Original article

Hyperthermia treatment prevents angiotensin II-mediated atrial fibrosis and fibrillation via induction of heat-shock protein 72

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Abstract

We tested the hypothesis that atrial fibrosis and atrial fibrillation (AF) evoked by angiotensin II (AII) could be prevented by the induction of heat-shock protein 72 (HSP72) by hyperthermia (HT). In cultured atrial fibroblasts isolated from male Sprague–Dawley rats, HT (42 °C) was applied for 30 min. AII (100 nmol/L) was added to the medium 8 h later. HT induced the expression of HSP72, which was associated with the attenuation of AII-induced extracellular signal-regulated kinase (ERK1/ERK2) phosphorylation, α-smooth muscle actin (α-SMA) expression, transforming growth factor-β_1 secretion, collagen synthesis, and expression of collagen type I and tissue inhibitor of metalloproteinases-1. A small interfering RNA targeting HSP72 abolished these anti-fibrotic effects of HT. In male Sprague–Dawley rats in vivo, an osmotic mini-pump was subcutaneously implanted for continuous infusion of AII (400 ng/kg/min). Whole-body HT (43 °C, 20 min) was applied 24 h before and 7, 14, and 21 days after the start of the AII infusion. Repeated HT led to the induction of HSP72 expression, which resulted in an attenuation of AII-induced left atrial fibrosis. In an electrophysiological study using isolated perfused heart, continuous AII caused slowing of interatrial conduction without affecting atrial refractoriness. In AII-treated hearts, extrastimuli from the right atrial appendage resulted in a high incidence of repetitive atrial responses, which were suppressed by treatment with HT. Our results suggest that HT treatment is effective in suppressing AII-mediated atrial fibrosis and AF via induction of HSP72 at least in parts, and is thus expected to be a novel strategy for prevention of AF.
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1. Introduction

Atrial fibrillation (AF) is the most common arrhythmia in the clinical setting and is promoted by activation of the renin–angiotensin system [1,2]. Angiotensin II (AII) has been reported to participate in the maintenance of AF by the induction of atrial structural remodeling, which is characterized by interstitial fibrosis [3,4]. Two-thirds of the cell population of the heart is composed of fibroblasts [5]. In response to the infusion of AII, it has been reported that fibroblasts proliferate, and myofibroblasts

that contain α-smooth muscle actin (α-SMA), a contractile protein, appear, in association with an increase in collagen volume and interstitial fibrosis [6]. The phosphorylation of extracellular signal-regulated kinases (ERK1/ERK2) has been shown to play a critical role in these processes [1].

Heat-shock protein 72 (HSP72) is an important endogenous protective protein that functions as a molecular chaperone [7], and we have shown that induction of cardiac HSP72 by hyperthermia (HT) or geranylgeranylacetone (GGA) protects the rat heart against ischemia/reperfusion injury [8–11]. Interesting clinical observations of the suppressive effects of HSP72 on AF have been recently reported. Mandal et al. [12] demonstrated, in patients undergoing elective coronary artery bypass surgery,

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that the preoperative HSP72 content in right atrial tissue obtained at surgery was higher in patients who did not develop postoperative AF than in those who developed AF. Inductions of heat-shock responses, particularly HSP27 induction, have been shown experimentally to protect the heart against atrial fibrillation, although the specific roles of HSP72 have not yet been elucidated [13,14].

Based on these observations, we tested the hypotheses: (1) that HT treatment could suppress the intracellular signals that stimulate fibrosis in cultured rat atrial fibroblasts exposed to AII via induction of HSP72; and (2) that HT treatment induction could prevent atrial fibrosis and reduce atrial vulnerability to developing AF in rats exposed in vivo to a continuous subcutaneous infusion of AII.

2. Methods

All experimental procedures were in accordance with the guidelines of the Physiological Society of Oita University, Japan, for the care and use of laboratory animals, which follow the guidelines of the National Institute of Health (USA).

2.1. Isolation and culture of adult rat atrial cardiac fibroblasts, small interfering RNA targeting HSP72, and thermal treatment

Adult male Sprague–Dawley rats (250 to 300 g) were anesthetized with sodium pentobarbital (50 mg/kg, IP) and the left atrium (LA) was removed and minced in phosphate-buffered saline (PBS) [15,16]. The pieces were washed three times with PBS, incubated in 0.02% EDTA for 5 min at 37 °C with shaking, and centrifuged at 500 rpm for 2 min to remove EDTA. The pieces were then incubated in 5 mL of Hanks' balanced salt solution (HBSS) containing collagenase at a concentration of 2 mg/mL (Type IV; Cooper Biochemical) for 10 min in a 37 °C water bath shaken at 120 rpm. The supernatant was discarded, and the remaining pieces of tissue were treated with dispase (1000 IU/mL; Godo Shusei) in 5 mL of Ca^{2+}- and Mg^{2+}-free HBSS, with stirring for 20 min at 37 °C. Dispase treatment was repeated twice more, after which the free-floating cells were stored in Dulbecco's modified Eagle's medium (DMEM). The cardiac myocytes were pelleted by centrifugation at 200 rpm for 2 min, and the supernatant containing the fibroblasts was collected. The fibroblasts were pelleted at 1000 rpm for 10 min and resuspended in DMEM supplemented with kanamycin and 10% fetal bovine serum (FBS). After a 30-min period of attachment to of the fibroblasts to tissue culture plates, cells that were weakly attached or unattached were rinsed off and discarded. The fibroblasts were cultured in DMEM containing 10% FBS at 37 °C under 5% CO_2 and 95% room air. After 2 to 3 days, confluent cultures were passaged by trypsinization and replated at a split ratio of 1:3. Fibroblasts at passage 2 were used in the experiments. Some of the atrial fibroblasts were transfected with small interfering RNA (siRNA) targeting HSP72 (HSP72 siRNA) at a concentration of 100 nmol/L with siPORT NeoFX Transfection (Ambion) [17]. Control cells were transfected with negative control siRNA. Twenty-four hours after siRNA transfection, the

cells were equilibrated in serum-free DMEM 24 h before incubation under the conditions of HT (42 °C for 30 min) or normothermia (NT, 37 °C for 30 min). Preliminary observations had demonstrated that HSP72 expression reached a peak 8 h after HT. Therefore, 8 h after each thermal treatment, some fibroblasts were collected to assess HSP72 expression. AII (100 nmol/L) or vehicle was added to the medium of the remaining cells. Incubated cells were collected 8 min after AII addition for the evaluation of ERK1/ERK2 phosphorylation, and 24 h after AII addition for the evaluation of the expression of α-SMA, collagen type I, metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinases-1 (TIMP-1) by Western blot analysis. The expression of endoplasmic reticulum (ER) chaperone including glucose regulated proteins 78 and 94 (GRP78 and GRP94) was also evaluated by Western blot analysis.

2.2. Western immunoblotting

Whole cell lysates were prepared in cell lysis buffer and homogenized by sonication [8–10]. Equal amounts of protein, assayed using the Bradford method [18], were loaded on 10% SDS/PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane. After transfer and blocking with 5% nonfat milk, the membranes were incubated with anti-HSP72 monoclonal antibody (Stressgen), anti-Phospho-p44/42 MAP kinase (Thr202/Tyr204) (phospho-ERK1/ERK2) antibody (Cell Signaling), anti-p44/42 MAP kinase (ERK1/ERK2) antibody, monoclonal anti-α-SMA antibody (Sigma), anti-collagen type I antibody (Santa Cruz), anti-MMP-1 antibody (Oncogene), anti-TIMP-1 antibody (Santa Cruz), anti-GRP78 and GRP94 antibody (Stressgen), or monoclonal anti-vimentin antibody (Sigma) overnight at 4 °C. The proteins were detected by enhanced chemiluminescence with exposure to Hyperfilm (Amersham Pharmacia). The amount of protein on the immunoblots was quantified using National Institutes of Health software (NIH Image).

2.3. Inositol phosphate production

Atrial fibroblasts were labeled for 18 h with *myo*-[³H] inositol (2 μCi/mL; GE Healthcare) before HT or NT treatment. Eight hours after each thermal treatment, the cells were washed in medium containing 10 mmol/L LiCl for 20 min, and then incubated with AII (10 or 100 nmol/L) or vehicle for 30 min. Some labeled cells were incubated with candesartan, an AII type 1 (AT1) receptor antagonist, at a concentration of 10 nmol/L for 5 h, followed by the addition of AII (100 nmol/L). Inositol phosphates (IP) and total inositol fractions were resolved on a Dowex AG1-X8 formate column (Bio-Rad), and IP accumulation was estimated by determining the ratio of IP radioactivity to the sum of IP plus inositol radioactivity [19].

2.4. Immunocytochemistry

Atrial fibroblasts cultured on glass coverslips (Lab-Tek II, Nalge Nunc Int) were transfected with siRNA for 24 h, incubated with serum-free DMEM for 24 h, and then incubated under the

conditions of HT or NT. AII (100 nmol/L) or vehicle was subsequently added to the medium. Twenty-four hours after the addition of AII or vehicle, the atrial fibroblasts were fixed in 4% paraformaldehyde for 30 min and washed twice with PBS. The cells were permeabilized in 0.5% Triton X-100/PBS for 30 min, blocked with 1% goat serum/PBS for 1 h, and incubated with anti-α-SMA antibody (Sigma) overnight. After washing twice with PBS, the atrial fibroblasts were incubated with anti-mouse IgG conjugated to FITC (fluorescein isothiocyanate, Sigma) and mounted in Vectashield mounting media containing DAPI (4′,6-diamino-2-phenylindole) for nuclear staining (Vector Laboratories, Burlingame) [16].

2.5. TGF-β₁ secretion

The secretion of transforming growth factor β_1 (TGF-β_1) from atrial fibroblasts 24 h after AII addition was measured in the supernatant using a TGF-β_1 quantitative ELISA kit (R&D systems Inc.) [20].

2.6. Collagen synthesis

Atrial fibroblasts were transfected in the presence or absence of HSP72 siRNA for 24 h and incubated with serum-free DMEM for 18 h in medium containing L-[³H]proline (1 μCi/mL; PerkinElmer Life and Analytical Science), followed by HT or NT treatment. Eight hours later, AII (100 nmol/L) or vehicle was added to the medium for 24 h, and cells were collected. The supernatant was replaced with ice-cold 10% trichloroacetic acid for 20 min at 4 °C. The precipitate was rinsed with deionized water, and then solubilized in 0.3 N NaOH-0.1% SDS for 2 h at 37 °C. The cell lysate was added to liquid scintillant, and the incorporated radioactivity was measured [21].

2.7. Continuous infusion of AII and thermal treatment

An osmotic mini-pump (Alzet 2ML4, Alzet) was implanted subcutaneously into male Sprague–Dawley rats weighing 250 to 300 g anesthetized with sodium pentobarbital (50 mg/kg, IP), for constant infusion of AII or vehicle [6]. Twenty-four hours before the start of AII or vehicle infusion (day 0), HT (42 °C for 20 min) or NT (37 °C for 20 min) was applied as previously described [8]. Briefly, rats were anesthetized with pentobarbital (20 mg/kg, IP) and placed, with their heads on a pillow to avoid aspiration of water, for 20 min in a bath in which the water temperature was maintained at 43 °C. Rectal temperature was monitored throughout the thermal treatment experiments. Application of HT at 43 °C elevated the rectal temperature to 41 °C within 10 min and the temperature remained between 41 and 42 °C during HT application. In AII-treated rats, AII was infused at a constant rate of 400 ng/kg/min. The thermal treatment was repeated on days 7, 14, and 21. Systolic blood pressure (SBP) was measured by tail cuff on days 1, 8, 15, 22, and 28. Our preliminary experiments had revealed that AII-treated rats given repeated HT (AII-HT group) showed a reduction in SBP compared with AII-treated rats given repeated NT (AII-NT group). Therefore, a cocktail containing hydrala-

zine (1.5 mg/kg/day), hydrochrolothiazide (5 mg/kg/day) and reserpine (0.1 mg/kg/day) was mixed into the drinking water of a subgroup of AII-treated rats given NT (AII-NT-HHR group) [22]. LA was removed from the rats at days 1, 8, 15, 22, and 28, and frozen in liquid nitrogen and kept at −80 °C. They were used to assay the expression of HSP72 by Western blotting or measurement of atrial natriuretic peptide (ANP) mRNA by real-time quantitative RT-PCR. At day 28, hemodynamic parameters were measured. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). A PE-50 tube was placed via the left ventricular (LV) apex into the LV cavity. Hemodynamic parameters were analyzed utilizing Chart v5.3 software (AD Instruments).

2.8. Real-time quantitative RT-PCR

ANP mRNA was amplified by PCR and quantified using real-time quantitative PCR [23]. In brief, total cellular RNA was prepared from LA using TRIzol (Lifetech). The cDNA was synthesized from 150 ng of total RNA using a ReverTra-Dash reverse transcriptase kit (Toyobo). The sequences of the specific primers were as follows: ANP (sense), 5′-ATGGGCTCCTTC-TCCATCACC-3′; ANP (anti-sense), 5′-TCCGCTCTGGGC-TCCAATCCTGT-3 ; ribosomal 28S (sense), GAATCCGC-TAAGGAGTGTGTAACA; ribosomal 28S (anti-sense), CTCC-AGCGCCATCCATTT. A primer for ribosomal RNA was used as an internal control. Using an ABI PRISM 7000 sequence detector (Applied Biosystems), PCR amplification was performed in a 50 μL volume containing 100 ng cDNA template in PCR master mix (Roche). ANP mRNA and ribosomal RNA values were calculated from standard curves obtained using two-fold serial dilutions of cDNA from the tissues, and ANP mRNA amounts were normalized to ribosomal RNA.

2.9. Electrophysiological studies

On day 28, hearts were isolated and perfused retrogradely using a Langendorff apparatus with Krebs–Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.9; pH 7.4) equilibrated with a 95% O₂/5% CO₂ gas mixture at 36.5 °C and at a constant pressure of 75 mm Hg. Two sets of Teflon-coated (except their tips) silver bipolar electrodes, each with an interelectrode distance of 1 mm, were placed on the appendages of the right atrium (RA) and LA. The distance between the electrodes on the RA and LA was set at 10 mm. The effective refractory periods (ERP) of the RA and LA were measured by the S2 extrastimulus method using 8 regularly paced beats with cycle lengths (CL) of 200, 150, 120, and 90 ms and four times the threshold current for 2 ms. The interatrial conduction time (IACT) was measured during RA pacing at CL of 200, 150, 120, and 90 ms. AF vulnerability was tested by the S3 extrastimulus method. The intervals of S1 to S2 and S2 to S3 were the same and decremented from 100 ms to the ERP of the RA. Following extrastimuli, we evaluated the appearance of a repetitive atrial response (RAR), defined as rapid, irregular atrial excitations continuing over six beats.

2.10. Histological studies

LA was carefully removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Deparaffined sections (5 μm) were stained with Masson trichrome. Microscopic images were digitized using Photoshop 7.0 (Adobe) and analyzed using NIH Image. Connective tissue was differentiated on the basis of its color and expressed as a percentage of the reference tissue area.

2.11. Statistical analysis

Data are expressed as the mean±SE. Serial changes in SBP were analyzed by two-way ANOVA followed by the Bonferroni–Donn test, unless otherwise specified. The relative density of each protein, the relative incorporated radioactivity of [^{3}H]proline, the relative ratio of IP radioactivity to the sum of IP plus inositol radioactivity, the hemodynamic parameters, ANP mRNA expression, and the percentages of fibrosis, ERP, and IACT were analyzed using one-way ANOVA followed by the Bonferroni–Dunn test. The incidence of RAR was analyzed by the Chi-square test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. HSP72 expression in atrial fibroblasts

Fig. 1A shows representative bands of HSP72 protein in the fibroblasts and the relative density, assessed 8 h after each thermal treatment. When compared with NT-treated cells, HT resulted in a 6.1-fold increase in HSP72 content ($P<0.01$, data not shown). Pretreatment with HSP72 siRNA reduced HT-induced HSP72 protein levels by approximately 60% ($P<0.01$).
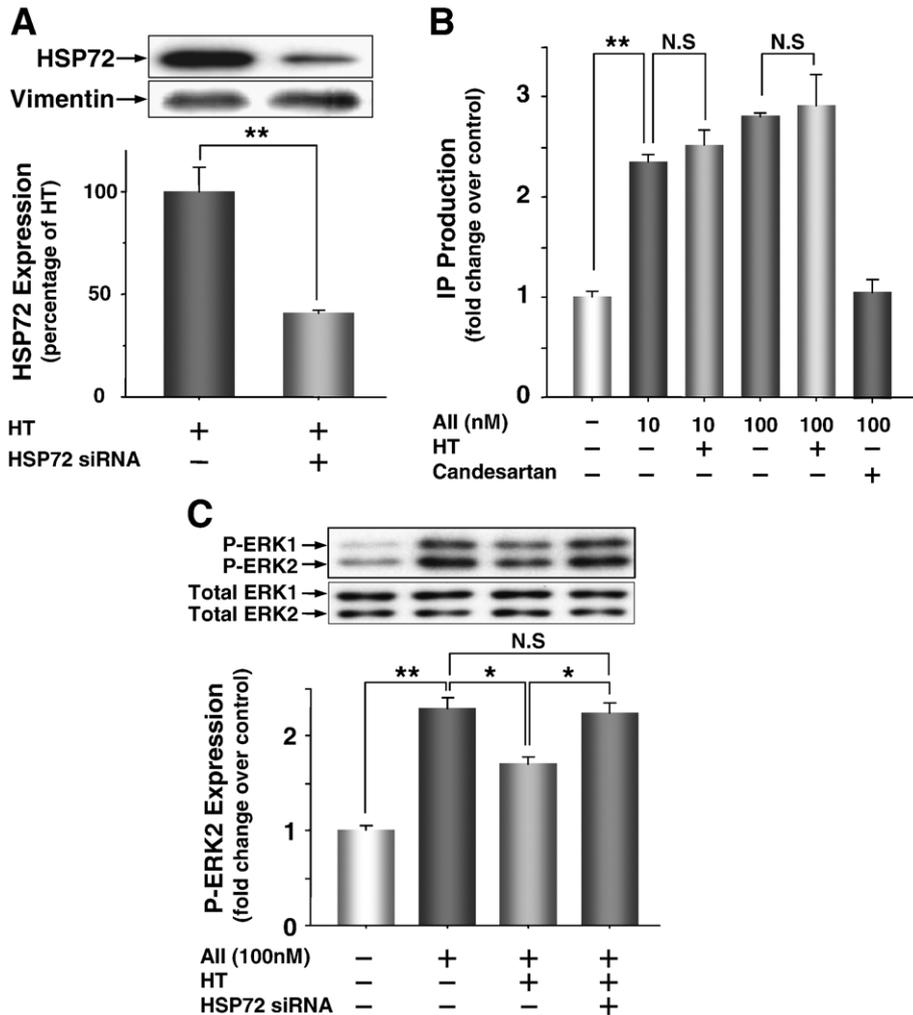

Fig. 1. (A) Expression of heat-shock protein 72 (HSP72) in atrial fibroblasts analyzed by Western blot. Above: Representative bands of HSP72 8 h after the application of hyperthermia (HT, left) and HT in the presence of HSP72 siRNA. Vimentin is an internal standard. Below: Quantitative expression of HSP72 (relative density). (B) Angiotensin II (AII)-induced inositol phosphate (IP) production in atrial fibroblasts. Data are relative radioactivity. (C) Extracellular signal-regulated kinase 1/2 (ERK1/ERK2) phosphorylation in atrial fibroblasts analyzed by Western blot. Above: Representative bands of phosphorylated-ERK 1/2 (P-ERK 1/2, top) and total ERK 1/2 (bottom). Below: Quantitative expression of P-ERK2 (relative density). Data are mean±SE. In each experiment (A–C), four independent cultures were evaluated. *$P<0.05$, **$P<0.01$, NS=not significant.

3.2. Activation of AT1 receptor

The application of AII (10 or 100 nmol/L for 30 min) increased IP production in the fibroblasts ($P<0.01$). While pretreatment with HT did not inhibit AII-induced increases in IP production, pretreatment with candesartan (10 nmol/L) suppressed AII-induced increases in IP production (Fig. 1B).

3.3. ERK1/ERK2 phosphorylation

Fig. 1C demonstrates the phosphorylation of ERK1/ERK2 and the relative density of phosphorylated ERK2 in the fibroblasts, when assessed 8 min after the addition of AII to the culture medium. In NT-treated cells, AII enhanced the phosphorylation of ERK1 and ERK2 2.3-fold ($P<0.01$) and 2.5-fold ($P<0.01$), respectively. The increases in ERK1/ERK2 phosphorylation were attenuated in HT-treated cells ($P<0.05$). Pretreatment with HSP72 siRNA abolished the suppressive effects of HT on AII-induced ERK1/ERK 2 phosphorylation ($P<0.05$, relative densities for ERK1 are not shown).

3.4. α-SMA expression

Fig. 2A illustrates α-SMA expression in fibroblasts by immunocytochemical analysis using laser microscopy. In NT-treated cells, AII caused a remarkable increase in expression of α-SMA in the cytoplasm, when assessed 24 h after AII application. Pretreatment with HT attenuated AII-induced α-SMA expression; the effects were abolished by pretreatment with HSP72 siRNA. Fig. 2B shows representative bands and the relative density of α-SMA by Western blot analysis. In NT-

treated cells, AII induced a 1.5-fold increase in α-SMA content ($P<0.01$). HT attenuated the AII-induced α-SMA expression ($P<0.05$). Pretreatment with HSP72 siRNA abolished the suppressive effects of HT on AII-induced α-SMA expression ($P<0.05$).

3.5. TGF-$β_1$ secretion, collagen synthesis, and expression of collagen type I, MMP-1, TIMP-1, and ER chaperone

When assessed 24 h after the addition of AII, TGF-$β_1$ secretion had increased by 45% ($P<0.01$, Fig. 3A). The increase in AII-induced TGF-$β_1$ secretion was attenuated by HT ($P<0.05$). Pretreatment with HSP72 siRNA abolished the suppressive effects of HT on TGF-$β_1$ secretion ($P<0.05$). Fig. 3B shows collagen synthesis evaluated by proline incorporation. In NT-treated cells, AII increased collagen synthesis by 1.4-fold ($P<0.01$). HT attenuated the AII-induced increase in collagen synthesis ($P<0.01$); the effect was counteracted by pretreatment with HSP72 siRNA ($P<0.05$). Figs. 3C–E show representative expression and the relative densities of collagen type I, MMP-1, and TIMP-1, respectively. In NT-treated cells, addition of AII resulted in a 2.4-fold increase in collagen type I content ($P<0.01$). Pretreatment with HT attenuated the AII-induced collagen type I expression ($P<0.01$); this effect was counteracted by pretreatment with HSP72 siRNA ($P<0.05$). As shown in Fig. 3D, the expression of MMP-1 was not significantly influenced by AII, HT, or HSP72 siRNA. In contrast, an AII-induced increase in TIMP-1 expression ($P<0.01$) was attenuated by pretreatment with HT ($P<0.05$, Fig. 3E). The expression of ER chaperone, represented by GRP78 and GRP94, was also evaluated (Figs. 3F and G). Both GRP78 and GRP94 were upregulated by
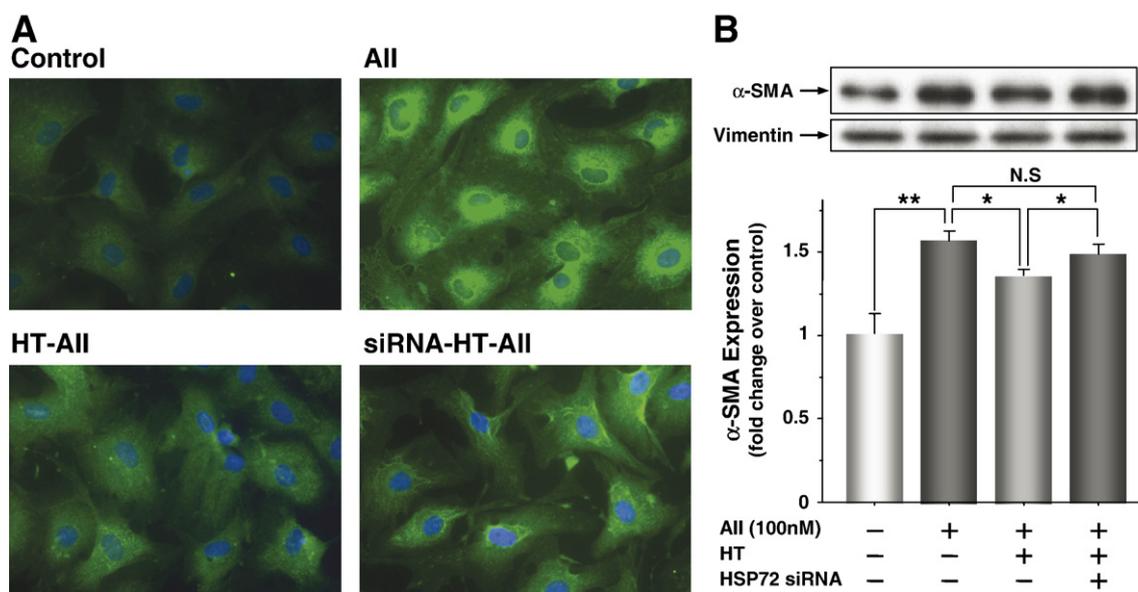

Fig. 2. Expression of α-smooth muscle actin (α-SMA) in atrial cardiac fibroblasts. (A) Immunocytochemical staining of atrial fibroblasts (passage 2) using specific α-SMA primary antibodies. (B) Above: Representative bands of α-SMA by Western blot analysis. Vimentin is an internal standard. Below: Quantitative expression of α-SMA protein (relative density). AII=angiotensin II. HT=hyperthermia. Data are mean±SE. Four independent cultures were evaluated. *$P<0.05$, **$P<0.01$, NS=not significant.
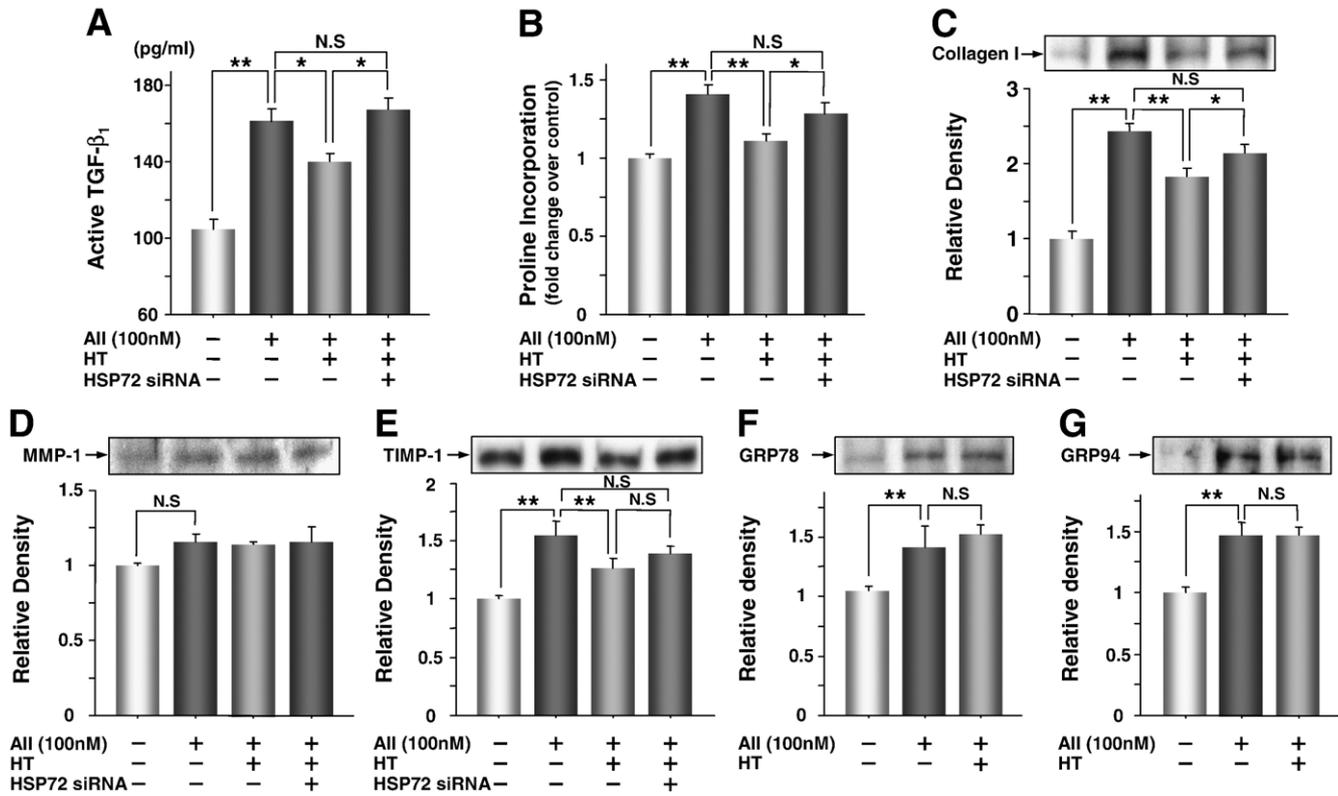
Fig. 3. (A) Transforming growth factor-β_1 (TGF-β_1) secretion. (B) [^{3}H] proline incorporation. (C–G) Representative bands and quantitative expressions (relative density) of collagen type I, metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinases-1 (TIMP-1), glucose regulated proteins 78 (GRP78), and GRP94. AII=angiotensin II. HT=hyperthermia. Data are mean±SE. In each experiment (A–G), four independent cultures were evaluated. *$P<0.05$, **$P<0.01$, NS=not significant.

AII, while the pretreatment with HT did not influence the expression levels.

3.6. Time courses of SBP and cardiac HSP72 expression, and hemodynamic parameters on day 28

Fig. 4 shows the time courses of SBP and cardiac HSP72 expression in the LA (days 1, 8, 15, 22, and 28). Repeated HT application (AII-HT group) effectively induced HSP72 expression in the LA (days 1, 8, 15, and 22), when compared with the AII-NT-HHR group ($P<0.05$, by ANOVA). Hemodynamic parameters on day 28 are show in Table 1. Body weight and ventricular weight were lower, but the ratio of ventricular/body weight was greater, in the AII-NT-HHR group compared with the NT-treated control group ($P<0.05$). With respect to the hemodynamic parameters, LV pressure, dP/dt max, dP/dt min, LV end diastolic pressure (LVEDP), and LV developed pressure (LVDP) were greater in the AII-NT-HHR group compared with the NT-treated control group ($P<0.01$). HT did not affect the weight or the hemodynamic parameters.

3.7. ANP mRNA expression

In the AII-NT-HHR group, ANP mRNA expression in LA was increased to 177% when compared with the control group (100%, $P<0.01$); this effect was suppressed by HT (133%, $P<0.05$, Table 1).

3.8. Atrial fibrosis

Fig. 5 shows representative histological sections of the LA isolated from the three groups. Compared with the control group (Fig. 5A), extensive and heterogeneous interstitial fibrosis,
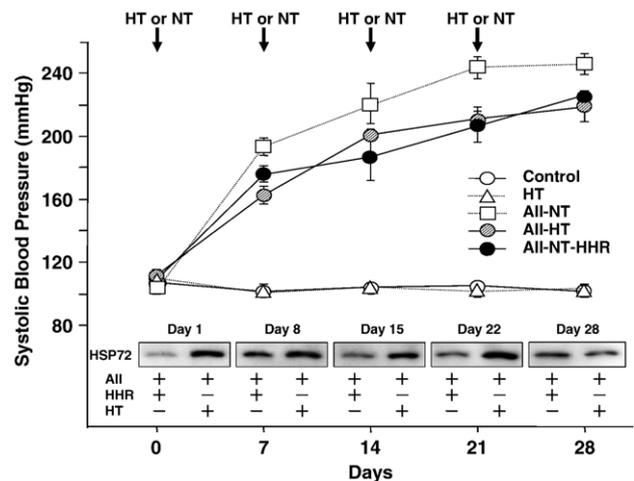

Fig. 4. Time course of systolic blood pressure (SBP) and HSP72 expression. Whole body hyperthermia (HT) or normothermia (NT) was applied at days 0, 7, 14, and 21. SBP was measured at days 0, 7, 14, 21, and 28 after the start of continuous AII infusion (400 ng/kg/min). In the panel, representative bands of HSP72 protein in the AII-NT-HHR group and the AII-HT group at days 1, 8, 15, 22, and 28 are shown. Data points represent means±SE. n=7–8 in each group.

Table 1
Characteristic and hemodynamic parameters, and tissue ANP mRNA expression in the control, AII-NT-HHR, and AII-HT groups

	Control (n=6)	AII-NT-HHR (n=6)	AII-HT (n=6)
Body weight (BW, g)	452.5±11.3	296.4±6.9*	300±5.6*
Ventricular weight (VW, g)	1.10±0.02	0.98±0.01*	0.98±0.02*
VW(mg)/BW(g)	2.45±0.08	3.33±0.07*	3.25±0.07*
LV pressure (mm Hg)	108.5±3.6	213.9±3.8**	211.5±3.1**
dP/dt max (mm Hg/s)	4399±216	8709±229**	8789±536**
dP/dt min (mm Hg/s)	−3650±239	−6024±79**	−5922±341**
LV end diastolic pressure (mm Hg)	6.7±0.6	7.2±0.3**	7.2±0.2**
LV developed pressure (mm Hg)	101.7±3.6	206.5±4.0**	204.2±3.3**
Left atrial ANP mRNA (% control)	100±10	177.5±18.9**	133.0±10.6#

Data are mean±SEM. *$P<0.05$, **$P<0.01$ vs. control. #$P<0.05$ vs. AII-NT-HHR.

AII=angiotensin II; HHR=cocktail constituted with hydralazine (1.5 mg/kg/day), hydrochrolothiazide (5 mg/kg/day), and reserpine (0.1 mg/kg/day); HT=hyperthermia; LV=left ventricular.

demonstrated using Masson trichrome staining, was observed in the AII-NT-HHR group ($P<0.01$, Fig. 5B). The interstitial fibrosis induced by AII was attenuated in the AII-HT group ($P<0.05$, Figs. 5C and D).

3.9. Electrophysiological studies

Figs. 6A and B depict the ERP of the RA and LA determined at the four basic CL (BCL) of 200, 150, 120 and 90 ms, respectively. In both the RA and the LA, the ERP in the control, AII-NT-HHR, and AII-HT groups were slightly shortened with a reduction in BCL. However, no significant differences in ERP were observed among the three experimental groups. The IACT was also evaluated at the same four BCL (Fig. 6C). Compared with the control group, the IACT in the AII-NT-HHR group was prolonged at all BCL tested ($P<0.05$ for each CL). This prolongation was not observed in the AII-HT group. Fig. 7A shows two representative RAR induced by the S3 extrastimuli that were observed in the AII-NT-HHR group, while Fig. 7B shows the incidence of RAR in the three groups. RARs were never induced in hearts from the control group. In the AII-NT-HHR group, the S3 extrastimuli induced RAR in seven out of eight rats (88%). In the AII-HT group, the induction of RAR was observed in three out of eight rats (38%). Thus, pre-treatment with HT resulted in a reduction in the incidence of RAR in the AII-treated hearts ($P<0.05$).

4. Discussion

In the present study, using cultured fibroblasts isolated from the LA of adult male rats, we observed that HT induced the
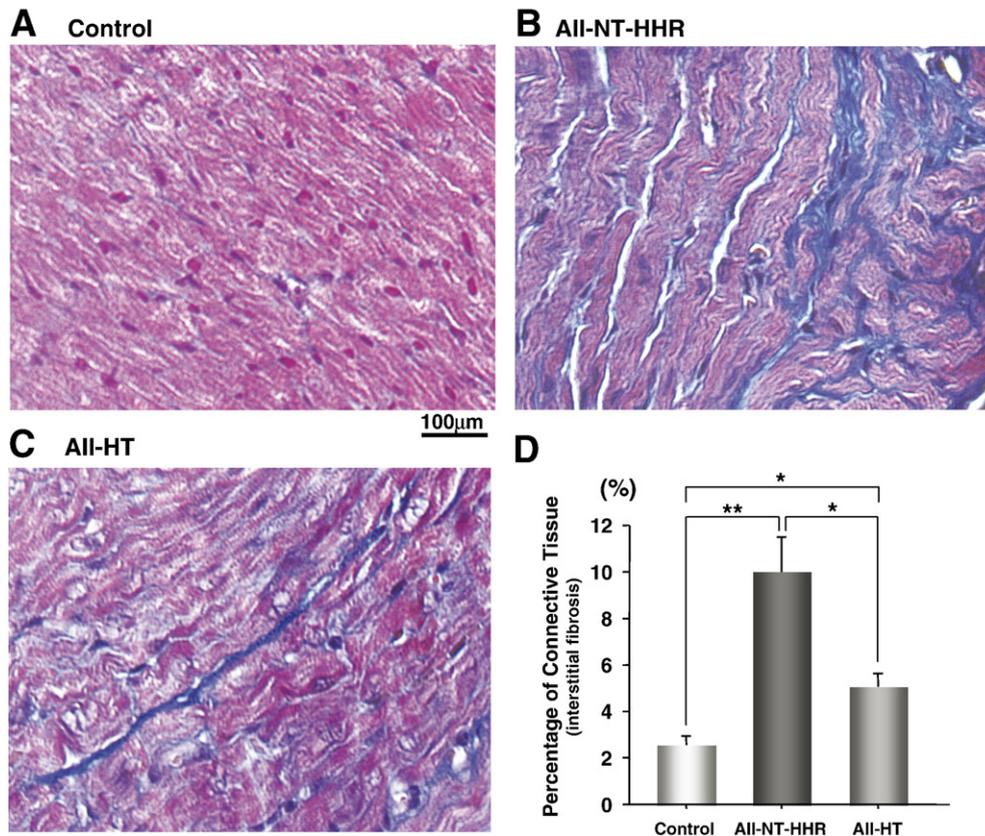

Fig. 5. Histology of the left atrial (LA) free wall. (A–C) Representative atrial tissue sections stained by Masson trichrome staining. (D) Histogram of mean interstitial fibrosis of the LA in the control, AII-HT and AII-NT-HHR groups. Data are mean±SE. $n=7$–8 in each group.
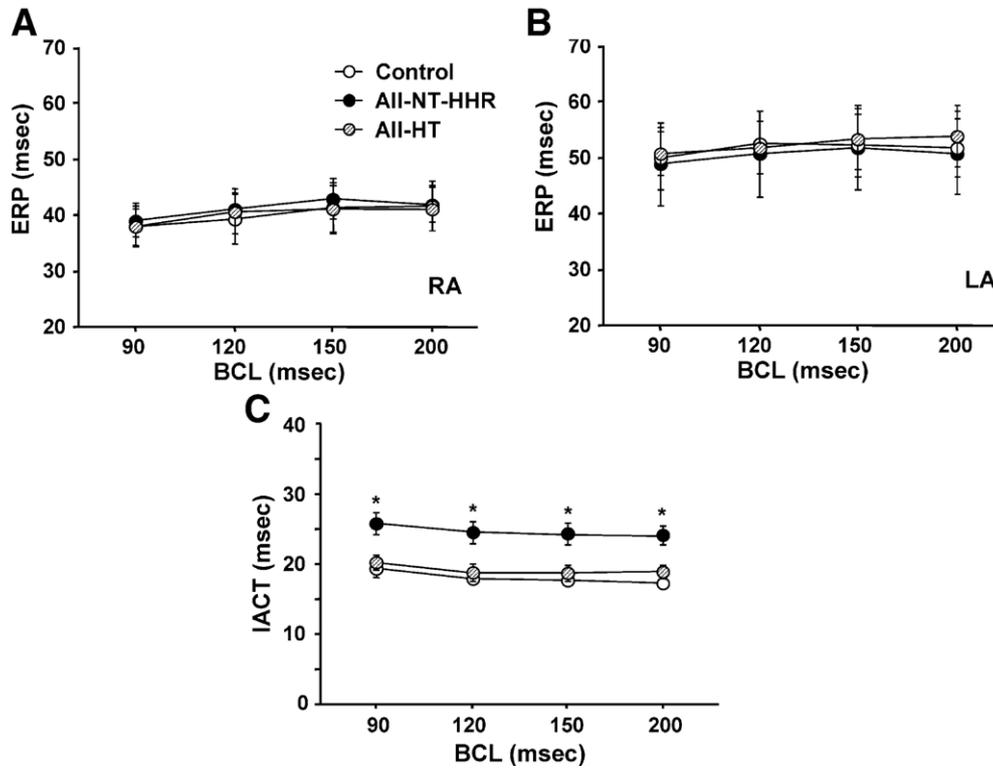
Fig. 6. Electrophysiological characteristics. (A) Effective refractory period (ERP) of the right atrium (RA). (B) ERP of the left atrium (LA). (C) Interatrial conduction time (IACT). ERP and IACT were measured at the basic cycle lengths (BCLs) of 200, 150, 120, and 90 ms. Data are mean±SE. $n=7$–8 in each group. $*P<0.05$ vs. control and AII-HT groups.

expression of HSP72, which was associated with the attenuation of AII-induced fibrotic signals, including ERK1/ERK2 phosphorylation. The siRNA targeting HSP72 abolished the most of the antifibrotic effects produced by HT, i.e. ERK1/ERK2 phosphorylation, α-SMA expression, TGF-β$_1$ secretion, collagen synthesis, and collagen type 1 expression, suggesting a significant role of HSP72 in antagonizing against AII-induced promotion of fibrotic processes. In rats in vivo, repeated whole-body HT induced atrial HSP72 expression and resulted in the attenuation of AII-induced LA fibrosis. In isolated-perfused hearts, continuous AII infusion caused interatrial conduction slowing without affecting atrial ERP. We evaluated the atrial

vulnerability by the incidence of RARs induced by the extrastimuli, because a similar method has been recently established for the evaluation of atrial vulnerability [24]. Atrial burst pacing has been frequently used to induce AF. However, burst pacing sometimes induces nonspecific AF and atrial tachycardia, even in control hearts. In fact, in our isolated-perfused heart, preliminary experiments showed induction of nonspecific AF by atrial burst pacing in some of the control hearts. That is why we applied an S3 extrastimuli protocol, in which we confirmed that RARs were never induced in NT-treated control hearts. In AII-infused hearts, atrial extrastimuli resulted in a high incidence of RAR, which were suppressed by
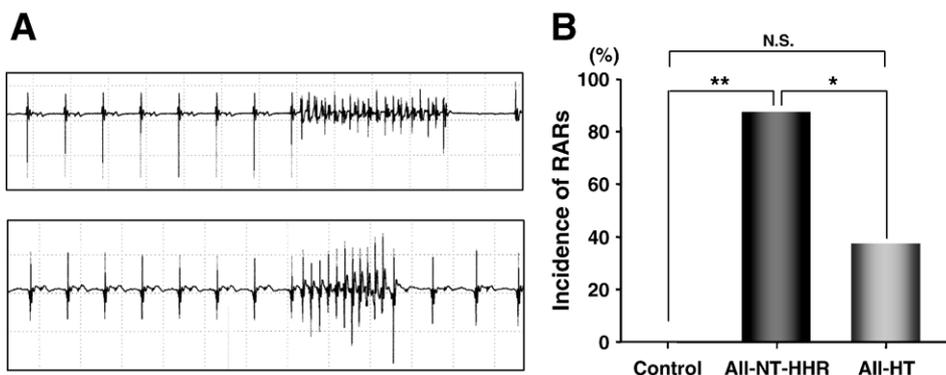

Fig. 7. Repetitive atrial response (RAR). (A) Two representative RAR observed in the AII-NT-HHR group. (B) Incidence of RAR. $*P<0.05$, $**P<0.01$, NS=not significant.

pretreatment with HT. RARs were seen as rapid and irregular atrial excitations, mimicking AF. The electrophysiological mechanisms responsible for RAR are unclear. It has been reported, however, that pre-existing nonuniformity in heterogeneous interstitial fibrosis can easily break the single activation wavefront into multiple wavefronts [25]. Therefore, it is suggested that slowed and nonuniform conduction due to heterogeneous interstitial fibrosis may contribute to the formation of multiple wavefronts, leading to the occurrence of RAR.

Cardiac fibroblasts are the predominant secretory cells producing extracellular matrix (ECM) proteins, key mediators of cardiac interstitial fibrosis [5]. This is why we used atrial fibroblasts rather than atrial cardiomyocytes. In response to AII, ERK1/ERK2 phosphorylation and subsequent cellular proliferation occur via direct stimulation of the AT1 receptor [26–28]. Consistently, AII caused ERK1/ERK2 phosphorylation in cultured atrial fibroblasts, which was attenuated by pretreatment with HT. Activation of the AT1 receptor by AII is characterized by conformational changes [19]. In contrast to candesartan, HT did not reduce IP production, i.e. it did not induce conformational changes in the AT1 receptor in cultured fibroblasts in response to AII, suggesting that HT might work below the level of the AT1 receptor.

In in vivo experiments, the atrial tachypacing-induced model has been established to represent clinical AF [29–33]. In some reports, characteristic shortening of atrial ERP, induced by short-term atrial tachypacing, was suppressed by pharmacological inhibition of the renin–angiotensin system [29,34]. Interestingly, in a canine AF model with rapid atrial pacing at 400 beats/min for 5 weeks, rather than shortening the atrial ERP, extensive interstitial fibrosis was found in the RA free wall in association with a gradual conduction prolongation in the atria [4]. Because long-term treatment with candesartan, which started 1 week before the pacing and continued for 5 weeks during tachypacing, prevented atrial fibrosis, Kumagai et al. [4] attributed the interatrial conduction slowing to interstitial fibrosis, which led to the initiation and maintenance of AF in their model, and concluded that candesartan can prevent the promotion of AF by suppressing the development of structural remodeling. When compared with atrial tachypacing, our approach using continuous AII infusion without pacing may have some advantages, because continuous AII infusion causes LA wall stretch (reflected as a slight increase in LVEDP) without heart failure, and causes atrial interstitial fibrosis without affecting atrial ERP. These processes are more likely to underlie the mechanisms for newly developed AF in patients with hypertension.

Besides the fibrotic signals evaluated in our experiments using cultured fibroblasts, a large number of factors must be involved in the AII-induced fibrosis observed in experiments in vivo. For instance, cardiac function is of importance in the development of atrial fibrosis and AF [3]. In our continuous AII infusion model, no overt heart failure was observed. No difference in LV function was observed between the AII-HHR and AII-HT groups, suggesting that improvement of LV function is unlikely to be coupled to the preventive effects of HT on AF. Regarding atrial function, it was impossible for us to directly measure the hemodynamic parameters of the atrium. Alterna-

tively, we estimated the ANP mRNA expression levels of the LA, because stretch and/or AII have been demonstrated to increase atrial ANP mRNA expression [35]. In the present study, the ANP mRNA levels were increased in the AII-HHR group; this effect was suppressed by the pretreatment of HT. It can therefore be postulated that AII-induced LA pressure overload, which was not reflected in LVEDP but in the LA ANP mRNA level, might be attenuated by HT treatment. However, substantial improvement of LV function, which did not appear in our measured indices, might underlie the improvement of atrial function. The ER is an organelle that participates in folding secretory and membrane proteins, and ER chaperones are upregulated in response to stress [36]. To investigate the involvement of ER stress in AII-induced fibrotic processes, we evaluated the levels of the major ER chaperones, GRP78 and GRP94. The expression of both GRP78 and GRP94 proteins was upregulated in response to AII. Pretreatment of HT, however, did not influence the expression levels of these proteins. To our knowledge, there have been no reports describing the role of ER stress in atrial fibrosis induced by AII.

Information is limited with respect to the effects of induction of the heat-shock response on AF substrate. Brundel et al. [13] reported that tachypacing applied to HL-1 (atrial myocytes) induced myolysis, which was prevented by the overexpression of HSP27 but not of HSP72. Because myolysis of atrial myocytes is reported to be associated with the progression of AF [37], the authors concluded that upregulation of HSPs, particularly HSP27, protects tachypaced atrial myocytes from myolysis, leading to a novel therapeutic approach in AF. Subsequently, Brundel et al. [14], using HL-1 cells, reported that atrial tachypacing-induced remodeling, including progressive reduction in the Ca^{2+} transient, L-type Ca^{2+} current, and action potential duration in myocytes, was suppressed by HT or GGA. They also demonstrated that the ERP shortening induced by 1 week of atrial tachypacing in dogs was suppressed by HT or GGA. By using the transfection technique in HL-1 cells, Brundel et al. concluded that HSP27 rather than HSP72 plays a predominant role in HT- or GGA-induced protection against AF. There are distinctly different experimental approaches between the studies of Brundel et al. [13,14] and our studies. In experiments in vitro, while they evaluated myolysis [13] and electrical remodeling [14] by introducing tachypacing to HL-1 myocytes, we evaluated the fibrotic signals evoked by direct application of AII using isolated atrial fibroblasts. In addition, in experiments in vivo, Brundel et al. did not evaluate structural remodeling represented by atrial fibrosis. These differences in experimental design may explain the disparity in the observations between our work, which shows that HSP72 induction is protective, and that of Brundel et al., which showed that induction of HSP27 but not HSP72 is protective against AF. Nevertheless, combining both sets of observations leads us to conclude that induction of the heat-shock response is expected to be effective in preventing AF via prevention of both electrical remodeling and structural remodeling.

There are several limitations to the present study. Firstly, continuous infusion of AII caused marked elevation of arterial BP. Therefore, we could not separate the direct effects of AII on

the fibroblasts that caused fibrotic change from the secondary outcomes derived from increased BP. Secondly, although we confirmed the suppressive effects of HSP72 on various fibrotic signals in cultured fibroblasts by using siRNA targeting HSP72, its inhibitory effects were incomplete. In addition, the effects of HSP72 induction could not be isolated in experiments in vivo. Besides the HSP family, HT is known to induce broad classes of protective proteins [7,38]. The anti-fibrotic effects of HT observed in vivo, therefore, could not be solely explained by the effects of HSP72 induction. Finally, although we evaluated several fibrotic factors, including ERK1/2 phosphorylation, it is impossible to directly connect these factors with antifibrotic effects of HSP72 induction. Based on our observations, it is conceivable that HSP72 inhibits AII-induced ERK1/ERK2 phosphorylation, a critical upstream signal, subsequently resulting in both prevention of fibroblasts to differentiate into myofibroblast in association with an acquisition of α-SMA and inhibition of TGF-β$_1$ secretion, collagen synthesis, and collagen type 1 expression. However, the possibility that HSP72 suppresses each of these fibrotic factors cannot be excluded.

In conclusion, our results suggest that the induction of the heat-shock response prevents atrial fibrosis and AF that are mediated by the activation of the renin–angiotensin system, at least partly through expression of HSP72. The mechanisms of induction of the heat-shock response, either via HT or pharmacologic stimuli, may be effective in preventing atrial fibrosis and AF where AII is involved.

Acknowledgments

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