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Atrial Natriuretic Peptide mRNA Regulation by p38-MAPK in the Perfused Amphibian Heart

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Key Words

Cell signaling • Amphibian heart • Atrial natriuretic peptide • p38-MAPK • Signal transduction

Abstract

Atrial natriuretic peptide (ANP) is a cardiac hormone involved in the regulation of fluid balance and blood pressure homeostasis. Using conventional reverse transcription and polymerase chain reaction and amplification, preproANP transcript from *Rana ridibunda* heart ventricle was cloned and characterized. Sequence analysis of the complete cDNA revealed an open reading frame for a 146-residue peptide, carrying a 17-residue ring at the C-terminus, characteristic of the natriuretic peptide family. Perfusion of *Rana ridibunda* myocardium under diverse stimuli such as mechanical, hyperosmotic or oxidative stress induced a rapid increase in the transcripts, which is accompanied by a significant increase in phosphorylated p38-MAPK levels. Furthermore, 1 μM SB203580, the selective p38-MAPK inhibitor, was found to abolish this increase suggesting the involvement of the p38-MAPK signalling pathway in the regulation of ANP mRNA expression.

Introduction

Natriuretic peptides (NPs), acting mainly via particulate guanylate cyclase receptors, are polypeptide hormones with important cardioinhibitory effects [1]. They are secreted by the myocardium and are involved in sodium secretion and blood pressure control in a wide range of organisms [2-4]. The family of natriuretic peptides consists of three structurally similar peptides: the atrial natriuretic peptide (ANP), the brain natriuretic peptide (BNP) and the C-type natriuretic peptide (CNP) [4]. They all have a characteristic 17 amino-acid residue ring structure formed by an intra-molecular disulfide bridge between two cysteine residues [5-6]. The sequencing of the NPs has revealed an elevated structural homology between different species, confirming their high conservation [4]. Cardiac natriuretic peptides are secreted upon relevant stimuli such as stretch of the atrial myocytes. However, the ventricular myocytes become progressively the major site of peptide secretion during cellular stresses, which are related to hypertrophy [7-8].

Among the various signal transduction pathways activated by diverse stressful stimuli the mitogen activated protein kinases (MAPKs) are included. Three subfamilies of the MAPKs have been clearly identified and

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extensively studied in mammalian experimental models: the extracellularly responsive kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs/SAPKs), and the p38-MAPKs [9-11]. The ERK pathway is primarily responsive to growth factors, mitogens, hypoxia or osmotic stress [12-15] and appears to be involved predominantly in anabolic processes. JNKs are only weakly activated by growth factors but show a strong response to various cellular stresses [16-17], reperfusion following ischaemia [18-19], mechanical stretch [20-21], and osmotic shock [22-23]. The third MAPK member p38 is activated by various environmental stresses, including hyperosmolarity [10, 24].

Data implicating p38-MAPK as a regulator of the hypertrophic response have largely been obtained in cultured neonatal rat cardiomyocytes. The G-protein-coupled receptor agonists phenylephrine and endothelin-1 are potent activators of p38-MAPK in cardiomyocytes [25]. Over-expression of activated MKK3 or MKK6 (upstream activators of p38-MAPK) has been shown to induce hypertrophy and atrial natriuretic factor expression *in vitro*, while pharmacological inhibition of p38-MAPK activity by the antagonists SB203580 or SB202190 was shown to decrease agonist-stimulated cardiomyocyte hypertrophy in culture [25-27]. Pharmacological or dominant negative inhibition of p38-MAPK signalling pathway significantly reduced agonist-induced B-type natriuretic peptide promoter activity in vitro [28-29] and inflammatory cytokines were shown to upregulate BNP expression via the same mechanism [30]. However, in additional studies, it was reported that p38-MAPK inhibition was not sufficient to decrease agonist-induced cardiomyocyte hypertrophy under certain conditions, suggesting a more specialized role for p38-MAPK signalling in vitro [25, 31-32]. Therefore, certain forms of stress that have been found to induce p38-MAPK phosphorylation, lead to the transcriptional activation of genes that contribute to appropriate responses, such as the expression of the ANP gene [33].

Hearts of ectotherms face a far greater range of environmental variants, such as anoxia/hypoxia, osmolarity and temperature, than those of endotherms. Aquatic animals are more sensitive than mammals to the stretch-mediated mechanisms and therefore they have to synthesize and release factors that, in turn, could prevent their hearts from overloading [34-36]. Conceivably, the role of cardiac NPs is very important in these organisms [3]. Particularly in amphibians, the heart is secreting an atrial natriuretic peptide very similar to the mammalian one [35] and at the same time is the target organ with specific binding sites for NPs, indicating that the frog heart is an important target of these hormones [36]. Remarkably, NPs were found to decrease the isoprenaline elevated calcium current in isolated ventricular myocytes [34]. Thus, the amphibian heart represents a suitable experimental model for the elucidation of the physiological and molecular mechanisms involved in load-induced hypertrophy of cardiac muscle.

The MAPKs (ERK, JNKs and p38-MAPK) have been recently characterized in our laboratory, in the isolated perfused *Rana ridibunda* heart and it is well established that p38-MAPK is strongly phosphorylated under diverse stressful conditions [37-41]. At the same time, immunohistochemical studies provide evidence that, under hyperosmotic conditions, the presence of ANP is enhanced in the ventricular myocardium and p38-MAPK mediates this response but the exact mechanism remains to be elucidated [39].

In the present study, we carried out cDNA cloning of ANF from the ventricle of *Rana ridibunda* ventricular myocardium. The nucleotide sequence analysis of the full-length cDNA revealed that it encodes a protein consisting of 145 amino acid residues with a well-conserved C-terminal domain that constitutes the mature atrial natriuretic peptide. Our results also demonstrate for the first time that phosphorylation of p38-MAPK under oxidative stress and mechanical overload accompanies the increase in ANF transcripts. This study provides evidence of a direct link between p38-MAPK signaling pathway and ANF transcription in the amphibian heart.

Materials and Methods

Materials

Rabbit polyclonal antibodies to either dually phosphorylated or total (phosphorylation state independent) p38-MAPK levels were obtained from Cell Signalling Technology (Beverly, MA, USA). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala 751 84, Sweden). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene NH, USA). SB 203580 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, California 94547, USA). X-OMAT AR 13x18 cm film was purchased from Eastman Kodak Company (New York, 14650, USA). Unless otherwise specified, most other biochemicals used were obtained from Sigma Chemical Co. (St Louis, USA).

Animals - Heart perfusions

Frogs (*Rana ridibunda* Pallas) weighing 120-150g 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).

Animals (*R. ridibunda*), were anaesthetized by immersion to 0.05% (w/v MS 222) and sacrificed by decapitation. The hearts were excised and mounted onto the aortic cannula of a conventional Langendorff perfusion system as previously described [38]. Briefly, perfusions were performed in a non-recirculating Langendorff mode at a pressure of 4.5 kPa (31.5 mmHg) with bicarbonate-buffered saline (23.8 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. At the end of the equilibration period, hearts were perfused with 30 µM H_2O_2 for 2 min (the time point of the respective maximal p38-MAPK activation) or at a pressure of 60mmHg for 2, 5, and 15 min. Hearts perfused with 0.5 M sorbitol for 15 min after the equilibration period were used as positive controls. Perfusions were also conducted in the presence of 1 µM SB203580 both during the equilibration period and the perfusion with 30 µM H_2O_2 for 2 min, elevated pressure for 2 min, or sorbitol for 15 min.

At the end of the perfusions, atria were removed and the ventricles, after being immersed in liquid N_2, were pulverized and powders were stored at –80°C.

RNA isolation

Total RNA was isolated from heart ventricles using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was extracted with phenol-chloroform and finally precipitated with isopropyl alcohol. RNase-free water was used to dissolve RNA while yield and purity was determined by measuring the optical density at 260nm and 280 nm. The integrity of the RNA was assessed by formaldehyde–agarose gel electrophoresis followed by ethidium bromide staining.

Cloning of ANF cDNA in Rana ridibunda ventricular tissue

After extraction, 2 µg of total RNA were reverse-transcribed to cDNA using 200 U Superscript II Rnase H^- Reverse Transcriptase (Invitrogen) with oligo-dT primer (MWG Biotech AG) according to the manufacturer's protocol. Briefly, total RNA was reverse-transcribed by incubation with 20 µl reverse transcription mixture containing 5 pmole oligo $(dT)_{12-18}$ primer, 0.5 mM dNTPs, 1x First-Strand Buffer, 10 mM DTT and 200 U Superscript II Rnase H^- Reverse Transcriptase at 42°C for 50 min. The reverse transcriptase was then inactivated by heating at 70°C for 15 min. The cDNAs encoding ANF were amplified by polymerase chain reaction (PCR) from single stranded cDNA templates using the primers, 5'-GCAAGCTTATAGGAGAAGGAT-3' (nucleotide position -1 to -21) and 5'-ATTCCCATACAAGACAAAACA-3' (nucleotide position 750 to 770) (Table 1). These primers were designed based on the nucleotide sequences in the flanking regions of the ANF cDNA from *Rana catesbiana* (GenBank Accession no. D01043). Polymerase chain reaction (PCR) amplifications were carried out in reaction mixtures (25 µl) containing 1 x PCR buffer, 0.2 mM dNTPs, 0.24 µM sense and antisense primers, 1.0 U Taq DNA polymerase (Finnzymes) and 2 µl cDNA. The PCR using a Tpersonal thermocycler (Biometra) consisted of 30 cycles with denaturation at 94°C for 30s, annealing at 45°C for 30s and extension at 72°C for 60s. The initial denaturation was carried out at 94°C for 4min and the final elongation step, at 72°C for 5 min.

The PCR product (10 µl) was ligated into pCR 2.1-TOPO and transformed into *Escherichia coli* competent cells for ampicillin selection. Individual bacterial colonies were picked and incubated overnight in LB medium at 37 °C under constant agitation. The size of the insert in transformed colonies was checked by Hind III, Xba I restriction digestion of extracted plasmid DNA (MO BIO Laboratories, Inc Miniprep kit). Glycerol stocks were created from transformed colonies and the plasmid DNA was then extracted (MO BIO Laboratories, Inc Maxiprep kit) and sequenced in both directions using M13F and M13R primers (Lark Technologies, Ltd., Essex, UK). The BLAST and the Multalin programmes were used to determine the homology of the nucleotide and amino acid sequence of *Rana ridibunda* ANF to other species ANF.

RT-PCR

A semiquantitative method for the reverse transcription of RNA was followed as described above and 2 µl of cDNA were used for the amplification reactions, which were performed for 30 sec at 94°C, 30 sec at 45°C, and 45 sec at 72°C (30 cycles) followed by an elongation step for 5 min at 72°C. A different set of primers was used for these amplification reactions, designed on the basis of high homology between several species [42]. Sense primers for ANP were 5'-CGAGGAGGTCCAGCATACAACTCC -3', corresponding to bp 125–148, and antisense primers were 5'-CTAGAACCTACGGCCACAACCCAT -3', corresponding to bp 476–499 (GenBank Accession no. D01043) (Table 1), which yielded a 375-bp PCR product. Amplified product was electrophoresed onto 1.2% agarose gels, stained with ethidium bromide, and the signal intensity was quantified using an imaging analyzer (Gel analyzer, v 1.0).

Tissue extractions

Heart powders were homogenized with 3 ml/g of buffer [20 mM Tris-HCl, pH 7.5, 20 mM *β*-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 10 mM benzamidine, 200 µM leupeptin, 120 µM pepsatin A, 10 µM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 µM phenyl methyl sulphonyl fluoride (PMSF), 0.5% (v/v) Triton X-100] and extracted on ice for 30 min. The samples were centrifuged (10000 g, 5 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 by using the BioRad Bradford assay.

SDS-PAGE and immunoblot analysis

Proteins (100μg) were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). 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 antibody (1:1000, diluted in TBS-T containing 5% (w/v) BSA) according to the manufacturer's instructions. After washing in 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, 1h, at 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 following the instructions of the software (Gel analyzer, v 1.0).

Statistical evaluations

All data are presented as mean ± S.E. Comparisons between control and treatments were performed using the Student's paired t test. A value of $P<0.05$ was considered to be statistically significant.

Results

Cloning of ANF cDNA

Total RNA was extracted from ventricles of *R. ridibunda* hearts and a full-length cDNA encoding prepro-ANP was isolated by PCR with oligonucleotide primers that were designed on the basis of the nucleotide sequence of *R. catesbiana* ANF cDNA (GenBank Accession no. D01043). The cDNA nucleotide and its deduced amino acid sequence are shown in Fig. 1. A total of 771bp nucleotides were obtained for the *R. ridibunda* ANF cDNA and the sequence was deposited at GenBank (Accession no AY743415). An open reading frame of 145 amino acid residues follows the initiation codon ATG at position +1. In this cDNA sequence, purine compounds at positions -3 and +4 indicate the consensus sequence for efficient eukaryotic translation initiation. In addition, as is the case for a number of immediate-early gene transcripts, the *R. ridibunda* mRNA contains ATTTA motifs in the 3´-untranslated region (underlined in Fig. 1), which could contribute to the instability of the transcript.

The amino acid sequence encoded in the open reading frame is deduced as shown in Fig.1. The N-terminal region of the preproANP contains a 27-residue sequence exhibiting a highly hydrophobic structure, which can be considered as signal peptide. Cleavage of this peptide would result in the proANP of 118 amino acid residues. The amino acid sequence between Cys-125 and Cys-141 corresponds to the ring structure characteristic of the natriuretic peptide family. A mature ANP form comprises 30 amino acid residues at the C-terminus (Fig. 1). As expected, comparison of *R. ridibunda* preproANP sequence with other known species, showed significant similarities in the entire sequence (Fig. 2 and data not shown). BLAST analysis showed the greatest homology (76%) with *R. catesbiana,* and lower homology with other species like *Homo sapiens* (42%) and *Mus musculus* (40%). When the amino acid sequence of the mature atrial natriuretic peptide is aligned the homology reaches 100% with *R. catesbiana,* 84% with *Mus musculus* and 80% with *Homo sapiens.*

Primer	Sequence 5'-3'	Length
β-actin-S*	ATCTGGCATCACACCTTCTAC	21-mer
β-actin-AS*	CTCCTGCTTGCTGATCCAC	19-mer
ANFrog-F	GCAAGCTTATAGGAGAAGGAT	21-mer
ANFrog-R	ATTCCCATACAAGACAAAACA	21-mer
ANF-F	CGAGGAGGTCCAGCATACAACTCC	24-mer
ANF-R	CTAGAACCTACGGCCACAACCCAT	24-mer

Table 1. Sequences of the oligo nucleotides used in the present study * (*β*-actin primers were as designed by Takase M, Ukena K, Tsutsui K. 2002. Brain Research 950: 288-296).

p38-MAPK activation and PreproANP expression by diverse stresses in the perfused amphibian heart

Primers ANF-F and ANF-R were designed based on the sequence of *R. catesbiana* and the expected 375-bp fragment was obtained corresponding to a portion of the atrial natriuretic factor. ANF mRNA transcipts were shown to be expressed in the myocardium perfused with normal bicarbonate-buffered saline (controls, Figs. 3 and 4). In the myocardium perfused with either 30 μM H_2O_2 for 2 min or 0.5 M sorbitol for 15 min, the levels of mRNA encoding ANP increased significantly. Interestingly, the selective p38-MAPK inhibitor SB203580 even at a relatively low concentration (1 μM), abolished the preproANP mRNA induced by oxidative stress (Fig. 3a, b).

In parallel, as expected, both, oxidative stress as exemplified by 30 μM H_2O_2 for 2 min and hyperosmotic stress as exemplified by 0.5 M sorbitol for 15 min induced a rapid increase in p38-MAPK phosphorylation levels (9.61±0.95-fold and 10.26±1.02-fold relative to control

Fig. 1. Nucleotide and deduced amino acid sequence of *R. ridibunda* prepro-ANP. Initiation and stop codons are bold underlined. The deduced amino acid sequence are displayed under the corresponding nucleotide sequence and the mature ANP sequence is underlined. TGC codons for Cys125 and Cys141 are shaded and ATTTA motifs in the 3´-untranslated region are bold.

```
GCAAGCTTATAGGAGAAGGATTCGGGTGCAAGAAACTAAAGGGAAACATCTACCAAGGGCA -1

ATGGGGACCTCATTCGTTGGGTACTTTACATTTGTACTGCTTCTTCTGGCATTGACCAAA    60
 M  G  T  S  F  V  G  Y  F  T  F  V  L  L  L  L  A  L  T  K    20

GTTCGAGGTGGTCCAGTATACAACCCCCCCCTGTCTTCAGACTTGTCCGATTTAAAGGGG   120
 V  R  G  G  P  V  Y  N  P  P  L  S  S  D  L  S  D  L  K  G    40

CTTCTAGAACGTTTAGAAGACAGACTCCCGGTAGAAGACGCAGAGGCTCCAGTTCAAGAC   180
 L  L  E  R  L  E  D  R  L  P  V  E  D  A  E  A  P  V  Q  D    60

ACCTTTGCTCCAAATTATGATTCCTCTGACTCTTCGAACTCAGCACCATCCTGGACTGGG   240
 T  F  A  P  N  Y  D  S  S  D  S  S  N  S  A  P  S  W  T  G    80

GAAGCAGCCCGGCCCGGGGCCGACATGGCGTATAACAGAGGGTCCTGGACACAGCAAGAG   300
 E  A  A  R  P  G  A  D  M  A  Y  N  R  G  S  W  T  Q  Q  E   100

AAATCATCTCCGCTGAGGAACAAACTTCGAGAGTTGCTGAATGCTCCCCGCAGCATGAGG   360
 K  S  S  P  L  R  N  K  L  R  E  L  L  N  A  P  R  S  M  R   120

AGATCATCCGACTGCTTTGGATCTCGAATTGACAGAATCGGCGCTCAAAGCGGCATGGGG   420
 R  S  S  D  C  F  G  S  R  I  D  R  I  G  A  Q  S  G  M  G   140

TGCGGCCGCAGGTTCTAGGAGGATACGCTATGGATTTTAGAAGAACCTAATCTTCGCCAT   480
 C  G  R  R  F  -                                              145

CGAGGGGATCTTGTGCCGTTATTTAGGGATGTCGTCTTCGTACAGACACTCTGCACCAAC   540
CACAGCCCTCCTCATTATTTAAATCCTATTTATTTTTCTACATTGTAACAACTTCCGTCA   600
CCATAGTCACCAAGTGGTTCCAGTAACACTTTGCCAGGTTTATAGATCAGATTACTGCTG   660
CGGATTTTGCCCGGGGCACCGTATTCTTTTGTTTTGTCTTGNATGGGAAT             710
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Fig. 2. Comparison of preproANP amino acid sequence of *R. ridibunda*, *R. catesbiana* and *X. laevis*. Differences between the amino acid sequences of *R. ridibunda* and *R. catesbiana* are shaded. The conserved amino acids among the different species are noted with *.

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                    10         20         30         40         50
Rana ridibunda      MGTSFVGYFT FVLLLLALTK VRGGPVYNPP LSSDLSDLKG L - LERLEDRL
Rana catesbiana     MGTSFVGYLT FVLLLLALTK VRGGPAYNSP LSSDLSDLKG L - LERLEDRL
Xenopus             MQTSFVGYFT LIFFLL SLMK AKGSPAYS SYLSSDLTDLKN MTLERLEDRY
                     *  * *** **  *      **  *  *    * *  *    ** *** * * *     * *** * * *

                    60         70         80         90        100
Rana ridibunda      PVEDAE APVQ DTFAPNYDSS DSSNSAPSWT GEAARPGADM AYNRGS -WTQ
Rana catesbiana     PVEE VE TPVQ DI FA PNYDS A DSSNSAPSL T YEAARPGADM MYNRGS -WTQ
Xenopus             TAEE PMAPSQ DLFAQNYDAA DSSNSAPSWT GEA I RPQSD I  I YNKGSSWET
                      *       * * *  **  * * *    * *** * ***   *  * *  **   *    * *  **  *

                   110        120        130        140
Rana ridibunda      QEKSSPLRNK LRELLNAPRS MRRSSDCFGS RIDRIGAQSG MGCG -RRF
Rana catesbiana     Q EKSSPLRNK LRELLNAPRS MRRSSDCFGS RIDRIGAQSG MGCG -RRF
Xenopus             PDKLSRLQSQ LRELLN SPRS LRRSSDCFGQ RIDRIGAQSG MGCNSHRF
                      * *  *     * *** **  * * *   ** *** * * *  *** *** * * * * * *     **
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values, P<0.001, respectively) (Fig. 3c, top panel, 3d). Furthermore, the selective p38-MAPK inhibitor SB203580 (1 μM) abolished the kinase activation induced by 30 μM H_2O_2 (Fig. 3c, top panel). To ensure that equal amounts of protein were loaded, western blot for total p38-MAPK was also performed (Fig. 3c, bottom panel).

Similarly, in the myocardium perfused at elevated pressure for 2 minutes, preproANP mRNA was increased significantly. During short lasting stretch, the levels of ANF mRNA correlated positively with those of phosphorylated p38-MAPK (Fig. 4a, top panel, 4c, top panel). In particular, mechanical overload induced a strong phosphorylation of p38-MAPK within 30 sec [38], with maximal values being attained within 2 min of stretch (8.03±0.56-fold, relative to control, P<0.001) (Fig. 4c, top panel, 4d). These findings are in line with previous results on co-immunolocalisation of phospho-p38-MAPK and active ANP in osmotically stressed *R. ridibunda* hearts [39]. However, when the perfusion at high pressure was sustained for 5 or 15 minutes, levels of ANF mRNA were

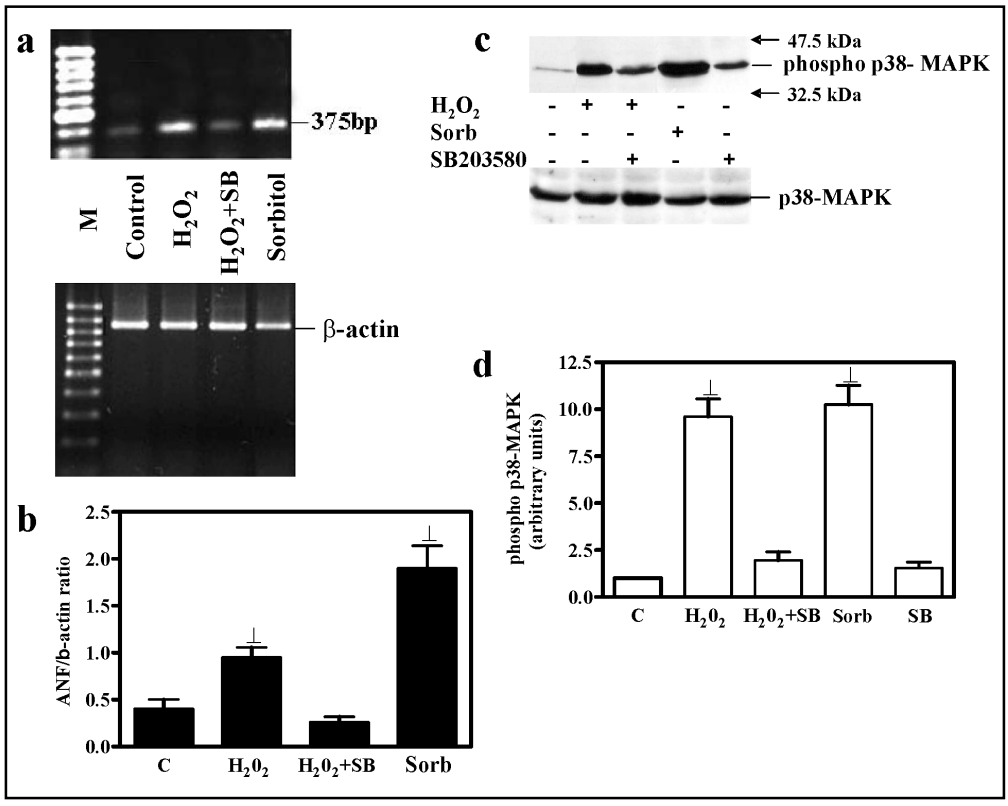

Fig. 3. Effects of H_2O_2 and sorbitol on p38-MAPK phosphorylation and the expression of preproANP mRNA. (a) RT-PCR analysis of RNA isolated from the ventricles of myocardiun perfused in the presence of H_2O_2, sorbitol, SB203580 and in controls (top panel) and amplification of β-actin cDNA from the same reverse transcribed-RNAs used in top panel (bottom panel). (b) densitometric analysis of the RT-PCR fragments expressed as ANF/β-actin ratio. Results are means±SE of 3 independent experiments. The PCR product of ANF (375bp) and β-actin (900bp) were analysed in 1.2% and 1.0% agarose gel electrophoresis, respectively. (c) Western blotting using antibodies specific for the dually phosphorylated p38-MAPK (top panel) and total p38-MAPK, (bottom panel). (d) densitometric analysis of phospho p38-MAPK bands by laser scanning. RT-PCR and Western blots shown are representative of 3 independent experiments, with similar results.

not further increased (Fig. 4a, top panel), while those of phosphorylated p38-MAPK were sustained (Fig. 4c, top panel). Therefore, long lasting stretch of myocytes is accompanied by increased levels of phosphorylated p38-MAPK but not of its putative target gene.

We also examined the possible involvement of p38-MAPK signaling pathway on the ANF mRNA induction under mechanical overload. To this end, we perfused the amphibian myocardium under mechanical stress, for 2 min, either in the absence or presence of 1μM SB203580. The results of this study are shown in Fig. 5. As can be seen, this p38-MAPK selective inhibitor abolished both, the ANF mRNA levels and the kinase phoshorylation induced by stretch, confirming a p38-MAPK mediated regulation of ANF expression.

Discussion

Ectotherms modulate their metabolic demands according to the status of their environment. These changes in metabolic demands require compensatory changes in cardiac output and the myocardium has been shown to be osmotically sensitive to the tonicity of its surrounding bathing solutions [43] with an electrophysiological behaviour also profoundly affected by temperature [44]. Therefore, the amphibian heart constitutes an intriguing candidate experimental system for studying the effects of environmental stress on multiple aspects of physiology at the cellular and molecular level. Among the various signal transduction pathways involved in the responses to environmental stresses, MAPKs have been shown to play a significant role [10, 11, 45-46]. In particular, p38-MAPK has been characterized as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature [47-48].

DeBold and Salerno [49] were the first to report the production of a hormone involved in the regulation of extracellular fluid volume and electrolyte balance by atria of various animal species. In amphibians, immunoreactive atrial natriuretic peptide (ANP) is detected in both, atrial and ventricular cardiac myocytes [50]. Important sequence homologies between the C-terminal regions of mammalian and amphibian ANP have been demonstrated

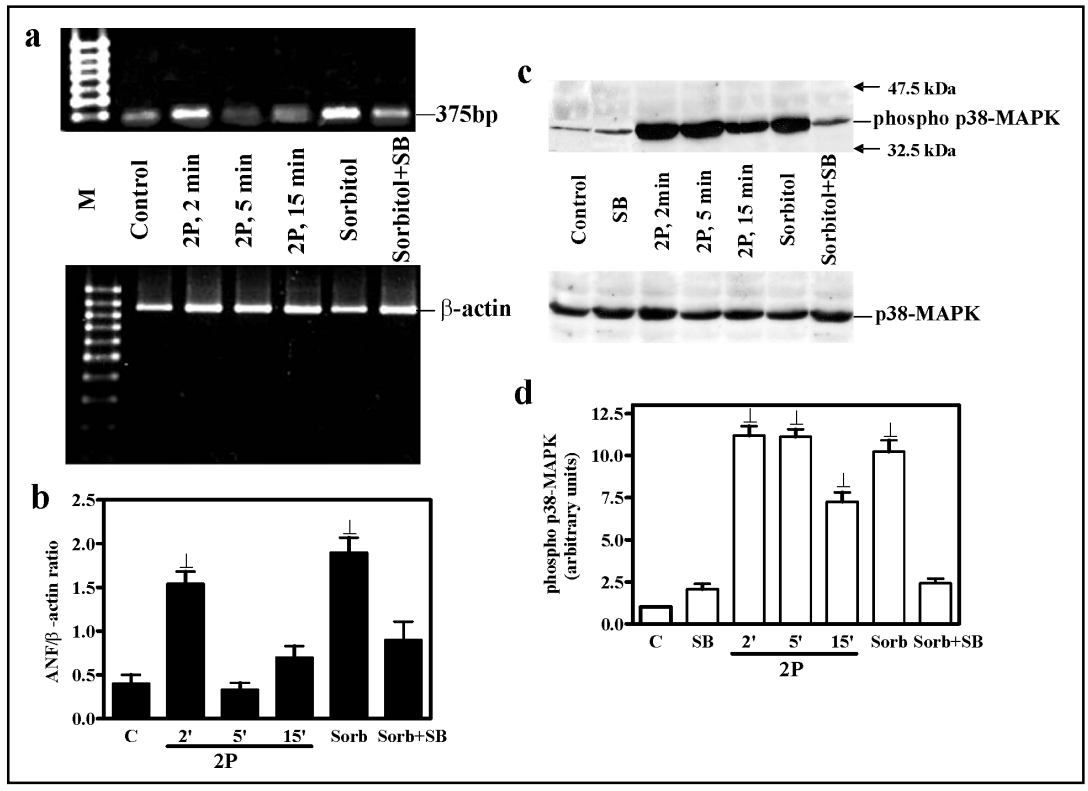

Fig. 4. Effects of elevated pressure on p38-MAPK activation and the expression of preproANP mRNA. (a) RT-PCR analysis of RNA isolated from the ventricles of myocardium perfused under double pressure (2P) for 2, 5 and 15 minutes, with 0.5 M sorbitol for 15 min either in the absence or presence of 1 μM SB203580 and in controls (top panel) and amplification of *β*-actin cDNA from the same reverse transcribed-RNAs used in top panel (bottom panel) (b) densitometric analysis of the RT-PCR fragments expressed as ANF/*β*-actin ratio. Results are means of 3 independent experiments. The PCR product of ANF (375bp) and *β*-actin (900bp) were analyzed in 1.2% and 1.0% agarose gel electrophoresis respectively. (c) Western blotting using antibodies specific for the dually phosphorylated p38-MAPK (top panel) and total p38-MAPK (bottom panel). (d) Densitometric analysis of phospho p38-MAPK bands by laser scanning. RT-PCR and Western blots shown are representative of 3 independent experiments, with similar results.

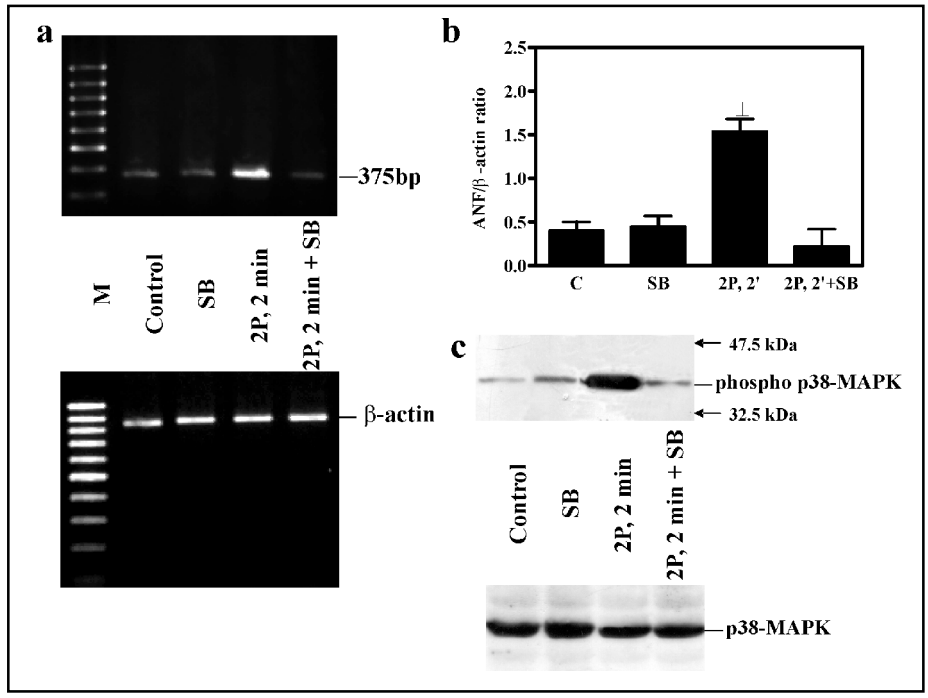

Fig. 5. Effects of SB203580 on p38-MAPK activation and the expression of preproANP mRNA under mechanical overload. (a) RT-PCR analysis of RNA isolated from the ventricles of myocardiun perfused under double pressure (2P) for 2 min in the absence or presence of 1 μM SB203580 and in controls (top panel) and amplification of *β*-actin cDNA from the same reverse transcribed-RNAs used in top panel (bottom panel) (b) densitometric analysis of the RT-PCR fragments expressed as ANF/*β*-actin ratio. Results are means of 3 independent experiments. The PCR product of ANF (375bp) and *β*-actin (900bp) were analyzed in 1.2% and 1.0% agarose gel electrophoresis respectively. (c) Western blotting using antibodies specific for the dually phosphorylated p38-MAPK (top panel) and total p38-MAPK (bottom panel).

by several investigators [35, 51], and confirmed in the present study, after cloning of the full length mRNA of ANF from *R. ridibunda* myocardium comprising a total of 771bp nucleotides. The *R. ridibunda* ANF mRNA contains several ATTTA motifs in the 3´-untranslated region (underlined in Fig.1), a characteristic of a number of immediate-early gene transcripts, which require no protein synthesis to be activated. Tandem repeats of the sequence ATTTA are a common feature of the 3' -untranslated region of several genes that code for protooncogenes, nuclear transcription factors, and cytokines and can function as regulators of mRNA stability. In most cases, ATTTA motifs contribute to the destabilization of mRNA [52] but under certain conditions, stabilization has been observed [53]. Most likely, this motif represents a binding site for specific proteins and could define a mechanism of ANF expression further supporting the role of this peptide as one of the earliest markers of cardiac differentiation and a sensitive indicator of cardiac stress.

Our experiments have also shown that ANF mRNA transcipts are expressed in the ventricular myocardium perfused with normal bicarbonate-buffered saline (controls, Figs. 3 and 4). In the myocardium perfused with either 30 μM H_2O_2 for 2 min or 0.5 M sorbitol for 15 min, the levels of mRNA encoding ANP increased significantly. Furthermore, this increase was attenuated when the perfusion media contained 1 μM SB203580, a selective p38-MAPK inhibitor (Fig. 3a, b). On the other hand, mechanical overload induced a significant accumulation of preproANP mRNA, within 2 min, whereas long lasting stretch led to an attenuation of this response (Fig. 4a, b).

The transcription of ANF -among other NPs- in frog ventricular cardiomyocytes and its increase under environmental stress, clearly suggests an elaborated system of regulation of electrolytes and water balance. This is fundamental for amphibians, which live in aquatic environments and face *in vivo* various exogenous stressors. Therefore, the synthesis and release of ANP from the ventricular myocardium under such stressful conditions can prevent itself from overloading.

Since hyperosmotic, mechanical, as well as oxidative stress was also found to induce p38-MAPK phosphorylation in the isolated perfused amphibian myocardium (Figs. 3, 4 and 5), our findings indicate a direct involvement of p38-MAPK signalling pathway in the regulation of ANP encoding gene under such stressful stimuli *in vivo*. Furthermore, SB203580 (1μM) was found to abolish p38-MAPK phosphorylation induced by the diverse forms of stress tested (Figs. 3, 4 and 5). Although several investigators propose that pyridinylimidazoles inhibit the enzymatic activity rather than the activation (phosphorylation) of p38-MAPK [54-55], we have clearly demonstrated that SB203580 inhibits stimulus-induced phosphorylation of p38-MAPK. Recent studies support our finding, suggesting that this inhibition may be due to its binding to the inactive form of the kinase, resulting to a significant reduction of the kinase activation rate [38, 56-57]. Among the several isoforms of the kinase which have been identified, only two are strongly inhibited by SB203580: α and β_1 [58] and presumably the above two isoforms contribute to the responses obtained in the isolated perfused *R. ridibunda* heart, under hyperosmotic, mechanical or oxidative stress.

The differential time-dependent responses of p38-MAPK and preproANP mRNA under mechanical stress found in the present study, could be explained by the fact that in addition to this target, many other p38-MAPK substrates *in vivo*, such as MAPKAPK2 and 3, HSP27/29 and ATF2 have been identified [11, 41, 59]. Therefore, the sustained kinase activation under such stressful conditions could represent the necessity of maintenance and amplification of the initial signal for a compensatory protective cellular response. Remarkably, reduced p38-MAPK signalling in the mammalian heart promotes myocyte growth through mechanisms involving enhanced calcineurin-NFAT signalling [60]. Another possibility could be that long-lasting stretch induced response is eventually converted to a pro-death signal, possibly through the p38-MAPK signalling pathway [61].

In conclusion, we have characterized the preproANP mRNA expressed in *R. ridibunda* ventricular tissue under diverse stressful conditions. Our experiments have confirmed its role as an immediate-early gene and cardioprotective agent. In particular, we have demonstrated the regulated expression of ANF under oxidative, hyperosmotic and mechanical stress through the p38-MAPK signaling pathway.

Abbreviations

MAPK (mitogen-activated protein kinase); ERK (extracellularly responsive kinase); JNK (c-Jun N-terminal kinase); p38-MAPK/RK (p38 reactivating kinase); PAGE (polyacrylamide gel electrophoresis); TBS (Tris-buffered saline); ANP (atrial natriuretic peptide); ANF (atrial natriuretic factor, precursor of ANP); RT-PCR (reverse transcribed polymerase chain reaction).

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