) Nonidet P-40. After extraction on ice for 10 min, samples were centrifuged (2700 ***g***, 10 min, 4°C) and supernatants were discarded. The remaining pellets were resuspended in 80 μl of buffer B [20 mmol $l^{-1}$ Hepes, pH 7.9, 400 mmol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ EGTA, 0.1 mmol $l^{-1}$ EDTA, 1.5 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ NaF, 1 mmol $l^{-1}$ $Na_3VO_4$, 20 mmol $l^{-1}$ β-glycerophosphate, 20% (v/v) glycerol, 0.2 mmol $l^{-1}$ DTT, 0.5 mmol $l^{-1}$ PMSF, 2 μg $ml^{-1}$ leupeptin, 4 μg $ml^{-1}$ aprotinin]. After extraction at 4°C for 1 h, samples were centrifuged (23 000 ***g***, 10 min, 4°C). Supernatants (nuclear fraction) were collected and both the cytoplasmic and nuclear protein fractions were boiled with 0.33 volumes of SDS-PAGE sample buffer [0.33 mol $l^{-1}$ Tris-HCl, pH 6.8, 10% SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) Bromophenol Blue]. Protein concentrations were determined using the Bradford assay reagent.

### Evaluation of caspase 3 activation

Additionally, heart powders were homogenized with 3 ml $g^{-1}$ CHAPS buffer [50 mmol $l^{-1}$ Hepes, pH 6.5, 2 mmol $l^{-1}$ EDTA, 0.1% (w/v) CHAPS, 20 μg $ml^{-1}$ leupeptin, 10 μg $ml^{-1}$ pepstatin A, 10 μg $ml^{-1}$ aprotinin, 5 mmol $l^{-1}$ DTT, 1 mmol $l^{-1}$ PMSF]. Following homogenization with a micropestle, samples were repeatedly frozen (3 times, –80°C) and left to thaw. Homogenates were then centrifuged (20 800 ***g***, 20 min, 4°C) and supernatants were boiled with 0.33 volumes of SDS-PAGE sample buffer. Protein concentrations were determined using the Bradford assay.

### Immunoblotting

Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bis-acrylamide slab gels [or 15% (w/v) acrylamide, 0.413% (w/v) bis-acrylamide slab gels for caspase 3 blots] and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Non-specific binding sites were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS-T [20 mmol $l^{-1}$ Tris-HCl, pH 7.5, 137 mmol $l^{-1}$ NaCl, 0.05% (v/v) Tween 20] for 30 min at room temperature. Subsequently, membranes were incubated overnight with the appropriate primary antibody (1:1000) at 4°C. After washing in TBS-T (4×5 min), blots were incubated with the respective HRP-conjugated secondary antibody [1:5000 dilution in TBS-T containing 1% (w/v) BSA] for 1 h at room temperature. After washing the blots in TBS-T (4×5 min), bands were detected using ECL, exposed to super RX film and quantified by laser scanning densitometry (Gel Analyzer v. 1.0, Biosure, Athens, Greece).

### Statistical evaluation

Western blots shown are representative of at least three independent experiments. All data are presented as means ± s.e.m. Comparisons between controls and treatments were performed using Student's unpaired *t*-test. A value of $P<0.05$ was considered to be statistically significant. All values were normalized against the respective total protein levels. Phosphorylation of p38-MAPK, JNKs, Hsp27 and c-Jun, as well as PARP fragmentation, in control hearts, was set at one and their detected phosphorylation as well as the respective PARP fragmentation, in treated hearts, was expressed as 'fold' activation over control hearts.

## RESULTS

Temperature is one of the most significant ecological stressful factors that may affect the survival of organisms, especially of ectotherms. So as to identify any potential viability loss of the cardiac tissue under the interventions used in the present study, the electromechanical activity of the hearts, protein loss into the perfusate and perfusate pH were determined. No significant change was observed in the contractile tension measured at 25°C (Fig. 1A left panel and Fig. 1B) while after 6 h perfusion at 42°C, a 20% decrease was observed (Fig. 1A right panel and Fig. 1B) compared with the initial recording (Fig. 1A middle panel and Fig. 1B). Regarding the protein loss detected in the perfusate, it was undetectable at 25°C (data not shown), whereas at 42°C the loss detected during the first 5 h of the perfusion period was not significant while it reached 1/10 of the initial protein content after 6 h (data not shown). Given that the electromechanical properties of the heart remained largely unchanged throughout the experiments and taking into consideration the fact that the total protein loss was not significant, any effects observed can be characterized and classified as a response to hyperthermia and not as a consequence of any tissue viability loss. In addition to this, no variations were detected in the pH stability of the perfusate during the above interventions.

Given that p38-MAPK and JNKs comprise the principal members of the MAPK superfamily, which participate in the transduction of stress signals, an effort was made to characterize their phosphorylation pattern in samples from isolated amphibian hearts perfused under hyperthermic conditions (42°C). Thus, immunoblotting analysis with an antibody against the dually phosphorylated form of p38-MAPK revealed the robust but transient increase in the phosphorylation levels of this kinase, which were maximal at 1 h [4.503(±0.577)-fold relative to control, $P<0.01$] decreasing thereafter (Fig. 2A upper panel and Fig. 2B). By performing a respective analysis for the time profile of Hsp27 phosphorylation, a known p38-MAPK substrate, we found that it too was increased, with maximal values being attained at 1 h [2.261(±0.327)-fold relative to control, $P<0.01$] and decreasing thereafter (Fig. 2C upper panel and Fig. 2D). Equal protein loading was verified by reprobing the membranes with an anti-actin antibody (Fig. 2A,C bottom panels). Perfusion at 42°C also led to moderate phosphorylation of JNKs in a prolonged manner. Thus, a rapid onset of JNKs phosphorylation was observed, which was maximally induced after 0.5 h [2.812(±0.156)-fold relative to control, $P<0.001$] of perfusion, remaining elevated for at least 4 h [2.641(±0.217)-fold relative to control, $P<0.01$; Fig. 3A upper panel and Fig. 3B]. The well established JNKs substrate c-Jun was similarly found to be phosphorylated with maximal values attained at 3 h of perfusion at 42°C [5.367(±0.081)-fold relative to control, $P<0.001$; Fig. 3C upper panel and Fig. 3D]. Looking at phosphorylation of p38-MAPK, Hsp27, JNKs and c-Jun in samples from hearts perfused at 25°C for corresponding times, no significant activation signal was detected compared with the control (data not shown).

Subsequently, with heat stress having been established to compromise cell survival, we decided to investigate the possible

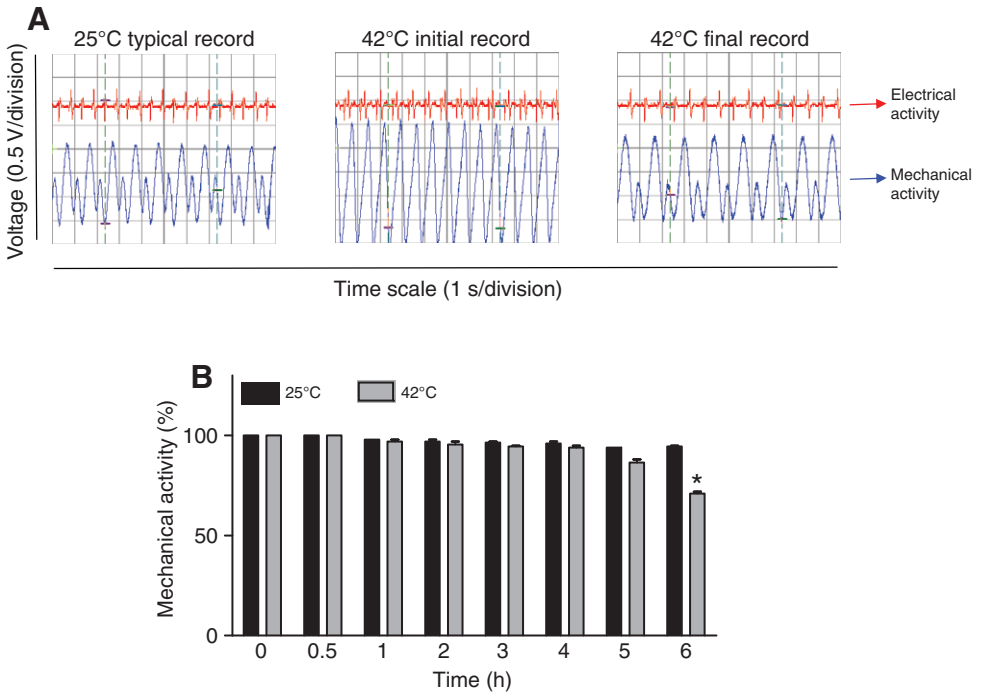


Fig. 1. (A) Representative records of the isolated perfused *Rana ridibunda* heart electromechanical activity. Isolated hearts were perfused at either 25 or 42°C for up to 6 h with bicarbonate buffer pH 7.35, and the electrical as well as mechanical activity were monitored throughout using an appropriate setting as described in Materials and methods. (B) Time course of the mechanical activity (%) of the isolated perfused heart at the two different temperatures studied. **P*<0.05 *vs* the respective value at time zero.

occurrence of apoptosis under the interventions used in this study. No activation of caspase 3 was observed, as evidenced by the detection solely of pro-caspase 3 and not of its cleaved fragment using an anti-caspase 3 primary antibody that recognizes pro-caspase 3 as well as its cleaved active forms (Fig. 4A upper panel). The proteolytic processing of PARP was also investigated, a marker routinely used to monitor apoptotic cell death (Strasser et al., 2000). Interestingly, 4 h perfusion at 42°C was found to markedly increase the generated PARP fragment band intensity [6.054(±0.103)-fold relative to control, $P<0.001$; Fig. 4B upper panel and Fig. 4C]. This result is indicative of hyperthermia triggering apoptosis in a caspase 3-independent manner. While investigating caspase 3 activation as

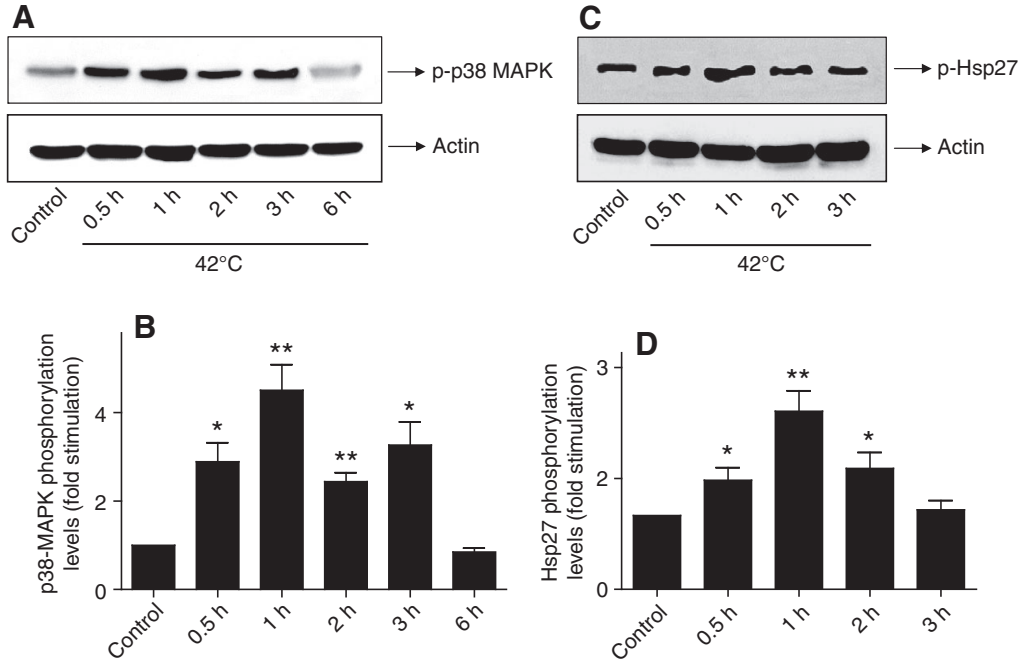


Fig. 2. Time profile of hyperthermia (42°C)-induced p38-MAPK (A) and Hsp27 (C) phosphorylation in samples from isolated perfused *Rana ridibunda* hearts. (A) Upper panel: phospho-p38-MAPK was detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration at 25°C followed by 0.5 h perfusion at 25°C) or hearts perfused after equilibration at 42°C for increasing periods of time as indicated. (C) Upper panel: phospho-Hsp27 was detected in corresponding samples. As a control for equal protein loading, an anti-actin antibody was used (A,C: bottom panels). Densitometric analysis of phospho-p38 (B) and phospho-Hsp27 (D) bands was performed by laser scanning. Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. from at least three independent experiments. **P*<0.05 and ***P*<0.01 *vs* control (untreated) hearts.

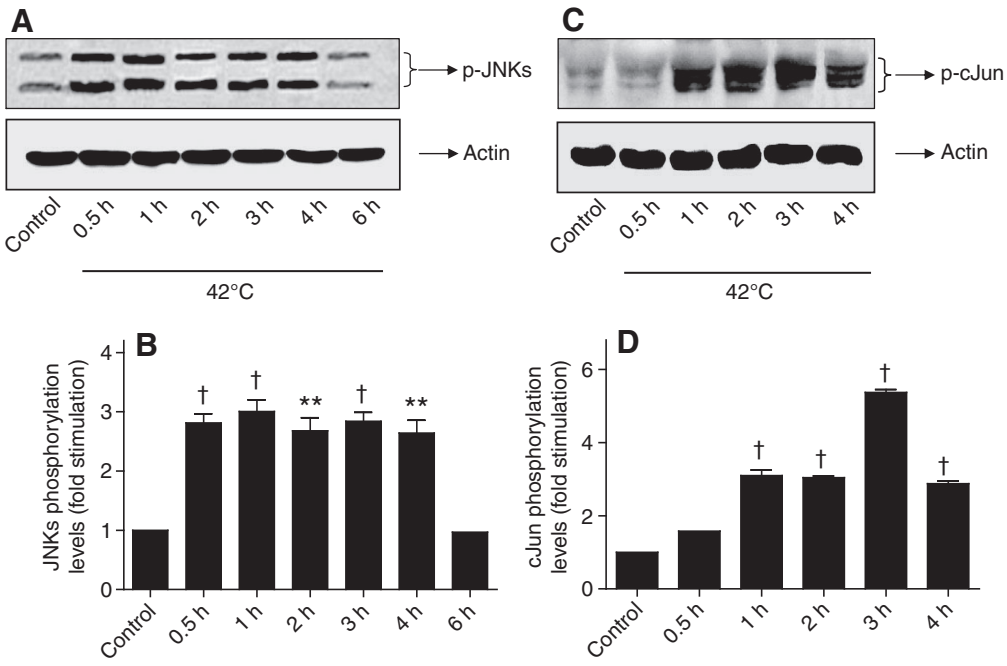


Fig. 3. Time course of phosphorylation of JNKs and c-Jun in samples from isolated perfused *Rana ridibunda* hearts. (A) Upper panel: phospho-JNKs were detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration at 25°C followed by 0.5 h perfusion at 25°C) or hearts perfused after equilibration at 42°C for increasing periods of time as indicated. (C) Upper panel: phospho-c-Jun was detected in corresponding samples. The membranes were re-probed with an anti-actin antibody so as to verify equal protein loading (A,C: bottom panels). Densitometric analysis of phospho-JNKs (B) and phospho-c-Jun (D) bands was performed by laser scanning. Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. $^{**}P<0.01$ and $^{\dagger}P<0.001$ *vs* control (untreated) hearts.

well as PARP fragmentation in samples from hearts perfused at 25°C for corresponding times, no signal different to the basal control levels shown was detected (data not shown).

Furthermore, in order to investigate the signaling mechanisms regulating this response, the effect of a number of inhibitors was assessed. In particular, we initially studied the effect of two known ROS counteracting enzymes: (a) catalase and (b) superoxide dismutase (SOD) as well as the potential role of ion channels that are involved in cellular pH regulation. To this end, the effects of HOE642, an inhibitor of the $Na^+/H^+$ exchanger 1 (NHE1) and ouabain, known to inhibit $Na^+/K^+$-ATPase, were investigated. Hearts were perfused with the inhibitors alone (data not shown) and the respective amount of DMSO alone (data not shown), as well as at 42°C in the presence or absence of the inhibitors. Interestingly, both antioxidants were found to almost ablate p38-MAPK phosphorylation (catalase by ~70±2.33% and SOD by ~77±3.57%, $P<0.001$; Fig. 5A upper panel and Fig. 5B,C), implicating ROS in the observed effect. In addition to this, HOE642 alone induced p38-MAPK phosphorylation [6.516(±0.313)-fold relative to control, $P<0.001$]. After subtracting this effect, HOE642 was shown to significantly inhibit the kinase phosphorylation (by ~70±2.65%, $P<0.001$) while ouabain had no such effect (Fig. 5A upper panel and Fig. 5B,C), implicating NHE1 in the mechanism activating the p38-MAPK pathway. By blotting with an antibody raised against actin, we once more confirmed equal protein loading (Fig. 5A bottom panel).

Notably, when examining the mechanism controlling the response of JNKs to hyperthermia, regulation of phosphorylation of the kinases was revealed to have a time-dependent profile. In particular, although catalase and SOD considerably suppressed phosphorylation of JNKs at both time points examined (at 1 h: catalase by ~71±3.37% and SOD by ~66±2.71%, $P<0.01$; and at 4 h: catalase by ~63±2.55% and SOD by ~51±2.81%, $P<0.001$; Figs 6 and 7A upper panels and Fig. 7B,C), ouabain was found to partially inhibit activation of JNKs only after 1 h perfusion (by ~49±2.47%, $P<0.01$; Fig. 6A upper panel and Fig. 6B,C), while HOE642 had an inhibitory effect solely at 4 h (by ~51±2.37, $P<0.001$; Fig. 7A upper panel and Fig. 7B,C). Thus, different effectors appear to mediate phosphorylation and hence activation of JNKs, as an early and a late response.

Given our interest in the actual biological impact of the signaling mechanisms regulating the transduction of the hyperthermic effect, we next investigated the effect of these inhibitors on the proteolytic cleavage of PARP and hence on apoptosis. As shown in Fig. 8A upper panel and Fig. 8B, catalase, SOD and HOE642 were all found to almost ablate the detrimental effect of hyperthermia while this was not the case in the presence of ouabain. Since this profile was similar to the effect of these inhibitors on phosphorylation of JNKs (at 4 h) we decided to probe into the potential involvement of JNKs in the observed apoptotic phenotype. Accordingly, in the presence of two established JNKs inhibitors, SP600125 and AS601245, PARP cleavage was hindered, indicating a principal role of JNKs in conferring apoptosis (Fig. 8A,B, last two lanes).

## DISCUSSION

Ectothermic vertebrates experience abrupt and profound variations in their body temperature with subsequent physicochemical adaptations, so as to ensure preservation of their biological functions. In this context, temperature is a key determinant of cellular activity, affecting principal factors of survival, including the electrophysiological behavior of cardiac muscle (Gennser et al., 1990). Therefore, the amphibian heart constitutes an ideal experimental setting for investigation of the signal transduction

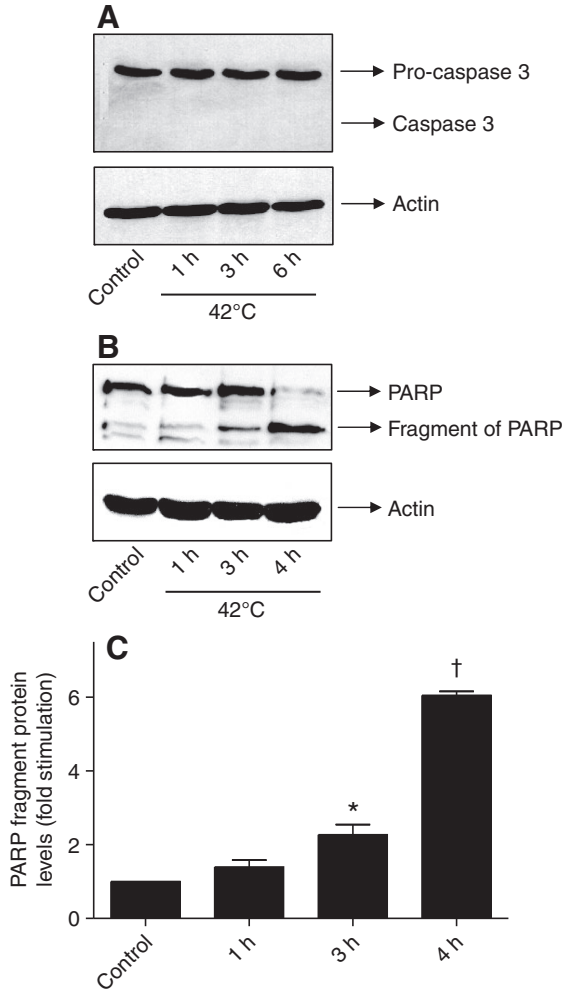


Fig. 4. Profile of caspase 3 and poly(ADP-ribose) polymerase (PARP) cleavage in samples from isolated *Rana ridibunda* hearts perfused after equilibration at 42°C for increasing periods of time as indicated. (A) Upper panel: pro-caspase 3 immunoreactivity was observed with no cleaved active forms of caspase 3 detected. (B) Upper panel: proteolytic processing of PARP was observed and quantification of its fragment was performed by laser scanning densitometry (C). The membranes were re-probed with an anti-actin antibody so as to verify equal protein loading (A,B: bottom panels). Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. **P*<0.05 and †*P*<0.001 *vs* control hearts (0.5 h equilibration followed by 1 h perfusion at 25°C).

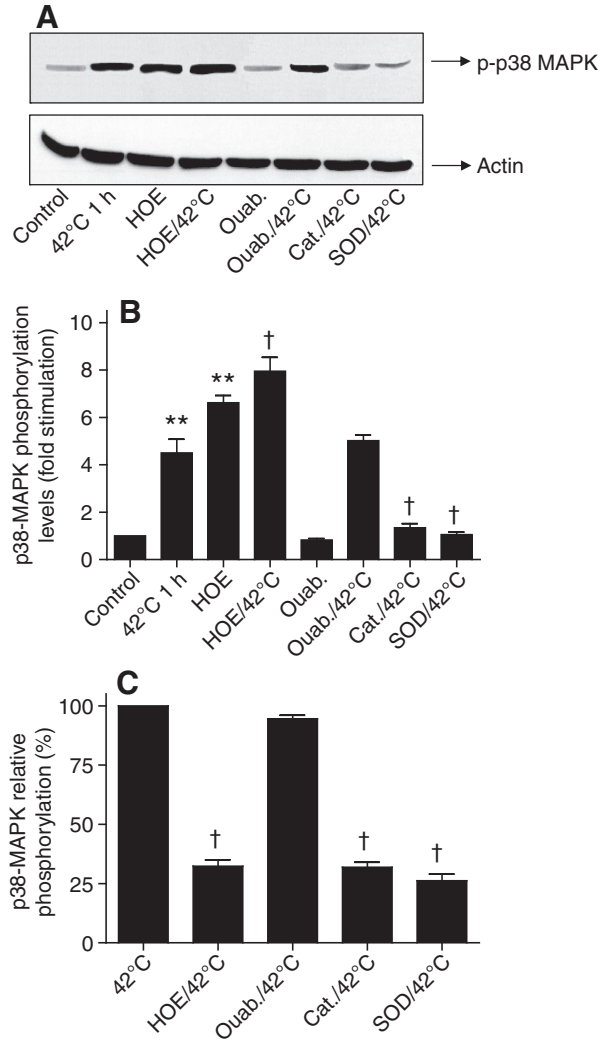


Fig. 5. Effect of HOE642 (HOE), ouabain (Ouab.), catalase (Cat.) and superoxide dismutase (SOD) on hyperthermia-induced phosphorylation of p38-MAPK (A: upper panel). The phosphorylated form of the kinase was detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration followed by 1 h perfusion at 25°C) or hearts perfused after equilibration for 1 h at 42°C in the presence or absence of HOE642 (5 μmol $l^{-1}$), ouab (100 μmol $l^{-1}$), catalase (150 U $ml^{-1}$) and SOD (30 U $ml^{-1}$). The effects of HOE642 and ouabain alone were also assessed. As a control for equal protein loading an anti-actin antibody was used (A: bottom panel). Densitometric analysis of phospho-p38-MAPK (B) was performed by laser scanning. (C) Relationship of the net p38-MAPK phosphorylation levels induced by hyperthermia in the presence of the agents assessed to the hyperthermia-induced p38-MAPK phosphorylation levels. Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. ***P*<0.001 *vs* control hearts and †*P*<0.001 *vs* hearts perfused at 42°C.

mechanisms regulating the response to such adverse environmental conditions.

Hyperthermia or heat shock has been shown to induce a stress response that also triggers the activation of a superfamily of protein kinases which are ubiquitously expressed and highly conserved throughout evolution: the mitogen-activated protein kinases (MAPKs) (Kyriakis and Avruch, 1996). Among the MAPK subfamilies, JNKs and p38-MAPK are collectively known as the principal stress-activated protein kinases (SAPKs) (Tibbles and Woodgett, 1999). These kinases transduce extracellular stimuli to the cytoplasm or nucleus, where they interact with their respective substrates, eliciting a cellular response favoring survival or apoptosis (Bogoyevitch, 2000).

In the present study, the observed p38-MAPK activation (Fig. 2) may serve a cardioprotective role against thermal stress in the isolated perfused amphibian heart, as has previously been reported in other experimental models under variable forms of stress (Lavoie et al., 1995; Clerk et al., 1998; McFalls et al., 2004). Through its detected hyperthermia-induced phosphorylation, Hsp27, a well-established p38-MAPK substrate, may contribute to the preservation of cytoskeletal stability under harsh conditions (Huot et al., 1996) and promote inhibition of the mitochondrial apoptotic pathway *via* ablation of cytochrome *c* release (Paul et al., 2002). What is more, Hsp27 has been shown to prevent apoptosis by suppressing the

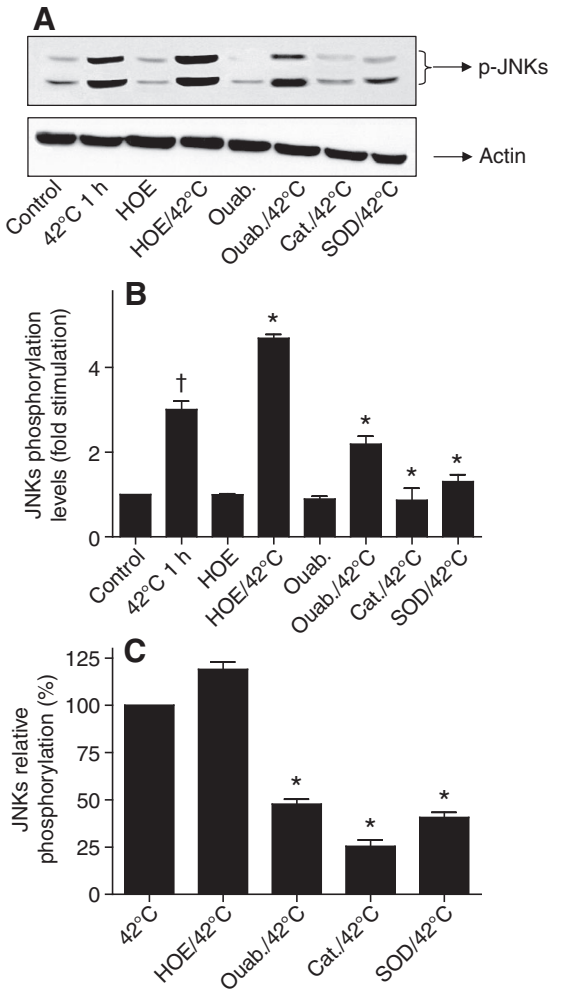


Fig. 6. Effect of HOE642, ouabain, catalase and SOD on hyperthermia-induced phosphorylation of JNKs (A: upper panel). Phosphorylated JNKs were detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration followed by 1 h perfusion at 25°C) or hearts perfused after equilibration for 1 h at 42°C in the presence or absence of HOE642 (5 μmol $l^{-1}$), ouabain (100 μmol $l^{-1}$), catalase (150 U $ml^{-1}$) and SOD (30 U $ml^{-1}$). The effects of HOE642 and ouabain alone were also assessed. As a control for equal protein loading, an anti-actin antibody was used (A: bottom panel). Densitometric analysis of phospho-JNKs (B) was performed by laser scanning. (C) Relationship of the net JNK phosphorylation level (%) induced by hyperthermia in the presence of the agents assessed to the hyperthermia-induced JNKs phosphorylation level. Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. †$P$<0.001 *vs* control hearts and *$P$<0.01 *vs* hearts perfused at 42°C.

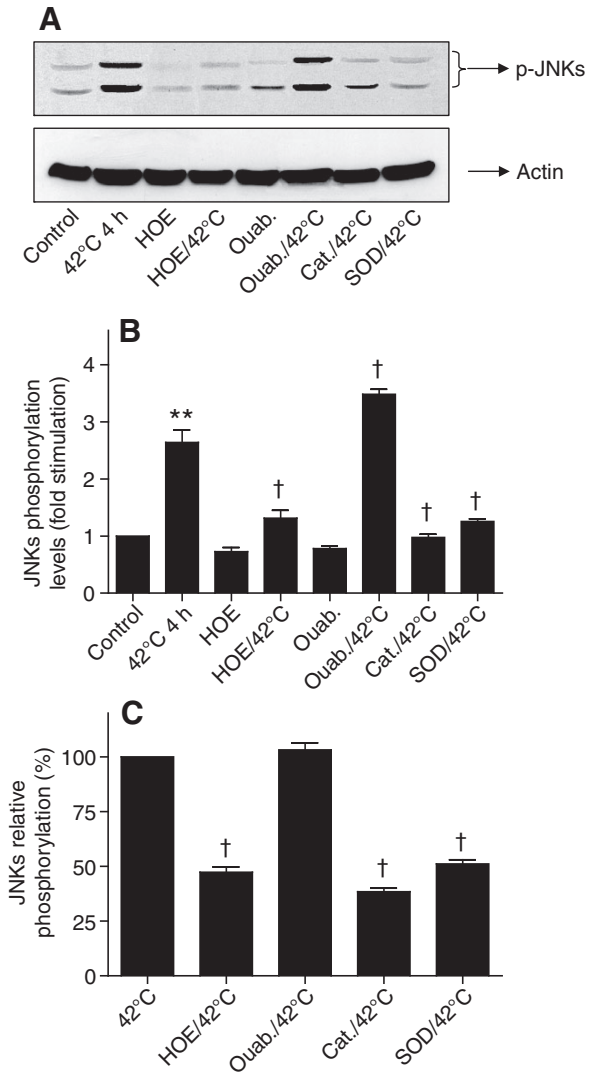


Fig. 7. Effect of HOE642, ouabain, catalase and SOD on hyperthermia-induced phosphorylation of JNKs (A: upper panel). Phosphorylated JNKs were detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration followed by 4 h perfusion at 25°C) or hearts perfused after equilibration for 4 h at 42°C in the presence or absence of HOE642 (5 μmol $l^{-1}$), ouabain (100 μmol $l^{-1}$), catalase (150 U $ml^{-1}$) and SOD (30 U $ml^{-1}$). The effects of HOE642 and ouabain alone were also assessed. As a control for equal protein loading, an anti-actin antibody was used (A: bottom panel). Densitometric analysis of phospho-JNKs (B) was performed by laser scanning. (C) Relationship of the net JNK phosphorylation level (%) induced by hyperthermia in the presence of the agents assessed to the hyperthermia-induced JNK phosphorylation level. Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. **$P$<0.001 *vs* control hearts and †$P$<0.001 *vs* hearts perfused at 42°C.

proteolytic maturation of caspases (Mehlen et al., 1996; Samali and Cotter, 1996). Consistent with these results, our research group has previously demonstrated the p38-MAPK→Hsp27 pathway to exert a protective effect on the amphibian heart under conditions of oxidative stress (Gaitanaki et al., 2003) or extracellular alkalosis (Stathopoulou et al., 2006). Our group has also previously reported the induction of both p38-MAPK and Hsp27 phosphorylation by hyperthermia at an earlier time point (15 min), indicating the biphasic profile of this signaling cascade and its potential immediate protective effect (Gaitanaki et al., 2007). Quite interestingly, in a previous study of ours, probing into the short-term p38-MAPK response to hyperthermia, the kinase activation was found to be maximal at 5 min reaching basal levels by 1 h of treatment (Aggeli et al., 2002). This apparent discrepancy could be attributed to the amphibian heart being seasonally acclimatized to heat (animals were collected at the end of the summer period). In this case, the observed transient activation of p38-MAPK could preserve a cellular homeostasis. However, in the present study, using samples from animals collected during winter, the p38-MAPK activation profile was found to be significantly prolonged in order for the kinase to exert its potential cardioprotective role.

In addition, Adler and colleagues have noted heat shock as a potent inducer of phosphorylation of JNKs and c-Jun in 3T3-4A mouse fibroblast cell line (Adler et al., 1995). Our findings, showing a moderate and sustained activation profile for JNKs correlating with the detected phosphorylation pattern of their substrate c-Jun (Fig. 3), are also in accordance with the study of Kyriakis and Avruch, who reported JNKs-dependent phosphorylation of the c-Jun transcription factor, which led to an increase in its transactivating activity (Kyriakis and Avruch, 1996). Despite a number of studies reporting a protective role for JNKs under stressful conditions, their ultimate biological effect appears to be dependent on the nature of the stimulus involved, as well as on the duration and extent of their activation, since there are also reports of them conferring apoptosis. In an attempt to decipher the impact of activation of p38-MAPK and JNKs on our experimental model and with heat shock known to trigger apoptosis (Creagh et al., 2000), we next tried to identify any features characteristic of apoptosis. Caspases are the primary regulators and effectors of this form of programmed cell death (Bohm and Schild, 2003; Regula and Kirshenbaum, 2005); however, no cleavage of caspase 3-indicative of apoptosis was detected (Fig. 4A). Nevertheless, the cleaved fragments of PARP detected with immunoblotting (Fig. 4B,C) provided evidence that apoptosis does occur. PARP functions as a system detecting DNA breaks, which enables the activity of DNA repair enzymes (Burkle, 2001; Scovassi and Diederich, 2004). Further studies are required in order to highlight the exact mechanism regulating this effect since the notion that caspases are not the sole effectors of apoptosis has already been established (Regula and Kirshenbaum, 2005).

Various reports have confirmed that thermal stress induces a plethora of biochemical compensatory responses including inhibition of RNA processing and translation, inhibition of DNA synthesis, protein denaturation, disruption of cytoskeletal components as well as alterations in ion membrane permeability affecting ion flux (Lindquist, 1986; Fujita, 1999). Hyperthermia has also been found to disturb the cellular redox status inducing oxidative stress (Privalle and Fridovitch, 1987; Davidson et al., 1996; Flanagan et al., 1998). In particular, Bruskov and colleagues have pointed to the generation of ROS by hyperthermia, creating an abnormal electrolyte milieu affecting $H^+$, $Na^+$ and $K^+$ ion movements, leading eventually to DNA damage (Bruskov et al., 2002).

Correlating the above, formation of ROS was verified by investigation of the regulation of hyperthermia-induced p38-MAPK phosphorylation (Fig. 5). With SOD being well known to function as a defense system against the superoxide anion [$O_2^-$] (McCord and Fridovich, 1969) and catalase established to counteract hydrogen peroxide ($H_2O_2$) (Fridovich, 1999), our results implicate $O_2^-$ and $H_2O_2$ as mediators of the observed p38-MAPK cascade activation. Numerous studies report that ROS alter membrane ion pump function in cardiac muscle, affecting mainly cardiac sodium channels and ion exchangers (Giordano, 2005). Accordingly, by using cariporide (HOE642), we found the NHE1, a primary pH regulatory effector (Aronson, 1985), to participate in the signaling network transducing the particular hyperthermic stimulus. NHE1 function consists of proton extrusion triggered by the transmembrane sodium gradient but has also been reported to limit ROS-induced damage in the cardiac muscle (Teshima et al., 2003; Fantinelli et al., 2006). As far as activation of JNKs is concerned, fluctuations in intracellular ion dynamics and the formation of ROS was shown to affect it at an early stage with the activity of the transmembrane $Na^+/K^+$-ATPase initially recruited, while as a late response, it was the activity of the sarcolemmal NHE1 pump that appeared to cross-talk with the JNKs pathway, exchanging intracellular $H^+$ with $Na^+$ (Figs 6 and 7, respectively).

Given the contradictory studies noting the salutary or detrimental effects of JNKs in terms of cell fate (Davis, 2000; Aoki et al., 2002; Dougherty et al., 2002), the above novel findings along with the confirmation of JNKs involvement in triggering apoptosis under the experimental conditions investigated (Fig. 8) constitute the first commentary on how hyperthermia is transmitted and converted to an apoptotic signal in the isolated perfused amphibian heart. On the other hand, our results also highlight a possible cytoprotective role of p38-MAPK, under these interventions in this particular experimental setting, correlating with other studies underscoring p38-MAPK function as a mediator of survival (Liu et al., 2001; Park et al., 2002).

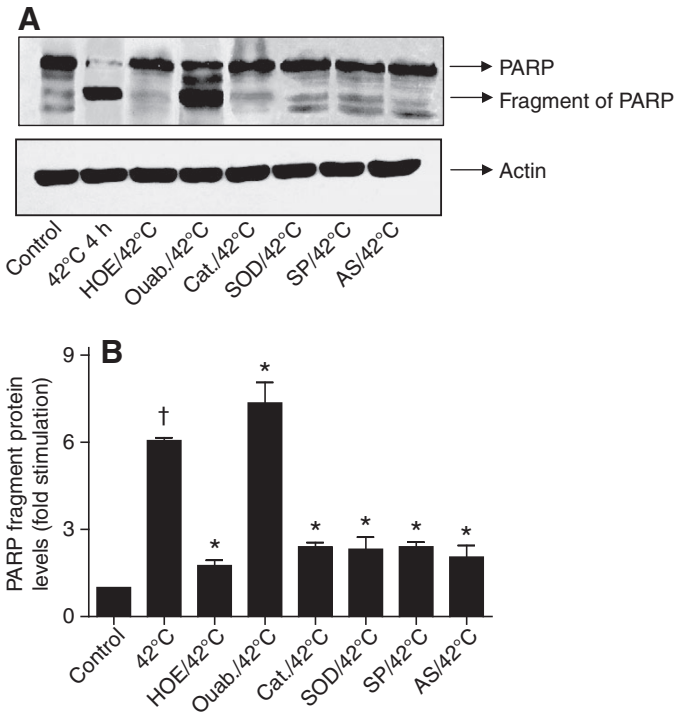


Fig. 8. Effect of HOE642, ouabain, catalase, SOD, SP600125 (SP) and AS601245 (AS) on hyperthermia-induced cleavage of PARP (A: upper panel). Proteolytical processing of PARP was detected in extracts (50 μg of protein) from control hearts (0.5 h equilibration followed by 4 h perfusion at 25°C) or hearts perfused after equilibration for 4 h at 42°C in the presence or absence of HOE642 (5 μmol $l^{-1}$), ouab (100 μmol $l^{-1}$), catalase (150 U $ml^{-1}$), SOD (30 U $ml^{-1}$), SP (10 μmol $l^{-1}$) and AS (1 μmol $l^{-1}$). To confirm equal protein loading the membranes were re-probed with an anti-actin antibody (A: bottom panel). Densitometric analysis of the band corresponding to the fragment of PARP was performed by laser scanning (B). Western blots shown are representative of at least three independent experiments while data are means ± s.e.m. for at least three independent experiments. †$P$<0.001 *vs* control hearts and *$P$<0.001 *vs* hearts perfused at 42°C.

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