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$CoCl_2$ induces protective events *via* the p38-MAPK signalling pathway and ANP in the perfused amphibian heart

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Summary

Mitogen-activated protein kinases (MAPKs) constitute one of the most important intracellular signalling pathways. In particular, the p38-MAPK subfamily is known to be activated under various stressful conditions, such as mechanical or oxidative stress. Furthermore, cobalt chloride ($CoCl_2$) has been shown to mimic hypoxic responses in various cell lines and cause overproduction of reactive oxygen species (ROS). In the current study, we investigated the effect of $CoCl_2$ on p38-MAPK signalling pathway in the perfused *Rana ridibunda* heart. Immunoblot analysis of the phosphorylated, and thus activated, form of p38-MAPK revealed that maximum phosphorylation was attained at 500 μmol l^{-1} $CoCl_2$. A similar profile was observed for MAPKAPK2 and Hsp27 phosphorylation (direct and indirect p38-MAPK substrates, respectively). Time course analysis of p38-MAPK phosphorylation pattern showed that the kinase reached its peak within 15 min of treatment with 500 μmol l^{-1} $CoCl_2$. Similar results were obtained for Hsp27 phosphorylation. In the presence of the antioxidants Trolox or Lipoic acid, p38-MAPK $CoCl_2$-induced phosphorylation was attenuated. Analogous results were obtained for Hsp27 and MAPKAPK2. In parallel, mRNA levels of the ANP gene, a hormone whose transcriptional regulation has previously been shown to be regulated by p38-MAPK, were examined (semi-quantitative ratiometric RT-PCR). $CoCl_2$ treatment significantly increased ANP mRNA levels, whereas, in the presence of antioxidants, the transcript levels returned to basal values. All the above data indicate that $CoCl_2$ stimulates compensatory mechanisms involving the p38-MAPK signalling cascade along with ANP.

Key words: $CoCl_2$, hypoxia, frog heart, p38-MAPK, ANP, cardioprotection, oxidative stress, signalling.

Introduction

Amphibians as ectotherms encounter adverse environmental conditions to which they have to adjust in order to survive. These organisms have penetrated into harsh environments, adjusting to wide variations in osmolality and ionic strength of their body fluids as well as to oxygen deprivation (Shoemaker, 1992; Holden and Storey, 1997). Preservation of cardiac muscle performance under such stressful conditions is crucial for survival. In particular, when exposed to hypoxic conditions, amphibians face the challenge of maintaining intracellular acid–base status, defending high energy phosphate homeostasis and metabolic substrates in the ventricular muscle (Hermes-Lima and Storey, 1996; Rocha and Branco, 1998; Andersen et al., 2003). The tolerance that these organisms exhibit has motivated a plethora of studies in an effort to elucidate the mechanisms regulating their respective responses.

Given their physiology, it was of great interest to evaluate amphibian heart response to chemically induced hypoxia, i.e. to cobalt chloride. $CoCl_2$ is a well-known hypoxia mimetic agent (Goldberg et al., 1988) that has been demonstrated to act as a pro-oxidant, inducing an increase in the levels of reactive oxygen species (ROS) (Zou et al., 2001; Xi et al., 2004; Kotake-Nora and Saida, 2006) in diverse cell types (Tomaro et al., 1991; Chandel et al., 2000). Co (II) has been identified as an oxidative stress-inducing factor producing ROS *via* a Fenton-type reaction (Moorhouse et al., 1985; Wang et al., 1993).

ROS at low levels are known to act as signalling molecules (Kamata and Hirata, 1999; Li and Jackson, 2002; Droge, 2002) while having deleterious effects in high concentrations (Droge, 2002). Cells possess multiple antioxidant systems so as to counteract ROS, including specific enzymes such as catalase (CAT), which is classified as an enzyme known to convert H_2O_2 to H_2O and molecular oxygen (Fridovich, 1999), and superoxide dismutase (SOD), whose main role is to convert the superoxide anion (O_2^-) to H_2O_2 (McCord and Fridovich, 1969).

Nitric oxide (NO) is another member of the free radical family that diffuses rapidly and does not readily react with most biomolecules (Lancaster, 1994). However, NO reaction with O_2^- generates peroxynitrite ($OONO^-$), which is highly cytotoxic (Beckman and Koppenol, 1996). Therefore, in the context of, for example, SOD competing with NO for binding to the superoxide anions, it becomes evident that SOD exerts its cardioprotective function by regulating formation of other ROS as well (peroxynitrite in this case). Given the interaction between these reactions one can deduce that, in this context, this is also the case for CAT.

Oxidative stress (exemplified by perfusion with H_2O_2) has also been found to transcriptionally upregulate atrial natriuretic peptide (ANP) levels in the amphibian heart (Vassilopoulos et al., 2005). There is also data implicating NO in the mechanism of this cardiac peptide hormone regulation (Sanchez-Ferrer et al., 1990; Carnio et al., 2004). Therefore, ANP appears to constitute another factor involved in the cardiac compensatory response to stimuli that could disturb extracellular fluid volume and electrolyte balance (Glass et al., 1996; Silberbach and Roberts, 2001), including redox perturbations.

Oxidative stress may trigger activation of a plethora of signalling cascades (Kannan and Jain, 2000) including mitogen-activated protein kinase (MAPK) pathways (Feuerstein and Young, 2000). The three major subfamilies characterized in mammals are: the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs) and p38 reactivating kinase (p38-MAPK) (Kyriakis and Avruch, 2001; Pearson et al., 2001). The respective MAPKs have also been identified in the amphibian heart (Aggeli et al., 2001a). These kinases interact with their substrates in both the cytoplasm and the nucleus, transducing a variety of molecular signals (Bogoyevitch, 2000). Amphibian heart p38-MAPK, in particular, has been demonstrated to be activated by various forms of environmental stress, including mechanical and hyperosmotic as well as thermal (Aggeli et al., 2001b; Aggeli et al., 2002). Additionally, certain antioxidants (SOD and CAT) have been demonstrated to reverse H_2O_2-induced amphibian heart p38-MAPK activation (Gaitanaki et al., 2006a). H_2O_2-induced (oxidative stress) p38-MAPK interacts with MAPK-activated-protein-kinase 2 (MAPKAPK2), a kinase that subsequently phosphorylates the small heat shock protein Hsp27 (Gaitanaki et al., 2003). Phosphorylation of Hsp27 contributes to stabilization of the actin cytoskeleton, protecting cells against unfavourable stressful conditions (Paul et al., 2002; Concannon et al., 2003). p38-MAPK is also involved in ANP gene expression, a cardiac hormone that has been found to regulate adaptation to hypoxia (Drexler et al., 1989; Loennechen et al., 2001).

The overall aim of the present study was to assess and characterize the nature of the signal transduction mechanisms triggered by $CoCl_2$ in the perfused amphibian heart, focusing on p38-MAPK cascade and ANP expression. Overall, our results indicate for the first time that $CoCl_2$ simulates oxidative stress conditions in this particular experimental setting. Thus, we have detected p38-MAPK pathway activation along with an enhanced p38-MAPK-dependent presence of ANP, under the conditions examined, possibly implicating them in the mechanism regulating the compensatory response to this form of stress.

Materials and methods

Chemicals and drugs

Nitrocellulose (0.45 μmol l^{-1}) was obtained from Schleicher & Schuell (Keene, NH, USA). The enhanced chemiluminescence kit was from Amersham International (Uppsala, Sweden). Bradford protein assay was from Bio-rad (Hercules, CA, USA). SB203580, a p38-MAPK specific inhibitor, was obtained from Alexis Biochemicals (Lausen, Switzerland) and stock solution (10 mmol l^{-1}) was prepared in dimethyl sulphoxide (DMSO).

Rabbit polyclonal antibodies specific for dually phosphorylated p38-MAPK (#9211), MAPKAPK2 (#3041) as well as Hsp27 (#2401) were purchased from Cell Signalling (Beverly, MA, USA). The anti-actin (A2103) antibody was from Sigma-Aldrich (St Louis, MO, USA). Secondary antibodies were from DakoCytomation (Glostrup, Denmark). Pre-stained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Super RX film was purchased from Fuji photo film GmbH (Dusseldorf, Germany). Most general laboratory reagents used were purchased from Sigma-Aldrich. The antioxidants used (Trolox and Lipoic acid) were synthesized and kindly provided by Dr Koufaki's group at the Institute of Organic and Pharmaceutical Chemistry at the National Hellenic Research Foundation. Catalase (C-30) and Superoxide Dismutase (SOD)-(S2515) were from Sigma-Aldrich.

Animals

Frogs (*Rana ridibunda* Pallas) 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 freshwater 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

Frogs were anaesthetized by immersion in 0.01% (w/v) MS222 and sacrificed by decapitation. The hearts were excised and mounted onto an aortic cannula. Perfusions were performed in a non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with a bicarbonate-buffered saline (23 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 7.35 at 25°C) supplemented with 10 mmol l^{-1} glucose and equilibrated with 95%:5% O_2:CO_2. Heart and perfusate temperatures were maintained at 25°C using a water-jacketed apparatus. All hearts were equilibrated for 15 min under these conditions. Following the equilibration period, hearts were perfused with various concentrations of $CoCl_2$ for 15 min or with 500 μmol l^{-1} $CoCl_2$ for periods of time ranging

from 0.5 to 60 min. After the 15 min equilibration hearts were also perfused with 500 μmol l^{-1} $CoCl_2$ for 15 min in the presence of Trolox (Tr) or Lipoic acid (LA) (both at 10 μmol l^{-1} final concentration). Control hearts for the effect of these antioxidants were also perfused with Tr or LA alone. Perfusions were also performed in the presence of 1 μmol l^{-1} SB203580, both in the equilibration period and in parallel with 500 μmol l^{-1} $CoCl_2$, for 15 min. To investigate ROS scavenging by CAT and SOD, perfusions were conducted in the presence of 150 U ml^{-1} of CAT or 12.5 U ml^{-1} of SOD after the equilibration period, along with 500 μmol l^{-1} $CoCl_2$ for 15 min. Control hearts were also perfused with 150 U ml^{-1} of CAT or 12.5 U ml^{-1} of SOD alone. At the end of the perfusions, atria were removed and the ventricles, after immersion in liquid N_2, were pulverized under liquid N_2. Powders were stored at –80°C. During all the treatments the electromechanical heart activity was monitored with no significant changes observed. No viability loss was detected in any experimental intervention.

Protein extraction

Heart powders were homogenized with 3 ml g^{-1} of buffer G [20 mmol l^{-1} Tris-HCl pH 7.5, 20 mmol l^{-1} β-glycerophosphate, 2 mmol l^{-1} EDTA, 10 mmol l^{-1} benzamidine, 20 mmol l^{-1} NaF, 0.2 mmol l^{-1} Na_3VO_4, 200 μmol l^{-1} leupeptin, 10 μmol l^{-1} trans-epoxy-succinyl-L-leucylamido-(4-guanidino) butane (E-64), 5 mmol l^{-1} dithiothreitol (DTT), 300 μmol l^{-1} phenylmethylsulphonyl fluoride (PMSF) and 0.5% (v/v) Triton X-100]. After extraction on ice for 15 min, samples were centrifuged (13 400 ***g***, 5 min, 4°C), and the supernatants boiled with 0.33 vol. of sodium dodecyl sulphate (SDS) sample buffer [SB4X: 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 BioRad Bradford assay reagent

Immunoblotting

Proteins (50 μg) were separated by SDS-PAGE on 10% (w/v) polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes. Nonspecific binding sites were blocked with 1% (w/v) bovine serum albumin (BSA) in TBST [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, the membranes were incubated overnight with the appropriate primary antibody (1:1000) at 4°C. After washing in TBST (3×5 min) blots were incubated with the respective horseradish peroxidase-conjugated secondary antibody 1:5000 in TBST containing 1% (w/v) BSA (60 min, room temperature, R_T). After washing in TBST (3×5 min), bands were detected using enhanced chemiluminescense (ECL), exposed to Super RX film (Fuji photo film GmbH Dusseldorf, Germany) and quantified by laser scanning densitometry (Gel Analyzer v. 1.0).

RNA preparation, cDNA synthesis and ratiometric reverse transcription PCR (RT-PCR)

The expression of endogenous ANP and β-actin was determined by ratiometric reverse transcription of total RNA followed by semi-quantitative PCR analysis. Total RNA was extracted from heart ventricles using Trizol (Invitrogen Life Technologies, CA, USA), according to the manufacturer's instructions. For cDNA synthesis, 2 μg of total RNA was denatured in the presence of 5 pmol oligo-dT primer in a reaction volume of 13.5 μl at 65°C for 5 min. Reverse transcription was performed with M-MLV Reverse Transcriptase (Invitrogen Life Technologies), first strand buffer (Promega, Madison, WI, USA), dithiothreitol (Promega) and deoxy-nucleotide triphosphates (dNTPs) (Promega). The first strand reaction was incubated at 37°C for 1 h. Termination of the reaction was achieved by inactivation of the reverse transcriptase at 70°C for 5 min. PCR for ANP was performed using 1.5 U Taq (Bioron GmbH, Ludwigshafen, Germany) with sense 5′-CGA GGA GGT CCA GCA TAC AAC TCC-3′ and antisense 5′-CTA GAA CCT ACG GCC ACA ACC CAT-3′ primers (GenBank accession no. D01043). These primers amplify a 375-base pair (bp) PCR product. After a 5 s denaturation at 94°C, PCR was carried out for 30 cycles (94°C for 30 s, 45°C for 30 s and 72°C for 45 s), and then a final extension was done at 72°C for 5 min. PCR (30 cycles) for β-actin was performed using the following primers: sense 5′-ATC TGG CAT CAC ACC TTC TAC-3′ and antisense 5′-CTC CTG CTT GCT GAT CCA C-3′ (Takase et al., 2002). cDNA samples derived from 'control' and treated cells were always amplified simultaneously. PCR products were separated on a 1.2% for ANP and 1% for β-actin (w/v) agarose gel supplemented with ethidium bromide (EtBr) at a final concentration of 100 μg l^{-1}. Band intensities were determined and quantified by scanning densitometry using an appropriate image analysis programme (Gel Analyzer v. 1.0). All values were normalized for the amount of β-actin mRNA and estimation of fragment band size (ANP 375 bp, β-actin 900 bp) was performed by comparison with GeneRuler 100 bp DNA ladder (Fermentas Life Sciences Inc., Hanover, USA).

Statistical evaluations

Western blots shown are representative of at least three independent experiments. Each data point is presented as the mean ± s.e.m. of samples from at least three separate hearts perfused under the respective conditions. Comparisons between control and treatments were performed using one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. A value of at least $P<0.05$ was considered to be statistically significant. p38-MAPK, MAPKAPK2 and Hsp27 activation in 'control' hearts was set at 1 and their stimulated activation in treated hearts was expressed as '-fold' activation *versus* control hearts.

Results

Activation of p38-MAPK was studied in extracts from amphibian hearts perfused under control conditions or in the presence of $CoCl_2$ (a hypoxia mimetic agent). The kinase dual phosphorylation, thus activation, was assessed *via*

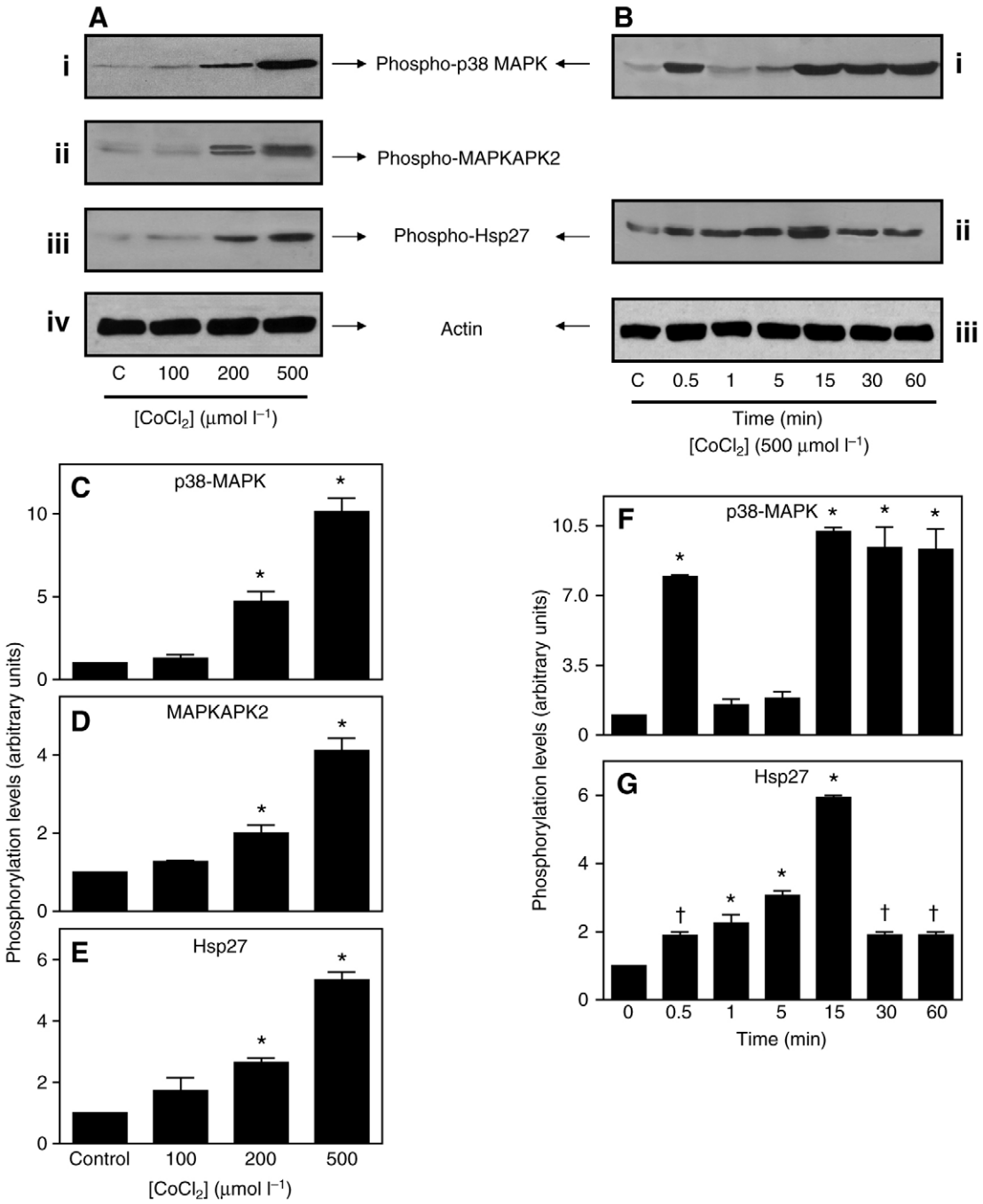

Fig. 1. Western blots showing dose–response (A) and time course (B) of p38-MAPK, MAPKAPK2 and Hsp27 phosphorylation in the amphibian heart after $CoCl_2$ treatment. (Ai) Phospho-p38-MAPK was detected in extracts (50 μg of protein) from control hearts (C) or hearts perfused for 15 min with the indicated concentration of $CoCl_2$. (Aii) Phospho-MAPKAPK2 was detected in identical samples. (Aiii) Phospho-Hsp27 was detected in identical samples. An anti-actin antibody was used as a control for equal protein loading (Aiv). (Bi) Phospho-p38-MAPK was detected in extracts (50 μg of protein) from control hearts (C) or hearts perfused with 500 μmol l^{-1} $CoCl_2$ for increasing periods of time. (Bii) Phospho-Hsp27 was detected in identical samples. An anti-actin antibody was used as a control for equal protein loading (Biii). (C–G) Densitometric analysis of phospho-p38 (C,F), phospho-MAPKAPK2 (D) and phospho-Hsp27 (E,G) bands was performed by laser scanning. Western blots shown are representative of at least three independent experiments; data are means ± s.e.m. for at least three independent experiments. *P<0.001 and $^{\dagger}P$<0.05 *vs* control (untreated) hearts.

immunoblotting using a specific antibody raised against phospho-p38-MAPK at Thr180 and Tyr182 residues. Initially, we determined the dose-dependent profile of the $CoCl_2$ effect on p38-MAPK phosphorylation. A notable increase in p38-MAPK phosphorylation levels was observed at 200 μmol l^{-1} $CoCl_2$ (3.1±0.3-fold relative to control hearts, P<0.001) (Fig. 1Ai,C).

Maximal activation (10.25±1.6-fold relative to control hearts, $P<0.001$) of the kinase was attained after perfusion with 500 μmol l^{-1} $CoCl_2$ for 15 min (Fig. 1Ai,C), which was comparable to the maximum activation induced by either 0.5 mol l^{-1} sorbitol (Aggeli et al., 2002) or 30 μmol l^{-1} H_2O_2 (Gaitanaki et al., 2003). MAPKAPK2 being an established p38-MAPK substrate (Rouse et al., 1994), we looked into its activation under the same experimental conditions. The kinase phosphorylation pattern was found to be similar to that of p38-MAPK, maximized at 500 μmol l^{-1} $CoCl_2$ (4.2±0.61-fold relative to control hearts, $P<0.001$) (Fig. 1Aii,D). Hsp27, which is phosphorylated by MAPKAPK2 (Stokoe et al., 1992), was subsequently examined. In particular, Hsp27 exhibited a response that did not differ from the aforementioned ones, with robust phosphorylation (5.1±0.5-fold relative to control hearts, $P<0.001$) also at 500 μmol l^{-1} $CoCl_2$ (Fig. 1Aiii,E). Equal protein loading was confirmed by probing with an anti-actin antibody (Fig. 1Aiv).

Next, so as to determine the time course of $CoCl_2$ effect on p38-MAPK and Hsp27 activation, the kinase-stimulated phosphorylation was examined in samples from hearts perfused with 500 μmol l^{-1} $CoCl_2$ for time periods ranging from 0.5 to 60 min (Fig. 1B). Under these conditions, p38-MAPK phosphorylation levels were rapidly elevated from as early as 0.5 min (8.1±0.77-fold relative to control, $P<0.001$) decreasing thereafter with a second maximal peak at 15 min (10.12±1.8-fold relative to control, $P<0.001$) that was sustained for at least 60 min (Fig. 1Bi,F). Hsp27 phosphorylation was observed to be equally prolonged, being statistically significant from 0.5 min, reaching maximal values at 15 min (5±0.67-fold relative to control, $P<0.001$) (Fig. 1Bii,G). Equal protein loading was once more confirmed using the anti-actin antibody (Fig. 1Biii).

$CoCl_2$ activity as an oxidative stress stimulator is depicted in Fig. 2. In the presence of two different antioxidant agents, i.e. Trolox (Tr) and Lipoic acid (LA), the phosphorylation of p38-MAPK and its substrates was considerably attenuated. In particular, Tr inhibited $CoCl_2$-induced p38-MAPK phosphorylation by ~85±3.5% and LA was equally effective, with only a 20±1.5% of the $CoCl_2$-stimulated activation of the kinase remaining in its presence (Fig. 2Ai,B). While Tr almost abrogated MAPKAPK2 phosphorylation by $CoCl_2$ (inhibition by ~95±2.4%) the latter was markedly reduced in the presence of LA (by ~60±3.1%) (Fig. 2Aii,C). Analogously, Tr was found to inhibit Hsp27 $CoCl_2$-induced phosphorylation by ~75±4% with LA conferring a ~50±3.7% inhibition (Fig. 2Aiii,D). Note that in order to determine the net effect of the antioxidants or inhibitors used in parallel with $CoCl_2$, any observed effect that these compounds have on their own has to be deducted.

It has been established for both Tr and LA that they counteract a number of chemically reactive molecules derived from oxygen including H_2O_2, hydroxyl radicals, nitric oxide, peroxynitrite, superoxide radicals etc. (Kagan et al., 1992; Scott et al., 1994; Brookes et al., 1998). In an attempt to specify the species of ROS inducing the observed response, after perfusion

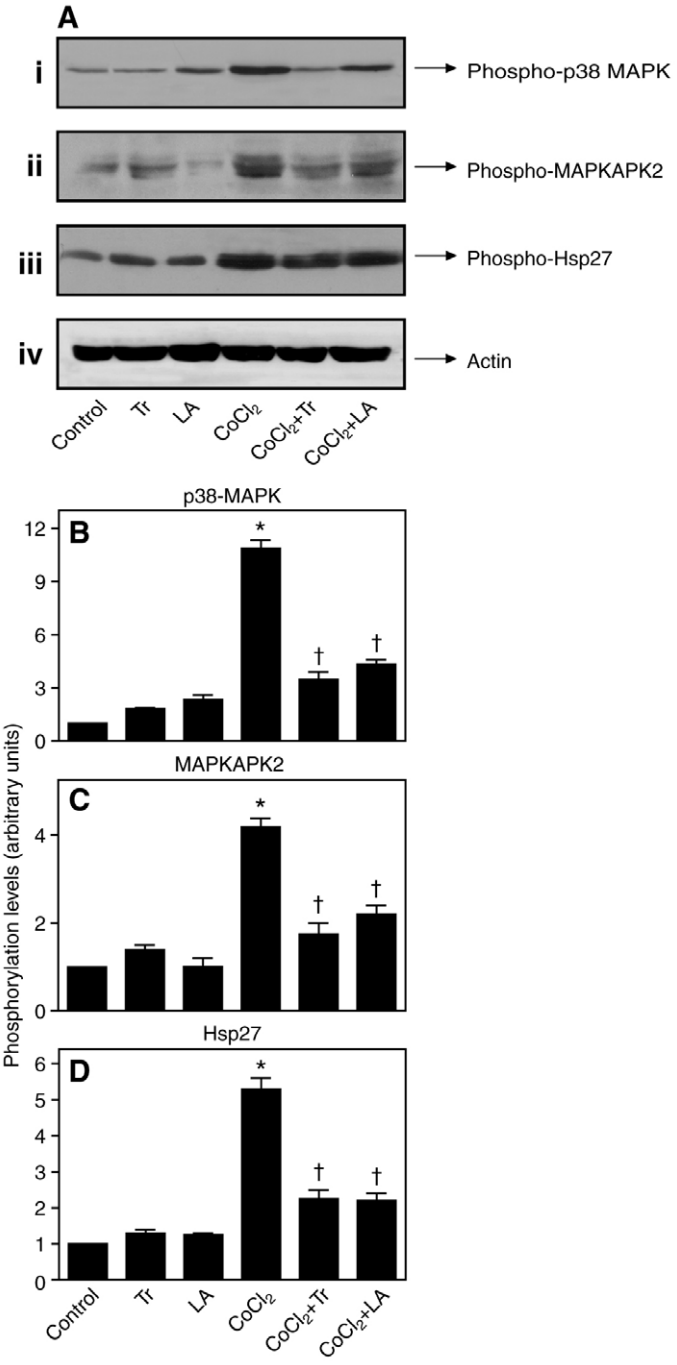

Fig. 2. Western blots showing the effect of Trolox (Tr) and Lipoic acid (LA) on $CoCl_2$-induced phosphorylation of p38-MAPK (Ai), MAPKAPK2 (Aii) and Hsp27 (Aiii) after $CoCl_2$ treatment. The phosphorylated forms of the kinases were detected in extracts (50 μg of protein) from control hearts (control) or hearts perfused for 15 min with 500 μmol l^{-1} of $CoCl_2$ in the presence or absence of TR (10 μmol l^{-1}) and LA (10 μmol l^{-1}). The effect of Tr and LA alone was also assessed. An anti-actin antibody was used as a control for equal protein loading (Aiv). Densitometric analysis of phospho-p38 (B), phospho-MAPKAPK2 (C) and phospho-Hsp27 (D) was performed by laser scanning. Western blots shown are representative of at least three independent experiments; data are means ± s.e.m. for at least three independent experiments. *$P<0.001$ *vs* control (untreated) hearts; †$P<0.05$ *vs* $CoCl_2$-treated hearts.

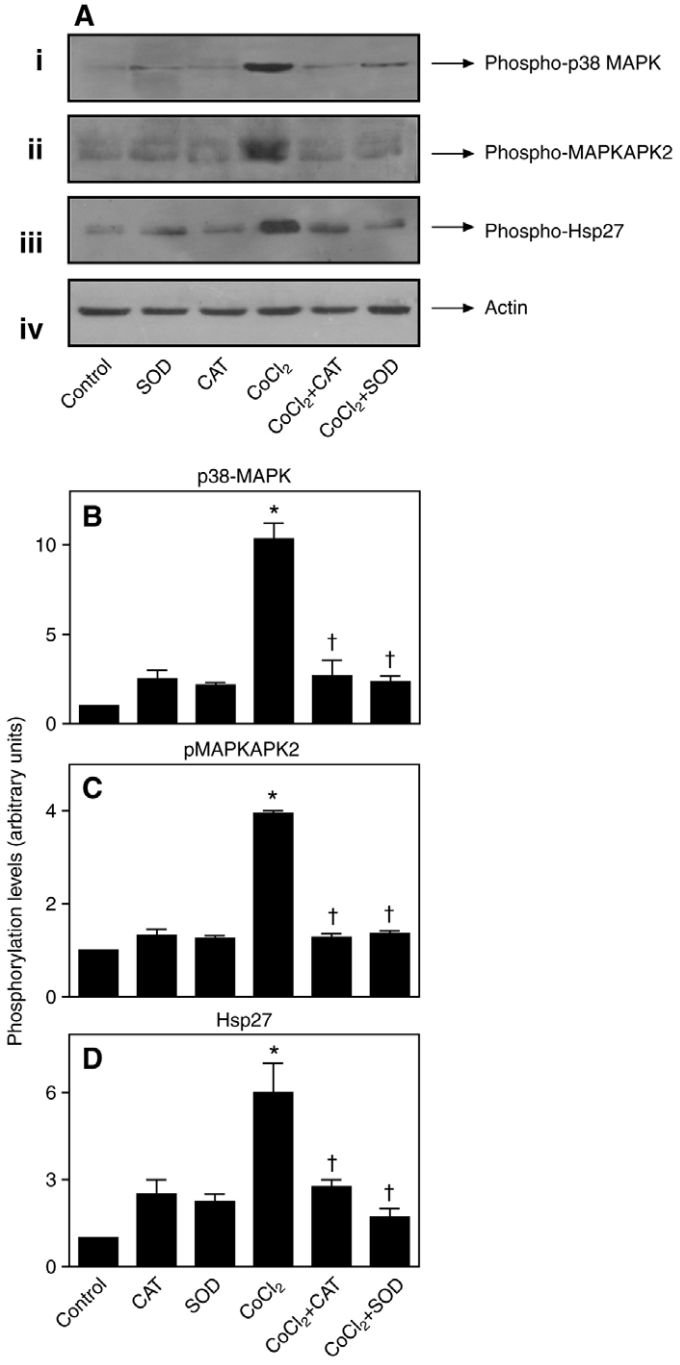

Fig. 3. Western blots showing the effect of superoxide dismutase (SOD) and catalase (CAT) on $CoCl_2$-induced phosphorylation of p38-MAPK (Ai), MAPKAPK2 (Aii) and Hsp27 (Aiii) after $CoCl_2$ treatment. The phosphorylated forms of the kinases were detected in extracts (50 μg of protein) from control hearts (control) or hearts perfused for 15 min with 500 μmol l^{-1} of $CoCl_2$ in the presence or absence of SOD (12.5 U ml^{-1}) and CAT (150 U ml^{-1}). The effect of SOD and CAT alone were also assessed. An anti-actin antibody was used as a control for equal protein loading (Aiv). Densitometric analysis of phospho-p38 (B), phospho-MAPKAPK2 (C) and phospho-Hsp27 (D) was performed by laser scanning. Western blots shown are representative of at least three independent experiments; data are means ± s.e.m. for at least three independent experiments. *P<0.001 *vs* control (untreated) hearts; †P<0.001 *vs* $CoCl_2$-treated hearts.

of the amphibian heart with $CoCl_2$, we next examined the potential antioxidant effect of catalase and superoxide dismutase. CAT is a known hydrogen peroxide (H_2O_2) scavenger (Fridovich, 1999; Halliwell, 1999) while SOD is known to mainly remove the superoxide anion (O_2^-) (McCord and Fridovich, 1969). Accordingly, both antioxidants were found to almost ablate p38-MAPK (Fig. 3Ai,B), MAPKAPK2 (Fig. 3Aii,C) as well as Hsp27 (Fig. 3Aiii,D) phosphorylation, thus activation. Blotting with the antibody raised against actin, we once more confirmed equal protein loading (Fig. 3Aiv).

Following a study by our group (Vassilopoulos et al., 2005) indicating p38-MAPK-mediated ANP transcript upregulation by direct oxidative stress (perfusion with H_2O_2), we next tried to evaluate mRNA levels of this natriuretic peptide, using semi-quantitative ratiometric PCR, in samples from hearts perfused with $CoCl_2$, in the presence or absence of Tr (10 μmol l^{-1}), LA (10 μmol l^{-1}) and 1 μmol l^{-1} SB203580 (a known specific p38-MAPK inhibitor). Perfusion for 15 min with 500 μmol l^{-1} $CoCl_2$ was found to significantly increase ANP mRNA levels (~4.3±0.35-fold relative to control, P<0.001) (Fig. 4A, top panel; Fig. 4B). We also assessed β-actin mRNA levels as an internal control (Fig. 4A, bottom panel). ANP response at the transcriptional level was almost abrogated by both antioxidants tested, as well as SB203580 (Fig. 4).

Discussion

Many species of ectotherms, when subjected to wide and rapid variations in oxygen availability, have a well-developed capacity to survive prolonged periods of complete anoxia (Storey and Storey, 1990; Driedzic and Gesser, 1994), e.g. as part of their winter hibernation in northern latitudes (Hermes-Lima and Storey, 1996). Hypoxia is defined as a state when oxygen tension drops below normal limits, playing a pivotal role in pathological conditions including cardiovascular diseases (Giaccia et al., 2003; Jiang et al., 2007; Muir et al., 2007). In particular, myocardial hypoxia is associated with major cellular ionic and metabolic changes leading to mechanical impairment (Frist et al., 1978; Kentish, 1986; Allen and Orchard, 1987).

Nevertheless, it should be noted that frog cardiac muscle has been shown to be resistant to hypoxia in terms of mechano-energetic properties (Allen and Orchard, 1987). Thus, it seemed exquisitely interesting to investigate the response of the amphibian heart to perfusion with $CoCl_2$, an agent that has been widely reported to act as a chemical inducer of a hypoxic effect (Goldberg et al., 1988). Data addressing which signalling pathways are activated in hypoxia are controversial. However, recent studies have indicated an emerging role for MAPKs, especially p38-MAPK and JNKs (Greenway and Storey, 2000; Cowan and Storey, 2003). Focusing on p38-MAPK, we first examined the dose- and time-dependent profile of this kinase activation along with those of MAPKAPK2 and Hsp27 (Fig. 1). Both of the latter proteins consist downstream components of the p38-MAPK signalling transduction pathway (Kyriakis and Avruch, 2001) and have been shown to exert a cytoprotective

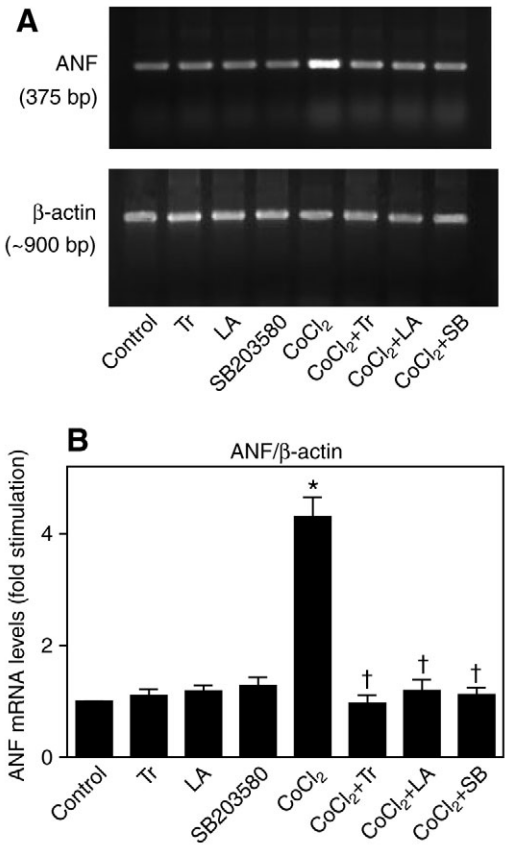

Fig. 4. Effect of Trolox (Tr), Lipoic acid (LA) and SB203580 (SB) on atrial natriuretic peptide (ANP) mRNA levels in samples from $CoCl_2$-perfused amphibian hearts. (A) RT-PCR analysis of RNA isolated from the ventricles of cardiac muscle perfused with 500 μmol l^{-1} $CoCl_2$ in the presence or absence of Tr (10 μmol l^{-1}), LA (10 μmol l^{-1}) and SB203580 (1 μmol l^{-1}). Amplification of ANP (top) and β-actin (bottom) cDNA in identical samples was performed. (B) After densitometric analysis of the PCR products the results were normalized for β-actin and presented as fold stimulation, expressed as ANP/β-actin ratio. Results are means ± s.e.m. for at least three independent experiments. *P<0.001 *vs* control (untreated) hearts; †P<0.001 *vs* $CoCl_2$-treated hearts. The PCR products of ANP (375 bp) and β-actin (900 bp) were analysed in 1.2% and 1% (w/v) agarose gel electrophoresis, respectively.

effect. Hsp27, in particular, is widely known to help maintain the cytoskeleton integrity and hence contribute to cardioprotection under stressful conditions (Huot et al., 1996).

Many reports show that $CoCl_2$ can mimic the hypoxic response in many aspects (Goldberg et al., 1988) but also function as an oxidative stress-inducing factor since Co (II) can react with H_2O_2 by a Fenton-type reaction to produce ROS (Moorhouse et al., 1985; Wang et al., 1993). This water-soluble substance has been demonstrated to increase ROS generation, and thus oxidative stress, by a non-enzymatic, non-mitochondrial mechanism (Chandel et al., 2000) in divergent cell types (Tomaro et al., 1991; Chandel et al., 2000). The concentration used (500 μmol l^{-1}) is applied routinely in studies investigating this compound's effect (Zou et al., 2002).

Among the various products of redox reactions one can distinguish ROS (oxygen-derived) as well as RNS (nitrogen-derived) free radicals. ROS include the superoxide anion, hydrogen peroxide and lipid peroxides, while nitric oxide and peroxynitrite belong to the RNS (Kyaw et al., 2004). In excessive quantities, these reactive species formed by aerobic organisms constitute a threat for the integrity of various biomolecules, but at physiological concentrations they are actually required for normal cell function (Nordberg and Arner, 2001), playing a significant physiological role in intracellular signalling and redox regulation. NO has been identified as a signalling molecule (Furchgott, 1995) and a modulating factor of gene expression (Bogdan, 2001). Furthermore, myocardial protection in terms of ischemic preconditioning may be mediated by ROS or NO (Baines et al., 1997; Lochner et al., 2002) *via* activation of downstream protein kinase cascades, i.e. p38-MAPK (Rakhit et al., 2001). Beneficial biological effects exerted by NO also include: vasodilatation, inhibition of superoxide accumulation, attenuation of platelet adhesion and protection of cardiac myocyte function (Cotton et al., 2002). Although NO can even ameliorate post-ischemic reperfusion myocardial injury it may also confer deleterious effects *via* reaction with O_2^- and formation of peroxynitrite, a strong oxidizing agent (Oishi et al., 2006). In particular, peroxynitrite ($OONO^-$) has been identified as a toxic metabolite of NO that causes myocardial dysfunction, but there are other reports of its protective function *via* conversion to S-nitrosothiols in blood and tissues (Vinten-Johansen, 2000). Low concentrations of peroxynitrite have been found to protect against ischemia-reperfusion-induced arrhythmias (Altug et al., 2001), and its prolonged vasorelaxant action under physiological conditions (Dowell and Martin, 1997) shares many characteristics in common with NO (Jothianandan and Furchgott, 1996). In particular, these may be attributed to its oxidant properties or the induction of cGMP levels and activation of K^+-ATP-dependent (K_{ATP}) channels (Moro at el., 1994). Beguin et al. (Beguin et al., 2005) have also demonstrated a protective role for the ATP-sensitive potassium channel in delayed hypoxia preconditioning. What is more, K_{ATP} channels have been identified in various tissues including the myocardium, where opening of the mitochondrial K_{ATP} channels especially stimulates generation of ROS that exert significant physiological roles as intracellular messengers (Irani, 2000; Pain et al., 2000; Krenz et al., 2002), conferring cardioprotection (Nagata et al., 2003). Further supporting evidence for this scenario suggests that mito K_{ATP} channel opening leads to the release of ROS that may stimulate p38-MAPK activity, resulting in the observed salutary effect on the myocardium (Yue et al., 2002).

The sustained and prolonged timewise phosphorylation pattern of p38-MAPK and Hsp27 observed in the present study, could probably underlie the pivotal role of this intracellular pathway in maintaining cellular homeostasis under the stressful conditions examined. Our group has previously shown that the p38-MAPK→MAPKAPK2→Hsp27 pathway transduces the oxidative stress signal as exemplified by perfusion with H_2O_2

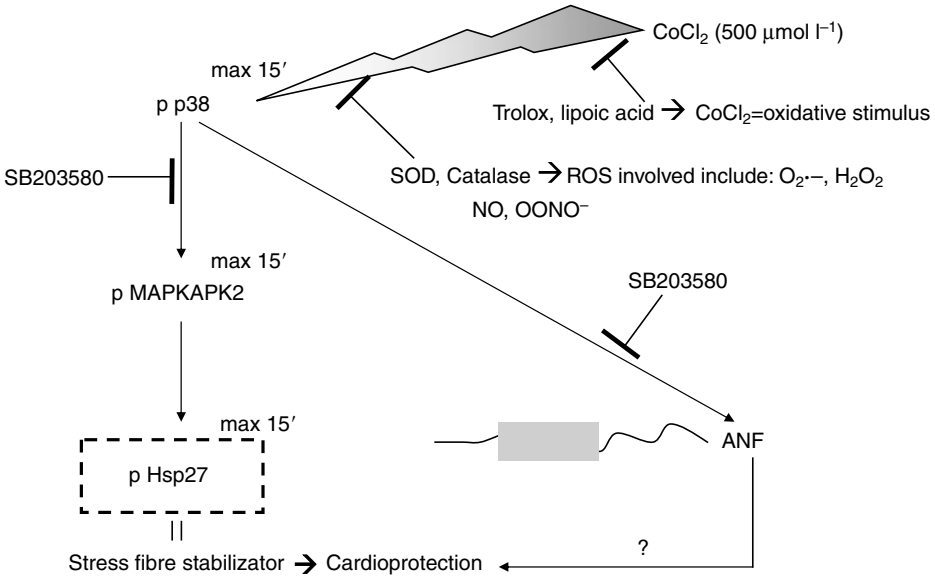

Fig. 5. A hypothetical schematic model for regulation of p38-MAPK, MAPKAPK2 and Hsp27 phosphorylation in $CoCl_2$-perfused amphibian hearts. → activation, –| inhibition.

(Gaitanaki et al., 2003). Hsp27 phosphorylation being the endpoint of this response, we may deduce that it mediates cytoprotection, since it has been reported to act as a molecular chaperone, to stabilize F-actin fibers and to inhibit the mitochondrial apoptotic pathway by preventing cytochrome *c* release (Guay et al., 1997; Paul et al., 2002; Concannon et al., 2003). The biphasic phosphorylation profile of p38-MAPK (Fig. 1Bii,D) could reflect the key role played by the kinase in transuding the particular signal in a rapid but at the same time, prolonged manner.

Our group has previously shown that complete hypoxia (anoxia) does not result in p38-MAPK activation (Aggeli et al., 2001a). Given the property of $CoCl_2$ to produce ROS (Moorhouse et al., 1985; Wang et al., 1993), an effort was made to elucidate the nature of this compound's effect on p38-MAPK pathway. In order to decipher whether perfusion with $CoCl_2$ represents an indirect oxidative stress stimulus, two different antioxidants were used: both trolox (a vitamin E analogue) and lipoic acid (a disulphide compound) inhibited $CoCl_2$-induced p38-MAPK phosphorylation (Fig. 2Ai,B), being equally effective in reducing MAPKAPK2 (Fig. 2Aii,C) and Hsp27 (Fig. 2Aiii,D) $CoCl_2$-stimulated phosphorylation levels. These agents are known to exert their biological antioxidant activity directly by free radical quenching as well as indirectly *via* promoting recycling of other cellular antioxidants (Packer et al., 1995).

Subsequently, in order to further identify and determine which reactive oxygen species actually mediate the observed response of p38-MAPK pathway, we investigated the effect of superoxide dismutase and catalase. These antioxidant enzymes function to protect cells from the lethal effects of excessive ROS formation (McCord and Fridovich, 1969; Kirkman and Gaetani, 1984). Accordingly, as shown in Fig. 3, SOD as well as CAT significantly attenuated the $CoCl_2$ effect. SOD constitutes a defense system against the superoxide anion (O_2^-) (McCord and Fridovich, 1969) while CAT removes hydrogen peroxide (H_2O_2) (Fridovich, 1999; Halliwell, 1999). Nevertheless, given the complexity, crosstalk and multiplicity of the metabolic pathways responsible for the generation as well as the detoxification of free radicals, the likelihood of other species being involved arises. Thus, although SOD and CAT are classified and extensively used as scavengers of superoxide and hydrogen peroxide, respectively (Li et al., 2005), one cannot exclude their role as limiting factors of peroxynitrite formation. SOD in particular, competes with NO over the fate of the superoxide anions, thus minimizing $OONO^-$ formation, and the redox equilibrium attained depends on reaction rates, targets concentration, rate constants etc. (Beckman et al., 1992). In addition to this, peroxynitrite is involved in the nitration of protein tyrosine residues, a reaction where SOD is once more involved as a catalyst (Ischiropoulos et al., 1992).

Therefore, our results indicate that under the experimental conditions studied, O_2^-, H_2O_2, NO and possibly $OONO^-$ may influence, directly or indirectly, triggering of p38-MAPK cascade activation. Our findings are further substantiated by previous reports noting the rapid response of p38-MAPK signal transduction pathway to oxidative stress, i.e. in the case of direct exposure to H_2O_2 both in our experimental model (Gaitanaki et al., 2003) and in rat neonatal cardiac myocytes (Clerk et al., 1998). The molecular mechanisms triggered during the amphibian heart response to cobalt perfusion have been evaluated to be specific to this metal, since our group has demonstrated that perfusion with copper, another metal that can induce rapid generation of ROS *via* a Haber–Weiss or Fenton-like reaction (Gutteridge, 1985; Shi and Dalal, 1992; Gaetke and Chow, 2003), does not stimulate p38-MAPK phosphorylation through O_2^- or H_2O_2 (Gaitanaki et al., 2006b).

The amphibian cardiac muscle is secreting an atrial natriuretic peptide (ANP) very similar to the mammalian one (Netchitailo et al., 1988) in order to prevent heart overloading under stressful conditions (Gisbert and Fischmeister, 1988; Netchitailo et al., 1988; Cerra et al., 2003). Given the fact that direct oxidative stress (i.e. H_2O_2) has been found to elevate ANP mRNA levels *via* the p38-MAPK signalling cascade in the frog heart (Vassilopoulos et al., 2005), an attempt was made to investigate ANP response to $CoCl_2$ perfusion. Accordingly, $CoCl_2$ was found to stimulate ANP mRNA levels to a significant degree, a response ablated by Trolox, Lipoic acid and SB203580 (Fig. 4). Thus, it is tenable to postulate

that ANP transcript levels appear to be modulated by reactive species in a p38-MAPK-dependent manner. De Vito et al. (De Vito et al., 2003) have proposed in their study a role for ANP to aleviate oxidative stress-induced damaging effects in rat aortic smooth muscle cells, while Kiemer et al. (Kiemer et al., 2002) have found ANP to be a defensive effector counteracting ischemia–reperfusion injury, which is known to reproduce oxidative stress conditions. Furthermore, several studies mark ANP regulation by free radicals, especially nitric oxide, which is known to be released by cardiac myocytes (Sanchez-Ferrer et al., 1990; Carnio et al., 2004). Supporting our results, Chun et al. (Chun et al., 2003) have reported ANP transcriptional upregulation by $CoCl_2$ in H9c2 cardiac myoblasts. In agreement with the above results, Thuerauf et al. (Thuerauf et al., 1998) have also shown a role for p38-MAPK in ANP gene transcriptional regulation in cardiac myocytes. The sustained p38-MAPK activation by $CoCl_2$ might act in concert with ANP transcriptional upregulation to ensure a compensatory response to $CoCl_2$ perfusion. ANP could therefore function as a sensitive marker of cardiac stress, also mediating cardioprotection.

Evidently, further studies are needed in order to fully delineate the exact nature of reactive species mediating the observed $CoCl_2$-induced effect in the perfused amphibian heart. ROS have been regarded as toxic byproducts of aerobic metabolism following reoxygenation after periods of ischemia or hypoxia (McCord, 1985). However, accumulating data now suggest that ROS play a vital physiological role in the context of signal transduction mechanisms regulating redox cellular homeostasis (Irani, 2000). Among the factors contributing to cardioprotection under oxidative stress conditions, recent studies have emphasized the role of nitric oxide, peroxynitrite and the mito K_{ATP} channels, which have as end-effectors the ROS generated or signalling molecules that are 'activated' (i.e. p38-MAPK cascade members or ANP). Hypoxia itself has been recently shown to confer a salutary effect against prolonged ischemia in the rat *via* NO and K_{ATP} channels (Beguin et al., 2005), with p38-MAPK implicated in triggering this delayed PC effect (Beguin et al., 2007).

A hypothetical schematic model outlining the amphibian heart response to perfusion with $CoCl_2$ can be seen in Fig. 5. Our results are indicative of p38-MAPK cascade and ANP playing a significant role in concert, contributing to protection of the amphibian heart against $CoCl_2$ perfusion.

In conclusion, the hypoxia mimetic agent $CoCl_2$ has been found to exert an oxidative stimulus effect in our experimental model. The novel findings of the present study implicate the p38-MAPK pathway to serve a cytoprotective role in this context, by mediating both Hsp27 phosphorylation and ANP mRNA upregulation. The precise identification and characterization of the physiological role played by this network of kinases, in order to achieve compensation for any adverse environmental conditions encountered by these organisms *in vivo* remain to be determined. Overall, the amphibian heart constitutes an excellent experimental model for the investigation of mechanisms triggered in response to $CoCl_2$-stimulated oxidative stress. Amphibians have developed strategies to adapt to and overcome hypoxic as well as oxidative stress conditions that they encounter, either due to low oxygen availability or abrupt changes in environmental temperature or body dehydration levels (Hermes-Lima and Storey, 1996; Hermes-Lima and Storey, 1998; Greenway and Storey, 2000). Therefore, any data concerning their response to such adverse conditions is of high physiological interest and gives insight to the signal transduction mechanisms triggered at the whole organ level rather than in a cell culture.

List of abbreviations

ANP	atrial natriuretic peptide
CAT	catalase
ECL	enhanced chemiluminescence
ERKs	extracellular signal-regulated kinases
Hsp27	heat shock protein 27
JNKs	c-jun N-terminal kinases
MAPKAPK2	MAPK activated protein kinase2
MAPKs	mitogen-activated protein kinases
p38-MAPK	p38 reactivating kinase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ROS	reactive oxygen species
SOD	superoxide dismutase
TBS	Tris-buffered saline

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