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# Effects of various oxidants and antioxidants on the p38-MAPK signalling pathway in the perfused amphibian heart

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## Abstract

We investigated the effects of different antioxidants such as L-ascorbic acid, catalase, and superoxide dismutase (SOD), on the p38-MAPK activation induced by oxidative stress in the isolated perfused amphibian heart. Oxidative stress was exemplified by perfusing hearts with 30 $\mu$M $H_2O_2$ for 5 min or with the enzymatic system of xanthine/xanthine oxidase (200 $\mu$M/10 mU/ml, respectively) for 10 min. $H_2O_2$-induced activation of p38-MAPK (7.04 $\pm$ 0.20-fold relative to control values) was totally attenuated by L-ascorbic acid (100 $\mu$M) or catalase (150 U/ml). These results were confirmed by immunohistochemical studies in which the phosphorylated form of p38-MAPK was localised in the perinuclear region and dispersedly in the cytoplasm of the ventricular cells during $H_2O_2$ treatment, a pattern that was abolished by catalase or L-ascorbic acid. p38-MAPK was also activated (2.34 $\pm$ 0.17-fold) by perfusing amphibian hearts with the reactive oxygen species (ROS)-generating system of xanthine/xanthine oxidase and this activation sustained in the presence of 150 U/ml catalase (2.16 $\pm$ 0.26-fold), 50 U/ml SOD (2.02 $\pm$ 0.07) or 100 $\mu$M L-ascorbic acid (2.18 $\pm$ 0.10), but was suppressed by the combination of 150 U/ml catalase and 50 U/ml SOD. Finally, our studies showed that xanthine/xanthine oxidase induced the phosphorylation of the potent p38-MAPK substrates MAPKAPK2 (3.14 $\pm$ 0.27-fold) and HSP27 (5.32 $\pm$ 0.83-fold), which are implicated in cell protection, and this activation was reduced by the simultaneous use of catalase and SOD. (Mol Cell Biochem **291:** 107–117, 2006)

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

*Abbreviation*: CAT, catalase; DMSO, dimethylsulfoxide; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; Hsp, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl methyl sulfonyl fluoride; p38-MAPK/RK, p38 reactivating kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBS, Tris-buffered saline; X, xanthine; XO, xanthine oxidase

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# Introduction

Reactive oxygen species (ROS) have been proven to play a major role in the regulation of heart physiology, as they are involved in the determination of the myocardial responses such as apoptosis or survival/hypertrophy via diverse signalling pathways [1–4]. The myocardial ROS production is common during hypoxia, since the highly reduced mitochondrial redox state would promote electron donation to residual $O_2$ to form superoxide anion ($O_2^{\bullet -}$) [5, 6]. Furthermore, ROS are known to rise after hypoxia/re-oxygenation through the activities of enzymes such as xathine oxidase, P-450 cytochrome oxidase and NADPH oxidase [6, 7].

The understanding of the oxidative stress consequences in the mammalian heart is of great theoretical and practical importance. Nevertheless, an approach of this issue focusing on lower vertebrates may also be of extreme interest. It should be considered that in such organisms the mechanisms of oxidative stress induction are similar with the ones mentioned above, though their physiology is quite different. Amphibians, in particular, are adapted to survive under stressful conditions arising either in low oxygen pressures or in water environments contaminated with transition metals, or during alterations of their metabolic rate in response to changes in temperature, food disposability and body dehydration [8–10]. As a result they have developed an effective antioxidant defence, reinforced by increased expression of antioxidant enzymes and amplified levels of antioxidant substances, such as glutathione and L-ascorbic acid [8, 9, 11, 12].

Various reports have documented the involvement of the mitogen-activated protein kinase (MAPK) signalling pathways in redox-stressed cells and tissues, including mammalian cardiac myocytes and intact myocardium [13–17]. It has been indicated that the three well established MAPK family members (JNKs, ERKs and p38-MAPK) play a significant role in the determination of either an anti-apoptotic or pro-apoptotic myocyte fate, which follows the oxidative stress induction [3, 4, 16, 18, 19].

p38-MAPK is implicated in one of the most important stress-activated signalling pathways, since it is activated by various forms of environmental stress, including hyperosmolarity, oxidative stress and heat shock [20, 21]. The respective MAPK subfamily in the amphibian heart has been previously characterised in our laboratory [22–25]. In particular, amphibian p38-MAPK has been found to be stimulated by mechanical overload, but most potently, by hyperosmotic and thermal stresses as well as $H_2O_2$ [16, 23, 24].

Activated p38-MAPK is characterised by its localisation in both, the cytoplasm and the nucleus, where it interacts with its substrates [15, 23]. A variety of p38-MAPK substrates has been identified including several transcription factors and other protein kinases, such as MAPK-activated protein kinase 2 (MAPKAPK2), which phosphorylates the small heat shock protein HSP27 [16, 26, 27]. In various cell types, phosphorylation of HSP27 is associated with stabilisation of the actin cytoskeleton, protecting cells against damage [28–30] and with its binding to cell proteins leading to prevention of their degradation [31].

In our previous studies [16, 17], the effect of oxidative stress on the phosphorylation of the amphibian *Rana ridibunda* heart MAPKs was examined by exposure of the perfused hearts to variant concentrations of $H_2O_2$. Considering that *in vivo* oxidative stress is mediated not only by $H_2O_2$, which is the most prevalent oxidant, but also by numerous and variant oxidative species, it is interesting to investigate the consequences of oxidative stress in the amphibian heart using enzymatic agents known to induce the production of such species in various cell types.

On this basis, amphibian hearts were subjected to perfusion with various oxidants in the absence or presence of antioxidants. L-ascorbic acid, which is biosynthesised in amphibians *in vivo* [11], was used in order to identify its possible antioxidative impact on the activation of p38-MAPK in the heart by oxidative stress. In addition, given that catalase is a $H_2O_2$ scavenger and superoxide dismutase (SOD) is a superoxide scavenger, these antioxidant enzymes were used in order to identify any specific role of each ROS in the p38-MAPK activation. Finally, we examined the effect of the enzymatic system of xanthine/xanthine oxidase on the activation of the p38-MAPK signalling pathway in the amphibian heart. This system, even though there is no report stating that it is active in the frog heart, was selected since it is known to produce a form of ROS other than $H_2O_2$, the superoxide anion [6, 7].

# Materials and methods

*Materials*

Most biochemicals used were purchased from Applichem GmbH (Ottoweg 10b, D-64291 Darmstandt, Germany). Catalase (from bovine liver, C-30), SOD (from bovine erythrocytes, S-2515), xanthine (X-0626) and xanthine oxidase (from microorganisms, X-2252) were obtained from Sigma Chemical Co (St Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala 751 84, Sweden) and alkaline phosphatase Kwik kit from Lipshaw (Pittsburgh, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, California 94547, USA). Nitrocellulose (0.45 $\mu$m) was obtained from Schleicher & Schuell (Keene N.H. 03431, USA).

Rabbit polyclonal antibodies specific for the total (phosphorylation state independent) and dually-phosphorylated p38-MAPK (#9212 and #9211, respectively), as well as, for the total and phosphorylated (Thr334) MAPKAPK2 (#3042

and 3041, respectively) and the phosphorylated (Ser82) small heat shock protein 27 (HSP27) (#2401) were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-actin antibody (A-2103) was from Sigma Chemical Co. Prestained molecular mass markers were from New England Biolabs (P7708S; Ipswich, MA, USA). HPR-conjugated anti-rabbit antibody was from DAKO A/S (Glostrup, Denmark). X-OMAT AR 13 × 18 cm and Elite chrome 100 films were 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).

*Heart perfusions*

Animals were anaesthetized by immersion in 0.05% (w/v) MS222 and sacrificed by decapitation. The hearts were excised and mounted onto the aortic cannula of a conventional Langendorff perfusion system. Perfusions were performed in a non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with Krebs bicarbonate-buffered saline (23.8 mM $NaHCO_3$, 103 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 1.8 mM $MgCl_2$, 0.6 mM $NaH_2PO_4$, pH 7.4 at 25 °C) supplemented with 10 mM glucose and equilibrated with 95% $O_2$/5% $CO_2$. The temperature of the hearts and perfusates was maintained at 25 °C by using a water-jacketed apparatus. All hearts were equilibrated for 15 min under these conditions.

Hearts were assigned to ten groups. The protocols for these distinct experimental groups are illustrated in Table 1. In the control group (C), hearts were perfused for 30 min under the physiological conditions mentioned above (the equilibration period included). As positive controls, hearts perfused with 30 $\mu$M $H_2O_2$ for 5 min were used. To test the impact of $H_2O_2$ scavenging by catalase, equilibrated hearts were treated with 30 $\mu$M $H_2O_2$ in the presence of 150 U/ml catalase for 5 min (group $H_2O_2$ + CAT). Moreover, the antioxidant activity of L-ascorbic acid was investigated through the study of three experimental groups: in the $H_2O_2$ + ASC group the hearts were treated with L-ascorbic acid (100 $\mu$M) during the equilibration period and the following perfusion with 30 $\mu$M $H_2O_2$ for 5 min, in the $H_2O_2$/ASC group L-ascorbic acid (100 $\mu$M) was present only during the equilibration period and subsequently absent during the perfusion with 30 $\mu$M $H_2O_2$ for 5 min and in the ASC group hearts were perfused for 20 min with normal perfusion buffer containing L-ascorbic (100 $\mu$M).

In the last experimental protocol, after the equilibration period, hearts were perfused with the ROS-generating enzymatic system xanthine/xanthine oxidase (200 $\mu$M/10 mU/ml, respectively) for 10 min, in the absence (group X/XO) or the presence of 150 U/ml catalase (group X/XO + CAT) or 150 U/ml catalase and 50 U/ml SOD (group X/XO + CAT + SOD). Perfusions were also performed for 10 min after equilibration with 50 U/ml SOD in the absence (group SOD) or the presence of the aforementioned enzymatic system (group X/XO + SOD). Finally, in the X control group and the X/XO-ASC group equilibrated hearts were treated for 10 min with 200 $\mu$M xanthine or with the system of xanthine-xanthine oxidase plus 100 $\mu$M L-ascorbic acid, respectively. During the perfusions in the presence of L-ascorbic acid, catalase and xanthine/xanthine oxidase the perfusion apparatus was covered with aluminium foil for photoprotection.

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

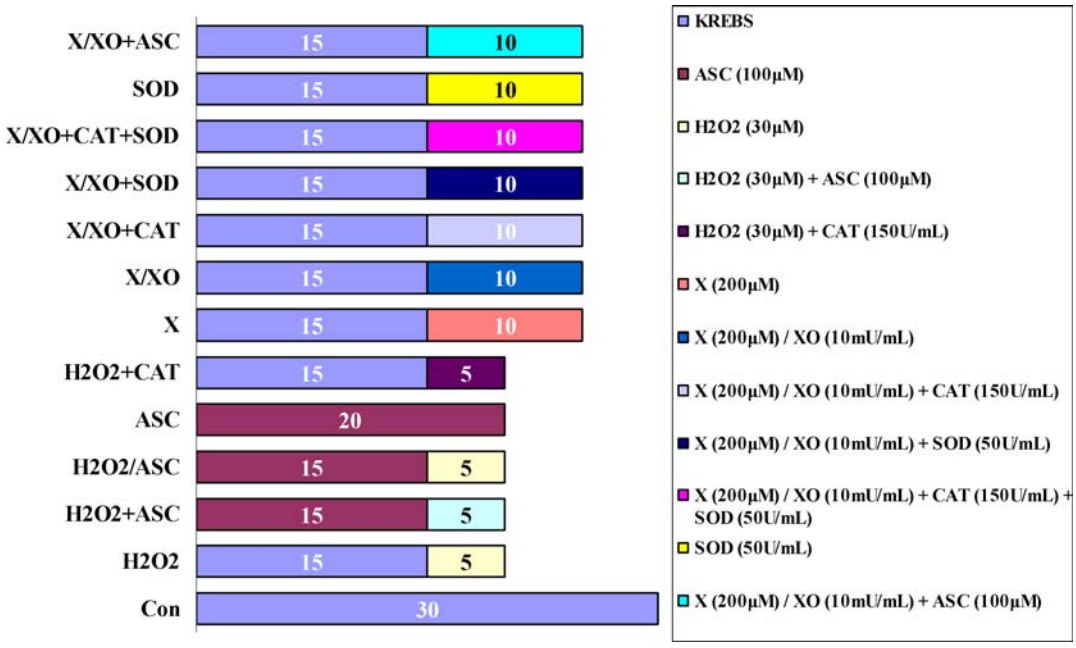
*Tissue extractions*

Heart powders were homogenised with 3 ml/g of buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 10 mM NaF, 1 mM $Na_3VO_4$, 1.5 mM $MgCl_2$, 20 mM $\beta$-glycerophosphate, 1 mM dithiothreitol (DTT), 2 $\mu$g/ml leupeptin, 0.5 mM phenyl methyl sulphonyl fluoride (PMSF), 4 $\mu$g/ml aprotinine] and extracted on ice for 30 min. The samples were centrifuged (10,000 rpm, 10 min, 4 °C) and the supernatants boiled with 0.33 volumes of SDS-PAGE sample buffer [0.33 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) Bromophenol Blue]. Protein concentrations were determined using the BioRad Bradford assay.

*SDS-PAGE and immunoblot analysis*

Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide or 15% (w/v) acrylamide, 0.413% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 $\mu$m). Membranes were then incubated in TBS-T [20 mM Tris-HCl, pH 7.5, 137 mM 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

*Table 1.* Experimental groups




TBS-T (4 × 5 min) the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies [1:5000 dilution in TBS-T containing 1% (w/v) BSA, 1 h, room temperature]. The blots were washed again in TBS-T (4 × 5 min) and the bands were detected by using the enhanced chemiluminescence (ECL) reaction with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

*Immunolocalisation of phospho-p38 MAPK*

At the end of the perfusions, atria were removed and ventricles were immersed in isopentane pre-cooled in liquid $N_2$ and stored at –80 °C. Tissues were sectioned with a cryostat at a thickness of 5–6 $\mu$m, fixed with ice-cold acetone (10 min, at room temperature) and specimens were stored at –30 °C until use. Tissue sections were washed in TBS-T [containing 0.1% (v/v) Tween 20] and non-specific binding sites were blocked with 3% (w/v) BSA in TBS-T (1 h, at room temperature). Specimens were incubated with primary antibody specific for phospho-p38 MAPK, diluted in 3% (w/v) BSA in TBS-T (overnight, 4 °C), according to the method previously described [24]. All sections were immunostained by the alkaline phosphatase method using a Kwik kit, according to the manufacturer's instructions. The alkaline phosphatase label was visualised by exposing the sections to Fast Red chromogen and nuclei were counterstained with Haematoxylin. Slides were mounted, examined with a Zeiss Axioplan microscope and photographed with a Kodak Elite chrome 100 film.

*Statistical evaluations*

All data are presented as means ± 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 was set at 1, and the stimulated kinase and HSP27 phosphorylation in treated hearts was expressed as "–fold" activation over control hearts.

## Results

In a previous study [16] we had shown that oxidative stress, as exemplified by $H_2O_2$, strongly induces the phosphorylation (hence activation) of p38-MAPK and its signalling pathway. This response was found to be specific as it was shown with experiments using the selective p38-MAPK inhibitor SB203580 (1 $\mu$M). Based on these results we have further examined the effect of the antioxidants L-ascorbic acid and catalase on the $H_2O_2$-induced p38-MAPK activation.

We first tried to assess the antioxidant capacity of L-ascorbic acid. Therefore, we perfused amphibian hearts with 100 $\mu$M L-ascorbic acid either during the equilibration period

or simultaneously perfusing the hearts with 30 $\mu$M $H_2O_2$ for 5 min. p38 MAPK activation was studied by immunoblot analysis using a specific antibody raised against the dually phosphorylated form of the kinase at the Thr and Tyr residues of the Thr-Gly-Tyr motif, since this form is known to be the active one [20]. The results of this study revealed that $H_2O_2$-induced activation of p38-MAPK (7.04 $\pm$ 0.20-fold relative to control values; $p < 0.001, N = 6$) was completely inhibited by L-ascorbic acid and this effect was observed both, in hearts perfused with 100 $\mu$M L-ascorbic acid before or at the same time with the oxidative factor (Figs. 1A, top panel and 1B). These data suggest that L-ascorbic acid can be an effective antioxidant for the protection of the amphibian heart against oxidative stress as exemplified by $H_2O_2$. Blots assayed with a total p38-MAPK (phosphorylation state independent) antibody were used as an equivalent loading control (Fig. 1A, bottom panel).

Catalase, an enzyme acting as a $H_2O_2$ scavenger [32], was another antioxidant used in this study. Amphibian hearts were perfused with 30 $\mu$M $H_2O_2$ for 5 min in the presence of 150 U/ml catalase and this resulted in the complete abrogation of the $H_2O_2$-induced activation of p38-MAPK, confirming that exogenous oxidative stress is a stimulator of this signalling pathway. Catalase alone did not affect p38-MAPK activation (Figs. 1C, top panel and 1D). Equivalent protein loading was verified by probing identical samples with an antibody recognising total p38-MAPK levels (Fig. 1C, bottom panel).

These results were confirmed by immunohistochemical studies in which the localisation pattern of the phosphorylated p38-MAPK was investigated under conditions of oxidative stress, in the absence or presence of the antioxidants mentioned above. For this purpose, frog hearts were perfused with 30 $\mu$M $H_2O_2$ for 5 min in the absence or presence of 150 U/ml catalase, or they were perfused with 100 $\mu$M L-ascorbic acid for 15 min before the addition of $H_2O_2$. After the removal of atria, the ventricle was sectioned and the respective specimens were processed using an antibody specific for the phosphorylated form of p38-MAPK. Neither in control hearts (Fig. 2A) nor in specimens incubated either with the secondary antibody or the chromogen alone, was any immunoreactivity detected (data not shown). However, in specimens from hearts perfused with 30 $\mu$M $H_2O_2$ strong immunoreactivity staining for the phosphorylated p38-MAPK was observed within the cytoplasm as well as in the perinuclear region (Fig. 2B). On the contrary, in specimens obtained from hearts perfused with the oxidative factor in the presence of the $H_2O_2$-scavenger catalase the perinuclear localisation of the phosphorylated form of p38-MAPK disappeared, while it was barely detectable in the cytoplasm (Fig. 2C). A similar observation was also made in heart tissues preconditioned with 100 $\mu$M L-ascorbic acid before being perfused with $H_2O_2$ (Fig. 2D).

In another set of experiments, oxidative stress was simulated by using the xanthine/xanthine oxidase enzymatic system, which is known to be involved in ROS production [6, 33]. Perfusion of the amphibian heart with 200 $\mu$M xanthine/10 mU/ml xanthine oxidase for 10 min activated p38-MAPK moderately compared to the $H_2O_2$ induced phosphorylation (2.34 $\pm$ 0.17-fold relative to control values; $p < 0.001$, $N = 5$), whereas xanthine alone did not affect significantly the kinase activation (Figs. 3A, top left panel and 3B). We next examined whether such an effect was due to the ROS produced by this system and for this reason the same heart perfusions were conducted in the presence of catalase (150 U/ml), SOD (50 U/ml) or in the simultaneous presence of catalase and SOD. $H_2O_2$ scavenging activity of catalase had little impact on the p38-MAPK phosphorylation induced by xanthine/xanthine oxidase (2.16 $\pm$ 0.26-fold relative to control values; $p < 0.01, N = 5$) (Figs. 3A, top left panel and 3B). Similar results were obtained when SOD, a superoxide anion scavenger, was used along with the system of xantine/xanthine oxidase (p38-MAPK activation: 2.02 $\pm$ 0.07-fold relative to control values; $p < 0.001$, $N = 3$), whereas SOD alone had no effect on the kinase phosphorylation levels (Figs. 3A, top right panel and 3B). However, the scavenging of every radical form by the combination of catalase and SOD abolished the xanthine/xanthine oxidase-induced activation of the kinase (Figs. 3A, top left panel and 3B). Perfusions with the xanthine/xanthine oxidase system were also performed in the presence of L-ascorbic acid (100 $\mu$M), but this agent did not reduce significantly the p38-MAPK phosphorylation induced by this system (2.18 $\pm$ 0.10-fold relative to control values; $p < 0.001, N = 3$) (Figs. 3A, top right panel and 3B). Actin protein levels of identical samples were also detected so as to confirm the equal protein loading (Fig. 3A, bottom panels).

We also investigated whether the oxidative stress induced by the xanthine/xanthine oxidase system affected the phosphorylation state of two potent members of the p38-MAPK signalling pathway, MAPKAPK2 and HSP27. Therefore, we conducted an immunoblot analysis using antibodies specifically raised against the phosphorylated forms of MAPKAPK2 (Thr334) and HSP27 (Ser82). The results of these experiments revealed that the enzymatic system of xanthine/xanthine oxidase induced a strong phosphorylation of MAPKAPK2 (3.14 $\pm$ 0.27-fold relative to control values; $p < 0.01$, $N = 3$) and that this phosphorylation was not significantly affected by 150 U/ml catalase (2.82 $\pm$ 0.26-fold relative to control values; $p < 0.01, N = 3$) or 50 U/ml SOD (2.95 $\pm$ 0.16-fold relative to control values; $p < 0.01$, $N = 3$) (Figs. 4A, top panel and 4B). SOD alone did not activate MAPKAPK2, while the combination of catalase and SOD abolished the xanthine/xanthine oxidase-induced MAPKAPK2 phosphorylation (Figs. 4A, top panel and 4B). Identical samples were probed with an antibody recognising total

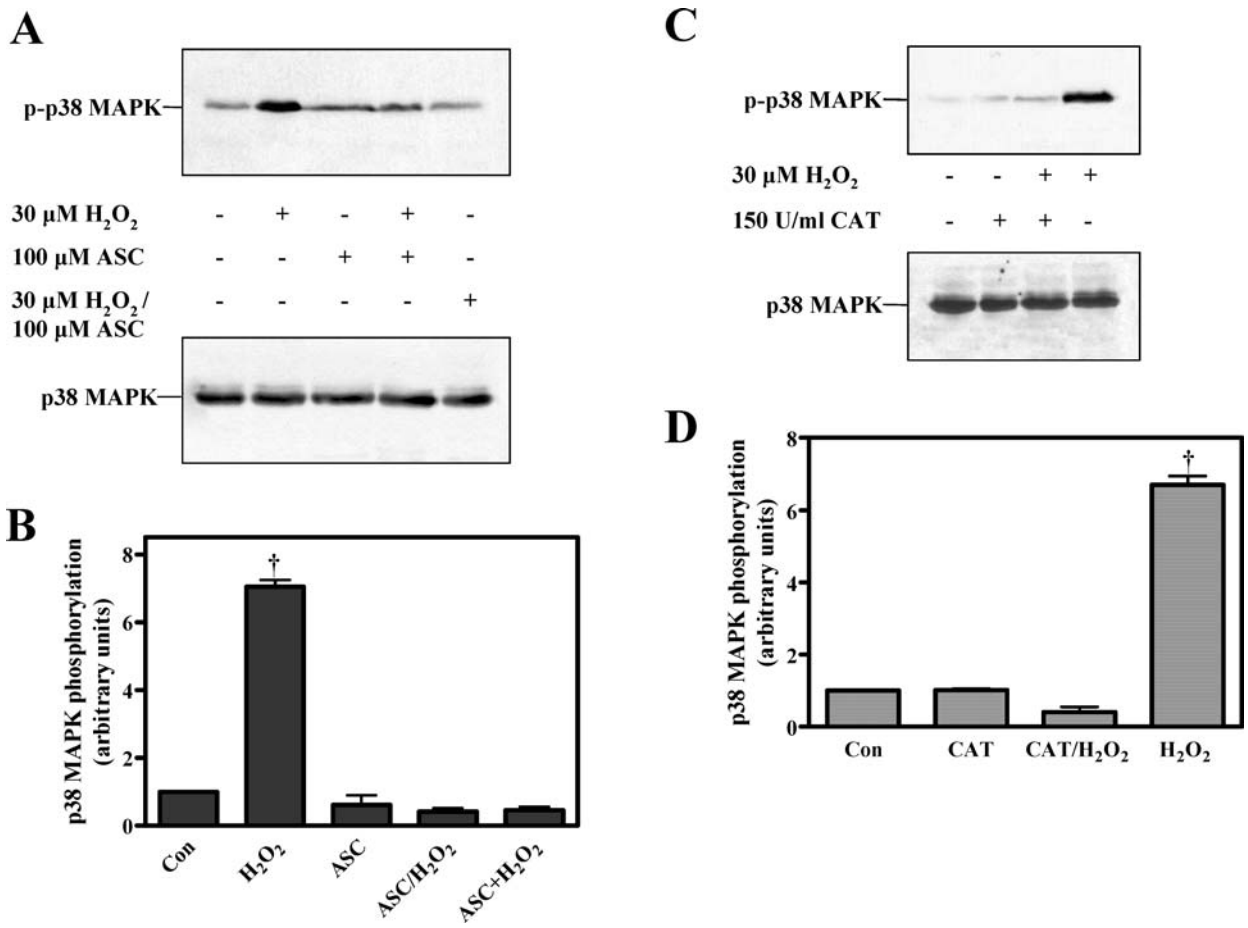



*Fig. 1.* Effect of L-ascorbic acid and catalase on the $H_2O_2$-induced p38-MAPK phosphorylation. (A, top panel): Protein (100 $\mu$g) from *Rana ridibunda* hearts perfused with or without (Con) 30 $\mu$M $H_2O_2$ for 5 min in the absence or presence of 100 $\mu$M L-ascorbic acid (ASC) was assessed by immunoblot analysis using a phosphospecific anti-p38-MAPK antibody. (C, top panel): The p38-MAPK phosphorylation was also measured in samples obtained from hearts perfused with 30 $\mu$M $H_2O_2$ for 5 min in the absence or presence of 150 U/ml catalase (CAT). (A and C, bottom panels): Immunoblots of identical samples for total p38-MAPK levels were included as a control for protein loading. (B, D): Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for at least three independent experiments performed with similar findings. $^{\dagger}p < 0.001$ *vs* control value.

MAPKAPK2 levels so as to confirm the equivalent protein loading (Fig. 4A, bottom panel).

As far as HSP27 is concerned, perfusions in the presence of xanthine alone induced a moderate HSP27 phosphorylation ($2.64 \pm 0.43$; $p < 0.05$, $N = 3$), while the xanthine/xanthine oxidase system induced a strong phosphorylation of this heat shock protein ($5.32 \pm 0.83$-fold relative to values; $p < 0.05$, $N = 3$). These phosphorylation levels were slightly but not significantly decreased when 150 U/ml catalase were added to the perfusate ($4.62 \pm 0.58$-fold relative to control values; $p < 0.001$, $N = 4$) (Figs. 5A, top panel and 5B). 50 U/ml SOD alone did not stimulate HSP27 phosphorylation, while SOD in the presence of xanthine/xanthine oxidase did not attenuate significantly the HSP27 phosphorylation induced by this enzymatic system (data not shown). However, the simultaneous use of 150 U/ml catalase and 50 U/ml SOD decreased the HSP27 phosphorylation induced by xanthine/xanthine oxidase ($2.02 \pm 0.29$-relative to control values; $p < 0.05$, $N = 4$) to levels similar to the ones induced by xanthine alone (Figs. 5A, top panel and 5B), indicating that the combination of these two antioxidant enzymes abolishes the xanthine/xanthine oxidase-induced HSP27 activation. Actin protein levels of identical samples were also detected so as to confirm the equal protein loading (Fig. 5A, bottom panel). This result supports the suggestion that the superoxide anion, which is a SOD substrate, is the main factor activating p38-MAPK signalling pathway when hearts are perfused with the enzymatic system of xanthine/xanthine oxidase.

## Discussion

Amphibians, like other ectotherms, are organisms, which need to continuously adapt to adverse environmental

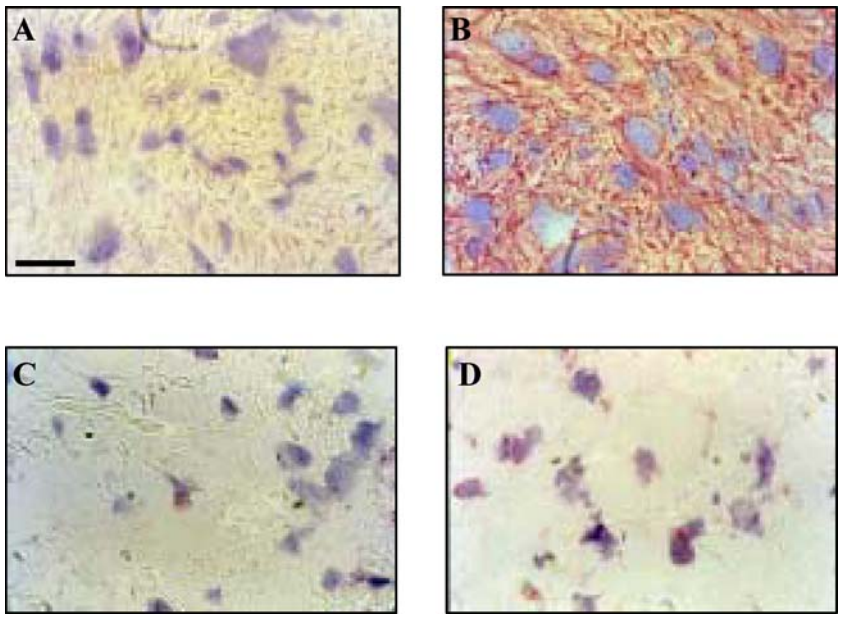



*Fig. 2*. Immunohistochemical localisation of phospho-p38-MAPK in the ventricle of isolated amphibian heart perfused without (control heart) (A) or with 30 $\mu$M $H_2O_2$ for 5 min in the absence (B) or presence of 150 U/ml catalase (C). Specimens from hearts preconditioned with 100 $\mu$M L-ascorbic acid for 15 min before perfusion with 30 $\mu$M $H_2O_2$ for 2 min (D) were also used for the phospho-p38-MAPK immunolocalisation. Cryosections were incubated with a phosphospecific anti-p38-MAPK (1:200 dilution) antibody and processed as described in *Materials and Methods*. Immunolocalisation deposits are visualized with Fast Red chromogen. Representative photographs of three independent experiments are shown. Scale bar 20 $\mu$m.

conditions such as temperature fluctuations and low oxygen availability. Under these conditions amphibians are subjected to different kinds of stress, among of which is oxidative stress.

Oxidative stress is generally thought to be the effect of high ROS concentrations. The severity of the stress depends on the kind of the species, with the hydroxyl radical ($^{\bullet}OH$) being more reactive than the superoxide anion ($O_2^{\bullet -}$) and $H_2O_2$ [34]. ROS can react with and modify the cellular components, causing severe damage which can finally lead to cell death [7]. Therefore, cells have developed antioxidant mechanisms including low molecular weight molecules (like ascorbic acid and glutathione) [7, 32, 34] and specific enzymes, such as SOD, which converts superoxide anion to $H_2O_2$, and catalase, which catalyses the dismutation of $H_2O_2$ to water and molecular oxygen.

ROS at low levels can also act as signalling molecules [6, 7, 35] and one of the signal transduction pathways activated by such a stimulus is that of p38-MAPK. This pathway can be either protective or pro-apoptotic [19, 36] and it has been extensively studied in the mammalian cardiovascular system, where oxidative stress is induced under conditions such as ischemia/reperfusion [6, 14, 37].

In addition, the p38-MAPK signalling pathway is also activated by oxidative stress (in the form of $H_2O_2$) in the amphibian heart [16, 17]. This activation was maximal at low concentrations of $H_2O_2$ (30 $\mu$M) and it was implicated in the regulation of the atrial natriuretic peptide gene expression, a protective cardiac hormone involved in the regulation of fluid balance and blood pressure homeostasis [17]. In continuation of these studies, we examined the effects of different antioxidants on the $H_2O_2$-induced p38-MAPK activation in the *Rana ridibunda* heart.

For this purpose, we used L-ascorbic acid, a naturally occurring antioxidant that has also been found in amphibians [11], and the $H_2O_2$-scavenger, catalase. L-ascorbic acid attenuated the $H_2O_2$-induced p38-MAPK phosphorylation (Figs. 1A and 1B) and the fact that it is an endogenous amphibian product [11] renders this agent as an important antioxidant for these animals. This result is in agreement with studies in various cell types in which the oxidative stress-induced p38-MAPK phosphorylation (either caused by $H_2O_2$ or other factors) is blocked by the presence of L-ascorbic acid [38–40].

Catalase, which is naturally located in peroxisomes and the mitochondrial membrane of cardiac myocytes [32, 41], was utilised extracellularly in this study so as to eliminate the factor used to exert the exogenous oxidative stress. Indeed, the use of catalase abolished the p38-MAPK activation induced by $H_2O_2$ (Figs 1C and 1D) confirming that this agent is a stimulator of the p38-MAPK signalling pathway. This result is in agreement with previous studies, in which catalase had been used extracellularly [42].

The above-mentioned data were supported with our immunolocalisation studies of the phosphorylated form of p38-MAPK in which the use of catalase or L-ascorbic acid

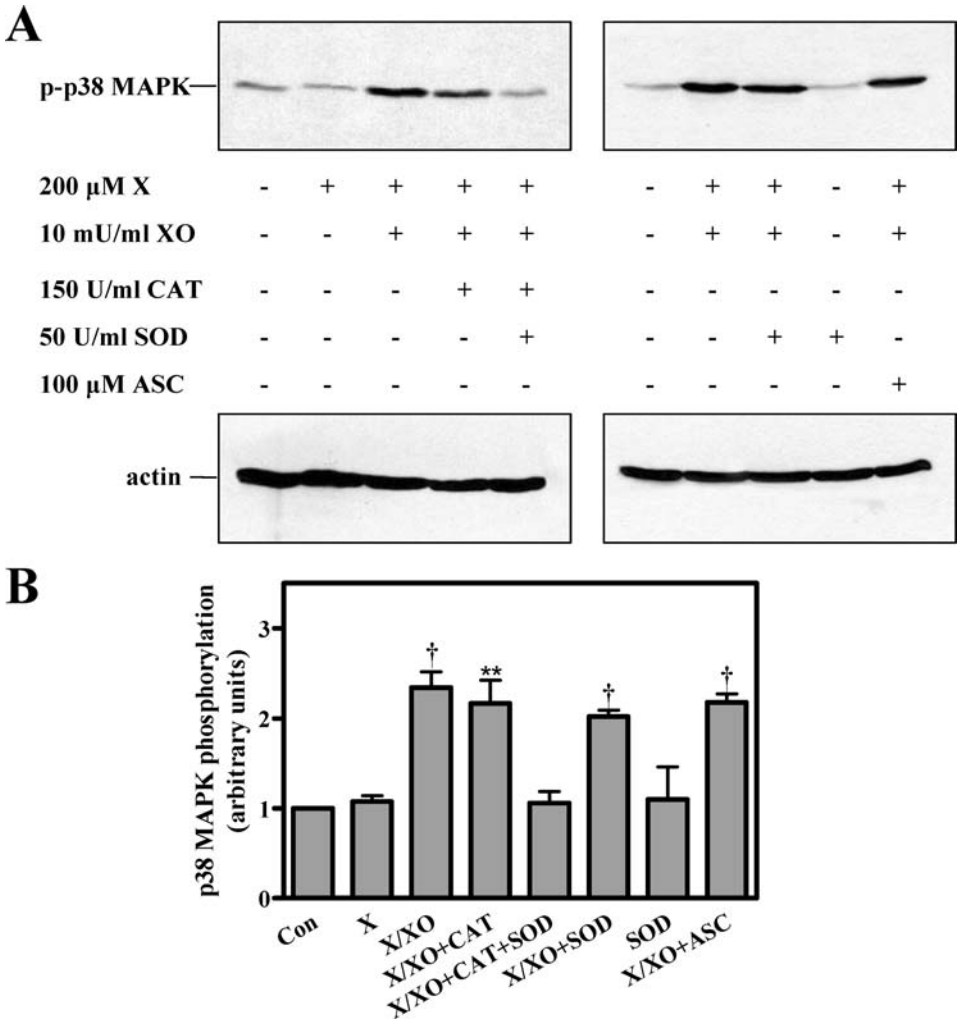



*Fig. 3.* Effect of the enzymatic system xanthine/xanthine oxidase on the p38-MAPK phosphorylation in the absence or presence of different antioxidants. (A, top left panel): Phospho-p38-MAPK was detected in extracts (50 $\mu$g) from control hearts (Con) and hearts perfused for 10 min with 200 $\mu$M xanthine (X) in the absence or presence of 10 mU/ml xanthine oxidase (XO). Samples of hearts perfused for 10 min with X/XO and 150 U/ml catalase (CAT) with or without 50 U/ml SOD, were also tested for p38-MAPK phosphorylation. (A, top right panel) p38-MAPK phosphorylation was also assessed in samples from hearts perfused for 10 min with 50 U/ml SOD, in the absence or presence of X/XO, or with X/XO in the presence of 100 $\mu$M L-ascorbic acid (ASC). (A, bottom panels): Identical samples were probed with an anti-actin antibody in order to confirm equal loading. (B): Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for five independent experiments. $^{**}p < 0.01$ *vs* control value, $^{\dagger}p < 0.001$ *vs* control value.

eliminated the immunocomplexes detected when hearts were treated with $H_2O_2$ (Fig. 2) [16]. This result also confirms that p38-MAPK activation is specifically stimulated by oxidative stress.

In the present study we also tried to assess whether oxidative factors other than $H_2O_2$ can induce the p38-MAPK signalling pathway. In order to achieve this, we performed perfusions with the enzymatic system of xanthine/xanthine oxidase, which mainly generates superoxide anion [7, 32, 33]. In our experimental model, xanthine/xanthine oxidase activated p38-MAPK. This activation was not abolished by L-ascorbic acid, which was effective in the case of $H_2O_2$, but it was totally attenuated by the combination of SOD and catalase (Fig. 3). This indicates that the main kinase activator produced by this system was the superoxide anion, which was eliminated by SOD and was converted to $H_2O_2$, which in turn was scavenged by catalase. This is further strengthened by the fact that catalase alone could not revert the xanthine/xanthine oxidase-induced p38-MAPK phosphorylation. The observed p38-MAPK activation when SOD was used simultaneously with xanthine/xanthine oxidase can be attributed to the $H_2O_2$ produced by this enzyme activity.

p38-MAPK phosphorylation induced by xanthine/ xanthine oxidase is moderate when compared to that induced by $H_2O_2$ and this can be ascribed to the levels of the oxidative factors generated by the specific concentration of xanthine

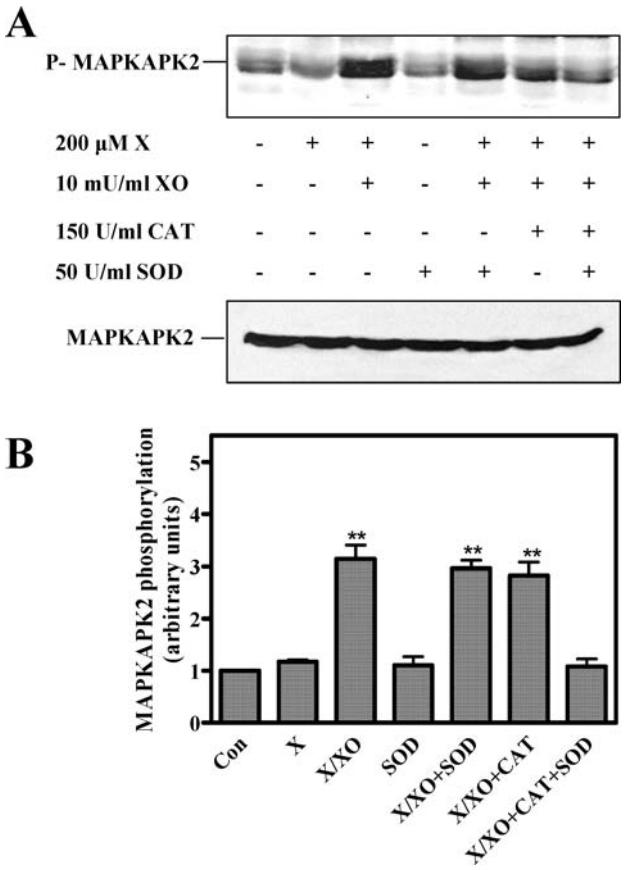



*Fig. 4*. Phosphorylation of MAPKAPK2 by the xanthine/xanthine oxidase system. (A, top panel): Extracts (100 $\mu$g) from hearts perfused without (Con) or with 200 $\mu$M xanthine (X) in the absence or presence of 10 mU/ml xanthine oxidase (XO) for 10 min were assayed for MAPKAPK2 phosphorylation through immunoblot analysis using an antibody specific for the phosphorylated form of MAPKAPK2. Extracts from hearts perfused for 10 min with SOD (50 U/ml), in the absence or presence of X/XO, or with X/XO and catalase (CAT) (150 U/ml), in the absence or presence of SOD, were also immunoassayed for phospho-MAPKAPK2. (A, bottom panel): Equal protein loading was assessed in identical samples using an antibody against total MAPKAPK2. (B): Densitometric analysis of phospho-MAPKAPK2 bands by laser scanning. Results are means ± S.E.M. for three independent experiments. $^{**}p < 0.01$ *vs* control value.

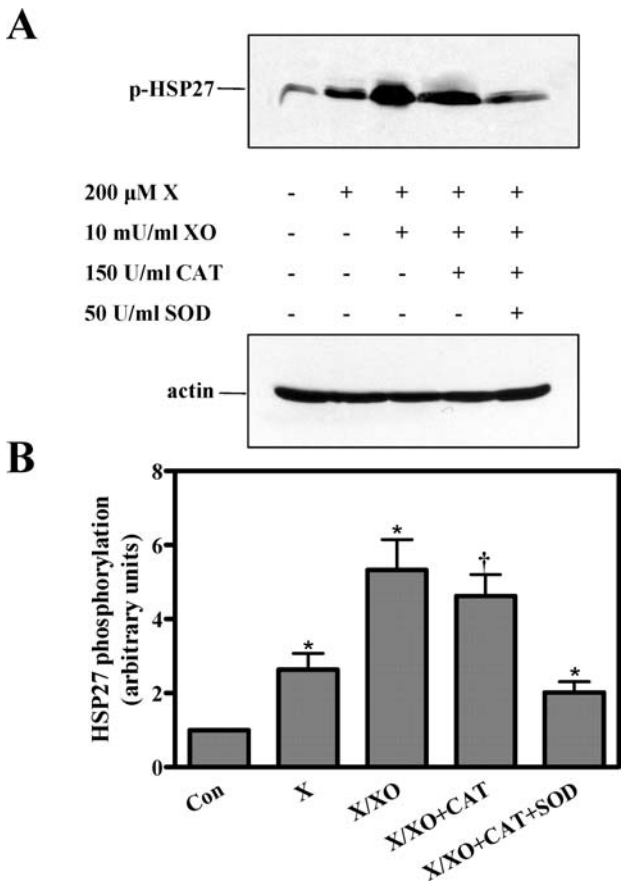



*Fig. 5*. Phosphorylation of HSP-27 by the xanthine/xanthine oxidase system. (A, top panel): Protein (100 $\mu$g) from hearts perfused for 10 min with 200 $\mu$M xanthine (X) without or with 10 mU/ml xanthine oxidase in the absence or presence of catalase (CAT) (150 U/ml) or catalase and SOD (50 U//ml) was used to perform Western blot analysis with antibodies specific for the phosphorylated HSP27. As negative controls (Con), samples from hearts perfused with the physiological bicarbonate-buffered saline were included. (A, bottom panel): Identical samples were assayed with an actin antibody as a control for protein loading (B): Densitometric analysis of phospho-HSP27 bands by laser scanning. Results are means ± S.E.M. for at least three independent experiments performed with similar findings. $^{*}p < 0.05$ *vs* control value, $^{\dagger}p < 0.001$ *vs* control value.

(200 $\mu$M) and/or activity of xanthine oxidase (10 mU/ml) used, which may not be as high as it is demanded for an intense p38-MAPK activation. In addition, superoxide anion, when compared with $H_2O_2$, is less potent in penetrating biological membranes [34] and, therefore, the triggering of the p38-MAPK signalling pathway is not so powerful. The involvement of xanthine/xanthine oxidase system in the p38-MAPK activation has also been documented in other reports [43, 44].

We also investigated two well-known downstream members of the p38-MAPK signalling pathway, MAPKAPK2 and HSP27 [20]. MAPKAPK2 phosphorylates HSP27, which is implicated in cytoprotection since it interacts with and stabilizes F-actin fibers under conditions of stress and inhibits the mitochondrial apoptotic pathway by preventing the release of cyt *c* [28–30].

In the amphibian heart, MAPKAPK2 and HSP27 have also been shown to be substrates of the p38-MAPK after stimulation with $H_2O_2$ [16]. These two proteins were also phosphorylated in response to the oxidative stress exerted by xanthine/xanthine oxidase (Figs. 4 and 5) and, additionally, signal amplification towards HSP27 was observed.

In summary, our results indicate that the p38-MAPK signalling pathway is activated by different forms of oxidative

stress and this is confirmed by the fact that a variety of antioxidant factors can attenuate this activation. Furthermore, this activation seems to be beneficial for cardiac myocytes since it is associated with the motivation of protective cellular mechanisms, like that of the small heat shock protein, HSP27.

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