Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload.

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Research Article

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Rapid Transcriptional Activation and Early mRNA Turnover of Brain Natriuretic Peptide in Cardiocyte Hypertrophy

Evidence for Brain Natriuretic Peptide as an "Emergency" Cardiac Hormone against Ventricular Overload

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Abstract

We previously demonstrated that brain natriuretic peptide (BNP) is a cardiac hormone mainly produced in the ventricle, while the major production site of atrial natriuretic peptide (ANP) is the atrium. To assess the pathophysiological role of BNP in ventricular overload, we have examined the gene expression of BNP, in comparison with that of ANP, in a model of cardiac hypertrophy using cultured neonatal rat ventricular cardiocytes. During cardiocyte hypertrophy evoked by endothelin-1, phenylephrine, or PMA, the steady state level of BNP mRNA increased as rapidly as the "immediate-early" induction of the c-*fos* gene expression, and reached a maximal level within 1 h. Actinomycin D, a transcriptional inhibitor, completely diminished the response, while the translational blockade with cycloheximide did not inhibit it. In contrast, ANP mRNA began to increase 3 h after the stimulation, and accumulated during cardiocyte hypertrophy. The BNP secretion from ventricular cardiocytes was also stimulated more rapidly than the ANP secretion. Furthermore, the turnover of BNP mRNA was significantly faster than that of ANP mRNA, being consistent with the existence of AUUUA motif in the 3′-untranslated region of BNP mRNA. These results demonstrate that the gene expression of BNP is distinctly regulated from that of ANP at transcriptional and posttranscriptional levels, and indicate that the characteristics of the BNP gene expression are suitable for its possible role as an "emergency" cardiac hormone against ventricular overload. (*J. Clin. Invest.* 1995. 96:1280–1287.) Key words: natriuretic peptides • ventricular cardiocytes • gene expression • messenger RNA • cardiac hypertrophy

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic peptide; BNP, brain NP; ET-1, endothelin-1; HP-GPC, high performance gel permeation chromatography; -LI, like immunoreactivity; MLC-2, myosin light chain-2; PKC, protein kinase C.

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Introduction

The identification of atrial natriuretic peptide (ANP)[1] in the cardiac atrium (1, 2) uncovered a new functional role of the heart as an endocrine organ regulating body fluid homeostasis and blood pressure control (3–5). ANP is mainly produced in and released from the atrium, and the plasma ANP concentration elevates in volume-overloaded states including congestive heart failure (6–8). In addition, the gene expression of ANP in the ventricle is markedly induced during the process of cardiac hypertrophy upon ventricular overload, and significantly contributes to the increase in the plasma ANP concentration in various cardiovascular disorders (9–11).

Brain natriuretic peptide (BNP), originally isolated from the porcine brain (12), is a second member of natriuretic peptide family (3–5). We previously demonstrated that BNP is predominantly synthesized in and secreted from the cardiac ventricle (13–15). We have further shown that the ventricular gene expression of BNP is substantially augmented in response to ventricular overload in congestive heart failure, idiopathic cardiomyopathy, or hypertensive heart disease with cardiac hypertrophy (14–17). Although the plasma BNP concentration is approximately one-sixth of the plasma ANP concentration in healthy men, it markedly elevates in patients with congestive heart failure in parallel with its severity and surpasses the plasma ANP concentration in severe cases (14, 18–20). Furthermore, we have recently demonstrated that the plasma BNP concentration increases rapidly and tremendously, in contrast to the modest change of the plasma ANP concentration, in the early clinical course of acute myocardial infarction (21, 22). These findings indicate that the biosynthesis and secretion of BNP are distinctly regulated from those of ANP in response to ventricular overload, and suggest that BNP may have a discrete pathophysiological role in the maintenance of cardiovascular homeostasis.

The augmented productions of BNP and ANP in the hypertrophied myocardium can be considered as a compensation mechanism against ventricular overload, since BNP and ANP serve to reduce both cardiac preload and afterload by their natriuretic, diuretic, and vasodilatory actions (23–25). It will be of great importance to characterize the gene expressions of BNP and ANP during the development of cardiac hypertrophy, which also constitutes one of the principal adapting mechanisms against increased ventricular workload (26). The cellular mechanisms of the cardiac adaptations to ventricular overload, especially the expressions of various cardiac-specific genes, have been intensively examined using the cell culture models of cardiac hypertrophy, which closely reproduce the characteristics of in vivo hypertrophy (27–34).

To assess the pathophysiological significance of BNP in the

compensation mechanism against cardiac overload, we examined the regulation of the BNP gene expression, in comparison with that of ANP, during the process of cardiocyte hypertrophy in cultured neonatal rat ventricular cardiocytes.

Methods

Cell culture. Apical halves of cardiac ventricles from 2- to 4-d-old Wistar rats were recovered and minced in a chilled balanced salt solution (116 mM NaCl, 20 mM Hepes, 12.5 mM NaH_2PO_4, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.35) (28). Ventricular cardiocytes were dispersed in the balanced salt solution containing 0.04% collagenase II (Worthington Biochemical Corp., Freehold, NJ) and 0.06% pancreatin (GIBCO Laboratories, Grand Island, NY) with agitation for 20 min at 37°C. The digestion steps were repeated six times and the collected cell suspensions were mixed with 1/10 vol of chilled FCS (Hazleton Biologics, Lenexa, KS) and pelleted by centrifugation. The pellets were combined in chilled FCS and kept at 4°C.

The differentiation of myocytes from nonmyocytes was performed by the discontinuous Percoll gradient method (28). The discontinuous gradient of Percoll (Sigma Chemical Co., St. Louis, MO) consisting of 40.5 and 58.5% was prepared in the balanced salt solution described above, and ventricular cells were suspended in the layer of 58.5% was prepared in the balanced salt solution described above, and ventricular cells were suspended in the layer of 58.5% Percoll. After centrifugation at 3,000 rpm for 30 min at 15°C, the cardiomyocytes selectively migrated to the interface of the discontinuous layers.

The purified myocytes were plated at a density of 3.6×10^4 cells/cm^2 (2.0×10^6 cells/10-cm dish) in the gelatin-coated culture dishes, in DME (Flow Laboratories, Irvine, United Kingdom) supplemented with 10% FCS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin). Following 30-h incubation, the cells were maintained in serum-free DME for 10 h. After the preconditioning period, the cultures were incubated in serum-free DME containing 1 mg/ml bovine serum albumin (Sigma Chemical Co.) with the following agents or vehicles: synthetic endothelin-1 (ET-1) (Peptide Institute, Osaka, Japan); phenylephrine, PMA, actinomycin D and cycloheximide (Sigma Chemical Co.); H-7 (Seikagaku Kogyo, Tokyo, Japan); calphostin C (a gift from Kyowa Hakko Kogyo, Tokyo, Japan).

High performance gel permeation chromatography (HP-GPC). The conditioned media were collected with 1/100 vol of 1 mg/ml Triton X-100, and acidified with 1/200 vol of trifluoroacetic acid at 4°C. Peptides were extracted from the medium samples using a Sep-Pak C_{18} cartridge (Millipore Corp., Milford, MA). HP-GPC was performed on a TSK-GEL G2,000 SW column (7.5 × 600 mm) (Toyo Soda, Tokyo, Japan), eluted with 10 mM trifluoroacetic acid containing 0.3 M NaCl and 30% acetonitrile as a solvent as previously reported (14, 35). An aliquot of each fraction was subjected to the RIAs for BNP and ANP without extraction.

RIAs for BNP and ANP. The BNP-like immunoreactivity (-LI) was determined by its specific RIA as previously reported (13). The minimal detectable quantity was 0.8 fmol/tube. The cross-reactivities with α-rat ANP and rat C-type natriuretic peptide, a third member of natriuretic peptide family (3, 4), in the RIA for rat BNP were $< 0.1\%$ on a molar basis. The ANP-LI was also measured by its specific RIA (9). The minimal detectable quantity was 0.3 fmol/tube, and the cross-reactivities with rat BNP and rat C-type natriuretic peptide in the RIA for rat ANP were $< 0.1\%$ on a molar basis.

Probe preparations. The following probes were used for Northern blotting hybridizations: a 368-bp HincII–StuI fragment of rat ANP cDNA (9), a 468-bp fragment of rat BNP cDNA (16), a 484-bp human c-*fos* genomic probe (Takara Shuzo Co., Kyoto, Japan), and a 500-bp fragment of rat myosin light chain-2 (MLC-2) cDNA prepared as follows.

The rat MLC-2 cDNA probe was prepared by the cDNA synthesis and polymerase chain reaction (36), utilizing poly(A)$^+$ RNA isolated from the rat heart. The synthetic primers were corresponding to the rat MLC-2 cDNA sequence (37), with the addition of EcoRI and PstI cleavage sites at the 5′-ends: 5′-GAATTCTGCAGATGTCACCAA-AGAAAGCCAAG-3′ (sense) and 5′-GAATTCTGCAGTCAGTCC-TTCTCTTCTCCGTG-3′ (antisense). The sequence of the amplified product was confirmed to be identical to that of rat MLC-2 cDNA by the dideoxy chain termination method (36). The probe contains the entire coding sequence of rat MLC-2 cDNA.

These probes were labeled by the random priming method (36) with [α-^{32}P]dCTP (Amersham International, Little Chalfont, United Kingdom). Specific activity was $\sim 1 \times 10^9$ cpm/μg DNA.

Total RNA extraction and Northern blotting analyses. Total cellular RNA was extracted from cultured ventricular cardiocytes by the guanidinium thiocyanate CsCl method as previously reported (38). 2-μg aliquots of total cellular RNA were size-fractionated by electrophoresis on a 1.4% agarose-formaldehyde gel, and transferred to Biodyne A nylon membranes (Pall Corp. Glen Cove, NY). The membranes were prehybridized in 50 mM sodium phosphate buffer (pH 7.0) containing 50% formamide, 5 × SSC, 5 × Denhardt's solution (36), 0.1% SDS, and 250 μg/ml salmon sperm DNA at 42°C for 12–24 h and hybridized with ^{32}P-labeled probes in the same solution mixture at 42°C for 20 h. For the human c-*fos* genomic probe, prehybridization was performed at 65°C for 12h in the solution containing 4 × SSC, 10 × Denhardt's solution, 0.5% SDS, and 250 μg/ml salmon sperm DNA, followed by the hybridization at 42°C for 20 h in the solution composed of 50% formamide, 4 × SSC, 5 × Denhardt's solution, 0.5% SDS, 10% dextran sulfate, and 250 μg/ml salmon sperm DNA. The membranes were washed serially, with the final wash in 0.1 × SSC, 0.1% SDS at 42°C. The exposure time on autoradiography was $\sim$ 10 h (ANP), 16 h (BNP), 8 h (MLC-2), and 60 h (c-*fos*) with intensifying screens, respectively, and relative amount of each mRNA was determined by densitometric scanning in the linear response range of the x-ray films.

Statistical analysis. Quantitative data were described as mean±SE, and statistical analyses were performed using Student's *t* test.

Results

Secretions of BNP and ANP from cultured neonatal rat ventricular cardiocytes. The serial dilution curves of the conditioned media of cultured neonatal rat ventricular cardiocytes were parallel to the standard curves of rat BNP and α-rat ANP in the RIAs for BNP and ANP, respectively (data not shown). Cultured ventricular cardiocytes in the control serum-free DME stably secreted BNP-LI as well as ANP-LI over 48 h. The amounts of BNP-LI and ANP-LI in the control media after a 48-h incubation were 2.81±0.257 and 4.02±0.527 pmol/dish, respectively (Fig. 1, *A* and *B*).

To further characterize the BNP and ANP secretions from cultured ventricular cardiocytes, the molecular forms of BNP-LI and ANP-LI in the conditioned media were analyzed using HP-GPC coupled with RIAs (Fig. 2). BNP-LI in the control conditioned media consisted of two components with approximate molecular masses of 10 and 5 kD, in which the 5-kD form was predominant (Fig. 2 *A*). The elution position of 5-kD BNP-LI was identical to that of synthetic rat BNP with 45 amino acids (39), which is a circulating form of rat BNP (40). The 10-kD component corresponded to rat proBNP (13). As for ANP-LI, two components of 15 and 3 kD emerged in the elution positions of γ-rat ANP (proANP) and synthetic α-rat ANP (circulating form) (13), respectively (Fig. 2 *A*). The two components of ANP-LI were almost equivalent in quantity.

Distinct stimulation of BNP and ANP secretions during ET-1–induced cardiocyte hypertrophy. The treatment of cultured ventricular cardiocytes with ET-1 evoked apparent increase in cell size around 24 h after the stimulation (data not shown). The BNP-LI and ANP-LI secretions into culture media from

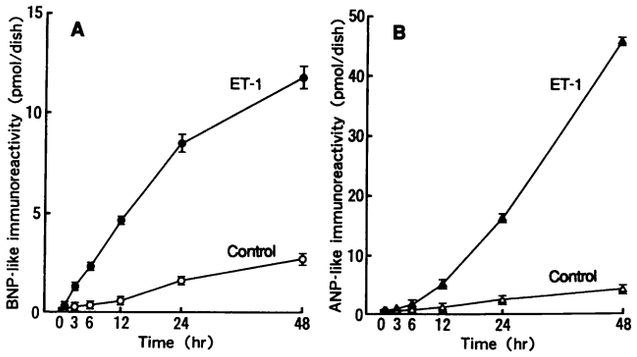

Figure 1. Distinct stimulation of BNP and ANP secretions by the treatment of ventricular cardiocytes with ET-1. Ventricular cardiocytes were incubated with 10^{-8} M of ET-1 or vehicle. The medium samples were removed at indicated times and subjected to the RIAs for BNP and ANP. Results are presented as the mean value of triplicate assay samples for each time point, with error bars representing SE. (*A*) BNP-LI, closed circles; ET-1, open circles; vehicle. (*B*) ANP-LI, closed triangles; ET-1, open triangles; vehicle.

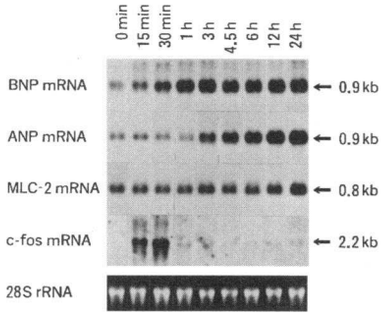

Figure 3. Differential gene expressions of BNP, ANP, MLC-2, and c-*fos* during ET-1–evoked cardiocyte hypertrophy. After indicated times of incubation with ET-1 (10^{-8} M), the cells were harvested for the isolation of total RNA and the steady state levels of various mRNAs were assessed by Northern blotting analyses. The same membrane (2 μg of total RNA/lane) was reused for the hybridizations.

ventricular cardiocytes were stimulated in a dose-dependent fashion during ET-1–induced cell hypertrophy. Maximal response of BNP-LI release in the 48-h incubation was 3.7-fold vs. control, which was obtained with ET-1 concentrations of $10^{-10} - 10^{-8}$ M. As for the ANP-LI release, maximal induction (10-fold vs. control) also occurred with $10^{-10} - 10^{-8}$ M of ET-1. The 50% effective concentration values were $\sim 3 \times 10^{-11}$ M. As shown in Fig. 2 *B*, BNP-LI and ANP-LI in the conditioned media of ET-1–treated hypertrophied ventricular cells exhibited essentially the same HP-GPC profiles as those in the control conditioned media.

We next examined the temporal profile of the BNP and ANP secretions during the course of ET-1–induced cardiocyte hypertrophy. The BNP-LI secretion was rapidly induced by the treatment with ET-1, and the fold increase vs. control reached its maximum within 3 h (Fig. 1 *A*). Thereafter, the maximally increased BNP-LI release was maintained until 24 h after the treatment. In contrast, the stimulation of the ANP-LI release was observed from several hours after the treatment and was time dependently augmented during myocardial cell hypertrophy (Fig. 1 *B*).

Differential gene expressions of BNP and ANP during cardiocyte hypertrophy. To further investigate the differential activation of the BNP and ANP productions in cultured ventricular cardiocytes, we examined the temporal profiles of the BNP mRNA and ANP mRNA levels during ET-1–evoked cardiocyte hypertrophy. The treatment of ventricular cardiocytes with ET-1 evoked cardiocyte hypertrophy, which was characterized by well-known phenotypic and genetic features of cardiocyte hypertrophy (28, 29, 31), as follows. As demonstrated in Fig. 3, the stimulation of ventricular cardiocytes with ET-1 (10^{-8} M) evoked rapid and transient increase in the c-*fos* mRNA level within 15 min. The MLC-2 mRNA level gradually increased from 12 h after the stimulation, followed by increase in cell size observed obviously around 24 h.

In this course of cardiocyte hypertrophy, the steady state level of BNP mRNA immediately increased within 15 min after the treatment with ET-1, reaching a maximal level at 1 h (386±30.4% of control cells, $P < 0.01$, $n = 12$) (Figs. 3 and 4). On the other hand, ANP mRNA began to increase 3 h after the treatment with ET-1, and gradually accumulated during myocardial cell hypertrophy.

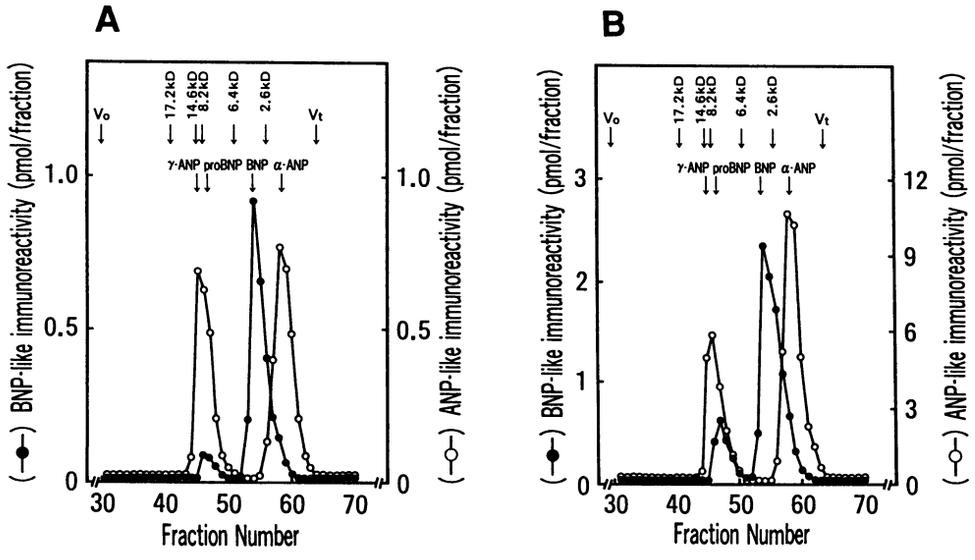

Figure 2. Molecular forms of BNP and ANP secreted from cultured ventricular cardiocytes. Ventricular cardiocytes were incubated with 10^{-8} M of ET-1 or vehicle for 48 h. (*A*) Vehicle. (*B*) ET-1. Closed circles; BNP-LI, open circles; ANP-LI. Arrows denote elution positions of polypeptide molecular weight calibration kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), void volume (*Vo*), and total volume (*Vt*). Elution positions of synthetic rat BNP, synthetic α-rat ANP, purified rat proBNP, and purified γ-rat ANP are also indicated.

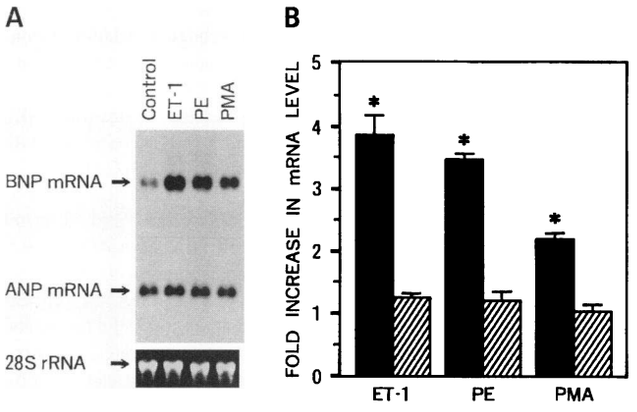

Figure 4. Rapid induction of BNP gene expression via protein kinase C activation. Ventricular cardiocytes were treated with ET-1 (10^{-8} M), phenylephrine (*PE*) (10^{-4} M), PMA (10^{-6} M) for 1 h. Total RNA extraction and Northern blotting analyses on BNP mRNA and ANP mRNA were performed as described in Methods. Representative results are shown in Panel *A*, and the quantitative analyses by the densitometric scanning are shown in Panel *B*. The data are presented as fold-increases against control (mean values with SE). Filled bars; BNP mRNA, hatched bars; ANP mRNA, $*P < 0.001$ compared with control.

Involvement of protein kinase C (PKC) activation in rapid induction of BNP gene expression. We further analyzed the BNP gene expression in cardiocyte hypertrophy evoked by the treatment with agents other than ET-1. The treatment with phenylephrine (10^{-4} M) or PMA (10^{-6} M) induced cardiocyte hypertrophy with increase in cell size and augmented expression of the MLC-2 gene around 24 h (not shown). As was the case with ET-1, rapid increase in the steady state level of BNP mRNA was induced within 1 h by the treatment with phenylephrine (348±8.55% vs. control, $P < 0.01$, $n = 3$) or PMA (218±8.73%, $P < 0.01$, $n = 3$) (Fig. 4, *A* and *B*), while there were no significant changes in the ANP mRNA level at 1 h. The mRNA level of ANP increased later during the establishment of cell hypertrophy, as observed by the treatment with ET-1 (data not shown).

Since ET-1, phenylephrine, and PMA were known to activate PKC in the intracellular signaling pathway, we examined the effects of PKC inhibitors (41) on the BNP gene expression. H-7 (a nonspecific PKC inhibitor) and calphostin C (a highly selective PKC inhibitor) both clearly suppressed the ET-1–induced rapid increase in the BNP mRNA level at 1 h (Fig. 5). The BNP mRNA level in cardiocytes simultaneously treated with ET-1 and H-7, or ET-1 and calphostin C was 55.3±4.13% ($P < 0.01$, $n = 4$) and 49.7 ± 7.75% ($P < 0.01$, $n = 4$), respectively, as compared with that of ET-1–treated cardiocytes. These findings suggest that the rapid induction of the BNP gene expression during cardiocyte hypertrophy is mediated via, at least in part, PKC activation.

Rapid transcriptional activation of BNP gene in protein synthesis–independent mechanism. As seen in Fig. 6, actinomycin D, an inhibitor of gene transcription, completely diminished the ET-1–induced increase in the BNP mRNA level (ET-1 + AMD: 79.6±10.4% of control cells, $n = 3$). On the other hand, the treatment with ET-1 could evoke rapid increase in the BNP mRNA level even in the presence of cycloheximide, a protein synthesis inhibitor (ET-1 + CHX: 91.2±3.90% of ET-1–treated cells, $n = 3$). Thus, the rapid induction of the

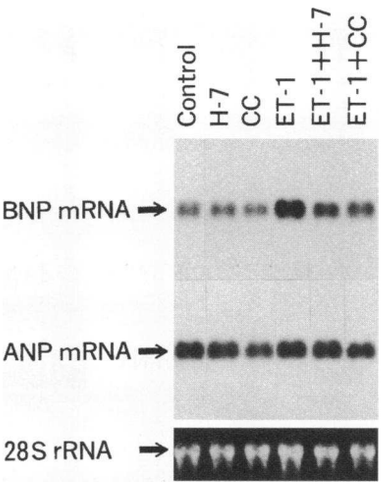

Figure 5. Inhibition of ET-1-induced rapid BNP gene expression by treatment with protein kinase C inhibitors. Ventricular myocytes were simultaneously treated with ET-1 (10^{-8} M) and H-7 (10 μg/ml), or ET-1 (10^{-8} M) and calphostin C (*CC*) (10^{-6} M) for 1 h, and their effects on ET-1–evoked BNP gene expression were evaluated by Northern blotting analyses.

BNP gene expression by the treatment with ET-1 occurs through the transcriptional up-regulation, and this induction does not require de novo synthesis of transacting regulatory proteins.

Rapid turnover of BNP mRNA in cultured ventricular cardiocytes. Fig. 7 (*A* and *B*) shows the turnover of BNP mRNA and ANP mRNA in cultured ventricular cardiocytes. After the transcriptional inhibition by the treatment with actinomycin D, the steady state levels of BNP mRNA and ANP mRNA at the indicated times were measured to evaluate the disappearance rates of preexisting mRNAs.

We evaluated the turnover rates of BNP mRNA and ANP mRNA in ventricular cardiocytes in basal, serum-free condition (Fig. 7 *A*). In addition, we examined the mRNA turnover in hypertrophied cells, which were incubated with 10^{-8} M of ET-1 for 36 h and were further treated with actinomycin D (Fig. 7 *B*). In both states, BNP mRNA was rapidly degraded in ventricular cardiocytes, in contrast to ANP mRNA. In basal, serum-free condition, the BNP mRNA levels at 2, 6, and 12 h after the treatment were 67, 38, and 16% of control (0 h), respectively (mean of triplicated determinations, half life = 4 h) (Fig. 7 *A*). In hypertrophied condition, the BNP mRNA levels at 2, 6, and 12 h were 78, 54, and 31% of control (0 h), respectively (mean of triplicates, half life = 7 h) (Fig. 7 *B*).

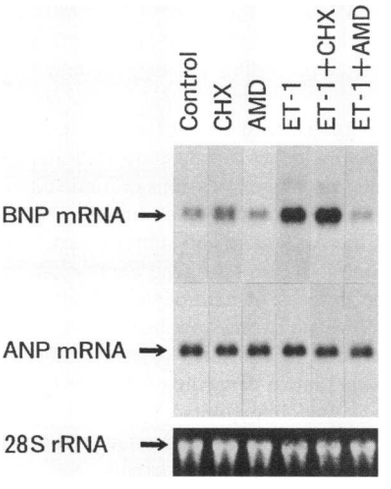

Figure 6. Rapid transcriptional activation of BNP gene in protein synthesis–independent mechanism. Ventricular cardiocytes were treated with cycloheximide (*CHX*) (10 μg/ml) or actinomycin D (*AMD*) (5 μg/ml), in the presence or absence of ET-1 (10^{-8} M). After 1-h incubation, total RNA was extracted for Northern blotting analyses as described in Methods.

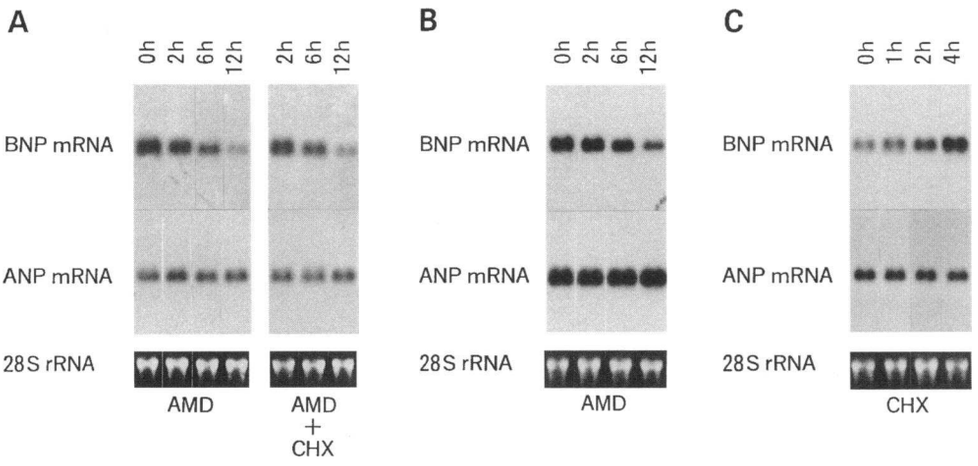

Figure 7. Rapid turnover of BNP mRNA through translation-dependent mechanism in cultured ventricular cardiocytes. (*A*) After the preincubation in serum-free DME, ventricular cells were treated with actinomycin D (*AMD*) (5 μg/ml), or simultaneously treated with actinomycin D and 10 μg/ml of cycloheximide (*AMD+CHX*). The steady state levels of BNP mRNA and ANP mRNA were examined at the indicated times, and the disappearance rates of preexisting mRNAs were evaluated by Northern blotting analyses. (*B*) Ventricular cardiocytes were treated with 10^{-8} M of ET-1 for 36 h. Hypertrophied myocytes were further treated with actinomycin D, and the mRNA disappearance rates were analyzed. (*C*) Ventricular cardiocytes were treated with 10 μg/ml of cycloheximide (*CHX*) for the indicated times, and the mRNA levels of BNP and ANP were examined.

In both states, the ANP mRNA level remained unchanged throughout the experiment period of 12 h (half-life > 12 h).

Turnover rate of BNP mRNA was significantly decreased in the presence of cycloheximide (Fig. 7 *A*), and the treatment with cycloheximide alone resulted in the increase in the steady state level of BNP mRNA; while it did not affect the ANP mRNA level (Fig. 7 *C*). These findings suggest that BNP mRNA is rapidly degraded through a translation-dependent destabilization mechanism in cultured ventricular cardiocytes.

Discussion

In the present study, we examined the regulation of the BNP gene expression, in comparison with that of ANP, in cultured neonatal rat ventricular cardiocytes. During the course of cardiocyte hypertrophy, the rapid induction of the BNP gene expression occurred as fast as the "immediate-early" induction of the c-*fos* gene expression, and reached a maximal level within 1 h. This rapid induction of the BNP gene expression was generated through transcriptional up-regulation, and did not require de novo synthesis of transacting regulatory factors. In contrast, the steady state level of ANP mRNA began to increase 3 h after the stimulation. Accumulation of MLC-2 mRNA and increase in cell size were obviously observed at 12–24 h. These results indicate that the gene expression of BNP is distinctly regulated from that of ANP during the process of cardiocyte hypertrophy, and that the induction of the BNP gene expression is one of the earliest events preceding the establishment of cardiocyte hypertrophy.

Recent studies have demonstrated the differential expressions of several cardiac-specific genes during cardiocyte hypertrophy (27–34). Rapid and transient expressions of immediate-early genes including cellular protooncogenes initially occur. Augmented expression of the ANP gene and subtype switching or up-regulation of the contractile protein gene expression are generated later during the process of cardiocyte hypertrophy. The time course of the BNP gene expression during cardiocyte hypertrophy elucidated in the present study suggests that the BNP gene should be classified into a new category of the inducible cardiac-specific genes, which exhibits characteristics of both "immediate-early" induction and sustained activation of gene expression during myocardial cell hypertrophy.

As was the case with ET-1, phenylephrine or PMA evoked cardiocyte hypertrophy and induced rapid increase in the BNP mRNA level during the course of cell hypertrophy. These results were well consistent with the recent reports (42, 43). Since ET-1, phenylephrine, and PMA activate PKC, the rapid induction of the BNP gene expression is likely to be a common feature seen in cardiocyte hypertrophy evoked by PKC activators. Furthermore, our present study demonstrated that PKC inhibitors significantly suppressed the rapid BNP gene expression induced by ET-1. These results suggest that PKC activation is one of the proximal signaling pathways in the rapid induction of the BNP gene expression, which is consistent with previous reports to show that PKC activation is a common signaling event in the inducible expressions of several cardiac-specific genes during cardiocyte hypertrophy (32, 34). It is known that ET-1 activates several signaling pathways in cardiocytes, such as inositol phospholipid hydrolysis, diacylglycerol formation, and increase in intracellular calcium concentration (31, 33, 34). Since the inhibitory effects of PKC inhibitors upon the ET-1–induced BNP gene expression were not complete even at high concentrations, it remains possible that intracellular events other than PKC activation may also lead to the rapid induction of the BNP gene expression. It will be of further necessity to determine whether activation of PKC is a crucial and restricted pathway to the induction of the BNP gene expression, and how the differential expressions of cardiac-specific genes occur during the process of cardiocyte hypertrophy.

The 5′-flanking promoter region of the ANP gene has been intensively studied (44–49), and the AP-1 (activator protein-1) binding site is shown to be a critical *cis*-element in the induction of the ANP gene expression in cardiac hypertrophy (49). Very recently, the 5′-flanking sequence of the rat BNP gene was characterized, and the promoter analysis indicated that the interaction between GATA element and GATA-4 protein is involved in the cardiac-specific and inducible expression of the rat BNP gene (50, 51). The AP-1 site and GATA element exist in the promoter regions of BNP and ANP in various species including humans (25, 50, 52, 53, Ogawa, Y., H. Itoh, O. Nakagawa, G. Shirakami, N. Tamura, K, Nagata, N. Yoshida, and K. Nakao manuscript submitted for publication), and may play pivotal roles in the gene expressions of BNP and ANP in the heart. However, the mechanism of the rapid transcriptional acti-

vation of BNP in cardiocyte hypertrophy, which we demonstrated in the present study, has not been elucidated. Further studies are necessary to clarify the precise mechanism of the differential gene regulation of BNP and ANP in cardiac hypertrophy.

The present study revealed that the turnover rate of BNP mRNA in cultured ventricular cardiocytes is much faster than that of ANP mRNA. In addition, the translational blockade with cycloheximide decreased the BNP mRNA turnover rate and caused the accumulation of BNP mRNA. BNP mRNA contains an AU-rich sequence including several repeat units of AUUUA motif in the 3′-untranslated region (4, 54). This feature is conserved among species, sharply distinguishing BNP mRNA from ANP mRNA. This AUUUA motif is generally found in the mRNAs of transiently expressed genes for cytokines, growth factors, or cellular protooncoproteins, and is involved in the translation-dependent mRNA degrading mechanism (55–57). BNP mRNA is likely to be rapidly degraded through this selective decay pathway. These observations indicate that the gene expression of BNP is distinctly regulated from that of ANP at a posttranscriptional level as well as at a transcriptional level.

Furthermore, the half-life of BNP mRNA was longer in ET-1–induced hypertrophied cells, as compared with that in control, unhypertrophied cells. Prolongation of BNP mRNA half-life is also observed in phenylephrine-evoked cardiocyte hypertrophy (43), and it has been reported that various vasoactive hormones regulate mRNA stability of crucial molecules in vascular smooth muscle cells or skeletal muscle cells (58, 59). Although the present study showed that the rapid induction of the BNP gene expression by ET-1 is mainly transcriptional (Fig. 6), the sustained elevation of the BNP mRNA level in established cardiocyte hypertrophy may occur not only at transcriptional level but also at posttranscriptional level.

The secretion of BNP from ventricular cardiocytes was immediately stimulated by the treatment with ET-1; while the ANP secretion gradually increased in a time-dependent fashion. The secretory patterns of BNP and ANP were in parallel with the changes of the BNP mRNA and ANP mRNA levels, respectively. These results support the notions that BNP and ANP are secreted from ventricular cells promptly after synthesis via a constitutive pathway, although they are stored in the secretory granules in the atrium and are secreted via a regulated pathway against secretagogues (60). Taken together with the fact that the main source of circulating BNP is the ventricle (14), the ventricular gene expression of BNP can be considered to directly and promptly lead to the alterations in the plasma BNP concentration.

We recently demonstrated that the plasma BNP concentration rapidly and tremendously increases, in comparison with that of ANP, in the clinical course of acute myocardial infarction (21, 22). The increase in the plasma BNP concentration was negatively correlated with the cardiac index, indicating that the changes of the plasma BNP concentration reflect the severity of ventricular dysfunction. Furthermore, we have also demonstrated that, in the rat model of experimental myocardial infarction, the ventricular BNP mRNA level and BNP-LI increase more rapidly than those of ANP not only in infarcted region but also in noninfarcted region (61). These findings suggest that hemodynamic overload directly induces the rapid ventricular expression of the BNP gene in the early course of acute myocardial infarction, and that the gene expressions of BNP and ANP are differentially regulated during the process of ventricular hypertrophy in vivo both in humans and in rats. The present study succeeded in reproducing this striking characteristics of the BNP gene expression using an in vitro culture model of cardiac hypertrophy, which allowed us to precisely examine the temporal profile and the regulatory mechanism of the BNP gene expression during cardiocyte hypertrophy.

Table I. Characteristics of BNP and ANP

	BNP	ANP
Gene expression		
Total mRNA content[9,11,14–16]		
Normal	Ventricle > atrium	Ventricle < atrium
Hypertrophy	Ventricle ≫ atrium	Ventricle ≥ atrium
Induction*	Rapid	Slow
mRNA turnover*	Rapid	Slow
Circulating hormone		
Plasma concentration[14,18,19]		
Basal in normal man	0.9±0.07 fmol/ml	6.4±0.9 fmol/ml
Increase in diseases	1,000-fold	100-fold
Clearance[14]	Slow (long-acting)	Rapid (short-acting)

Parentheses refer to the studies which demonstrated the characteristics of BNP and ANP. Asterisks refer to the present study.

Upon ventricular overload, the rapid induction of the BNP gene expression occurs in the ventricle, and BNP is promptly secreted into circulation. BNP elicits vasodilation, natriuresis, diuresis, and inhibition of renin-angiotensin-aldosterone system (24), and should serve to reduce both cardiac preload and afterload as an "emergency-rescue" hormone against ventricular overload. When the significant atrial overload simultaneously occurs, ANP stored in the atrium is also secreted. The amount of ANP stored in the atrium, however, may often be insufficient to meet the sudden deterioration of the hemodynamic conditions. BNP, rapidly produced in and secreted from the ventricle, should, therefore, work to reduce increased workload in such an adverse circumstance. Subsequently, the ANP gene expression in the heart gradually increases in response to cardiac overload, and BNP and ANP might cooperatively serve to compensate cardiovascular dysfunction in the chronic congestive heart failure.

Furthermore, the demonstrations of the natriuretic peptide receptors in rat and human hearts (62, 63) raise a possibility that BNP and ANP may possess some intracardiac actions during cardiac hypertrophy. We recently demonstrated the production of C-type natriuretic peptide and the expression of its specific receptor in vascular walls, and have proposed that natriuretic peptides should act as local regulators especially for vascular growth inhibition (35, 64, 65). BNP and ANP, produced in response to ventricular overload, may also be relevant to cardiac remodeling including hypertrophy as autocrine and/or paracrine factors.

Table I summarizes the characteristics of BNP and ANP which were elucidated in our present and previous studies. BNP and ANP have various distinct features in tissue specificity and inducibility of the gene expression, mRNA turnover, alteration in the plasma concentration, and clearance from systemic circulation. BNP possesses characteristics suitable for its possible role as an "emergency-rescue" cardiac hormone against ventricular overload. BNP and ANP have distinct and complementary roles in the maintenance of cardiovascular homeostasis as an exquisite dual cardiac natriuretic peptide system.

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