Increased Expression of Mineralocorticoid Receptor and 11 β -Hydroxysteroid Dehydrogenase Type 2 in Human Atria During Atrial Fibrillation

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ABSTRACT

Background: Atrial fibrillation (AF) is associated with the activation of the renin-angiotensin-aldosterone system in the atria. It is not clear whether the expression of a mineralocorticoid receptor (MR), or 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), conferring aldosterone specificity to the MR, in patients with AF is altered.

Hypothesis: Patients with AF may be associated with increased expression of MR and 11 β HSD2 in the atria.

Methods: Atrial tissue samples of 25 patients with rheumatic heart valve disease undergoing a valve replacement operation were examined. A total of 13 patients had chronic persistent AF (>6 mo) and 12 patients had no history of AF. The MR and 11 β HSD2 expression were analyzed at the mRNA and protein level. The localization of MR and 11 β HSD2 in atrial tissue was performed using specific immunohistochemistry staining.

Results: The results of real-time quantitative polymerase chain reaction (PCR) showed that AF groups, in comparison with sinus rhythm, had a higher mRNA expression level of MR or 11 β HSD2 (all $P < 0.01$). Both the MR and 11 β HSD2 protein expression level in atrial tissue were also significantly increased in patients with AF compared with patients with sinus rhythm ($P < 0.05$ or $P < 0.01$). The immunohistochemical staining of MR or 11 β HSD2 demonstrated that MR and 11 β HSD2 predominately located in the cytoplasm of myocardial cells in the atrium and the intensity and density of immunostaining appeared to be increased in the atria of patients with AF compared to those without AF.

Conclusions: Increasing expression of MR and 11 β HSD2 in the atria during AF is one of the molecular mechanisms for development of atrial interstitial fibrosis in patients with AF. These findings may have an important impact on the treatment of AF with aldosterone antagonists.

Introduction

Atrial fibrillation (AF) is a potential cause of thromboembolic events and is related to increasing disability and mortality. The development and maintenance of AF are associated with changes in cardiac structure, function, and electrical properties known as cardiac remodeling. Although the mechanisms behind this arrhythmia are complex and the process of atrial remodeling is still not completely understood, mounting evidence suggested that the renin-angiotensin-aldosterone system (RAAS) plays a major role in the pathogenesis of AF. Clinical trials^{1,2} have demonstrated that angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blocking agents (ARBs) reduced the incidence of AF.

Atrial fibrillation is associated with the activation of the atrial RAAS, resulting in elevating aldosterone levels. Goette³ showed that serum aldosterone levels are elevated in patients with persistent AF, whereas restoration of sinus rhythm by electrical cardioversion significantly lowers serum aldosterone. The risk of AF in patients with primary

aldosteronism is 12-fold higher than in patients with essential hypertension matched for age, gender, and systolic and diastolic blood pressure, which is independent of blood pressure.⁴ Aldosterone is a potent promoter of fibrosis, that may result in atrial fibrosis which has been appreciated as a morphologic substrate for AF. Therefore, it is rational to presume that aldosterone may mediate the process of atrial fibrosis and promote atrial structural remodeling. But aldosterone acts by binding to the mineralocorticoid receptor (MR), however the role of aldosterone and its receptor (MR) in AF is unclear.

The MR is overexpressed in cardiomyocytes of patients with congestive heart failure.⁵ RALES⁶ and EPHESUS⁷ have demonstrated that the addition of MR antagonists to optimal medical therapy substantially reduces the risk of both morbidity and mortality among patients with severe heart failure or acute myocardial infarction complicated by left ventricular dysfunction and heart failure. The RAAS activation in AF is similar to that in heart failure; it can therefore be hypothesized that the expression of MR in

patients with AF may change. But this speculation needs to be further studied.

The MR is not a specific receptor on aldosterone,⁸ because aldosterone and glucocorticoid cortisol bind to human MR with equal affinity.⁹ But aldosterone-responsive tissues also express the cortisol-inactivating enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), which confers aldosterone specificity to MR.¹⁰ Transgenic mice overexpressing 11 β HSD2 in cardiomyocytes developed cardiac fibrosis.¹¹ Therefore, 11 β HSD2 might be associated with the development of AF.

The purpose of the present study was to determine whether atrial expression of MR and 11 β HSD2 is altered in patients with AF, because that could have an important therapeutic impact on AF.

Methods

Patients

A total of 25 patients with rheumatic heart valve disease, 12 in sinus rhythm (no history of AF) and 13 in chronic, persistent AF (>6 months), with a mean age of 46.44 ± 12.42 years, underwent a valve replacement operation. Patients were excluded from the study on the basis of the following criteria: hypertension; hyperthyroidism; chronic obstructive pulmonary disease; coronary atherosclerotic heart disease; significant kidney, hepatic diseases, or cardiomyopathy; or New York Heart Association (NYHA) heart failure class IV. No ACEIs or ARBs were administered during the 2 months prior to heart surgery and all patients had taken spironolactone for more than 15 days before heart surgery. Heart function in these patients was at NYHA class II to III. After written informed consent was obtained,⁸ right and left atrial lateral wall tissue samples were obtained from these patients during the valve replacement operation. Tissue samples were quickly frozen and stored in liquid nitrogen for further analysis. They were divided into 4 groups based on sinus rhythm (SR) or AF and right atria (RA) or left atria (LA) samples: group A ($n = 12$), RA samples in SR; group B ($n = 13$), RA samples in AF; group C ($n = 7$), LA samples obtained in 7 patients of group A with SR; group D ($n = 8$), LA samples obtained in 8 patients of group B with AF (Table 1).

Real-time Quantitative Polymerase Chain Reaction

Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA) and was reverse-transcribed. Specific primer sequences and polymerase chain reaction (PCR) product length are as follows: MR (181 bp) Sense 5'-GAT TTG GCG AGA CCA GAG CA-3', Antisense 5'-AAG CGA ACG ATA CCA GAA ACT ACA-3'; 11 β HSD2 (187 bp) Sense 5'-GTG CTC ATC ACC GGC TGT G-3', Antisense 5'-GGC TAA TGT CTC CTG GTT TGG T-3'; β -actin Sense (626 bp) 5'-CCT CGC CTT TGC CGA TCC-3', Antisense 5'-GGA TCT TCA TGA GGT AGT CAG TC-3'.

Quantitative PCR was performed using the PE-5700 analyzer (PE Corp. American, Wilton, CT). The 20- μ L reaction mixture consisted of $MgCl_2$ 2 mmol/L, dNTPs 200 nmol/L (each), cDNA templates 2 μ L, target gene specific primers 0.2 μ L (each), Taq polymerase 1.5 u, SYBR Green I (1:1000, Molecular Probes, Carlsbad, CA) 0.4- μ L, 10 $\times$ PCR buffer 2- μ L. Amounts of β -actin were used to normalize cDNA contents. Initial denaturation at 95°C for 2.5 minutes was followed by 40 cycles with denaturation at 94°C for 45 seconds, annealing at 64°C for 45 seconds, and elongation at 72°C for 45 seconds. The fluorescence intensity, which reflected the amount of actually formed PCR product, was read at the end of each elongation step. Initial amounts of template mRNA were calculated by determining the time point at which the linear increase of PCR product started, relative to the corresponding points of a standard curve. Target genes mRNA expression level were quantified by normalizing them with that from β -actin.

Western Blotting

Total proteins (50 μ g per lane) were separated in SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% dry milk, then incubated with rabbit polyclonal anti-MR antibody or anti-11 β HSD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA 1:1000) for 24 hours at 4°C. After washing with TBST, the membrane was incubated with goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, 1:500) for 60 minutes. Negative controls were obtained by omitting the primary antibody. Monoclonal antibody against GAPDH (Santa Cruz Biotechnology) was used in every experiment for internal control. The images were obtained by exposure to film and were scanned. The densities of each band were analyzed by ImageJ software, then quantified by normalizing them with that from GAPDH.

Immunohistochemical Staining of MR and 11 β HSD2

Immunohistochemistry for localization of MR or 11 β HSD2 were performed in tissue specimens. Cryosections of atrial samples were obtained by embedding a small cube of atrial tissue in an optimal cutting temperature compound and cutting into 5- μ m sections by cryostat and then storing at -80°C before staining. Immunostaining was performed with antibodies, as specified above, directed against MR or 11 β HSD2 (dilution 1:100) following standard protocols. The specificity was controlled by omitting the primary antibody.

Statistics

All group quantitative data were expressed as mean $\pm$ SD. A Student *t* test was used to determine the differences between groups. Nonparametric statistics was evaluated using Wilcoxon rank sum test. Statistical analysis was performed with statistical software SPSS 13.0. (SPSS, Chicago, IL) Statistical significance was considered at $P < 0.05$.

Results

Patients Characteristics

In the AF groups, LA dimensions were significantly greater than that in the SR groups ($P < 0.01$, Table 1). The AF duration was similar between RA and LA specimens (Group B vs Group D) in the fibrillation groups. There were no differences in age, clinical diagnosis of etiology,

left ventricular ejection fraction, and left ventricular end-diastolic dimensions.

mRNA expression level of MR and 11 β HSD2: Both the mRNA expression level of MR and 11 β HSD2 in the atrium were increased significantly in the AF groups compared with those in the SR groups ($P < 0.01$, Table 2, Figure 1). But the mRNA expression level of MR and 11 β HSD2 were no

Table 1. Basic Clinical Characteristics of the Study Population

Parameters	Right Atrial Specimens		Left Atrial Specimens	
	SR, Group A	AF, Group B	SR, Group C	AF, Group D
n	12	13	7	8
Age, y	43.17 $\pm$ 13.32	49.46 $\pm$ 11.19	41.29 $\pm$ 16.13	50.00 $\pm$ 10.56
LAD, cm	4.74 $\pm$ 0.50	5.98 $\pm$ 0.63 ^a	4.74 $\pm$ 0.59	6.31 $\pm$ 0.54 ^b
AF duration, m	. . .	55.85 $\pm$ 40.39	. . .	77.25 $\pm$ 35.02
LVEF, %	60.92 $\pm$ 4.52	58.00 $\pm$ 3.14	61.86 $\pm$ 5.61	58.88 $\pm$ 2.03
LVEDd, cm	4.60 $\pm$ 0.91	4.44 $\pm$ 0.96	4.80 $\pm$ 1.15	4.44 $\pm$ 1.15
Valve disease				
MS	1	1	1	1
MS/MI	6	4	2	2
MS/MI/AI	2	4	2	3
MS/MI + AS/AI	3	4	2	2
NYHA (?/?)	9/3	9/4	5/2	5/3

Abbreviations: AF, atrial fibrillation; AI, aortic incompetence; AS, aortic stenosis; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; LVEDd, left ventricular end-diastolic dimension; MI, mitral incompetence; MS, mitral stenosis; NYHA, New York Heart Association.

^a $P < 0.01$ vs group A.

^b $P < 0.01$ vs group C.

Table 2. Relative Expression Amount of mRNA and Protein from MR and 11 β HSD2

Groups	n	MR		11βHSD2	
		mRNA	Protein	mRNA	Protein
RA specimens					
SR, group A	12	2.67 ± 1.09	0.86 ± 0.33	0.33 ± 0.12	0.71 ± 0.21
AF, group B	13	5.37 ± 1.15 ^a	1.65 ± 0.72 ^a	0.86 ± 0.14 ^a	1.18 ± 0.64 ^b
LA specimens					
SR, group C	7	2.70 ± 0.82	0.97 ± 0.37	0.37 ± 0.10	0.85 ± 0.15
AF, group D	8	5.19 ± 1.14 ^c	1.72 ± 0.62 ^d	0.95 ± 0.15 ^c	1.36 ± 0.58 ^d
^a P < 0.01, vs group A.					
^b P < 0.05, vs group A.					
^c P < 0.01, vs group C.					
^d P < 0.05, vs group C..

Figure 1. Relative expression amount of mRNA in atrium from (A) MR and (B) 11βHSD2. ^a $P < 0.01$ AF vs SR in both RA and LA specimens.

Figure 2. Western blots demonstrating differences in (A,B) MR and (C,D) 11βHSD2 protein expression between patients with SR and AF. (1,2) RA specimens with Sr. (3,4) LA specimens with Sr. (5,6) RA specimens with AF. (7,8) LA specimens with AF. ^a $P < 0.01$ AF vs SR in RA specimens. ^b $P < 0.05$ AF vs SR in LA specimens.

different between the LA and RA in both the fibrillation and sinus groups.

Protein expression level of MR and 11βHSD2: Both MR and 11βHSD2 protein expression in RA and LA were increased in the fibrillation groups in comparison to those in the SR groups ($P < 0.05$ or $P < 0.01$, Table 2, Figure 2). But there was no difference found in the protein expression of MRs and 11βHSD2 between RA and LA in both the fibrillation and sinus groups.

Immunolocalization of MR and 11βHSD2: The special immunohistochemical staining (Figure 3) of MR or 11βHSD2 demonstrated that MR and 11βHSD2 were abundant and predominately located in the cytoplasm of the myocardial cells of the atria. The density and the intensity of immunostaining of MR and 11βHSD2 appeared to be increased significantly in the atria of patients with AF compared to those without AF. Immunohistochemical staining also demonstrated that MR is coexpressed with 11βHSD2 in the human heart.

Figure 3. Immunohistochemical staining of (A) MR and (B) 11 β HSD2 in atrial tissue (magnification $\times 400$). (a) RA specimens with SR. (b) RA specimens with AF. (c) LA specimens with SR. (d) LA specimens with AF.

Discussion

Aldosterone, Mineralocorticoid Receptor, and Atrial Fibrillation

Mounting evidence demonstrates that fibrosis generates a major mechanism for AF.¹² Although the precise signaling processes involved in the development of atrial fibrosis are unknown, the potentially important role of RAAS in AF is presented in a recent article.¹³ Clinical trials showed a reduction in the development and recurrence of AF with ACEIs and ARBs.^{1,2,14–18} Surprisingly however, the association of aldosterone and AF, and aldosterone blockade for the prevention of AF have received little attention. Serum aldosterone levels are elevated in patients with persistent AF and decline after sinus rhythm is restored.³ Patients with primary hyperaldosteronism have an increased incidence of AF compared with age, gender, and blood pressure matched controls with essential hypertension.⁴ Aldosterone promotes adverse cardiac structural remodeling via cellular proliferation and induces myocardial and vascular fibrosis by binding MR.¹⁹ Our results indicate that MR expression increased at the mRNA and protein level in patients with AF. The MR was mainly localized in the myocardial cell cytoplasm of atrial tissue.

Although serum aldosterone levels are increased in patients with persistent AF, our results suggest that MR is overexpressed at the transcriptional and protein level. The results are parallel with Yoshida's study,⁵ in which the serum aldosterone level is increased and MR is overexpressed in patients with congestive heart failure. Further study is required to elucidate the mechanism of MR upregulation in the presence of high-aldosterone concentration in patients with AF.

11 β -Hydroxysteroid dehydrogenase type 2 and atrial fibrillation: Although aldosterone acts by binding to the MR, increments of only MR does not necessarily represent augmentation of aldosterone action, because MR is

not a specific receptor on aldosterone,⁸ and also aldosterone and glucocorticoid cortisol bind to human MR with equal affinity.⁹ Cardiomyocyte MRs are normally occupied by endogenous glucocorticoids that are present at higher levels than aldosterone in the blood under physiological conditions. However, in pathophysiological states, it has been hypothesized that mineralocorticoid aldosterone can access cardiac MR and produce cardiac damage. Earlier studies demonstrate that MR and 11 β HSD2 are prerequisites for cardiac aldosterone.²⁰ Our results demonstrated that 11 β HSD2 expression also increased in the same manner as MR at mRNA and protein levels in patients with AF. Immunohistochemistry showed that 11 β HSD2 also were mainly localized in the cytoplasm of the myocardial cells of atrial tissue, indicating MR is coexpressed with 11 β HSD2 in the human heart and thus possesses the cellular machinery required for direct aldosterone action.

Aldosterone-responsive tissues express 11 β HSD2, which converts endogenous glucocorticoids cortisol to their receptor-inactive 11-keto derivatives that have a low affinity for MR,¹⁰ thereby conferring aldosterone specificity to MR. Mutations in the 11 β HSD2 gene lead to apparent mineralocorticoid excess syndrome (AME), which manifest as severe hypertension and hypokalemia in a clinical setting.^{10,19} Various epithelial cells, vascular smooth muscle cells (VSMCs), and cardiomyocyte express MR. But the difference between epithelial (and VSMC) and nonepithelial MRs, such as myocardial cell MRs, is their response to occupancy by endogenous glucocorticoids. In epithelia, glucocorticoids mimic aldosterone, as in AME.^{10,19} In nonepithelial tissue such as cardiomyocytes, glucocorticoids do not activate MR, but act as antagonists.^{10,19} Although the mechanism underlying this difference is unknown, it may reflect tissue-specific differences in coactivator/corepressor recruitment by MR/glucocorticoid complexes.

Overexpression of 11 β HSD2 in the atria during AF produces pathophysiology, reflecting inappropriate occupancy and activation of MRs by circumventing the presumed tonic inhibitory (or physiological antagonist) role of glucocorticoids under physiological conditions. Transgenic mice with normotension overexpressing 11 β HSD2 in cardiomyocytes developed cardiac hypertrophy, fibrosis, and heart failure, and eplerenone, a selective aldosterone blocker, ameliorated this phenotype.¹¹ A previous study shows that the spontaneously hypertensive rat with cardiac fibrosis, which has been reported to have significantly high plasma aldosterone, has increased expression of MR and 11 β HSD2,²¹ which is similar to our results. New evidence shows that angiotensin II (ANG II) directly activates the MR gene transcription that can be blocked by the ARB, losartan, and MR antagonist, spironolactone, which is independent of aldosterone generation, suggesting the role of MR as a signal effector for ANG II.²² This study indicates that in addition to being an aldosterone-dependent mechanism, MRs promote atrial remodeling mediated by an ANG II dependent mechanism. These data and our study suggest that the upregulation of MR and 11 β HSD2 in the atria during AF may promote and mediate atrial interstitial fibrosis.

Potential Limitation

Some potential limitations may have influenced our results. All patients included in our study had significant valve diseases and underwent cardiac surgery; also these patients had received the aldosterone antagonist, spironolactone prior to surgery. The pathophysiologic mechanism of AF in non-valve disease patients without heart failure; or in lone AF patients without underlying heart diseases may be different. In contrast to autopsy and animal studies, we examined only tissue specimens from the right or left atrial lateral wall. Thus, no comment can be made regarding the distribution of changes in MR and 11 β HSD2 within the rest of the atria. Further studies are needed to confirm whether there is increasing expression of MR and 11 β HSD2 in patients with AF and without heart failure or underlying heart diseases. The aldosterone tissue levels were not determined in the study. The extent of fibrosis was not quantified. However, differences in atrial fibrosis between patients with and without AF were strikingly evident. The favorable effects of aldosterone antagonism on ventricular remodeling in heart failure have been demonstrated, but the role of aldosterone in AF deserves further study since it may contribute to the development of new effective strategies against atrial remodeling.

Conclusions

To the best of our knowledge, the present study demonstrates for the first time an increase in atrial expression of MR and 11 β HSD2 in patients with AF. Increasing expression

of MR and 11 β HSD2 in the atria is one of the causes of atrial interstitial fibrosis in association with AF. These findings may have an important impact on the treatment of AF with aldosterone antagonists. This is supported by the results of Shroff et al who showed that the selective aldosterone blocker, eplerenone, suppresses atrial tachyarrhythmias in heart failure.²³

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