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Downregulation of angiotensin converting enzyme II is associated with pacing-induced sustained atrial fibrillation

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Abstract **Atrial fibrillation (AF), the most common cardiac arrhythmia, is frequently accompanied by atrial interstitial fibrosis. Angiotensin II (Ang II) dependent signaling pathways have been implicated in interstitial fibrosis during the development of AF. However, Ang II could be further degraded by angiotensin converting enzyme II (ACE2). We examined expression of ACE2 in the fibrillating atria of pigs and its involvement in fibrotic pathogenesis during AF. Nine adult pigs underwent continuous rapid atrial pacing to induce sustained AF and six pigs were sham controls (i.e., sinus rhythm; SR). In the histological examinations, extensive accumulation of extracellular matrix in the interstitial space of the atria, as evidenced by Masson's trichrome stain, were found in fibrillating atria. The relative amount of collagen type I in the atria with AF was significantly increased as compared with that in the SR. Local ACE activity in the fibrillating atria was also markedly higher than that in the SR subjects. ACE2 gene and protein expression in the AF subjects were significantly decreased compared with those in the SR subjects, whereas expression of mitogen-activated/ERK kinase 1/2 (MEK1/2), extracellular signal-regulated protein kinase 2 (ERK2), and activated ERK2 were significantly greater in the AF subjects. We propose that decreasing ACE2 expression during AF may affect the Ang II-dependent signaling pathway. In addition, our results suggest that atrial fibrosis in AF may be induced by antagonistic regulation between ACE and ACE2 expression.**

Keywords: Angiotensin converting enzyme II; Atrial fibrillation; Fibrosis; Extracellular signal-regulated kinase; Renin-angiotensin system

1. Introduction

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice. Several studies have shown that AF causes atrial tissue structure remodeling [1–3] that is frequently accompanied by hypertrophic growth of cardiac myocytes and hyperplastic growth of cardiac fibroblasts and results in increased deposition of extracellular matrix (ECM) constituents leading to fibrosis [4]. Likewise, Li et al. [5] have shown in a heart failure model that AF can be promoted by atrial fibrosis that interferes with local conduction. Furthermore, atrial fibrosis alone is enough to promote AF occurrence is proved in animal model [2,6]. Several studies revealed that the renin-angiotensin system (RAS) is associated with the development of AF [7–10]. The cardiac effects of prolonged stimulation of angiotensin II (Ang II), an important effector peptide in the RAS, have been well documented and include the accumulation of interstitial fibrosis [11].

Angiotensin I (Ang I) is converted to Ang II by angiotensin converting enzyme (ACE), an important regulator of the RAS [12]. About 90% of patients with AF have clinical or subclinical cardiovascular disease that may alter cardiac pressure and volume load [13]. These can lead to myocardial stretch, which is a potent stimulus that increases local Ang II levels [14,15]. Yamazaki et al. [15] showed that mechanical stretch can induce cardiac hypertrophy by Ang II-dependent mechanisms by activation of the extracellular signal-regulated kinase (ERK)-mitogen activated protein kinase (MAPK) pathway. Activation of the ERK pathway by G protein–coupled receptor agonists, such as Ang II, can induce cellular differentiation processes and activation of fibroblasts that cause the development of interstitial fibrosis [16,17]. Ang II can also induce proliferation of fibroblasts and accumulation of ECM proteins via activation of ERK1/ERK2, members of a MAPK subfamily [18,19]. Furthermore, inhibitions of the generation or action of Ang II in atrial tissue can downregulate ERK1 and ERK2 expression, resulting in reduced fibrosis [20]. Enhanced activation of ERK1/ERK2 within interstitial heart cells, most likely due to activated RAS, may explain the presence of marked atrial fibrosis in association with AF [9].

In addition to being a potent vasopressor, Ang II also mediates a broad array of physiological and pathophysiological effects by binding to specific cell membrane receptors [21,22]. Human studies have demonstrated that the angiotensin II type 1 (AT1) receptors is largely responsible for many of the pathological effects of Ang II, inclusive of marked cardiac fibrosis [23]. Activation of AT1 receptor, which occurs in the atria with increased frequency in patients with AF, induces fibrosis, which promotes the occurrence of arrhythmia [24]. Several studies has shown that AT1 receptor antagonists could really

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Abbreviations: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme II; AF, atrial fibrillation; Ang I, angiotensin I; Ang II, angiotensin II; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; MEK1/2, mitogen-activated/ERK kinase 1/2; MI, myocardial infarction; RAS, renin-angiotensin system; SR, sinus rhythm

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decreased the rate of AF recurrences or prevent atrial structural remodeling, a contributing factor for the development of AF, in animal model or clinical trial [10,25–27].

Angiotensin converting enzyme II (ACE2), an enzyme identified in rodent and human with a more restricted distribution than ACE, is expressed mainly in heart and kidney [28,29]. ACE2 cleaves a single residue from Ang I to generate angiotensin 1–9 (Ang 1–9) and cleaves Ang II, the main effector of the RAS, to the vasodilator angiotensin 1–7 (Ang 1–7) [28,29]. Results from experiments with *ace2* mutant mice suggest that ACE2 negatively regulates activated RAS [28–30].

Thus, ACE2 may have an important role in fibrotic formation during AF. However, regulation of ACE2 in fibrillating atrium is largely unexplored. Therefore, the purpose of this study was to determine ACE2 expression in pace-inducing fibrillating atria and to test the hypothesis that downregulated ACE2 in the atria is associated with AF.

2. Materials and methods

2.1. Materials

Anti-ERK2 (#sc154), anti-phospho-ERK2 (#sc7976), and anti-ACE2 (#sc17719) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-MEK1/2 (#9121) antibody was purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin (#ac15) and collagen type I (#ab6308) were purchased from Abcam (Cambridge, MA). HRP-labeled secondary antibodies against goat IgG (#sc2020), mouse IgG (#sc2005), and rabbit IgG (#sc2004) were purchased from Santa Cruz Biotechnology. All other reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK).

2.2. Experimental model

Fifteen female pigs (average weight of 65 kg) were used. Of these, six were sham controls that were kept in sinus rhythm (SR), while the remaining nine pigs were subjected to experimentally induce sustained AF as described in our previous report [31,32]. In brief, Yorkshire–Landrace pigs were treated with intravenous anesthesia by thiamylal (2–3 mg/kg) (Kyorin Pharmaceutical, Tokyo, Japan) and then transvenously implanted with a high-speed pacemaker (Itrel-III, model 7425; Medtronic, Minneapolis, MN). A screw-in atrial lead (Model 4568; Medtronic) was positioned at the right atrial appendage via the left internal jugular vein under fluoroscopy. The pacemaker was set to pace the atria at a rate of 400–600 beats per minute in the AF group. During the period of atrial pacing, oral digoxin (0.25 mg per day) was given daily to minimize the possibility of developing congestive heart failure. Consistency of the atrial pacing was checked daily in the first week and weekly thereafter with a portable ECG monitor by a programmer turning the atrial pacing on and off. After 4–6 weeks of continuous pacing, the pigs exhibited sustained AF (i.e., AF was maintained $\geqslant$ 24 h after discontinuance of atrial pacing). In the SR control group, the pigs received a sham operation of pacemaker implantation without atrial pacing for 4 weeks. The pigs of AF and SR group were euthanized by high-dose intravenous barbiturate, the atrial pacemaker was removed and the animals were sacrificed for atrial tissue sampling. The experimental protocol conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and was approved by the animal welfare committees of the National Chiao Tung University and the National Taiwan University. All pigs were provided by the Animal Technology Institute Taiwan (ATIT) and housed at the animal facility at ATIT.

2.3. Tissue sampling and treatments

The left atrial appendages were excised from the isolated hearts and trimmed for pathological examination and frozen storage according to the protocol of our previous report [33]. For assays of ACE activity, RT-PCR and Western blotting, the excised specimens were immediately frozen in liquid nitrogen and stored at −80 °C until use for RNA or protein extraction. For pathological examination, the tissues were cut into small blocks about 10 × 5 mm and immersed in 4% phosphate-buffered formalin for 24 h. After dehydration, each section was cut into 5 μm-thick slices. Deparaffinized sections were stained with Masson's trichrome.

2.4. ACE activity assay

Atrial tissues were frozen immediately after collection and kept at −80 °C until use. Atrial tissues were minced in homogenization buffer consisting of 50 mM HEPES (pH 7.4), 0.3 M NaCl and 1 μM ZnCl, and homogenized at maximum speed for 30 s. Tissue homogenates were centrifuged at 3000×*g* for 10 min. The supernatant was then subjected to centrifugation at 14000×*g* for 60 min at 4 °C. The resulting membrane pellet was resuspended in the homogenization buffer containing 0.5% Triton X-100 and stored overnight at 4 °C. After additional centrifugation at 14000×*g* for 30 min, the supernatant was collected and used as the source of tissue ACE. Protein concentration was determined by the Bradford's method using bovine serum albumin (BSA) as a standard.

ACE activity was measured by the method of Cushman with slight modification [34]. Briefly, 60 μl of 0.4 mM hippuryl-histidyl-leucine (HHL) as substrate was added to 10 μl of sample (30 μg protein) and incubated at 37 °C for 30 min with continuous agitation at 450 rpm. HHL was prepared in 100 mM borate buffer (pH 8.3) containing 0.3 M NaCl. The reaction was stopped by heating at 65 °C for 15 min, and then was centrifuged at 12000×*g* for 5 min, and then 10 μl of supernatant was injected into the column, and the amount of hippuric acid (HA) liberated from the substrate was analyzed by high performance liquid chromatography (HPLC). One unit of ACE activity is defined as the amount of enzyme catalyzing the release of 1 μM of HA from HHL per minute at 37 °C under standard assay condition.

HPLC was performed on a Waters (Milford, MA) Alliance HPLC system equipped with a 2996 photodiode array detector and Waters 717 plus autosampler. Separations were accomplished on Waters Atlantis dC18 column (4.6 mm × 250 mm, 5 μm). The HPLC separation of HHL and HA was detected at 228 nm. The column was eluted (0.5 ml/min) with a two solvent system: (A) 0.05% trifluoroacetic acid (TFA) in water and (B) 0.05% TFA in acetonitrile (45%) for 20 min. External standard HA samples were prepared freshly on a daily basis and used for calculation of the concentration of HA.

2.5. Protein extraction and electrophoresis

Frozen atrial tissues (about 0.2 g) were homogenized in 1 ml of ice-cold lysis buffer containing 20 mM Tris–HCl, 1 mM dithiothreitol, 200 mM sucrose, 1 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.5 mM PMSF, and 1% (v/v) Triton X-100. The homogenate was then centrifuged at 12000×*g* at 4 °C for 10 min, and the supernatant was collected for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Aliquots containing 30 μg protein were resolved on 10% slab SDS–PAGE gels.

2.6. Western blotting

Protein extracts of the left atrial appendages separated by SDS–PAGE were electrophoretically transferred to PVDF membranes (Immobilon-P™; Millipore, Bedford, MA). Briefly, non-specific binding sites were blocked by incubating membranes in 5% non-fat milk. Primary antibodies against proteins were diluted as follows: 1:5000 for β-actin, 1:1000 for ACE2, ERK2, phosphorylated ERK2 and phosphorylated MEK1/2, and 1:2000 for collagen I. The secondary antibody was applied using a dilution of 1:2000. Substrates were visualized using ECL according to the manufacturer's instructions (Western Lightning Chemiluminescence Reagent Plus™; Perkin–Elmer, Boston, MA) and by exposing the membranes to X-Ray film (Super Rx Medical X-Ray Film; Fujifilm, Kanagawa, Japan). The bands were detected at the expected size. The band intensity was quantified using densitometric analysis by imaging software (Scion image™; Scion, Frederick, MD). The amounts of collagen I, ACE2, ERK2, phosphorylated ERK2, and phosphorylated MEK1/2 are expressed relative to the amount of β-actin in respective samples.

2.7. RNA isolation

Total cellular RNA of the left atrial appendages was extracted as recommended by the manufacturer of TRIzol™ (GIBCO BRL, Rockville, MD). Briefly, the TRIzol method consists of the addition of 1 ml

Table 1
Sequences of the primers used in this study for RT-PCR analysis

Gene	GenBank accession number	Forward/Reverse sequence (5′ → 3′)	Condition (cycle no.)	Size of PCR product (bp)
ACE2	AY623811	CAT GAT GAA ACA TAC TGT GAC C CTC CAA GAG CTG ATT TTA GG	94 °C, 30 s → 55 °C, 30 s → 72 °C, 45 s (32)	412
ERK2	M84489	CAG ACA TGA GAA CAT CAT TGG TTT GGA GTC AGC ATT TGG	94 °C, 30 s → 55 °C, 30 s → 72 °C, 45 s (32)	622
GAPDH	BC059110	GGT GAT GCT GGT GCT GAG TA TTC AGC TCT GGG ATG ACC TT	94 °C, 30 s → 55 °C, 30 s → 72 °C, 45 s (24)	413

of the TRIzol reagent to each homogenized tissue (about 100 mg). The mixture was vigorously agitated for 30 s and incubated at room temperature for 5 min. After this procedure, 200 μl chloroform was added to the tube, and the solution was centrifuged at 12000×*g* for 15 min. The aqueous phase was transferred to a clean tube, precipitated with 500 μl isopropyl alcohol, and centrifuged at 12000×*g* for 15 min. The resulting RNA pellet was then washed with 1 ml of 75% cold ethanol and centrifuged at 7500×*g* at 4 °C for 5 min. The pellet was dried at room temperature, resuspended in 20 μl of diethylpyrocarbonate (DEPC)-treated water, and stored at −80 °C. RNA was quantified by measuring absorbance at 260 nm and 280 nm and electrophoresed on a denaturing 1% agarose gel. The integrity and relative amounts of RNA were evaluated using ultraviolet visualization of ethidium bromide-stained RNA.

2.8. RT-PCR

Semi-quantitative RT-PCR was performed as our previously reported [35]. For cDNA synthesis, 3 μg RNA was supplemented in a total reaction volume of 20 μl with 1× reverse transcription (RT) buffer, 0.5 mM dNTPs, 2.5 μM oligo-dT (Invitrogen, Carlsbad, CA), 40 U/μl RNase inhibitor (Invitrogen), and 20 U/μl Superscript II™ reverse transcriptase (Invitrogen). After incubation for 60 min at 50 °C, the mixture was incubated for 15 min at 70 °C to denature the products. The mixture was then chilled on ice.

PCR primers for RT-PCR analysis were shown in Table 1. PCR reaction contains 2 μl cDNA, 1 μl each primer (10 μM), 5 μl of 10× PCR buffer, 2 μl of 10 mM dNTP, 1 μl of 5 U/μl Taq polymerase (Promega, Madison, WI) and 38 μl distilled water in a total volume of 50 μl. Thermal cycler (MiniCycler™; MJ Research, Waltham, MA) conditions were as follows: 1 cycle of 5 min at 94 °C, 24 or 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s, and 1 cycle of 3 min at 72 °C. The resulting PCR products were visualized on 2% agarose gels stained with ethidium bromide. The stained image was recorded by an image analyzer (Kodak DC290 Digital camera System™; Eastman Kodak, Rochester, NY), and the band intensity was quantified using densitometric analysis by Scion image™. The relative mRNA expression of the ACE2 and ERK2 genes was calculated as ratios to glyceraldehyde-3-phophate dehydrogenase (GAPDH) expression.

2.9. Statistical analysis

All values are expressed as means ± standard deviation (S.D.). A Student's *t*-test was used to evaluate differences between groups of discrete variables. A value of $P < 0.05$ was considered statistically significant.

3. Results

Nine pigs with sustained AF were examined for electrophysiological properties and histological characteristics as described in our previous reports [31,32]. In the histological examinations, enlarged intracellular spaces and extensive accumulation of ECM in the interstitial space of the atria, as evidenced by Masson's trichrome stain, were found in fibrillating atria (Fig. 1A and B). Expression of collagen I, a major component of ECM, was measured by quantitative Western blot analysis (Fig. 1C). The assay showed that the relative amount of collagen I was increased in the AF group compared to the SR group (3.44 ± 0.58 vs. 1.62 ± 0.47; $P < 0.01$) (Fig. 1D).

To evaluate the changes of local ACE activity during the AF, we measured the ACE activity in the atrial tissues with AF and SR by the method of HPLC-based assay (Fig. 2A). ACE activity was determined by hydrolysis of the synthetic substrate, HHL and the amount of HA released from the substrate were analyzed. The ACE activity of atrial tissues were increased in the AF group as compared with that in the SR group (4.04 ± 0.61 vs. 1.29 ± 0.57 U/mg; $P < 0.05$) (Fig. 2B).

Relative ERK2 and ACE2 mRNA levels in the left atrial appendages from the AF group and SR group were measured and compared by semi-quantitative RT-PCR (Fig. 3A). The relative amount of ERK2 mRNA in the atrial tissues of the AF group was greater than that in the SR group (1.14 ± 0.36 vs. 0.38 ± 0.27; $P < 0.01$) (Fig. 3B). However, the relative amount of ACE2 mRNA in the AF subjects was less than that in the SR group (0.48 ± 0.14 vs. 1.00 ± 0.28; $P < 0.01$) (Fig. 3C).

Relative protein expression of activated MEK1/2, ERK2, phosphorylated ERK2, and ACE2 in left atrial appendages sampled from the AF and SR groups was detected by Western blotting (Fig. 4A). Activated MEK1/2, an upstream activator kinase of ERK1/2, was raised in the AF group as compared with that in the SR group (4.03 ± 0.31 vs. 1.52 ± 0.35; $P < 0.05$) (Fig. 4B). Furthermore, phosphorylated and activated ERK2 in response to activated MEK1/2 can be expected. The result shows that phosphorylated ERK2 was increased in the atrial tissue of AF subjects as compared with SR subjects (3.35 ± 0.97 vs. 0.65 ± 0.04; $P < 0.01$) (Fig. 4C). Similar to the protein expression of phosphorylated ERK2, the relative amount of ERK2 in the AF group was also greater than that in the SR group (2.59 ± 0.51 vs. 1.16 ± 0.44; $P < 0.05$) (Fig. 4D). In contrast, myocardial tissue showed a decrease in the relative amount of ACE2 (1.20 ± 0.45 vs. 2.75 ± 0.17 in AF vs. SR; $P < 0.05$) (Fig. 4E).

4. Discussion

To our knowledge, our studies demonstrate for the first time that ACE2 contributes to the development of AF in a rapid pacing-induced model. Our results show that both the mRNA and protein level of ACE2 are significantly downregulated in the fibrillating atria of pigs.

Numerous studies on human patients and large animal models suggest the importance of atrial fibrosis in the develop-

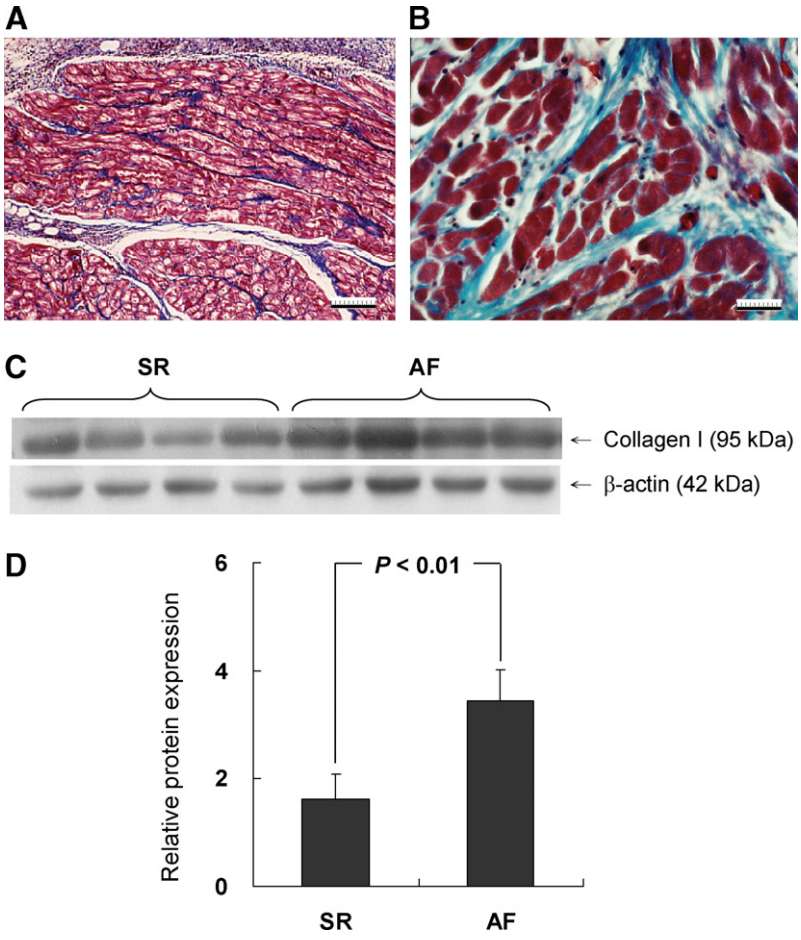

Fig. 1. Pathological examination and measurement of collagen I in left atrial appendages of pigs. (A) In fibrillating atria, extensive interstitial fibrosis was found, as evidenced by Masson's trichrome stain (original magnification, 40×; the scale bar represents 100 μm). (B) A higher-power view of (A) (original magnification, 200×; the scale bar represents 20 μm). (C) Western blot showing expression of collagen type I. (D) Relative protein expression of collagen type I in the AF group was significantly higher than that in the SR group ($P < 0.01$) as determined by densitometry of autoradiograms. The results for each experiment are normalized to the density of β-actin. All values in the histograms are expressed as the means ± S.D.

ment of AF [6,10,20,36,37]. In a previous study, we found that atrial myocytes of fibrillating atria in rapid pacing pigs showed abnormal sarcomeres and a loss of some contractile material [31]. In the present study, extensive interstitial fibrosis, as evidenced by Masson's trichrome stain, was observed in the atria with AF (Fig. 1C and D). Classical mechanism of AF was that AF is caused by single reentry circuit, or triggered by multiple functional reentrant circuits [38]. However, Nattel [39] has mentioned that some extrinsic substrates, e.g. atrial fibrosis, could possibly promote occurrence of single reentry circuit during progression of AF. No matter how AF begins, atrial fibrosis alone is enough to promote AF occurrence is proved in animal model [6].

Several studies have demonstrated that Ang II can stimulate collagen protein synthesis in cultured cardiac fibroblasts and increased collagen I mRNA expression in rat hearts [40,41]. Tharaux et al. [42] reported that Ang II activates the expression of collagen I though the ERK-MAPK signal pathway in fibrotic tissues. In the present study, the results show that upregulation of local ACE activity, activation of MEK/ERK-MAPK cascade and highly expression of collagen I protein in the atrial tissues with AF. Thus, it can be proposed that the upregulation of local ACE activity would lead to the raised Ang II levels which could further activate MEK/ERK-MAPK cascade and stimulate collagen I protein synthesis, which might explain the highly expression of collagen I protein and extensive interstitial fibrosis in the fibrillating atria. Goette et al. [9] has provided the similar results that the expression of atrial ERK1/ERK2 and ACE is found in patients with AF and activated ERK1/ERK2 can be reduced in the patients treated with ACE inhibitors. Dai et al. [43] also demonstrated that patients with AF show a marked atrial fibrosis and increased expression of ERK1/2 in atrial interstitial cells.

Experimental and clinical data have confirmed the proarrhythmic role of the RAS and demonstrated anti-arrhythmic effects of ACE inhibitors and AT1 receptor blockers [24,44–46]. Clinical observations indicate that pharmacological interventions capable of interfering with electrical and structural remodeling processes are of critical importance in the management of patients with AF [47]. Blockade of the RAS has been clearly established in the pathophysiology of a number of cardiac diseases, including hypertension, heart failure, and ventricular remodeling [48]. The beneficial effects of this RAS blockade may be a consequence of preventing the hypertrophic and proinflammatory actions of Ang II as well as the accumulation of the ECM [49,50]. In addition, Shi et al. [51] reported

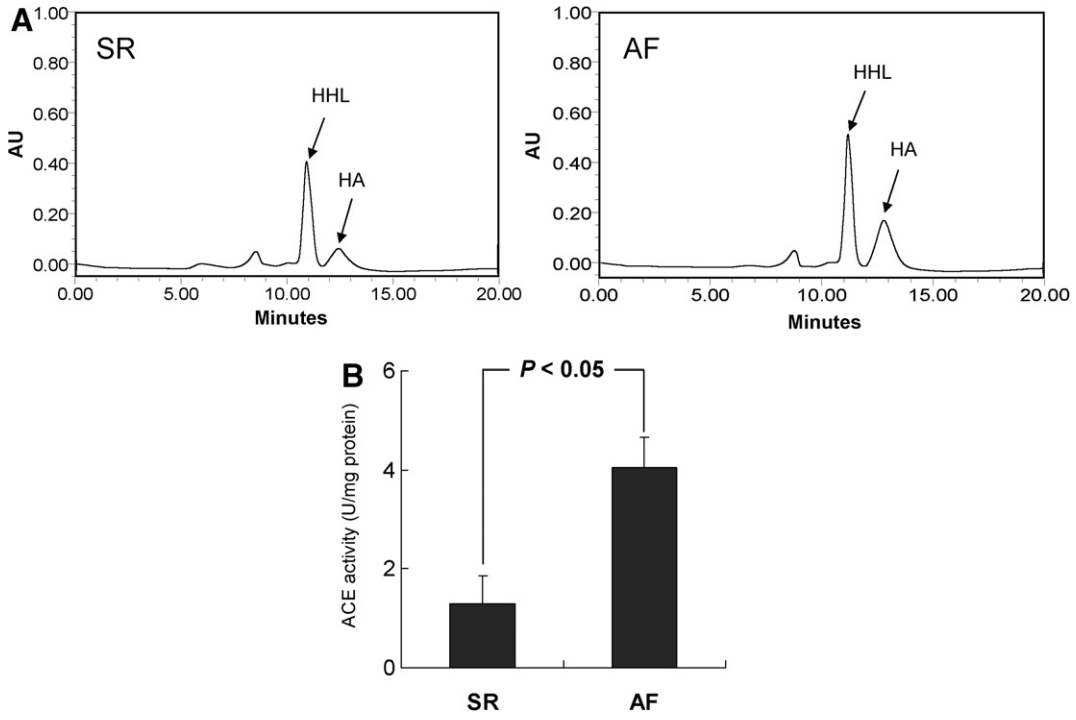

Fig. 2. ACE activity was measured with HPLC-based assay in the atrial tissues with AF and SR. (A) The left panel shows the HPLC separation of HHL hydrolysis catalyzed by ACE in the atrial tissues with SR. Chromatogram of reaction mixtures was showing HHL and HA peaks following HPLC injection. The right panel shows the generated HA and HHL were resolved and quantified by HPLC in reaction mixtures from the fibrillating atria. (B) ACE activities were calculated after HPLC assay and expressed in units per mg of protein. In the AF group ($n = 9$), ACE activity was higher than that in the SR group ($n = 6$) ($P < 0.05$). Data are expressed as the means ± S.D.

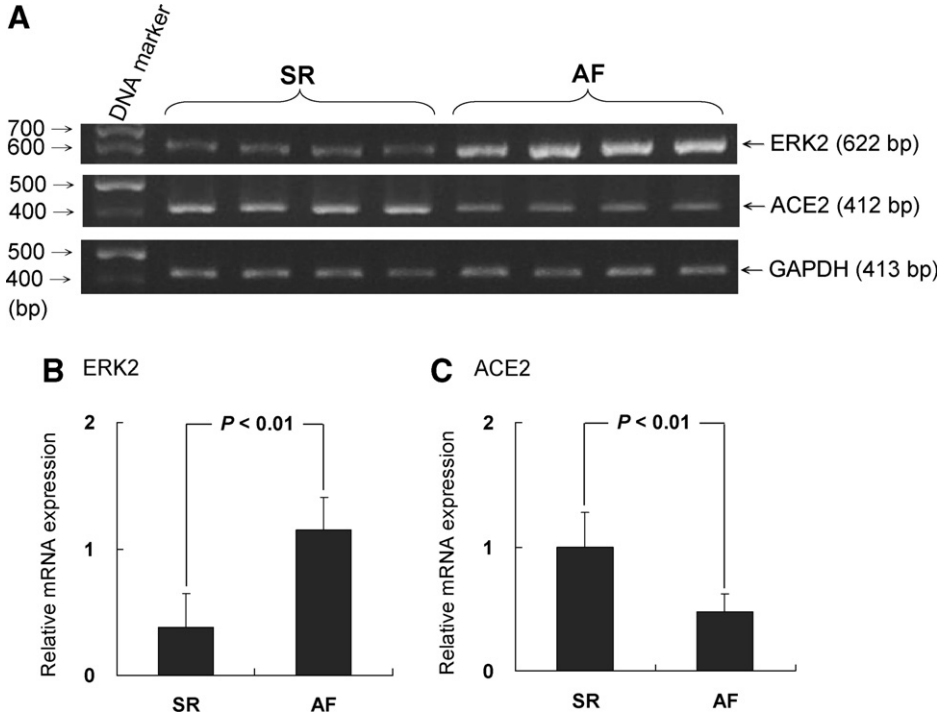

Fig. 3. The relative amounts of ERK2 and ACE2 mRNA in the atrial tissues with AF and SR. (A) Expression of ERK2 and ACE2 in both SR and AF tissues were determined by RT-PCR. The sizes of the PCR products for ERK2, ACE2 and GAPDH were 622 bp, 412 bp and 413 bp, respectively. Amplification of GAPDH is shown as a control. (B) In the AF group ($n = 9$), the relative amount of ERK2 mRNA was increased ($P < 0.01$) as compared with that of the SR group ($n = 6$). (C) In contrast with ERK2, the relative amount of ACE2 mRNA was decreased in the AF group ($P < 0.01$). The results for each experiment were normalized to the density of the GAPDH PCR product. Histograms of all values are expressed as the means ± S.D.

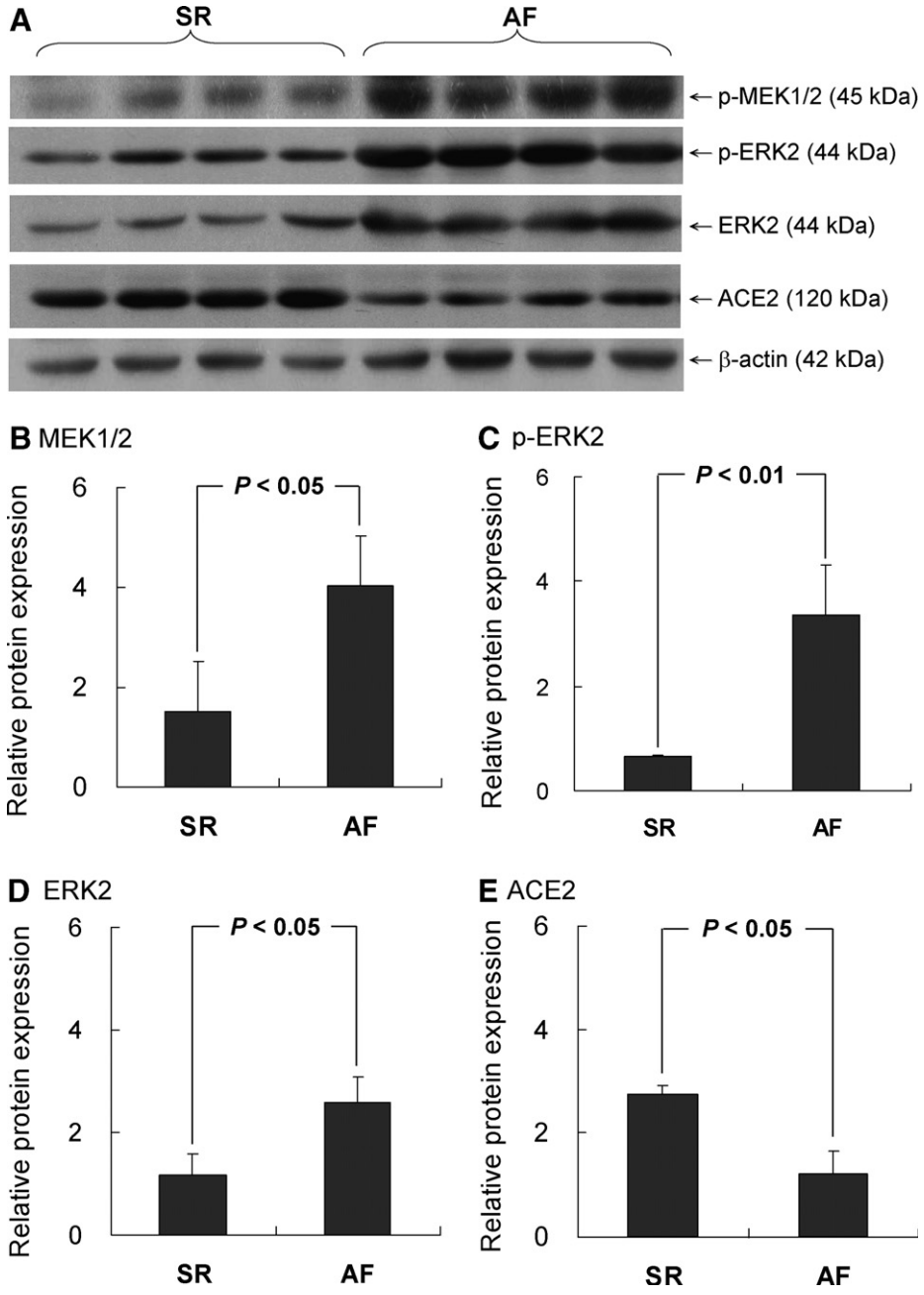

Fig. 4. Protein expression of phosphorylated MEK1/2, ERK2, phosphorylated ERK2, and ACE2 in the atria with AF and SR. (A) Protein expression in both SR and AF tissues were determined by Western blot using antibodies directed against phosphorylated MEK1/2, ERK2, phosphorylated ERK2, ACE2, and β-actin. (B) In the AF group ($n = 9$), the relative expression of phosphorylated MEK1/2 was higher than that in the SR group ($n = 6$) ($P < 0.05$). (C) Phosphorylated ERK2 was significantly increased ($P < 0.01$) in the AF group compared to the SR group. (D) Total ERK2 was more abundant in the AF group ($P < 0.05$) compared to the SR group. (E) In the AF group, the level of ACE2 expression was significantly less than that in the SR group ($P < 0.05$). The results were normalized to the density of β-actin. The values are expressed as the means ± S.D.

that when canines with heart failure (induced by high-frequency stimulation) are pretreated with an ACE inhibitor to inhibit the RAS, the ensuing fibrosis in the atrium is markedly reduced.

A newly discovered enzyme in the RAS pathway, ACE2, cleaves Ang II to produce the vasodilatory/anti-hypertrophic peptide Ang 1–7. These data give rise to the hypothesis that ACE2 provides a counter-regulatory system to Ang II, thereby contributing to the beneficial effects of the RAS blockade (Fig. 5). The potential role of Ang 1–7 as a cardioprotective peptide having vasodilator, anti-growth and anti-proliferative actions has been recognized relatively [19,52]. In addition, Ang 1–7 can downregulate both transcription and translation of the AT1 receptor [53]. Ang 1–7 also augments nitric oxide release, which has a key role in the regulation of cardiac fibrosis in response to myocardial ischemia, in part by antagonizing the action of Ang II [54]. Normal cardiac function in *ace*/*ace2* double-knockout mice suggests that a catalytic product of

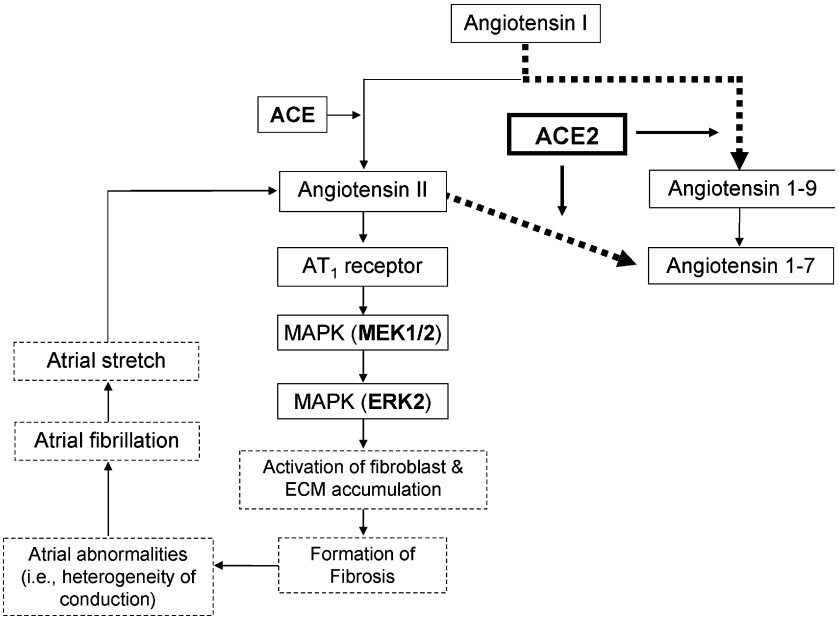

Fig. 5. Schematic overview of antagonistic regulation between ACE and ACE2 in the formation of fibrosis during AF formation and sustenance.

ACE triggers contractile impairment in the absence of ACE2, supporting the hypothesis that ACE2 is indeed a critical negative regulator of the cardiac effects of the RAS [30]. Huentelman et al. [55] showed that delivery of Lentivirusal ACE2 to Sprague–Dawley rat results in significant attenuation of the increased heart weight to body weight ratio and myocardial fibrosis induced by Ang II infusion. These observations demonstrated that ACE2 overexpression is protective for Ang II-induced cardiac hypertrophy and fibrosis. Our results show that ACE2 expression is significantly downregulated in the porcine atria with sustained AF, and we suggest that interstitial fibrosis might be due to an imbalance of the RAS caused by reduced expression of ACE2 during AF development. Recently, Ishiyama et al. [56] provided evidence that the AT1 receptor blockers, losartan and olmesartan, increase Ang peptide concentrations, return AT1 receptor expression to normal, and increase ACE2 expression in hearts suffering from myocardial infarction. These results argue that AT1 receptor blockade may upregulate ACE2 expression. Thus, we suggest that decreasing ACE2 expression during AF may affect the Ang II-dependent signaling pathway.

We propose that there are at least three potential mechanisms by which ACE2 may be cardioprotective via the prevention of cardiac fibrotic processes during AF: first, Ang II is cleaved to Ang 1–7 by ACE2, thereby attenuating Ang II-induced cardiac fibrosis; second, Ang 1–7 may reduce the effects of Ang II by downregulating expression of AT1 receptor; and third, the vasodilatory effect of Ang 1–7 might against the strong vasoconstriction effect of Ang II overproduction during AF to reduce long-term myocardial ischemia-induced cardiac fibrosis.

In conclusion, both ACE2 mRNA and protein are downregulated in the porcine induced by atrial rapid pacing. Our results reveal that decreased ACE2 level might be associated with fibrotic formation during AF. The significant upregulation of MEK1/2 and ERK2 in the atria with AF confirms previous observations linking ERK2 expression and AF, and supports the idea that MEK/ERK-MAPK pathway is activated by the Ang II-signaling pathway. The data further suggest that upregulation of ACE2, which increases the conversion of Ang II to Ang 1–7 to counterbalance the effects of ACE, may be a potential therapeutic strategy for AF.

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