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p42/p44 mitogen-activated protein kinases inhibit atrial natriuretic peptide mRNA transcription in gp130-mediated hypertrophic ventricular myocytes

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ABSTRACT

Objective: To understand the role of *ANP* mRNA transcription regulation in gp130-mediated cardiomyocyte hypertrophy, and the involved mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK, also called p42/p44 MAPK) signaling pathway. **Methods:** Isolated neonatal ventricular myocytes were treated with different concentrations of CT-1 (10^{-9} , 10^{-8} and 10^{-7} mol/L). MTT was used to analyze the viability and RT-PCR was used to detect *ANP* mRNA levels in cardiomyocyte. To inhibit p42/p44 MAPK activity in hypertrophic cardiomyocytes, the cells were pretreated with a specific MEK1 inhibitor. **Results:** CT-1 significantly induced *ANP* mRNA expression and the viability of cardiomyocytes in a dose- and time-dependent manner. Furthermore, blocking p42/p44 MAPK activity by the special MEK1 inhibitor upregulated the *ANP* mRNA. **Conclusions:** p42/p44 MAPK have an important role in suppressing *ANP* mRNA transcription and cell activity in gp130-mediated hypertrophic ventricular myocytes.

1. Introduction

Under normal physiological circumstances, atrial natriuretic peptide (*ANP*) is almost exclusively found in the atrium and is only in the ventricle during embryonic development. During periods of sustained increase in blood pressure and associated compensatory left ventricular hypertrophy, *ANP* is induced more in ventricular myocardial cells. Overexpression of *ANP* is an adaptation of cardiomyocyte, and multiple evidence

has shown that increasing expression of *ANP* is associated with the inhibition of hypertrophy^[1,2]. *ANP* inhibits cardiomyocyte size increase and protein synthesis in cultured cardiomyocyte when stimulated by angiotensin II or endothelin 1 (ET-1)^[3,4]. Evidence for *ANP* inhibition has also been observed in genetic models of spontaneous hypertension and related conditions of pressure and volume overload. The adaptation of myocardium is initially associated with an increase in gene transcription of *ANP in vivo*^[5-7].

The mitogen-activated protein kinase (MAPK) is a family of serine-threonine protein kinases that are activated by a number of extracellular stimuli. Three major subfamilies have been characterized, including extracellular signal-regulated kinase (ERK), also called p42/44 MAPK, p38 MAPK, and JNK, which mediate downstream effects via activation of appropriate transcription factors and play role in cellular proliferation, differentiation, and apoptosis^[8,9].

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Although it has been reported that p38 MAPK pathway is involved in *ANP* transcription^[10], furthermore, MAPK kinase (MEK) and p42/p44 MAPK are potential activators of hypertrophy^[9,11], little is known as to whether p42/p44 MAPK is involved in the expression of *ANP* gene.

Cardiotrophin-1 (CT-1), as a 21.5 kDa protein, was identified by expression cloning based on its ability to induce cardiomyocyte hypertrophy, and characterized as a member of the IL-6 family and a factor to induce cardiomyocyte hypertrophy^[12,13].

The binding of CT-1 to its receptor activates Janus kinases (JAKs) which then phosphorylate gp130, producing binding sites for proteins with src homology 2 (SH2) domains, such as growth factor receptor bound protein2 (GRB2). This is then the starting point for a Ras/Raf/MEK/ERK/p90 RSK cascade^[14].

In this study, CT-1-stimulated hypertrophic ventricular myocytes were used for studying *ANP* mRNA transcription under the suppression of the MEK/ERK (p42/p44 MAPK) pathway.

2. Materials and methods

2.1. Chemical compounds and reagents

Dulbecco's modified eagle medium (DMEM) medium (Boehringer Mannheim GmbH) prepared with positive pressure filtration sterilization and adjusted to pH 7.2 was kept at 4 °C. D-Hanks plus enzyme solution was prepared with pancreatin (DIFCO™, Becton Dickinson and Company; dilution 1:250), diluted to 0.8 g/L (pH 7.2, Ca²⁺ and Mg²⁺ free), filtrated through a microporous membrane (0.22 μm) and stored at -20 °C. 5-Bromodeoxyuridine (BrdU, SERVA Co. Ltd) was diluted to 10 mmol/L, then filtrated through a microporous membrane and stored at -20°C. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma- Aldrich, St Louis, MO, United States) was dissolved at a concentration of 5 mg/mL in DMSO. MEK/ERK1/2 activation inhibitor (PD098059, Sigma-Aldrich Co.) was prepared at a concentration of 5 mmol/L. Calphostin C (Cal C, a PKC inhibitor, Sigma-Aldrich Co.) was prepared at a concentration of 1 mmol/L. CT-1 (Alomone Labs Ltd., Jerusalem, Israel) was prepared at 5 mmol/L, 1 mmol/L and 10 μmol/L.

RNA extraction reagents were TRIzol reagent (Life Technologies, Carlsbad, CA, USA), lower melting point agarose (Promega Co., Madison, WI, USA); Tris-boric acid (TBE) electrophoresis buffer (10×): 108 g Tris base, 55 g boric acid and 40 mL of 0.5 mol/L EDTA, pH 8.0; 10 mg/mL

ethidium bromide (Sigma-Aldrich Co.). Before use, the agarose was diluted in 1×TBE.

2.2. Neonatal ventricular myocytes preparation and culture

Primary cultures of ventricular myocytes were prepared from 1-day-old Sprague-Dawley neonatal rats using an established lab protocol. The isolation procedure uses 0.8 g/L pancreatin, pH 7.2 (DIFCO™, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and 0.08% trypsin solution for 10 min to produce separated cells, which were seeded on glass coverslips (3 mm diameter), and beat spontaneously. Cells were incubated at 37 °C in air with 5% CO₂ and 98% humidity. The culture medium (DMEM) was changed every other day. After incubation, ventricular myocytes were seeded at a density of 3×10⁶ cells/mL in 25-mL culture flasks. Culture media were initially treated with 0.1 mmol/L bromodeoxyuridine (BrdU) for inhibition of non-cardiomyocyte proliferation. All rats were treated according to the Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission, People's Republic of China).

2.3. Colorimetric MTT cell viability assay

The colorimetric MTT assay was used to assess the viability of isolated ventricular myocytes. After treatment with different concentrations of CT-1, hypertrophic ventricular myocytes mixed with 10 μL of MTT (5 mg/mL) were incubated for 4 h at 37 °C and 5% CO₂ (myocyte density was 10⁵ cell/L). Then, 150 μL of DMSO (0.05 mol/L) were added with vibration for 10 min. The optical density (OD) value of each well was measured by a microplate spectrophotometer (Multiskan EX Primary EIA V.2.1-1) using a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.4. Extraction of total RNA from cultured ventricular myocytes and RT-PCR

Cultured ventricular myocytes were treated with 1 mL of TRIzol reagent for 5 min at room temperature. Purified total RNA was measured with an ultraviolet spectrophotometer by adjusting the OD₂₆₀/OD₂₈₀ ratio between 1.8 and 2.0. RNA concentrations were calculated by spectrophotometry at OD₂₆₀. 2 μg of total RNA was used as template to generate first strand cDNA by random priming using Promega RT System. *ANP* primers were: forward 5'-CAT CAC CAT CTT CCA GGA GCC-3'; reverse 5'-CCC CGC TTC ATC GGT CTG-3' (cDNA fragment with an amplified length of 186 bp). GAPDH was used as an internal control with the

following primers: forward 5'-CAT CAC CAT CTT CCA GGA GCC-3'; reverse 5'-TGA CCT TGC CCA CAG CCT TG-3' (cDNA fragment with an amplified length of 443 bp). The products of PCR were separated by 1.5%–2.0% agarose gel electrophoresis 0.5% (w/v) ethidium bromide (EB) and visualized under UV using gel imaging system (Bio-Rad Gel Doc1000, Bio-Rad). The *ANP* cDNA to *GAPDH* cDNA ratio was calculated based on the optical density of the bands. All measurements were repeated three times.

2.5. Statistical analysis

All results are presented as mean±standard error of the mean (SEM). All statistical analyses were performed using Sigma Plot for Windows, Version 10.0 (Systat Software, INC). Differences between experimental groups were analyzed using the Mann-Whitney *U*-Test. Values of $P < 0.05$ between groups were considered significant.

3. Results

3.1. CT-1 induced neonatal ventricular myocytes hypertrophy and viability

Compared with control ventricular myocytes on day 3, there was no change in the surface area of CT-1-treated ventricular myocytes at 10^{-10} mol/L (Figure 1A), but it increased when the cells were treated with 10^{-9} mol/L CT-1 (Figure 1B). A significant increase was observed with concentrations of 10^{-8} and 10^{-7} mol/L (Figure 1C and D, respectively) on day 3. Further studies revealed that the effect of CT-1 on cardiomyocytes viability was time-dependent by the MTT assay (Figure 1E). Moreover, when ventricular myocytes were incubated with 1×10^{-9} , 10^{-8} or 10^{-7} mol/L CT-1 for 72 h, significant differences were found compared with the control group (Figure 1F), and the effect was dose-dependent.

3.2. ANP mRNA expression in CT-1-induced hypertrophic ventricular myocytes

After a 24 h incubation with serum-free DMEM, ventricular myocytes were incubated with 10^{-9} , 10^{-8} or 10^{-7} mol/L CT-1. In the control group D-Hanks solution was added instead of CT-1. Total RNA was amplified by RT-PCR and *ANP* mRNA levels were analyzed. *ANP* mRNA levels increased to 146% and 213% in ventricular myocytes that were treated with 10^{-8} and 10^{-7} mol/L CT-1, respectively (Figure 2A and C). Next, total RNA of ventricular myocytes was extracted after 24,

72 and 120 h incubation with 10^{-8} mol/L CT-1 and analyzed by RT-PCR. The optical densities of the product bands were 1.16 ± 0.18 , 1.47 ± 0.17 and 1.97 ± 0.24 , respectively. A significant difference was found between the 72 h group and the 120 h group ($P < 0.01$, Figure 2B).

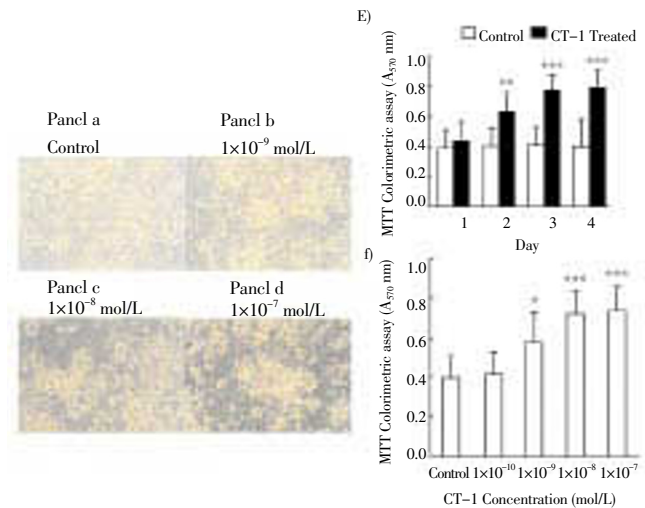


Figure 1. A) Isolated, cultured neonatal cardiomyocytes with culture medium only (day 3, 40 \times). B–D) Cardiomyocytes incubated with 10^{-9} , 10^{-8} or 10^{-7} mol/L Cardiotrophin-1 (CT-1) (day 3, 40 \times). E) CT-1 (10^{-8} mol/L) increased cardiomyocyte viability in a time-dependent manner. F) CT-1 enhanced cell viability in a dose-dependent manner. Control means cells were treated with D-Hanks solution. * means $P < 0.05$ versus control, ** means $P < 0.01$ versus control, *** means $P < 0.001$ versus control.

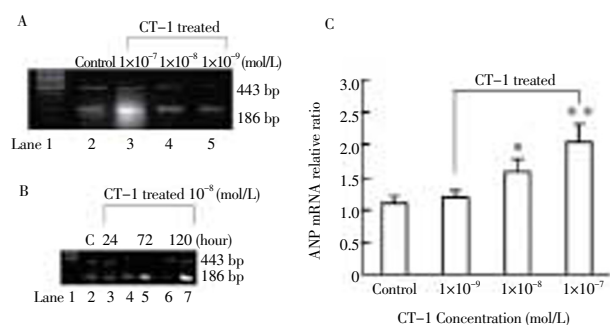


Figure 2. A) *ANP* mRNA level from ventricular myocytes with CT-1-mediated hypertrophy (443 bp = *GAPDH* mRNA band, used as the internal control; 186 bp = *ANP* mRNA band). Lane 1: DNA maker (100–1000 bp), lane 2: mRNA from untreated ventricular myocytes; lanes 3–5: mRNA from ventricular myocytes treated with 10^{-7} mol/L, 10^{-8} mol/L or 10^{-9} mol/L CT-1 for 72 h. B) Time course of *ANP* mRNA expression in CT-1 (10^{-8} mol/L)-treated ventricular myocytes. Lane 1: DNA maker (100–1000 bp); Lanes 2, 4, 6: controls (untreated cultured myocytes); Lane 3: 24 h treatment with CT-1; Lane 5: 72 h treatment with CT-1; Lane 7: 120 h treatment with CT-1. C) *ANP* mRNA increased in CT-1-treated cultured cardiomyocytes in a dose-dependent manner. * means $P < 0.05$ versus control, ** $P < 0.01$ versus control.

3.3. PD098059 inhibited MEK/ERK pathway to increase ANP mRNA expression and cell viability in cultured cardiomyocytes

To explore whether CT-1 induced ANP mRNA upregulation through the MEK/ERK-dependent pathways in ventricular myocytes, the specific inhibitor PD098059, was tested for the effect on CT-1-mediated ANP mRNA expression. To compare effects of different pathways, a protein kinase C (PKC) inhibitor calphostin C was used. Ventricular myocytes were pretreated with 5 mmol/L PD098059 or 1 mmol/L calphostin C for 30 min, followed by incubation with 10^{-8} mmol/L CT-1 for 72 h. The PD098059 group ($n=3$) showed a significant increase in ANP expression (187%) compared with the control group (Figure 3A and B). In contrast, the calphostin C group showed no significant difference in ANP expression compared with the control group ($n=3$, ANP/GAPDH ratio was 1.27 ± 0.17 and 1.04 ± 0.17 , respectively, Figure 3B).

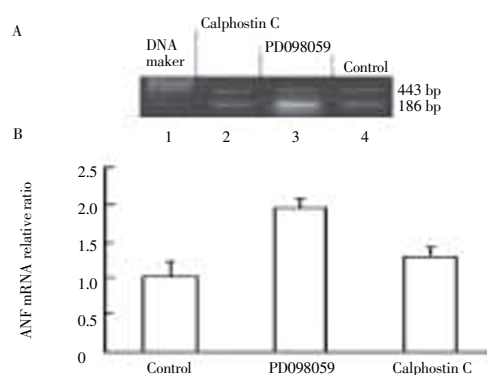


Figure 3. Effect of MEK1 inhibitor PD098059 and PKC inhibitor calphostin C on the CT-1-induced ventricular myocytes. A) Lane 1: Maker (100–1000 bp); Lane 2: cultured myocytes treated with calphostin C and CT-1 (10^{-8} mol/L); Lane 3: cultured myocytes treated with PD098059 and CT-1 (10^{-8} mol/L); Lane 4: control (cultured myocytes treated with 10^{-8} mol/L CT-1 only). B) Quantification of the results shown in A, normalized against APDH. ** means $P<0.01$ versus control.

4. Discussion

ANP gene transcription is active both in atrial and ventricular myocytes during embryonic and fetal development, but is switched off in the ventricular cells in the later stages of fetal development. The ANP-(4–28) and ANP-(5–28) peptides are the main ones expressed in myocardium granules^[15]. Normally, only minor amounts of ANP are produced in ventricular cells, but it can be

overexpressed during left ventricular hypertrophy under hemodynamic overload of pathogenic or sustained physical fitness^[16,17]. Previous studies have indicated that ventricular cardiomyocyte hypertrophy is associated with activation of ANP mRNA transcription, when stimulated by angiotensin, epinephrine or endothelin *in vivo*^[18] and in cultured neonatal ventricular myocytes *in vitro*^[19]. The expression of ANP increases quickly within the initial period of experimental mechanical overload^[20].

As the factor responsible for initiating hypertrophy during continuous stimulation, in the current study, ANP mRNA transcription was detected via CT-1-induced hypertrophy. CT-1 is known as a IL-6-related cytokines and a potent candidate to induce cardiac myocyte hypertrophy. CT-1 initially binds to membrane receptors that activate JAKs, which then phosphorylate gp130, producing binding sites for proteins with Src homology 2 (SH2) domains. Then, the Ras/Raf/MEK/ERK(p42/44 MAPK) /p90RSK cascade is activated, and subsequently phosphorylated and translocated into the nucleus^[21–23]. The result from our study revealed that the transcription of ANP mRNA in hypertrophic ventricular myocyte is significantly affected by the concentration and incubation time with CT-1.

Furthermore, to test the MEK and p42/p44 MAPK signaling pathway may also play roles in CT-1-mediated ANP mRNA expression, the specific inhibitor of p42/p44 MAPK PD098059 was used. Our present results showed that inhibition of p42/p44 MAPK enhanced the transcription of ANP mRNA, whereas, the CT-1-induced ANP mRNA expression can not be influenced by calphostin C, a specific inhibitor of PKC.

In conclusion, our study suggests that ANP mRNA transcription is inhibited by MEK-induced p42/p44 MAPK phosphorylation. Hence, p42/p44 MAPK may act as an inhibitor of ANP mRNA transcription in gp130-mediated ventricular hypertrophy.

Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Hayashi D, Kudoh S, Shiojima I, Zou Y, Harada K, Shimoyama M, et al. Atrial natriuretic peptide inhibits cardiomyocyte hypertrophy through mitogen-activated protein kinase phosphatase-1. *Biochem Biophys Res Commun* 2004; **322**: 310–319.

- [2] Kishimoto I, Rossi K, Garbers DL. A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylylcyclase-A) inhibits cardiac ventricular myocyte hypertrophy. *Proc Natl Acad Sci USA* 2001; **27**: 2703–2706.
- [3] Hayashi D, Kudoh S, Shiojima I, Zou Y, Harada K, Shimoyama M, Imai Y, et al. Atrial natriuretic peptide inhibits cardiomyocyte hypertrophy through mitogen-activated protein kinase phosphatase-1. *Biochem Biophys Res Commun* 2004; **322**: 310–319.
- [4] O'Tierney PF, Chattergoon NN, Louey S, Giraud GD, Thornburg KL. Atrial natriuretic peptide inhibits angiotensin II-stimulated proliferation in fetal cardiomyocytes. *J Physiol* 2010; **588**: 2879–2889.
- [5] Younes A, Boluyt MO, O'Neill L, Meredith AL, Crow MT, Lakatta EG. Age-associated increase in rat ventricular ANP gene expression correlates with cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 1995; **269**: H1003–H1008.
- [6] Perhonen M, Takala TE, Vuolteenaho O, M?ntymaa P, Leppaluoto J, Ruskoaho H. Induction of cardiac natriuretic peptide gene expression in rats trained in hypobaric hypoxic conditions. *Am J Physiol* 1997; **273**: R344–352.
- [7] Ohta K, Kim S, Hamaguchi A, Miura K, Yukimura T, Iwao H. Cardiac hypertrophy-related gene expression in spontaneously hypertensive rats: crucial role of angiotensin AT1 Receptor. *Jpn J Pharmacol* 1995; **67**: 95–99.
- [8] Nagai N, Klimava A, Lee WH, Izumi-Nagai K, Handa JT. CTGF is increased in basal deposits and regulates matrix production through the ERK (p42/p44mapk) MAPK and the p38 MAPK signaling pathways. *Invest Ophthalmol Vis Sci* 2009; **50**: 1903–1910.
- [9] Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, et al. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J* 2000; **19**: 6341–6350.
- [10] Vassilopoulos A, Gaitanaki C, Papazafiri P, Beis I. Atrial natriuretic peptide mRNA regulation by p38-MAPK in the perfused amphibian heart. *Cell Physiol Biochem* 2005; **16**: 183–192.
- [11] Tu VC, Bahl JJ, Chen QM. Distinct roles of p42/p44(ERK) and p38 MAPK in oxidant-induced AP-1 activation and cardiomyocyte hypertrophy. *Cardiovasc Toxicol* 2003; **3**: 119–133.
- [12] Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM. Expression clone of cardiotrophin-1, which induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci USA* 1995; **92**: 1142–1146.
- [13] Bristow MR, Long CS. Cardiotrophin-1 in heart failure. *Circulation* 2002; **106**: 1430–1432.
- [14] Miyake T, Alli NS, Aziz A, Knudson J, Fernando P, Megency LA, et al. Cardiotrophin-1 maintains the undifferentiated state in skeletal myoblasts. *J Biol Chem* 2009; **284**: 19679–19693.
- [15] Bialik GM, Abassi ZA, Hammel I, Winaver J, Lewinson D. Evaluation of atrial natriuretic peptide and brain natriuretic peptide in atrial granules of rats with experimental congestive heart failure. *J Histochem Cytochem* 2001; **49**: 1293–1300.
- [16] Takemura G, Fujiwara H, Horike K, Mukoyama M, Saito Y, Nakao K, et al. Ventricular expression of atrial natriuretic polypeptide and its relations with hemodynamics and histology in dilated human hearts. Immunohistochemical study of the endomyocardial biopsy specimens. *Circulation* 1989; **80**: 1137–1147.
- [17] Scharhag J, Schneider G, Urhausen A, Rochette V, Kramann B, Kindermann W. Athlete's heart: right and left ventricular mass and function in male endurance athletes and untrained individuals determined by magnetic resonance imaging. *J Am Col Cardiol* 2002; **40**: 1856–1863.
- [18] Lutz M, Jorg F, Rudolf JW, Heimo E. Predominant activation of endothelin-dependent cardiac hypertrophy by norepinephrine in rat left ventricle. *Am J Physiol Regul Integr Comp Physiol* 2002; **282**: R1389–R1394.
- [19] Dietz JR. Mechanisms of atrial natriuretic peptide secretion from the atrium. *Cardiovasc Res* 2005; **68**: 8–17.
- [20] Ruskoaho H. Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacol Rev* 1992; **4**: 479–602.
- [21] Pennica D, Wood WI, Chien KR. Cardiotrophin-1: a multifunctional cytokine that signals via LIF receptor gp130 dependent pathways. *Cytokine & Growth Factor Rev* 1996; **7**: 81–91.
- [22] Wu L, Zhao L, Zheng Q, Shang F, Wang X, Wang L, et al. Simvastatin attenuates hypertrophic responses induced by cardiotrophin-1 via JAK-STAT pathway in cultured cardiomyocytes. *Mol Cell Biochem* 2006; **284**: 65–71.
- [23] Fahmi A, Smart N, Punj A, Jabr R, Marber M, Heads R. p42/p44-MAPK and PI3K are sufficient for IL-6 family cytokines/gp130 to signal to hypertrophy and survival in cardiomyocytes in the absence of JAK/STAT activation. *Cell Signal* 2013; **25**: 898–909.