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**Cardiac BNP gene
activation by angiotensin II *in vivo***

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Abstract - The transcription factors involved in the activation of cardiac gene expression by angiotensin II (Ang II) *in vivo* are not well understood. Here we studied the contribution of transcriptional elements to the activation of the cardiac B-type natriuretic peptide (BNP) gene promoter by Ang II in conscious rats and in angiotensin II type 1 receptor (AT1R) transgenic mice. Rat BNP luciferase reporter gene constructs were injected into the left ventricular wall. The mean luciferase activity was 1.8-fold higher ($P<0.05$) in the ventricles of animals subjected to 2-week Ang II infusion as compared with vehicle infusion. Our results indicate that GATA binding sites at -90 and -81 in the rat BNP promoter are essential for the *in vivo* response to Ang II. The GATA factor binding to these sites is GATA-4. BNP mRNA levels and GATA-4 binding activity are also increased in the hypertrophied hearts of aged AT1R transgenic mice.

Cardiac hypertrophy is the primary adaptive mechanism for terminally differentiated cardiac myocytes to increased hemodynamic load (Lorell and Carabello, 2000). In addition to mechanical load, a number of neurohumoral and paracrine factors, such as angiotensin II (Ang II) and endothelin-1 (ET-1) participate in the adaptive response of cardiac myocytes. Ang II has direct growth-promoting effects on the cardiac, vascular and glomerular cells, in part via induction of the expression of various growth factors, extracellular matrix proteins and cytokines (Dostal and Baker, 1999; Kim and Iwao, 2000). There are two distinct G-protein-coupled receptor (GPCR) subtypes for Ang II, the AT₁ and AT₂ receptors, both of which are widely expressed in a variety of tissues including myocardium (Kim and Iwao 2000). Ang II activates, mainly via the AT₁ receptor, a large array of signal transduction cascades, including mitogen-activated protein (MAP) kinases and janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways that participate in the regulation of gene expression (Blume et al., 1999; Sano et al., 2000).

At the genetic level, initiation of myocyte hypertrophic growth is accompanied by a rapid and transient expression of immediate-early genes (*c-fos*, *c-jun* and *Egr-1*), followed by an activation of the fetal gene regulatory program with re-expression of genes for contractile proteins and atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) (Sugden and Clerk, 1998). The regulation of gene expression is controlled by interactions of transcription factors, which bind to *cis* regulatory elements of target genes to either activate or repress transcription. *In vitro*, Ang II activates a number of inducible transcription factors such as activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B) and nuclear factor of activated T-cells 3 (NF-AT3), which in turn affect the expression of genes that regulate cell growth and extracellular matrix protein biosynthesis (Molkentin et al., 1998; Purcell et al., 2001; Ruiz-Ortega et al., 2001). AP-1 elements have been reported to be required for *in vivo*

responsiveness of the ANP and AT_{1a} receptor promoters to pressure overload (Cornelius et al., 1997; Herzig et al., 1997; von Harsdorf et al., 1997). Additionally, the AT_{1a} receptor and the β -myosin heavy chain promoters contain GATA elements also required for the pressure overload-induced gene expression (Hasegawa et al., 1997; Herzig et al., 1997). Proximal GATA motifs are also sufficient to confer transcriptional activation of the BNP gene in response to acute nephrectomy-induced hemodynamic stress (Marttila et al., 2001). Moreover, the GATA pathway is involved in the BNP gene activation following *in vitro* mechanical stretching and in response to ET-1 treatment of cultured cardiac myocytes (Morin et al., 2001; Pikkarainen et al., 2003a). Finally, Ets-like gene-1 transcription factor (Elk-1) was recently reported to participate in ET-1 but not Ang II or mechanical stretch-induced activation of BNP transcription *in vitro* (Pikkarainen et al., 2003b). However, whether these or other transcription factors mediate Ang II-induced activation of cardiac gene expression *in vivo* is not known.

The aim of the present study was to identify the transcription pathways including promoter element(s) and their cognate binding proteins responsible for the activation of the cardiac BNP gene in response to Ang II *in vivo*. To examine which DNA sequences mediate the increase in BNP transcription in response to Ang II, BNP gene constructs were injected directly into the left ventricle of rats (Kass-Eisler and Leinwand, 1997; Marttila et al., 2001). The binding of the GATA, AP-1 and Ets factors to their sites in the BNP promoter was studied by gel mobility shift assays. BNP and GATA-4 levels were quantified in the ventricles of Ang II type 1 receptor transgenic (AT1R) mice which develop progressive cardiac hypertrophy in the absence of any blood pressure changes (Paradis et al., 2000). Together, the results point to an essential role for GATA-4 in mediating the *in vivo* genetic changes induced by Ang II in the heart.

MATERIALS AND METHODS

Animals. Male 2-month-old Sprague-Dawley rats (270-300 g), obtained from the Center for Experimental Animals at the University of Oulu, Finland, were used for Ang II infusion experiments. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu, Finland. The Ang II type 1 receptor (AT1R) transgenic (Tg) line 27 and their littermate wild type (Wt) used in this study were described previously (Paradis et al., 2000). These experiments were approved by the institutional animal ethical committee of ICRM, Montreal, Canada.

BNP luciferase gene constructs. Rat BNP luciferase reporter gene construct -534rBNPluc was prepared as previously described (Pikkarainen et al., 2002). Site-directed mutations (Stratagene) were introduced to two adjacent (-91 bp and -80 bp) GATA sites in -534rBNPluc (-534GATAmut), to an AP-1 site at -372 (-534AP-1mut) and to an Ets binding site at -498 (-534Etsmut) as previously described (Pikkarainen et al., 2003b). BNP minimal promoter constructs containing one or three copies of a GATA element have been previously described (Morin et al., 2000). The expression vector for rat AT1aR was a gift from Dr. Sadashiva S. Karnik (Miura et al., 2000).

In vivo injections and reporter gene assays. Rats were anesthetized with an intraperitoneal injection of 250 µg/kg (1.06 µmol/kg) medetomidine hydrochloride and 50 mg/kg (182 µmol/kg) ketamine hydrochloride. Plasmids (in 100 µl of 0.9 % NaCl) were injected directly into the left ventricular free wall close to the apex (Kass-Eisler and Leinwand, 1997; Marttila et al., 2001). The plasmid DNA consisted of 50 µg of construct DNA as well as 100 µg of β-galactosidase expression vector (pSVβ-gal, Promega Co) (Marttila et al., 2001). Ang II 33.3 µ

g/kg/h (31.5 nmol/kg/h) or 0.9 % NaCl as vehicle were administered by SC minipumps for 6 hours or 1 or 2 weeks in conscious rats as previously described (Suo et al., 2002). The administration of Ang II was started 1 week after the plasmid injections in the 6 hour experiments, and simultaneously with the injections in the 1 and 2 week experiments. In a separate series of experiments, Ang II 33.3 µg/kg/h (31.5 nmol/kg/h) was administered together with AT₁ receptor antagonist losartan 417 ng/kg/h (0.99 nmol/kg/h) by SC minipumps for 2 weeks. Reporter gene activities of the left ventricles were measured by using luciferase and β-galactosidase assays with luminometer (Luminoskan RS, Labsystems, Helsinki, Finland). Luciferase values were divided by β-galactosidase values to correct for variation in transfection efficiency (Marttila et al., 2001).

Hemodynamic measurements. For telemetric monitoring of blood pressure, rats were instrumented with a catheter in the descending aorta and coupled with a sensor and transmitter (TA11PA-C40; Data Sciences). Mean arterial pressure (MAP) and heart rate were measured every 10 minutes and averaged every hour (Suo et al., 2002).

Tissue Weights. Age-matched Wt and Tg mice were anesthetized, their body weights were determined, the heart and femur were isolated and tissue weights and femur length were measured as previously described (Paradis et al., 2000). The rat hearts were further dissected and the ventricles were frozen in liquid nitrogen and stored at -80°C until used.

Assays of BNP mRNA and peptide. RNA was isolated from left ventricles by the guanidine isothiocyanate-CsCl method (Chirgwin et al., 1979) or using TRIZOL reagent (Invitrogen, Burlington ON, Canada). Twenty µg of rat total RNA were used for Northern blot analyses. The membranes were hybridized using rat BNP cDNA (390 bp), GATA-4 (1417 bp), and

GATA-6 (1175 bp) probes labeled with ^{32}P -dCTP and a cDNA probe complementary to rat 18S ribosomal RNA (482 bp) (Magga et al., 1997; Hautala et al., 2001). The BNP radioimmunoassay was performed as previously described (Hautala et al., 2001). The mRNA levels of BNP, GATA-4 and ribosomal protein S16 in mouse ventricles were determined by quantitative PCR (QPCR) as previously described (Debrus et al., 2005). The oligonucleotides used for QPCR for BNP were CAGCTCTTGAAGGACCAAGG (forward) and AGAGACCCAGGCAGAGTCAG (reverse), for GATA-4 CAGCAGCAGTGAAGAGATGC (forward) and ATGTCCCCATGACTGTCAGC (reverse) and for ribosomal protein S16 ATCTCAAAGGCCCTGGTAGC (forward) and ACAAAGGTAAACCCCGATCC (reverse).

Nuclear protein extractions and gel mobility shift assays. Nuclear protein extractions and gel mobility shift assay method used for the detection of sequence-specific DNA-binding proteins has been described previously (Hautala et al., 2001). The specificity of the transcription factor binding was confirmed by competition with unlabeled oligonucleotides with intact or mutated binding sites as well as by supershift experiments using specific antibodies (Santa Cruz Biotechnology) against GATA-4 (cat #sc-1237-X), GATA-5 (cat #sc-7280-X), GATA-6 (cat #sc-7244-X), the members of activator protein-1 (AP-1) complex (c-Fos (cat #sc-52-X), c-Jun (cat #sc-45-X), Jun B (cat #sc-46-X), Jun D (cat #sc-74-X)), Ets-like gene -1 (p-Elk-1) (cat #sc-840-X) and Oct-1 (cat sc#-25399).

Cell cultures and transient transfections. Myoblast C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS). Transfections and luciferase assays were carried out as previously described (Morin et al., 2001). At 16 h post-transfections, the medium was changed to serum free and supplemented with 100.5 $\mu\text{g/l}$ (100 nmol/l) Ang II for 16 h.

Statistical analysis. The results are expressed as mean $\pm$ SEM. Student t-test or Mann-Whitney's U-test were used for comparison between two groups. Hemodynamic variables were analyzed with two-way repeated-measures analysis of variance (ANOVA), while ventricular weight (VW), body weight (BW) and VW/BW ratio were analyzed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Rat hemodynamics and left ventricular hypertrophy. Ang II administration for 6 hours increased MAP by 29 % which persisted throughout the 2-week infusion period (Table 1). The heart rate was not changed initially, but at 2 weeks, it was higher in the Ang II-infused than in the vehicle-infused rats. In agreement with the effects of Ang II on food intake and energy expenditure (Brink et al., 2001; Cassis et al., 2002), Ang II infusion for 2 weeks decreased body weight. The index of left ventricular hypertrophy, VW/BW ratio, was significantly increased at 2 weeks in the rats treated with Ang II (Table 1). The hemodynamics and the level of left ventricular hypertrophy were similar in 1-week (data not shown) and 2-week infusions of Ang II.

Rat ventricular BNP, GATA-4 and GATA-6 mRNA and ir-BNP levels during Ang II infusion. To examine the changes in BNP gene expression during Ang II infusion, we measured the levels of left ventricular BNP mRNA and BNP immunoreactivity. The left ventricular BNP mRNA levels were 4.5-fold higher at 6 hours and 1.8-fold at 2 weeks in the Ang II-infused rats compared to vehicle-infused animals (Figures 1A and 1B). The ir-BNP levels in the left ventricles increased 3.5-fold at both 6 hours and 2 weeks in response to Ang II infusion (Figure 1C). We also examined the left ventricular GATA-4 and GATA-6 mRNA levels, but there were not seen any changes during Ang II infusion (Figure 1A).

Activation of rat BNP promoter in response to Ang II infusion in vivo. Previous *in vitro* studies have shown that the proximal 5'-flanking region of 534 bp is sufficient to confer inducibility of the rat BNP promoter by Ang II, ET-1 and mechanical stretching in cultured neonatal ventricular myocytes (Morin et al., 2001; Tokola et al., 2001; Pikkarainen et al.,

2002; Pikkarainen et al., 2003a; Pikkarainen et al., 2003b). To determine whether Ang II infusion *in vivo* stimulates the transcription of BNP gene, we injected a plasmid construct containing 534 bp of the BNP gene 5' regulatory region linked in front of the luciferase reporter gene (Figure 2A) directly into the left ventricular wall and the rats were infused with vehicle or Ang II for 6 hours and 1 or 2 weeks. Ang II infusion for 6 hours did not activate the rat BNP promoter 534 bp fragment while the one- and two week infusions with Ang II resulted in a 2.0-fold and 1.8-fold higher ventricular luciferase activity, respectively, compared to infusion with vehicle (Figure 2B). These results suggest that the acute activation of BNP by Ang II occurs through stabilization of the mRNA as previously suggested (Suo et al., 2002) while chronic Ang II effect reflects increased BNP transcription.

GATA motif is essential for Ang II-responsiveness of the rat BNP promoter. To identify which *cis*-acting elements are involved *in vivo* in the activation of the rat BNP gene by Ang II, point mutations were introduced to the proximal GATA (-534GATAmut), AP-1 (-534AP-1mut) and Ets (-534Etsmut) binding sites (Figure 2A), and the responses to the 2-week Ang II infusions were evaluated. Since the presence of the -90 or -81 GATA site is sufficient to maintain the basal BNP promoter activity (Thuerlauf et al., 1994), both of the sites were mutated. The basal activities of the GATA and AP-1 mutant constructs were 30 % of the intact -534rBNPluc (GATA: 0.304 ± 0.095 vs. 1, $n=24$; AP-1: 0.302 ± 0.049 vs. 1, $n=29$), in agreement with previous *in vitro* (Grepin et al., 1994; Pikkarainen et al., 2002) and *in vivo* (Marttila et al., 2001) studies. The mutation of the Ets-binding site did not influence on basal transcriptional activity (1.187 ± 0.118 vs. 1, $n=16$). Mutations of the GATA sites resulted in a complete loss of inducible expression, while the mutation of the AP-1 or the Ets element at -498 did not have any significant effect (Figure 2C). The data indicate that the GATA binding sites are required for Ang II induced BNP promoter activity while the AP-1 and Ets binding

sites are nonessential.

Ang II-induced rat BNP promoter activity is inhibited by AT₁ receptor blockade. To determine whether the activation of rat BNP promoter is mediated by AT₁ receptors, rats were infused with Ang II in the presence or absence of the AT₁ receptor antagonist, losartan. The simultaneous infusion of losartan with the Ang II blocked the activation of the rat BNP promoter at 2 weeks (Figure 2D), suggesting that Ang II-induced *in vivo* effects on BNP transcriptional activity are mediated by AT₁ receptors. We have previously reported that AT₁ receptor blockade by losartan also abolishes Ang II-induced increase in the left ventricular BNP gene expression (Lako-Futo et al., 2003).

Transcription factor binding activity and mRNA levels in the rat cardiac ventricles. The binding of GATA factors, AP-1 and Ets-1 to the BNP promoter were studied by performing gel mobility shift assays. BNP GATA but not Oct-1 binding activity was significantly increased during Ang II infusion at 6 hours (Figures 3A and 3B), indicating a specific activation of GATA binding activity. Supershift analysis of the GATA binding showed antibody-induced supershifts of GATA-4 but not of GATA-5 or GATA-6 complexes (Figure 3A). The AP-1 binding was also significantly increased at 6 hours (Figures 4A and 4B), whereas the Ets factor binding was not influenced by the Ang II treatment (Figures 5A and 5B). The GATA-4 or the AP-1 binding activities were not increased anymore at 2 weeks. To study the effect of Ang II on transcript levels, we performed Northern blot analysis (Figure 1A). The levels of GATA-4 and GATA-6 mRNA remained unchanged in response to 6 hours and 2 weeks of Ang II infusions, suggesting that increased GATA-4 binding activity is due to posttranscriptional regulation.

Ang II transactivates BNP directly through cardiac AT₁ receptors independently of hypertension. In order to further determine whether the long term action of Ang II reflects Ang II-induced changes in blood pressure or is the consequence of Ang II-induced hypertrophy, BNP mRNA levels were examined in the heart of AT1R transgenic mice which overexpress the human AT₁ receptor specifically in the heart (Paradis et al., 2000). These animals develop cardiac hypertrophy with aging in the absence of any change in blood pressure. Furthermore, BNP increased in the ventricles of AT1R mice independently of cardiac hypertrophy. BNP mRNA levels increased significantly in the ventricles of younger mice without cardiac hypertrophy and of older AT1R transgenic mice with cardiac hypertrophy and tended to increase in younger mice without cardiac hypertrophy (Fig. 6A and B). Although the body weight decreased during Ang II infusion in rats (Table 1), there was no significant difference between the AT1R transgenic and littermate Wt mice (Fig. 6C). Interestingly, and consistent with the results obtained in rats infused with Ang II, the activation of BNP in AT1R mouse ventricles was associated with an increase in GATA-4 binding activity (Fig. 6D) in the absence of any changes at the mRNA level (Fig. 6E). Thus, in this model, BNP levels and GATA-4 activity correlated with Ang II-dependent hypertrophy. Finally, to determine whether Ang II acts directly at the level of GATA-4 to induce its activity, GATA dependent heterologous promoter constructs were tested for their response to Ang II. As shown in Fig. 6F, these reporters were activated in a GATA-dependent fashion by coexpressing AT1aR and Ang II stimulation in C2C12 cells.

DISCUSSION

The mechanisms regulating the genetic reprogramming in cardiac overload have been the subject of intensive research. In addition to mechanical stretch, a number of neurohumoral factors, such as G-protein-coupled receptor agonists Ang II, ET-1 and norepinephrine participate in the adaptive process and modify cardiac gene expression, myocyte growth and heart function (Kim and Iwao, 2000; Olson and Schneider, 2003). In the present study, to identify promoter elements necessary for the activation of the BNP gene, a hallmark of the genetic changes in diseased hearts (Tokola et al., 2001), the effect of mutations in various BNP promoter elements on its activation by Ang II was tested in rat heart *in vivo*. We show here for the first time that the well conserved proximal GATA element of the rat BNP promoter mediates the *in vivo* transcriptional response to Ang II. We also provide evidence that Elk-1 and AP-1 elements are not essential for the adaptive response of the BNP gene to Ang II in conscious rats. These results were extended by studies of BNP activation by Ang II in the cardiac ventricles of AT1R transgenic mice with cardiac hypertrophy and without hypertension and by *in vitro* analysis of direct effect of AT1R on GATA dependent transcription and GATA-4 activity. Together, the results support a prominent role for GATA-4 in the *in vivo* Ang II-mediated regulation of transcription.

In the heart, the nuclear targets mediating Ang II-induced activation of gene expression *in vivo* are not well understood. Cardiac AP-1 DNA binding activity has been reported to increase in response to Ang II infusion in conscious rats (Yano et al., 1998; Suo et al., 2002), as well as in hypertensive rats double transgenic for the human renin and angiotensinogen genes (Muller et al., 2000; Fiebeler et al., 2001). The increase in left ventricular AP-1 DNA binding activity can be prevented by AT₁ receptor antagonism in stroke-prone spontaneously

hypertensive rats (Izumi et al., 2000). In addition, Ang II has been suggested to be required for the activation of AP-1 as well as for GATA BNP binding since an AT₁ receptor antagonist losartan inhibited these DNA binding activities in response to direct left ventricular wall stretch in isolated perfused rat hearts (Hautala et al., 2002). Previously, site-specific mutation of the ANP AP-1-like element has been shown to eliminate the response of the promoter to aortic banding-induced pressure overload in dogs (von Harsdorf et al., 1997). The AP-1 site of the ANP promoter has also been reported to be an important factor in response to acute elevation of cardiac wall stress (Cornelius et al., 1997). In this study, mutation of the AP-1 element at -372 bp significantly decreased the basal activity of the rat BNP promoter, but it did not affect the BNP gene activation in response to Ang II infusion. Interestingly, the rat BNP promoter does not contain binding sites for NF- κ B (Tokola et al., 2001), which functions in concert with AP-1 (Barnes and Karin, 1997), and participates in the activation of human BNP promoter by mechanical stretch and ET-1 in cardiac myocytes *in vitro* (Liang et al., 2000).

We have recently identified a novel Ets binding sequence in the rat BNP gene proximal region on which nuclear protein binding is activated in ET-1-treated cultured cardiac myocytes (Pikkarainen et al., 2003b). This sequence binds Ets-like gene-1 transcription factor (Elk-1) and mediates ET-1-specific activation of transcription but does not contribute to Ang II- or mechanical stretch-induced promoter activity *in vitro* (Pikkarainen et al., 2003a,b). In agreement with these findings, our present study *in vivo* demonstrates that the binding affinity of the Ets factors to the BNP promoter did not change nor did the mutation of the Ets have any effect on the activation of the promoter in response to Ang II-induced hypertension. Despite these results, a role for these in BNP gene regulation that would be independent of their binding to DNA cannot be excluded. Indeed, several transcription factors have been

shown to have dual mechanisms of action either directly through DNA binding or through protein-protein interactions (discussed below).

GATA factors, especially GATA-4, control the tissue-specific expression of many cardiac genes, e.g. β -myosin heavy chain and troponin I, in addition to ANP and BNP genes (Murphy et al., 1997; Grepin et al., 1994; Thuerlauf et al., 1994; Hasegawa et al., 1997). GATA-4 is one of the earliest transcription factors expressed in developing murine cardiac cells (Heikinheimo et al., 1994). It continues to be expressed in adult cardiac myocytes and also in gonads, lung, liver, and small intestine (Arceci et al., 1993). GATA-4 knock-out mice die during embryonic development due to failure of ventral morphogenesis and heart tube formation (Kuo et al., 1997; Molkenstein et al., 1997). In the present study, mutation of the GATA elements located at -90 and -81 bp in the -534rBNP promoter completely abolished the activation of the promoter by Ang II. We also studied the binding of the three cardiac GATA factors to these sites in the BNP promoter by gel mobility shift assays. Supershifts showed that GATA-4 is the predominant GATA protein bound to the -90 and -81 bp sites in the BNP promoter. Our results also demonstrate that GATA-4 binding activity was increased in response to 6 hours but not 2 weeks of Ang II infusion. Thus, it seems that the binding activity does not need to be increased for GATA-4 activation. Indeed, the mechanisms of GATA-4 activation are complex and may involve post-translational modifications. It has been reported that phosphorylation can enhance both DNA binding (Wang et al., 2005) and transcriptional activity (Charron et al., 2001). Recruitment of co-factors, such as Fos, Jun, STATs, is another potential mechanism of GATA-4 activation (Pikkarainen et al., 2004; Peterkin et al., 2005; Tamsah and Nemer, 2005). The interaction of GATA-4 with members of the AP-1 family may be especially relevant to Ang II response. We previously showed that GATA-4 is able to recruit c-Fos to target promoters such as BNP (McBride et al., 2003). Thus, c-Fos and possibly other

AP-1 members, may act as Ang II-induced co-activator(s) of GATA-4 and may well modulate gene transcription via GATA binding sites. Therefore, our results do not exclude the participation of AP-1 members or Elk-1 in cardiac BNP gene activation *in vivo*, although mutations of AP-1 or Ets binding sites had no effect on the activation of the promoter in response to Ang II-induced hypertension. GATA-4 and GATA-6 mRNA levels in the left ventricles did not differ between the vehicle and Ang II groups, showing that the enhanced phosphorylation and the acute increase in DNA binding affinity of GATA-4 rather than its increased expression or synthesis result in the activation of BNP promoter.

In summary, our results show that GATA4 but not Elk-1 nor AP-1 binding sites are essential for BNP gene activation *in vivo* in response to Ang II-induced hypertension. Taken together with previous studies, GATA-4 appears to be essential for tethering other transcriptional activators to target promoters thus guiding the cardiac muscle cell-specific gene expression not only during basal conditions but also during pressure overload caused by Ang II.

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FIGURE LEGENDS

Figure 1. Acute and chronic Ang II treatments increase BNP gene expression at both mRNA and protein levels in the left ventricle. A. A representative Northern blot is showing changes of BNP, GATA-4 and GATA-6 mRNA levels in the left ventricle of rats treated with Ang II for 6 h and 2 weeks (w) by comparison to vehicle (Veh) treated rats. 18S, 18S ribosomal RNA, $n=10$ for each group, number of experiments=2. B. Left ventricular BNP mRNA levels expressed as the ratio of BNP to 18S ratio, $n = 10$ for each group, number of experiments=2. Results are expressed as mean $\pm$ SEM. $*P<0.05$, $***P<0.001$ Ang II vs. vehicle. C. Immunoreactive BNP (ir-BNP) concentrations were determined by radioimmunoassay in the left ventricle of rats treated with vehicle or Ang II for 6 h and 2 weeks, $n = 10$ in each group, number of experiments=2. Results are expressed as mean $\pm$ SEM. $*P<0.05$, $***P<0.001$ Ang II vs. vehicle.

Figure 2. Chronic but not acute treatment with Ang II increases the BNP promoter activity through a GATA element. A. Rat -534BNP luciferase constructs are presented. Site-directed mutations of the two GATA motifs and AP-1 and EBS elements are indicated by mut. B. The -534rBNP promoter activity (luciferase/ β -gal) was determined in the left ventricle of rats treated with vehicle or Ang II for 6 h, 1 weeks or 2 weeks, $n = 13-23$ per group, number of experiments=3-6. Open bars indicate vehicle and solid bars Ang II. Results are expressed as mean $\pm$ SEM. $*P<0.05$, $**P<0.01$ Ang II vs. vehicle. C. The -534rBNP and mutated promoter activities (luciferase/ β -gal) in the left ventricle of rats treated with vehicle or Ang II for 2 weeks are shown, $n = 13-29$ in each group, number of experiments=3-6. Open bars indicate vehicle and solid bars Ang II. Results are expressed as mean $\pm$ SEM. $*P<0.05$, $**P<0.01$ Ang II vs. vehicle. D. Ang II transactivation of BNP is blocked by AT₁ receptor

antagonist. The -534rBNP promoter activities (luciferase/ β -gal) in the left ventricle of rats treated for 2 weeks with vehicle, losartan, Ang II or both are presented. Results are expressed as mean $\pm$ SEM, $n = 6$ for each group, number of experiments=2.

Figure 3. Ang II increases the GATA-4 DNA binding activity. A. Gel mobility shift assay were performed with left ventricular nuclear extracts from rats treated with vehicle or Ang II for 6 h using a BNP GATA probe. Binding to the BNP GATA probe was competed with 50 fold molar excess of cold BNP GATA (lane 3), non-related DNA Oct-1 (lane 4), BNP/mutGATA (lane 5) or GATA consensus (lane 6) probes. The specificity of the GATA binding was determined by supershift assays by incubating the left ventricular nuclear extracts from Ang II-infused hearts of lane 7 with anti-GATA-4 (4, lane 8), anti-GATA-5 (5, lane 9) or anti-GATA-6 (6, lane 10) antibodies, $n=7$ per group, number of experiments=2. B. BNP GATA DNA binding activities in response to Ang II infusion at 6 h and 2 weeks. Results are expressed as mean $\pm$ SEM, $n = 7$ for each group, number of experiments=2. * $P<0.05$ Ang II vs. vehicle. The original gel shifts are not shown for 2-week experiments.

Figure 4. Ang II increases the AP-1 DNA binding activity. A. Gel mobility shift assay were performed with left ventricular nuclear extracts from rats treated with vehicle or Ang II for 6 h using a BNP AP-1 probe. Binding to the BNP AP-1 probe was competed with 50 fold molar excess of cold BNP AP-1 (lane 4), BNP/mut AP-1 (lane 5) or non-related DNA Oct-1 (lane 6) probes. The specificity of the AP-1 binding was determined by supershift assays by incubating the left ventricular nuclear extracts from Ang II-infused hearts with anti-c-Fos (lane 7), anti-c-Jun (lane 8), anti-JunB (lane 9), anti-JunD (lane 10) or anti-Oct 1 (lane 11) antibodies, $n=7$ per group, number of experiments=2. B. BNP AP-1 DNA binding activities in response to Ang II infusion at 6 h and 2 weeks. Results are expressed as mean $\pm$ SEM, $n = 7$

for each group, number of experiments=2, * $P<0.05$ Ang II vs. vehicle. The original gel shifts are not shown for 2-week experiments.

Figure 5. Ets DNA binding activity is not altered by Ang II treatment. A. Gel mobility shift assay were performed with left ventricular nuclear extracts from rats treated with vehicle or Ang II for 6 h using a BNP Ets probe. Binding to the BNP Ets probe was competed with 50 fold molar excess of cold BNP Ets (lane 4), BNP/mut Ets (lane 5) or non-related DNA Oct-1 (lane 6) probes. The specificity of the Ets binding was determined by supershift assays by incubating the left ventricular nuclear extracts from Ang II-infused hearts with anti-Ets (lane 6) or anti-Oct 1 (lane 7) antibodies, $n=7$ per group, number of experiments=2. B. BNP Ets DNA binding activities in response to Ang II infusion at 6 h and 2 weeks. Results are expressed as mean $\pm$ SEM, $n = 7$ for each group, number of experiments=2. The original gel shifts are not shown for 2-week experiments.

Figure 6. BNP is transactivated directly by Ang II through cardiac AT1R. BNP is increased significantly in the ventricles of AT1R transgenic (TG) mice independently of cardiac hypertrophy. A. The expression level of BNP was quantified in the ventricles of young (75 days old) and older (115 days old) AT1R transgenic and littermate wild type (Wt) mice by QPCR. B and C. The heart weight to femur length ratio (HW/FL) and the body weights were determined as described in Materials and Methods section. The results shown in A, B and C represent the mean $\pm$ SEM with $n = 9, 13, 14$ and 8 for young Wt, young AT1R, old Wt and old AT1R mice, respectively, * $P<0.01$ vs Wt. D. GATA-4 binding is increased in the ventricles of old AT1R mice with cardiac hypertrophy. Electrophoretic mobility shift assays (EMSA) was performed using nuclear extracts of ventricles of old AT1R transgenic and littermate Wt mice with the distal GATA containing atrial natriuretic peptide

(ANP) probe. GATA-4 antibody (aGATA-4) was used to confirm the identity of the DNA-binding complex in the ventricles of mice. GATA binding is indicated by an arrow head and the supershift by *. The EMSA experiment was performed twice with identical results. **E.** The mRNA expression level of GATA-4 is not increased in the ventricles of old AT1R mice with cardiac hypertrophy. The expression level of GATA-4 was quantified in the ventricles of old AT1R transgenic and littermate Wt mice by QPCR. The results shown represent the mean $\pm$ SEM, $n = 7$ in each group **F.** Ang II increases the activity of a BNP minimal promoter construct in C2C12 cells in a GATA dependent fashion. C2C12 cells were cotransfected with a BNP minimal promoter construct containing one or three copies of a GATA element with the AT1aR expression vector and then treated with or without 100.5 $\mu\text{g/l}$ (100 nmol/l) Ang II for 12 h. The results are shown as the mean $\pm$ SEM of at least six independent experiments and represent the fold activation of each reporter activity by Ang II in C2C12 cells.

Table 1. Effects of Ang II infusion on body and left ventricular weights and hemodynamics.

Parameter	Group	6 hours	2 weeks
Left ventricular weight (mg)	Vehicle	999 ± 8	1129 ± 8
	Ang II	993 ± 8	1193 ± 12***
Body weight (g)	Vehicle	297 ± 2	349 ± 3
	Ang II	287 ± 2	269 ± 4***
LVW/BW (mg/g)	Vehicle	3.36 ± 0.02	3.25 ± 0.03
	Ang II	3.46 ± 0.02	4.49 ± 0.04***
MAP (mmHg)	Vehicle	108 ± 3	107 ± 8
	Ang II	139 ± 1*	181 ± 5***
Heart rate (beats/min)	Vehicle	382 ± 5	355 ± 6
	Ang II	374 ± 7	417 ± 4***

Results are shown as mean ± SEM. For the mean arterial pressure (MAP) and heart rate measurements, $n = 10$ for both Ang II 33.3 µg/kg/h (31.5 nmol/kg/h) and vehicle-treated groups. For the left ventricular weight (LVW), body weight (BW) and LVW/BW ratio, $n = 70$ at 6 hours and $n = 114$ at 2 weeks for Ang II- and vehicle-treated groups. * $P < 0.05$, *** $P < 0.001$ Ang II vs. vehicle.

A

Lane	1	2	3	4	5	6	7	8	9	10
Ang II	-	+	+	+	+	+	+	+	+	+
Competitor	-	-	+	+	+	+	-	-	-	-
GATA Ab	-	-	-	-	-	-	-	4	5	6

Supershift →

GATA →

B