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Forskolin Modulates the Inhibitory Effect of C-Type Natriuretic Peptide on Hypoxia-Induced Atrial Dynamics and Hypoxia Inducible Factor 1 Alpha Activity

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Abstract

Our study investigated effects of C-type natriuretic peptide (CNP) on atrial dynamics and hypoxia inducible factor 1 alpha (HIF-1*α*) activity in perfused beating rat atria, under hypoxic conditions. Hypoxia significantly increased the levels of HIF-1*α*, concomitant with decreased trial dynamics. CNP (0.1 μmol/L) further decreased atrial dynamics under hypoxia and suppressed hypoxia-induced stimulation of HIF-1*α* expression. An adenylylcyclase (AC) activator, forskolin (0.1 μmol/L), significantly up-regulated atrial phosphodiesterase subtype 3A (PDE 3A) protein without affecting hypoxia-induced dynamics. In the presence of forskolin, the inhibitory effects of CNP on hypoxia-induced atrial dynamics and HIF-1*α* levels were significantly attenuated. Forskolin also prevented hypoxia-induced downregulation of PDE3A protein. These findings suggested that CNP inhibited atrial dynamics and HIF-1*α* activity in the isolated perfused beating rat atria under hypoxic conditions. Furthermore, both effects were modulated by the AC activator forskolin, through activation of CNP-PDE 3A signaling.

Keywords

C-Type Natriuretic Peptide, Hypoxia Inducible Factor-1*α*, Phosphodiesterase, Adenylyl Cyclase, Forskolin

1. Introduction

Hypoxia is a common phenomenon in most cardiovascular diseases, including

coronary artery disease, heart failure, myocardial hypertrophy and pulmonary hypertension [1] [2] [3]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that plays a major role in cellular adaptation to hypoxia [4]. It is composed of HIF-1α and HIF-1β subunits, and its activity is dependent on stability of the α-subunit [5] [6]. It was reported that cyclic adenosine monophosphate (cAMP)-dependent protein kinase (protein kinase A, PKA) phosphorylated Thr^{63} and Ser^{692} on HIF-1α *in vitro*, enhancing its transcriptional activity and increasing target gene expression of rat cardiomyocytes. PKA also stimulated binding of the coactivator p300 to HIF-1α, enhancing its transcriptional activity while counteracting inhibition by asparaginyl hydroxylation of the association of p300 with HIF-1α [7] [8]. Thus, cAMP promotes HIF-1 transcriptional activity and increases HIF-1α protein levels through PKA activation, exerting physiological and pathophysiological effects on the myocardium.

As an endocrine gland, the heart produces and secretes natriuretic peptides (NPs), such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Hypoxia potently stimulated cardiac ANP and BNP secretion [9] [10]. ANP and BNP conferred resistance in the ischemic heart to hypoxia and myocardial cell damage, resulting in cellular adaptation to hypoxia and cardioprotection, through activation of cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signaling [11] [12]. Several studies demonstrated that ANP and BNP markedly down-regulated HIF-1α during renal ischemia/reperfusion (I/R) injury in mice [13] [14]. However, effect of CNP on HIF-1α regulation in the atrium is unclear. Our study, therefore, investigated effects of CNP on hypoxia-induced HIF-1α levels in isolated beating rat atria. We also evaluated effects of the adenylyl cyclase (AC) activator, forskolin, on regulation of hypoxia-induced HIF-1α levels by CNP.

2. Materials and Methods

2.1. Preparation of Perfused Beating Rat Atria

Sprague-Dawley (SD) rats of both sexes were used, with mean weights of 250 - 300 g. Isolated perfused beating left atria were prepared as previously described [15] [16]. Soon after setting up each perfused atrium, transmural electrical field stimulation with a luminal electrode was started at 1.5 Hz (0.3 ms, 30 - 40 V), and the atrium was perfused with HEPES buffer solution using a peristaltic pump (1 mL/min), allowing atrial pacing for measurement of changes in atrial pulse pressure. The perfused atrium was supplied with sufficient oxygen during the entire process. The HEPES buffer contained (in mmol/L) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH), as well as 0.1% bovine serum albumin.

2.2. Hypoxic Atrial Model Preparation

The hypoxic atrial model was prepared as previously described [9]. Briefly, the atrial O_2 was replaced by N_2 gas and the normal HEPES buffer was replaced with N_2-saturated HEPES buffer.

Intra-atrial pressure was recorded using a Physiograph (Power Lab 2/20) via a pressure transducer (Statham P23Db, Oxnard, CA, USA) and pulse pressure was calculated by the difference between systolic and diastolic pressures. Pulse pressures were expressed as cm H_2O.

Changes in atrial pulse pressure (fold) = (value of pulse pressure − mean basal value of pulse pressure)/mean basal value of pulse pressure.

2.3. Experimental Protocol

Each atrium was perfused for 60 min to stabilize atrial dynamics and then the control cycle (12 min as an experimental cycle) was followed by infusion of hypoxic buffer for four cycles, monitoring changes in atrial dynamics. For western blot analysis, immediately after perfusion, the atrial tissue was frozen and stored at −80˚C until analyzed.

To investigate effects of CNP and forskolin on hypoxia-induced atrial dynamics, one cycle of hypoxia after the control was followed by three cycles of infused treatment agent plus hypoxia. The treatment agents used were CNP (0.1 μmol/L) and forskolin (0.1 μmol/L). In the control group, vehicle was introduced instead of treatment agent. Values obtained during the periods corresponding to control and experimental observations were compared.

2.4. Western Blot Analysis

Proteins derived from left atrial tissue were analyzed by western blotting. Atrial tissues were homogenized in radio-immunoprecipitation assay lysis buffer (Solarbio institute of Biotechnology, Shanghai, China), and protein concentrations were determined with a Bradford protein assay kit. Solubilized proteins were denatured in Lane Maker Loading buffer and proteins separated by 10% or 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein bands were then transferred to polyvinylidene difluoride filter membranes (Beyotime Institute of Biotechnology, China). Each membrane was blocked with a 5% skim milk in phosphate buffer (PBST) solution at room temperature. After 2 h the membranes were incubated with the appropriate primary antibodies, overnight at 4˚C. The primary antibodies used were anti-phosphodiesterase subtype 3A (PDE3A, 1:1000, Abcam Shanghai, Shanghai, China) or anti-HIF-1α (1:1000, Abcam Shanghai), using, rabbit polyclonal β-actin (1:1000; Com Win Biotech, Beijing, China) as a loading control for all lanes. The membranes were then washed and incubated with secondary antibodies (1:2000) at room temperature for 2 h. After washing membranes thoroughly with PBST, stained bands were visualized by the ECL method (ECL Western Blot Kit, Com Win Biotech) and band densities quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Statistical Analysis

The significance of differences among values was determined by one-way ANOVA followed by Dunnett's multiple comparison test. An unpaired t-test was also

applied. Statistical significance was defined as $P < 0.05$. All data were presented as means ± SEM.

3. Results

3.1. Effect of CNP on Hypoxia-Induced Atrial Dynamics

As shown in Figure 1, hypoxia significantly decreased pulse pressure in isolated perfused beating rat atria ($P < 0.05$ vs. control, (a)). CNP also substantially

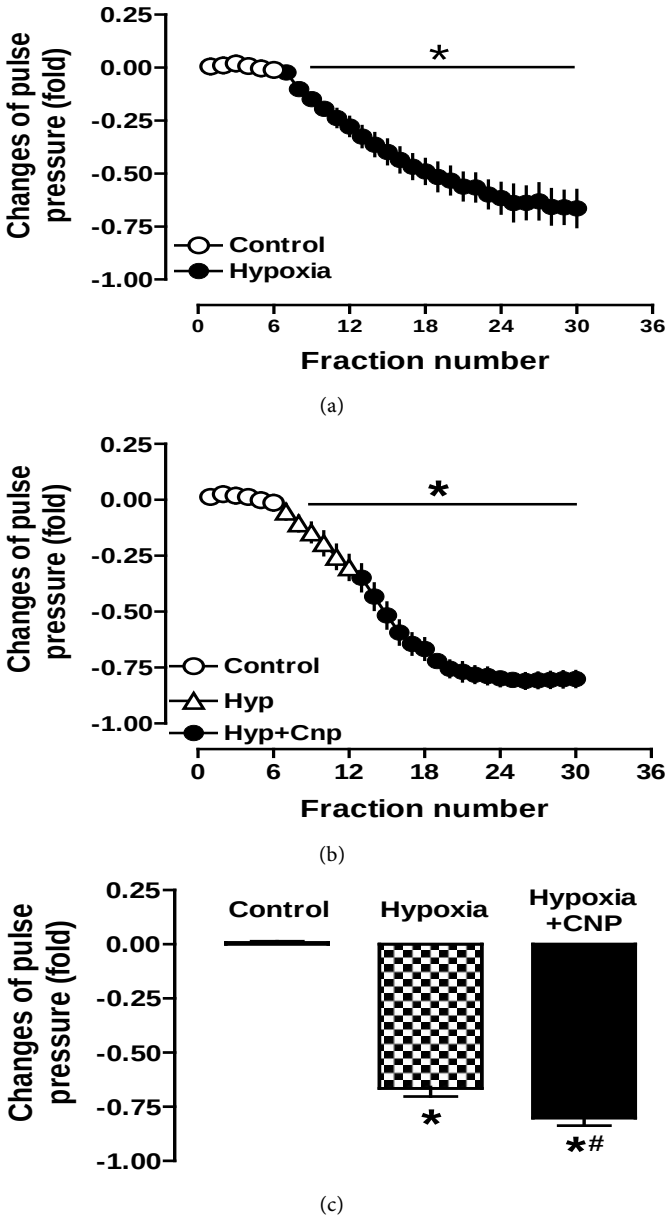

Figure 1. Effect of CNP (0.1 μmol/L) on hypoxia-induced pulse pressure (PP) in isolated perfused rat atria. (a) hypoxia-induced PP; (b) and (c) CNP modulated PP (data in (c) were derived from (a) and (b) control as well as the last cycle of the experimental period). Data were expressed as mean ± SEM, n = 6. *$P < 0.05$ vs. control; #$P < 0.05$ vs. hypoxia.

decreased pulse pressure in the hypoxic atria ($P < 0.05$ vs. control, (b)), a net effect that was greater than that of hypoxia alone ($P < 0.05$ vs. hypoxia alone, (c)). These data indicated that CNP had a negative inotropic effect in the hypoxic atrium.

3.2. Effects of Forskolin on CNP-Induced Suppression of Hypoxic Atrial Pulse Pressure

To determined effects of an AC activator, forskolin, on regulation of hypoxia-induced atrial dynamics by CNP, a series of experiments were performed with this agent in the perfused beating rat atria. As shown in Figure 2, forskolin did not affect hypoxia-induced atrial pulse pressure ($P < 0.05$ vs. control; $P > 0.05$ vs. hypoxia). In contrast, the AC activator dramatically attenuated the inhibitory effects of CNP on hypoxia-induced pulse pressure ($P < 0.05$ vs. hypoxia; $P < 0.05$ vs. CNP; $P < 0.05$ vs. forskolin). These results suggested that forskolin reversed the inhibitory effects of CNP on atrial dynamics in the beating hypoxic atrium.

3.3. Effects of Forskolin and CNP on Atrial PDE3A Levels under Hypoxia

PDE 3A regulates intracellular cAMP levels, so we examined levels of this protein to investigate the mechanism of forskolin-mediated reversal of inhibition by CNP of hypoxia-induced atrial pulse pressure. Atrial PDE3A levels were determined by western blotting in hypoxic beating atria that had been treated with or without forskolin and/or CNP. Forskolin significantly up-regulated atrial PDE 3A protein levels under hypoxic conditions ($P < 0.05$ vs. control group; $P < 0.05$ vs. hypoxia group, Figure 3). There were no significant changes in PDE 3A levels with hypoxia alone or with hypoxia plus CNP. However, in the presence of forskolin, CNP dramatically suppressed levels of PDE 3A in hypoxic atria ($P < 0.05$ compared with all other groups, Figure 3). This indicated that CNP inhibited forskolin-induced PDE3A activity activation in hypoxic atria.

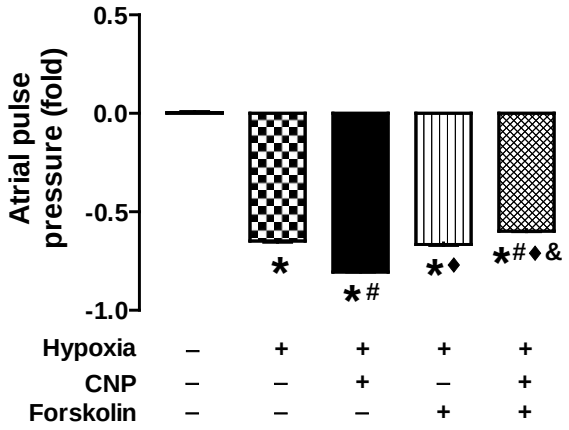

Figure 2. Effect of forskolin (0.1 μmol/L), an activator of adenylyl cyclase, on the regulation of CNP-induced pulse pressure in perfused beating rat hypoxic atria. Data were expressed as mean ± SEM, n = 6. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. hypoxia group; ♦$P < 0.05$ vs. CNP group; &$P < 0.05$ vs. forskolin group.

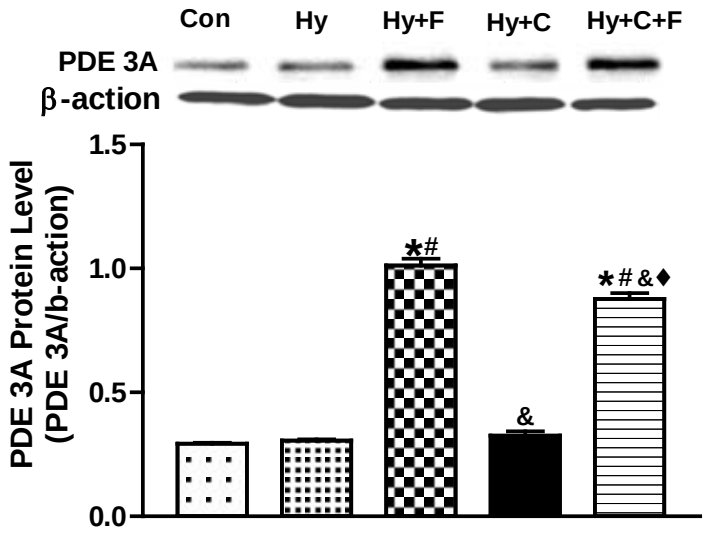

Figure 3. Effects of CNP (0.1 μmol/L) and forskolin (0.1 μmol/L) on hypoxia-induced atrial phosphodiesterase subtype 3A (PDE 3A) expression. Con, control; Hy, hypoxia; F, forskolin; C, CNP. Data were expressed as mean ± SEM, n = 5. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. hypoxia group; ♦$P < 0.05$ vs. CNP group; &$P < 0.05$ vs. forskolin group.

3.4. Effects of CNP and Forskolin on HIF-1α Levels in Hypoxic Atria

We next investigated regulation by CNP of hypoxia-induced increases in atrial HIF-1α, as well as the impact of forskolin. As shown in **Figure 4**, hypoxia substantially increased atrial levels of HIF-1α ($P < 0.05$ vs. control group) and this effect was completely abolished by CNP ($P < 0.05$ vs. hypoxia group). In addition, forskolin clearly augmented the hypoxia-induced increase in HIF-1α levels in the atria ($P < 0.05$ vs. control group; $P < 0.05$ vs. hypoxia group). This effect was dramatically attenuated by CNP, though HIF-1α levels remained elevated, as compared with hypoxic atria with CNP alone ($P < 0.05$ vs. control group; $P < 0.05$ vs. hypoxia group; $P < 0.05$ vs. CNP group). These results suggested that, in hypoxic atria, CNP suppressed upregulation of HIF-1α and that this effect could be modulated by the AC activator forskolin.

4. Discussion

In our study, in isolated perfused beating rat atria under hypoxic conditions, CNP inhibited atrial dynamics and suppressed HIF-1α levels. This effect of CNP was modulated by the AC activator forskolin, through activation of CNP-PDE 3A signaling.

It is well known that CNP can bind to B-type natriuretic peptide receptors (NPR-B) and negatively affect cardiac myocyte function through activation of the guanylyl cyclase (GC)-cGMP-PKG signaling pathway [17] [18] [19]. In addition, particulate GC (pGC) activation in atria by CNP led to increased pGC-cGMP-PDE 3 signaling and elevated cAMP levels [20]. In our study, hypoxia

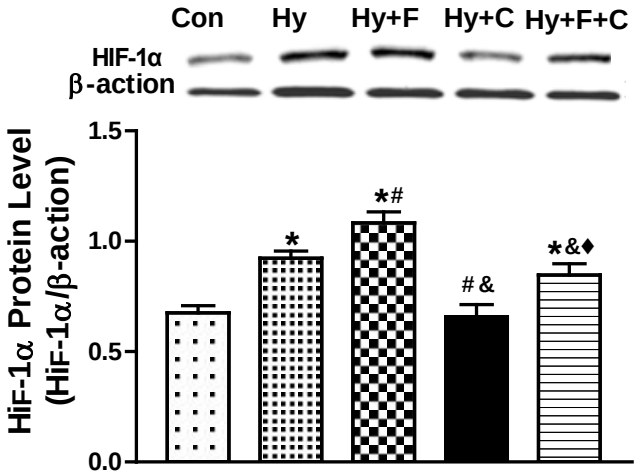

Figure 4. Effects of forskolin (0.1 μmol/L) and CNP (0.1 μmol/L) on hypoxia-induced atrial HIF-1*α* expression. Con, control; Hy, hypoxia; F, forskolin; C, CNP. Data were expressed as mean ± SEM, n = 5. **P* < 0.05 vs. control group; #*P* < 0.05 vs. hypoxia group; ♦*P* < 0.05 vs. CNP group; &*P* < 0.05 vs. forskolin group.

significantly inhibited atrial dynamics, an effect clearly augmented by CNP treatment. Furthermore, under hypoxia, the AC activator forskolin substantially increased atrial PDE 3A protein levels without affecting dynamics. Nevertheless, under hypoxia and in the presence of forskolin, the inhibitory effect of CNP on atrial dynamics was substantially attenuated, with concomitant suppression of the forskolin-induced increases in PDE 3A protein levels. These results indicated that CNP can have negative inotropic effects on atrial dynamics under hypoxia and that these actions can be modulated by forskolin, through activation of CNP-PDE 3A signaling. Our results agreed well with previous findings [17] [18] [19] [20].

Intracellular cAMP is levels are determined by the rate of cAMP generation, through activation of adenylyl cyclase, and its degradation by phosphodiesterases (PDEs) [21]. At least four families of PDEs, PDE 1, PDE 2, PDE 3 and PDE 4, were identified in the heart [22] [23] [24]. PDE 3, a cGMP-inhibited PDE subtype, represents one of the major cAMP-degrading PDEs in the human heart [25]. PKA enhances HIF-1*α* transcriptional activity [7] [8] and PDE 3 inhibition leading to an elevating of intracellular cAMP levels [20], which subsequently, activates PKA. Thus, PDE 3 may be involved in regulation of HIF-1*α* activity. In our study, hypoxia significantly increased atrial HIF-1*α* protein levels and this effect was augmented by forskolin, which also increased PDE 3A levels. The effect was, in contrast, blocked by CNP, without affecting PDE 3A levels. Nevertheless, the inhibitory effect of CNP on hypoxia-induced atrial HIF-1*α* protein expression was dramatically attenuated by forskolin. Under these conditions, there was concomitant suppression, by CNP, of the forskolin-induced increases in PDE 3A levels. These results indicated that the suppression by CNP of hypoxia-induced HIF-1*α* elevation in the perfused beating rat atria was modulated by

forskolin, through activation of CNP-PDE 3 signaling. Thus, PDE 3 is a potential regulatory target for modulating HIF-1*α* activity.

5. Conclusion

In conclusion, CNP inhibited atrial dynamics and HIF-1*α* activity in the isolated perfused beating rat atria under hypoxic conditions. These effects of CNP were modulated by the AC activator, forskolin, through increased CNP-PDE 3A signaling.

Acknowledgements

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