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QUARTERLY FOCUS ISSUE: HEART RHYTHM DISORDERS

Increased Expression of Mineralocorticoid Receptor in Human Atrial Fibrillation and a Cellular Model of Atrial Fibrillation

Chia-Ti Tsai, MD, PHD,* Fu-Tien Chiang, MD, PHD,*† Chuen-Den Tseng, MD, PHD,* Juey-Jen Hwang, MD, PHD,* Kuan-Ting Kuo, MD,*‡ Cho-Kai Wu, MD,* Chih-Chieh Yu, MD,* Yi-Chih Wang, MD,* Ling-Ping Lai, MD, PHD,*§ Jiunn-Lee Lin, MD, PHD*

Taipei, Taiwan

Objectives This study was designed to evaluate the status of steroidogenesis proteins and de novo synthesis of aldosterone in the atrium, and relationships of these factors to atrial fibrillation (AF).

Background The role of mineralocorticoid in the pathogenesis of AF is unknown.

Methods We studied atrial expression of steroidogenesis proteins and aldosterone level in patients with and without AF, and in HL-1 atrial myocytes. We also investigated the electrophysiologic effects and signal transduction of aldosterone on atrial myocytes.

Results We found basal expressions of mineralocorticoid receptors (MRs), glucocorticoid receptors, and 11-beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) but not 11-beta-hydroxylase (CYP11B1) or aldosterone synthase (CYP11B2) in human atria and HL-1 myocytes. There was no significant difference of mean atrial aldosterone level between patients with AF and those with normal sinus rhythm. However, patients with AF had a significantly higher atrial MR expression compared with those with normal sinus rhythm (1.73 ± 0.24-fold, $p < 0.001$). Using mouse HL-1 atrial myocytes as a cellular AF model, we found that rapid depolarization increased MR expression (1.97 ± 0.72-fold, $p = 0.008$) through a calcium-dependent mechanism, thus augmenting the genomic effect of aldosterone signaling as evaluated by MR reporter. Aldosterone increased intracellular oxidative stress through a nongenomic pathway, which was attenuated by nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenyleneiodonium, but not by MR-blockade spironolactone. Aldosterone increased expression of the alpha-1G and -1H subunits of the T-type calcium channel and thus increased the T-type calcium current (−13.6 ± 2.9 pA/pF vs. −4.5 ± 1.6 pA/pF, $p < 0.01$) and the intracellular calcium load through a genomic pathway, which were attenuated by spironolactone, but not by diphenyleneiodonium.

Conclusions Expression of MR increased in AF, thus augmenting the genomic effects of aldosterone. Aldosterone induced atrial ionic remodeling and calcium overload through a genomic pathway, which was attenuated by spironolactone. These results suggest that aldosterone may play a role in AF electrical remodeling and provide insight into the treatment of AF with MR blockade. (J Am Coll Cardiol 2010;55:758–70) © 2010 by the American College of Cardiology Foundation

The renin-angiotensin system (RAS) is involved in many cardiovascular diseases. Recent reports suggest that atrial fibrillation (AF) is associated with activation or up-regulation of RAS in the atria in humans (1) and in animal models of AF (2). Aldosterone, a mineralocorticoid, is a well-known component of the RAS, but its role in AF has not been investigated in detail. It has been shown that aldosterone levels are elevated in patients with persistent AF, and restoration of sinus rhythm with electrical cardioversion lowers serum aldosterone concentrations (3).

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Aldosterone is a biologic steroid. Aldosterone synthase belongs to the cytochrome P450 (CYP) family and is also called CYP11B2. It is the key enzyme for synthesizing aldosterone. Synthesis of the glucocorticoids, corticosterone, and cortisol requires a distinct isoenzyme closely

From the *Division of Cardiology, Department of Internal Medicine, †Department of Laboratory Medicine, ‡Department of Pathology, and the §Institute of Pharmacology, National Taiwan University, Taipei, Taiwan. Partially supported by grants from the National Taiwan University Hospital (94A03 and 96M27), the National Science Council (95-2314-B-002-087-MY3, 96-2314-B-002-187-MY3, and 98-2314-B-002-134-MY3), and the New Century Health Care Promotion Foundation.

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related to aldosterone synthase; this isoenzyme is called 11-beta-hydroxylase, or CYP11B1. Mineralocorticoid receptor (MR) is the nuclear receptor of aldosterone and is responsible for the genomic effects of aldosterone. The enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) tightly regulates MR binding and selectively metabolizes glucocorticoids to inactive metabolites, allowing aldosterone to occupy and activate the MR.

De novo cardiac synthesis of aldosterone by CYP11B2 in the ventricular myocardium is reported and has been implicated in the pathogenesis of ventricular hypertrophy and fibrosis (4). Unknown issues are whether proteins related to cardiac steroidogenesis are expressed, whether de novo synthesis of aldosterone occurs in the atrial myocardium, and whether expression of these proteins was altered in AF.

The aims of this study were 3-fold. First, we wanted to assess the expression of MRs, glucocorticoid receptors (GRs), 11bHSD2, CYP11B1, and CYP11B, as well as de novo cardiac synthesis of aldosterone in human atria and murine atrial HL-1 myocytes. Second, we determined whether expression of these proteins was altered in human AF and in a cellular model of AF. Third, we investigated the effect of aldosterone on HL-1 atrial myocytes and the possible signaling pathways.

Methods

See the Online Appendix for an expanded Methods section.

Human atrial tissues. Samples of right atrial appendages were obtained from consecutive patients with or without AF who were undergoing mitral and/or aortic valve replacement for significant valvular disease. Another study group without significant valvular heart disease, who had less left atrial enlargement, was also included.

HL-1 cell culture and rapid electrical-field stimulation (RES). The HL-1 atrial cell line was derived from adult mouse atria obtained from Louisiana State University, New Orleans, and cultured as previously described (5–7). RES of the cultured HL-1 cells was performed as previously described (6).

Transient transfection and MR reporter luciferase assay. Transient transfection of HL-1 cells with MR luciferase reporter was accomplished by using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions as previously described (5,7).

Measurement of myocardial aldosterone levels. The concentration of tissue aldosterone was measured by radioimmunoassay as previously described (8).

Extraction of ribonucleic acid and reverse transcription-polymerase chain reaction (RT-PCR). The extraction and quantification of messenger ribonucleic acid (mRNA) by means of reverse transcription-polymerase chain reaction were performed as previously described (6,7,9). The mRNA of cells of human adrenocortical cell line H295R (kindly provided by Dr. K. D. Wu) was used as the positive control for human CYP11B1 and CYP11B2. The mRNA from the mouse adrenal gland was used as the positive control for mouse CYP11B1 and CYP11B2 (HL-1 myocytes).

Measurement of transmembrane currents and sarcoplasmic reticulum calcium load. Transmembrane currents were measured by using a patch-clamp amplifier (8900, Dagan Corporation, Minneapolis, Minnesota) by applying a whole-cell recording technique as previously described (7). The sarcoplasmic reticulum (SR) calcium load was measured by rapid caffeine application as previously described (7).

Statistical analysis. Data are presented as mean ± SD and were analyzed by using the Student *t* test for 2-group comparisons, and 1-way analysis of variance for comparisons among more than 2 groups, with post hoc Student *t* tests with Bonferroni corrections for the p values. A p value <0.05 indicated a statistically significant difference.

Abbreviations and Acronyms

11bHSD2 = 11-beta-hydroxysteroid dehydrogenase type 2
AF = atrial fibrillation
BAPTA-AM = 1,2-bis (aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid acetomethyl ester
COL = procollagen type
CYP = cytochrome P450
GR = glucocorticoid receptor
ICaL = L-type calcium current
ICaT = T-type calcium current
IKr = rapidly activating delayed rectifier potassium current
MR = mineralocorticoid receptor
mRNA = messenger ribonucleic acid
NSR = normal sinus rhythm
RAS = renin-angiotensin system
RES = rapid electrical-field stimulation
ROS = reactive oxygen species
RT-PCR = reverse transcription–polymerase chain reaction
SR = sarcoplasmic reticulum

Results

Myocardial aldosterone levels in the human atrium. Table 1 shows the clinical characteristics of the study subjects with significant valvular heart disease undergoing valve replacement surgery. Mean left atrial dimension was larger in patients with AF than in subjects with normal sinus rhythm (NSR). We detected no significant difference in other clinical variables between these groups. The mean AF duration was 8.3 ± 5.6 (0.6 to 19.4) years for the patients with AF. The mean heart rate for the patients with AF was 77.3 ± 9.3 beats/min and 71.1 ± 6.3 beats/min for those with NSR ($p = 0.053$).

Histologically, we found a nonsignificant increase of extracellular matrix accumulation and a trend of increase of procollagen expression (COL1A1) in subjects with AF (extracellular matrix: 16.3 ± 4.4 % in subjects with NSR vs. 20.7 ± 5.3% in those with AF, $p = 0.157$; 1.28 ± 0.22-fold increase of COL1A1 expression in subjects of AF compared with those with NSR, $p = 0.066$, $n = 6$ for each group) (Online Fig. 1). Mean aldosterone levels in atrial tissues did not significantly differ between subjects with AF and those with NSR (6.12 ± 1.73 pg/mg protein in subjects with NSR vs. 5.83 ± 1.54 pg/mg protein in those with AF, $p = 0.643$, $n = 14$ for each group) (Fig. 1).

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Table 1 Patient Characteristics

	Age, yrs	Sex	VHD	HTN	DM	LVEF, %	LAD, mm	Drugs
SR (n = 14)								
	73	F	AR	1	0	56	43	C, Di
	66	M	MR	0	1	62	42	O, Di, D
	54	M	MR	0	0	63	48	D, Di
	83	F	AR, MR	0	0	51	55	Di, D
	59	M	MR	1	0	36	47	A, Di, D
	67	M	AR, MR	0	1	49	42	O, D
	52	F	MR	1	0	57	53	A, D
	43	M	AR	0	0	82	46	Di
	55	M	MR	0	1	50	54	A, Di, D, O
	72	F	AR	1	1	56	48	C, Di, D, O
	54	M	MR	0	1	65	41	D, O
	65	F	AR, TR	0	0	45	49	Di, D
	77	M	MR	0	0	71	45	C, Di
	72	F	MR, TR	0	0	53	54	Di, D
Total	63.7 ± 11.2	8 M/6 F				56.8 ± 11.4	47.6 ± 4.8	
AF (n = 14)								
	69	M	AR	0	0	64	54	Di, D
	63	F	MR	0	0	57	53	Di, D
	70	M	AR	1	0	47	57	C, Di, D
	65	M	MR	0	1	43	48	C, Di, O
	64	F	AR, MR	0	0	33	49	A, B, Di, D
	73	M	MR	1	0	56	54	Di, D
	58	M	MR	1	0	53	55	C, D
	71	M	MR, TR	0	0	73	57	C, Di, D
	73	M	MR, TR	0	0	67	65	B, Di, D
	43	M	MR	0	0	51	53	Di, D
	73	F	MR	0	0	55	46	Di, D
	79	F	MR	1	0	61	52	A, D
	59	M	AR	0	0	63	44	C, Di
	80	F	MR, AR	0	0	60	58	Di, D
Total	67.1 ± 9.6	9 M/5 F				55.9 ± 10.3	53.2 ± 5.4*	

*$p < 0.05$ versus sinus rhythm.

A = angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker; AF = chronic persistent atrial fibrillation; AR = aortic regurgitation; B = beta-blocker; C = calcium-channel blockers; D = digoxin; Di = diuretics; DM = diabetes mellitus; HTN = hypertension; LAD = left atrial dimension; LVEF = left ventricular ejection fraction; MR = mitral regurgitation; O = oral hypoglycemic agent; SR = sinus rhythm; TR = tricuspid regurgitation; VHD = valvular heart disease requiring valve replacement.

Mean aldosterone levels in atrial tissues also did not significantly differ between subjects with AF and those with NSR in the second study group without significant valvular heart disease (Online Appendix).

Basal expression of MRs, GRs, 11bHSD2, CYP11B1, and CYP11B2 in human atrium and murine atrial HL-1 cells. Levels of expression for CYP11B1 and CYP11B2 were high in the human adrenocortical cell line (Fig. 2A). We observed basal expression of MRs, GRs, and 11b-HSD2 but not CYP11B1 and CYP11B2 in the human atria (Fig. 2A).

In HL-1 cells, expression patterns for MR, GRs, 11b-HSD2, CYP11B1, and CYP11B2 were similar to those seen in the human atrium (Fig. 2B); again, expression of CYP11B1 and CYP11B2 was absent. Although some have reported that angiotensin II can induce CYP11B2 expression (10), we found no induction of CYP11B2 expression in HL-1 myocytes after stimulation with angiotensin II 1 μmol/l for as long as 24 h (data not shown).

Increased expression of MRs in human AF. When we compared expression levels for MRs, GRs, and 11b-HSD2 between patients with AF and those with NSR, we found that expression of MR (Fig. 3C) was higher in the AF group than in the other group (1.73 ± 0.24-fold, $p < 0.001$ by quantitative RT-PCR, n = 14 for each group). Mean expression levels of GRs (Fig. 3B) and 11b-HSD2 (Fig. 3A) did not notably differ.

The results were similar in the second study group without significant valvular heart disease (Online Fig. 2).

Rapid depolarization increased sustained elevation of intracellular calcium, and increased MR expression and aldosterone responsiveness in HL-1 myocytes. In confluent HL-1 cells, we noted rhythmic oscillation of the fluorescence signal (6). Under RES at 1.0 V/cm and 10 Hz, elevation of the fluorescence signal with fibrillatory-like morphology was observed (6). This finding indicated sustained elevation of intracellular calcium levels, a characteristic feature of atrial myocytes during AF.

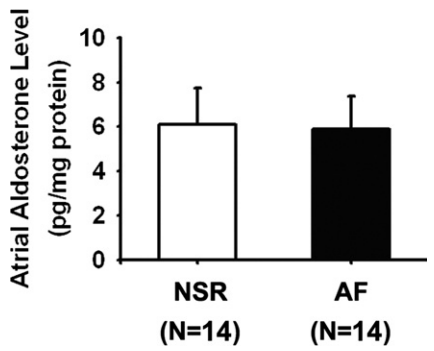

Figure 1 **Mean Atrial Tissue Aldosterone Level in Patients With NSR and Those With AF**

No significant difference of mean atrial tissue aldosterone level between patients with normal sinus rhythm (NSR) and those with atrial fibrillation (AF). Atrial tissue aldosterone level was measured by radioimmunoassay. Data represent mean ± SD and n = 14 for each group.

Figure 4 shows changes in the expression of MRs, GRs, and 11bHSD2 under RES. Expression of MR was up-regulated in a time-dependent manner (1.97 ± 0.72-fold after 24 h of stimulation, p = 0.008) (Fig. 4A). This effect was abolished by intracellular calcium chelator 1,2-bis (amino-phenoxy) ethane-*N,N,N′,N′*-tetraacetic acid acetomethyl ester (BAPTA-AM) (10 μmol/l) and the L-type calcium-channel blocker verapamil (10 μmol/l) (Fig. 4B), which indicated that it was calcium-dependent. Expressions of GR and 11bHSD2 were down-regulated, but the change was not statistically significant (Fig. 4A). There was no detectable aldosterone in the serum-free culture medium, either before or after RES. The up-regulation of MR by RES was also not attenuated by the MR-blockade spironolactone (Online Fig. 3).

It has been shown that calcium overload during rapid pacing induces oxidative stress via nicotinamide adenine dinucleotide phosphate oxidase (11). Therefore, it is possible that the up-regulation of MR by RES is both calcium- and oxidative stress–dependent. However, we found that RES-induced MR expression was not attenuated by nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenyleneiodonium or the reactive oxygen species (ROS) scavenger *N*-acetylcysteine (Online Fig. 4).

We also performed studies to prove that the effect of RES on MR expression was not due to the nonspecific effect of electrolysis or electrical current produced in the medium by RES. See the results in the Online Appendix (Online Fig. 5).

To study the functional importance of increased MR expression, we performed MR reporter luciferase assay. When HL-1 myocytes were subjected to aldosterone stimulation (0.1 μmol/l for 24 h), MR reporter luciferase activity increased 1.7-fold, which was attenuated by the MR-competitive antagonist spironolactone (1 μmol/l) (Fig. 4C, open bars). This result suggested the specificity of the MR reporter to aldosterone stimulation. When HL-1 myocytes were subjected to simultaneous aldosterone and RES stimulations, MR reporter luciferase activity increased 2.4-fold, which was also attenuated by the MR-competitive antagonist spironolactone (1 μmol/l) (Fig. 4C, solid bars). The increase of luciferase activity in response to aldosterone stimulation was higher in the presence than in the absence of RES (2.4 ± 0.5-fold with RES vs. 1.7 ± 0.3-fold without RES; p = 0.015), suggesting the functional importance of increased MR expression due to RES. The effect of RES to increase aldosterone responsiveness was also attenuated by intracellular calcium chelator BAPTA-AM (10 μmol/l) and the L-type calcium-channel blocker verapamil (10 μmol/l) (Online Fig. 6).

Electrophysiological effects of aldosterone on atrial myocytes. HL-1 myocytes had typical L- and T-type calcium currents (ICaL and ICaT), which were sensitive to

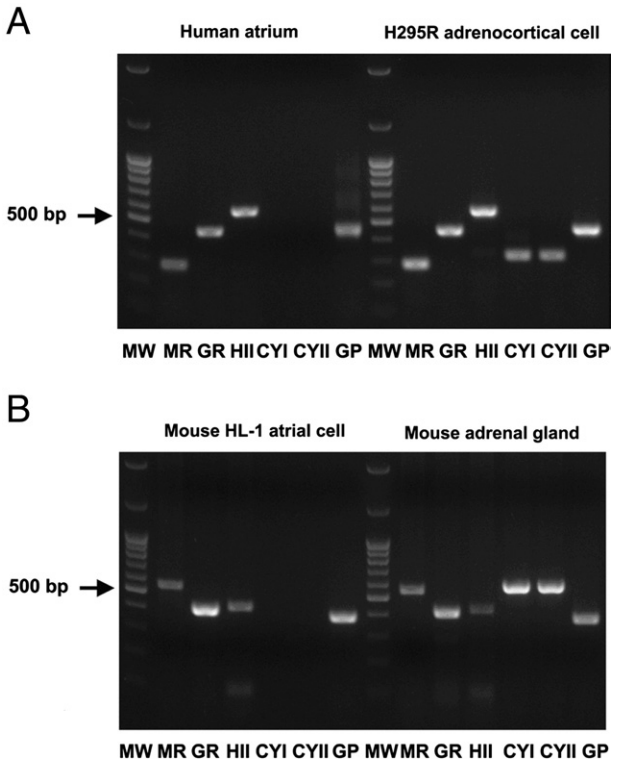

Figure 2 **Basal Expressions of Steroidogenesis Proteins in the Human Atrium and the Mouse HL-1 Atrial Myocytes**

Basal expressions of 11-beta-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and 11-beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) in the human atrium and the mouse atrial HL-1 myocytes. There are no expressions of CYP11B1 and CYP11B2 in the human atrium and the mouse HL-1 atrial myocytes. Total ribonucleic acid was isolated and reverse transcription-polymerase chain reaction products with specific primer pairs were visualized by electrophoresis. **(A)** The **left panel** shows the polymerase chain reaction results of a sample from right atrial appendage of 1 patient, and the **right panel** shows those of a positive control for human CYP11B1 and CYP11B2 from human adrenocortical H295R cells. **(B)** The **left panel** shows the polymerase chain reaction results of HL-1 atrial myocytes, and the **right panel** shows those of a positive control for mouse CYP11B1 and CYP11B2 from the mouse adrenal gland. bp = base pair; CYI = CYP11B1; CYII = CYP11B2; GP = glyceraldehyde 3-phosphate dehydrogenase; HII =11bHSD2; MW = molecular weight maker.

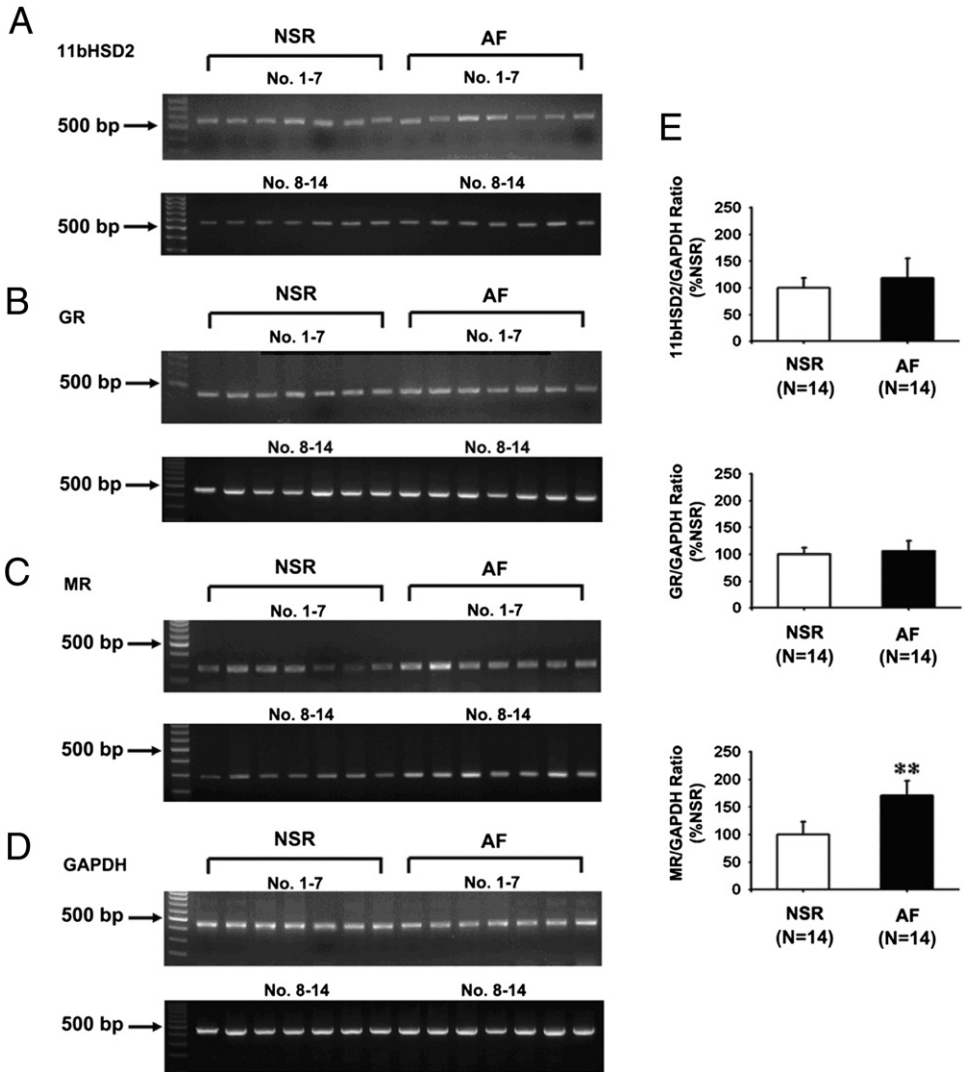

Figure 3 **Increased Expression of MR in Patients With AF**

Increased expression of the messenger ribonucleic acid (mRNA) level of MR without significant changes of those of GR and 11-beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) in patients with atrial fibrillation (AF). Total ribonucleic acid was isolated and reverse transcription-polymerase chain reaction products were visualized by electrophoresis. Both semiquantitative and quantitative reverse transcription-polymerase chain reaction were used to quantify mRNA levels of MR, GR, and 11bHSD2 normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **(A to D)** The reverse transcription-polymerase chain reaction results of the 11bHSD2 **(A)**, GR **(B)**, MR **(C)**, and GAPDH **(D)** in patients with AF and those with normal sinus rhythm (NSR). **(E)** The mean expression level of mRNA of 11bHSD2, GR, and MR in patients with AF and those with NSR. Data were normalized to GADPH level **(D)**, and represent mean ± SD (n = 14 for each group). **p < 0.01 versus patients with NSR. Abbreviations as in Figure 2.

nifedipine 3 μmol/l and nickel 1 μmol/l, respectively (7). Aldosterone (0.1 μmol/l for 24 h) increased ICaT current density (peak inward current: −13.6 ± 2.9 pA/pF for aldosterone treatment [n = 6] and −4.5 ± 1.6 pA/pF for control [n = 6]; $p < 0.01$), without a significant change in ICaL (Figs. 5A and 5B). Coadministration of MR-blockade attenuated the increase in ICaT density (peak inward current: −6.7 ± 1.8 pA/pF [n = 6]; $p < 0.01$ vs. aldosterone treatment) (Figs. 5A and 5B).

HL-1 myocytes also had typical rapidly-activating delayed rectifier potassium currents (IKrs), which were sensitive to the specific IKr blocker E4031 (1 μmol/l) (Fig. 6A). Aldosterone (0.1 μmol/l for 24 h) decreased IKr current density (peak tail current: 7.9 ± 4.6 pA/pF for aldosterone treatment [n = 6] and 18.2 ± 3.7 pA/pF for control [n = 6]; $p < 0.01$) (Figs. 6B and 6C). We observed no significant changes in other potassium currents (data not shown). Coadministration of MR-blockade spironolactone (1 μmol/l for 24 h) also attenuated the decrease in IKr current density (peak outward current: 15.5 ± 2.6 pA/pF [n = 6]; $p < 0.01$ vs. aldosterone treatment) (Figs. 6B and 6C). It has been shown that spironolactone also exerts MR-independent effects

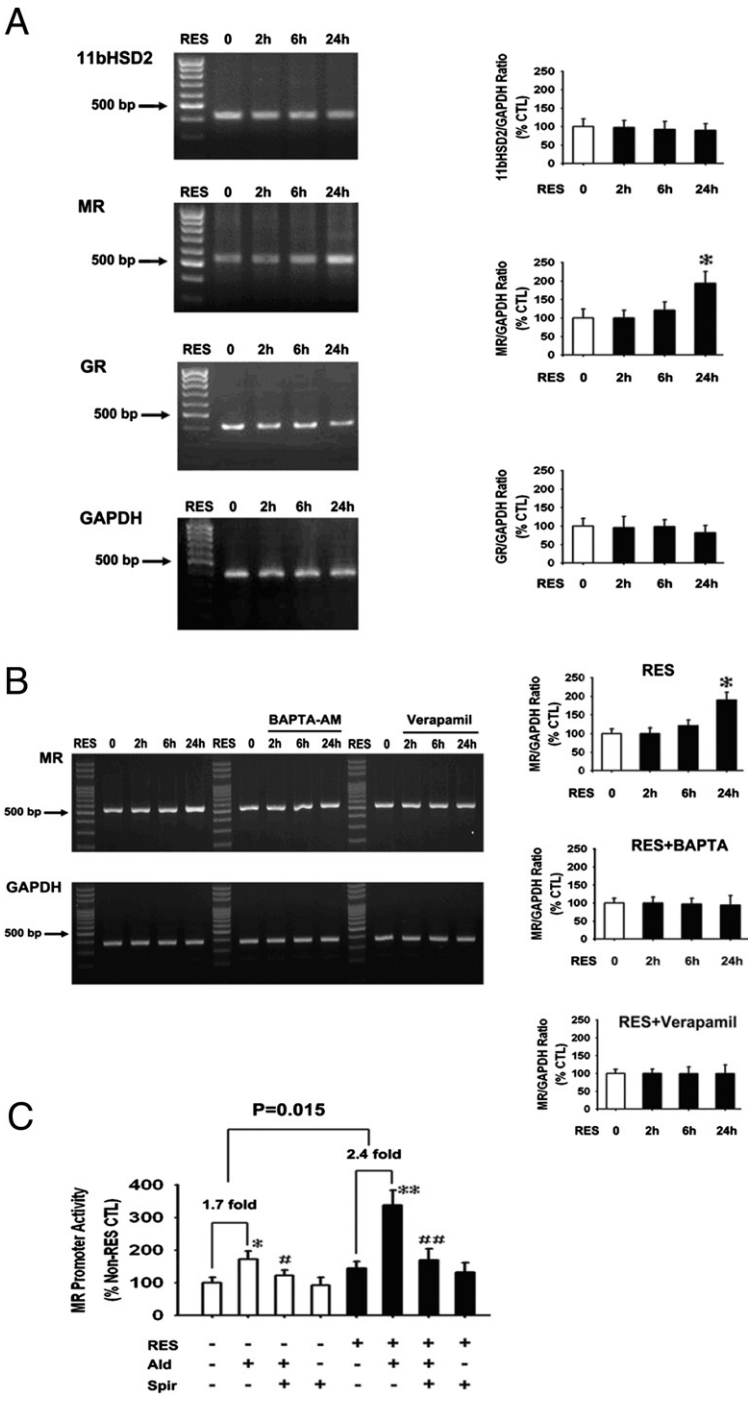

Figure 4 **REF Stimulation Increases MR Expression and Aldosterone Responsiveness in HL-1 Atrial Myocytes**

Rapid electrical-field stimulation (RES) increases MR expression and aldosterone responsiveness in HL-1 atrial myocytes. **(A)** Total ribonucleic acid (RNA) was isolated from HL-1 myocytes left untreated (0 h) or treated for indicated times with RES. Reverse transcription-polymerase chain reaction products were visualized by electrophoresis. The messenger RNA levels of MR, GR, and 11-beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) were quantified and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **(B)** Total RNA was isolated from HL-1 myocytes left untreated (0 h) or treated for indicated times with RES + 1,2-bis (aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid (10 μmol/l) and RES + verapamil (10 μmol/l). The mRNA levels of MR were quantified and normalized to that of GAPDH. **(C)** HL-1 atrial myocytes were transiently transfected with MR luciferase reporter and the plasmid of renilla luciferase-thymidine kinase (pRL-TK) vector for an internal control, then subjected to RES alone, RES plus aldosterone (Ald) (0.1 μmol/l) simultaneously, RES + aldosterone (0.1 μmol/l) plus spironolactone (Spir) (1 μmol/l), and RES plus spironolactone (1 μmol/l) for 24 h, and then luciferase activities were measured **(solid bars)**. Another set of myocytes with the same treatment but without RES were used for comparison **(open bars)**. Data represent mean ± SD of 3 independent experiments for reverse transcription–polymerase chain reaction study and 6 for luciferase study. *p $<$ 0.05 and **p $<$ 0.01 versus control; #p $<$ 0.05 and ##p $<$ 0.01 versus aldosterone-treated cells. CTL = control patients; other abbreviations as in Figure 2.

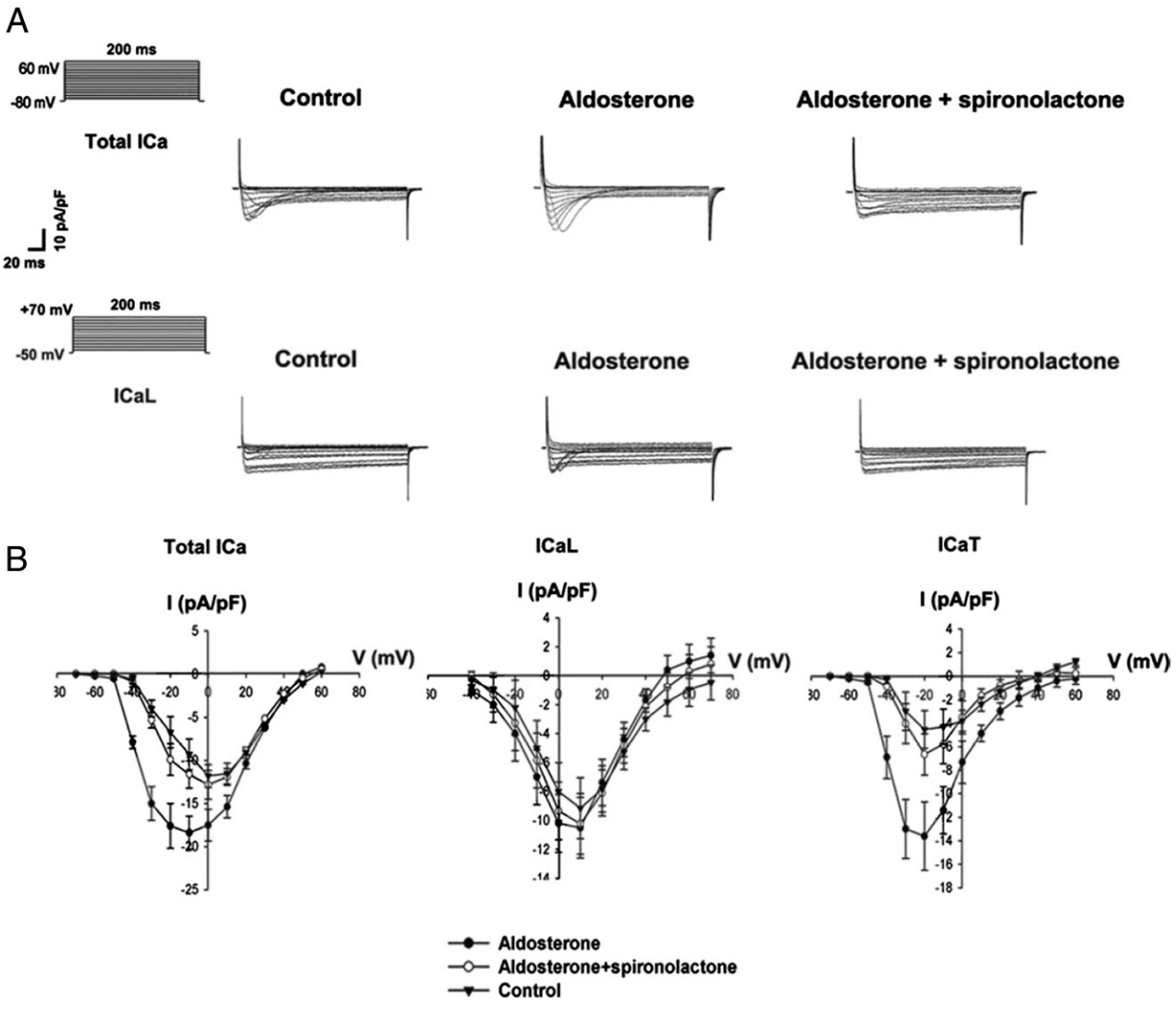

Figure 5 **Aldosterone Increases ICaT in HL-1 Atrial Myocytes**

Aldosterone increases T-type inward calcium current (ICaT) in HL-1 atrial myocytes, which is attenuated by spironolactone. **(A)** Representative recordings of whole-cell inward calcium currents are shown for the control cells, aldosterone-treated (0.1 μmol/l, 24 h) and aldosterone (0.1 μmol/l, 24 h) plus spironolactone (1 μmol/l, 24 h)-treated cells. Total calcium currents including L-type (ICaL) and ICaT were obtained by a family of depolarization steps to +60 mV from the holding potential −80 mV. The ICaL current was obtained by a family of depolarization steps to +70 mV from the holding potential −50 mV. The ICaT current was obtained after subtracting total calcium current by ICaL. **(Inset)** Voltage protocol. **(B)** Representative current density-voltage relationships of ICaL and ICaT in control cells, aldosterone-treated, and aldosterone plus spironolactone-treated cells (all n = 6).

blocking IKr potassium channels (12). We found that single spironolactone did not exert any effect on the current density of IKr in HL-1 atrial myocytes (Figs. 6B and 6C).

Aldosterone increased mRNA levels of the alpha-1G and -1H subunits of ICaT and decreased that of the ether-a-go-go-related (ERG) subunit of IKr (Figs. 7A and 7B). Co-administration of MR-blockade attenuated these effects (Figs. 7C and 7D). We noted no significant changes in the mRNA levels of channel subunits of other ionic channels, such as alpha-1C, SCN5A, KVLQT1, Kv4.2, Kir2.1, Kv 2.1, HCN1, and HCN2 (data not shown), after aldosterone stimulation.

Aldosterone also increased SR calcium load of HL-1 atrial myocytes (Fig. 8). Coadministration of MR-blockade spironolactone (1 μmol/l for 24 h) attenuated this change.

Oxidative stress and aldosterone effects on atrial myocytes. The aldosterone signaling mechanisms are very complex, including genomic and nongenomic effects. It has been shown that aldosterone promotes oxidative stress through the nongenomic effect and is MR-independent (13). On the other hand, other studies have also shown that MR antagonists reduce oxidative stress (14). Therefore, we evaluated the role of oxidative stress and the relative influence of

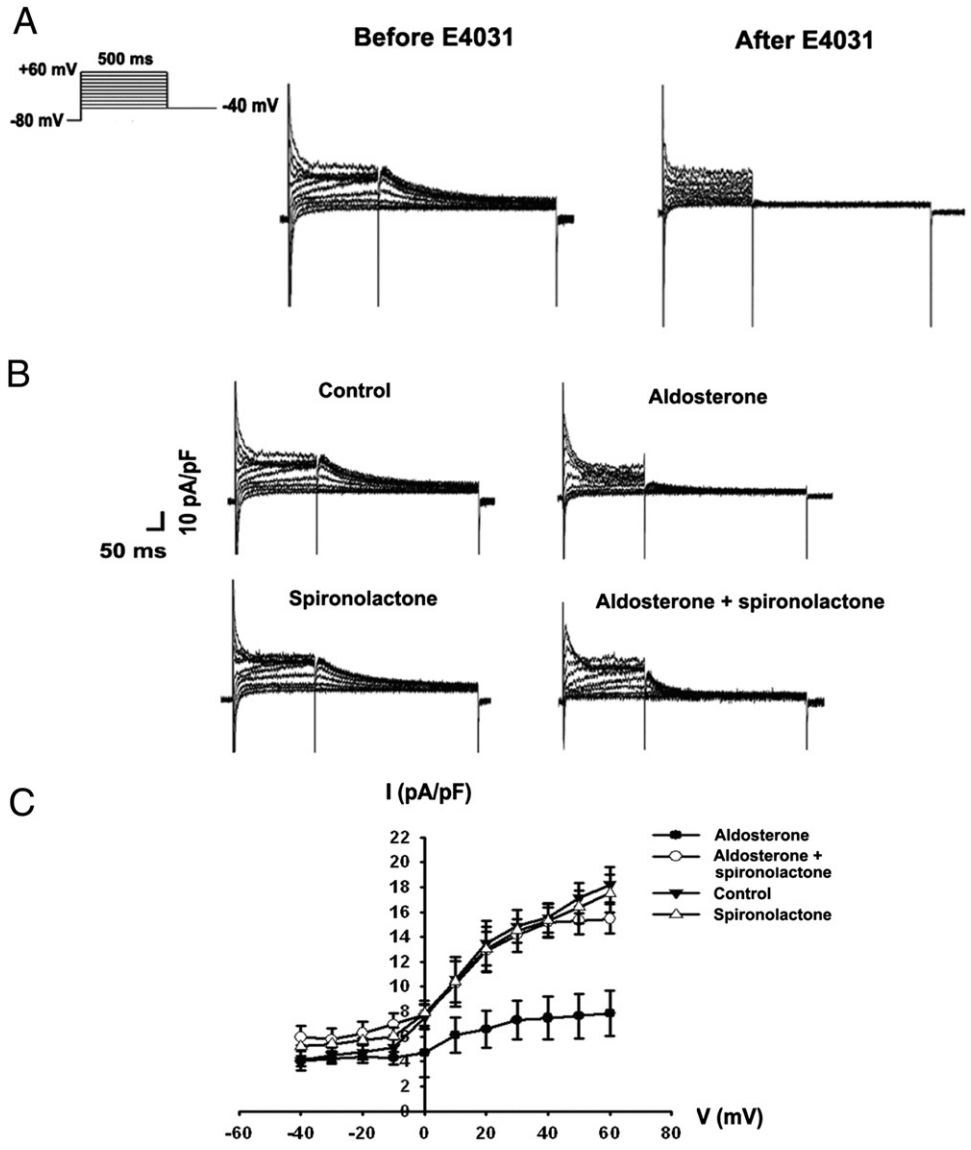

Figure 6 **Aldosterone Decreases IKr in HL-1 Atrial Myocytes**

Aldosterone decreases rapidly-activating delayed rectifier potassium current (IKr) in HL-1 atrial myocytes, which is attenuated by spironolactone. **(A)** IKr was obtained by a family of depolarization steps from −80 mV, and was measured as the tail currents at the repolarization step to −40 mV, which was sensitive to E4031 (1 μmol/l). **(B)** Representative recordings of IKr tail currents are shown for the control cells, aldosterone-treated (0.1 μmol/l, 24 h), aldosterone (0.1 μmol/l, 24 h) plus spironolactone (1 μmol/l, 24 h)-treated, and spironolactone-treated (1 μmol/l, 24 h) cells. **(C)** Representative current density-voltage relationships of IKr tail currents in control cells (n = 6), aldosterone-treated (n = 6), aldosterone plus spironolactone-treated (n = 6), and spironolactone-treated (n = 6) cells.

genomic and nongenomic mechanisms to mediate aldosterone effect on ionic remodeling.

We found that aldosterone time-dependently increased intracellular level of ROS, as evaluated by lucigenin-dependent chemiluminescence (7) (Online Fig. 7A). This effect was nongenomic, because it was not attenuated by the classical MR-blockade spironolactone. It was nicotinamide adenine dinucleotide phosphate oxidase-dependent, because it was attenuated by nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenyleneiodonium and the ROS scavenger *N*-acetylcysteine (Online Fig. 7B).

Therefore, it is possible that the effect of aldosterone on ionic channel expression may be through this ROS-dependent nongenomic effect. However, we found that the effect of aldosterone to modulate ionic channel gene expressions was not attenuated by N-acetylcysteine and diphenyleneiodonium (Online Fig. 8). As shown before, the effect of aldosterone to modulate ionic channel gene expressions was attenuated by MR-blockade. Taken together, aldosterone triggers electrical remodeling through the classical genomic, MR-dependent pathway, but not the nongenomic, ROS-dependent pathway.

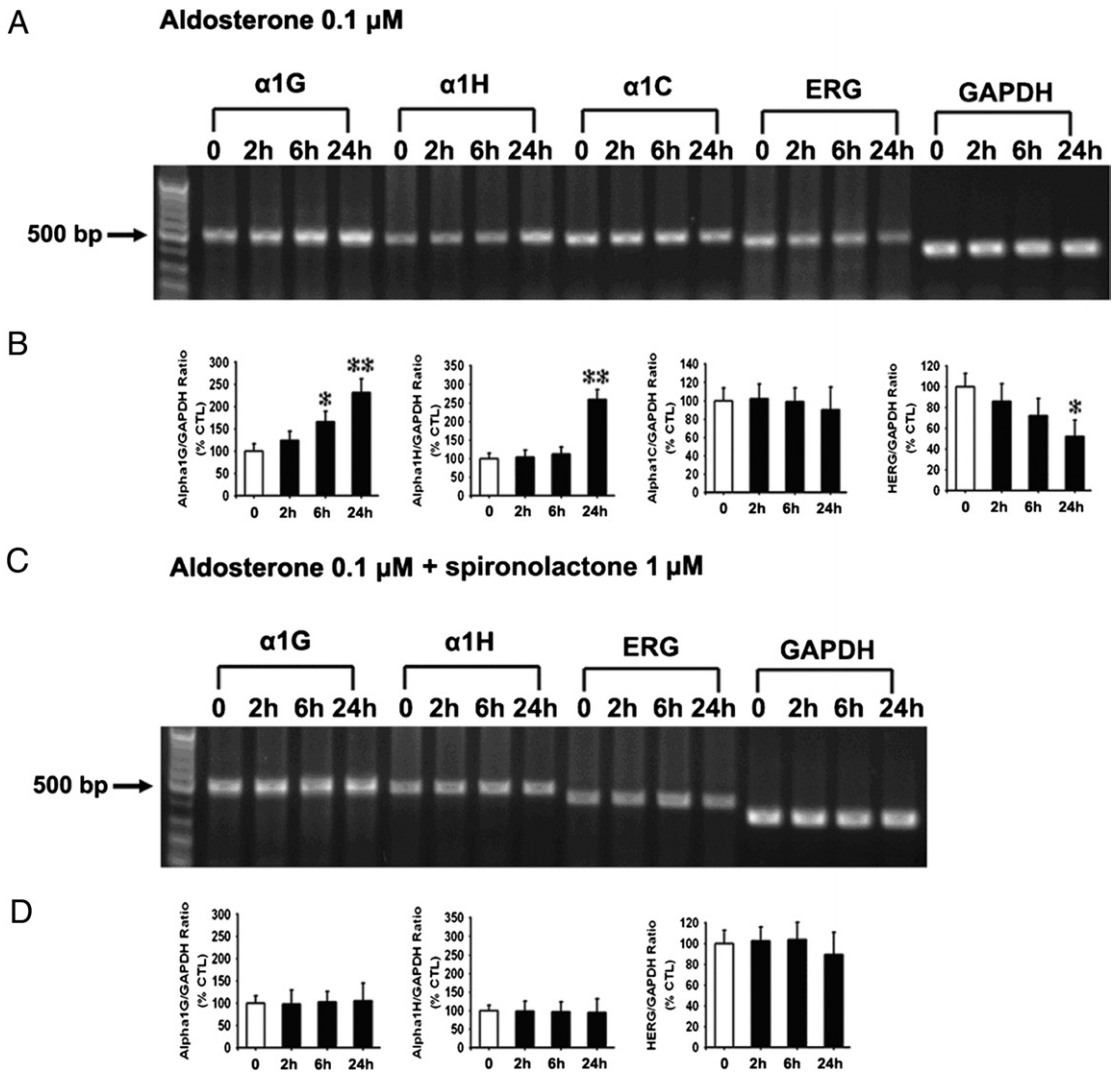

Figure 7 **Aldosterone Modulates Expressions of Ionic Channel Genes in HL-1 Atrial Myocytes**

Aldosterone increases expressions of the alpha-1G and -1H subunits of the T-type calcium channel and decreases expression of the ether-a-go-go-related (ERG) subunit of the rapidly-activating delayed rectifier potassium channel in HL-1 atrial myocytes, all of which are attenuated by spironolactone. **(A)** Total ribonucleic acid (RNA) was isolated from HL-1 myocytes left untreated (control subjects) or treated for indicated times with aldosterone (0.1 μmol/l). Reverse transcription-polymerase chain reaction products were visualized by electrophoresis. **(B)** The messenger RNA levels of alpha-1C, -1G, -1H, and ERG subunit proteins were quantified and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **(C)** Total RNA was isolated from HL-1 myocytes left untreated (control subjects) or treated for indicated times with aldosterone (0.1 μmol/l) plus spironolactone (1 μmol/l). Reverse transcription-polymerase chain reaction products were visualized by electrophoresis. **(D)** The messenger RNA levels of alpha-1G, -1H, and ERG subunit proteins were quantified and normalized to that of GAPDH. Data represent mean ± SD of 3 independent experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus control. Abbreviations as in Figure 2.

Discussion

Main findings. We first demonstrated that in patients with AF, atrial tissue MR expression was increased, although atrial tissue aldosterone levels were not altered. In the in vitro cellular model of AF, MR expression increased with rapid depolarization. We observed a concomitantly increased response to aldosterone stimulation, which was attenuated by MR-blockade. Aldosterone increased the inward T-type calcium current and SR calcium load. These changes suggest the potential role of an augmented aldosterone-MR effect in calcium overload during AF. Blockage of this effect with MR-blockade may be an option to prevent calcium overload and treat AF.

Expression of steroidogenesis proteins in the human atrium. To our knowledge, basal expression of the proteins of steroidogenesis in the human atrium and its relationship to AF has not been reported in the literature. Expression of CYP11B1 and CYP11B2 has been reported in failing or hypertrophic myocardium in humans but not in normal hearts (15). We found almost no basal expression of CYP11B1 or CYP11B2 in the atrial tissue of patients with either AF or normal sinus rhythm.

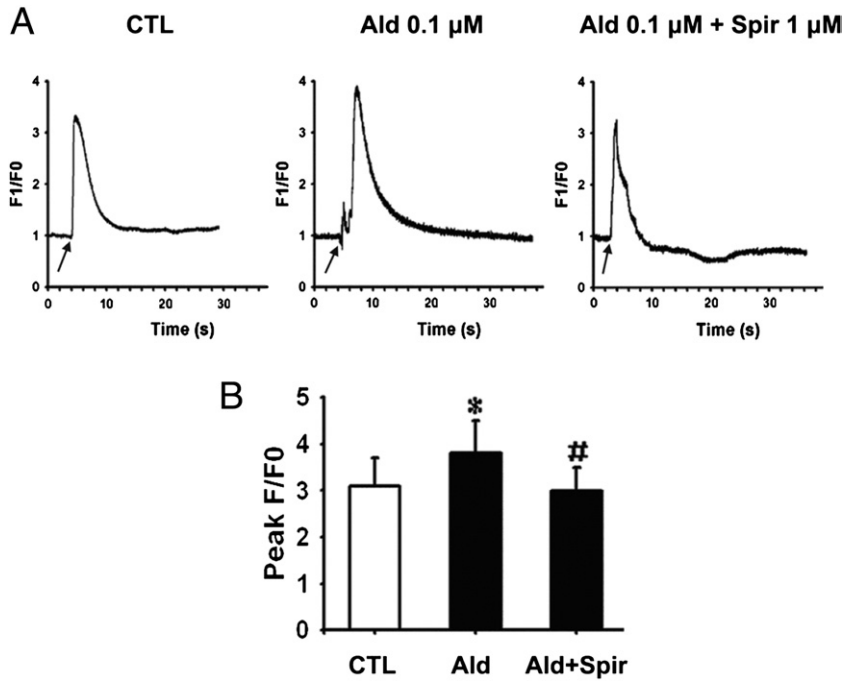

Figure 8 **Aldosterone Increases the SR Calcium Load in HL-1 Atrial Myocytes**

Aldosterone increases the sarcoplasmic reticulum (SR) calcium load in HL-1 atrial myocytes, which is attenuated by spironolactone. **(A)** The SR calcium load was measured by rapid caffeine application (50 mmol/l) and was reported as F1/F0. **Arrows** indicate caffeine application. **(B)** The bar graph shows the average peak normalized fluorescence level (peak F1/F0) after rapid caffeine application in the control cells (CTL), aldosterone-treated (Ald), and aldosterone plus spironolactone-treated (Ald+Spir) cells ($n = 6$). *$p < 0.05$ versus control; #$p < 0.05$ versus aldosterone-treated cells.

The aldosterone in the atrial tissue, as detected in our study, probably came from the systemic circulation (16).

Expression of MRs, GRs, and 11bHSD2 may be ubiquitous and were detectable in the human atrium, as we demonstrated. We further found that MR expression increased in the atrial tissue from patients with AF, which, to our knowledge, has not been reported in the literature. The status of MR expression has been investigated in the left ventricles of rats with myocardial infarction, but no significant change was found when compared with those from the left ventricles of normal rats (17).

Because the aldosterone detected in the atrial tissue comes from the systemic circulation, the abundance of atrial MR expression is the major factor determining the effect of serum aldosterone on the atrial tissue. MRs are the nuclear receptors for aldosterone and are responsible for its genomic effects. In the cellular model, we showed that rapid depolarization increased MR expression, which was similar to the findings in the human atrial samples. Using a promoter luciferase assay, we verified that increased MR expression was associated with increased aldosterone responsiveness. We also found that the effects of aldosterone, including increased ICaT, decreased IKr and increased SR calcium load on atrial myocytes, were mediated through the genomic effect, which were attenuated by the classical MR competitive antagonist spironolactone, but not through the nongenomic oxidative pathway (13).

The exact mechanism by which AF or rapid depolarization increases MR expression is interesting. In our cellular AF model, intracellular calcium concentration was elevated by rapid depolarization (6). This result is similar to that found in a rapid-pacing model of AF (18). We also showed that intracellular calcium chelator BAPTA-AM and the L-type calcium-channel blocker verapamil abolished rapid depolarization-induced increment of MR expression. Therefore, the mechanism may be calcium-dependent. Increased intracellular calcium due to rapid depolarization may trigger calcium-related signaling pathways (such as calcium-/calmodulin-dependent protein kinase or nuclear factor of activated T-cell pathway) and increases MR expression by means of a transcriptional mechanism. Although calcium overload and oxidative stress are interrelated processes (11), we did not demonstrate that rapid depolarization-induced MR expression through the oxidative stress pathway.

The proposed signaling pathway of AF-induced MR expression and the signaling pathway of aldosterone-induced ionic remodeling are illustrated in Figure 9.

Aldosterone, electrical remodeling, and mechanism of AF. As mentioned earlier, rapid depolarization-induced calcium overload triggers the observed increase in MR gene expres-

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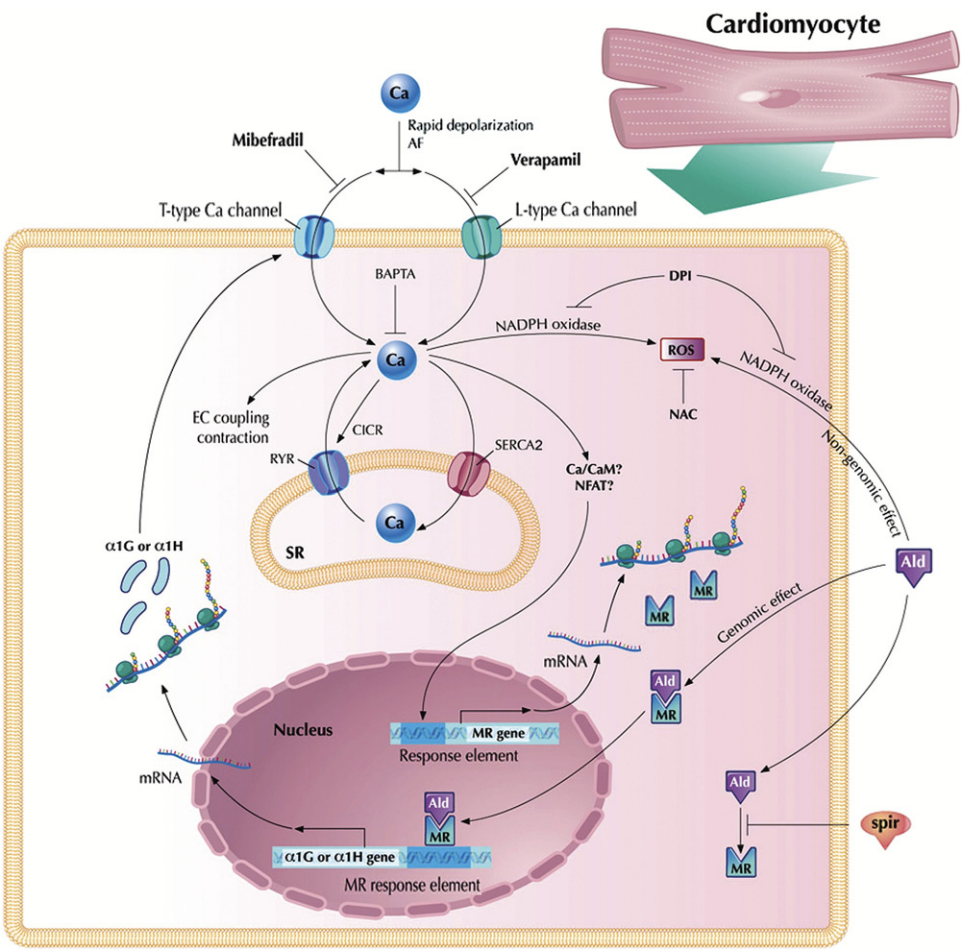

Figure 9 **A Summary Figure Demonstrating the Regulation of Signaling Cascades Proposed in the Present Study**

A summary figure demonstrates the signaling cascades of increased mineralocorticoid receptor (MR) expression by rapid-depolarization and those of aldosterone-induced change of ionic channel gene expression. Figure illustration by Rob Flewell. Ca = calcium; Ca/CaM = calcium/calmodulin; CICR = calcium-induced calcium release; DPI = diphenyleneiodonium; EC = excitation-contraction; NAC = *N*-acetylcysteine; NADPH = nicotinamide adenine dinucleotide phosphate; NFAT = nuclear factor of activated T-cells; ROS = reactive oxygen species; RYR = ryanodine receptor; SERCA2 = sarcoplasmic reticulum calcium adenosine triphosphatase 2; other abbreviations as in Figures 1, 3, 4, and 8.

sion and aldosterone responsiveness. This finding suggests that calcium influences aldosterone effects. However, we also showed that aldosterone increases ICaT and induces calcium overload. These results indicate a positive feedback vicious cycle (Fig. 9), which may contribute to the mechanism of calcium overload and electrical remodeling in AF (18,19). This is in accordance with the recent report that selective ICaT blocker mibefradil successfully prevents tachycardia-induced electrical remodeling (20). However, this drug is currently not available in the market due to its severe hepatic toxicity. In this regard, MR-blockade may be the potential available drug to prevent tachycardia-induced calcium overload and electrical remodeling.

Aldosterone, structural remodeling, and mechanism of AF. Recently it has been demonstrated that CYP11B2 T-344C promoter polymorphism, which is associated with a higher aldosterone level, was an independent predictor of AF in patients with heart failure (21). The prominent type of atrial remodeling in the presence of structural heart disease or heart failure is the structural remodeling. Therefore, aldosterone may play a role in the mechanism of atrial structural remodeling. The second part of the present study focuses on a cellular model of AF examining the effects of rapid depolarization. This is not a model of structural remodeling, because rapid depolarization or atrial tachycardia causes mainly electrical remodeling. Furthermore, the patients in the first part of the study had significant structural heart disease as reflected by an increased left atrial dimension, especially the group with significant valvular heart disease.

However, rapid depolarization per se could also induce structural remodeling (6). Our previous study showed that rapid depolarization of atrial myocytes during AF induced a paracrine secretion of angiotensin II, which may stimulate the nearby fibroblasts to produce extracellular matrix (6). We

further found that angiotensin II increased the expression of MR in cardiac fibroblasts, which might augment the response of cardiac fibroblasts to aldosterone stimulation (data not shown and provided upon request). However, we found that aldosterone itself did not increase the expression of pro-collagens, such as pro-collagen type 1 alpha 1 (COL1A1), type 2 alpha 1 (COL2A1), and type 3 alpha 1 (COL3A1), in cardiac fibroblasts (data not shown). Whether aldosterone stimulates collagen synthesis still remains controversial (22–25). Some studies showed a positive result (22,23), but others did not (24,25). Therefore, the role of aldosterone in atrial structural remodeling needs further studies.

Nevertheless, structural and electrical remodelings usually coexist. Rapid depolarization is present in all kinds of AF models and in all patients with AF. The result of the cellular study may imply that MR expression is up-regulated when rapid depolarization is present, irrespective of the presence or absence of structural remodeling.

Study limitations. First, most of the atrial tissue was obtained from patients suffering from significant valvular heart disease and left atrial enlargement. Although we also included the second study group with less left atrial enlargement, these patients did not have pure lone AF. Therefore, the results of the first part of the study cannot be extrapolated in AF patients with other underlying conditions, especially those with lone AF. Second, only right atrial samples were obtained in the first part of the study. We could not exclude the regional differences in the measured parameters. For example, the left atrium has lower metabolic reserve and increased wall stress compared with the right atrium, especially in patients with valvular abnormalities of the left heart. Finally, we did not find any difference of atrial aldosterone levels between the study groups. The atrial aldosterone might come from the systemic circulation. Use of diuretics is known to activate RAS and increase plasma aldosterone level (26). Therefore, a potential difference of atrial aldosterone level might be obscured due to use of diuretics, because most of the study subjects, either those with AF or those with NSR, were receiving diuretics.

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Reprint requests and correspondence: Dr. Ling-Ping Lai or Dr. Jiunn-Lee Lin, Division of Cardiology, Department of Internal Medicine, National Taiwan University Hospital, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan. E-mail: lplai2003@ntu.edu.tw or jiunnlee@ntu.edu.tw

REFERENCES

1. Goette A, Staack T, Rocken C, et al. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. J Am Coll Cardiol 2000;35:1669–77.
2. Li D, Shinagawa K, Pang L, et al. Effects of angiotensin-converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing-induced congestive heart failure. Circulation 2001;104:2608–14.
3. Goette A, Hoffmanns P, Enayati W, et al. Effect of successful electrical cardioversion on serum aldosterone in patients with persistent atrial fibrillation. Am J Cardiol 2001;88:906–9.
4. Tsybouleva N, Zhang L, Chen S, et al. Aldosterone, through novel signaling proteins, is a fundamental molecular bridge between the genetic defect and the cardiac phenotype of hypertrophic cardiomyopathy. Circulation 2004;109:1284–91.
5. Tsai CT, Lai LP, Kuo KT, et al. Angiotensin II activates STAT3 via Rac1 in atrial myocytes and fibroblasts: implication for the therapeutic effect of statin in atrial structural remodeling. Circulation 2008;117:344–55.
6. Tsai CT, Lai LP, Hwang JJ, et al. Renin-angiotensin system component expression in the HL-1 atrial cell line and in a pig model of atrial fibrillation. J Hypertens 2008;26:570–82.
7. Tsai CT, Wang DL, Chen WP, et al. Angiotensin II increases expression of α1C subunit of L-type calcium channel through a reactive oxygen species and cAMP response element binding protein dependent pathway in HL-1 myocytes. Circ Res 2007;100:1476–85.
8. Wu KD, Chen YM, Chu TS, et al. Expression and localization of human dopamine D2 and D4 receptor mRNA in the adrenal gland, aldosterone-producing adenoma, and pheochromocytoma. J Clin Endocrinol Metab 2001;86:4460–7.
9. Lai LP, Su MJ, Lin JL, et al. Down-regulation of L-type calcium channel and sarcoplasmic reticular Ca(2+)-ATPase mRNA in human atrial fibrillation without significant change in the mRNA of ryanodine receptor, calsequestrin and phospholamban: an insight into the mechanism of atrial electrical remodeling. J Am Coll Cardiol 1999;33:1231–7.
10. LeHoux JG, Lefebvre A. Novel protein kinase C-epsilon inhibits human CYP11B2 gene expression through ERK1/2 signaling pathway and JunB. J Mol Endocrinol 2006;36:51–64.
11. Bukowska A, Schild L, Keilhoff G, et al. Mitochondrial dysfunction and redox signaling in atrial tachyarrhythmia. Exp Biol Med 2008;233:558–74.
12. Caballero R, Moreno I, González T, et al. Spironolactone and its main metabolite, canrenoic acid, block human ether-a-go-go-related gene channels. Circulation 2003;107:889–95.
13. Callera GE, Touyz RM, Tostes RC, et al. Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src. Hypertension 2005;45:773–9.
14. Brown NJ. Aldosterone and vascular inflammation. Hypertension 2008;51:161–7.
15. Young MJ, Clyne CD, Cole TJ, et al. Cardiac steroidogenesis in the normal and failing heart. J Clin Endocrinol Metab. 2001;86:51216.
16. Gomez-Sanchez EP, Ahmad N, Romero DG, et al. Origin of aldosterone in the rat heart. Endocrinology 2004;145:4796–802.
17. Silvestre JS, Heymes C, Oubenaissa A, et al. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. Circulation 1999;99:2694–701.
18. Ausma J, Dispersyn GD, Duimel H, et al. Changes in ultrastructural calcium distribution in goat atria during atrial fibrillation. J Mol Cell Cardiol 2000;32:355–64.
19. Chou CC, Nihei M, Zhou S, et al. Intracellular calcium dynamics and anisotropic reentry in isolated canine pulmonary veins and left atrium. Circulation 2005;111:2889–97.
20. Fareh S, Bénardeau A, Thibault B, et al. The T-type Ca(2+) channel blocker mibefradil prevents the development of a substrate for atrial fibrillation by tachycardia-induced atrial remodeling in dogs. Circulation 1999;100:2191–7.
21. Amir O, Amir RE, Paz H, Mor R, Sagiv M, Lewis BS. Aldosterone synthase gene polymorphism as a determinant of atrial fibrillation in patients with heart failure. Am J Cardiol 2008;102:326–9.

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February 23, 2010:758–70

22. Zhou G, Kandala JC, Tyagi SC, Katwa LC, Weber KT. Effects of angiotensin II and aldosterone on collagen gene expression and protein turnover in cardiac fibroblasts. Mol Cell Biochem 1996;154:171–8.
23. Brilla CG, Zhou G, Matsubara L, Weber KT. Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. J Mol Cell Cardiol 1994;26:809–20.
24. Fullerton MJ, Funder JW. Aldosterone and cardiac fibrosis: in vitro studies. Cardiovasc Res 1994;28:1863–7.
25. Gekle M, Mildenberger S, Freudinger R, Grossmann C. Altered collagen homeostasis in human aortic smooth muscle cells (HAoSMCs) induced by aldosterone. Pflugers Arch 2007;454:403–13.
26. Rowland NE, Morian KR. Roles of aldosterone and angiotensin in maturation of sodium appetite in furosemide-treated rats. Am J Physiol 1999;276:R1453–60.

Key Words: atrial fibrillation ■ aldosterone ■ mineralocorticoid receptor ■ ionic remodeling ■ spironolactone.

APPENDIX

For an expanded Methods section and supplementary figures, please see the Online Appendix.