Original Article

AngiotensinII-Induced Ventricular Hypertrophy and Extracellular Signal-Regulated Kinase Activation Are Suppressed in Mice Overexpressing Brain Natriuretic Peptide in Circulation

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Atrial and brain (B-type) natriuretic peptides (ANP and BNP, respectively) are known to exert various cardio-protective effects. For instance, knocking out the expression of ANP, BNP, or their receptor, guanylyl cyclase-A, induces cardiac hypertrophy and/or fibrosis. The cardiac effects of elevated circulating natriuretic peptides are less well understood, however. We therefore compared angiotensin (Ang) II-induced cardiac hypertrophy and fibrosis in BNP-transgenic (Tg) mice, in which circulating BNP levels were elevated by increased secretion from the liver, and their non-Tg littermates. Left ventricular expression of Ang II type 1a receptor was similar in BNP-Tg and non-Tg mice, and there was no significant difference in the elevation of blood pressure elicited by chronic infusion or acute injection of Ang II. Nevertheless, cardiac hypertrophy and fibrosis were significantly diminished in BNP-Tg mice chronically infused with Ang II. In addition, ventricular activation of extracellular signal-regulated kinase (ERK) induced by acute injection of Ang II was also diminished in BNP-Tg mice, as was activation of ERK kinase (MEK). Conversely, expression of mitogen-activated protein kinase phosphatase (MKP) was significantly increased in the ventricles of BNP-Tg mice. Based on these findings, we conclude that elevated circulating BNP exerts cardioprotective effects via inhibition of a ventricular ERK pathway. The mechanism responsible for this inhibition likely involves 1) increased ventricular MKP expression and 2) inhibition of transduction mediators situated upstream of ERK. (Hypertens Res 2003; 26: 847–853)

Key Words: natriuretic peptide, cardiac hypertrophy, extracellular signal-regulated kinase, angiotensin II, transgenic mice

Introduction

For the past several years, atrial and brain (B-type) natriuretic peptide (ANP and BNP, respectively) have been used in the treatment of congestive heart failure (CHF) because their systemic infusion elicits such beneficial hemodynamic changes as arterial and venous dilatation, enhanced sodium
excretion, and suppression of the renin-angiotensin (Ang)-aldosterone and sympathetic nervous systems (1–6). That is to say, natriuretic peptides (NPs) act in the body to counteract the activity of the renin-Ang system. In that regard, the well documented (7) benefits of angiotensin converting enzyme (ACE) inhibition in hypertension, after myocardial infarction, and in CHF suggest that NPs might be expected to exert similar cardioprotective effects. Indeed, knocking out ANP, BNP, or their receptor, guanylyl cyclase-A (GC-A; NPR-A), is known to elicit cardiac hypertrophy and/or cardiac fibrosis (8–10). Moreover, we have recently demonstrated and characterized the crosstalk between GC-A signaling and Ang II type 1 (AT1) receptor signaling (11). By contrast, neither the direct cardiac effects of elevated circulating NPs nor the molecular mechanism of the crosstalk between the NP system and Ang II system are well understood. In the present study, therefore, we used BNP-transgenic (Tg) mice, which overexpress BNP in the liver and maintain a high level of circulating BNP, to examine the effects of elevated circulating BNP on Ang II-induced cardiac hypertrophy and fibrosis. Upon confirming that increased BNP expression has cardioprotective effects, we investigated as a possible mechanism the effects of BNP on the signaling pathway via an extracellular signal-regulated kinase (ERK) that is activated by Ang II.

Methods

Animals

Generation of BNP-Tg mice under the control of the human serum amyloid P component promoter, which is active only in the liver after birth, was performed as described previously (12). Tg mice overexpressing BNP may exhibit skeletal abnormalities of variable severity, depending on the plasma BNP concentration (13), and thoracocyrtosis resulting from enlargement of the vertebral bodies can affect cardiovascular function. In this study, therefore, we used a BNP-Tg line carrying about 20 copies of the transgene but exhibiting no apparent thoracocyrtosis. Their responses were compared to those of their non-Tg (control) littermates. All mice were 10 weeks old and male. BNP-Tg mice exhibited elevated circulating BNP levels due to BNP secretion from the liver: at 10 weeks of age, plasma BNP concentrations were about 2 pmol/ml in the BNP-Tg mice and < 0.16 pmol/ml in the non-Tg control mice. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Materials

Synthetic Ang II was purchased from Sigma (St. Louis, USA). Anti-Ang II type-1a receptor (AT1a) antibody was a gift from Dr. Hiromi Rakugi, Osaka University Medical School, Osaka, Japan. Anti-p44/42 mitogen-activated protein kinase (MAPK, ERK1/2), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-MAPK kinase (MEK1/2), and anti-phospho-MEK1/2 (Ser217/221) antibodies were from Cell Signaling Technology (Beverly, USA); anti-MAPK phosphatase (MKP)-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA); and anti-MKP-2 antibody was from Transduction Laboratories (Lexington, USA).

Chronic Ang II Infusion

Ang II (dissolved in 0.01 mol/l acetic acid) was subcutaneously infused at the rate of 0.6 mg/kg/day for 2 weeks using an osmotic minipump (Alzet model 2002; Alza Corp., Mountain View, USA) implanted in each mouse. After 1 week of Ang II infusion, systolic blood pressure (SBP) was measured in conscious mice using a noninvasive computerized tail-cuff method (BP-98A; Softron Corp., Tokyo, Japan).

Determination of Heart Weights and Interstitial Fibrosis

After infusing Ang II for 2 weeks, the weight of the whole heart (HW) was measured, and the ratio of HW to the body weights (HW/BW) was calculated and used as an index of cardiac hypertrophy. The left ventricles were then fixed in 10% formaldehyde and prepared for routine histology. To determine the degree of collagen fiber accumulation, we randomly selected 20 fields in three individual sections and calculated the ratio of the areas of van Gieson-stained interstitial fibrosis to the total left ventricular area using a KS400 image system (Zeiss, Oberkochen, Germany). Perivascular fibrosis was excluded in the present study.

Acute Ang II Injection

Mice were anesthesized by i.p. injection of sodium pentobarbital (75 mg/kg), after which polyethylene tubing (PE 10) was inserted into the right carotid artery and the left jugular vein. Arterial blood pressure was recorded continuously from the carotid artery using a polygraph system (Fukuda Denshi, Tokyo, Japan). Once the baseline had stabilized, Ang II (0–3.0 mg/kg) was injected over a period of 1 min via the jugular vein.

Protein Extraction and Western Blot Analysis

Frozen ventricles were homogenized on ice in cell lysis buffer (Cell Signaling Technology), and Western blot analysis was performed as previously described (14).

RNA Extraction and Northern Blot Analysis

Total RNA for Northern blots was extracted from frozen ventricles by the acid guanidinium phenol chloroform method. Northern blot analysis for AT1a and c-fos was per-
formed as previously described (15). A murine AT1a probe was prepared as previously reported (16), and a human c-fos genonomic probe was purchased from Takara Shuzo Co. (Kyoto, Japan).

Statistical Analysis

Data are expressed as the mean ± SEM. Analysis of variance (ANOVA) with post hoc Fisher’s tests was used to evaluate differences. Values of p < 0.05 were considered to indicate statistical significance.

Results

Expression of Ventricular AT1a

Before analyzing the responses to Ang II, we compared the basal expression of AT1a in the ventricles of BNP-Tg and non-Tg mice and found no significant difference in the expression of AT1a mRNA (Fig. 1A and B) or protein (Fig. 1A and C).

Effects of Chronic Ang II Infusion on SBP, HW/BW, and Interstitial Fibrosis

SBP was about 5 mmHg lower in conscious BNP-Tg mice than non-Tg mice (92.6 ± 1.0 vs. 97.7 ± 0.8 mmHg, p < 0.01), but chronic Ang II infusion (0.6 mg/kg/day) increased SBP to the same degree (by about 10 mmHg) in both groups (8.3 ± 0.3 and 10.9 ± 0.5 mmHg in BNP-Tg and non-Tg mice, respectively) (Fig. 2A). Among vehicle-treated mice, HW/BW ratios tended to be smaller in BNP-Tg than non-Tg mice, but the difference was not significant (4.04 ± 0.21 vs. 4.32 ± 0.07 mg/g, p = 0.26); there was also no difference in the levels of left ventricular interstitial fibrosis (0.55 ± 0.03 and 0.62 ± 0.03% in BNP-Tg and non-Tg, respectively). Chronic Ang II infusion significantly increased HW/BW ratios (5.13 ± 0.17 mg/g) (Fig. 2B) and interstitial fibrosis (1.41 ± 0.09%) (Fig. 2C–E) in non-Tg mice, but had little effect in BNP-Tg mice (4.07 ± 0.14 mg/g and 0.77 ± 0.05% for HW/BW ratios and interstitial fibrosis, respectively).

Blood Pressure Elevation after Acute Ang II Injection

As shown in Fig. 3A, basal mean blood pressure (BP) measured under anesthesia from the carotid artery was 10–20 mmHg lower in BNP-Tg mice than in non-Tg mice (61.8 ± 3.1 vs. 75.3 ± 2.5 mmHg, p < 0.01). In both groups, BP rapidly increased after acute injection of Ang II (0.3 mg/kg), peaking within about 5 min (after an about 60 mmHg increase), and then declined over a period of about 10 min. The change in mean BP (∆mean BP)—i.e., the difference between the peak and basal mean BPs—also did not significantly differ between the two groups (60.7 ± 2.4 and 63.3 ± 5.1 mmHg in BNP-Tg and non-Tg mice, respectively) (Fig. 3B), nor did the area under the BP curve (over basal BP level, until 15 min after Ang II injection) (1.91 ± 0.16 and...
2.00 ± 0.18 in BNP-Tg and non-Tg mice, respectively) (Fig. 3C).

Fig. 3. Changes in mean blood pressure (BP) evoked by acute injection of Ang II (0.3 mg/kg). A: Representative traces showing arterial BP in an anesthetized BNP-Tg (right) and non-Tg (left) mouse. The arrows indicate the beginning of the Ang II injection. B and C: Changes in mean BP (Δmean BP), measured as the difference between peak and basal mean BP (B), and the areas under the BP curve (over basal BP level, until 15 min after Ang II injection) (C). Values are the means ± SEM (n = 8).

Fig. 4. Time course and dose-dependency of Ang II-induced activation of ventricular ERK in non-Tg mice. Phosphorylation of ERK1/2 was examined by immunoblotting with an anti-phospho-ERK1/2 antibody. A: Representative Western blots of phospho-ERK1/2 in ventricles harvested at the indicated times after injection with Ang II (0.3 mg/kg). B: Representative Western blots of phospho-ERK1/2 in the ventricles harvested 5 min after injection with the indicated dose of Ang II. C and D: Time course (C) and dose-response (D) curves showing relative levels of ventricular ERK1/2 activity. Phospho-ERK1/2 was measured densitometrically from immunoblots like those in panels A and B and normalized to the control level (at 0 min), which was assigned a value of 1. Values are the means ± SEM (n = 5–10); * p < 0.01 vs. control group.

Ventricular ERK Activation in Ang II-Injected Mice

Basal ERK activity did not differ between the ventricles of BNP-Tg and non-Tg mice, and chronic infusion of Ang II had little effect on ventricular ERK activity (data not shown). On the other hand, acute injection of Ang II (0.3 mg/kg) into non-Tg mice induced a significant increase in ERK1/2 activation, as indicated by the enzyme’s phosphorylation. Levels of phospho-ERK1/2 increased rapidly, reaching a peak within 5 min, and then returned to baseline within 30 min (Fig. 4A and C). The effect of Ang II on ERK activation was dose-dependent, with a > 25-fold increase at a dose of 3 mg/kg (Fig. 4B and D). Comparison with BNP-Tg mice showed that overexpression of BNP significantly (33%) diminished Ang II-induced ERK1/2 activation (Fig. 5).

Ventricular c-fos mRNA Expression

Acute injection of Ang II (0.3 mg/kg) into non-Tg mice induced significant increases in ventricular expression of c-fos mRNA that peaked within 30 min (Fig. 6A and B). Comparison with BNP-Tg mice showed that overexpression of BNP significantly (45%) diminished c-fos expression measured 30 min after Ang II injection (Fig. 6C and D).

Ventricular MKP Expression

To investigate the mechanism by which BNP inhibits the ERK-c-fos pathway in vivo, we assessed the expression of MKP-1 and -2 in the ventricles of BNP-Tg and non-Tg mice (Fig. 7). It is notable that the expression of both these enzymes was significantly (27% and 15%, respectively) induced in BNP-Tg mice (Fig. 7B and C).
Finally, we examined the effects of Ang II on the ventricular levels of activated (phospho-) ERK kinase (MEK1/2), the MAPK kinase directly responsible for activation of ERK1/2. We found that in both BNP-Tg and non-Tg mice, acute injection of Ang II (0.3 mg/kg) increased the levels of phospho-MEK1/2 within 5 min (Fig. 8A), but that the effect was significantly (33%) diminished in BNP-Tg mice (Fig. 8B).

Discussion

In the present study, we demonstrate that elevated circulating BNP inhibits the ventricular hypertrophy and fibrosis induced by Ang II in BNP-Tg mice. This finding suggests that NPs may not only improve the hemodynamics of CHF patients, but may also improve the long-term prognosis of CHF patients in the manner of ACE inhibitors through their cardioprotective effects.

Moreover, the data presented in this report show that elevated circulating BNP inhibits Ang II-induced activation of the ERK pathway. To our knowledge, this is the first report of crosstalk between NPs and the ERK pathway in the heart in vivo. A number of studies have demonstrated the key roles played by hypertrophic mediators in the ERK pathway (17–21). But while NPs and GC-A signaling are known to be involved in the regulation of cardiac hypertrophy and fibrosis (8–10), little was known about the in vivo mechanism by which NPs affect cardiac hypertrophy and fibrosis.

BNP-Tg mice exhibited reduced cardiac hypertrophy and fibrosis when chronically infused with Ang II. Although basal BP in BNP-Tg mice was lower than that in non-Tg mice, Ang II increased BP to the same degree in both groups; moreover, ventricular expression of AT1a was the same in BNP-Tg and non-Tg mice. This means that differences in extracellular stimuli—i.e., humoral and mechanical factors—cannot account for the antihypertrophic and antifibrotic effects of BNP.
necrotic effects of BNP. What, then, is the mechanism of the antihypertrophic and antifibrotic actions of BNP?

The MAPK family members ERK, p38 MAPK, and c-Jun N-terminal kinases (JNKs) are known to be very important mediators of cardiac hypertrophy (17–25). In particular, the ERK pathway has been very frequently investigated due to its importance as a hypertrophic mediator. We therefore examined the effects of BNP on Ang II-induced activation of the ERK pathway and found that Ang II-induced ERK activation was significantly diminished in the ventricles of BNP-Tg mice, as was expression of c-fos, one of the immediate early response genes up-regulated in cardiac hypertrophy (26–28). In addition, because ANP was previously shown to induce expression of MKP-1 in glomerular mesangial cells, renal tubular cells, and endothelial cells (29–31), we assessed the ventricular expression of MKP-1 and -2 and found it to be significantly higher in BNP-Tg than in non-Tg mice. With regard to ventricular expression of MKP-1, Hirono et al. reported that Ang II increases MKP-1 gene expression via negative feedback in cultured cardiac myocytes (32). But in our study, there was a difference in the expression of MKPs between BNP-Tg and non-Tg mice before Ang II infusion. This finding indicated that BNP increases ventricular MKP expression independent of any Ang II-related mechanism. MKPs dephosphorylate and thus inactivate ERK (33). In fact, in a previous study, Tg mice overexpressing MKP-1 showed no ERK activation and attenuated cardiac hypertrophy in response to aortic banding and catecholamine infusion (21). We therefore suggest that BNP-induced expression of MKPs in the ventricle reduces ERK activation at the same location, which in turn represses hypertrophy and fibrosis. It should be noted, however, that MKP-1 also inhibits activation of two other MAPKs, p38 MAPK and JNK (21), which might also have contributed to the antihypertrophic and antifibrotic effects of BNP.

Ang II-induced activation of MEK, the MAPK kinase directly responsible for activation of ERK, was also significantly repressed in mice overexpressing BNP. Suhusini et al. reported that in baby hamster kidney (BHK) cells, cGMP-dependent protein kinase inhibits the Ras-MAPK pathway by phosphorylating c-Raf kinase on Ser43, thereby inhibiting its activation (34). Because BNP increases the level of cGMP via GC-A, we suggest that BNP stimulates phosphorylation of c-Raf kinase, which would inhibit its activation and in turn inhibit activation of MEK. It thus appears that BNP exerts its cardioprotective effects largely by acting upstream of ERK, on both MKP and MEK.

We also recently reported that phosphorylation of ERK and immune-mediated renal injury are attenuated in the kidneys of experimental nephritic BNP-Tg mice, and NPs inhibit ERK phosphorylation induced by Ang II in cultured mesangial cells (14). Several lines of evidence have shown that Ang II contributes to the progression of renal injury in experimental glomerulonephritis (GN), as demonstrated by the alleviation of renal injury in GN associated with the inhibition of Ang II generation as well as the pharmacologic blockade or genetic disruption of AT1 (35–37). Taken together, these findings indicate that BNP inhibits the ERK phosphorylation induced by Ang II in the nephritic kidney, and consequently attenuates renal injury in GN. This mechanism of the renoprotective effect of BNP is compatible with the mechanism of the cardioprotective effect of BNP shown in the present study. It seems likely that MKPs are also up-regulated in the kidney of BNP-Tg mice.

In summary, we have shown that Ang II-induced ventricular hypertrophy and fibrosis are diminished in mice overexpressing BNP and that these cardioprotective effects are attributable to the inhibition of ventricular activation of the MEK-ERK transduction pathway. Based on these findings, it is hoped that the cardioprotective efficacy of BNP will be further examined in clinical studies.

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