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Cu^{2+} and acute thermal stress induce protective events *via* the p38-MAPK signalling pathway in the perfused *Rana ridibunda* heart

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Summary

In the present study, we investigated the induction of the p38-MAPK signalling pathway by copper, as exemplified by $CuCl_2$, in the isolated perfused heart of the amphibian *Rana ridibunda*. We found that p38-MAPK phosphorylation by $CuCl_2$ occurs in a dose-dependent manner, with maximum activation (8.73±1.43-fold relative to control values) attained by perfusion with 500 μmol l^{-1} $CuCl_2$ for 15 min, while this activation sustained even after 60 min of reperfusion with normal bicarbonate buffer. $CuCl_2$ also induced the phosphorylation of the small heat shock protein 27 (Hsp27) in a p38-MAPK dependent manner, as revealed by experiments using the p38-MAPK inhibitor SB203580. p38-MAPK and Hsp27 phosphorylations were also strongly induced by hyperthermia (42°C), while the simultaneous use of hyperthermia and $CuCl_2$ had a synergistic effect on p38-MAPK activation. Furthermore, perfusions with the potent antioxidant L-ascorbic acid (100 μmol l^{-1}), the antioxidant enzymes catalase (CAT) (150 U ml^{-1}) or superoxide dismutase (SOD) (30 U ml^{-1}) in the presence of 500 μmol l^{-1} $CuCl_2$ did not attenuate the $CuCl_2$-induced p38-MAPK activation, implying that at least the reactive oxygen species (ROS) scavenged by these agents are not implicated in this kinase activation. The p38-MAPK phosphorylation induced by the combined action of $CuCl_2$ and hyperthermia was partially inhibited by catalase, indicating that hyperthermia possibly activates the kinase through the production of H_2O_2. Caspase-3, an effector protease of apoptosis, remained inactive in hearts perfused at normal or hyperthermic conditions, in the absence or presence of 500 μmol l^{-1} $CuCl_2$. All the above results suggest that, in the amphibian *Rana ridibunda* heart, p38-MAPK activation by copper has a possible protective role through the small Hsp27.

Key words: oxidative stress, thermal stress, copper, antioxidants, p38-MAPK, Hsp27, amphibian heart, *Rana ridibunda*, signal transduction.

Introduction

Mammalian cardiac muscle is an obligate aerobic organ and under anaerobic conditions cannot produce enough energy to maintain essential cellular processes; thus, a constant supply of oxygen is indispensable for sustaining cardiac function and viability (Giordano, 2005). On the other hand, ectothermic vertebrates, like amphibians, face a wide range of seasonal variations in their natural environment (Hoffman and Katz, 1997; Hutchison and Dupr, 1992), such as low oxygen pressure or temperature imbalances (Hermes-Lima and Storey, 1996; Hermes-Lima and Storey, 1998; Greenway and Storey, 2000), which are related to increased production of reactive oxygen species (ROS), while their skin is a site for gas exchange and high permeability to water (Katz et al., 1986). As a result, these organisms have developed effective antioxidant defences so as to adapt to the adverse environmental conditions (Hermes-Lima and Storey, 1996; Hermes-Lima and Storey, 1998).

Copper, a heavy transition metal, can exist in both an oxidized, cupric (Cu^{2+}), or reduced, cuprous (Cu^{+}) state (Linder and Hazegh-Azam, 1996) and is able to move across cell membranes or through ion channels (Handy et al., 2002) into the cells. As a redox-active metal, copper plays important catalytic roles as a cofactor in many enzymes such as copper-zinc superoxide dismutase and cytochrome oxidase, which renders it an essential trace element for all aerobic organisms (Solomon and Lowery, 1993; Gaetke and Chow, 2003). At elevated cellular copper levels, rapid generation of ROS is induced *via* a Haber-Weiss or Fenton-like reaction (Gutteridge, 1985; Shi and Dalal, 1992; Gaetke and Chow, 2003). Interestingly, copper catalyzes the formation of hydroxyl radicals, which lead to oxidative damage of lipids, proteins and nucleic acids (Gaetke and Chow, 2003). In addition, this metal can be toxic by direct binding to protein sulfhydryl groups, which results in enzyme inactivation or altered protein conformation (Jeon et al., 2000). Recent studies have demonstrated that copper accumulates in the tissues of the

amphibian *Rana ridibunda* (Papadimitriou and Loumbourdis, 2003), the experimental model used in the present study.

Another well-established environmental parameter with profound effects on cellular biochemistry, morphology and function, is temperature. In fact, this variable determines the activity of cellular multi-enzyme systems, the kinetics and flux throughout biochemical pathways and, in particular, the metabolic rates. Amphibians normally experience rapid and intense variations in body temperature (T_b) and appear to tolerate such changes with minimal metabolic cost. They utilise physiological mechanisms such as controlled evaporative water loss or peripheral vasodilation/constriction to affect small changes in T_b (Lillywhite, 1971; Shoemaker et al., 1989). In case of extreme temperature changes, thermal stress results in elevated generation of ROS as well as alterations in intracellular antioxidant capacity (Bagnyukova et al., 2003; Gius et al., 2004). Interestingly, mitochondria undergo a temperature-dependent uncoupling of the respiratory chain leading to generation of ROS, thereby increasing the risk of oxidative cell damage (Schiaffonati et al., 1990; Salo et al., 1991).

The disturbances in the physiological context of a cell can trigger the activation of diverse intracellular signal transduction pathways, which determine the cell fate by regulating gene expression and the activation of protective or pro-apoptotic proteins. Among these signalling pathways the mitogen-activated protein kinase (MAPK) ones are included (Kyriakis and Avruch, 2001) and various reports have documented their involvement in thermal- and redox-stressed cells and tissues, including cardiac myocytes and intact myocardium (Turner et al., 1998; Bogoyevitch, 2000; Gaitanaki et al., 2003; Gaitanaki et al., 2006; Vassilopoulos et al., 2005; Aggeli et al., 2002; Aggeli et al., 2006). However, the factors that modulate these pathways have not been clarified in any system studied to date.

In the present study, we describe the effect of copper on the phosphorylation of p38-MAPK in the amphibian heart. The simultaneous impact of copper and acute thermal stress, as well as the effect of various antioxidants on the p38-MAPK signal transduction pathway, are also determined.

Materials and methods

Materials

Most biochemicals used were purchased from Applichem GmbH (Ottoweg 10b, D-64291 Darmstandt, Germany). Catalase (from bovine liver, C-30) and Cu/Zn SOD (from bovine erythrocytes, S-2515) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala 751 84, Sweden). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH, USA). Rabbit polyclonal antibodies specific for the dually-phosphorylated p38-MAPK (#9211) and the phosphorylated (Ser 82) Hsp27 (#2401) were purchased from Cell Signaling Technology (Beverly, MA, USA). A rabbit monoclonal antibody specific for caspase-3 (#9665) that detects the endogenous levels of full-length (35 kDa) and large active fragments (17/19 kDa) of caspase-3 resulting from cleavage at Asp175 was also purchased from Cell Signaling Technology. Anti-actin antibody (A-2103) was from Sigma Chemical Co. The p38-MAPK-inhibitor SB203580 was obtained from Alexis Corporation (CH-4415 Lausen, Switzerland) and stock solutions (10 mmol l^{-1}) prepared in dimethyl sulfoxide (DMSO). Prestained molecular mass markers were from New England Biolabs (P7708S; Ipswich, MA, USA). Horse radish peroxidase (HRP)-conjugated anti-rabbit antibody was from DAKO A/S (Glostrup, Denmark). X-OMAT AR (13×18 cm) film was purchased from Eastman Kodak Company (New York, 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 fresh water and received humane care in accordance to 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 a week after arrival.

Heart perfusions

Animals were anesthetized by immersion in 0.05% (w/v) MS222 and their hearts were excised and preserved in an ice-cold solution similar to the one used for the perfusion. Then, the hearts were 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). The standard perfusion buffer used was the Krebs 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. The temperature of the hearts and perfusates was maintained at 25°C using a water-jacketed apparatus. All hearts were equilibrated for 30 min under these conditions. All ingredients used were directly diluted in the Krebs bicarbonate-buffered saline.

Control hearts (Con) were perfused for 30 min at 25°C with the bicarbonate-buffered saline described above. As positive controls, we used hearts perfused with 50 μmol l^{-1} H_2O_2 for 2 min, after a 30 min equilibration period with the normal Krebs buffer. To examine the effect of the trace metal Cu^{2+}, hearts were equilibrated as previously and perfused for 15 min with increasing concentrations of $CuCl_2$ (50–500 μmol l^{-1}) diluted in the bicarbonate-buffered saline. Perfusions were also conducted in the presence of the specific p38-MAPK inhibitor SB203580 (1 μmol l^{-1}), during both the equilibration period and the perfusion with 500 μmol l^{-1} $CuCl_2$ for 15 min. In another series of experiments, hearts were re-perfused with normal bicarbonate-buffered saline for time periods varying from 2 to 60 min, after the p38-MAPK maximal activation was

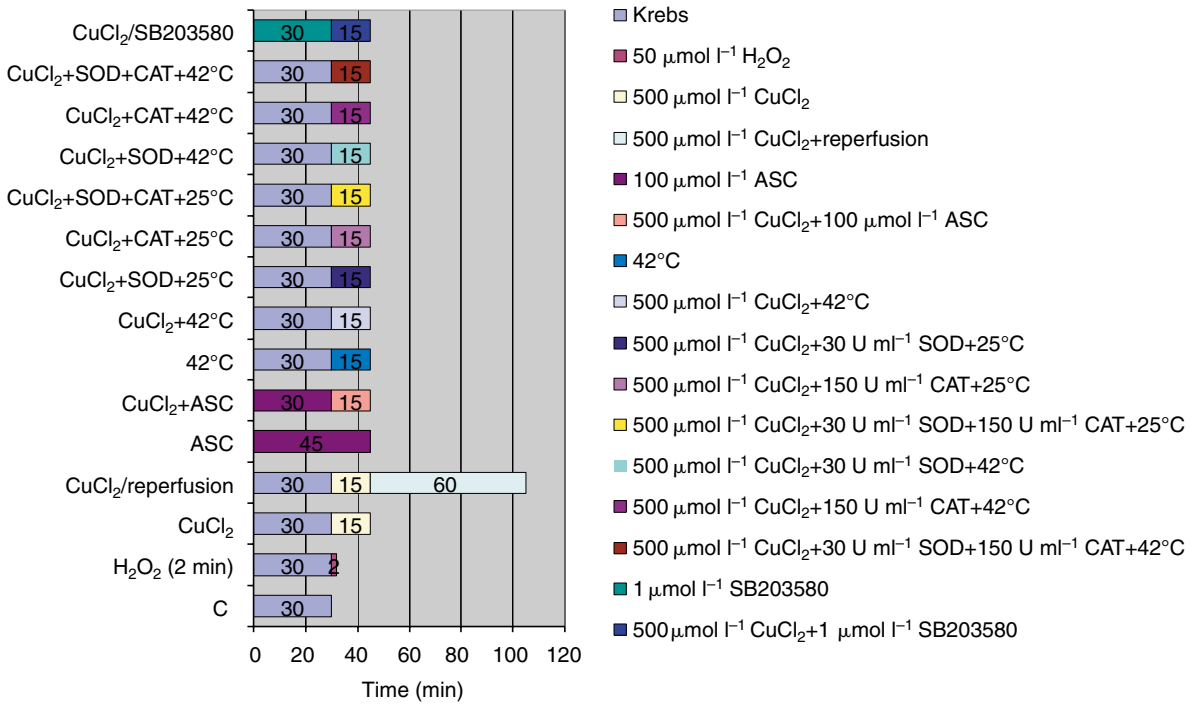

Fig. 1. Perfusion protocols. For further details, see Materials and methods; for abbreviations, see List of abbreviations.

reached. Moreover, the potent antioxidant activity of L-ascorbic acid (ASC) was investigated by perfusing hearts with 100 μmol l^{-1} ASC during both the equilibration period and the following perfusion with 500 μmol l^{-1} $CuCl_2$ for 15 min. Hearts were also perfused for 45 min with normal perfusion buffer containing ASC (100 μmol l^{-1}). In other experiments, after the 30 min equilibration period, temperature was increased up to 42°C, by using a second water-jacketed apparatus, and perfusions were conducted for 15 min under these conditions in the absence or presence of 500 μmol l^{-1} $CuCl_2$. In the last series of experiments, hearts, after being equilibrated with normal bicarbonate-buffered saline, were perfused for 15 min with 500 μmol l^{-1} $CuCl_2$ in the presence of catalase (CAT; 150 U ml^{-1}), SOD (30 U ml^{-1}) or CAT+SOD, at either 25°C or 42°C. All experimental conditions are illustrated in Fig. 1.

At the end of the perfusions, atria were removed and the ventricles frozen by immersion in liquid N_2, then pulverized under liquid N_2. Tissue powders were stored at –80°C.

Tissue extractions

Heart powders were homogenized with 3 ml g^{-1} of buffer [10 mmol l^{-1} Hepes, pH 7.9, 10 mmol l^{-1} EGTA, 0.1 mmol l^{-1} EDTA, 10 mmol l^{-1} NaF, 1 mmol l^{-1} Na_3VO_4, 1.5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} β-glycerophosphate, 1 mmol l^{-1} dithiothreitol (DTT), 2 μg ml^{-1} leupeptin, 0.5 mmol l^{-1} phenyl methyl sulphonyl fluoride (PMSF), 4 μg ml^{-1} aprotinine] and extracted on ice for 30 min. The samples were centrifuged (10000 ***g***, 10 min, 4°C) and the supernatants boiled with 0.33 vol. SDS-PAGE sample buffer [0.33 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 Bio-Rad Bradford assay.

SDS-PAGE and immunoblot analysis

Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bis-acrylamide or 15% (w/v) acrylamide, 0.413% (w/v) bis-acrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in TBS-T [20 mmol l^{-1} Tris-HCl, pH 7.5, 137 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 primary antibody according to the manufacturer's instructions. After washing in TBS-T (4×5 min) the blots were incubated with peroxidase-conjugated anti-rabbit IgG antibodies [1:5000 dilution in TBS-T containing 1% (w/v) BSA for 1 h at room temperature]. The blots were washed again in TBS-T (4×5 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.

Statistical evaluations

All data are presented as means ± 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. All values were normalized against total protein levels. Kinase and Hsp27 phosphorylation in 'control' hearts were set at 1, and the stimulated kinase and

Hsp27 phosphorylation in treated hearts were expressed as '-fold' activation over control hearts.

Results

In the present study, we examined the effects of copper on the p38-MAPK phosphorylation levels. To this end, hearts were perfused with normal bicarbonate-buffered saline containing $CuCl_2$ at concentrations varying from 50 μmol l^{-1} up to 500 μmol l^{-1}, for 15 min. The phosphorylation state of p38-MAPK, which is activated by dual phosphorylation of Thr and Tyr residues within a Thr-Gly-Tyr motif, was assessed by immunoblotting using a specific antibody for this dually phosphorylated form of the kinase (Aggeli et al., 2001a). The results of this study revealed that $CuCl_2$ induced the activation of p38-MAPK in a dose-dependent manner. As can be seen in Fig. 2Ai,B, 50 μmol l^{-1} of this trace metal, in the form of $CuCl_2$, induced a strong p38-MAPK phosphorylation (4.78±1.04-fold relative to control values), whereas 500 μmol l^{-1} $CuCl_2$ induced maximal activation of the kinase (8.73±1.43-fold relative to control values). As a positive control, extract from hearts perfused with 50 μmol l^{-1} H_2O_2 for 2 min was used (Fig. 2Ai). Equal protein loading was confirmed by detecting actin protein levels of identical samples (Fig. 2Aii).

We also investigated whether copper affected the phosphorylation state of a potent member of the p38-MAPK signalling pathway, Hsp27. Hsp27 is phosphorylated at up to three sites (Ser15, Ser78 and Ser82) by MAPK-activated protein kinase 2 (MAPKAPK2) and the related kinase MAPKAPK3, which in turn are activated and phosphorylated by p38-MAPK (Stokoe et al., 1992; Rouse et al., 1994; Gaitanaki et al., 2003). The phosphorylation state of Hsp27 was assessed by immunoblot analysis using a rabbit polyclonal antibody that detects the phosphorylated Hsp27 at Ser82. As can be seen in Fig. 2C,D the phosphorylation of Hsp27 follows a similar dose-dependent pattern to the one observed for p38-MAPK. What is more, both the p38-MAPK and Hsp27 phosphorylations induced by 500 μmol l^{-1} $CuCl_2$ were abolished by 1 μmol l^{-1} of the selective p38-MAPK inhibitor, SB203580 (Fig. 3), confirming that Hsp27 is indeed a downstream member of the p38-MAPK pathway.

We also examined the effect of reperfusion with normal bicarbonate-buffered saline, for time periods varying from 2 up to 60 min, after a 15 min perfusion with 500 μmol l^{-1} $CuCl_2$, (Fig. 4Ai,B). The results revealed that p38-MAPK phosphorylation by 500 μmol l^{-1} $CuCl_2$ was not reversible for the periods of reperfusion examined. On the contrary, a reperfusion period of 2 min with normal buffer almost doubled the activation levels of the kinase (18.44±1.2-fold relative to control values) and p38-MAPK phosphorylation remained at this high level for up to 60 min of reperfusion (21.03±0.15-fold relative to control values). Equivalent protein loading was confirmed by probing identical samples with an antibody recognizing actin (Fig. 4Aii).

In order to examine the effect of thermal stress on $CuCl_2$-induced p38-MAPK phosphorylation, hearts were subjected to hyperthermia (42°C) for 15 min, either in the presence or absence of 500 μmol l^{-1} of this trace metal. The results obtained clearly showed that hyperthermia alone induced a strong p38-MAPK phosphorylation (8.41±1.67-fold relative to

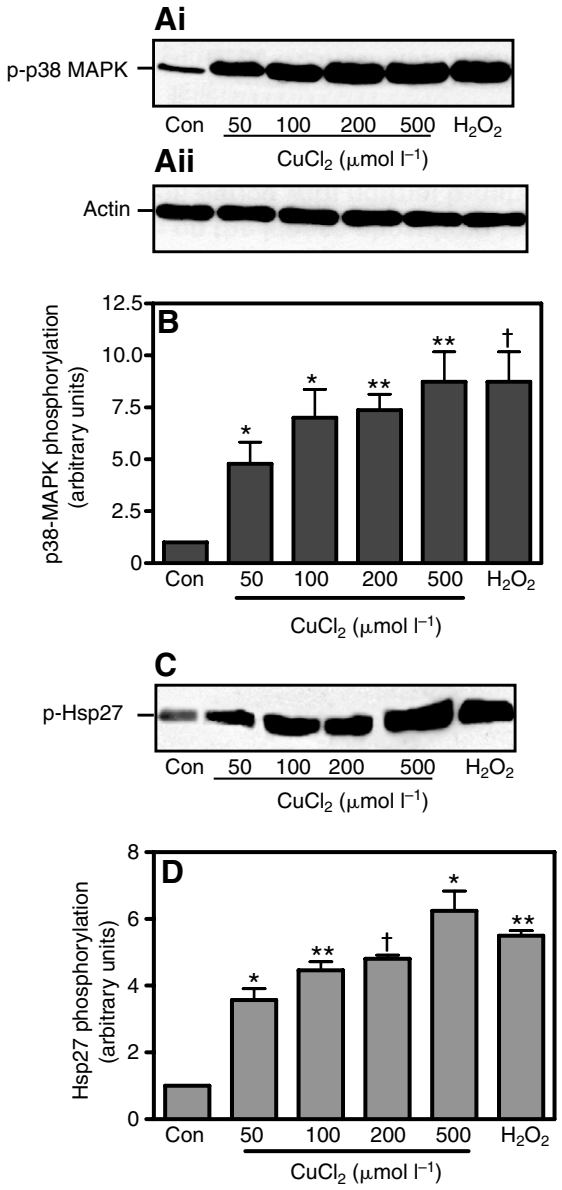

Fig. 2. Phosphorylation of p38-MAPK and Hsp-27 by $CuCl_2$. (A,C) Protein (A, 50 μg or C, 100 μg) from *Rana ridibunda* hearts perfused in the absence (Con) or presence of increasing concentrations of $CuCl_2$ (50–500 μmol l^{-1}) for 15 min was analysed by immunoblotting with anti-p38-MAPK (Ai) and anti-Hsp27 (C) phosphospecific antibodies. As a positive control, extracts from hearts perfused with 50 μmol l^{-1} H_2O_2 for 2 min were included. Identical samples were assayed with an anti-actin antibody as a control for protein loading (Aii). (B,D) Densitometric analysis of phospho-p38-MAPK (B) and phospho-Hsp27 (D) bands, by laser scanning. Results are means ± s.e.m. for three independent experiments. *P<0.05, **P<0.01, $^{\dagger}P$<0.001 *vs* control value.

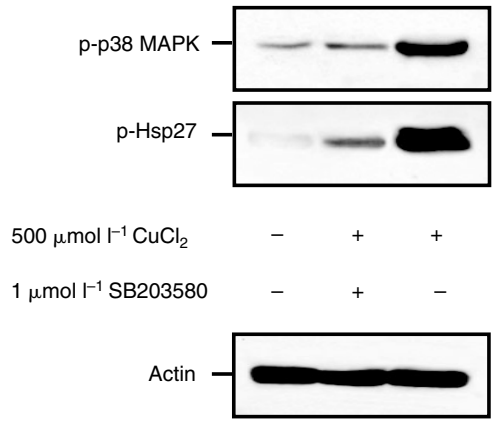

Fig. 3. Effect of the selective inhibitor SB203580 on the p38-MAPK and Hsp27 phosphorylation induced by $CuCl_2$. Protein (50 μg, top and bottom, and 100 μg, middle) from hearts perfused without or with 500 μmol l^{-1} $CuCl_2$ for 15 min in the absence or presence of 1 μmol l^{-1} SB203580 was analysed by immunoblotting with phosphospecific anti-p38-MAPK (top), phosphospecific anti-Hsp27 (middle) or anti-actin (bottom) antibodies. Western blots shown are representative of four independent experiments performed with similar results.

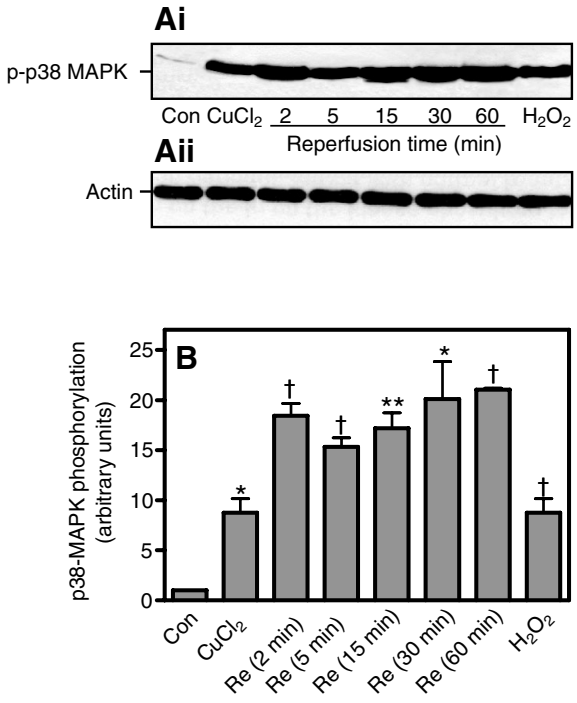

Fig. 4. Time course of p38-MAPK phosphorylation in the amphibian heart, in response to reperfusion after $CuCl_2$ treatment. (A) Phospho-p38-MAPK was detected in extracts (50 μg protein) from control hearts (Con), hearts perfused with 500 μmol l^{-1} $CuCl_2$ for 15 min or hearts perfused for the indicated times with normal bicarbonate-buffered saline following the 15 min perfusion with 500 μmol l^{-1} $CuCl_2$. As a positive control, extract from hearts perfused with 50 μmol l^{-1} H_2O_2 for 2 min was used (Ai). Equal loading was assessed, as previously, using an actin antibody (Aii). (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± s.e.m. for three independent experiments. Re, reperfusion. $^{*}P<0.05$, $^{**}P<0.01$, $^{\dagger}P<0.001$ *vs* control value.

control values), while in the simultaneous presence of $CuCl_2$ this activation was almost doubled (15.29±0.48-fold relative to control values) (Fig. 5Atop,B). Equal protein loading was confirmed by detecting actin protein levels of identical samples (Fig. 5Abottom).

In parallel, the effect of these stressful conditions on the Hsp27 phosphorylation was tested. The results of these experiments revealed that $CuCl_2$ or hyperthermia (42°C) alone induced a significant increase in the Hsp27 phosphorylation ($CuCl_2$: 6.24±0.59-fold relative to control values; hyperthermia: 7.86±0.40-fold relative to control values), whereas the combined effect of $CuCl_2$ and hyperthermia did not result in an additive Hsp27 phosphorylation (6.69±0.58-fold relative to control values) (Fig. 5C,D).

In order to determine whether $CuCl_2$-induced p38-MAPK phosphorylation was due to ROS produced in our experimental model by this trace metal, we performed perfusions in the presence of different antioxidants. First, we perfused amphibian hearts with the potent antioxidant L-ascorbic acid (100 μmol l^{-1}) in either the absence or presence of 500 μmol l^{-1} $CuCl_2$. The results of this study revealed that $CuCl_2$-induced activation of p38-MAPK (8.73±1.43-fold relative to control values) was not changed by L-ascorbic acid (11.24±0.57-fold relative to control values) (Fig. 6Atop,B). Heart perfusions with L-ascorbic acid alone showed that this agent had no effect on the activation of p38-MAPK. Blots assayed with an anti-actin antibody were used as a control for equivalent protein loading (Fig. 6Abottom).

Furthermore, hearts were perfused with 500 μmol l^{-1} $CuCl_2$ in the presence of the superoxide anion scavenger SOD (30 U ml^{-1}), the H_2O_2 scavenger catalase (CAT; 150 U ml^{-1}) or their combination, at either 25°C or 42°C. At 25°C, the scavenging enzymes SOD and CAT, either alone or in combination, had no impact on the p38-MAPK phosphorylation levels induced by $CuCl_2$ (SOD: 10.26±0.24-fold; CAT: 10.36±0.25-fold; SOD/CAT: 9.85±1.37-fold) (Fig. 7Ai,B). As far as the combination of hyperthermia and $CuCl_2$ is concerned, the use of SOD in the absence or presence of catalase did not attenuate p38-MAPK phosphorylation, but interestingly it induced a slight increase in the kinase activation when compared to the one induced by $CuCl_2$ at 42°C ($CuCl_2$: 15.23±0.48-fold; $CuCl_2$/SOD: 18.42±0.32-fold; $CuCl_2$/SOD/CAT: 17.50±0.77-fold relative to control values) (Fig. 7Ai,B). However, when only catalase was used at the above-mentioned conditions the phosphorylation of p38-MAPK was decreased to the levels induced by $CuCl_2$ at 25°C ($CuCl_2$/CAT at 42°C: 9.58±0.78-fold, *vs* $CuCl_2$ at 25°C: 8.73±1.43-fold) (Fig. 7Ai,B). Protein equal loading was assessed by actin immunoblot analysis of respective samples (Fig. 7Aii).

Finally, we examined whether perfusions for 15 min with

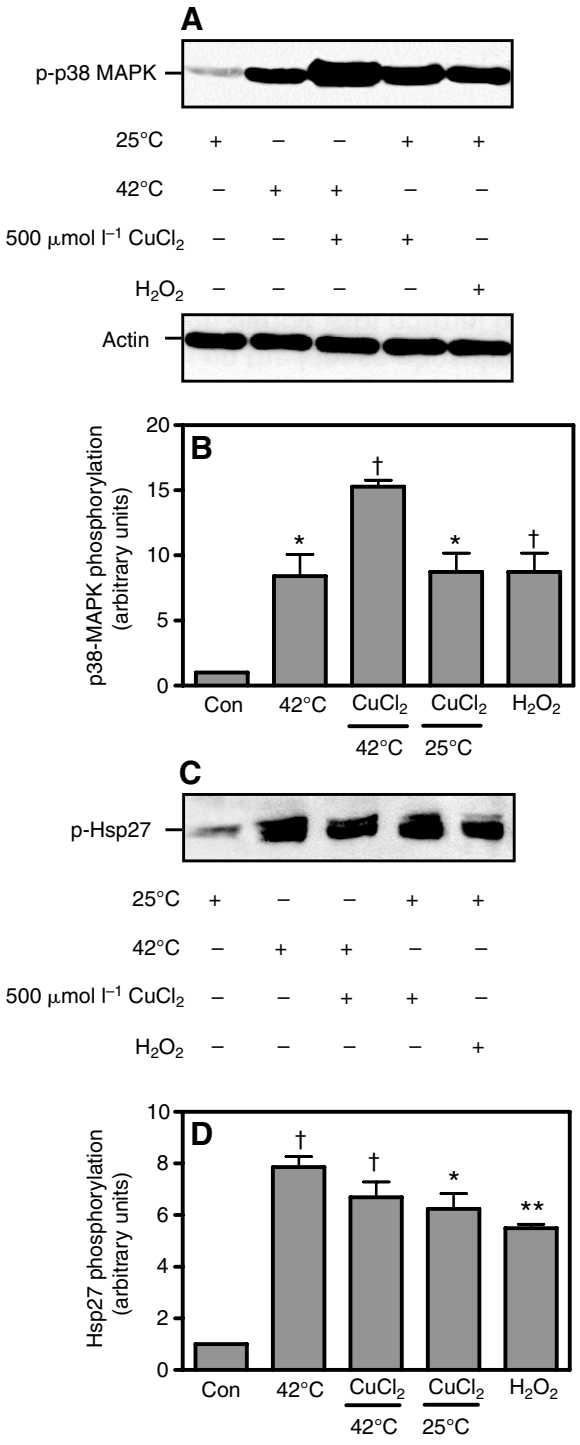

Fig. 5. Phosphorylation of p38-MAPK and Hsp27 by the combined effects of $CuCl_2$ and hyperthermia (42°C). (A,C) Phosphorylated p38-MAPK (A,top) and Hsp27 (C) levels were detected in extracts (50 and 100 μg of protein, respectively) from *Rana ridibunda* hearts perfused for 15 min with normal bicarbonate-buffered saline maintained at 25°C (Con) or at 42°C, either in the absence or presence of 500 μmol l^{-1} $CuCl_2$. As a positive control, extracts from hearts perfused with 50 μmol l^{-1} H_2O_2 for 2 min were included. Identical samples were analysed using an actin antibody as a control for protein loading (A,bottom). (B,D) Densitometric analysis of phospho-p38-MAPK (B) and phospho-Hsp27 (D) bands by laser scanning. Results are means ± s.e.m. for three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{\dagger}P<0.001$ *vs* control value.

500 μmol l^{-1} $CuCl_2$, in the absence or presence of the antioxidant enzymes CAT and SOD, either at normal temperature or under hyperthermia, induced the activation of the pro-apoptotic marker caspase-3. For this reason we performed immunoblotting using a specific antibody that recognizes both the inactive pro-caspase and the active fragmented form of the protease. Our results revealed that under all conditions tested, only the full-length pro-caspase was detected, suggesting therefore, that caspase-3 was not activated (Fig. 8).

Discussion

We had previously reported that, in the amphibian heart, p38-MAPK phosphorylation is induced by different kinds of stress such as mechanical, thermal and oxidative (Aggeli et al., 2001a; Aggeli et al., 2001b; Aggeli et al., 2002; Gaitanaki et al., 2003; Gaitanaki et al., 2006). In particular, in the latter case, the stress was exerted by using different oxidative factors such as H_2O_2 and the enzymatic system of xanthine/xanthine oxidase (Gaitanaki et al., 2003; Gaitanaki et al., 2006; Vassilopoulos et al., 2005).

In the present study, we sought to investigate whether p38-MAPK signalling pathway in the amphibian heart is also activated by copper, in the form of $CuCl_2$, a trace metal known to be associated with the induction of oxidative stress (Gutteridge, 1985; Shi and Dalal, 1992; Gaetke and Chow, 2003). Our results revealed that $CuCl_2$ activates p38-MAPK in a dose-dependent manner (Fig. 2), with maximum activation attained by 500 μmol l^{-1} of this agent. These results are in agreement with several previous studies showing that $CuCl_2$ leads to significant p38-MAPK phosphorylation in diverse kinds of tissues and cell types, including the mantle and gill tissues of the mussel *Mytilus galloprovincialis* (Kefaloyianni et al., 2005), trout hepatoma cells (Burlando et al., 2003), human bronchial epithelial cells (Samet et al., 1998) and human pulmonary artery endothelial cells (Li et al., 2005). The concentrations of the agent and the time-points where maximum p38-MAPK activation was observed varied among individual studies, reflecting the differences in the experimental models.

The effect of $CuCl_2$ on kinase phosphorylation levels was doubled after a 2 min reperfusion period with normal bicarbonate-buffered saline and was sustained even after 60 min of $CuCl_2$ withdrawal (Fig. 4). This considerable effect of reperfusion on p38-MAPK activation may be due to the fact that Cu^{2+} could not be removed from the heart tissue and this might enhance the kinase activation.

Recent studies have also revealed that hyperthermia is likely to lead to the activation of multiple protein kinases, including p38-MAPK (Woessmann et al., 1999; Aggeli et al., 2002), which may then regulate the stress response either by facilitating the repair of damaged proteins and other cellular

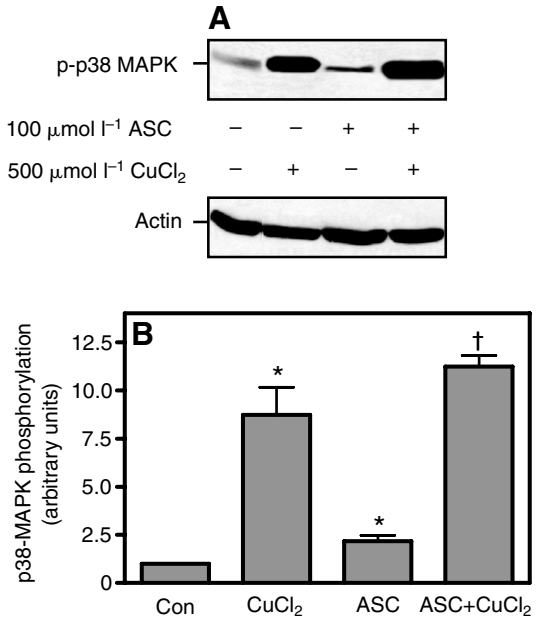

Fig. 6. Effect of L-ascorbic acid on the $CuCl_2$–induced p38-MAPK phosphorylation. (A) Protein (50 μg) from *Rana ridibunda* hearts perfused without (Con) or with 100 μmol l^{-1} L-ascorbic acid (ASC) for 15 min, in the presence or absence of 500 μmol l^{-1} $CuCl_2$, was analysed by immunoblotting with a phosphospecific anti-p38-MAPK antibody (top). Equal loading was verified by blotting identical samples with an anti-actin-specific antibody (bottom). (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± s.e.m. for three independent experiments performed with similar findings. *P<0.05, †P<0.001 *vs* control value.

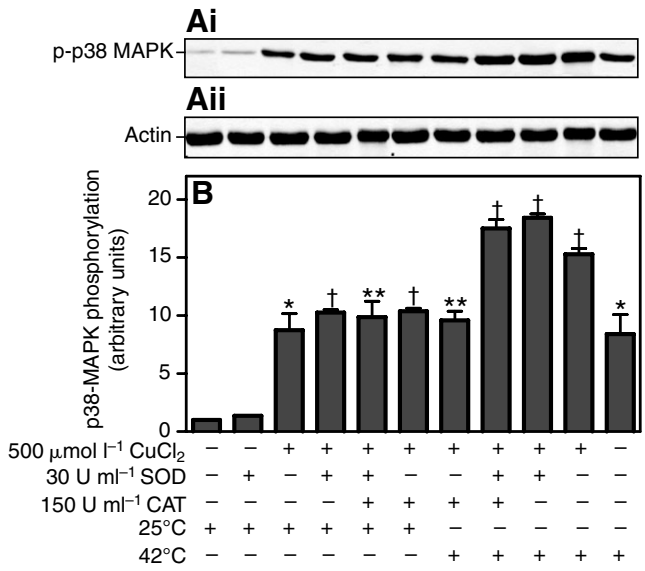

Fig. 7. Effect of different antioxidants on the p38-MAPK phosphorylation induced by $CuCl_2$ in the absence or presence of hyperthermia (42°C). Phospho-p38-MAPK was detected in extracts (50 μg protein) from control hearts (Con), hearts perfused for 15 min with 30 U ml^{-1} SOD alone or with 500 μmol l^{-1} $CuCl_2$, either in the absence or the presence of 30 U ml^{-1} SOD, 150 U ml^{-1} CAT or the combination of SOD+CAT, at 25°C. In addition, hearts were perfused for 15 min with 500 μmol l^{-1} $CuCl_2$ and identical combinations of antioxidant agents but at 42°C (Ai). Actin protein levels of identical samples were detected so as to confirm equal protein loading (Aii). Densitometric analysis of phospho-p38-MAPK bands by laser scanning was performed (B). Results are means ± s.e.m. for three independent experiments. *P<0.05, **P<0.01, †P<0.001 *vs* control value.

components or by inducing apoptosis (Woessmann et al., 1999). In their natural environment, amphibians could be simultaneously affected by both thermal stress, due to elevated ambient temperatures, and increased levels of copper, because of water pollution. Therefore, we examined the combined impact of $CuCl_2$ and hyperthermia (42°C) in the amphibian heart. Our results confirmed that thermal stress activates p38-MAPK in the frog heart and that its combination with $CuCl_2$ has an additive effect on the kinase phosphorylation (Fig. 5), a result that agrees with our previous studies performed in the mantle tissue of *Mytilus galloprovincialis* (Kefaloyianni et al., 2005).

In order to determine whether the p38-MAPK activation by $CuCl_2$ at normal temperature (25°C) or at 42°C was due to the production of oxidative factors, we conducted perfusions in the abovementioned conditions in the presence of SOD, catalase or both of them. At 25°C, neither SOD nor catalase had any significant effect on the $CuCl_2$-induced p38-MAPK activation, suggesting that the ROS scavenged by these enzymes are not implicated in the kinase activation at this temperature. In the case of the combined action of hyperthermia and $CuCl_2$, only catalase attenuated the p38-MAPK activation down to the level of the $CuCl_2$-stimulated p38-MAPK activation at 25°C and this

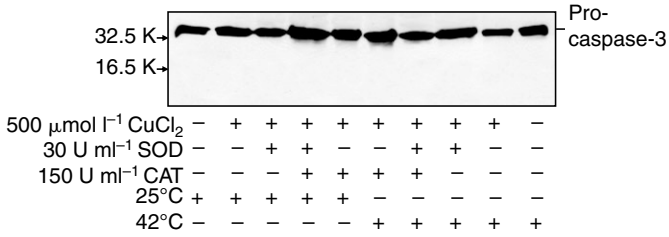

Fig. 8. Absence of caspase-3 activation by $CuCl_2$, at 25°C or at 42°C, in the absence or presence of CAT and SOD. Immunoblot analysis using a specific antibody that recognizes both the inactive full-length pro-caspase-3 and the fragmented active form of the protein led to the detection of only pro-caspase-3 in samples (100 μg protein) from hearts perfused without or with 500 μmol l^{-1} $CuCl_2$ for 15 min, either in the absence or the presence of 30 U ml^{-1} SOD, 150 U ml^{-1} CAT or the combination of SOD+CAT, at 25°C or at 42°C. Western blot shown is representative of three independent experiments performed with similar results. The positions of marker proteins are shown on the left.

result is indicative of the H_2O_2 production during hyperthermia (Fig. 7). This also agrees with a previous study, which reports the induction of oxidative stress in *Rana ridibunda* after great

change in the animal's body temperature (Bagnyukova et al., 2003). By contrast, when catalase was combined with SOD in the aforementioned conditions, the activation of p38-MAPK was enhanced in comparison to the one obtained when hearts were perfused with $CuCl_2$ at 42°C (Fig. 7). This last result could be attributed to the fact that, in the presence of H_2O_2, Cu and Cu/Zn SOD can induce the formation of the carbonate anion radical from bicarbonate, the standard buffer used in this study (Liochev and Fridovich, 2002; Liochev and Fridovich, 2006). This ROS could also participate in the activation of the p38-MAPK signalling pathway in the amphibian heart.

Another potent antioxidant tested was L-ascorbic acid, which other studies have shown that naturally exists in the amphibians (Singh and Sinha, 1990). Previously, we had demonstrated that this factor attenuated the H_2O_2-induced p38-MAPK activation in the perfused amphibian heart, but it had no impact on the kinase phosphorylation levels induced by xanthine/xanthine oxidase system (Gaitanaki et al., 2006). In the present study, L-ascorbic acid also did not abolish the $CuCl_2$-induced p38-MAPK phosphorylation at 25°C (Fig. 6), indicating that, in the case of $CuCl_2$, this agent does not function as an antioxidant.

Since at normal temperature (25°C), p38-MAPK activation by copper is not mediated by oxidative factors, it could be suggested that this stimulation is possibly mediated by conformational changes in membrane proteins that participate in signal transduction pathways (Wu et al., 1999; Letelier et al., 2005). In addition, copper has been proposed to inhibit the activity of the antiporter Na^+/K^+-ATPase (Li et al., 1996; Handy et al., 2002). In the amphibian heart, inhibition of Na^+/K^+-ATPase by ouabain also induces a strong activation of p38-MAPK (Stathopoulou et al., 2006), indicating that copper could stimulate the kinase through this mechanism.

Hsp27, the well-known downstream member of the p38-MAPK signalling pathway (Kyriakis and Avruch, 2001), was also investigated. This protein is implicated in cytoprotection since it interacts with and stabilizes F-actin fibers under stressful conditions and inhibits the mitochondrial apoptotic pathway by preventing cytochrome *c* release (Guay et al., 1997; Paul et al., 2002; Concannon et al., 2003). $CuCl_2$, at either 25°C or 42°C, induced a strong Hsp27 phosphorylation (Figs 2, 5), but did not activate caspase-3 (Fig. 8). Hsp27 phosphorylation follows a similar pattern as the one for p38-MAPK when hearts were perfused with increasing doses of $CuCl_2$ (Fig. 2) and is p38-MAPK dependent as exemplified with experiments using the kinase's specific inhibitor SB203580 (Fig. 3). However, no additive response was observed in the case of the simultaneous use of $CuCl_2$ and hyperthermia (Fig. 5), possibly because hyperthermia on its own is quite a strong activator of Hsp27.

In conclusion, our results demonstrate that in *Rana ridibunda* heart, $CuCl_2$ differentially activates the p38-MAPK signalling pathway depending on temperature. Overall, this p38-MAPK cascade activation seems to be beneficial for cardiac myocytes since it is associated with the motivation of protective cellular mechanisms, like that of the small Hsp27.

List of abbreviations

ASC	ascorbic acid
BMK1	big MAPK1
BSA	bovine serum albumin
CAT	catalase
Con	control
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinase
HRP	horse radish peroxidase
Hsp	heat shock protein
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
PAGE	polyacrylamide gel electrophoresis
PMSF	phenyl methyl sulfonyl fluoride
p38-MAPK	p38-mitogen-activated protein kinase
ROS	reactive oxygen species
SOD	superoxide dismutase
TBS	Tris-buffered saline

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