Stretch-Activated Atrial Natriuretic Peptide Secretion in Atria with Heat Shock Protein 70 Overexpression

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The mechanical overload of the heart is known to induce the expression of atrial natriuretic peptide (ANP) and heat-shock protein 70 (HSP70) in the heart. However, the relationship between these two substances remains unknown. In the present study, we characterized ANP secretion from mouse atria and examined a possible role that HSP70 may play in the regulation of ANP synthesis and secretion by using atria in transgenic mice in which HSP70 was overexpressed. We generated transgenic mice harboring the human *HSP70* gene under the transcriptional control of human myosin heavy-chain promoter. In these mice, the transgene was overexpressed in the heart. Both atrial ANP messenger RNA and its concentration in the HSP70 transgenic mice were measured; these were not significantly different from those in wild-type mice. In isolated perfused nonbeating atria, basal secretion of ANP was similar in both groups. When atrial volume was increased by changing atrial pressure, extracellular fluid (ECF) translocation and ANP secretion proportionately increased. Changes in atrial volume and ECF translocation and ANP secretion were positively correlated. However, these parameters did not significantly differ between the two groups. Endothelin-1 (ET-1), the strongest paracrine stimulus of ANP secretion, accentuated stretch-activated ANP secretion without significantly changing mechanically stimulated ECF translocation, as compared with that in the wild-type mice. The increased ANP secretion due to ET-1 in the transgenic mice was similar to that in the wild-type mice. The results suggest that both atrial stretching and ET-1 are important stimuli to ANP secretion from mouse atria, and the responsiveness of the ANP system to those stimuli are unlikely coupled to the pathway involving HSP70. Exp Biol Med 228:200–206, 2003

Key words: HSP70; atrium; atrial natriuretic peptide; secretion; endothelin-1; stretch; synthesis

This work was supported by the Korea Health 21 R&D Project, Ministry of Health & Welfare (HMP-01-PJ1-PG1-01CH06-0003), Republic of Korea.

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Received March 18, 2002.
Accepted October 16, 2002.

1535-3702/03/2282-0200$15.00

The cardiac atrium is well known to be an endocrine gland that participates in the regulation of body fluid and electrolyte balance by secreting atrial natriuretic peptide (ANP) (1). ANP is secreted mainly from the atria into blood stream in response to atrial stretch, hypoxia, endothelin-1 (ET-1), and protein kinase C activator (2–4). ANP secretion results in diuresis, natriuresis, vasorelaxation, and an inhibition of aldosterone release (1, 2). The genetic expression of ANP mRNA in the atrium gradually increases with aging, whereas in the ventricle, such expression decreases abruptly after birth (5, 6). However, ANP mRNA in the ventricle can be reactivated by an increased cardiac workload as observed in work induced cardiac hypertrophy (7).

Heat shock proteins (HSPs) constitute a universal intracellular response to stress. There are five major groups of HSPs based on molecular size. Much of the work on HSPs has focused on HSP70. HSP70 and HSP90 are molecular chaperones required for the proper folding and trafficking of many proteins involved in signal transduction (8, 9). HSP70 also has been shown to regulate the binding of secretory precursor polypeptides to the membrane of the endoplasmic reticulum (10), suggesting that HSP70 may play a role in regulating hormonal secretion. Knowlton *et al.* (11) and Izumo *et al.* (12) have shown that a single myocardial stretch or pressure overload can stimulate the expression of myocardial HSP70. Other factors known to increase HSP70 synthesis are heat shock, acute myocardial ischemia, acute hypoxia, and cooling (13, 14). Peptide hormones such as ANP (15), insulin (16), and vasopressin (17) are other triggers for HSP70 synthesis. In general, HSPs are the major proteins participating in the adaptation to stress (13), resistance of the heart to ischemia, and reperfusion after heat shock (18, 19).

The mechanical overloading of the heart has been shown to induce the expression of HSP70 and/or ANP (12, 20). Izumo *et al.* (12) reported that the induction of HSP70 and cellular proto-oncogenes are early responses to pressure

overload, whereas the reinduction of the genes normally expressed only in perinatal life, such as the gene for ANP, is a later event. The rapid induction of HSP70 with aortic banding has been interpreted as evidence for a role of HSP70 in myocardial hypertrophy (12, 20) even though the exact role of HSP70 in cardiac hypertrophy and its mechanism are not well defined. In contrast, endogenous ANP suppressively regulates the development of cardiac hypertrophy (21). Kiemer *et al.* (15) also reported that during perfusion and reperfusion period, rat liver ANP causes increases in HSP70 protein concentration and its mRNA level. Therefore, HSP70 may have an opposite action on cardiac hypertrophy and may possibly affect ANP system. In this study, we tested whether overexpression of HSP70 in the mouse heart influences the synthesis of ANP and its secretion. For this purpose, we first characterized the ANP secretion from isolated perfused nonbeating mouse atria.

Materials and Methods

Animals. Transgenic mice that had a fusion gene for the α-cardiac myosin heavy-chain promoter were created by using the human *hsp70* gene and standard methods described previously (22). HSP70 overexpression in the heart was confirmed by Western blot analysis and a reverse-transcriptase polymerase chain reaction (RT-PCR). The atria from the transgenic mice (12–15 weeks of age) and male littermates were used. This study was approved by our Institution's Animal Care and Use Committee and was conducted according to applicable animal care guidelines.

Isolated Perfused Atrial Preparation. Mice were sacrificed by cervical dislocation, and an isolated perfused atrial preparation was made by using a method described before (23, 24). Briefly, the heart was rapidly removed and placed in oxygenated physiologic saline solution. The left atria were dissected through the atrioventricular sulcus of the heart. A Tygon cannula containing two small catheters sealed within it was carefully inserted into the left atrium and was secured by using ligatures around the atrioventricular sulcus. The cannulated atrium was fitted into an organ chamber containing buffer solution at 36.5°C and was fixed with a watertight silicone rubber cap. The atrium was immediately perfused with HEPES buffer solution by using a peristaltic pump at a rate of 0.1 ml/min. The composition of buffer solution was NaCl 118 m*M*, KCl 4.7 m*M*, $CaCl_2$ 2.5 m*M*, $MgCl_2$ 1.2 m*M*, $NaHCO_3$ 25 m*M*, HEPES 20 m*M*, glucose 10 m*M*, and bovine serum albumin (BSA) 0.1%; the pH was adjusted to 7.40 ± 0.03 by using 1.0 *N* NaOH. The perfusate was equilibrated with oxygen through a gas chamber and the pericardial buffer solution, which contained [^{3}H]-inulin to measure the translocation of extracellular fluid (ECF) through the atrial endocardium, was oxygenated through silicone tubing coils located in the pericardial space. The pericardial space of the organ chamber was tightly sealed and was then connected with a calibrated microcapillary tube by which changes in atrial volume were monitored. After a 30-min stabilization, the perfusate was continuously collected at 4-min intervals at 4°C throughout the experiments. After two collection periods, atrial distension was induced for 4 min at 20-min intervals by elevating the outflow catheter tip (2, 6, or 10 cm of H_2O), and atrial contraction was induced by lowering the position of catheter tip to the basal level.

To test the effect of ET-1 on ANP secretion, HEPES buffer containing ET-1 (10^{-8} *M*) was perfused into the atrial lumen, and the protocol just described was performed.

Measurement of ECF Translocation. ECF translocated from the atria was measured as described previously (24). At the start of the atrial perfusion, the pericardial buffer solution contained [^{3}H]-inulin at a concentration of 15.0 μCi/ml. Radioactivities in the perfusate and pericardial buffer were measured by using a liquid scintillation counter, and the amount of ECF translocated through the atrial wall was calculated as follows: ECF translocated in microliters per minute per gram of tissue wet weight = total radioactivity in the perfusate in counts per minute per minute divided by radioactivity in the pericardial reservoir in counts per minute per microliter multiplied by atrial wet weight in grams.

Radioimmunoassay (RIA) of Immunoreactive ANP. The concentrations of immunoreactive ANP in perfusates and tissue homogenates were measured by using RIA, as described previously (23–25). RIA was performed in Tris-acetate buffer (0.1 *M*, pH 7.4) containing 0.2% neomycin, 1.0 m*M* EDTA, 50 benzoyl arginine ethyl ester units per milliliter of soybean trypsin inhibitor, 200 kallilkrein-inhibiting units per milliliter of aprotinin, 0.4% phenylmethyl sufonylfluoride (PMSF), 0.02% sodium azide, and 1% bovine serum albumin (BSA). The standards and samples were incubated with anti-ANP antibody and [^{125}I]-ANP for 24 hr at 4°C. Free and antibody-bound forms were separated by using a charcoal suspension. RIA was performed on the day of the experiments, and all samples in an experiment were analyzed in a single assay. The molar concentration of ANP released was calculated as follows (24, 25): ANP released in micromoles = ANP secretion in picograms per minute per gram per ECF translocated in microliter per minute per gram × 3,060. The denominator 3,060 refers to the molecular mass of ANP, that is, 1-28, because the ANP secreted was found to be predominantly processed ANP (24, 25).

Western Blot Analysis. To determine the expression of HSP70 in various tissues, positive and negative littermates of the HSP70 transgenic mice were sacrificed after they were screened for the transgene. Total tissue lysates were extracted by using extraction buffer (which contained 10 m*M* Tris-HCl with 1 m*M* EDTA, 150 m*M* NaCl, 1% Triton X-100, 1 m*M* PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 50 m*M* NaF, and 0.2 m*M* Na_3VO_4) and were sonicated briefly. Lysates were separated by using a SDS-polyacrylamide gel electrophoresis and were transferred onto a nitrocellulose filter by using standard techniques. After immunoblotting was performed, the membrane with the anti-HSP70 antibody (W27; Santa Cruz Biotechnology,

Santa Cruz, CA) was incubated with a peroxidase-conjugated anti-mouse secondary antibody (Jackson Immuno-Research Laboratories, West Grove, PA), and was finally visualized by using an enhanced chemiluminescence detection kit (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ). The amount of loaded proteins was measured by using Ponceau S stain.

RT-PCR for HSP70. RT-PCR was performed as described previously (22, 26, 27). We suspended 5 μg of total RNA from the atria in 20 μl of RT buffer containing 10 m*M* Tris (pH 8.3); 50 m*M* KCl; 5 m*M* $MgCl_2$;1 m*M* each of dATP, dCTP, dGTP, and dTTP; 20 units of ribonuclease inhibitor; 2.5 μ*M* random hexamers; and 150 units of Moloney leukemia virus RT (Perkin Elmer, Branchburg, NJ). mRNA was reverse transcribed at room temperature for 10 min and at 42°C for 30 min. The reaction was stopped by means of heat inactivation at 99°C for 5 min and then by chilling on ice. The PCR buffer, which contained 10 m*M* Tris (pH 8.3); 50 m*M* KCl; 2 m*M* $MgCl_2$; 200 μ*M* each of dATP, dCTP, dGTP, and dTTP; 2.5 units of Taq polymerase; and 1.5 p*M* human HSP70 specific primers (5′-GGTGTGTAACCCCATCATCAGC-3′ and 5′-TCATCTCTGCATG TAGAAACCG-3′), was added to make a final volume of 50 μl. The temperature profile of the amplification consisted of the following: a 30-sec denaturation at 95°C, a 1-min annealing at 58°C, and a 1-min 30-sec extension at 72°C for 30 cycles. PCR products were separated on 2% agarose gels, and bands were visualized by ethidium bromide staining. PCR products obtained from heart of HSP70 transgenic mice were confirmed by sequence analysis and were used as a positive control. PCR without RT was also performed as a negative control.

Measurement of ANP mRNA. Atrial ANP mRNA was measured by means of Northern blot analysis as described previously (26). Total RNA from the atria was extracted by using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer's protocol. RNA samples were quantitated by means of spectrophotometry at 260 nm. For Northern blot analysis, 15 μg of RNA was denatured with glyoxal, separated by size on 1.2% agarose gels, and transferred to a GeneScreen (NEN Research Products, Boston, MA). A complementary DNA probe for rat ANP was made by using RT-PCR. Probes were radiolabeled with the random priming method according to the manufacturer's instructions (Prime-a-Gene; Promega, Madison, WI). Specific activities of probes were typically 2–3 × 10^9 dpm/μg. Hybridizations were performed at 65°C for 20 hr in 4× SSC, 2× Denhardt's solution, 0.1% SDS, and 10 μg/ml salmon sperm DNA. Blots were washed at 65°C with SCC twice and 0.1% SDS, and signals were visualized by means of autoradiography at −70°C with an exposure of 1–5 days with an intensifying screen. In all experiments, the integrity, equivalent loading, and complete transfer of the RNA samples were established by means of ultraviolet shadowing of the blot prior to hybridization.

Statistics. The statistical significance of the differences was tested by using an analysis of variance (ANOVA) followed by the Duncan multiple range test. A P value of less than 0.05 indicated a significant difference. The results are given as the mean ± SEM.

Results

ANP mRNA and Its Concentration in Hearts with HSP70 Overexpression. HSP70 protein was mainly overexpressed in the heart of transgenic mice, as shown Figure 1. The PCR product of HSP70 in the atria was observed only in the transgenic mice and not in the wild-type mice (Fig. 2). The wet weights of the atria and ventricles in the mice with HSP70 overexpression were not significantly different from those in the wild-type mice. The concentrations of ANP in the left atria of the transgenic mice (n = 6) were 101.33 ± 5.94 ng/mg wet weight, which was significantly higher than that of the right atria (81.17 ± 5.02 ng/mg wet weight, $P < 0.01$; Fig. 3A). The concentrations of ANP in the left ventricles of the transgenic mice (n = 6) were 4.58 ± 0.62 ng/mg wet weight, which was significantly lower than that of the right ventricles (12.02 ± 0.96 ng/mg wet weight, $P < 0.001$; Fig. 3B). However, no differences in the ANP concentrations in the atria and ventricles were found between the two groups (Fig. 3, A and B). The genetic expression of ANP mRNA in the atria was also similar between the two groups (n = 3; Fig. 3C).

Stretch-Activated ANP Secretion. Changes in atrial pressure by 2, 6, or 10 cm of H_2O from the basal level resulted in atrial stretch and contraction, which increased atrial volume (323.9 ± 125.6, 661.5 ± 113.0, or 1391.1 ± 180.8 μl/g wet weight, respectively; Fig. 4B). Proportional increases in atrial volume induced by the elevation of atrial pressure were not significantly different between the two groups.

The basal rate of ANP secretion from the atria of the wild-type mice before atrial stretch was 4.55 ± 1.40 ng/min/g (n = 7 at the second period), which was not different from that of transgenic mice (2.92 ± 0.64 ng/min/g, n = 9; Fig. 4C). Atrial distension induced by increased atrial pressure caused proportional increases in ANP secretion, with peak values of 5.18 ± 1.38, 10.98 ± 2.26, and 22.52 ± 4.10 ng/min/g; these values were not different from those of the

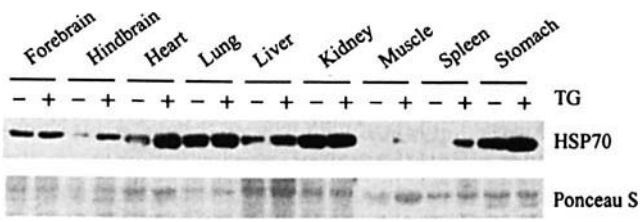

Figure 1. Expression of HSP70 protein in various tissues of transgenic and wild-type mice. Total tissue extracts from HSP70 transgenic (TG, +) mice and wild-type (TG, −) were prepared and analyzed for HSP70 protein by using anti-HSP70 antibody. The amount of loaded protein was equal in all sets of samples, as shown in the bottom panel of the Ponceau S stains. HSP70 was mainly overexpressed in the hearts of the transgenic mice.

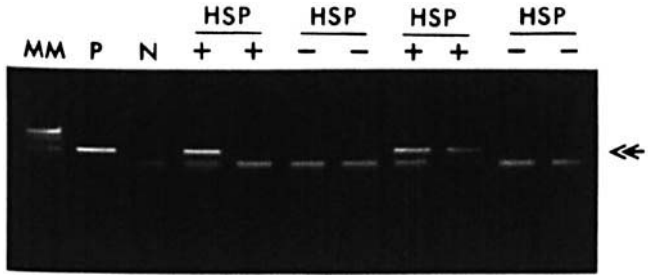

Figure 2. RT-PCR analysis of atrial mRNA in transgenic and wild-type mice. The HSP70 PCR product in the atria was observed only in the transgenic mice and not in the wild-type mice. The arrow indicates the size of the HSP70. HSP+, HSP70 transgenic mice; HSP−, wild-type mice; MM, DNA molecular weight size marker (Hae III); N, negative control; P, positive control.

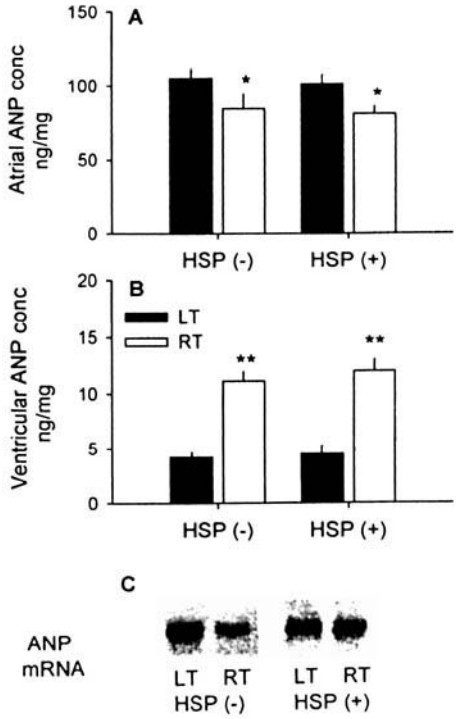

Figure 3. Tissue concentrations of atrial (n = 6; A) and ventricular ANP (n = 5; B), and atrial ANP mRNA (n = 3; C) in the transgenic and wild-type mice. Atrial ANP concentrations on the left side were higher than those of the right side. Ventricular ANP concentrations on the left side were lower than those of right side. However, no significant differences in tissue ANP concentration were found between the two groups. Solid column, the left side; open column, the right side. LT, the left side; RT, right side. An asterisk indicates significant difference from left side, $P < 0.01$; A double asterisk indicates significant difference from left side, $P < 0.001$.

transgenic mice (5.84 ± 1.22, 12.01 ± 3.13, and 21.57 ± 5.71 ng/min/g, respectively).

We previously showed that in isolated perfused beating and nonbeating atria of rats and rabbits, ANP secretion is regulated by two-step sequential mechanism (24). Therefore, to determine whether atrial ANP is sequentially secreted from atrial myocytes to the lumen in mice, the translocation of ECF was simultaneously measured with ANP secretion by assessing the clearance of [^{3}H]-inulin. The

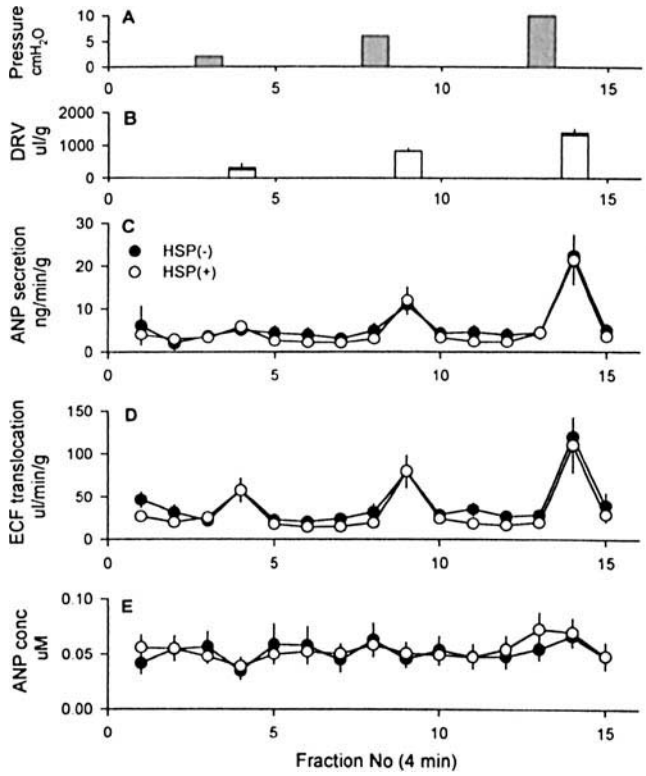

Figure 4. Increased atrial pressure (A) and changes in atrial volume (B), ANP secretion (C), ECF translocation (D), and ANP concentration (E) in isolated perfused nonbeating atria from transgenic (●, n = 9) and wild-type (○, n = 7) mice. Atrial pressure was increased at 20-min intervals from the basal level to 2, 6, or 10 cm of H_2O, and perfusate was collected at 4-min intervals. ANP secretion and ECF translocation proportionately increased in response to increased atrial volume. ANP conc, ANP concentration; DRV, distension and reduction volume; Fraction No, fraction number in the 4-min interval.

basal rate of ECF translocation in the wild-type mice was 31.73 ± 8.21 μl /min/g (n = 7 at the second period), which was not different from that of the transgenic mice (20.24 ± 3.51 μl/min/g, n = 9; Fig. 4D). Mechanical stimulation caused proportional increases in ECF translocation, with peak values of 58.07 ± 11.84, 79.71 ± 15.63, and 120.0 ± 22.68 μl/min/g, which were not different from those of the transgenic mice (57.76 ± 13.98, 79.88 ± 18.73, and 110.54 ± 32.20 μl/min/g, respectively). ECF translocation and ANP secretion caused by changes in atrial pressure increased during the period of atrial contraction from the level at distension to the basal level, but ECF did not increase during the period of atrial distension. The concentration of ANP secreted into atrial lumen in relation to ECF translocation was not significantly changed by the increased atrial pressure (Fig. 4E).

Figure 5 shows the relationships between changes in atrial volume, ECF translocation, and ANP secretion in response to increased atrial pressure in the two groups. Changes in atrial volume were proportional to changes in atrial pressure (Fig. 5A). Changes in ECF translocation and ANP secretion positively correlated with changes in atrial volume (Fig. 5, B and C), and ANP secretion was also found

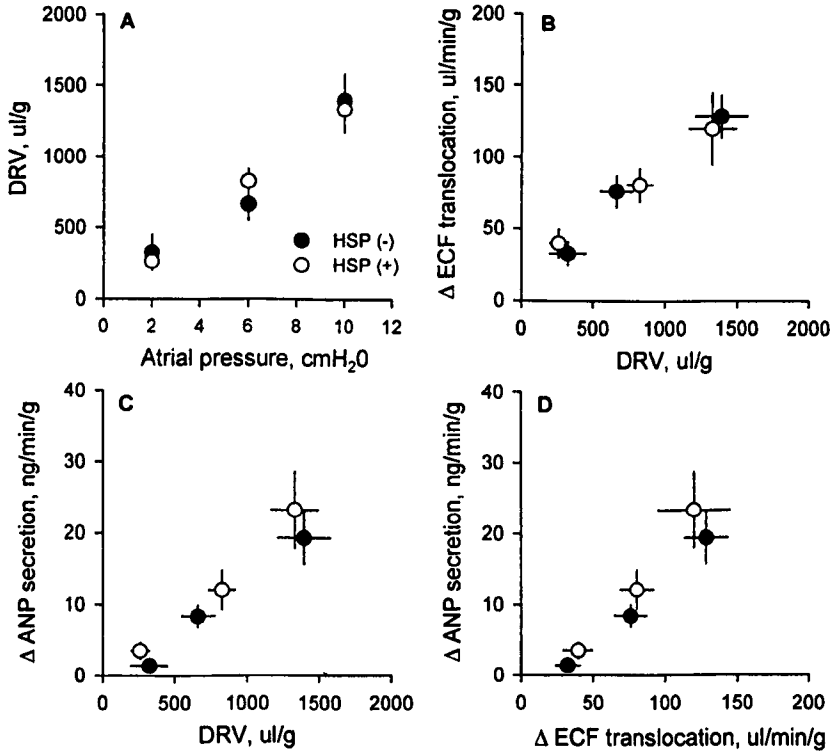

Figure 5. Relationship between intraatrial pressure, volume change, mechanically stimulated ECF translocation, and ANP secretion in the transgenic and wild-type mice. Mechanically stimulated ECF translocation and ANP secretion were calculated by subtracting the previous two values from the two peak values obtained after each pressure application, as shown in Figure 4. These parameters were positively correlated. No significant differences between the two groups were found. ANP conc, ANP concentration; DRV, distension and reduction volume; Fraction No, fraction number in the 4-min interval. ●, wild-type mice; ○, transgenic mice.

to be a function of ECF translocation (Fig. 5D). However, differences in these parameters between the two groups were not significant.

ANP Secretion Stimulated by ET-1. To test whether ET-1 stimulates stretch-activated ANP secretion in mice, the atria were perfused with HEPES buffer containing ET-1 (10^{-8} *M*). In wild-type mice, ET-1 increased basal ANP secretion and accentuated stretch-activated ANP secretion without significant differences in atrial volume and ECF translocation. Therefore, the relationship between mechanically stimulated ECF translocation and stretch-activated ANP secretion shifted upward and leftward (Fig. 6B). In the transgenic mice, an accentuation of stretch-activated ANP secretion by ET-1 was also observed, but no significant differences were found between the two groups (Fig. 6D). Figure 7 shows the comparison of stretch-activated ANP secretion in relation to mechanically-stimulated ECF translocation in response to ET-1; differences between the two groups were not significantly different.

Discussion

Although the mouse is an useful animal for creating genetic models using molecular biology techniques, only a very few reports are available on the ANP system due to the difficulty in performing such experiments. The present study attempted to characterize the ANP secretion from mice atria. Measurements of pressure-induced volume changes, ECF translocation, and ANP secretion from isolated perfused nonbeating atria were successful and reproducible. In species comparisons, the basal secretion of ANP in the mice was similar to that of rats and rabbits (24, 28). However, the basal rate of ECF translocation in the mice was 31.73 ± 8.21 μl/min/g, which was higher than that of rats and rabbits (6.79 ± 0.82 and 2.85 ± 0.65 μl/min/g, respectively) (24, 28). The larger volume of ECF translocated may have lowered the basal ANP concentration in mice as compared with rats and rabbits. The discrepancy in the basal ECF translocation among different species is likely due to differences in the atrial wall thickness. The isolated atria model appears to be an elegant model, and with a better hypothesis, much interesting work should be able to be done.

Cho *et al.* (24) proposed that the process of ANP secretion involves two steps: initially, ANP is released from atrial myocytes into the extracellular space by atrial distension, which is followed by the translocation of ECF containing ANP into the lumen by atrial contraction. Therefore, the secretion of atrial ANP is closely correlated with volume changes of atria and the translocation of ECF (24, 25). Atrial stretch (2, 3) and contraction from stretch are essential for the secretion of ANP (23). In the present study, the enhancement of ECF translocation and ANP secretion by increased atrial volume were apparent during atrial contraction from distension but not during atrial distension. A positive correlation was detected between atrial volume changes, mechanically stimulated ECF translocation, and stretch-activated ANP secretion. Our observation suggests that ANP secretion from mice atria perhaps involves the two-step mechanism reported in rats and rabbits (24, 28, 29).

To determine whether the stretch-activated ANP secre-

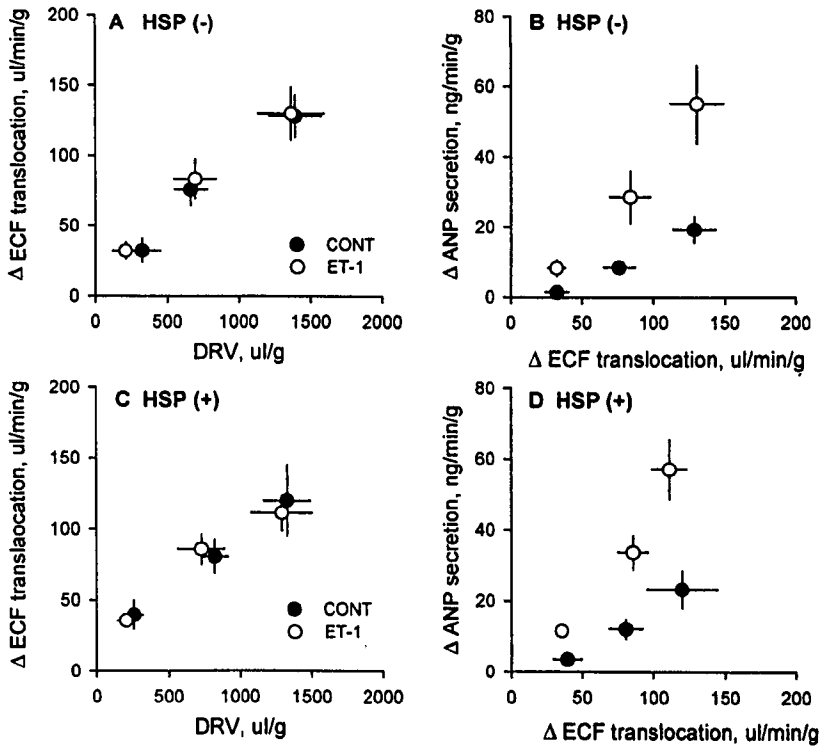

Figure 6. Responsiveness to ET-1 (10^{-8} *M*) on atrial volume change, mechanically stimulated ECF translocation, and ANP secretion in wild-type mice ($n = 8$; A and B) and transgenic mice ($n = 8$; C and D). ET-1 augmented stretch-activated ANP secretion without changing mechanically stimulated ECF translocation or atrial volumes in wild-type and transgenic mice. Therefore, ET-1 shifted the relationship between ANP secretion and ECF translocation upward and leftward (B and D). ●, control atria; ○, atria perfused with ET-1.

tion from mice atria responds to ET-1, a potent vasoconstrictor produced by vascular endothelial cells was perfused into mice atria. ET-1 increased both basal and stretch-activated secretions of ANP. These findings are consistent with those of others (30). However, ET-1 did not affect atrial volume changes or mechanically stimulated ECF translocation. Therefore, the mass of ANP released was markedly increased by ET-1. From these data, we suggest that ET-1 facilitated the initial step, i.e., the release of ANP from atrial myocytes into the surrounding extracellular space. This result also supports the hypothesis that ANP secretion in mice takes place in two steps.

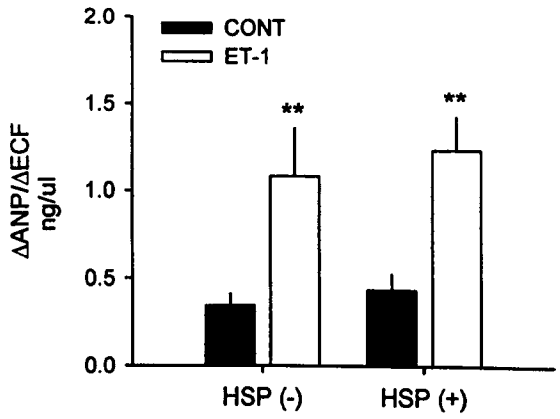

Figure 7. Comparison of stretch-activated ANP secretion in terms of ECF translocation due to ET-1 in the transgenic and wild-type mice. The accentuation of stretch-activated ANP secretion due to ET-1 in the transgenic mice was not significantly different from that of the wild-type mice. Solid column, the control atria; open column, atria perfused with ET-1. A double asterisk indicates significant difference from control atria, $P < 0.01$.

In the present study, no correlation was demonstrated between overexpression of HSP70 and the ANP secretion. The results suggest that the synthesis and secretion of ANP in mouse atria may not be regulated by HSP70, i.e., the two systems may be operated independently of each other. However, this was rather unexpected because the genes encoding these two proteins express in sequence after mechanical stretch. Alternatively, the impact of HSP70 on the ANP system may take place early in life such that the biological adaptation may be acquired before the mouse grows older. Thus, in adult mice used in this study, the functional connection between HSP70 and the ANP system may no longer be detectable. The genetic manipulation may also not be sufficient enough to produce any measurable functional changes in the ANP system. For example, an additional unknown factor may be necessary and not readily available in this genetic manipulation.

In summary, the ANP secretion pathway was characterized in isolated mouse atria and the influence of HSP70 overexpression was evaluated. The ANP secretion in mouse atria was comparable with other species and appeared to occur in two sequential steps. The results suggest that atrial stretch and ET-1 are important stimuli to ANP secretion from mouse atria. The responsiveness of ANP system to those two stimuli was not altered by HSP70 overexpression. Regardless, the mouse model presented in this study will be very useful in assessing the functional consequences of genetic alterations with respect to their influence on atrial ANP systems.

The authors thank Professor Hye Kyung Lee in the Department of Pharmacology (University of Ulsan College of Medicine) for giving valuable comments. We also thank Keum Nim Koh and Kyung Sun Lee for their assistance in the screening of the transgenic mice.

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