C-type natriuretic peptide prevents angiotensin II-induced atrial connexin 40 and 43 dysregulation by activating AMP-activated kinase signaling

DA-ZHI DING1*, YA-NAN JIA1*, BO ZHANG2, CHENG-MING GUAN1, SHUAI ZHOU3, XIANG LI2 and XUN CUI2,4

1Department of Cardiology, Institute of Clinical Medicine; 2Department of Physiology, College of Medicine; 3Key Laboratory of Organism Functional Factors of The Changbai Mountain, Ministry of Education; 4Cellular Function Research Center, Yanbian University, Yanji, Jilin 133002, P.R. China

Received December 3, 2018; Accepted August 14, 2019

DOI: 10.3892/mmr.2019.10744

Abstract. C-type natriuretic peptide (CNP), from the family of natriuretic peptides (NPs), has been shown to induce antihypertrophic and antifibrotic effects in cardiomyocytes. However, the roles of CNP in the atrial dysregulation of connexin (Cx)40 and Cx43 remain to be elucidated. The present study aimed to investigate the effects of CNP on angiotensin (Ang) II-induced Cx40 and Cx43 dysregulation in isolated perfused beating rat left atria. A rat isolated perfused beating atrial model was used and the protein levels were determined via western blotting. Ang II significantly upregulated NF-κB, activator protein-1, transforming growth factor-β1 (TGF-β1), collagen I and matrix metalloproteinase 2, leading to atrial fibrosis, and downregulated expression of Cx40 and Cx43. The changes in Cx40 and Cx43 induced by Ang II were abolished by CNP through upregulation of phosphorylated AMP-activated kinase a1 (AMPK) and downregulation of TGF-β1. The effects of CNP on AMPK and TGF-β1 levels were inhibited by KT5823 and pertussis toxin, inhibitors of protein kinase G (PKG) and NP receptor type C (NPR-C), respectively. Thus, CNP can prevent Ang II-induced dysregulation of Cx40 and Cx43 through activation of AMPK via the CNP-PKG and CNP-NPR-C pathways in isolated beating rat atria. The present findings suggested that CNP may be therapeutically useful for clinical conditions involving cardiac dysregulation of Cx expression-related diseases.

Introduction

C-type natriuretic peptide (CNP) is the third member of the family of natriuretic peptides (NPs) originally identified in porcine brains (1). It is also widely expressed in the vasculature endothelium, where it regulates vascular tone (2,3), and in the myocardium (4). Cardiac production of CNP, and its possible autocrine and paracrine functions, have been demonstrated in patients with heart failure (5,6) and endogenous CNP secreted from cardiomyocytes and fibroblasts reduces the deleterious pathological changes occurring during heart failure (7). In addition, activation of the CNP/NP receptor type B (NPR-B) pathway following myocardial infarction has been reported induce antiproliferative and antihypertrophic effects in cardiac cells (8,9). CNP administration improved cardiac function and attenuated cardiac remodeling after myocardial infarction in rats in vivo, and these effects have been attributed to its antifibrotic and antihypertrophic actions (9,10).

The cardiac gap junction is the most important intercellular communication structure in cardiomyocytes, and is indispensable for effective function of the heart (11). Among various gap junctional proteins, connexin (Cx)43 is abundant in ventricular as well as atrial myocytes, and plays a major role in inter-myocyte connections (12). Cx40, another connexin isoform in the heart, is normally restricted to the atrium (13). In the infarct border zone and the failing or hypertrophied heart, myocardial Cx43 demonstrated decreased or non-anisotropic expression patterns, and these altered expression profiles have been designated ‘gap junction remodeling’ (14,15). Gap junction remodeling is considered to impair intercellular communication and myocardial function. As a novel antifibrotic and antihypertrophic agent, CNP and its specific receptor NPR-B may be important for regulation of cardiac hypertrophy and remodeling. However, the effects of CNP on atrial Cx40 and Cx43 dysregulation remain unclear. Angiotensin (Ang) II may play a central role in the etiology and pathophysiology
of cardiovascular diseases, including cardiac hypertrophy and remodeling in humans (16). In a previous study, it was observed that excessive Ang II induced significant dysregulation of Cx40 and Cx43 expression in isolated perfused beating rat atria (17). Therefore, the present study investigated the effects of CNP on Ang II-induced Cx40 and Cx43 dysregulation in isolated perfused beating rat left atria.

Materials and methods

Preparation of cultured atrial fibroblasts. All experimental procedures were approved by the Yanbian University Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (18). In total, 60 Sprague-Dawley rats (weight, 250-300 g; age, 18 weeks; female to male ratio, 3:7) were obtained from Yanbian University. The rats were acclimatized for one week in the Animal Experimental Center of Yanbian University [animal license no. SCXK(ji)2012-006] with 45-65% humidity, at a constant temperature 24-2°C and under a 12-h light/dark cycle. Rats were given a free access to food and water. The rats were anesthetized with pentobarbital sodium via intraperitoneal injection (90 mg/kg), then decapitated and sterilized with alcohol. The hearts were dissected under aseptic conditions, and the atria were separated and placed in phosphate-buffered saline (PBS). The left atria were digested with 0.1% collagenase type II (Gibco; Thermo Fisher Scientific, Inc.) in a 37°C water bath. The isolated cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 20% fetal bovine serum (HyClone; GE Healthcare Life Sciences). Cells were identified using vimentin staining as described below to confirm that >90% of cells were atrial fibroblasts. When the cell growth approached 95% confluency, the second to fourth passages were used in experiments.

Fibroblasts were seeded in 6-well plates and cultured for 24 h. The cells were divided into two groups: Control group and Ang II [100 nmol/l, as previously described (19)] group. After 24 h, the cells were collected for western blotting analysis.

Identification of atrial fibroblasts by immunofluorescence staining. Adherent cells were identified as atrial fibroblasts via inverted microscopy. Second-generation atrial fibroblasts (1x10^4) were inoculated into glass cover slips wells (24 mm), and sequentially incubated with 4% paraformaldehyde for 20 min and 0.5% Triton X-100 for 20 min at room temperature. Cell slides were then blocked with 5% BSA for 2 h at 37°C, and incubated overnight with anti-vimentin antibody (1:200; cat. no. ab8069; Abcam) at 4°C. Subsequently, the slides were incubated with anti-mouse IgG-FITC (1:50; cat. no. ZF-0312; ZSGB-BIO) at room temperature for 1 h under light-proof conditions. The cell nuclei were stained with DAPI for 20 min at room temperature. Finally, fluorescence images were obtained with a fluorescence microscope (U-RFL-T; Olympus Corporation).

Preparation of perfused beating rat atria. Sprague-Dawley rats of both sexes with a weight range of 250-300 g were anesthetized with pentobarbital sodium by intraperitoneal injection (90 mg/kg), then decapitated and sterilized with alcohol. Isolated perfused beating left atria were prepared as previously described (20). After preparation of each atrium, transmural electrical field stimulation was applied with a luminal electrode at 1.5 Hz (0.3 ms, 30-40 V) and the atrium was perfused with HEPES buffer using a peristaltic pump (1.0 ml/min) to enable atrial pacing for measurement of pulse pressure changes. Oxygen was continuously supplied and the temperature of the atrium was maintained at 36°C. The HEPES buffer contained (in mmol/l) 118 NaCl, 4.7 KCl, 2.5 CaCl_2, 1.2 MgCl_2, 25 NaHCO_3, 10.0 glucose and 10.0 HEPES (pH 7.4 with NaOH), together with 0.1% BSA.

Experimental protocols. The rats were randomly divided into eight groups (n=5/group) as follows: Control group; Ang II group; CNP group; Ang II + CNP group; Ang II + cANP_{1-23} (NPR-C activator) group; Ang II + CNP + KT5823 [protein kinase G (PKG) inhibitor] group; and Ang II + CNP + pertussis toxin (inhibitor of NPR-C) group; Ang II + CNP + KT5823 + pertussis toxin.

Each atrium was perfused for 60 min to stabilize the atrial dynamics. After a control cycle (12 min as an experimental cycle), the treatment cycle was followed by seven cycles of treatment agent infusion. The treatment agents were as follows: i) Control group, each atrium was perfused with normal buffer for seven cycles; ii) Ang II group, three cycles of normal buffer were followed by five cycles of Ang II [5.0 µmol/l, as used in our previous study (17)]; iii) Ang II + CNP group, three different doses of CNP (0.03, 0.1 or 0.3 µmol/l) were used, and each atrium was perfused for one cycle of CNP after two cycles of normal buffer, and then perfused with CNP + Ang II for five cycles. In addition, two cycles of normal buffer were followed by six cycles of CNP alone (0.1 µmol/l); iv) Ang II + cANP_{1-23} group, after two cycles of normal buffer, one cycle of cANP_{1-23} (3.0 µmol/l) was followed by five cycles of cANP_{1-23} + Ang II; v and vi) Ang II + CNP + KT5823 and Ang II + CNP + pertussis toxin groups, one cycle of KT5823 (0.3 µmol/l) or pertussis toxin (0.06 µmol/l) after one cycle of normal buffer, one cycle of KT5823 or pertussis toxin + CNP was followed by five cycles of KT5823 or pertussis toxin + CNP + Ang II; effects of KT5823 + pertussis toxin + CNP + Ang II were also tested. Immediately after perfusion, the atrial tissues were collected, frozen in liquid nitrogen, and stored at -80°C for subsequent western blot analysis.

Western blot analysis. Proteins extracted from fibroblasts and left atrial tissue samples were analyzed via western blotting. The proteins were lysed at 4°C in RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) mixed with protease and phosphatase inhibitors. Protein concentration was determined using the bicinchoninic acid assay (cat. no. P0010; Beyotime Institute of Biotechnology) Solubilized proteins (mass of protein loaded per lane, 40 µg) were separated via 8-10% SDS-PAGE, and the protein bands were transferred to polyvinylidene difluoride filter membranes (Beyotime Institute of Biotechnology). The membranes were blocked with 5% skimmed milk powder in PBS at room temperature for 2 h. Subsequently, the membranes were incubated at 4°C overnight with a rabbit anti-CNP polyclonal antibody (1:500; cat. no. E-AB-30982;
Elabscience), rabbit anti-NPR-B antibody (1:2,000; cat. no. ab139188; Abcam), rabbit anti-NPR-C antibody (1:2,000; cat. no. ab177954; Abcam), rabbit anti-Cx40 polyclonal antibody (1:1,000; cat. no. ab101929; Abcam), rabbit anti-Cx43 polyclonal antibody (1:1,000; cat. no. ab11370; Abcam), rabbit anti-transforming growth factor-β1 (TGF-β1) polyclonal antibody (1:1,000; cat. no. ENT4632; Elabscience), rabbit anti-collagen I monoclonal antibody (1:1,000; cat. no. ab138492; Abcam), rabbit anti-matrix metalloproteinase 2 (MMP2) monoclonal antibody (1:1,000; cat. no. ab92536; Abcam) or rabbit anti-p-AMP-activated protein kinase a1 polyclonal antibody (anti-p-AMPK; 1:500; cat. no. PA5-17831; Thermo Fisher Scientific, Inc.). All membranes were also incubated with a rabbit anti-β-actin monoclonal antibody (1:1,000; cat. no. ENM0028; Elabsence Biotechnology Co., Ltd.) as a loading control. The membranes were also incubated with appropriate horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000; cat. no. AEP003; Elabscience Biotechnology Co., Ltd.) for 2 h at room temperature. After thorough washing of the membranes with phosphate-buffered saline containing 0.1% Tween-20, the antibody-bound bands were visualized with an ECL Plus western blotting detection system (ECL Western Blot Kit; Beijing CoWin Biotech Co., Ltd.) and the band densities were quantified using ImageJ software (version 1.48; National Institutes of Health).

**Histopathological analysis.** Immediately after perfusion in the control, Ang II and Ang II + CNP groups, each atrium was placed in a pre-dosed fixative (10% formalin, Bouin's fixative) overnight at room temperature, dehydrated with ethanol, embedded in paraffin, sectioned at 3-4-µm thickness and subjected to Masson's three-color dye staining using a Masson's staining kit (Beijing Solarbio Science & Technology Co. Ltd.) for 10 min at room temperature. The stained sections were examined via optical microscopy (five fields analyzed/sample), and images acquired at x100 magnification. The collagen volume fraction (collagen area/total area x100%) was determined using Image-Pro Plus 6.0 software (Media Cybernetics Inc.).

**Statistical analysis.** Data were analyzed with Prism 5.0 software (GraphPad Software, Inc.). Significant differences were determined by an unpaired t-test or two-way ANOVA followed by a Bonferroni post hoc tests. Data were presented as means ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Identification of atrial fibroblasts. Vimentin is an intermediate fiber in interstitial cells that forms part of the cytoskeleton. It has been reported that cardiac fibroblasts can be identified...
by positive expression of vimentin (21). The results revealed green fluorescence for vimentin-positive cells in the highly purified atrial fibroblasts (Fig. 1).

**Figure 2.** Effect of Ag (100 nmol/l) on (A) CNP, (B) NPR-B and (C) NPR-C protein levels in cultured rat atrial fibroblasts. Data were expressed as mean ± standard error of the mean; n=5. *P<0.05 vs. Cont. Ag, angiotensin II; CNP, C-type natriuretic peptide; NPR, natriuretic peptide receptor; Cont, control.

**Figure 3.** Effect of CNP on Ang II-induced upregulation of TGF-β1 expression in beating rat atria. Data were expressed as mean ± standard error of the mean; n=5. *P<0.05 vs. Cont; #P<0.05 vs. Ang II. C/CNP, C-type natriuretic peptide; Ag, Ang II; TGF, transforming growth factor; Cont, control.

**Figure 4.** Effect of CNP on Ang II-induced upregulation of (A) collagen I and (B) MMP2 expression in isolated perfused beating rat atria. Data were expressed as mean ± standard error of the mean; n=5. *P<0.05 vs. control; #P<0.05 vs. Ang II. C/CNP, C-type natriuretic peptide; Ag/Ang II, angiotensin II; MMP, matrix metalloproteinase.

**Effects of Ang II on CNP, NPR-B and NPR-C levels in cultured atrial fibroblasts.** To determine the effects of
Ang II on the regulation of CNP, NPR-B and NPR-C protein expression, a series of experiments were performed on cultured rat atrial fibroblasts. Ang II significantly increased CNP protein expression (P<0.05 vs. control; Fig. 2A), as well as NPR-B and NPR-C protein expression (P<0.05 vs. control; Fig. 2B and C) in rat cultured atrial fibroblasts. These results suggest that Ang II can promote the production of CNP and lead to upregulation of its receptors in rat atrial fibroblasts.

Effects of CNP on Ang II-induced TGF-β1 expression and atrial fibrosis. Excessive Ang II-induced TGF-β1 can lead to cardiac fibrosis and remodeling. Therefore, another series of experiments were performed to examine the effects of CNP on Ang II-induced TGF-β1 expression and atrial fibrosis. Ang II significantly increased TGF-β1 expression (P<0.05 vs. control; Fig. 3) concomitantly with upregulation of collagen I and MMP2 levels (P<0.05 vs. control; Fig. 4A and B); these effects were completely blocked by CNP pretreatment (P<0.05 vs. control; P<0.05 vs. Ang II; Figs. 3 and 4). In addition, Masson's staining revealed that Ang II induced widespread fibrous tissue in interstitial areas compared with the control (collagenous fibers were stained blue; Fig. 5A and B), and this effect was also abolished by CNP pretreatment (Fig. 5A and B). These

Figure 5. Effect of Ang II-induced myocardial fibrosis in beating rat atria. (A) Fibrosis was evaluated by Masson's trichrome staining, with myocardial cells stained red, and collagenous fibers stained blue. (B) Collagen deposition was quantitatively analyzed as collagen volume fraction (%). Data were expressed as mean ± standard error of the mean; n=5. *P<0.05 vs. Cont; #P<0.05 vs. Ag. Cont, control; C/CNP, C-type natriuretic peptide; Ag, angiotensin II.
results suggested that CNP induced inhibitory effects on Ang II-induced activation of TGF-β1 and fibrosis in beating rat atria.

**Effect of CNP on Ang II-induced dysregulation of Cx40 and Cx43.** It was demonstrated that Ang II can upregulate TGF-β1, leading to cardiac fibrosis and subsequent cardiac remodeling. Therefore, to examine whether Ang II induces dysregulation of atrial connexin proteins, and the effects of CNP on this process, the levels of Cx40 and Cx43 were analyzed. Ang II significantly decreased atrial expression of Cx40 and Cx43, and the effects were markedly inhibited by CNP in a dose-dependent manner (P<0.05 vs. control; P<0.05 vs. Ang II; Fig. 6A and B). These results indicated that CNP prevented Ang II-induced gap junction remodeling in beating rat atria.

**Effect of CNP on p-AMPK expression.** In the light of the inhibitory effects of AMPK on Ang II-induced cardiac hypertrophy and fibrosis, p-AMPK expression was analyzed. CNP significantly increased p-AMPK expression (P<0.05 vs. control, P<0.05 vs. Ang II; Fig. 7). Furthermore, the NPR-C activator cANP, an agonist of NPR-C, mimicked the effect of CNP on atrial p-AMPK expression (P<0.05 vs. control, P<0.05 vs. Ang II; Fig. 7) in beating rat atria. Activation of AMPK expression
by CNP, as well as by cANP<sub>4-23</sub>, was almost completely abolished by KT5823 and pertussis toxin, inhibitors of PKG and NPR-C, respectively (P<0.05 vs. CNP and cANP<sub>4-23</sub>, respectively). In addition, the preventive effects of CNP on Ang II-induced dysregulation of Cx40 and Cx43 were clearly attenuated by KT5823 and pertussis toxin (P<0.05 vs. control, P<0.05 vs. Ang II; P<0.05 vs. CNP; Fig. 9A and B). These results suggested that CNP prevented Ang II-induced dysregulation of connexin expression by inhibiting TGF-β1 activity through CNP-PKG and CNP-NPR-C signaling in beating rat atria.

Discussion

The present study demonstrated that, in isolated perfused beating rat atria, CNP prevented Ang II-induced dysregulation of Cx40 and Cx43 protein expression via activation of AMPK, thereby inhibiting Ang II-induced upregulation of TGF-β1 and atrial fibrosis through both the CNP-PKG and CNP-NPR-C pathways.

As well as being the most abundant NP in the brain, CNP is also synthesized in cardiac fibroblasts, and may serve a role as an autocrine regulator against excessive cardiac fibrosis (22). Protective roles of CNP on cardiovascular diseases have also been identified (8,23). In the present study, Ang II significantly increased the CNP protein level concomitantly with upregulation of NPR-B and NPR-C expression in adult rat left atrial cultured fibroblasts. In addition, exogenous CNP completely abolished the fibrosis induced by Ang II in isolated beating rat atria. These findings are consistent with the aforementioned studies.

In our previous study, it was observed that excessive Ang II stimulated atrial TGF-β1 expression through activation of p38-MAP kinase and nuclear transcription factors, ultimately leading to atrial dysregulation of Cx40 and Cx43 expression (17). In the present study, Ang II also significantly increased atrial TGF-β1 protein expression and upregulation of collagen I as well as MMP2, ultimately leading to atrial fibrosis. Thus, Ang II-induced fibrosis caused downregulation of Cx40 and Cx43. These results are consistent with previous studies (14,15,24). In addition, it was found that CNP completely abolished Ang II-induced upregulation of atrial TGF-β1, extracellular matrix proteins and downregulation of Cx40 as well as Cx43. These results demonstrate that CNP can play a protective role against Ang II-induced connexin protein dysregulation by suppressing TGF-β1 expression in perfused beating rat atria. The present data are consistent with previous studies that CNP prevents cardiac hypertrophy, fibrosis, and remodeling (10,24-26).

A previous study demonstrated that stimulation of AMPK phosphorylation led to inhibition of TGF-β1/Smad3-mediated myofibroblast differentiation (27), and prevented Ang II-induced cardiac hypertrophy and fibrosis (28,29). Similarly, in the present study, CNP dramatically increased atrial AMPK phosphorylation, thereby suppressing Ang II-induced upregulation of TGF-β1, and consequently preventing Ang II-induced dysregulation of Cx40 and Cx43 expression. In addition, CNP-induced upregulation of AMPK phosphorylation and downregulation of TGF-β1 were abolished by not only KT5823, a PKG pathway inhibitor, but also by pertussis toxin, an NPR-C inhibitor. These results suggested that CNP can stimulate AMPK phosphorylation and prevent Ang II-induced dysregulation of Cx40 and Cx43 expression via PKG as well as

Figure 9. Effects of KT5823 and pertussis toxin on the regulation of Ang II-induced atrial (A) Cx40 and (B) Cx43 expression. Data were expressed as mean ± standard error of the mean, n=5. *P<0.05 vs. Cont; †P<0.05 vs. Ang II; ‡P<0.05 vs. CNP. Cx, connexin; Cont, control; Ag/Ang II, angiotensin II; C, C-type natriuretic peptide; K, KT5823, an inhibitor of PKG; P, pertussis toxin, an inhibitor of NPR-C.
NPR-C signaling in beating rat atria. These results are consistent with previous findings that CNP acts via the CNP-NPR-B and CNP-NPR-C pathways to play an important protective role against cardiac remodeling (4).

In conclusion, CNP prevented Ang II-induced dysregulation of Cx40 and Cx43 expression through upregulation of p-AMPK and downregulation of TGF-β1 via the CNP-PKG and CNP-NPR-C pathways in isolated perfused beating rat atria. These findings suggest that CNP is potentially useful for clinical applications involving cardiac dysregulation of connexin expression-related diseases.

Acknowledgements

Not applicable.

Funding

The present study work was supported by The National Natural Science Foundation of China (grant nos. 81360061 and 81660089).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DD performed atrial perfusion experiments and cell culture experiments, BZ performed immunofluorescence staining, YJ and XC designed the experiments and wrote the manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Yanbian University Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.