Supplemental material

Detailed methods

Animals

Male New-Zealand rabbits (2.0-2.5 kg) were randomly divided into 6 groups (n=8 for each group): normal control (N), sham control (S), rapid atrial pacing (RAP) alone (P), RAP +losartan 10 mg.kg-1 daily (D1), RAP + losartan 20 mg.kg-1 daily (D2), and RAP + losartan 30mg.kg-1 daily (D3).

Left Atrium Rapid Pacing

Rabbits were anesthetized with 30 $mg.kg^{-1}$ pentobarbital sodium (i.v.), and then intubated and ventilated with a volume-cycled ventilator (Model HX-200, TAIMENG, Chengdu, China). The left thoracic cavity was opened via sternum from the second intercostals to the fourth intercostals, and then the heart was exposed by a dilator. A custom-designed set of electrodes, comprising a pair of electrodes with a distal hook for pacing and a pair of electrodes with an interelectrode distance of 15 mm aligned proximally for recording, were sutured to the epicardial surface of the left atrium. The reason to choose left atrium for RAP is because of its higher inducibility of AF than right atrium.[1] The distal ends of these electrodes leads were tunneled subcutaneously and exposed on the back, and connected to a pacemaker (output of 6V with 1.0 ms pulse duration, Guangzhou Academy of Sciences, China) in the jacket. The pacemaker was programmed to provide RAP at 1000 ppm and this pacing rate was maintained continuously for 4 weeks with a brief period of break for measurement of electrophysiological and mechanical parameters. Rabbits in normal control (Group N) were not subjected to surgery while those in sham control (Group S) were operated with the identical surgery procedure but RAP. When surgery was completed, the rabbits were given antibiotics and then allowed to recover for 5 days. Postoperative care included the administration of antibiotics and analgesics. In Groups D_1, D_2, and D_3, oral administration of losartan (Merck & CO., Inc., USA) started at the same time as RAP, and continued for 4 weeks during the pacing period. The same amount of normal saline was given to rabbits in Groups N, S, and P.

ECG recordings

ECG was recorded before and after the pacing. During the 4 weeks of pacing ECG was measured every day to ensure that the pacemakers were working properly. Atrial effective refractory period (AERP) of the left atrial appendage was measured at basic cycle lengths (BCLs) of either 120 ms or 200 ms. Five basic drive stimuli were followed by 1 single premature stimulus, and all stimuli were twice the diastolic threshold. The interval between S1 and S2 was decreased in steps of 2 ms, and AERP was determined to be the shortest S1-S2 interval resulting in a propagated atrial response.

Atrium samples

At the end of the experiment all rabbits were sacrificed and the hearts were removed and weighed immediately. Left atrium were then quickly removed and cut into three (upper, meddle, lower) sections. Each section was divided equally into four pieces. Three pieces from each section were randomly chosen to form a new part for radioimmunity assay of AngII accumulation and hydroxyproline content analysis. One part was paraffin-embedded for

Masson's trichrome staining. The remaining parts were quickly frozen in liquid nitrogen and maintained at -80^{o}C until being used for mRNA and protein analysis. The whole procedure was performed in cold conditions.

Masson Trichrome Staining for Collagen

Stain deparafinized sections taken out of water in Hansen's iron hematoxylin for I-5 minutes. After 5 minutes the nuclei should be a rich black. Stain in Masson's fuchsin-ponceau mixture, diluted 10 times with water acidulated to 0.2 per cent with acetic acid, for 5 minutes or more. Rinse in distilled water acidulated in the same fashion. If the city supply be not too alkaline, tap water may be used. Usualy a few drops of glacial acetic acd in tap water will work well. Treat for 15 seconds to 30 minutes with phosphotungstic acid orange G. A few minutes usually suffice amply. Rinse in distilled water acidulated in the same fashion as before. Stain for 5 minutes in Masson's light green solution dfluted 10 times with water acidulated as above. Rinse in distilled water acidulated in the same fashion for 5 minutes to eliminate the phosphotungstic acid and to differentiate the various color tones. Dehydrate in the usual manner with ascending percentages of alcohol, clear in xylol and mount in balsam. To quantitate atrial collagen content, images were captured with a digital camera and the red pixel content of the myocardium was measured using Adobe Photoshop 5.5 and Scion Images for Windows Beta 4.0.2 software as described. The analyses were performed by at least two independent investigators on coded specimens in a blinded fashion.

Left atrium collagen content

Hydroxyproline content was measured as an index of the amount of collagen, which reflects the degree of myocardial fibrosis. The specimens were minced and then homogenized for 2 min at 4^{o}C in sufficient deionized water to yield a 10% mixture (weight/volume). The hydroxyproline content of homogenates was assayed as described by Jamall et al. Briefly, Atrial samples were homogenized and hydrolyzed in 6 N HCl at 110 ^{o}C for 18 h. After filtration of the hydrolysate through a 0.45-mm milli-pore filter, chloramine T was added to a final concent-ration of 2.5 mmol/L. The mixture was then treated with 400 mmol/L paradi-methyl-amino-benzaldehyde and incub-ated at 60 ^{o}C for 30 min. After cooling to room temper-ature, samples were read spectrophotometrically at 560 nm against a reagent blank containing no tissue and compared with a standard curve of known amount of hydroxyproline. The hydroxyproline content of the liver was expressed as mg/g wet weight.

Radioimmunity assay of AngII accumulation

Tissues (50 mg) from each sample was homogenated in cool acetic acid, centrifuged and torrefied per the Kit instructions (^{125}ICAMP RIA Kit, Medical college of SUN Yet-San University, Guangzhou China), and assayed with a γ-immunity indicator (FM2000, Xi'an, China).

Cell culture

Cardiac fibroblasts were isolated from the hearts of adult New-Zealand rabbits.[2, 3] Left atriums were minced, and cells were dispersed with collagenase (Boehringer Mannheim, Indianapolis, IN) and pancreatin (Gibcobrl, Gaithersburg, MD). After five separated sequential digestions, the cell suspensions were combined, centrifuged, and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Cardiac fibroblast were

plated onto 150-mm cell culture dishes for 12 hours at 37°C to permit attachment of cells, after which the media were changed to remove unattached cells. Cultured cells were characterized by immunocytochemistry and were immunopositive for a-smooth muscle actin, vimentin, and □bronectin but not for desmin or α-sarcomeric actin. Fibroblasts were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5g/L glucose, supplemented with 10% v/v fetal bovine serum (FBS), penicillin–streptomycin (Gibco, 100units/mL and 100 μg/mL, respectively). Cells were subcultured and passed as they reached 80-90% confluency. The purity of fibroblasts used in these experiments was found to be 90% using routine phenotyping methods.[2, 3]

The primary cardiac fibroblasts passage 2（P2）were used for studies as described below. Cells were serum starved for 24 hours, followed by treatment with:A) 0.005% dimethylsulfoxide (DMSO) (control group, DMSO was used to dissolve PD123319 and 0.005% was the □nal concentration in the incubation solution); B) 1 μM AngII for 48 hr; C) 1 μM AngII + 10 μM losartan (AT_1 receptor antagonist) for 48 hr; D) 1 μM AngII + 100 μM PD123319 (AT_2 antagonist, sigma) for 48 hr; Cells were treated with losartan, or PD123319 at 1 hour before AngII stimulation. Then cells were harvested. The protein expression of TGF-β1, P-Smad2/3, Smad4，collagen I, Smad7 and Arkadia were determined by Western blotting. What follows next is we investigated whether the Ubiquitin-dependent protein degradation involved in the regulation of Smad7 expression. The fibroblasts were treated with (100 mmol/L) of lactacystin (specific proteasome inhibitor) for 1 h before cells treated with AngII, losartan and PD123319(which was explained above). In addition, to study the Smad7 expression mechanism involving AT1 post-receptor signaling pathway, the fibroblasts were treated with: A) 0.005% DMSO(control group); B) 1 μM angiotensin II for 48 hr; C) 1 μM angiotensin II+10 μg/ml neutralizing TGF-β antibody for 48 hr; D) 1 μM angiotensin II+100 μM PD98059 (ERK1/2 inhibitor) for 48 hr; and E) 1 μM angiotensin II+10 μM losartan for 48 hr; F) 1 μM AngII + 100 μM PD123319. Cells were treated with neutralizing TGF-β antibody, PD98059, losartan, or PD123319 at 1 hour before AngII stimulation. The expression of Smad7 was analyzed by Western blotting and immunochemistry.

Small interfering RNA (siRNA) transfection

Silencer siRNAs targeting Smad2, Smad3, or Smad7 were synthesized by Ambion based on the sequences of rabbit Smad2 (ID#: AAGW02032355), Smad3 (ID# AAGW02025187) and Smad7 (ID# AAGW02032373) and were used in knockdown experiments. To demonstrate that the transfection does not induce nonspecific effects on gene expression a control siRNA (CsiRNA. ID# NM001082253) , which has no homology to known sequences from rabbitor humans, was used. The adult rabbit cardiac fibroblasts were cultured to approximately 80–90% confluence and then transfected with 400 pmol siRNA using Lipofectamine 2000 in a 6-well plate according to the manufacturer's instructions. Clones of Smad2, Smad3 or Smad7 siRNA that presented at least 90% inhibition of target genes were chosen for further analysis. For experimental procedures, second to third passages of each clone were used. The morphology of knockdown cells was monitored during culture under an inverted microscope. After 24 hours of transfection, cells were treated with or without AngII (1 μmol/L) / losartan (10 μmol/L) for 48 hours. Levels of Smad2/3, Smad7 and collagen I expression were determined by western blot.

Smad7 expression plasmid and transfection

The Smad7 expression plasmid pcDNA3-FLAG-Smad7 was constructed as previously described.[4] Fibroblasts were grown to 50% confluence in 100-mm dishes. After a 4-hour

incubation with serum-free medium, cultures were transfected with 1.0 μg pcDNA3-FLAG-Smad7 or 1.0 μg empty pcDNA3 vector in six-well plates by using Lipofectin (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. After transfection, cells were treated with AngII (1 μM) for 48 hours in media. In this experiment, total DNA in each well was adjusted to the same amount using vector DNA. All assays were performed in triplicate, and the results are presented as the means (±the standard errors) of three independent transfections.

Quantitative Real Time Polymerase Chain Reaction

Total RNA was extracted with TRIZOL reagent (Gibco-BRL Life Technologies). cDNA was synthesized with SYBR ExScript™ RT-PCR kit(TOYOBO, Japan) according to the protocol provided by the manufacturer. PCR primers for TGF-β1 and Smad7 were designed with Primer Express software (Applied Biosystems). glyceraldehydes-3-phosphatedehydrogease(GAPDH) served as references to normalize input amounts of RNA for all samples. The following primers were used. 5'-ACA TTG ACT TCC GCA AGG AC-3' (sense) and 5'-TAG TAC ACG ATG GGC AGT GG-3' (antisense) for the TGF-β1 gene; 5'-GTG GCA TAC TGG GAG GAG AA-3' (sense) and 5'-GAT GGA GAA ACC AGG GAA CA -3' (antisense) for the Smad7 gene. 5'-GCA CCG TCA AGG CTG AGA AC-3'(sense) and 5'-ATG GTG GTG AAG ACG CCA GT-3' (antisense) for the GAPDH gene. Real-time PCR was performed using a ABI7300 Real-time PCR system (Applied Biosystems, CA) with SYBR green fluorophore.[5, 6] Reactions were performed in a total volume of 20 uL including 10uL 2 X QuantiTect Sybr Green PCR-Kit (Qiagen), 6 umol/L of each primer and 2 uL of the previously reverse-transcribed cDNA template. The cycles for PCR were as follows: 1 cycle of 95℃ for 10 s, 40 cycles of 5 s at 95℃, 5 s at 60℃, 31 s at 60℃ and a final 7 min at 72℃. Melting curve analysis was always included to validate the specificity of the PCR products. All tests were done in triplicate to ensure reproducibility. Gene-specific expression was analyzed using iCycler iQ Optical System Software Version 3.0a (BioRad Laboratories, Ltd). A melt curve analysis was performed after every run to ensure a single amplified product for every reaction. All reactions were performed in at least duplicate for every sample. Threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). GAPDH was used as an internal control. mRNA -fold change relative to GAPDH was calculated with the comparative Ct method of $2^{-\Delta\Delta Ct}$.[6]

Immunohistochemistry

Immunohistochemistry was performed using a microwave-based antigen retrieval technique as described previously.[7] Briefly, cells after culturing in 6-chamber glass slides, the biopsies were embedded in paraffin, and 2-μm-thick sections parallel to the long axis were cut and mounted two sections per slide. The sections were deparaffinized and endogenous peroxidase blocked by 0.5% H_2O_2 in absolute methanol. In order to reveal antigens, sections were boiled for 10 min in 0.1 mM Tris/HCl and 0.5 mM EGTA, pH 9. Non-specific binding was blocked by 1% BSA (bovine serum albumin). Sections were incubated overnight at 4°C with a primary antibody against Smad7 (1:200, Santa Cruz Biotechnology) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton-X100. Negative controls were incubated with serum instead of primary antibody. After washing, the sections were incubated with horseradish peroxidase-conjugated Secondary antibodies(Cell signaling Technology, USA), for 1 h at 20°C. The peroxidase was visualized by reaction with 0.05% 3,3'-diaminobenzidine tetrahydrochloride dissolved in PBS

with 0.1% H_2O_2 before counterstaining with Mayer's Haematoxylin and alcoholic eosin. For analysis of Smad7 in cultured Cardiac fibroblasts, positive stain for Smad7 was counted in 500 cells and expressed as percentage. All examinations were performed blindly on coded slides.

Western blotting

Low-molecular weight marker (Cell Signaling Technology, CST) and 50 ug of protein from samples were separated on 10% or 12% SDS gels by SDS-PAGE. Separated protein was transferred on a polyvinylidene difluoride(PVDF) membrane that was blocked at room temperature for 1 h in Tris-buffered saline with 0.2% Tween 20(TBS-T) containing 5% skim milk and probed with primary antibodies overnight at 4℃. The diluted concentration of the primary antibody: TGF-β1: 1:200(Santa Cruz, Calif); phosphorylated Smad2/3: 1:250(Santa Cruz, Calif); Smad4: 1:200(Santa Cruz, Calif); Smad7: 1:200(Santa Cruz, Calif); collagen I: 1:250(Santa Cruz, Calif); arcadia: 1:200(Santa Cruz, Calif); β-actin: 1:500(Santa Cruz, Calif); Secondary antibodies(Cell signaling Technology, USA) included horseradish peroxidase (HRP)-labeled were duluted 1:1000/2000 with 0.2% TBS-T with 1% skim milk and incubated for 1 h at room temperature. Protein bands on Western blots were visualized ECL Plus (Amersham, Arlington Heights, IL) according to the manufacturer's instructions and were developed on film. Relatively even protein loading was confirmed by immunoblotting against actin.

Statistical Analysis

Data were expressed as the mean±S.E. ANOVA and Student's *t* test were used to determine statistical significance. A two-tailed probability (*P*) of $\leqslant 0.05$ was considered statistically significant.

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