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Atrial natriuretic peptide inhibits cardiomyocyte hypertrophy through mitogen-activated protein kinase phosphatase-1

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Abstract

Cardiac hypertrophy is formed in response to hemodynamic overload. Although a variety of factors such as catecholamines, angiotensin II (AngII), and endothelin-1 (ET-1) have been reported to induce cardiac hypertrophy, little is known regarding the factors that inhibit the development of cardiac hypertrophy. Production of atrial natriuretic peptide (ANP) is increased in the hypertrophied heart and ANP has recently been reported to inhibit the growth of various cell types. We therefore examined whether ANP inhibits the development of cardiac hypertrophy. Pretreatment of cultured cardiomyocytes with ANP inhibited the AngII- or ET-1-induced increase in the cell size and the protein synthesis. ANP also inhibited the AngII- or ET-1-induced hypertrophic responses such as activation of mitogen-activated protein kinase (MAPK) and induction of immediate early response genes and fetal type genes. To determine how ANP inhibits cardiomyocyte hypertrophy, we examined the mechanism of ANP-induced suppression of the MAPK activation. ANP strongly induced expression of MAPK phosphatase-1 (MKP-1) and overexpression of *MKP-1* inhibited AngII- or ET-1-induced hypertrophic responses. These growth-inhibitory actions of ANP were mimicked by a cyclic GMP analog 8-bromo-cyclic GMP. Taken together, ANP directly inhibits the growth factor-induced cardiomyocyte hypertrophy at least partly via induction of MKP-1. Our present study suggests that the formation of cardiac hypertrophy is regulated not only by positive but by negative factors in response to hemodynamic load.

Keywords: Angiotensin II; Atrial natriuretic peptide; Cardiac hypertrophy; Cardiomyocyte; Cyclic GMP; Endothelin-1; Growth factor; MAPK phosphatase-1; Mitogen-activated protein kinase; Vasoactive peptide

Since cardiac myocytes virtually lose their proliferative ability soon after birth, they respond to external stimuli not by increasing the cell number but by increasing the individual cell volume, called hypertrophy. Although cardiac hypertrophy has been considered to be a beneficial adaptive response of the heart to the increased workload, the hypertrophic heart often leads to dilated cardiomyopathy and eventually causes congestive heart failure after sustained overload [1]. Recent clinical studies have demonstrated that the increased ventricular mass is an independent risk factor for cardiac morbidity and mortality [2]. Therefore, it has become even more important to elucidate the molecular mechanism of how cardiac hypertrophy is formed.

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A variety of factors have been implicated in the pathogenesis of cardiac hypertrophy [3]. Vasoactive peptides such as angiotensin II (AngII) [3–7] and endothelin-1 (ET-1) [8,9] have been reported to induce cardiomyocyte hypertrophy by autocrine or paracrine mechanisms. These factors are produced and secreted in the heart in response to the increased overload and induce cardiomyocyte hypertrophy. Antagonists of these factors effectively inhibit the load-induced cardiomyocyte hypertrophy [6–9]. The intracellular signaling pathways initiated by these growth factors and the resultant hypertrophic responses in cardiomyocytes have also been intensively investigated. AngII and ET-1 activate various protein kinases including protein kinase C and the mitogen-activated protein kinase (MAPK) family. Activation of these protein kinases induces expression of many specific genes [10–12] and an increase in protein synthesis [12].

Among the genes that are upregulated in the hypertrophied heart, atrial natriuretic peptide (ANP) has unique features. ANP reduces the hemodynamic load as a potent vasorelaxing and diuretic–natriuretic peptide, which indirectly inhibits the development of cardiac hypertrophy [13]. In addition to the indirect effects of ANP via hemodynamics, ANP has recently been reported to have direct growth-inhibitory effects in various cell types such as vascular smooth muscle cells, glomerular mesangial cells, endothelial cells, cardiac fibroblasts, and astrocytes [14–19]. In these cells, 8-bromo-cyclic GMP (8-Br-cGMP), a cell permeable analog of cyclic GMP (cGMP), mimicked the effects of ANP, indicating that the growth-inhibitory effects of ANP are dependent on cGMP. Furthermore, it has recently been reported that ANP induces expression of MAPK phosphatase-1 (MKP-1) [20]. MKP-1 is a dual serine/threonine and tyrosine phosphatase and specifically inactivates MAPK family members [21]. Overexpression of MKP-1 blocks the MAPK-dependent gene expression and inhibits cell proliferation [22–24]. These observations suggest that ANP may exert its inhibitory effects on cell growth through inactivation of MAPK family members by induction of MKP-1. Three members of the MAPK family, the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38MAPKs, have been shown to be activated by hypertrophic stimuli in cardiac myocytes and have been implicated in the development of cardiac hypertrophy [25–30].

In the present study, we examined whether ANP has direct inhibitory effects on growth factor-induced cardiomyocyte hypertrophy. Pretreatment with ANP suppressed the growth factor-induced increase in the cell volume and the protein synthesis of cardiomyocytes. ANP also inhibited hypertrophic responses such as activation of MAPK and induction of immediate early response genes and fetal type genes. In addition, ANP strongly induced expression of MKP-1 in cardiac myocytes and overexpression of *MKP-1* suppressed AngII-induced gene expressions. These results suggest that anti-hypertrophic effects of ANP are, at least in part, mediated by inactivation of MAPK via induction of MKP-1. These inhibitory actions of ANP were mimicked by a cGMP analog, 8-Br-cGMP.

Materials and methods

Reagents. [γ-^{32}P]ATP and [^{3}H]phenylalanine were purchased from Du Point-New England Nuclear. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco-BRL. Polyclonal antibodies against MKP-1 were purchased from Santa Cruz Biotechnology. A rat ANP [1–28], AngII, myelin basic protein (MBP), and other reagents were purchased from Sigma.

Cell culture. Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats as described previously [6]. Except for the reporter gene assay, cells were plated at a field density of 1×10^3 cells/mm^2 on 35-mm culture dishes and cultured in DMEM with 10% FBS for the first 24 h, and then the culture medium was changed to DMEM containing 0.1% FBS. After 48 h of serum starvation, cardiomyocytes were stimulated by various agents. For transfection and reporter gene assay, cells were plated at the same density, and subjected to transfection after 24 h of culture in DMEM with 10% FBS.

Immunofluorescence. Immunostaining of cardiomyocytes with MF20, a monoclonal antibody against sarcomeric myosin heavy chain (MHC), was performed as described previously [7]. An anti-mouse immunoglobulin G conjugated with tetramethyl rhodamine isothiocyanate was used as the secondary antibody. The cell size of cardiomyocytes was measured by directly tracing the stained areas on a photograph.

[^{3}H]Phenylalanine incorporation. Protein synthesis was assessed by measuring the [^{3}H]phenylalanine incorporation as previously described [7]. Cardiac myocytes were cultured for 2 days without serum and then incubated for 24 h with AngII, ET-1 or vehicle. [^{3}H]Phenylalanine (1.0 μCi/ml) was added 3 h before the harvest. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), incubated 30 min with 1 ml of 10% trichloroacetic acid, and washed twice with PBS. Precipitates were solubilized for 30 min in 800 μl of 1 N NaOH, and radioactivity was measured by liquid scintillation spectroscopy.

Northern blot analysis. Total cellular RNA was extracted from cardiac myocytes by acid-guanidine phenol–chloroform method. Ten micrograms of total RNA was size-fractionated by 1.2% agarose gels and transferred to nylon membranes. Northern blot analyses were performed using the *c-fos* and *ANP* cDNA as probes as described previously [26,31]. The cDNA of rat *MKP-1* was isolated by the polymerase chain reaction method with a pair of primers corresponding to the amino acids 174–181 and 342–349.

Transfection and reporter gene assay. The luciferase reporter plasmids (3 μg/dish) containing ~1800 bp 5′ flanking region of the *brain natriuretic peptide* (*BNP*) gene (a kind gift from Dr. Y. Saito, Kyoto) were transiently transfected into cultured cardiac myocytes using standard calcium phosphate method. Cells were washed with PBS at 12 h after transfection and culture medium was changed to the medium containing 0.1% FBS. After 24 h of serum starvation, cells were treated with various reagents. Cells were harvested at 48 h after stimulation in 150 μl of extraction buffer (100 mM tricine, 10 mM $MgSO_4$, 2 mM EDTA, pH 7.8, and 1 mM dithiothreitol) and luciferase activities were measured by Berthold Lumat LB9501 luminometer. Next, 1 μg of luciferase reporter plasmids containing the human *c-fos* promoter

(pFC2) [32] or rat β *myosin heavy chain* (*βMHC*) promoter (−354 to +33) [33] and 3 μg of *MKP-1* expression plasmid DNA were co-transfected into cultured cardiac myocytes using lipofectin, Tfx-50 (Promega, WI, USA), according to the manufacturer's instructions. At 4 h after the transfection, the culture medium was changed to 0.1% FCS-containing DMEM and 24 h later, cardiac myocytes were exposed to 10^{-6} M Ang II for 4 h. Differences in transfection efficiency were corrected by β-galactosidase activities of co-transfected *SV40-βgal* plasmids (0.5 μg/dish).

Immunoprecipitation and Western blot analysis. Cardiomyocytes were lysed with lysis buffer (1% Triton X-100, 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 100 μM sodium orthovanadate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride PMSF, and 1 mM aprotinin) and protein extract was immunoprecipitated with a polyclonal anti-MKP-1 antibody. Immunoprecipitates were subjected to SDS–PAGE and immunoblotted with the same anti-MKP-1 antibody. The anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody and immune complexes were visualized using the ECL detection kit according to the manufacturer's directions.

Assay of ERK activity. The activity of ERKs was examined by "in gel assay" using MBP-containing gel as described previously [7]. In brief, cells were lysed with 100 ml Buffer A (25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EGTA, and 1 mM PMSF) and 25 μl of cell lysates was applied to an SDS–polyacrylamide gel containing 0.5 mg/ml MBP. ERKs in the gel were denatured in 6 M guanidine–HCl and renatured in 50 mM Tris–HCl, pH 8.0, containing 0.04% Triton X-100, and 5 mM of 2-mercaptoethanol. The activity of ERKs was assayed by incubating the gel with [γ-^{32}P]ATP. After incubation, the gel was washed, dried, and subjected to autoradiography.

Statistical analysis. All results are expressed as means ± SEM. One-way ANOVA and Fisher's exact test for post hoc analyses carried out multiple comparisons among three or more groups. A value of $P < 0.05$ was considered statistically significant.

Results

ANP inhibited AngII- or ET-1-induced cardiomyocyte hypertrophy

To examine whether ANP directly inhibits the development of cardiomyocyte hypertrophy, cultured cardiomyocytes were pretreated with ANP and then stimulated by AngII or ET-1. AngII or ET-1 enhanced the cell size of cardiomyocytes by approximately 2.6- or 3.2-fold, respectively (Figs. 1A and B). Pretreatment of cardiomyocytes with ANP (10^{-7} M) for 2 h significantly inhibited the AngII- or ET-1-induced increase in the cell size (Figs. 1A and B). A cGMP analog 8-Br-cGMP (10^{-3} M) also significantly blocked the vasoactive peptide-induced increase in the cell size (Fig. 1B). We also examined the protein synthesis in cardiomyocytes which were pretreated with ANP and subsequently stimulated by the vasoactive peptides. AngII or ET-1 stimulation increased the phenylalanine incorporation in cardiomyocytes by approximately 1.5- or 1.8-fold, respectively (Fig. 1C), which is consistent with previous results [5,7,9]. Pretreatment with ANP significantly reduced the AngII- or ET-1-induced increase in phenylalanine incorporation (Fig. 1C). 8-Br-cGMP also significantly inhibited the vasoactive peptide-induced phenylalanine incorporation (Fig. 1C). To confirm the relationship between ANP and cGMP in cardiomyocytes, we examined the concentrations of cGMP in the culture media after treatment of cardiomyocytes with ANP. The ANP treatment increased the cGMP concentrations in a dose-dependent manner, suggesting that ANP induces the cGMP generation, and secretion from cardiomyocytes (Fig. 1D). Furthermore, we investigated whether changes in the cGMP activity influence the inhibitory actions of ANP on vasoactive peptide-induced hypertrophic responses. Pretreatment with a selective inhibitor of the cGMP-specific phosphodiesterase (ZAPRINAST), which increases the cGMP concentration by blocking its metabolism, enhanced the inhibitory effect of ANP on AngII- or ET-1- induced increase in phenylalanine incorporation (Fig. 1E). On the other hand, pretreatment with a cGMP-dependent protein kinase inhibitor (KT5823), which blocks signals from cGMP, suppressed it (Fig. 1E). These effects of ZAPRINAST or KT5823 on actions of ANP were statistically significant in case of ET-1, though not of Ang II (Fig. 1E). These results suggest that ANP has a direct inhibitory effect on vasoactive peptide-induced cardiomyocyte hypertrophy in a cGMP-dependent manner.

ANP inhibited AngII- or ET-1-induced hypertrophic responses in cardiomyocytes

We next examined the effects of ANP on AngII- or ET-1-induced hypertrophic responses such as specific gene expressions. AngII induced expression of the *c-fos* gene in cardiac myocytes and the induction was inhibited by the pretreatment with 10^{-7} M ANP (Fig. 2A). The inhibitory effect of ANP on the *c-fos* gene induction was mimicked by the pretreatment with 10^{-3} M 8-Br-cGMP (Fig. 2A). We also examined the effects of ANP on the induction of fetal cardiac genes by AngII or ET-1. Both AngII and ET-1 increased the expression levels of the *ANP* gene, and pretreatment with ANP or 8-Br-cGMP inhibited AngII- or ET-1-induced increase in the *ANP* mRNA levels (Fig. 2B). To elucidate whether ANP inhibits the induction of fetal genes at the transcriptional level, we examined the effects of ANP on the *BNP* promoter activity. AngII or ET-1 activated the *BNP* promoter by approximately 2.5- or 3.5-fold, respectively (Fig. 2C). This transcriptional activation was significantly inhibited by the pretreatment of cardiomyocytes with ANP (10^{-7} M) or 8-Br-cGMP (10^{-3} M), although ANP or 8-Br-cGMP had no effects on the basal promoter activity of *BNP* (Fig. 2C). These results suggest that ANP inhibits the vasoactive peptide-induced reprogramming of gene expression in cardiomyocytes in a cGMP-dependent manner.

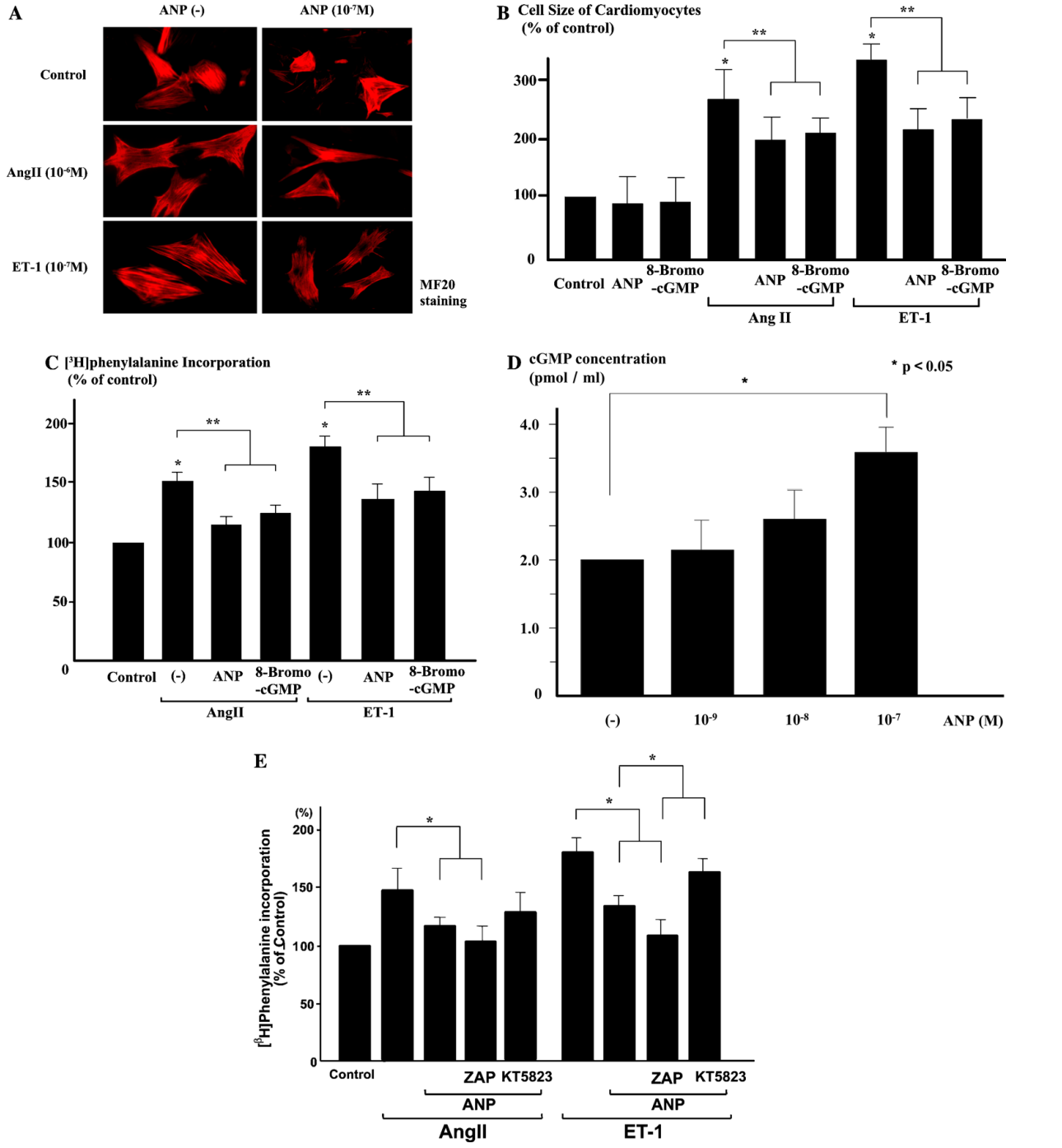

Fig. 1. ANP inhibited AngII- or ET-1-induced increase in the cell size and the protein synthesis of cardiomyocytes. (A,B) After pretreatment with ANP (10^{-7} M) or 8-Br-cGMP (10^{-3} M) for 2 h, cultured cardiomyocytes were stimulated with 10^{-6} M AngII or 10^{-7} M ET-1 for 24 h and then the cells were immunostained with MF20, an anti-sarcomeric MHC antibody, 24 h later. The cell size of cardiomyocytes was measured by directly tracing the stained areas on a photograph. Data represent the average percentages against the control (=100%, vehicle) from three independent experiments (mean ± SE). Statistical differences ($P < 0.05$) between the non-treated control and the Ang II or ET-1 treatment are denoted by ∗, and those between no pretreatment and ANP or 8-Br-cGMP pretreatment are shown by ∗∗. (C) After pretreatment with ANP (10^{-7} M) or 8-Br-cGMP (10^{-3} M) for 2 h and subsequent stimulation with AngII (10^{-6} M) or ET-1 (10^{-7} M) for 24 h, [^{3}H]phenylalanine (1 μCi/ml) was added 3 h before harvest. The effects of ANP or 8-Br-cGMP on the protein synthesis were evaluated by measuring the [^{3}H]phenylalanine incorporation. The total radioactivity of incorporated [^{3}H]phenylalanine was determined by liquid scintillation counting. Data represent the average percentages against the control (=100%, vehicle) from three independent experiments (means ± SE). Statistical differences ($P < 0.05$) between the non-treated control and the AngII or ET-1 treatment are denoted by ∗, and those between no pretreatment and ANP or 8-Br-cGMP pretreatment are shown by ∗∗. (D) After serum starvation of cultured cardiomyocytes with 0.1% FBS for 48 h and subsequent treatment with ANP at indicated concentrations for 24 h, the concentrations of cGMP in the culture media were examined. Statistical differences ($P < 0.05$) from the non-treated control are denoted by ∗. (E) After pretreatment with ANP (10^{-7} M) along with ZAPRINAST (ZAP) and KT5823 (10^{-8} M each) for 2 h and subsequent stimulation with AngII (10^{-6} M) or ET-1 (10^{-7} M) for 24 h, [^{3}H]phenylalanine (1 μCi/ml) was added 3 h before harvest. The evaluation of the [^{3}H]phenylalanine incorporation is similar to that in (C).

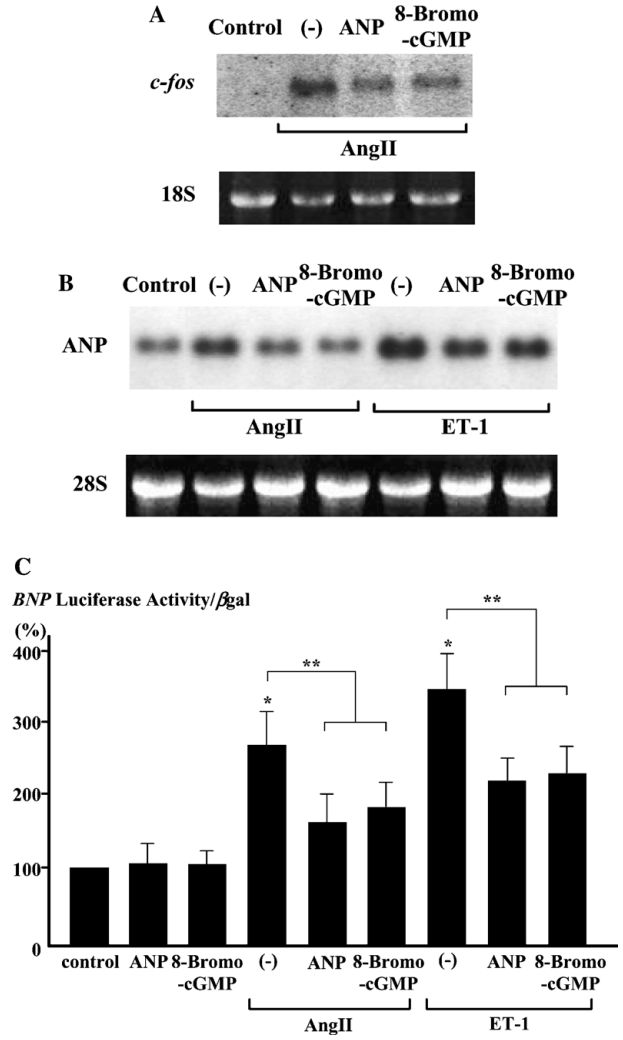

Fig. 2. ANP inhibited AngII- or ET-1-induced hypertrophic responses in cardiomyocytes. (A) Expression of the *c-fos* gene was examined by Northern blot analysis. Cardiomyocytes were pretreated for 2 h with ANP (10^{-7} M) or 8-Br-cGMP (10^{-3} M) and stimulated with AngII (10^{-6} M) for 30 min. A representative autoradiogram is shown. (B) The *ANP* gene expression was examined by Northern blot analysis. Cardiomyocytes were pretreated for 24 h with ANP (10^{-7} M) or 8-Br-cGMP (10^{-3} M) and stimulated with AngII (10^{-6} M) or ET-1 (10^{-7} M) for 2 h. A representative autoradiogram is shown. (C) The *BNP* promoter activity was examined by transient transfection assay. The results are indicated as means ± SEM of three independent experiments ($n = 9$) compared with unstimulated controls (100%). Statistical differences ($P < 0.05$) between the non-treated control and the AngII or ET-1 treatment are denoted by ∗, and those between no pretreatment and the ANP or 8-Br-cGMP pretreatment are shown by ∗∗.

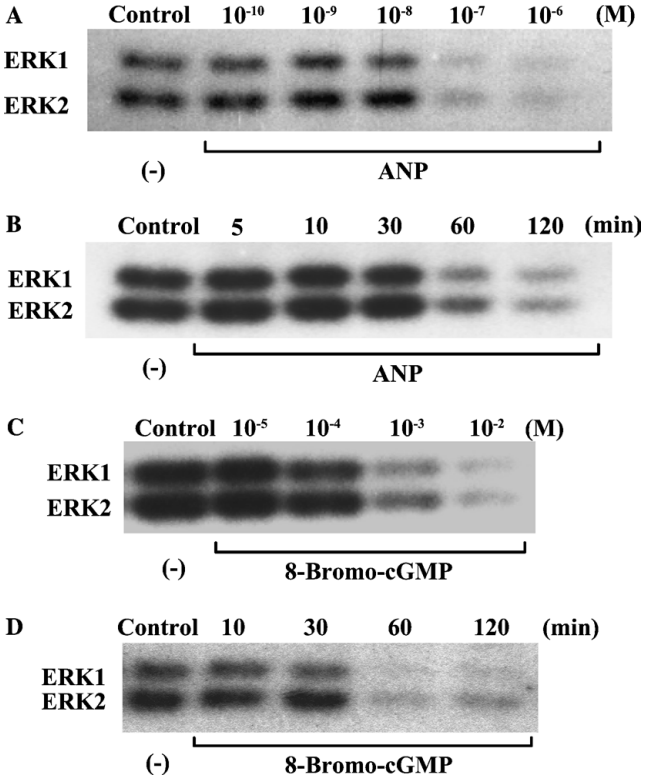

Fig. 3. ANP suppressed basal activities of ERKs in cardiomyocytes. (A) Cultured cardiomyocytes were treated with various concentrations of ANP (10^{-10}–10^{-6} M) for 2 h and the activities of ERKs were measured by the in-gel kinase assay described in "Materials and methods." (B) Cultured cardiomyocytes were treated with 10^{-7} M ANP and ERK activities were examined for indicated periods of time. (C) Cultured cardiomyocytes were treated with various concentrations of 8-Br-cGMP (10^{-5}–10^{-2} M) for 2 h and the activities of ERKs were measured. (D) Cultured cardiomyocytes were treated with 8-Br-cGMP (10^{-3} M) and ERK activities were examined for indicated periods of time. Representative autoradiograms are shown.

ANP decreased basal activities of ERKs in cardiomyocytes in a cGMP-dependent manner

We next examined the effects of ANP on MAPK, which has been reported to be important for the induction of cardiac hypertrophy [10,11]. The in-gel assay revealed that basal activities of both 44 kDa (ERK1) and 42 kDa (ERK2) ERKs were reduced by the treatment with ANP in a dose-dependent manner (Fig. 3A) and a significant decrease in ERK activities was observed at 60 min and reached the minimum level at 120 min after the 10^{-7} M ANP treatment (Fig. 3B). 8-Br-cGMP decreased basal activities of ERKs in a dose-dependent manner (Fig. 3C) with the same time course as ANP (Fig. 3D). These results suggest that ANP represses the basal ERK activity in a cGMP-dependent manner in cardiac myocytes.

ANP inhibited vasoactive peptide-induced activation of MAPKs in a cGMP-dependent manner

We next examined whether ANP represses the vasoactive peptide-induced activation of MAPK. Treatment of cardiomyocytes with 10^{-6} M AngII or 10^{-7} M ET-1 for 10 min markedly increased the ERK activity as reported before [7,9]. This vasoactive peptide-induced increase in the ERK activity was significantly inhibited by the pretreatment with 10^{-7} M

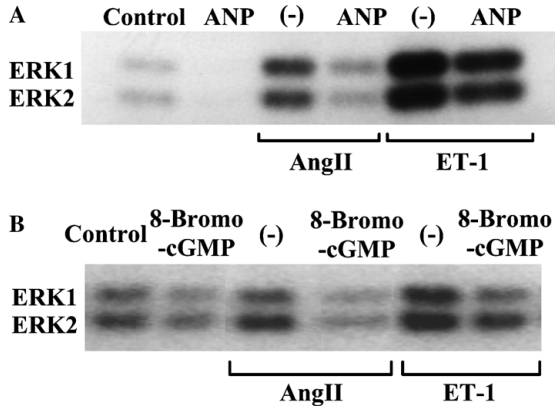

Fig. 4. ANP inhibited AngII- or ET-1-induced activation of MAPK in cardiomyocytes. (A) Cultured cardiomyocytes were pretreated with ANP (10^{-7} M) for 2 h and stimulated with AngII or ET-1 for 10 min. ERK activities were measured by the in-gel kinase assay. (B) Cultured cardiomyocytes were stimulated with AngII or ET-1 for 10 min and the effects of 8-Br-cGMP (10^{-3} M) pretreatment for 2 h on ERK activities were examined by the in-gel kinase assay. Representative autoradiograms are shown.

ANP for 2 h (Fig. 4A). The effects of 8-Br-cGMP on vasoactive peptide-induced ERK activation were also examined. Pretreatment of cardiomyocytes with 10^{-3} M 8-Br-cGMP decreased the AngII- or ET-1-induced ERK activation by approximately 70% and 60%, respectively (Fig. 4B).

ANP induced MKP-1 expression in cardiomyocytes

MAPK is inactivated by a dual phosphatase, MKP-1 [21], and the induction of MKP-1 has been implicated in the growth-inhibitory effects of ANP in mesangial cells [20]. To elucidate the mechanism by which ANP inhibits the development of cardiomyocyte hypertrophy, we examined whether MKP-1 is induced in cardiomyocytes by ANP. ANP significantly increased expression levels of the *MKP-1* gene and the mRNA levels of *MKP-1* peaked at 30 min and returned to the basal level at 120 min after the treatment with ANP (10^{-7} M) (Fig. 5A). 8-Br-cGMP (10^{-3} M) also induced MKP-1 by the same time course (Fig. 5A). The protein content of MKP-1 was also examined by Western blot analysis. The MKP-1 protein was dramatically induced by ANP or 8-Br-cGMP with its peak at 120 min after the treatment (Fig. 5B). These results clearly indicate that ANP induces expression of MKP-1 in cardiomyocytes in a cGMP-dependent manner.

MKP-1 blocked vasoactive peptide-induced hypertrophic responses

To elucidate the significance of the increase in the *MKP-1* gene expression, we examined the effects of

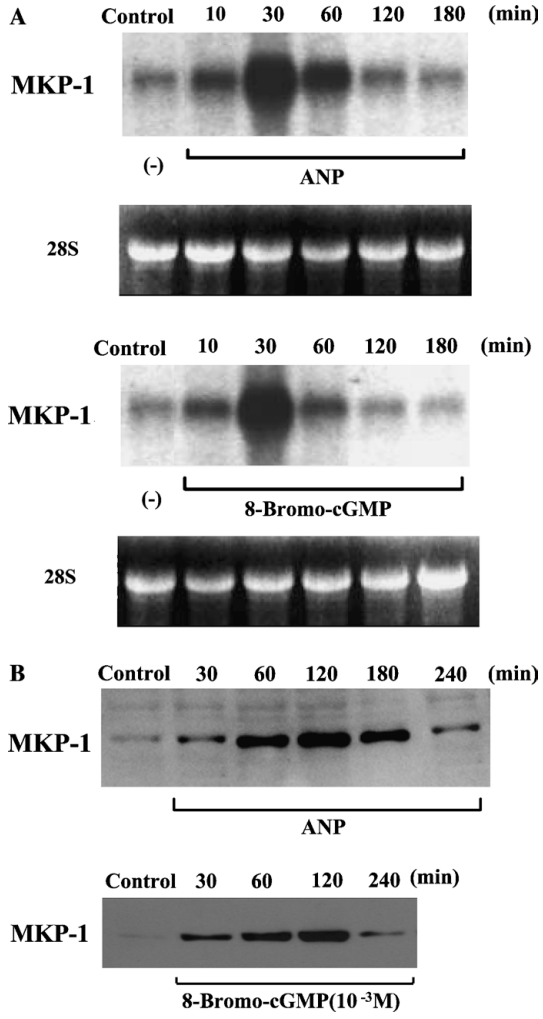

Fig. 5. ANP induced expression of MKP-1 in cardiomyocytes. (A) Cultured cardiomyocytes were treated with ANP or 8-Br-cGMP for indicated periods of time and expression of the *MKP-1* mRNA was examined by Northern blot analysis. (B) Cultured cardiomyocytes were treated with ANP or 8-Br-cGMP for the indicated periods of time and the protein content of MKP-1 was examined by immunoblot analysis. Representative autoradiograms are shown.

overexpression of the *MKP-1* gene on hypertrophic responses such as induction of the *c-fos* and *βMHC* genes. We co-transfected *c-fos* or *βMHC* promoter-containing luciferase reporter plasmids and *MKP-1* expression plasmids into the cultured cardiomyocytes, and examined the luciferase activity after stimulation with AngII. AngII activated the *c-fos* gene transcription in cardiomyocytes (Fig. 6A). Overexpression of the *MKP-1* mRNA significantly suppressed the AngII-induced increase in the *c-fos* gene transcription as well as the non-treated, basal transcription (Fig. 6A). AngII also increased the luciferase activity of the *βMHC* reporter gene and overexpression of *MKP-1* significantly suppressed the AngII-induced increase in the *βMHC* gene transcription (Fig. 6B). Furthermore, overexpression of *MKP-1* significantly suppressed AngII- or ET-1-induced increase in

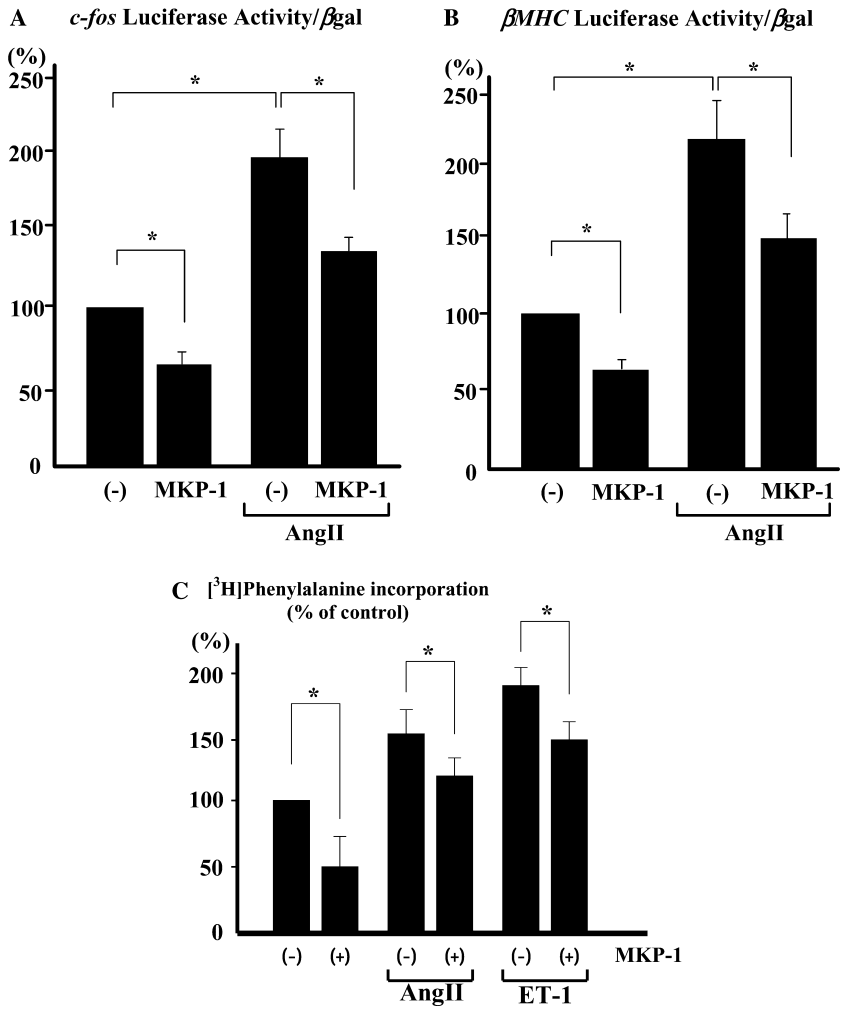

Fig. 6. MKP-1 blocked AngII- or ET-1-induced hypertrophic responses in cardiomyocytes. (A,B) One microgram of MKP-1 plasmid DNA was co-transfected with 1 μg of *c-fos* (A) or *βMHC* (B) luciferase reporter plasmids into cultured cardiomyocytes using lipofectin, Tfx-50. At 4 h after the transfection, the culture medium was changed to 0.1% FBS-containing DMEM. After this serum starvation for 24 h, cardiomyocytes were incubated with Ang II (10^{-6} M) for 4 h. Luciferase activities were measured using a luminometer and the data are shown as means ± SEM of three independent assays ($n = 18$) ($^{*}P < 0.05$). (C) After the transfection and the starvation same as those in (A,B), cardiomyocytes were incubated with Ang II (10^{-6} M) and ET-1 (10^{-7} M) for 24 h. [^{3}H]Phenylalanine (1 μCi/ml) was added 3 h before harvest. The evaluation of the [^{3}H]phenylalanine incorporation is similar to that in Fig. 1C. Statistical differences ($P < 0.05$) from the non-transfected control are denoted by ∗.

phenylalanine incorporation (Fig. 6C). These findings suggest that an increase in the *MKP-1* mRNA levels inhibits vasoactive peptide-induced hypertrophic responses such as an increase in protein synthesis and specific gene expressions.

Discussion

In the present study, we have obtained several results as follows. (i) ANP directly inhibits the vasoactive peptide-induced increase in the cell size and the protein synthesis of cardiomyocytes through the cGMP-dependent pathway. (ii) ANP also inhibits the vasoactive peptide-induced hypertrophic responses such as reprogramming of gene expressions and activation of MAPK. (iii) ANP upregulates expression of MKP-1 in cardiomyocytes. (iv) Overexpression of *MKP-1* inhibits the vasoactive peptide-induced hypertrophic responses.

ANP was originally identified as a natriuretic and diuretic peptide predominantly produced and secreted from atrial cells. Many studies demonstrated that ANP regulates sodium and water homeostasis via changes in the glomerular filtration rate and inhibition of the renin and aldosterone secretion [13]. In addition to these effects on the circulatory system, ANP has been shown to have a direct vasorelaxing effect which counteracts the vasoconstrictive factors such as AngII and ET-1 [13]. Moreover, it has recently been demonstrated that ANP acts as a growth-inhibitory factor that antagonizes the growth-promoting effects of AngII or ET-1 in various cell types including vascular smooth muscle

cells, glomerular mesangial cells, astrocytes, endothelial cells, and cardiac fibloblasts [14–19]. These findings suggest that ANP antagonizes various effects of vasoactive and/or growth-promoting factors. We therefore hypothesized that ANP might antagonize cardiomyocyte hypertrophy-promoting effects of vasoactive peptides. In the present study, in fact, ANP inhibited the AngII- or ET-1-induced increase in protein synthesis and hypertrophic responses such as expression of *c-fos* and fetal type genes and activation of MAPK in cardiomyocytes.

ANP and the other natriuretic peptides function through a family of membrane receptors called natriuretic peptide receptors, NPR-A, NPR-B, and NPR-C [13]. NPR-A and NPR-B have three domains, an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular domain. An intracellular domain is consisting of a kinase domain and a guanylyl cyclase domain, which generates cGMP upon ligand binding [13]. On the other hand, NPR-C or clearance receptor contains the extracellular and transmembrane domains but lacks the intracellular domain, and is thought to be mainly responsible for the internalization and degradation of ligands [13]. Our present data strongly suggest that the anti-hypertrophic actions of ANP are mediated by guanylyl cyclase-linked NPR-A because a cGMP analog 8-Br-cGMP showed the effects similar to ANP and also because ANP exhibits relatively higher binding affinity for NPR-A than for NPR-B. Another recent report demonstrated that the inhibitory action of ANP on hypertrophic response was not suppressed by a cGMP-dependent protein kinase inhibitor KT5823, implying the involvement of additional cGMP-independent pathways [34]. This result and our present results are controversial, which may be at least partially due to differences in cardiomyocytes used in assays (adult vs. neonatal) and the ways how to stimulate them (concentration, duration, etc.). Nevertheless, we consider our data clearly and for the first time demonstrated a series of evidence that the cGMP analog mimicked the effects of ANP on vasoactive peptide-induced cardiomyocyte hypertrophy, indicating the importance of the cGMP-dependent pathway for this anti-hypertrophic action of ANP. The growth-inhibitory effects of ANP on glomerular mesangial cells [20] and vascular smooth muscle cells [18] are also thought to be mediated by cGMP-dependent pathways, although the anti-proliferative actions of ANP on astrocytes [19] are reported to be mediated by clearance receptors, suggesting that modes of inhibitory actions of ANP may depend on cell types nonetheless.

Although the mechanism by which AngII or ET-1 induces cardiomyocyte hypertrophy is not fully understood, protein kinases especially the MAPK family have been reported to play a pivotal role in the development of cardiac hypertrophy [10,11,27–30]. Three subfamilies of MAPKs such as ERKs, JNK, and p38MAPK have been reported to be involved in cardiac hypertrophy as follows. (i) Hypertrophic stimuli such as AngII and ET-1 activate all three members of MAPKs [5–7,9,26,29]. (ii) Anti-sense oligonucleotides against ERKs inhibit the phenylephrine-induced increase in cell size [12]. (iii) Selective activation of JNK by a constitutively active form of MKK7/JNKK2 leads to cardiomyocyte hypertrophy [30]. (iv) Activation of p38MAPK induces cardiomyocyte hypertrophy while that of JNK exhibits inhibitory effects [27]. (v) p38MAPK is necessary for the maintenance of hypertrophic response in a longer period but not for the immediate morphological responses [29]. Taken together, although precise roles of individual MAPKs are still controversial at present, these results suggest that activation of the MAPK pathways plays a critical role in the development of cardiomyocyte hypertrophy. In this respect, MKP-1, the recently identified dual protein phosphatase with selectivity for MAPKs, is of quite interest as a negative regulator of the MAPK pathways [21]. MKP-1 has been shown to be widely expressed in various cell types and to be capable of dephosphorylating phosphothreonine and phosphotyrosine residues of ERKs, JNK, and p38MAPK [21,24,32]. Because expression of MKP-1 is rapidly induced by many growth factors and cytokines that also induce activation of ERKs, JNK, and p38MAPK, MKP-1 has been implicated for the feedback loop serving to downregulate the MAPK activities in response to external stimuli [21,24,32]. Recently, MKP-1 has been reported to be induced by ANP in glomerular mesangial cells [20]. MKP-1 was also induced by ANP in cardiomyocytes in this study, although ANP did not activate ERKs in cardiomyocytes (data not shown). ANP also reduced the basal MAPK activity and inhibited vasoactive peptide-induced activation of MAPK in cardiomyocytes through the cGMP-dependent pathways. Taken together, our present study suggests that ANP inhibits the vasoactive peptide-induced cardiomyocyte hypertrophy at least in part by inhibiting activation of MAPK through upregulation of MKP-1.

In addition to the induction of MKP-1, there are possible mechanisms by which ANP inhibits the growth-promoting processes in various cell types. In astrocytes, ANP has been shown to inhibit ERKs by attenuating the MEK activity, although the precise signaling pathway leading to the inactivation of MEK remains to be identified [19]. In mesangial cells, ANP inhibits the ET-1-induced JNK activation possibly by attenuating the ET-1-induced increase in intracellular Ca^{2+} concentration [33]. It has been reported that ANP inhibits the norepinephrine-induced growth of cardiac myocytes by a cGMP-mediated inhibition of norepinephrine-stimulated Ca^{2+} influx [35]. Although MKP-1 was strongly induced in cardiomyocytes by ANP, we cannot rule out

the possibilities that some other mechanisms are also involved in the growth-inhibitory effects of ANP in cardiomyocytes.

Although a variety of molecules that promote the development of cardiac hypertrophy have been well examined [3–8], little is known about the molecules that inhibit cardiac hypertrophy. ANP is quite unique in that it exhibits growth-inhibitory effects on cardiomyocytes. Simultaneous induction of both growth-promoting and growth-inhibiting factors in the myocardium suggests that cardiac growth in response to hemodynamic overload is controlled by complex regulatory mechanisms. Although the precise mechanism by which ANP inhibits the development of cardiac hypertrophy remains to be further clarified, understanding the physiological and pathological actions of ANP on cardiac cells may allow the development of novel therapeutic strategies for modulating the hypertrophy of cardiomyocytes and the overall remodeling of the myocardium.

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