Inhibitory Regulation of Hypertrophy by Endogenous Atrial Natriuretic Peptide in Cultured Cardiac Myocytes

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Abstract—Atrial natriuretic peptide (ANP) may function as an endogenous regulator of cardiac hypertrophy, because the natriuretic peptide receptor has been found in the heart and because mice lacking its receptor have been shown to have a markedly elevated ventricular mass. We examined the role of endogenous ANP in cardiac hypertrophy in vitro. The effects of the blockade of endogenous ANP by its receptor antagonist, HS-142–1, on cell hypertrophy were investigated with the use of cultured neonatal rat ventricular myocytes. HS-142–1 increased the basal and phenylephrine (PE, 10^{-5} mol/L)–stimulated protein syntheses in a concentration-dependent manner (1 to 300 μg/mL). A significant increase in the cell size of myocytes was also induced by this antagonist. In addition, the expression levels of skeletal α-actin, β-myosin heavy chain, and ANP genes, markers of hypertrophy, were partially elevated by treatment with HS-142–1 (100 μg/mL) under nonstimulated or PE-stimulated conditions. A cGMP-specific phosphodiesterase inhibitor, zaprinast (5×10^{-4} mol/L), and a cGMP analogue (10^{-4} mol/L) suppressed the basal and PE-stimulated protein syntheses. Our observations suggest that endogenous ANP inhibits cardiac myocyte hypertrophy under basal and PE-stimulated conditions, probably through a cGMP-dependent process. ANP may play a role as an autocrine factor in the regulation of cardiac myocyte growth. (Hypertension. 2000;35:19-24.)

Key Words: hypertrophy ■ atrial natriuretic peptide ■ autocrine-paracrine ■ myocytes

Atrial natriuretic peptide (ANP) is a cardiac hormone that has an important role in the regulation of body fluid homeostasis and systemic blood pressure.1,2 Once in the circulation, ANP binds to its specific receptor, mainly in the vascular tissue, kidney, and adrenal gland, and increases cellular cGMP levels.3 The cGMP production induces vasodilatation, natriuresis, and diuresis.

Subsequent studies have revealed the existence of natriuretic peptide receptors in cardiac cells.4,5 Therefore, apart from acting as a circulating hormone, ANP may have some function as an autocrine and/or paracrine factor. However, the local actions of ANP on the heart itself have not been fully elucidated. Oliver et al6 recently demonstrated with the use of knockout mouse models that the complete absence of one subtype of natriuretic peptide receptors causes marked cardiac hypertrophy, suggesting the possibility that endogenous ANP suppressively regulates the development of cardiac myocyte hypertrophy. With regard to the direct effect of ANP on cardiac hypertrophy, only one study7 reported that exogenous ANP inhibits cardiac myocyte hypertrophy in the limited conditions. Therefore, we conducted the present study to examine the direct effect of endogenous ANP on cell hypertrophy in cultured ventricular myocytes of neonatal rats. We used a specific antagonist for natriuretic peptide receptors, HS-142–1, which competitively and selectively inhibits ANP binding to its biological (guanylyl1 cyclase [GC]-containing) receptor.8 Several studies have used this antagonist to examine the roles of endogenous ANP in vivo and in vitro.9–12 We also investigated whether endogenous ANP influences the expression of fetal-type contractile protein genes in addition to the protein synthesis in cultured cardiac myocytes. The participation of cellular cGMP in the effect of ANP on protein synthesis was also examined.

Methods

Cell Cultures

Primary cultures of neonatal ventricular myocytes were prepared as described previously.13 Briefly, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced, and dispersed with 0.1% collagenase type II (Worthington Biochemical Corp). To segregate myocytes from nonmyocytes, a discontinuous gradient of Percoll (Sigma Chemical Co) was prepared. After centrifugation, the upper layer consisted of a mixed population of nonmyocyte cell types, and the lower layer consisted almost exclusively of cardiac myocytes. After the myocytes were incubated twice on uncoated 10-cm culture dishes for 30 minutes to remove any remaining nonmyocytes, the nonattached viable cells were plated on gelatin-coated 24-well culture plates or 10-cm culture dishes and then cultured in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies). After a 24-hour incubation in DMEM with...
FCS, the culture medium was changed to serum-free DMEM, and all experiments were performed 24 hours later. This purification procedure has well been established,14,15 and in fact, >95% of the cells obtained by this method were cardiomyocytes.

Protein Synthesis and Cell Size Measurement
The effect of various agents on the protein synthesis in cultured cardiac myocytes was evaluated by the incorporation of [14C]phenylalanine (14C-Phe) into cells, according to the method described by Simpson16 with some modifications. Myocytes were plated on 24-well plates mainly at a density of 6x10^4 cells/cm². Several experiments were performed at a density of 1.5x10^4 cells/cm². After the preconditioning period, HS-142–1 (a gift from Kyowa Hakko Kogyo, Tokyo, Japan), phenylephrine (PE, Research Biochemicals, Inc), endothelin-1 (ET, Peptide Institute), FCS, zaprinast (Biomol Research Laboratories), 3-isobutyl-1-methylxanthine (IBMX, Nakalai Tesque), 8-bromo-cGMP (Sigma), 8-bromo-cAMP (Sigma), and/or rat ANP (Peptide Institute) were added, and 0.3 μCi of 14C-Phe was also added. After the cells were incubated for 24 hours, the radioactivity of aliquots of the trichloroacetic acid--insoluble material was determined by a liquid scintillation counter.

For cell size measurement, 2 or 3 fields in phase-contrast pictures of cultured cardiac myocytes were randomly chosen and photographed, and 50 individual cell surface areas were measured by planimetry.

Northern Blot Analysis
After a 24-hour incubation with treatment of HS-142–1 and/or PE, the cultured myocytes were submitted for RNA extraction. Total RNA was extracted from cultured cells with TRIzol Reagent (Life Technologies). Northern blot analyses were performed with oligonucleotide probes for rat skeletal α-actin mRNA, β-myosin heavy chain (β-MHC) mRNA, and 18S ribosomal RNA and with a cDNA probe for rat ANP mRNA, according to the method previously reported.13,17,18

Measurement of Cellular cGMP
After preincubation, myocytes grown in 24-well plates were treated for 10 minutes with various concentrations of rat ANP, rat brain natriuretic peptide (BNP, Peptide Institute), and/or HS-142–1 in the presence of 5x10^-4 mol/L IBMX, as described previously.19 The reaction was stopped by rapid aspiration of the medium and the addition of ice-cold 70% ethanol. After each ethanol sample was evaporated by a centrifugal evaporator, the dry residue was dissolved in a assay buffer. The cGMP levels were determined by a radioimmunoassay performed with a cGMP assay kit (Yamasa Shoyu Co), as previously reported.20

Measurement of Immunoreactive ANP and BNP
After cardiac myocytes were treated with HS-142–1 and/or PE for 24 hours, the culture medium was aspirated and stored at -80°C. The radioimmunoassay for rat ANP and BNP was performed as previously reported.21

Statistical Analysis
Unpaired t test was used for comparison between the 2 groups. The significance of differences among >3 groups was evaluated by an unpaired ANOVA, and probability values were calculated by the Fisher method. A value of P<0.05 was accepted as statistically significant.

Results
To investigate the secretion levels of ANP and BNP from cultured neonatal rat ventricular myocytes, we examined the immunoreactive (ir)-ANP and ir-BNP concentrations in the medium of cells cultured without FCS. As shown in Table 1, the basal releases of ir-ANP and ir-BNP from myocytes for 24 hours were ~10 pmol and 1 pmol per 10^5 cells, respec-

| TABLE 1. Secretion Levels of ir-ANP and ir-BNP From Cultured Cardiac Myocytes |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | ir-ANP, pmol/10^5 Cells | ir-BNP, pmol/10^5 Cells |
| HS-142-1 Cells                  | HS-142-1 Cells | HS-142-1 Cells | HS-142-1 Cells | HS-142-1 Cells |
| Nonstimulated                   | 7.4±1.1        | 14.0±0.8*       | 0.84±0.04       | 1.22±0.13†      |
| 10^-5 mol/L, PE                 | 13.7±0.9       | 21.1±1.0*       | 1.42±0.24       | 2.26±0.24†      |
| 10^-7 mol/L, ET                 | 14.8±1.4       | 24.5±1.4*       | 1.31±0.16       | 2.17±0.18†      |
| 5% FCS                          | 20.9±4.5       | 49.2±2.9*       | 2.27±0.17       | 3.68±0.25*      |

Values are mean±SE of 4 measurements. Cells were incubated without (-) or with HS-142-1 (100 μg/mL) for 24 hours.

*P<0.01 and †P<0.05 vs HS-142-1 (-).

Figure 1. A, Stimulatory effect of ANP on the production of cellular cGMP in cultured cardiac myocytes. B and C, Inhibitory effect of HS-142–1 on the cellular cGMP levels in the basal (B) and ANP (10^-5 mol/L)-stimulated (C) myocytes. Values are mean±SE of 6 measurements. **P<0.01 vs basal level; ††P<0.01 vs ANP alone.
The expression level of skeletal α-actin mRNA in cardiac myocytes treated with HS-142–1 was significantly higher than that of the controls (Figure 3B). PE elevated the skeletal α-actin gene expression, but a further increase in its level was not obtained by the addition of HS-142–1. The expression of β-MHC mRNA was elevated by HS-142–1 under PE-treated conditions (Figure 3C). The mRNA level of ANP was increased by HS-142–1 under both nonstimulated and PE-stimulated conditions (Figure 3D).

To elucidate whether the inhibitory effect of endogenous ANP on myocyte hypertrophy is causally linked to the increase in cellular cGMP, we examined the effects of 2 phosphodiesterase inhibitors on protein synthesis in cultured cardiac myocytes. A cGMP-specific phosphodiesterase inhibitor, zaprinast, and a nonspecific inhibitor, IBMX, elevated the cellular cGMP levels at doses $\geq 10^{-4}$ mol/L, which is the cell density used in other experiments in the present study. However, the increased uptake of 14C-Phe by treatment with 50 µg/mL HS-142–1 was suppressed significantly by $10^{-6}$ mol/L ANP (Figure 5C). When cultured cells were prepared at a low density (1.5×$10^6$ cells/cm²), the 14C-Phe incorporation levels under both basal and PE-stimulated conditions were decreased by $10^{-6}$ mol/L ANP (Figure 5B).

**Discussion**

The present study has demonstrated for the first time that the blockade of endogenous ANP induces the hypertrophy of cultured neonatal rat ventricular myocytes. HS-142–1, a natriuretic peptide receptor antagonist, clearly increased the protein synthesis and cell size of cardiac myocytes under basal and PE-stimulated conditions. In the presence of an excessive dose of exogenous ANP, however, HS-142–1 failed to increase the protein synthesis (Figure 5C). This result indicates that the effect of HS-142–1 observed in the present study is not due to nonspecific stimulation but really due to the competitive and specific inhibition of ANP binding.

Three receptor subtypes for natriuretic peptides are presently known.22,23 Two of these receptors have GC activity and are called GC-A and GC-B.3,22 Although HS-142–1 is a competitive antagonist for both GC-A and GC-B,8 the observed increase in protein synthesis by this antagonist in the present study appears to be mainly through a GC-A blockade, because rat ventricular myocytes have been reported to produce predominantly GC-A.9 Among the 3 members of the natriuretic peptide family (ANP, BNP, and C-type natriuretic peptide), both BNP and ANP combine with GC-A.3,22 In fact, we confirmed that BNP has an effect that is almost equivalent to the effect of ANP on the production of cellular cGMP in cultured cardiac myocytes. However, the secretion level of ANP in neonatal rat ventricular myocytes was much higher than that of BNP. Therefore, the induction of cell hypertrophy by HS-142–1 obtained in the present study may be due mainly to a blockade of endogenous ANP.

Oliver et al6 recently reported that hypertension and cardiac hypertrophy were found in GC-A knockout mice; these mice lacking GC-A had elevated blood pressure and hearts exhibiting marked hypertrophy with interstitial fibrosis. Mice homozygous for disruption of the pro-ANP gene have no circulating or tissue ANP, and they exhibit increased heart weight and blood pressure when maintained on intermediate salt diets.24 Transgenic mice overexpressing ANP have a low heart weight under normoxic conditions and a blunted right ventricular hypertrophy response to hypoxia-induced pulmonary hypertension.25 These observations suggest that ANP may be closely associated with the progression of myocardial hypertrophy. However, the issue of whether the influence of ANP on cardiac hypertrophy is a direct effect of its peptide or secondary to the change of blood pressure levels was not resolved by these studies using knockout or transgenic mice.
With regard to the direct effect of ANP on cardiac hypertrophy, a very recent report (Calderone et al 7 ) has shown that exogenous ANP and cGMP inhibit the protein synthesis of neonatal cardiomyocytes under limited conditions, that is, in norepinephrine-stimulated cells cultured at a low density (1 to $2 \times 10^4$ cells/cm$^2$). The study of Calderone et al and another study 26 showed that the inhibitory effect of cGMP was absent in cells cultured at a high density ($1 \times 10^5$ cells/cm$^2$). Therefore, the study of Calderone et al could not determine whether endogenous ANP may have an inhibitory effect on myocyte hypertrophy. Our present study demonstrates that endogenous ANP has a direct action on myocyte hypertrophy, independent of the hemodynamic change, in the cells cultured even at a high density ($1 \times 10^5$ cells/cm$^2$). In addition, the present results have shown that myocyte hypertrophy produced by the blockade of endogenous ANP by HS-142–1 is partially accompanied by increases in the expression of skeletal $\alpha$-actin, $\beta$-MHC, and ANP genes, which are genetic markers for cardiomyocyte hypertrophy. These results indicate that endogenous ANP may influence the growth of neonatal cardiac myocytes with qualitative changes.

Regarding the mechanism of inhibitory effect of ANP on cellular hypertrophy, we have obtained some evidence of a causal relation between cGMP production and inhibition of cell hypertrophy by ANP. ANP markedly increased the cGMP levels in cells, and a cGMP-specific phosphodiesterase inhibitor and a cGMP analogue suppressed the basal and PE-stimulated protein syntheses. These results suggest that

**Figure 3.** Effect of HS-142-1 on the expression of skeletal $\alpha$-actin, $\beta$-MHC, and ANP genes in cultured cardiac myocytes. A, Representative image of Northern hybridization in cells incubated without or with HS-142–1 (100 $\mu$g/mL) and/or PE ($10^{-5}$ mol/L). B to D, Quantitative analysis of skeletal $\alpha$-actin, $\beta$-MHC, and ANP gene transcripts in nonstimulated (open bars) and PE-stimulated (solid bars) myocytes. Values shown were corrected by using the density of the corresponding 18S ribosomal RNA. Data are mean±SE of 4 measurements. *$P<0.05$ and **$P<0.01$ vs control; †$P<0.05$ and ††$P<0.01$ vs PE alone.

**Figure 4.** Effects of phosphodiesterase inhibitors (A) and cGMP and cAMP analogues (B) on protein synthesis in cultured cardiac myocytes. Cells were incubated without (-) or with zaprinast ($5 \times 10^{-4}$ mol/L), IBMX ($5 \times 10^{-4}$ mol/L), 8-bromo-cGMP (Br-cGMP, $10^{-4}$ mol/L), or 8-bromo-cAMP (Br-cAMP, $10^{-3}$ mol/L) under unstimulated (open bars) and PE ($10^{-5}$ mol/L)-stimulated (solid bars) conditions. Values are mean±SE of 8 measurements. **$P<0.01$ vs control; †$P<0.05$ and ††$P<0.01$ vs PE alone.

**Figure 5.** Effect of exogenous ANP on protein synthesis in cultured cardiac myocytes incubated at a high density ($6 \times 10^4$ cells/cm$^2$) (A and C) and low density ($1.5 \times 10^4$ cells/cm$^2$) (B). Cells were incubated under unstimulated (open bars) and PE ($10^{-5}$ mol/L)-stimulated (solid bars) conditions. In panel C, cells were incubated without (control) or with ANP ($10^{-6}$ mol/L) and/or HS-142–1 (50 $\mu$g/mL). Values are mean±SE of 6 to 8 measurements. **$P<0.01$ vs control; †$P<0.05$ and ††$P<0.01$ vs PE alone; §§$P<0.05$ vs HS-142–1 alone in nonstimulated conditions; and $\cdot\cdot\cdot P<0.05$ vs HS-142–1 alone in PE-stimulated conditions.
endogenous ANP and exogenous ANP inhibit the protein synthesis in cardiac myocytes, probably through a cGMP-dependent process. We cannot deny the possibility that the increase in the cellular cGMP level after treatment with a cGMP-specific phosphodiesterase inhibitor may be derived not only from endogenous ANP but also from endogenous nitric oxide. However, the cGMP production in the presence of a considerable amount of ANP is thought to be derived mostly from ANP, because the cellular cGMP production in the presence of 10^{-8} mol/L ANP was inhibited almost completely by HS-142–1.

In the present study, both the increase in protein synthesis by HS-142–1 and its decrease by phosphodiesterase inhibitors were greater in PE-stimulated myocytes than in non-stimulated cells. These data indicate that the effects of endogenous ANP and cGMP are probably accelerated by the stimulation with PE. Some studies have shown that ANP also inhibits the catecholamine-induced and growth factor–induced DNA synthesis of cultured rat cardiac fibroblasts.4,7,27 Although ANP expression is minimal in normal adult ventricular myocardium, cardiac overload and hypertrophy induce ANP production in the ventricles.28–31 In human failing or hypertrophied hearts, the expression of ANP is remarkably induced, and considerable levels of its peptide are detected in the ventricles.28–30 These findings lead to the possibility that endogenous ANP plays a role as an autocrine and/or paracrine inhibitory regulator against excessive cardiac cell growth in some pathological states, such as heart failure and cardiac hypertrophy. However, the present study was performed with the use of cultured neonatal, not adult, cardiac myocytes of rats, as in many other studies. Alternatively, it is possible that ANP regulates the myocardial growth during development, in view of the fact that fetal and neonatal myocardium express the ANP gene and peptide even in the normal state.13,28,32 Further investigations are necessary to clarify the physiological and pathophysiological effects of endogenous ANP on cardiac myocytes.

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