Spironolactone Prevents Aldosterone Induced Increased Duration of Atrial Fibrillation in Rat

Carina Lammers¹, Theresa Dartsch¹, Mathias C. Brandt¹, Dennis Rottländer¹, Marcel Halbach¹, Gabriel Peinkofer¹, Simon Ockenpoehler¹, Marco Weiergraeber⁴, Toni Schneider^{2,3}, Hannes Reuter¹, Jochen Müller-Ehmsen¹, Jürgen Hescheler², Uta C. Hoppe^{1,3} and Carsten Zobel¹

¹Department of Internal Medicine III, University of Cologne, Cologne, ²Institute for Neurophysiology, ³Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, ⁴Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM), Bonn

Key Words

Electrical remodeling • Structural remodelling • Myocardial hypertrophy

Abstract

Background: Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice. The Renin-Angiotensin-Aldosterone-System plays a major role for the atrial structural and electrical remodelling. Recently elevated aldosterone levels have been suggested to increase the risk for the development of AF. **Methods:** Rats were treated with aldosterone by means of an osmotic minipump (0.5µg/h) over a period of 4 weeks. AF was induced by transeptal burst pacing. Action potentials (AP) were recorded from left atrial preparations with microelectrodes. Atrial collagen was quantified by histological studies. **Results:** Aldosterone treatment resulted in hypertrophy as indicated by an increased ratio of heart weight/tibia length and doubled the time until the AF converted spontaneously into sinus rhythm (85.8±13.4 s vs. 38.3±6.9 s, $p<0.01$). This was associated with a significant shortening of the AP (APD₉₀ 26.2±1.1 vs. 31.2±1.9, $p<0.05$) and an increased protein expression of Kir2.1 and Kv1.5.

Atrial collagen deposition was significantly greater in aldosterone-treated rats. The alterations could be prevented by additional application spironolactone. **Conclusions:** The results of the present study suggest that in addition to the structural remodelling aldosterone also promotes AF by altering repolarising potassium currents leading to action potential shortening.

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Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice, affecting 0.4-2% of the population. The disease increases the risk for the occurrence of thromboembolic complications and results in a reduction of cardiac performance by 10-20% [1]. The renin-angiotensin-aldosterone system (RAAS) plays a major role for the atrial structural and electrical remodelling and its inhibition by angiotensin-converting enzyme inhibitors or angiotensin type 1 (AT₁) receptor antagonists has been suggested to be beneficial [2]. However, the results from the recently published ANTIPAF trial

[3] contradict the earlier reports by showing no effect of the AT₁ receptor antagonist olmesartan on the recurrence of paroxysmal atrial fibrillation. The well-known phenomenon described as “aldosterone escape” [4] leads to increased aldosterone levels despite the blockade of the RAAS by ACE inhibitors and/or AT₁ receptor antagonists. Aldosterone excess leads to an aldosterone-receptor mediated atrial fibrosis independent of increased wall stress due to hypertension [5]. The role of aldosterone for the development and maintenance of AF is further underlined by the observation that patients with primary hyperaldosteronism show a 12-fold greater AF risk compared with hypertensive controls [6]. Moreover, serum aldosterone levels are elevated in patients with persistent AF and decline after sinus rhythm is restored [7], and increase after the onset of AF, indicating a possible vicious cycle between AF and aldosterone [8]. Furthermore, in human atrial tissue from patients with AF and in a cellular AF model increased expression levels of the mineralocorticoid receptor have been observed [9]. Recently Reil et al. [10] were able to show that longterm aldosterone treatment in rats resulted in promotion of atrial fibrillation, which was related to atrial fibrosis, myocyte hypertrophy and conduction disturbances.

Results from animal models [11] subsequently confirmed in human studies [12] indicate a central role of I_{Ca} changes in AF related remodelling of repolarisation. However, more recent work also points to an important contribution of alterations in K⁺-currents [13]. Specifically changes in I_{K1} [14, 15] and I_{Kur} [15, 16] have been implicated to alter repolarisation in atrial fibrillation.

Therefore, we hypothesized that aldosterone might be involved in electrical remodelling resulting in the maintenance of AF. To address this hypothesis we investigated the duration of atrial fibrillation induced by transesophageal burst pacing as well as the electrical remodelling in a rat model of long term aldosterone treatment.

Materials and Methods

Animal Model and Implantation of Osmotic Minipumps

Male Wistar rats (23 rats, mean body weight 223g) (Charles River) were treated with aldosterone (Sigma-Aldrich) or solvent (polyethylene glycole 400, PEG 400, Sigma-Aldrich) over a period of 4 weeks via implanted osmotic minipumps (ALZET, Pump Model 2004). Aldosterone was dissolved in PEG 400 (aldosterone release 0.5 µg/h). Pumps were implanted subcutaneously under anaesthesia using 50 mg/kg ketamine (Ketavet®, Sanofi) and 7 mg/kg xylazine (Rompun®, Bayer). The

animals were either fed with the aldosterone antagonist spironolactone (100 mg/kg⁻¹ body weight/d⁻¹) (Roche) with the chow (Altromin) or with a control diet (Altromin). Rats were separated into 4 groups: Solvent control (CON) – 5 animals, aldosterone (ALD) – 8 animals, spironolactone (SPI) – 5 animals and aldosterone+spironolactone (ALD+SPI) – 5 animals. The rats had free access to food and water and were maintained in a constant environment with a conventional 12 h/ 12 h light-dark cycle starting at 6 am. All experiments were carried out in accordance with the Directive of the European Commission 86/609/EEC and were approved by the local authorities on animal care (8.87-50.10.35.08.111).

Blood Pressure Measurement

Systolic blood pressure (SBP) was determined after 4 weeks of drug treatment with the non-invasive tail cuff method (Harvard Apparatus). Measurements were repeated three times calculating the mean values.

Induction of AF by burst pacing

AF was induced by burst pacing after 4 weeks of drug or vehicle treatment, as described earlier [17]. Rats were anaesthetized by intra-peritoneal injection of pentobarbital sodium (50 mg/kg). The animals were monitored for adequacy of anesthetic depth, which was based on the absence of movement and the reaction to paw-pinch. Ventilation was initiated after intubation by an 18-G vein catheter for artificial respiration, tidal volume was set at 2.5 ml/kg and respiratory rate at 100 strokes/min. The ECG was continuously monitored. A clinically available 6-French quad-polar electrodes catheter (Finder) was inserted into the esophagus under the monitoring of an esophageal electrogram and positioned at a site where the lowest level of voltage could just capture the atrium. The pacing pulse used for the induction of AF was rectangular in shape of 60 V (about 1.5 times of the diastolic threshold voltage) and 6 ms width. The atrium was paced at a cycle length of 12 ms (83 Hz) for 30 s (=burst pacing) via the distal electrodes pair of the catheter using an electrical stimulator. After inducing AF by burst pacing, its duration was measured until the ECG showed sinus rhythm again. Burst pacing and measurement of AF was repeated three times, with a five minute break in between. Burst pacing was without effect on the ECG parameters and the animals showed no other signs of cardiac damage like ectopic electrical activity or changes in heart rate. At the end of the procedure the animals were euthanized by intraperitoneal injection of 100 mg/kg pentobarbital and excision of the hearts which were snap frozen in liquid nitrogen for later use.

Atrial action potential recordings

At day 28 after induction of atrial fibrillation by burst pacing, left atria were excised for action potential recordings in 3 animals per studied group. Rats were killed by cervical dislocation and thoracotomy was performed. To rapidly minimize energy demand of cardiomyocytes, hearts were perfused in situ by ice cold Tyrode solution (136 mmol/l NaCl, 5.4 mmol/l KCl, 0.33mmol/l NaH₂PO₄, 1 mmol MgCl₂, 10 mmol/l Glucose, 5 mmol/l HEPES, 30 mmol/l 2,3-butanedione monoxime

Fig. 1. Typical transesophageal and surface electrogram recordings during sinus rhythm (A) and after induction of AF (B).

(BDM) and 0.9 mmol/l Ca_2Cl , pH 7.4). Left atria were cut off and stored in Tyrode solution for 30 min to recover from preparation. Afterwards, atria were transferred into the recording solution (DMEM without serum, bubbled with 5% CO_2 and 95% O_2 at 37°C; concentrations of inorganic salts in mmol/l: CaCl_2 1.8, MgSO_4 0.8, KCl 5.3, NaHCO_3 44, NaCl 110, NaH_2PO_4 0.9; Gibco/Invitrogen, San Diego, USA). Before measurements were carried out, atria were superfused by the recording solution for at least 30 min to ensure steady-state conditions. Intracellular action potential (AP) recordings were performed at 37 °C with sharp glass electrodes (15–40 M Ω when filled with 3 mol/l KCl) made of borosilicate glass capillaries (World Precision Instrument, Sarasota, USA) as described before [18]. 4 to 10 APs were recorded from each preparation. A stimulation frequency of 2 Hz was applied with a SD9 square pulse stimulator (Grass Technologies, West Warwick, USA), using a unipolar custom made stimulation electrode. Signals were amplified with a SEC-10LX amplifier (npi electronic, Tamm, Germany) and acquired with the Pulse software (HEKA, Lambrecht/Pfalz, Germany). Data were analyzed offline with the Mini Analysis program (Synaptosoft, Fort Lee, USA).

Western Blot

Total proteins were isolated from left atria of each animal of the four studied groups (see above) and Western blot analysis was performed as prescribed previously [19]. The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Hoefer Scientific Instruments), transferred to nitrocellulose membrane (Bio-Rad Laboratories), and blocked in 5% milk. Primary antibodies were incubated over night at 4°C in buffer according to the manufacturer's instructions followed by an anti-rabbit α -peroxidase secondary antibody (Sigma Aldrich) to allow visualization by chemiluminescence (Lumi-Light Western blotting Substrate, Roche Diagnostics) on a X-ray film (Curix, AGFA Healthcare). Antibodies: Anti-Kir2.1, Anti-Kir2.3 and Anti-Kv1.5 (Alomone) at dilutions of 1:200. GAPDH (1:6000) (Abcam) was used as control for protein loading.

Fig. 2. The mean AF time in rats treated with aldosterone was significantly increased compared to control animals. Spironolactone prevented the increase in AF time. * $p < 0.05$ vs CON. ♦ $p < 0.05$ vs ALD.

Histology

Left atrial tissue was fixed in a solution of 4 % buffered formalin for 24 hours, embedded in paraffin and sectioned at a thickness of 5 μm . Sections were deparaffinised in xylol and a descending alcohol sequence and brought into distilled water. Subsequently, the slices were exposed to a Picro-sirius red solution for one hour. Tissue sections were then washed in acetic acid for 10 min. Slices were dehydrogenated in an ascending alcohol sequence and xylol. In the end the slices were embedded in a mounting medium (Entellan). Three images were analyzed per animal with Adobe Photoshop 7.0. The reader was blinded to group assignment.

Statistics

Data are presented as means $\pm$ SEM. One-way ANOVA and Newman-Keuls *Post-hoc* test were applied to identify significant effects. $P < 0.05$ was considered significant.

Results

Blood pressure and cardiac hypertrophy

Systolic blood pressure was measured on day 28 in all groups to evaluate the role of pressure overload for the expected alterations. Aldosterone treatment did not induce a significant increase in the systolic blood pressure (CON 129 ± 5 mmHg, ALD 134 ± 10 mmHg, ALD+SPI 132 ± 2 mmHg, SPI 139 ± 7 mmHg, $p > 0.05$). Despite the lack of blood pressure increase by aldosterone, there was significant myocardial hypertrophy in the aldosterone group (heart weight/tibia length: CON 0.34 ± 0.01 g/cm vs. ALD 0.38 ± 0.004 g/cm, $p < 0.05$). Spironolactone prevented aldosterone-induced hypertrophy (ALD+SPI 0.32 ± 0.006 g/cm, $p < 0.05$ vs ALD). Spironolactone alone was without influence on the ratio

of heart weight to tibia length (SPI 0.33 ± 0.008 g/cm, $p>0.05$ vs CON)

Atrial fibrillation

AF was repeatedly (three times) induced in all animals of each group as described in the method section. Figure 1 shows typical transesophageal and surface electrogram recordings during sinus rhythm and after induction of AF. In rats treated with aldosterone the time until spontaneous conversion into sinus rhythm was more than doubled (85.8 ± 13.4 s vs. 38.3 ± 6.9 s, $p<0.01$, Fig. 2) compared to control animals. Rats additionally treated with spironolactone showed AF duration not different from the control group (43.4 ± 4.8 s, $p>0.05$ vs. ALD). Spironolactone itself had no significant effect on the AF time.

Action potential recordings

Measurements of left atrial action potentials (AP) revealed stable and similar resting membrane potentials and amplitudes in all groups (Table 1 and Fig. 3). The repolarisation was dominated by the characteristic fast phase 1 with an APD_{50} of 13.8 ± 2.5 ms and an APD_{90} of 31.2 ± 1.9 ms in control rats. Animals treated with aldosterone showed a significant reduction in APD_{90} (26.2 ± 1.1 ms, $p<0.05$ vs. CON), while the APD_{50} was not significantly altered. The combined exposure of aldosterone and the aldosterone antagonist spironolactone prevented the reduction in APD_{90} (32.0 ± 1.5 ms, $p>0.05$ vs. CON) Spironolactone itself did not influence any parameter of the recorded APs in a significant manner.

Altered ion channel expression

To address the changes on a cellular level which might be responsible for the extended AF time and the shortening of the APs we focused on the expression of potassium channels that are involved in the repolarisation phase of the AP. The protein expression of Kir2.1, a subunit of the inward rectifier potassium channel I_{K1} , was significantly increased in animals which received aldosterone compared to control animals (Fig. 4). The aldosterone antagonist spironolactone was able to prevent the increase in the expression of Kir2.1. Moreover, Kir2.3 which also contributes to I_{K1} in atrial tissue tended to be increased in the presence of aldosterone in a spironolactone sensitive manner. However, these differences did not reach statistical significance (Fig. 5). I_{Kur} , the ultra-rapid activating delayed rectifier potassium current is carried by Kv1.5 channel subunits, which were significantly up-regulated in aldosterone treated animals

	APD_{50} (ms)	APD_{90} (ms)
CON	13.76 ± 0.57	31.22 ± 1.91
ALD	12.32 ± 1.03	$26.24\pm1.15^*$
ALD + SPI	11.66 ± 0.73	32.03 ± 1.45
SPI	12.11 ± 1.08	29.87 ± 2.60

Table 1. Left atrial action potential parameters from recordings of 3 animals per group with 4 to 10 APs from each preparation. Mean $\pm$ SEM. * $p<0.05$ vs. CON.

Fig. 3. Overlay of normalized typical intracellular left atrial action potential recordings. Aldosterone treatment reduced action potential duration, while spironolactone prevented this effect.

Fig. 4. Protein expression of Kir2.1 was significantly increased in the presence of aldosterone. Additional treatment with spironolactone prevented the increase in protein expression. Quantified band densities are depicted in the upper panel after normalization to the internal standard GAPDH and to CON. The lower panel depicts compiled representative Western blots of Kir2.1 and the loading control GAPDH. * $p<0.05$ vs CON. ♦ $p<0.05$ vs ALD.

Fig. 5. Protein expression of Kir2.3 tended to be increased in the presence of aldosterone. Quantified band densities are depicted in the upper panel after normalization to the internal standard GAPDH and to CON. The lower panel depicts compiled representative Western blots of Kir2.3 and the loading control GAPDH.

Fig. 6. Protein expression of Kv1.5 was significantly increased in the presence of aldosterone. Additional treatment with spironolactone prevented the increase in protein expression. Quantified band densities are depicted in the upper panel after normalization to the internal standard GAPDH and to CON. The lower panel depicts compiled representative Western blots of Kv1.5 and the loading control GAPDH. * $p < 0.05$ vs CON. ♦ $p < 0.05$ vs ALD.

(Fig. 6). Rats treated with aldosterone and spironolactone had Kv1.5 protein expression levels similar to control rats.

Structural remodeling

We next evaluated tissue fibrosis. The Figure 7 shows representative photomicrographs of Picrosirius red-stained left atrial sections from the studied groups. A significant increase in interstitial collagen deposition could be observed in rats treated with aldosterone. Additional application of spironolactone prevented the increase in collagen content significantly.

Discussion

The major findings of the present study are as follows: (1) Longterm aldosterone treatment leads to an increased duration of AF induced by transesophageal burst pacing in rats which is sensitive to spironolactone. (2) Shortening of the AP due to the increased expression of repolarising potassium currents and fibrosis of the atrial tissue likely contribute to the stabilization of AF in the presence of aldosterone.

In the multiple re-entry theory, the stability of AF is determined by the number of wavelets in the atria [20]. The wavelength depends on the refractory period and

Fig. 7. The upper panel depicts representative images of Picrosirius red stained left atrial tissue from the four studied groups. Original magnification $\times 400$. The lower panel shows the mean quantified collagen content. * $p < 0.05$ vs CON. ♦ $p < 0.05$ vs ALD

the conduction velocity [21]. Therefore, the shorter the wavelength, the more wavelets in the atria, and thus AF will be more stable [20]. Consequently, the abbreviation

of the action potential and the shortening of the atrial refractory period observed in AF increase the stability of AF itself. Therefore the observation that the action potentials are abbreviated after treatment with aldosterone suffices to explain the increased duration of AF induced by burst pacing.

Potassium channel mutations underlying familial AF are predominantly characterized by gain of function changes and are usually predicted to promote repolarisation, shorten the atrial action potential, and facilitate re-entry [22]. Gain of function mutations of Kir2.1, encoding for the inward rectifier potassium current I_{K1} , have been shown to be associated with AF in simulation studies, [23] in a mouse model [24] and in humans [25]. I_{K1} dominates the late repolarisation of the action potential and has been shown to be increased in patients with AF and in animal models [14, 15, 26] due to an upregulation of Kir2.1 on mRNA [26] and protein level [26]. Therefore a contribution of aldosterone-mediated increase in protein expression of Kir2.1 to the observed action potential shortening in the studied rat model seems at least likely.

Kv1.5 is encoding for I_{Kur} , the ultrarapid potassium outward current. The data regarding I_{Kur} in human AF subjects are inconsistent with no change [14] or decreases in current, [15] Kv1.5 subunit mRNA [27] or protein expression reported [15, 16]. The influence of I_{Kur} on the duration of the action potential is difficult to predict, since it depends on the level of electrical remodelling [28]. In a right atrial tissue from patients with sinus rhythm inhibition of I_{Kur} results in a shortening of the AP, while in atrial tissue from patients with AF the action potential is prolonged [28]. Transgenic mouse ventricular myocytes with overexpression of a truncated K^+ -channel (Kv1DN) show a prolonged action potential which could be abbreviated by injection of adenoviral vectors expressing wild type Kv1.5 into the myocardium [29]. Similar observations have been made in cultured rat neonatal ventricular myocytes where overexpression of Kv1.5 also resulted in action potential abbreviation [30]. In contrast to our model APD₅₀ and APD₉₀ were reduced. The discrepancy might be explained by differences in the magnitude of Kv1.5 overexpression or differences in the composition of ion currents underlying the action potential in cultured neonatal ventricular myocytes and adult atrial tissue. In a rat model of rapid pacing an enhanced atrial mRNA expression of Kv1.5 correlated with a shortening of APD₉₀ [31]. Thus, most lines of evidence suggest that

upregulation of Kv1.5 protein contributed to the action potential shortening in our rats treated with aldosterone.

Recent evidence indicates that aldosterone has both rapid non-genomic and slow genomic effects [32]. Multiple signalling pathways have been shown to be activated by aldosterone including the mitogen-activated protein kinases [33], diacylglycerol and inositol-1,4,5-triphosphate [34], different subtypes of protein kinase C [34], calcineurin [35], serum- and glucocorticoid-induced kinase 1 (SGK1) [36] and cAMP [37]. The detailed mechanisms by which aldosterone influences ion channel expression have not been elucidated yet. Expression levels could either be influenced by genomic actions mediated by the intracellular mineralocorticoid receptor or by the intracellular signalling pathways listed above.

The 5'-non-coding region of the Kv1.5 gene contains a cAMP response element and a cAMP dependent upregulation of the Kv1.5 transcript could be demonstrated in cardiac cells [38] suggesting a possible mechanism for the upregulation of Kv1.5 by aldosterone. Alternatively SGK1 might be involved in the increased abundance of Kv1.5 protein since inhibition of the Nedd4-4 ubiquitination pathway by SGK1 leads to stabilization of Kv1.5 in the plasma membrane [39]. Ras and the mitogen-activated protein kinase (MAPK) pathways have been described to be involved in the regulation of Kir2.1 expression by affecting the subcellular localization of the channel [40].

Conclusions

Aldosterone has been shown to exert deleterious cardiac effects in various experimental and clinical settings [6, 41]. Particularly, the structural remodelling of the atria during AF fibrillation has convincingly been shown to be sensitive to the mineralocorticoid receptor antagonist spironolactone [42]. The results of the present study now extend these previous observations supporting the notion that in addition to the structural remodelling aldosterone promotes AF by electrical remodelling, i.e. by altering repolarising potassium currents leading to action potential shortening.

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