Angiotensin-II down-regulates cardiac natriuretic peptide receptor-A mediated anti-hypertrophic signaling in experimental rat hearts

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Atrial natriuretic peptide (ANP) exerts anti-hypertrophic effects in the heart via natriuretic peptide receptor-A (NPR-A). However, ANP mediated anti-hypertrophic activity is decreased in the cardiomyopathic conditions. In the present investigation the in vivo effects of angiotensin II (Ang II), a hypertrophic agonist have been studied on the ventricular expression level of NPR-A in Wistar rat hearts. NPR-A expression at the protein and mRNA levels were found to be markedly reduced by 5-fold respectively in Ang II infused rats heart as compared with sham rat hearts. Moreover, cGMP production in response to ANP was reduced by 77% in the isolated cardiac membrane preparation from the Ang II infused rat hearts. Losartan treatment reversed NPR-A expression and responsiveness to ANP. This study suggests that Ang II down regulates cardiac NPR-A activity by suppressing Npr1 gene transcription.

Keywords: Angiotensin-II, Atrial natriuretic peptide, Guanylyl cyclase activity, Left ventricular hypertrophy, Natriuretic peptide receptor-A

Angiotensin II (Ang II) is a potent inducer of cardiac hypertrophic growth, and chronic stimulation of Ang II promotes cardiac remodeling and fibrosis. Inhibition of Ang II/AT1R (AngII type 1 receptor) signaling by angiotensin-converting enzyme (ACE) blockers or AT1R antagonists prevents heart failure progression and reduces mortality in patients. Ang II has been shown to down regulate the NPR-A gene expression by activating protein kinase C and/or by stimulating protein tyrosine phosphorylation.
phosphatase activity in vitro. Further, the cGMP production in response to ANP was suppressed in the presence of Ang II in cultured smooth muscle cells. These in vitro studies suggested that Ang II (vasoconstrictor) interact and antagonize the biological effects of ANP/NPR-A system. However, in vivo studies have not been carried out to examine the interaction between Ang II and ANP/NPR-A system. In the present investigation, in vivo effects of Ang II have been evaluated on the levels of ventricular NPR-A and compared ventricular expression levels of NPR-A at the protein and mRNA in sham operated (control) and Ang II infused rats hearts. In addition, the responsiveness of cardiac membrane bound NPR-A to ANP is also studied in membrane preparations isolated from the sham operated control and Ang II infused rats hearts.

Materials and Methods

Materials—TriZol reagent, RT-PCR kit and PCR master mix were obtained from GeNei (Bangalore, India). Gene-specific primers were purchased from Ocimum Biosolutions Inc. (Netherland). Antibodies for NPR-A, and β-actin were purchased from Santa Cruz Biotechnology (San Diego, CA). Angiotensin (Ang II), ANP and cGMP kit were obtained from Sigma Aldrich, USA. All other chemicals used were of reagent grade.

Animals—Wistar male rats weighing approximately 120-150 g were used in this study. The animals were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India and maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (M/s. Hindustan Lever Ltd., Bangalore, India) and water ad libitum. All the experiments were designed and conducted according to the ethical norms approved by Institutional animal ethics committee guidelines of University of Madras, Chennai.

Implantation of the ALZET mini-osmotic pump—The animals were anesthetized by ketamine (80 mg/kg) and xylazine (10 mg/kg). The ALZET mini-osmotic pump (model 1002, Alzet, Inc., Palo Alto, California, USA) filled with Ang II (800 ng/kg/min) was carefully implanted subcutaneously in animals. For subcutaneous placement, a small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissues apart in the neck region of the heart. The pump was then inserted into the pocket with the flow moderator pointing away from the incision. The pump starts functioning immediately after the insertion and delivers subcutaneously Ang II (0.25 µL/hr).

Ang II-induced left ventricular cardiac hypertrophy in experimental rats—The animals were randomly divided into following three groups of 6 each: Gr. I sham operated rats served as controls, received vehicle saline alone through a mini-osmotic pump; Gr. II rats received Ang II (800 ng kg/min; solvent, saline) through mini-osmotic pump was implanted subcutaneously into neck of rats as described previously for 7 days; and Gr. III rats received losartan (10 mg/kg/day in drinking water) along with Ang II (as of group II rats) the treatment lasted for 7 days.

At the end of experimental period, the animals were sacrificed, heart was immediately removed and placed in ice-cold saline to remain in diastole to remove blood and the blood vessels and atrial tissue were trimmed off. The left ventricular (LV) tissue was separated from right ventricle (RV) at the interventricular septum, and were blotted and weighed. LV weight to body weight (BW) ratio was calculated as an index of cardiac hypertrophy. The LV tissue was used for mRNA isolation, cellular fraction and guanylyl cyclase (GC) assays. For the histological analysis, the LV samples were fixed in 10% phosphate buffered formalin. Paraffin embedded specimens were cut into 5 µm thick sections and stained with Hematoxylin–Eosin (H&E).

Reverse transcription-polymerase chain reaction (RT-PCR) analyses of hypertrophy markers, natriuretic peptides and its receptor gene—Total RNA was isolated from the LV tissues of control and experimental group rats hearts, using TRIzol reagent according to the manufacturer’s protocol. To remove genomic DNA contamination, RNA samples were treated with RNase free DNase I (1 unit/µg RNA) at 37 ºC for 30 min. The RNA integrity was confirmed by visualization of distinct 28 S and 18 S bands after electrophoresis on 1.5 % agarose gel (not shown). RT-PCR was performed with GeNei M-MuLV RT-PCR kit with following oligonucleotide gene-specific primers: β-MHC (NM_17240) sense primer : 5'-TTCAAAGGCTCAGGTCTCAGG-3' and anti-sense primer : 5'-GGCAACACCCATGTCTCA-GTTC-3' (product size: 202bp); α-skeletal actin (NM_019212) sense primer : 5'-CTCTCTCTCCT
CAGGACGACAATC-3' and anti-sense primer : 5'-CA GAATGCGTGGC TTATATGTTTC-3' (product size: 207bp); e-fos (NM_022197) sense primer : 5'-CCAAC TTATCCCCACGGTGAC-3' and anti-sense primer : 5'- TGGAATCTCGTGCTGCAAC-3' (product size: 381bp); ANP (NM_012612) sense primer : 5'-GAAC CTGCTAGACC CT-3' and anti-sense primer : 5'-CCTAGTCACCTGAGGCT-3' (product size: 312bp); BNP (NM_031545) sense primer : 5' AAG CTGCTGAGCTGATAAGA-3' and anti-sense primer 5'-GTTACAGCCCAAACGACTG AC-3' (product size: 451bp); GAPDH (XR_009165) sense primer : 5'-TTGCAG GCTGGGTCCTCATTG TCA-3' (product size: 401bp). The intensity of the bands were quantified using image density analysis software. The expressions were normalized with GAPDH.

Western-blot analysis of NPR-A—LV tissues were homogenized in an ice-cold 10 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 800 g, and the supernatant was separated and stored at -70 °C until use. Tissue homogenate (50 µg proteins) was mixed with equal amount of sample loading buffer and separated under reducing condition using 10% SDS-PAGE. The separated proteins were transferred at 100 Volts to a polyvinylidenedifluoride (PVDF) membrane. The membrane was blocked with 1x Tris-buffered saline-Tween 20 (TBST), pH 7.5, containing 5% BSA for 45 min at room temperature and incubated at 4 °C overnight with specific antibodies to NPR-A and β-actin at a dilution of 1:600 and 1:10000 in 1x TBST containing 3% BSA. After three washes with 1x TBST for 5 min each, the membrane was incubated for 1 h in HRP-conjugated anti-rabbit antibody at a dilution of 1:5000, washed three times with 1x TBST. Protein antibody complexes were detected by the addition of substrate (diaminobenzidine).

Immunofluorescence analysis of NPR-A—Paraffin embedded tissue sections of 5 µm thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% BSA in TBS for 2 h. The non inflated sections were then immunostained with primary antibody, NPR-A at a concentration of 1:100 dilution with 5% BSA in TBST and incubated overnight at 4 °C. After washing the slides thrice with TBST, the sections were incubated with FITC conjugated secondary antibody (Sigma Aldrich, USA), diluted 1:5000 with 3% BSA in TBST and incubated for 1 h at room temperature. The slides were counterstained with propidium iodide (Sigma Aldrich, USA). Images of propidium iodide—red (excitation 529 nm; emission 550 nm) and NPR-A-FITC (excitation 488 nm; emission 520 nm) stained sections were visualized using a fluorescent microscope.

Preparation of membranes and guanylyl cyclase (GC) activity assay—LV membrane fraction was isolated as described earlier. The membrane fractions containing approximately 50 µg protein was added to 100 µL of assay buffer containing 25 mM HEPES, 4 mM MnCl₂, 2 mM IBMX, 1 mM BSA, 5 units of creatinephosphokinase, 7.5 mM creatine phosphate, 0.5 mM GTP, and different concentration of peptides and incubated for 3 min. The reactions were stopped with 400 µL of ice-cold 50 mM sodium acetate solution containing 5 mM EDTA. The samples were then centrifuged, supernatant was collected and cGMP assay was performed using a direct cGMP immunoassay kit (Sigma Aldrich, USA) as previously described. The ANP concentration was chosen based on the preliminary studies. About 1µM concentration was used to represent the saturating concentration and this dose elicits maximal hormone-stimulated GC activity. The results were expressed as picomoles cGMP/mg protein.

Statistical analysis—Statistical analysis was performed using GraphPad Prism (version 5.0; GraphPad Software, San Diego, USA). The results are presented as mean ± SE. The statistical differences between the groups were determined by Student's t test. The probability value of P < 0.05 was considered significant.

Results

Ang II inhibits left ventricular NPR-A expression—Representative Western blot and densitometric analysis of left ventricular (LV) NPR-A protein expression in sham operated control and Ang II infused rat hearts are shown in Fig. 1A and B. Immunoblot analysis clearly showed that a marked reduction in the level of NPR-A (5-fold; P<0.001) protein in the left ventricular tissue of Ang II-infused rat hearts as compared to that of control left ventricular tissues. The NPR-A expression was increased (P<0.01) in the losartan treatment group compared to Ang II infused group. Representative...
RT-PCR and densitometric analysis of mRNA expression level of Npr1 gene in left ventricular tissue of sham operated control and Ang II infused rat hearts is shown in Fig. 1 C and D. mRNA expression level of Npr1 gene was markedly reduced (5-fold; P<0.001) in the Ang II-infused rat hearts as compared with control rat hearts. The reduced mRNA expressions of Npr1 gene (P<0.01) was remarkably reverted back to normal levels by the losartan treatment. Ang II-induced down regulation of NPR-A expression was further confirmed by immunofluorescence analysis (Fig. 2). Ang II-infused rats LV sections (Fig. 2 d-f) exhibited significant decreased green fluorescence as compared with control sections (Fig. 2 a-c), which signifies the decreased expression of NPR-A. The decreased expression of NPR-A was remarkably reverted back to its normal level in the losartan treated group of rats (Fig. 2 g-i), which was evident from its increased green fluorescence.

Ang II treatment inhibits Left ventricular NPR-A dependent guanylyl cyclase (GC) activity—To examine the functional consequences of decreased expression of Npr1 gene, the ANP-induced GC activity of the NPR-A receptor was studied in the membrane preparations isolated from the experimental rat hearts. The GC activity in the LV membrane preparation of control and Ang II-infused and losartan treated rat hearts in absence and presence of ANP was determined (Table 1). A significant decrease in basal NPR-A dependent GC activity (59%, P<0.01) was observed in the Ang II infused rat heart as compared to control rat heart. Saturating concentration of ANP stimulated GC activity was significantly decreased (P<0.001) in the Ang II-infused rat hearts as compared to control LV heart membrane preparation, and the decreased activity was significantly (P<0.01) reversed by AT1R blocker treatment.

Administration of Ang II caused left ventricular hypertrophy in rats—The RT-PCR analyses of the mRNA expression of hypertrophy markers are depicted in Fig. 3A. Ang II-infused rat hearts displayed significantly increased expression of β-MHC (4-fold, P<0.01), α-skeletal actin (4-fold, P<0.01), and c-fos (5-fold, P<0.001) as compared to control rat hearts. The increased expression of the marker genes were remarkably prevented by losartan treatment. RT-PCR mRNA expression analysis of
natriuretic peptide genes in sham operated control, Ang II-infused, and losartan treated rat hearts is shown in Fig. 3B. The expressions of ANP and BNP genes were markedly increased \((P<0.001; \text{ respectively})\) in the LV of Ang II-infused rats as compared with control rat hearts, while the increased expressions of ANP and BNP \((P<0.01 \text{ and } <0.001)\) were reversed to normal level by the losartan treatment. The increased heart size caused by Ang II was accompanied with a 50\% increase in the ratio of LVW/BW (sham operated: 0.17±0.014 vs Ang II-infused: 0.255±0.033; \(P<0.01\)) (Fig. 3C). Further, histopathological analysis confirmed the induction of hypertrophy in group II rats (Fig. 3D, a-b). ANG II infused rats (group II) left ventricular heart showing the enlarged cardiac muscle compared to sham operated control cardiac muscle.

**Discussion**

The results of the present study show that Ang II, acting through AT1R, down-regulates \(Nprl\) gene
expression, which modulates the cardiac hypertrophy. This mechanism was supported by decreased expression of Npr1 and its cGMP producing activity. Additionally, activation of hypertrophy marker gene in the Ang II induced left ventricular heart and activation of hypertrophy markers. Previous studies have shown that decreased vascular Npr1 mRNA expression along with increased ACE and AT1 receptors expressions in the two-kidney one-clip (2K1C) and N\(^G\)-nitro-L-arginine methyl ester (L-NAME) hypertension models\(^{26}\). Further, NPR-A dependent guanylyl cyclase (GC) activity was also decreased in the aortic banded mice model\(^{27}\). Involvement of Ang II/AT1R signaling in cardiac remodeling and hypertrophy was observed in 2K1C, L-NAME and aortic banded model. A recent study also demonstrated that Npr1\(^{-/-}\) Knockout (mice lacking NPR-A) mice develops marked cardiac hypertrophy and fibrosis and this phenomenon was greatly attenuated by deletion or pharmacological blockade of AT1R, suggesting an interaction between these two signaling pathways\(^{28}\). Furthermore, a negative correlation between NPR-A expression and increased AT1R has been reported in heart failure patients\(^{25,28}\).

In the present study, it was observed that the pathological concentration of Ang II, directly down regulates the NPR-A expression, suggesting that Ang II/AT1R mediated signaling. Previous studies have shown that ANP-dependent activation of GC activity and the intra cellular cGMP accumulation in the cells. The ligand (ANP) mediated GC activity of the NPR-A receptor was studied in the membrane preparations isolated from the experimental rat hearts. The results of the present study suggests that ANP- induced NPR-A dependent GC activity was markedly down regulated in the Ang II-infused rat hearts (Table 1). Moreover, losartan treatment completely abolished the Ang II mediated repression of NPR-A dependent GC activity, suggesting the involvement of Ang II/AT1R mediated signaling. Previous studies have
shown that Ang II down regulates \textit{Npr1} gene expression in cultured mouse mesangial cells in a dose dependent manner by activating protein kinase C\textsuperscript{19} and or by stimulating protein tyrosine phosphatase activity\textsuperscript{20}, suggesting that Ang II/AT1R mediated downstream signaling pathway is playing major role in suppressing the \textit{Npr1} gene expression.

As expected, Ang II administrated rats have displayed an increased LVW/BW ratio as compared to control rats. Moreover, an increased mRNA expression of hypertrophic marker genes such as c-fos (5-fold), β-MHC (4-fold), and α-skeletal actin (4-fold) (Fig. 3) were also observed in the ventricular tissues of the Ang II-infused rats, which further confirmed the induction of LVH in these rats\textsuperscript{10}. The expression of c-fos\textsuperscript{29}, β-myosin heavy chain\textsuperscript{30}, and skeletal actin\textsuperscript{31} are considered as a hallmark indicators of LVH development and cardiac remodeling. Further, the histopathology results also confirmed the induction of cardiac hypertrophy in the Ang II infused rat hearts. Chronic infusion of Ang II has been shown to promote the development of cardiac hypertrophy and fibrosis in animal models\textsuperscript{32}. It is well known that physiological effects of Ang II are mediated through AT1R\textsuperscript{33}. Furthermore, studies have shown that treatments with ACE inhibitors or AT1R blocker effectively ameliorate myocardial remodeling in experimental animals\textsuperscript{34,35}. In present study, losartan treated (group III) rats showed significantly decreased expression of hypertrophic marker genes along with reduced LVH/BW ratio as compared with that of Ang II-infused rats. Based on these results, it is tempting to conclude that Ang II mediated down regulation of NPR-A could be a one of the mechanisms of the decreased responsiveness of the ANP/NPR-A system in cardiomyopathic diseased conditions.

In conclusion, the results of this study suggests that Ang II down regulates cardiac NPR-A receptor activity by suppressing \textit{Npr1} gene transcription, and this could be a reason for the impaired local cardiac anti-hypertrophic activity of ANP in diseased state.

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