Experimental studies on new therapeutic approaches in atrial fibrillation

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Experimental Studies on New Therapeutic Approaches in Atrial Fibrillation

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. L. L. G Soete
volgens het besluit van de College van Decanen,
in het openbaar te verdedigen
op donderdag 14 November 2013 om 16.00 uur

door

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Chapter 1:

General Introduction

General Introduction

1% of the European population suffers from atrial fibrillation (AF) and the number of AF-patients is expected to double or triple within the next two to three decades.¹⁻⁴ The clinical presentation of AF is highly variable, ranging from the complete absence of symptoms to palpitations, heart failure, and hemodynamic collapse. Importantly, AF increases the risk of stroke about 5 times.^{5,6} AF doubles mortality and causes marked morbidity.⁷ Symptoms and complications of AF result from the irregular and often rapid ventricular response, loss of atrial contraction as well as from AF-associated hypercoagulability.

Studies, which compared rhythm control (cardioversion and subsequent prophylactic therapy against AF-recurrence) with rate control strategies showed that none of these two treatment strategies is superior. Rhythm control is more costly and inconvenient than rate control.⁸ However, it is very likely that safer and more effective anti-arrhythmic therapy would cause a clear trend towards an advantage of rhythm control.

Although strokes account for most of the functional impairment associated with AF, the arrhythmia can also decrease quality of life directly. In the Canadian Trial of Atrial Fibrillation (CTAF) study, quality of life improved after pharmacological antiarrhythmic treatment.⁹ The post-cardioversion EMERALD (European and Australian Multicenter Evaluation Research on Atrial Dofetilide) study showed that dofetilide improved quality of life 1 month after electrical cardioversion.¹⁰ The randomized MANTRA-PAF (Medical ANtiarrhythmic Treatment or Radiofrequency Ablation in Paroxysmal Atrial Fibrillation) trial compared pulmonary vein isolation with

radiofrequency ablation to antiarrhythmic drug therapy as a first-line rhythm control intervention in 294 patients. At 24-month follow-up, significantly more patients in the ablation group were free from symptomatic AF. Quality of life was significantly better in the ablation group at 12 and 24 months. However, total AF burden was not significantly different between both groups.¹¹ Similar information has emerged from the results of the RAAFT II (Radiofrequency Ablation for Atrial Fibrillation Trial).¹² Therefore, achieving sinus rhythm in AF is a central goal of treatment in AF patients. In order to develop new treatment strategies for AF, it is important to understand the underlying pathophysiological mechanisms of AF. Different forms of AF might require different types of treatment.

Mechanisms of AF

Theories of the mechanism of AF involve two main processes: ectopic focal discharges in one or several rapidly depolarizing foci and reentry involving one or more circuits (Figure 1). Both, reentry circuits as well as ectopic focal discharges can either be localized or they can intermittently change their location.

Figure 1: Different types of AF (modified from Eckstein et al.)

Focal atrial fibrillation

Rapid activation originating from the pulmonary veins initiates AF in many patients with paroxysmal AF.^{13,14} Although some experimental studies have attempted to identify arrhythmogenic mechanisms in the pulmonary veins,¹⁵⁻¹⁸ the prominent role of the pulmonary veins in paroxysmal AF remains poorly understood. Histological studies have demonstrated cardiac muscle with preserved electrical properties extending into the pulmonary veins.¹⁹⁻²² Ectopic focal discharges as a trigger and perpetuator of AF are supported by experimental models of aconitine-induced AF,^{23,24} in which the arrhythmia only persisted, when the ectopic focal discharges were present. The focal origin appears to be more important in patients with paroxysmal AF than in those with persistent AF, and ablation of such foci may be curative. A better understanding of the mechanisms initiating 'focal' AF may help to develop safer and better ablation strategies and other therapeutic pharmacological modalities. Independent of the mechanisms involved, spontaneous atrial premature beats represent a potent trigger for AF initiating AF-episodes in most cases.^{25,26}

Multiple-wavelet hypothesis

The multiple-wavelet hypothesis as the mechanism of reentrant AF was proposed by Moe and colleagues,²⁷ who suggested that continuous wave front and wavebreak interactions results in wavebreak thereby providing a steady source of fibrillation waves. The number of wavelets present at any time depends on the refractory period, mass, and conduction velocity in different parts of the atria. A large atrial mass with a short refractory period and delayed conduction may favor the occurrence of a large number of wavelets, stabilizing AF.

Atrial electrophysiological, structural and autonomic remodeling

AF itself and several other conditions lead to an electrophysiological, structural and autonomic remodeling processes creating an arrhythmogenic substrate for AF. The complex interaction between electrophysiological, structural and autonomic remodeling processes in AF is summarized in figure 2. Sympathetic activation increases calcium-entry and the spontaneous release of calcium from the sarcoplasmic reticulum leading to atrial ectopies (trigger loop). Increased vagal activation together with AF induced atrial electrical remodeling shortens action potential duration facilitating reentry and thereby promoting AF. In the structural loop, atrial stretch during different conditions including congestive heart failure, hypertension or obstructive sleep apnea activates numerous profibrotic pathways resulting in atrial structural alterations and conduction disturbances, also facilitating reentrant mechanisms. The circular positive-feedback enhancement of these pathophysiological changes explains the general tendency of AF to become more stable with time.

Figure 2: Interaction between electrophysiological, structural and autonomic remodeling (modified from Schotten et al.²⁸).

Electrical remodeling and atrial fibrillation

AF itself causes shortening in atrial refractoriness, thereby perpetuating AF and contributing to early AF recurrence after cardioversion, progressive drug resistance of longer-lasting AF, and progression from paroxysmal to more persistent forms of AF (AF begets AF).^{29,30} Calcium enters atrial cells with each action potential. During rapid atrial rates, increased calcium-loading initiates autoprotective mechanisms that reduce calcium entry by calcium current inactivation and L-type calcium current downregulation. That decreases calcium loading by reducing action potential duration. Decreased action potential duration reduces wavelength thereby stabilizing reentrant circuits perpetuating AF.^{28,31-34}

Structural remodeling and atrial fibrillation

Structural remodeling is characterized by increased atrial fibrosis formation and myocyte hypertrophy. Atrial tissue fibrosis impairs electrophysiological cell-to-cell coupling and conduction disturbances.^{28,31-36} Longterm AF leads to myocyte hypertrophy and increased endomysial fibrosis associated with electrical dissociation within the epicardial layer as well as between the epicardial layer and the endocardial bundle network.³⁵ Besides AF alone, also other conditions cause structural alterations in the atrium. Longterm hypertension leads to atrial inflammation and fibrosis resulting in conduction abnormalities and increased inducibility of AF.³⁷ Congestive heart failure is associated with the development of a structural remodeling characterized by increased fibrosis formation and alterations in gap junction expression promoting heterogeneity of conduction and reentry.³⁸ Long-term obstructive sleep apnea has been shown to be associated with significant atrial remodeling characterized by atrial enlargement and site-specific and widespread conduction abnormalities, and longer sinus node recovery in humans.³⁹

Atrial autonomic nervous system and AF

Beta-adrenoceptor activation increases calcium-entry and the spontaneous release of calcium from the sarcoplasmic reticulum. This promotes delayed afterdepolarisation-related ectopic firing representing a potent trigger for AF.^{28,40-43} Vagal discharges enhance acetylcholine dependent potassium currents (I_{KACH}) reducing action potential duration thereby stabilizing reentry circuits perpetuating AF. In contrast, activation of sympathetic activity showed variable effects on action potential duration and atrial effective refractory period.²⁸

Very early studies suggested that exercise-induced AF may be sympathetically driven; in contrast, the parasympathetic nervous system may be contributing to AF in

young patients with no structural heart disease.⁴⁴ Several observations suggest that the autonomic nervous system plays an important role in both the initiation and the maintenance of AF. Studies in lone AF patients and in animal models of both intermittent rapid atrial pacing and congestive heart failure have indicated that AF onset is associated with simultaneous sympathovagal activation rather than with an increase in vagal or sympathetic drive alone.⁴⁵⁻⁴⁸ Ogawa et al, using direct nerve recordings from the stellate ganglia and vagal nerves, have shown increased sympathetic and vagal nerve discharges before the onset of atrial arrhythmias in dogs with pacing-induced congestive heart failure.⁴⁷ Indeed, the atrial tachyarrhythmias in this model were prevented by prophylactic ablation of the stellate ganglion and the T2 to T4 thoracic sympathetic ganglia.⁴⁷ The combination of vagal and sympathetic activity may provoke triggered activity (mainly late phase 3 EADs) acting as a potent initiator and perpetuator of AF. Combined sympatho-vagal activation can create a state with short action potential duration (parasympathetic stimulation) and an increase in calcium-load of the cells (sympathetic stimulation). This may explain observations that simultaneous sympathovagal discharge is associated with the onset of AF paroxysms in both patients and animal models (described above).

Additionally, in persistent AF patients and tachycardia-induced atrial remodeling in dogs atrial sympathetic hyperinnervation occurs,⁴²⁻⁴⁵ possibly promoting AF persistence and recurrence. In a dog model of pacing-induced congestive heart failure, Ng et al. recently demonstrated increased sympathetic and parasympathetic nerve growth in the left atrium; nerve growth was most pronounced in the pulmonary veins and the posterior wall of the left atrium.⁴¹ Jayachandran et al. demonstrated a heterogeneous increase in sympathetic innervation in the atria of dogs subjected to rapid atrial pacing for prolonged periods of time.⁴² There is also evidence of

sympathetic hyperinnervation in patients with persistent AF.⁴³ Interestingly, the cardiac autonomic system plays also a crucial role for the acute atrial electrical remodeling during atrial tachypacing since shortening in atrial effective refractory period could be inhibited by ganglionated plexi ablation.⁴⁵

Treatment strategies for AF

As mechanisms of AF differ dependent on the underlying substrate, individualized therapy of AF might be a promising way to treat AF. Different strategies to treat AF are available today:

Antiarrhythmic drugs

Antiarrhythmic drugs have been traditionally defined as membrane-active agents, which modulate the opening and closing of ion channels, change the function of membrane pumps, and activate or block membrane receptors.⁴⁹ This essentially results in antiarrhythmic electrophysiological changes characterized by an increase in refractoriness (by potassium channel blockers (class III antiarrhythmic drugs), e.g. sotalol, dofetilide, amiodarone) of the myocardium or a decrease in conduction velocity through the myocardium (by sodium channel blockers (class I antiarrhythmic drugs), e.g. flecainide). Additionally, some drugs, mainly sodium channel blockers, may decrease the firing rate of ectopic focal discharges. However, under certain conditions, anti-arrhythmic drugs do not only fail to display anti-arrhythmic effects but can also be proarrhythmic.⁴⁹ Novel anti-arrhythmic drugs with conventional anti-arrhythmic mechanisms are under investigation in AF, including newer multiple-channel blockers with a better safety profile and specific agents targeting atrial selective repolarization to avoid ventricular proarrhythmic effects.⁴⁹

Upstream therapy

‘Upstream’ therapies to attenuate the development of a structural or electrical arrhythmogenic substrate is another approach to treat AF. Angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), statins, and omega-3 polyunsaturated fatty acids (PUFAs) have theoretical advantages as potential therapeutic strategies.^{28,30,34,50-53} In patients with structural heart disease ACE-inhibitors and angiotensin inhibitors could reduce new onset AF.⁵²⁻⁵⁶ However, clinical studies failed to show sufficient efficacy of these approaches in patients without structural alterations. One year of angiotensin receptor blocker therapy per se does not reduce the number of AF episodes in patients with documented paroxysmal AF without structural heart disease.⁵⁷

Identification of novel pathways involved in the atrial remodeling process might provide new strategies to influence the development of an arrhythmogenic atrial structural substrate.

Pulmonary vein ablation

Catheter-based pulmonary vein ablation or isolation, surgically based maze operations, and atrioventricular node ablation for rate control may be effective nonpharmacological treatment strategies especially in younger individuals with paroxysmal AF and near-normal hearts. The European Society of Cardiology guidelines on management of AF released in 2010 have upgraded pulmonary vein ablation to first line therapy in patients with paroxysmal AF.⁵⁸ The surgical maze procedure is presently limited to patients undergoing other heart surgery, e.g. mitral valve repair or replacement. However, as these operations become minimally invasive and highly effective, they are likely to be more widely used.^{59,60}

Modulation of the autonomic system

Modulation of the autonomic nervous system might be a promising strategy to protect the myocardium from proarrhythmic autonomic influences and the development of electrical, autonomic and structural atrial remodeling. The most promising new strategy to modulate autonomic nervous system is renal sympathetic denervation.

Activation of renal efferent sympathetic nerve fibers increases sodium and water retention, reduce renal blood flow and elevate renin release from juxtaglomerular apparatus, regulating blood pressure.⁶¹ Renal nerves contain sensory afferent fibers, which enable communication with the central nervous system. Activation of renal afferent nerves itself elevate sympathetic nervous outflow to the kidney and other downstream organs (see Figure 4).

Figure 3: (modified by Schlaich et al.⁶⁴).

Recently, a catheter-based approach has been developed for renal sympathetic denervation (RDN). Special ablation catheters are inserted percutaneously via the femoral artery and advanced into the renal arteries. After placement, the catheter is withdrawn from distal to proximal segments and 4-8 ablations are administered within each artery. Both, a proof-of-concept trial (The Symplicity HTN-1 Trial ⁶²), including 45 patients with therapy-resistant hypertension (blood pressure $\geq 160/90$ mmHg despite receiving ≥ 3 anti-hypertensive drugs) and a multicentre, prospective, randomized trial (The Symplicity HTN-2 Trial ⁶³) including 106 patients demonstrated that RDN significantly lowered blood pressure. Mechanistically, it has been observed that the procedure resulted in a 47 % reduction of renal norepinephrine spillover measured with a radiochemical tracer methodology using 3H-norepinephrine.^{62,64} Interestingly, firing of single sympathetic vasoconstrictor fibers, measured by single muscle sympathetic nerve activity were also reduced.⁶⁵ These findings indicate a combined modulation of efferent and afferent signaling at the kidney by RDN. RDN may show antiarrhythmic effects by several mechanisms. Modulating of the central autonomic drive and of neuronal remodeling associated with AF (as described above) by RDN may reduce atrial arrhythmogenesis. Additionally, blood pressure reduction by RDN may attenuate the development of an atrial structural substrate.

Aim and outline of the study

A better understanding of AF mechanisms is crucial to identify promising targets for pharmacological and device based therapy to prevent AF-initiation and perpetuation. Up to now, the arrhythmogenic substrates due to different conditions like paroxysmal or permanent AF, congestive heart failure, hypertension, atrial dilation and aging, have already been studied in several animal models.³¹ However, the underlying mechanisms of arrhythmogenic triggers or electrical and structural alteration (substrate) associated with recently identified risk factors for AF like diabetes or obstructive sleep apnea are less understood. Additionally, although focal discharges are discussed to occur during AF and might represent an important factor for the perpetuation of AF, the interaction of ectopic focal discharges with re-entry mechanisms during AF is unknown. A better understanding of these conditions might be important to optimize new treatment strategies of patients with AF.

The aim of this study was to develop and characterize animal models for AF maintained by (1) diabetes, (2) obstructive sleep apnea and (3) ectopic discharges to identify and test potential new antiarrhythmic treatment approaches:

- 1) Diabetes has been identified as a risk factor for AF. In a rat model for type 2 diabetes, we identified the underlying mechanisms involved in the development of the atrial arrhythmogenic substrate. We characterized the structural and electrophysiological alterations by electrophysiological measurements, magnetic resonance imaging, biochemical and histological measurements. Subsequently, the effect of pharmacological inhibition of cathepsin A, an alternative angiotensin splicing enzyme, which is highly upregulated in atria in diabetes, was investigated (Chapter 2).

- 2) Obstructive sleep apnea is associated with recurrence of AF after electrical cardioversion, pharmacological antiarrhythmic treatment and ablation based therapy. In pigs, the effect of obstructive respiratory events on atrial electrophysiology and AF-inducibility was investigated (Chapter 3). In this model, the effects of available AADs (Chapter 4) and modification of the autonomic system by renal denervation (Chapter 5) was tested.
- 3) The role of ectopic focal discharges for the inducibility and maintenance of AF is unknown. In goats, ectopic focal discharges were induced by epicardial application of aconitine and the interaction between AF and ectopic focal discharges were characterized. Additionally, the effect of the sodium blocker flecainide in this model was studied (Chapter 6).

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Chapter 2

CathepsinA-Inhibition Reduces Susceptibility to Atrial Tachyarrhythmia and Protects Atrial Emptying Function in Old Zucker Diabetic Fatty Rats.

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Submitted

Abstract

Introduction: Type 2 diabetes (T2D) is an independent risk factor for atrial fibrillation (AF) and stroke and is associated with decreased exercise tolerance and cardiomyopathy. Cathepsin A (CathA) is a lysosomal enzyme involved in bradykinin and angiotensin metabolism. We tested the hypothesis that CathA-inhibition protects against atrial remodeling in old Zucker diabetic fatty-rats (ZDF), an animal model for T2D. **Method:** Three groups of rats were studied: ZDF receiving vehicle (n=20), ZDF receiving CathA-inhibitor (SAR); 50 mg/kg by gavage; n=20), and their age matched lean littermates (LEAN, n=20). Left atrial (LA) emptying function (magnetic resonance imaging) and atrial electrophysiological parameters were measured before the hearts were harvested for histological and biochemical analysis. **Results:** At the age of 9.5 months, ZDF-rats showed increased LA-CathA-activity, an increase in AF susceptibility, and reduced atrial contractile function. CathA-inhibition decreased the median duration of LA-tachyarrhythmia (12.0 ± 1.7 s vs. 1.2 ± 0.47 s, $p < 0.01$) induced by burst pacing. Total atrial activation times and regions of slowest conduction (local conduction times longer than 2ms, $4.3 \pm 1.7\%$ vs. $0.3 \pm 0.5\%$, $p < 0.01$) were reduced. Cardiac magnetic resonance imaging revealed better LA-emptying function parameters (active percent emptying, $29 \pm 1\%$ vs. $23 \pm 2\%$, $p < 0.01$) and normal LA-dimensions after CathA-inhibitor-treatment compared with untreated ZDF. Inhibition of CathA-activity resulted in reduced atrial bradykinin degradation and decreased LA-fibrosis formation, Cx43 lateralization and osteopontin gene expression in ZDF-rats. **Conclusion:** CathA-inhibition prevents the development of arrhythmogenic remodeling and contractile dysfunction in Zucker diabetic fatty-rats. These data suggest a causal relationship between atrial CathA-activity and atrial remodeling in T2D.

Introduction

Atrial fibrillation (AF) is the most common type of sustained tachyarrhythmia and is associated with significant morbidity and mortality.¹ Type 2 diabetes (T2D) is a strong independent risk factors for atrial fibrillation and stroke.^{2,3,4} In a rat model, diabetes has been shown to produce an arrhythmogenic substrate in the atrium with an increase in AF susceptibility.^{5,6} Additionally, an abnormal response to exercise is frequently observed in asymptomatic diabetic patients without any clinical evidence for further cardiovascular disease.^{7,8,9} The reduced exercise tolerance might be related to atrial and/or ventricular contractile dysfunction frequently seen in diabetic patients.¹⁰

Cathepsin A (CathA) is a lysosomal serine protease, which is also found in the extracellular space. It has been suggested to play an important role in degradation of extracellular peptides and inflammation.^{11,12} CathA belongs to a group of enzymes mediating alternate routes for angiotensin formation and bradykinin breakdown. Evidence of upregulation of alternative angiotensin-generating enzymes like chymase or cathepsins has been described in many pathologies e.g. in myocardial infarction, congestive heart failure or diabetes.^{13,14,15} Pharmacological inhibition of CathA by the natural product ebelactone B¹⁶ and antisense oligonucleotides suppressing the expression of CathA¹⁷ increased renal bradykinin levels and prevented deoxycorticosterone acetate-salt hypertension in rats. Treatment with SAR¹⁸, an orally available CathA inhibitor, has been shown to result in a reduction of cardiac hypertrophy in apolipoprotein E knockout mice with continuous angiotensin II infusion and prevented AF in rats with ventricular ischemia/reperfusion, a rat model for atrial structural remodeling¹⁸.

As activation of the renin-angiotensin system might also be involved in atrial remodeling during diabetes⁶, we tested the protective effect of the selective and potent orally available CathA inhibitor SAR ((S)-3-[[1-(2-Fluoro-phenyl)-5-methoxy-1H-pyrazole-3-carbonyl]-amino]-3-(2-methylphenyl)-propionic acid, characterized as 2a in¹⁸) on atrial emptying function and atrial electrical and structural arrhythmogenic remodeling in old Zucker Diabetic Fatty rats (ZDF), an animal model for T2D.

Methods

Animal model

Zucker diabetic fatty-rats (ZDF/Gmi-fa/fa, n=40) and their age matched lean littermates (male, 200 – 250g body weight, 10 weeks old, LEAN, n=20) were purchased from Charles River and housed three per cage under standardized conditions (room temperature 24°C, relative humidity 60%, lighting times 12 hours dark/light cycle). They had free access to a standardized diet, sodium content 0.2 %, Altromin, Lage, Germany) and tap drinking water. ZDF-rats were randomized in 2 groups: placebo treated ZDF-rats (ZDF-placebo, n=20) and CathA-inhibitor (SAR)¹⁸ treated ZDF-rats (ZDF-SAR, n=20). SAR was synthesized at Sanofi-Aventis, Frankfurt am Main, and administered daily via oral gavage (50 mg/kg/d, Vehicle: 0,5% HEC / Tween 5%, volume administered: 1 ml/kg). ZDF-placebo rats were treated with vehicle only. After 38 weeks, animals were anesthetized and cardiac function was assessed by cardiac magnet resonance imaging (MRI). One week later, electrophysiological measurements were performed in open chest experiments and the animals were sacrificed. Hearts were rapidly removed, trimmed free from non

cardiac tissues, and weighed. Left atrial (LA) tissue was frozen in liquid nitrogen for biochemical analysis or stored in formalin for histological examination.

All animal studies conform with the guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Electrophysiological studies

All measurements were performed during general anesthesia by intraperitoneal injection of pentobarbital at the age of 9.5 months. Surface ECG (lead II) was recorded via subcutaneous needle electrodes. To analyze atrial conduction, a custom made mapping electrode with 4*5 unipolar electrodes (1.0mm interelectrode distance) was placed on the LA-free wall. Unipolar signals were recorded using a custom made channel mapping amplifier (filtering bandwidth 0.1-408Hz, sampling rate 1kHz, A/D resolution 16 bits). Unipolar pacing was performed from the surface of the right atrium (pulse width of 1ms at twice the diastolic threshold). Maps from 5 consecutive beats were analyzed. Local activation times were identified by maximum negative dV/dt in each unipolar electrogram. Local activation time differences were calculated between neighboring electrodes (conduction times). Conduction times of $\geq 3\text{ms}$ (equivalent to conduction velocities $\leq 33\text{cm/s}$) were considered as being prolonged.¹⁹ Total-atrial activation time was defined as the time difference between the right atrial activation time, determined from a MAP-catheter (Franz-like electrode) next to the pacing electrode, and the latest left atrial activation time recorded by the mapping electrode during right atrial-pacing (cycle length: 150ms). Atrial effective refractory period (AERP) was measured at a basic cycle length of 150ms at the free wall of the LA. The mean of 3 AERP measurements was used for analysis.

Susceptibility to atrial tachyarrhythmia/atrial fibrillation (AT/AF) induced by repetitive 1s bursts of stimuli (very rapid pacing, cycle length: 10 ms). When atrial electrograms showed a rapid atrial rate, cycle length < 70ms and duration >5 beats, AT/AF was diagnosed. The duration of the longest of three subsequent episode of AT/AF induced during this protocol was measured and was taken as AF/AT-duration.

Magnetic resonance imaging (MRI)

All scans were acquired with a 7-T magnetic resonance imaging (MRI) scanner (Bio Spec 70/30) in all rats at the age of 9.5 months (anesthesia: 1.5-2.5% isoflurane). Multi-slice short-axis cine imaging was performed from the upper left atrial roof to the apex with a slice thickness of 1.2 mm and an inter-slice gap of 0 mm. Orthogonal long axis cine imaging of the whole heart, two chamber and four chamber views, were acquired. Boundaries of the left ventricle and left atrium were obtained in each short-axis image. The LV-volumes were then determined by Simpson's rule. LA-area was manually encircled. The point of insertion of the mitral valve leaflets was taken as the atrioventricular border. Pulmonary veins were excluded at their ostia and the left atrial appendage was excluded at its base. All dimensions were measured throughout the cardiac cycle.²⁰ The cardiac cycle was divided into 10 equal phases with an interphase time difference of the spontaneous cycle length / 10. The MRI-images of all LAs were analyzed by the same blinded investigator.

LV-enddiastolic volume (LVedV) and end-systolic volume (LVesV) were derived from the LA-volume/time curves for maximal and minimal values. LV-ejection fraction (EF) was subsequently computed. Minimal LA- (LA min) and maximal LA-volume (LAmx) and their difference (cyclical change volume) were determined from the LA-volume/time curves. The minimal volume at the end of rapid passive emptying (LAre) and the volume before active emptying (LApc) were determined from the volume-

time curves as described elsewhere.²¹ LA-emptying function parameters (total percent emptying $((L_{Amax}-L_{Amin})/L_{Amax})$, active percent emptying $((L_{Apc}-L_{Amin})/L_{Apc})$, passive percent emptying $((L_{Amax}-L_{Apc})/L_{Amax})$ were computed according to Jarvinen et al..²⁰

Measurement of LV-pressure and blood pressure

LV-enddiastolic pressure (LVedP) was measured in anaesthetized rats using Millar Tip catheters (Millar Instruments Inc, Houston, USA) introduced from the right arterial carotis and advanced into the left ventricle. In conscious rats, blood pressure was measured using the tailcuff method.

Gene expression analysis

Gene expression analysis was performed by realtime PCR. Total RNA was extracted from whole LA by homogenizing in RLT buffer and by using RNeasy Mini columns (Qiagen, Hilden, Germany). Genomic DNA impurities were removed by DNase treatment (DNA removal kit; Ambion, Austin, TX), and cDNA was synthesized by reverse transcription (Life Science Technologies, Darmstadt, Germany). Quantitative real-time RT-PCR was performed using the Taqman primers on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA) and c(t) values obtained by using a respective software (SDS version 1.9). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. For each analysis, mean values were used from 8–10 animals per group. Probes used to amplify the transcripts were as follows (purchased by Applied Biosystems): CathA (Rn01424035_g1); angiotensin converting enzyme (ACE, Rn00561094_m1); angiotensin receptor 1a, (AGTR1a, Rn01435427_m1); angiotensin receptor 1b (AGTR1b, Rn02132799_s1), Chymase (Rn00565319_m1); osteopontin (OPN,

Rn00681031_m1); tissue growth factor β 1 (TGF β 1, Rn00572010_m1); collagen 1a1 (Col1a1, Rn01463848_m1); matrix metalloproteinase-2 (MMP2, Rn01538174_m1); tissue inhibitors of metalloproteinases1 (TIMP, Rn00561661_m1); atrial natriuretic peptide (ANP, Rn00561661_m1); glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn99999916_s1).

CathepsinA activity, angiotensin and bradykinin degradation

LA-tissue was homogenized in 25mM MES pH 5.5 containing 10 μ M E64. Homogenates were then mixed with SAR or DMSO in a multiple assay plate. After incubation for 15 minutes at room temperature a bradykinin peptide carrying a N-terminal BodipyFL label (Jerini Peptide Technology) was added to the assay mixture to a final concentration of 2 μ M. After incubation for 15min at room temperature the reaction was stopped by the addition of 130mM Hepes pH 5.5 and containing 0.013% Triton X-100, 0.13% Coating Reagent (Caliper Life Sciences), 6.5% DMSO and 20 μ M ebelactone B (Sigma, #E0886). Uncleaved substrate and product were then separated by a microfluidic capillary electrophoresis on a LabChip® 3000 Drug Discovery System (12-Sipper-Chip, Caliper Life Sciences) and quantified by determination of the respective peak areas. Substrate turnover was calculated by dividing product peak area by the sum of substrate and product peak areas. The difference in bradykinin turnover in the presence and absence of SAR at pH 5.5 (optimum of carboxypeptidase activity) was used to quantify CathA-activity. Additionally, bradykinin turnover at pH 7.4 was quantified in all groups. Angiotensin was quantified in LA-preparations by ELISA.

Histology and connexins

Separated LA fixed in 4% formaldehyde for 24h and imbedded in paraffin were used for histological examination and gap junction analysis. Tissue sections of 5µm were fixed at 56°C overnight, deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) to determine cardiomyocyte diameter and cell distances. To visualize tissue fibrosis amount, the sections were stained with Picro-Sirius Red. The percentage of the LA consisting of interstitial collagen was calculated as the ratio of Picro-Sirius-Red positively stained area over total LA-tissue area, excluding blood vessels and the epi- and endocardial plane, using ImageJ 1.37a (National Institute of Health, USA). Fibrosis was quantified on three sections per atrium (6–8 fields per section).

Immunostaining for connexin43 (Cx43) in formalin fixed left atrial tissue from 9 LEAN, 9 ZDF-placebo and 8 ZDF-SAR rats was performed like described elsewhere.²² Briefly, expression of Cx43 at the lateral and polar membrane of cardiomyocytes from rat atria was investigated. Per animal 2-3 sections were analyzed, and per section 5-10 cells, so that each data point of the columns is representing 100-250 cells.

Blood samples

The concentration of BNP32 (fluorescent ELISA) and osteopontin was determined in plasma using a radio immunoassay (Phoenix Pharmaceuticals Inc., USA). Serum ACE-activity was determined by a fluorimetric assay according to Santos et al.

Statistical Analysis

The data are expressed as mean $\pm$ SEM. Statistical significance was assessed by analysis of variance (ANOVA), followed by the unpaired Student's t tests (two-tailed) for 2 groups. If necessary, Dunnett's test was employed. P-values of <0.05 were considered statistically significant.

Results

Increase in atrial Cathepsin A activity in ZDF

LA-CathA-activity was significantly increased in ZDF-placebo, and this increase was effectively inhibited by SAR treatment (Figure 1). This was associated with increased CathA-gene expression in ZDF-placebo compared to LEAN (1.49 ± 0.14 -fold, $p < 0.001$).

Figure 1: CathepsinA-activity (SAR-sensitive bradykinin turnover) in left atrial preparations in LEAN, in ZDF-placebo and in ZDF-SAR.

Metabolic and hemodynamics parameters

Metabolic and hemodynamic parameters from these experiments are summarized in Table 1. At the age of 9.5 months, increased concentration of glucose and HbA1c in serum of ZDF-placebo was accompanied with severe signs of nephropathy and dyslipidemia. SAR-treatment did not change metabolic parameters or renal function in ZDF-rats at the age of 9.5 months. Cardiac hypertrophy index, expressed as heart weight / 100g body weight, was neither elevated in hearts from ZDF-placebo nor from SAR-treated ZDF-rats in comparison to LEAN. Body weights between untreated and SAR-treated ZDF-rats were not different. However, LEAN were heavier than ZDF-rats.

LV-EF and LV-dimensions determined by MRI did not differ between ZDF and LEAN, but LV-enddiastolic pressure was increased in ZDF, indicating the development of moderate diastolic dysfunction with preserved systolic function in old ZDF-rats. Blood pressure measured in conscious rats (tail-cuff-methode) revealed no differences between ZDF-placebo and LEAN, but heart rate tended to be lower in ZDF-rats. SAR-treatment in old ZDF-rats did not significantly change blood pressure nor heart rate in ZDF-rats. Lower LVedP and plasma BNP-levels (121.3 ± 1.5 ng/ml vs. 159.9 ± 11.3 ng/ml in ZDF, $p < 0.001$) were preserved in SAR-treated ZDF-rats. LA-dimensions determined by LA-volume/heart weight indices and metabolic parameters were not affected by SAR treatment.

Table 1: General Characteristics

	LEAN	ZDF- placebo	ZDF-SAR
Characteristics			
Bodyweight (g)	472.7±7.8 **	337.5±6.3	328.1±6.7
Heart weight (g)	1.25±0.01 *	1.06±0.03	0.99±0.01
LV-weight (g)	0.91±0.02 *	0.77±0.09	0.71±0.04
LA-weight (g)	0.022±0.001 *	0.015±0.001	0.015±0.002
Heart weight/100g body weight	0.27±0.02	0.32±0.02	0.31±0.01
LV-weight/100g body weight	0.21±0.01	0.23±0.01	0.22±0.01
LA-weight/100g body weight	0.0046±0.0002	0.0043±0.0003	0.0045±0.0007
Diabetes and metabolism			
Glucose (mmol/l plasma)	6.26±0.16 **	28.68±0.97	28.21±0.97
HbA1c (%)	4.15±0.03 **	10.1±0.07	9.49±0.01
Albumin/creatinin-ratio	0.07±0.01 **	12.96±1.75	9.37±1.01
Creatinine-clearance	7.4±0.2 **	9.3±0.3	9.3±0.2
LDL (mmol/l)	0.30±0.01 **	0.63±0.06	0.66±0.06
Triglyceride (mmol/l)	1.37±0.06 **	5.02±0.34	4.28±0.19
Blood pressure (tailcuff)			
Blood pressure (mmHg)	141.3±2.2	141.5±3.1	141.5±3.9
Heart rate (bpm)	339.2±8.0	327.1±6.8	329.8±7.7
Ventricular haemodynamics			
LVedP (mmHg)	5.1±0.3 *	7.6±0.7	5.2±0.5 *
LV-function (MRI)			
EF (%)	66.6±0.93	64.6±0.65	63.6±0.58
EDV (µl)	711±15.6 *	646±21.9	605±8
ED-Vol. Index (µl/g BW)	1.52±0.03 **	1.87±0.07	1.83±0.04
ESV (µl)	239±9.5	229±9.8	220±4.3
ES-Vol. Index (µl/g BW)	0.51±0.02 *	0.66±0.03	0.66±0.02
LA-dimension (MRI)			
LAD (cm)	0.52±0.02 *	0.48±0.01	0.47±0.02
LAV (ml)	0.43±0.01 **	0.33±0.01	0.32±0.01
LAV-index (µl/g BW)	0.91±0.02	0.94±0.03	0.97±0.02
LAV-index (µl/g HW)	0.29±0.02	0.31±0.03	0.31±0.02

p<0.01, vs. ZDF-placebo **

p<0.05, vs. ZDF-placebo *

Atrial electrophysiology

Short episodes of AF/AT could be induced in all rats investigated in our study. The median duration of AF/AT was increased in ZDF-placebo in comparison to LEAN (Figure 2 A). Total atrial activation time was significantly longer (Figure 2 B) and the percentage of regions with slow conduction during rapid pacing was higher (Figure 2 C) in ZDF-placebo compared to LEAN. AERP was prolonged in ZDF-placebo when compared to LEAN (Figure 2 D). Importantly, SAR-treatment reduced median AF/AT-duration in ZDF-rats to values comparable to the LEAN animals. SAR-treatment did not attenuate AERP-prolongation in ZDF-rats but reduced total atrial activation time and the percentage of regions with slow conduction.

LA-emptying function

LA-emptying function parameters are shown in figure 3. LA-fractional shortening was reduced in ZDF-placebo when compared to LEAN ($15.4 \pm 1.5\%$ vs. $17.3 \pm 0.9\%$, $p < 0.001$). Global LA-emptying function as assessed by total percent emptying was not changed (Figure 3 B). In contrast, active percent emptying was diminished in old ZDF-placebo compared to LEAN (Figure 3 E). SAR-treatment attenuated changes in LA-fractional shortening and active percent emptying. During the MRI-scan, heart rate was lower in ZDF-placebo and ZDF-SAR compared to LEAN (261 ± 3.34 bpm, 260 ± 5.67 bpm and 281 ± 5.37 bpm respectively) ($p < 0.05$). However, since atrial emptying function is inversely correlated to heart rate, this would even lead to underestimation of the differences between LEAN and ZDF-placebo and ZDF-SAR and would not influence the comparison between ZDF-placebo and ZDF-SAR.

Figure 2: Atrial electrophysiological measurements in rats. (A) AF-duration induced by burst-pacing (top) and representative atrial electrograms in ZDF-placebo and in LEAN (bottom). **(B)** Total atrial activation time, **(C)** areas of slow conduction and **(D)** left atrial effective refractory period (AERP) at a stimulation frequency of 400/min (basic cycle lengths (BCL): 150ms) are shown at the right.

Figure 3: Atrial emptying function in rats. (A) Representative makroskopic and MRI-images and (B) LA-volume/time curves showing the % maximal phasic LA-volume change in LEAN and in ZDF-placebo. Cardiac cycle is divided in 10 phases. (A: minimal LA-volume, B: maximal LA-volume, C: minimal volume at the end of rapid passive emptying, D: max volume before active emptying.) (C) Total percent emptying, (D) passive percent emptying and (E) active percent emptying determined out of the LA-volume/time curve are shown at the right.

LA-structural remodeling

In sirius red–stained sections, the degree of atrial tissue fibrosis was increased in ZDF-placebo LA when compared to LEAN (shown in Figure 4) as were myocyte diameters and myocyte-myocyte distances within bundles as a measure of enhanced extracellular LA-matrix formation. SAR-treatment in ZDF-rats prevented the increase in myocyte-myocyte distances and total atrial tissue fibrosis but did not modify increased cell-diameter in ZDF-rats.

SAR reduced LA-gene expression of the profibrotic and proinflammatory adhesion protein osteopontin, which was highly upregulated in ZDF-placebo when compared to LEAN (Table 2). Osteopontin plasma concentration was significantly reduced in ZDF-SAR (8.4 ± 0.4 ng/ml, $p < 0.01$) when compared to ZDF-placebo (12.1 ± 1.4 ng/ml). Other profibrotic markers like TGF β 1 and MMP2 and Col1a1 gene expression were clearly detectable but kept unchanged on mRNA level in ZDF-placebo or ZDF-SAR.

Connexin 43

In LEAN we found the typical intracellular distribution of Cx43, with a clear accentuation of Cx43 at the cell poles, and nearly no Cx43 at the lateral sides of the cells. However, in ZDF-placebo rats there was a significantly enhanced lateral Cx43 immunostaining, whereas polar staining remained unchanged. This lateralization of Cx43 was significantly attenuated by SAR. In figure 5, the pattern of Cx43 immunostaining in LA-tissue is shown.

Figure 4: Atria histological analysis. Representative histological images of **(A)** sirius red stained and **(B)** H&E stained left atrial preparations in LEAN, ZDF-placebo and ZDF-SAR. Results for **(C)** tissue fibrosis amount, **(D)** myocyte diameter and **(E)** myocyte distances are shown at the right.

Figure 5: Atrial connexin 43 expression. Representative immunohistochemical staining for Cx43 in left atrial preparations in **(A)** LEAN, **(B)** ZDF-placebo and **(C)** ZDF-SAR. Quantification of positively stained **(D)** lateral and **(E)** polar cardiomyocyte membrane.

Components of the renin-angiotensin system and bradykinin-degrading activity

LA-total bradykinin-degrading activity was increased in ZDF-placebo compared to LEAN and reduced to LEAN values in ZDF-SAR (Figure 6A). LA-tissue angiotensin II levels were neither altered in ZDF nor in SAR-treated ZDF when compared to LEAN (Figure 6B). Serum ACE-activity was slightly higher in both, ZDF-placebo (107.1 ± 2.1 U/l, $p < 0.01$) and ZDF-SAR rats (109.1 ± 1.5 U/l, $p < 0.01$) when compared to LEAN (90.3 ± 0.9 U/l, $p < 0.01$). In LA-tissue, ACE, chymase or ATRa and ATRb mRNA expression were unchanged in ZDF-placebo with no effect of SAR treatment (Table 2).

Figure 6: (A) Total bradykinin turnover and **(B)** angiotensin II content of left atrial tissue in LEAN, ZDF-placebo and ZDF-SAR.

Table 2: Left atrial gene expression data

	LEAN	ZDF-placebo	ZDF-SAR
Gene expression/GAPDH	mean [%of mean ZDF]	mean [%of mean ZDF]	mean [%of mean ZDF]
Markers of the renin-angiotensin system			
ACE	82±10	100±9	98±13
Agtr 1a	91±15	100±18	85±13
Agtr1b	68±11	100±35	99±37
Chymase	89±41	100±36	82±26
Markers of LA-structural remodeling			
Osteopontin	5±2	100±40 ⁺	54±12 ⁺⁺
TGFβ1	67±2	100±14	86±21
Col1a1	91±15	100±18	83±13
MMP2	98±12	100±11	97±15
TIMP	18±5	100±37 ⁺	54±12

* p<0.01 vs. ZDF-placebo, ⁺ p<0.01 vs. LEAN

Discussion

In ZDF-rats, a rat model for T2D, susceptibility to AT/AF was increased and LA-emptying function parameters were impaired while LV systolic function remained unchanged. These findings were accompanied by upregulation of the lysosomal enzyme CathA in LA. Inhibition of CathA by SAR attenuated arrhythmogenic and functional atrial remodeling without influencing T2D-related metabolic changes, blood pressure, LV-systolic function, plasma ACE-activity or LA-angiotensin II levels in ZDF. CathA-inhibition reduced serum BNP-levels, LA-gene expression of profibrotic and proinflammatory factor osteopontin, and LA-bradykinin-degrading activity.

The proarrhythmic substrate for AF in ZDF-rats

In ZDF, increased susceptibility to AT/AF was associated with longer total atrial activation times, more areas of local atrial conduction slowing and prolonged AERP when compared to LEAN. Local heterogeneities in conduction have been demonstrated to underlie the high AF susceptibility in structurally remodeled atria in a variety of animal models.^{19,23} In ZDF-rats, myocyte-myocyte distances in the atrial myocardium as a measure of enhanced extracellular matrix formation were increased as were myocyte diameters. Additionally, enhanced ANP expression in LA of ZDF may contribute to atrial amyloidosis.^{25,26} Cx43 lateralization was more pronounced in atria of old ZDF-rats compared to LEAN, a finding also reported in patients with AF and various animal models for AF.^{22,27,28} These structural atrial changes together with the disorganization of connexins likely resulted in disruption of side to side electrical connections between muscle bundles obviously accounting for the impaired atrial conduction properties and increased susceptibility to AT/AF observed in old ZDF-rats compared to LEAN. Interestingly, this arrhythmogenic

substrate and these structural alterations of the extracellular matrix occurred without any changes in LA-dimensions in ZDF-rats. In contrast, prolongation of atrial refractoriness in ZDF-rats suggests that ion channel remodeling, as seen in tachycardia-induced atrial remodeling⁶, does not play an important role in the enhanced susceptibility for AT/AF in ZDF-rats.

Atrial contractile dysfunction in ZDF-rats

Experimental and clinical information about changes in ventricular function caused by pure diabetic conditions are controversially discussed.^{29,30,31} In our study, ZDF rats showed moderate signs for diastolic ventricular dysfunction with fully preserved systolic function. Additionally, we found an impairment of active LA-emptying function in ZDF-rats suggestive for a LA-hypocontractility in ZDF-rats. Those findings are in line with impairment of LA-emptying function revealed by echocardiography in patients with type 1 diabetes and preserved LV-function.³² Potentially, impaired LA-emptying function reduces exercise tolerance in diabetic patients.^{7,8,9} Besides, it might contribute to the enhanced risk for strokes in patients with T2D.¹⁰ Atrial contractile dysfunction reduces local blood flow velocity increasing the risk for thrombus formation. The mechanisms leading to impaired LA-emptying function in diabetes are unknown. A similar reduction of contractile function with unchanged or prolonged AERP was found in a dog model with congestive heart failure³³ and in goats with dilated atria.³⁴ Increased interstitial fibrosis amount in old ZDF-rats could account for abnormal myocardial stiffness and ultimately for atrial dysfunction. Another possibility is altered energetics of myofilaments as also found in patients with AF.³⁵

Role of CathA in atrial remodeling in ZDF-rats

In this report, we identify a relevant role of CathA for the LA-remodeling process in T2D. LA-CathA activity was increased in ZDF compared to LEAN. The novel CathA inhibitor SAR attenuated functional and arrhythmogenic atrial remodeling characterized by increased tissue fibrosis, myocyte-myocyte distances and lateralization of Cx43. Smaller myocyte-myocyte distances and attenuation of lateralization of Cx43 increase coupling between the atrial bundle network and are likely involved in the reduction of AF/AT-duration and conduction disturbances in ZDF-SAR compared to ZDF-placebo. As there were no changes in atrial refractoriness related to treatment with SAR an effect of the compound on ion channel composition does not likely contribute to the protective effect of SAR.

LA-gene expression and plasma concentration of the profibrotic and proinflammatory marker osteopontin (OPN) was markedly increased in ZDF-rats. CathA-inhibition by SAR was associated with a reduction in LA-gene expression and plasma concentration of OPN. OPN is a secreted multifunctional phosphoprotein, which plays an important role in the pathogenesis and fibrosis formation and inflammation in CHF and diabetic cardiopathy.³⁶ Its expression is accelerated by mechanical stress including pressure/volume loading and angiotensin II infusion in rats.^{37,38} OPN knockout prevented diastolic dysfunction and interstitial fibrosis following myocardial infarction.³⁹ Additionally, streptozotocin-induced diabetic cardiomyopathy and cardiac fibrosis were attenuated in OPN-deficient mice, suggesting that OPN is a key factor for the remodeling process in the heart especially under diabetic conditions.⁴⁰ Both, CathA and OPN are involved in inflammation occurring rapidly (within 24h) after onset of ventricular tachypacing in dogs. However, these inflammatory changes are transient and disappear gradually.⁴¹ In line with this, we did not observe indicators for enhanced inflammation in ZDF rats. However, inflammatory processes

might well have contributed to extracellular matrix changes at an early stage of T2D in ZDF. Thus, inhibition of OPN expression by SAR may be of value in the treatment and prevention of diabetic atrial cardiomyopathy.

We found arguments to believe that the action of SAR differs substantially from inhibitors involved in the renin-angiotensin system. First, atrial tissue angiotensin II concentration and gene expression of different players of the local renin-angiotensin system were not modified by SAR, suggesting a mode of action different to simple modulation of local renin-angiotensin system. Furthermore, unlike established targets in the cardiac renin-angiotensin and kinin system like angiotensin converting enzyme, CathA is only weakly expressed in the vasculature and has an acidic pH optimum of activity.¹¹ Besides, angiotensin I and bradykinin have been described as substrates for CathA in vitro.¹¹ CathA-inhibition by SAR resulted in a pronounced decrease in bradykinin-degrading activity in LA-homogenates which may well explain the observed atrial protective effects of CathA-inhibition in ZDF-rats. Bradykinin elicits positive inotropic effects in isolated LA-preparation of the guinea pig heart, which could explain the attenuation of LA-emptying function in SAR-treated ZDF.⁴² Also, increase of bradykinin has been shown to attenuate cardiac hypertrophy and fibrosis.⁴³ Thus, reduction of bradykinin turnover might well explain the beneficial effects of the CathA inhibitor.

Limitations

We investigated the effect of SAR in ZDF-rats at one specific time point at the end of the study. The exact time course of the beneficial effects of SAR treatment thus remains to be studied. We did not investigate whether the compound tested is able to reverse preexisting structural and functional atrial remodeling. Further intensive studies are necessary to identify the full pathway involved in the beneficial effect of SAR in diabetic conditions.

Conclusion

CathA gene expression and activity in LA were increased in old ZDF rats. CathA-inhibition attenuated increased myocyte-myocyte-distances and lateralization of Cx43. CathA-inhibition decreased bradykinin-degrading activity in LA-homogenates and attenuated elevated LA-gene expression and plasma level of profibrotic and proinflammatory marker OPN which might explain the protective effect of SAR. This was associated with reduced susceptibility to AF and improved LA-emptying function parameters. The atrial protective effect of CathA-inhibition was neither associated with changes in markers of the renin-angiotensin system, blood pressure, ventricular or renal function nor with changes in LA-dimensions. These findings provide evidence for CathA playing a role for cardiac and especially atrial remodeling in T2D and emphasize the potential use of CathA-inhibitors for atrial upstream therapy in patients with T2D.

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Chapter 3

Negative Tracheal Pressure During Obstructive Respiratory Events Promotes Atrial Fibrillation by Vagal Activation.

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Abstract

Introduction: Obstructive sleep apnea (OSA) causes negative tracheal pressure (NTP) and is associated with atrial fibrillation (AF). This study aimed to determine the mechanism of atrial electrophysiological changes during tracheal occlusion with or without applied NTP and to evaluate the role of vagal activation, Na⁺/H⁺-exchanger (NHE) and ATP-dependent potassium channels (K_{ATP}).

Methods: 17 closed-chest pigs were anesthetized with urethane and an endotracheal tube was placed to apply NTP (up to -100 mbar) comparable to clinically observed OSA in patients by a negative pressure device for a time period of 2 minutes. Right atrial refractory periods (AERP) and AF-inducibility were measured transvenously by a monophasic action potential recording and stimulation catheter.

Results: All tracheal occlusions with and without applied NTP resulted in comparable increases in blood pressure and hypoxemia. NTP shortened AERP (157.0±2.8 ms to 102.1±6.2 ms, p<0.0001) and enhanced AF-inducibility during AERP-measurements from 0% at baseline to 90% (p<0.00001) during NTP. Release of NTP resulted in a prompt restoration of sinus rhythm and AERP returned to normal. NTP-induced AERP-shortening and AF-inducibility were prevented by atropine or vagotomy. Neither the NHE-blocker cariporide nor the K_{ATP}-channel-blocker glibenclamide abolished NTP-induced AERP-shortening. By contrast, tracheal occlusion without applied NTP caused comparable changes in blood gases but did not induce AERP-shortening or AF-inducibility.

Conclusions: NTP during obstructive events is a strong trigger for AF compared to changes in blood gases alone. NTP caused AERP-shortening and increased susceptibility to AF mainly by enhanced vagal activation. AERP-shortening was not prevented by K_{ATP}-channel-blockade or NHE-blockade.

Introduction

Patients with obstructive sleep apnea (OSA) show a high prevalence of atrial fibrillation (AF) ranging from 32 to 49% ¹⁻³. OSA was the strongest predictor of recurrent AF following catheter ablation ⁴ and patients with untreated OSA have a higher risk of recurrence of AF after successful cardioversion ⁵. The finding that appropriate treatment of OSA with continuous positive airway pressure (CPAP) is associated with lower recurrence of AF suggests a causal association of OSA inducing AF ⁶. Several mechanisms causing AF in OSA have been discussed including hypoxemia and acidosis. These changes in blood gases might influence the parasympathetic and sympathetic tone leading to a substrate for AF. While enhanced vagal tone is known to induce AERP-shortening, increased sympathetic tone and autonomic dysbalance may enhance spontaneous triggered activity that could induce AF ⁷. Severe bradycardia and atrioventricular block are frequently seen in OSA and suggest vagal activation ⁸. However, other mediators beyond hypoxemia might be relevant in humans. Obstructive apneas are caused by collapse of the upper airway during sleep resulting in repetitive forced inspiration against the obstructed upper airway. Correspondingly substantial negative changes in intratracheal pressure down to -100 mbar were observed in OSA patients ^{3,9,10}. The resulting intrathoracic pressure changes might stretch heart chambers and lead to changes in transmural pressure gradients particularly in the thin walled atria. The influence of negative tracheal pressure (NTP) on atrial electrophysiology has not been investigated, yet. We hypothesized that the NTP during obstructive events and not changes in blood gases is responsible for increased AF-susceptibility in OSA. Therefore, we investigated electrophysiological changes in the pig right atrium during airway obstructions with or without NTP generated by a negative pressure device. Atropine followed by vagotomy was used to study the role of vagal activation.

Additionally, we characterized the role of hypoxia and stretch activated Na^+/H^+ -exchanger subtype 1 (NHE) and ATP-dependent potassium channel (K_{ATP})¹¹⁻¹³ by cariporide and glibenclamide.

Methods

All animal studies were performed in accordance to the German law for the protection of animals. Furthermore, the investigation conforms with the guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental model for OSA

In 17 chest-closed male castrated pigs (25–30 kg) of the German Landrace (anesthetized with 20% urethane (0.8 ml/kg i.v. load, 0.4 ml/kg/h maintainance) and 4% alpha-chloralose (0.4 ml/kg i.v. load, 0.1 ml/kg/h maintainance)), a tracheotomy was performed to place an endotracheal tube. This tube was used for tracheal occlusion and to apply different levels of negative tracheal pressures by a negative pressure device. The negative pressure device consisted of a negative pressure container (50 L) and a vacuum pump controlled by a manometer. The tracheal tube was connected to the vacuum container and a solenoid valve was opened and the tracheal tube and the vacuum container created a closed system. In pilot experiments, spontaneous breathing attempts during tracheal occlusion without NTP just created fluctuating tracheal pressures down to -40 mbar without causing electrophysiological changes. The Mueller maneuver (forced inspiration against airway obstruction) is used in the clinical setting to simulate conditions, particularly negative thoracic pressure, during obstructive sleep apnea¹⁴. As a modification of

this maneuver, we applied NTP at -100 mbar during tracheal occlusion corresponding to NTPs found in patients with OSA^{3,9,10}.

Right atrial pressure changes were investigated by a catheter which was advanced via the femoral vein (Gould, P23 series pressure transducer, Hato Rey, Puerto Rico). Blood pressure was measured by a TIP-catheter (Millar PC 350; Millar Instruments, Houston, Texas, USA) in the femoral artery. Temperature was measured via a rectal probe and maintained around 37.0°C by a heating lamp. Blood gas analysis (pO₂, pCO₂, pH, O₂-Saturation) was performed before, at the end and 15 min after release of tracheal occlusion without or with applied NTP in 7 pigs. In all pigs of group 1, the cervical vagi were dissected and bilateral vagotomy was performed.

Experimental design

In figure 1, the electrophysiological protocol (Fig. 1 A) and the experimental design (Fig. 1 B) are shown. Atrial effective refractory period (AERP) measurements and monophasic action potential (MAP) recordings were performed and AF-inducibility was investigated during normal breathing and at every 30 sec during 2 minutes of either tracheal occlusion without applied NTP or 2 min of tracheal occlusion with applied NTP at -100 mbar. Recovery of the AERP was shown by AERP measurement at 5 and 15 min between the different tracheal occlusion maneuvers. After the first run, vehicle was given to all animals and a second complete run including AERP-measurement, MAP recordings and AF-inducibility during normal breathing and during tracheal occlusion with and without NTP was performed. After those two complete runs at baseline atropine was administered first followed by vagotomy and finally glibenclamide was given (group 1, n=6). In group 2 (n=5), glibenclamide was given first followed by atropine. Three pigs received cariporide followed by atropine (group 3). An additional run of the electrophysiological protocol

was performed in exactly the same manner 15 min after each drug or bilateral vagotomy. A group of 3 pigs served as time vehicle control (control group).

Figure 1: Flow chart of the electrophysiological protocol (EP-protocol): (A) A complete EP-protocol included AERP-measurement, MAP-recording and AF-inducibility during normal breathing and every 30 sec during 2 min of tracheal occlusion without and with applied NTP at -100mbar with a recovery period of 15min between the different NTP maneuvers. **(B)** The experimental design is shown.

Electrophysiological examinations

Atrial effective refractory period (AERP) measurements at a representative basic cycle length (BCL) of 300 ms were performed before, during, and after the tracheal occlusion as described previously ¹⁵. Briefly, atrial responses to the pacing procedure were visualized by monophasic action potential (MAP) recordings from the endocardium of the right atrium by a MAP pacing catheter (combined MAP- & stimulation catheter, 7F, Foehr Medical Instruments GMBH, Seeheim, Germany). The catheter was inserted via a femoral vein. The tip of the catheter was advanced to the lateral right atrium to record a stable and sharp MAP-signal. This catheter was left at one location to ensure AERP determination at a fixed anatomic site throughout the experiment. A conditioning train of 5 basic stimuli (S1) at twice-diastolic pacing threshold was followed by an interpolated extrastimulus (S2, pulse duration 1 ms) starting about 30 ms below the expected AERP with a 5 ms increment (UHS 20, universal heart stimulator; Biotronik, Berlin, Germany). The shortest coupling interval able to elicit a propagated atrial response was taken as the AERP.

For analysis of right atrial MAP-signals, MAP electrode and position of the electrode were the same as used for AERP measurements (see above). A particular effort had to be made to obtain atrial MAPs of sufficient quality. MAP-signals were just used for analysis, if a regular baseline and amplitude of the MAPs could be maintained for at least 30 sec and if the shape of the MAP was stable during NTP-procedures. Right atrial MAP duration was evaluated from 70% repolarisation during regular pacing (BCL=300ms).

During the AERP-measurement procedure the shortest premature S2-extrastimulus resulting in a propagated response frequently induced episodes of AF. When atrial

MAP-signals showed an irregular rapid activation (cycle length < 200 ms, duration >5 seconds), AF was diagnosed.

Each NTP-maneuver was repeated twice in all animals. The mean percentage of NTP-maneuvers triggering AF was used to describe right atrial vulnerability. After vagotomy, AF-inducibility was additionally tested by 5 times 1 second burst pacing (BCL: 50ms).

Drugs

Glibenclamide (3mg/kg, synthesized at Sanofi-Aventis) was dissolved in 0.5 ml DMSO and 2.5 ml of polyethyleneglycol (PEG) 400 (Riedel-de Haen, Seelze, Germany). Cariporide (3mg/kg, synthesized at Sanofi-Aventis) was dissolved in saline. All solutions were injected over 5 minutes. Vehicle as a control was injected 15 min before the second baseline NTP-run in all animals.

Statistics

Data are presented as mean $\pm$ SEM. For comparisons of single repeated measures only, a paired Student's t-test was used. For multiple repeated measures comparisons with the same baseline, repeated-measures ANOVA was used, followed by Dunnet's test to compare individual mean differences if ANOVA was significant. A p value of 0.05 was considered significant.

Results

Effect of NTP on hemodynamic parameters and blood gases

We investigated hemodynamic changes (Fig. 2 A, upper panel) and changes in blood gases (Fig. 2 A, lower panel) during tracheal occlusion with (left) and without (right) applied NTP. During 2 min of tracheal occlusion with applied NTP at -100 mbar, blood pressure did not change significantly and right atrial pressure decreased to -16.5 ± 2.5 mmHg vs. 4.3 ± 0.3 mmHg at baseline ($p < 0.001$). By contrast, atrial pressure did not change significantly during tracheal occlusion without applied NTP. Release of tracheal occlusion resulted in a comparable increase in blood pressure after tracheal occlusion with or without applied NTP. Blood gas analysis during tracheal occlusion revealed significant increases in $p\text{CO}_2$, whereas $p\text{O}_2$ (Fig. 2 A, lower panel), SO_2 (Fig. 2 B) and pH (Fig. 2 C) were decreased. Changes in blood gases did not differ significantly between tracheal occlusion with or without applied NTP. Moderate bradycardia developed during tracheal occlusion with applied NTP (51.4 ± 4.1 bpm vs. 79.6 ± 2.6 bpm before NTP, $p < 0.0001$) but not during tracheal occlusion without applied NTP (Fig. 2 D). Release of tracheal occlusion resulted in a comparable increase in heart rate after tracheal occlusion with or without applied NTP (149.9 ± 17.4 bpm and 150.8 ± 12.3 bpm, respectively, n.s., data not shown). Ten minutes after release of tracheal occlusion, blood gases and hemodynamic parameters returned to normal in all animals.

Figure 2: Effect of 2 minutes of tracheal occlusion with (left) and without (right) applied negative tracheal pressure at -100 mbar (NTP, continuous line) on hemodynamics and blood gases (dotted line): changes in systolic (Syst.) and diastolic (Dias.) blood pressure and atrial pressure (at the top) and pO₂ and pCO₂ (at the bottom) are shown (A). Below, changes in SO₂ (B), pH (C) and heart rate (HR) (D) are shown. (* p<0.01, ** p<0.001, * p<0.0001 vs. before tracheal occlusion)**

Effect of NTP on AERP, MAP-duration and AF-inducibility

Figure 3 shows an individual example of the effect of tracheal occlusion with (left) and without (right) applied NTP on AERP (Fig. 3 A) and MAP-duration (Fig. 3 B). Two minutes of tracheal occlusion with -100 mbar NTP significantly shortened the AERP from 161.2 ± 2.6 ms to 96.3 ± 9.0 ms ($p < 0.0001$) and shortened the MAP at 70% of repolarisation from 168.9 ± 2.1 ms to 102.2 ± 7.2 ms ($p < 0.0001$, representative MAPs are shown in Fig. 3). By contrast, two minutes of tracheal occlusion without applied NTP had no significant effect on AERP (155.5 ± 1.8 ms vs. 160.0 ± 2.2 ms before occlusion, n.s.) or MAP-duration. Progressive NTP-induced AERP- and MAP-shortening was associated with increased inducibility of AF by premature stimuli during the AERP-measurement from 0% during normal breathing or during tracheal occlusion without applied NTP to 91% during tracheal occlusion with applied NTP ($p < 0.00001$) (Fig. 3 C). Release of tracheal occlusion resulted in a prompt restoration of sinus rhythm within 2.4 ± 1.1 min and NTP-induced electrophysiological changes were completely reversible in all animals. AF was not inducible by premature stimuli during normal breathing or tracheal occlusion without applied NTP. Additionally, in 3 control animals (data not shown), 4 repetitive full electrophysiological protocols including tracheal occlusions with and without applied NTP were conducted over a time frame similar to those of the treated animals. NTP-induced changes in electrophysiology and recovery at each repetition over time were constant.

Figure 3: Changes in atrial electrophysiology during 2 min of tracheal occlusion with (left) or without (right) applied negative tracheal pressure (NTP) at -100 mbar. An individual example of changes in AERP is shown in figure 3 **(A)** and corresponding original recordings of monophasic action potentials (MAP) at baseline and at 2 minutes of tracheal occlusion are shown in figure 3 **(B)**. In figure 3 **(C)**, an individual example of corresponding MAP signals during representative AERP-measurements at the end of 2 minutes of tracheal occlusion is shown. Applied NTP at -100 mbar resulted in a progressive shortening of AERP and MAP and AF was inducible by a premature beat during the S1-S2 AERP measurement procedure.

Effect of vagal blockade, NHE-blockade and K_{ATP}-channel-blockade

An individual example of the effect of vagal blockade and K_{ATP}-channel-blockade on AERP during tracheal occlusion with applied NTP is shown in figure 4. The effect of vagal blockade, K_{ATP}-channel-blockade and NHE-blockade on NTP-induced AF-inducibility and AERP is summarized in figure 5 and table 1.

Table 1: Atrial Effective Refractory Period (AERP, ms) During Tracheal Occlusion With or Without Applied Negative Tracheal Pressure (NTP, -100 mbar). Effect of Vagal Blockade, Glibenclamide and Cariporide.

		Tracheal Occlusion With NTP at - 100mbar		Tracheal Occlusion Without Applied NTP		
		Before Occlusion	2 min of Occlusion	Before occlusion	2min of Occlusion	
Group 1						
	Baseline	161.2±2.6	96.3±9.0 *	160.0±2.2	155.5±1.8	n=6
	+ Atropine	153.7±6.7	152.0±6.7 ++	151.2±3.5	145.3±4.6	n=6
	+ Vagotomy	158.7±5.8	156.2±5.71 ++	148.7±0.9	150.3±4.6	n=6
	+ Glibenclamide	152.9±5.6	180.6±5.2 +‡	158.7±3.0	146.2±6.9	n=6
Group 2						
	Baseline	151.4±4.3	109.8±6.6 *	151.4±8.6	148.1±8.6	n=5
	+ Glibenclamide	142.8±6.6	103.0±8.9 ‡	146.0±10.7	142.0±9.1	n=5
	+ Atropine	153.7±6.1	179.4±7.4 +&	150.2±8.2	145.4±6.1	n=5
Group 3						
	Baseline	152.1±2.9	92.1±2.9 *	153.3±1.3	153.3±2.4	n=3
	+ Cariporide	153.7±1.7	83.7±1.7 ‡	150.3±1.7	153.9±1.9	n=3
	+ Atropine	145.3±1.6	143.7±3.3 +			n=3

Values are shown as mean ± SEM. Negative tracheal pressure, NTP. Atrial effective refractory period, AERP

*, p<0.0001 vs. AERP before tracheal occlusion

+, p<0.01 vs. AERP-shortening at NTP at baseline

++, p<0.001 vs. AERP-shortening at NTP at baseline

‡, p<0.01 vs. AERP after glibenclamide or cariporide

&, p<0.01 vs. AERP after atropine + vagotomy

Atropine (group 1, n=6) did not change the AERP significantly at normal breathing, but fully prevented the NTP-induced AERP-shortening (Fig. 4 A and B) and MAP-shortening at 70% of repolarisation (at -100 mbar NTP: 161.2 ± 3.8 ms vs. 168.9 ± 2.9 ms at baseline, n.s.). After atropine, AF was only inducible in 17% of all tracheal occlusion maneuvers with applied NTP (vs. 91% during tracheal occlusion with applied NTP before atropine, $p < 0.0001$) (Fig. 5). Bilateral vagotomy was performed in all atropine treated pigs and showed no significant additional effect on the AERP over atropine alone. Also, AF was not inducible, neither by premature beats (S2) nor by burst pacing.

Glibenclamide was given after (group 1, Fig. 4 A, B and C) and before vagal blockade (group 2; Fig. 4 D, E and F). Glibenclamide did not change the AERP at normal breathing in neither group. NTP-induced AERP-shortening, AF-duration (2.3 ± 0.8 min vs. 2.5 ± 1.3 min at baseline, n.s.) and AF-inducibility (Fig.5) were not inhibited by glibenclamide when given before vagal blockade in group 2. Glibenclamide given after vagal blockade further increased AERP (180.6 ± 5.2 ms vs. 152.9 ± 5.1 ms before NTP, $p < 0.01$) and MAP at 70% of repolarisation (176.2 ± 6.4 ms vs. 156.2 ± 3.1 ms before NTP, $p < 0.05$) during tracheal occlusions with applied NTP towards the end of the NTP procedure. A similar increase in AERP was seen during tracheal occlusion with applied NTP, when atropine was given after glibenclamide (179.4 ± 7.4 ms vs. 153.7 ± 6.1 ms before NTP, $p < 0.01$) so that the combinations of atropine and glibenclamide independently of the order of administration showed about the same prolongation of the AERP.

Cariporide (group 3, table 1) did not change the AERP at normal breathing and did not inhibit NTP-induced AERP-shortening, AF-duration (2.5 ± 1.8 min vs. 2.4 ± 1.0 min at baseline, n.s.) MAP-shortening at 70% of repolarisation (156.2 ± 2.5 ms vs. 82.4 ± 6.2 ms at -100 mbar of NTP, $p < 0.0001$) or AF-inducibility (Fig. 5). Again, vagal blockade after cariporide completely prevented NTP-induced AERP-shortening and AF-inducibility.

Figure 4: Effect of vagal blockade and glibenclamide on AERP during tracheal occlusion with applied NTP at -100 mbar. Representative time curves of a pig of group 1 (A-C) and a pig of group 2 (D-F) are shown. Effect of NTP (continuous line) on right AERP (dotted line) at baseline and after atropine followed by glibenclamide (group 1) or after glibenclamide followed by atropine (group 2).

Figure 5: Percent of tracheal occlusions with applied NTP at -100 mbar with inducible AF and effect of vagal blockade, glibenclamide and cariporide. Tracheal occlusion without applied NTP for 2 minutes did not cause AF-inducibility in the pig right atrium. (* $p < 0.001$ vs. respective baseline).

Discussion

We report that application of negative tracheal pressure (NTP) during tracheal occlusion but not tracheal occlusion without applied NTP reproducibly and reversibly shortened the AERP and atrial MAP-duration and strongly enhanced inducibility of AF by single premature beats. Electrophysiological changes were mediated by vagal activation since they were completely prevented by atropine or vagotomy. Activation of Na^+/H^+ -exchanger or K_{ATP} -channels did not contribute to enhanced AF-inducibility. These findings support a role of NTP during obstructive events as the trigger for OSA-associated AF-susceptibility.

Changes in blood gases, atrial stretch and blood pressure

Hypoxemia, hypercapnia and acidosis are invariably linked with tracheal occlusion as occurring in OSA^{9 10}. In our experiments, changes in blood gases appeared during all obstructive maneuvers and did not differ between tracheal occlusion with or without applied NTP. Isolated hypercapnia during modified continuous ventilation without tracheal obstruction and NTP under autonomic blockade resulted in AERP-prolongation and reduced AF-vulnerability in a sheep model¹⁶. In superfused rabbit atria hypoxia prolonged the AERP¹⁷. In line with these observations, increased AF-inducibility due to pronounced AERP- and MAP-shortening only appeared during tracheal occlusion with applied NTP and not during tracheal occlusion without applied NTP but with hypoxia, acidosis and hypercapnia. This indicates that AERP-shortening and AF-inducibility is caused by NTP and not by changes in blood gases alone in our model. Possibly two minutes of tracheal occlusion in our model were too short to cause the AERP-prolonging effect observed in previous studies with longer hypoxemic and hypercapnic episodes¹⁶. Ineffective inspiratory efforts against the occluded upper airway in OSA generate marked increases in negative tracheal

pressure, resulting in negative intrathoracic pressure. Tracheal occlusion with applied NTP at -100 mbar as observed in OSA patients ^{9,10} resulted in a right atrial pressure down to -16 mmHg suggesting increases in atrial transmural pressure gradients and atrial distension.

Our results show that NTP and not changes in blood gases alone is the mechanism for the observed strong increase in AF-inducibility in our model for OSA. However, AF-episodes were fairly short. Pronounced blood pressure surges due to increased sympathetic activity during the arousal together with changes in blood gases during longterm OSA might lead by a variety of mechanisms to structural atrial remodeling that favors development of persistent AF ^{3,9,10,18-20}.

NTP as a potent trigger for vagally induced atrial fibrillation in OSA

Vagal activation was identified as the major mechanism involved in NTP-induced AERP-shortening. Atropine had no significant effect on AERP at normal breathing or tracheal occlusion without applied NTP, but it prevented NTP-induced AERP-shortening in all animals tested. Vagotomy after atropine had no additional effect over atropine alone. After vagotomy, AF was not inducible anymore during NTP-maneuvers, neither by premature beats, nor by burst pacing. Our findings are in line with previous studies showing that the vagal tone is increased during obstructive events in patients with OSA ⁸. Interestingly, ablation of ganglionated plexi or combined autonomic blockade inhibited the appearance of apnea induced AF during a 2 min apneic interval in dogs ²¹. This dog-model is used as a model for central apnea or Cheyne-Stokes respiration. Our pig model with applied NTP mimics conditions known from obstructive sleep apnea. Our results suggest that the NTP during inspiration against the obstructed airway in OSA is the trigger for vagally induced AF in OSA while changes in blood gases alone during simple apnea in

central sleep apnea just play a minor role for acute electrophysiological changes in the atrium. However our studies do not disclose how NTP-maneuvers enhance vagal activity. NTP could stimulate vagal afferent nerves in the lung, the heart and the atria. With respect to atrial afferent vagal activity so-called B-receptors are present in the atria acting as volume receptors⁸. An increase in the transmural pressure gradient during NTP might give an inadequate signal of volume overload. Activation of B-receptors is known to inhibit sympathetic activity and to result in vagal activation.

Hypoxia and/or stretch activated NHE-transport and K_{ATP}-channels

In order to study mechanisms beyond vagal activation, we characterized the role of the hypoxia and/or stretch activated Na⁺/H⁺-exchanger and K_{ATP}-channels in NTP-induced AERP-shortening. We investigated the effect of cariporide, a blocker of the cardiac NHE¹³, and of glibenclamide, a blocker of K_{ATP}-channels¹¹. Blockade of the cardiac NHE subtype 1 by cariporide did not prevent NTP-induced AERP-shortening in our study. Activation of the NHE occurs in ventricular myocardium during hypoxia and ischemia to regulate intracellular pH. It is known to be proarrhythmic possibly by changes in intracellular calcium¹². NHE is an important mechanism underlying short-term but not long-term atrial electrophysiological remodeling from rapid atrial rates and from atrial ischemia²². Our data argues against a role for NHE subtype 1 for NTP-induced AERP-shortening. The role of K_{ATP}-channels seems to be more complex as suggested by the differential effects of glibenclamide, a blocker of K_{ATP}-channels. Glibenclamide given after vagal blockade further prolonged the AERP in the course of the NTP procedure indicating activation of K_{ATP}-channels. K_{ATP}-channels may be opened by hypoxia and opening may be enhanced by increased transmural pressure gradients and stretch in the atria during the NTP-procedure

since these channels were reported to be stretch-sensitive ¹¹. By contrast, glibenclamide given before atropine did not prevent NTP-induced AERP-shortening although one would assume that K_{ATP} -channels were opened by hypoxia and/or stretch, too. The absence of an obvious effect by glibenclamide when given before atropine is in line with the observation that glibenclamide did not prevent tachypacing-induced AERP-shortening in the atrium ²³. The differential effect of glibenclamide could be explained by previous findings showing that glibenclamide can favor the release of ACh in the atrium ²⁴. This means that a facilitation of ACh release by glibenclamide would result in a more pronounced AERP-shortening which could have neutralized an AERP-prolonging effect by blockade of K_{ATP} -channels in atrial cardiomyocytes. Atropine would completely eliminate such a vagal enhancing effect and an AERP-prolonging component of K_{ATP} -channel-blockade in atrial cardiomyocytes could be unmasked. In line with this assumption, the combination of atropine and glibenclamide resulted in almost the same AERP-prolongation independent of the order of administration. Additionally, tracheal occlusion without applied NTP after atropine plus glibenclamide did not cause AERP-prolongation.

Limitations

There are some limitations but also strengths of our study. Experimental conditions chosen to induce AERP-shortening were strong with regard to the duration of the challenge and the extent of NTP. Spontaneous breathing of our anesthetized pigs against the occluded trachea developed tracheal pressures of -40 mbar only possibly due to anesthesia, young age, weight and species. We applied NTPs comparable to those reported for subjects with OSA ^{9,10}. The NTP had to be applied continuously for technical reasons. The tracheal pressure changes caused by the breathing attempts of the animal during tracheal occlusion with applied NTP ranged

between -110 and -90 mbar. We applied a premature beat to induce AF. While AERP shortening would strongly facilitate reentry, a trigger is still needed to induce AF. In some animals, we observed that AF was induced by spontaneous atrial premature beats, but this spontaneous induction of AF was too rare for a systematic evaluation. A further limitation is that our investigations were made at a single fixed anatomical position in the right atrium although the left atrium may be more relevant for the induction of AF. We did not look at inhomogeneity in conduction, conduction velocity or spatial distribution of refractoriness in the atrium. Investigations in the left atrium and investigations of conduction inhomogeneities would require thoracotomy, which is not possible with urethane anesthesia. Thoracotomy would result in a more artificial situation which would not reflect OSA. Although we tried a lot, our pig-model did not allow to control blood gases content during NTP maneuvers.

Conclusions

In the present study we found that application of strong NTP during tracheal occlusion shortened AERP and MAP-duration and increased AF-vulnerability in a pig model for OSA. These electrophysiological changes were clearly mediated by vagal activation. By contrast, comparable changes in blood gases during tracheal occlusion without applied NTP did not have such an electrophysiological effect. The results strongly support the hypothesis that NTP during inspiration against the obstructed airway and not changes in blood gases alone is the most relevant mechanism for vagally induced AF-susceptibility in OSA. Additionally, the blockade of NHE-transport or K_{ATP} -channels did not attenuate NTP-induced AERP-shortening, but K_{ATP} -channels are activated upon NTP. Our pig model may be appropriate for the development of new treatment strategies to treat therapy resistant AF in OSA and can be used as a new model for vagal-AF.

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Chapter 4

Combined Blockade of Early And Late Activated Atrial Potassium Currents Suppresses Atrial Fibrillation in a Pig Model of Obstructive Apnea.

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Abstract

Background: Negative tracheal pressure (NTP) during tracheal obstruction in obstructive apnea increases vagal tone and causes pronounced shortening of the atrial effective refractory period (AERP) thereby perpetuating atrial fibrillation (AF). The role of different atrial potassium channels under those conditions has not been investigated. This study aimed to evaluate the atrial effects of blockade of the late activated potassium current (I_{Kr}) by sotalol, of blockade of the early activated potassium currents (I_{Kur}/I_{to}) by AVE0118 and of the multichannel blocker amiodarone during tracheal occlusions with applied NTP.

Methods: 21 pigs were anesthetized and an endotracheal tube was placed to apply NTP (up to -100 mbar) comparable to clinically observed obstructive sleep apnea for 2 minutes. Right AERP and AF-inducibility were measured transvenously by a monophasic action potential recording and stimulation catheter.

Results: Tracheal occlusion with applied NTP caused pronounced AERP-shortening. AF was inducible during all NTP-maneuvers. Neither blockade of I_{Kr} by sotalol, nor blockade of I_{Kur}/I_{to} by AVE0118 nor amiodarone affected NTP-induced AERP-shortening although they prolonged the AERP during normal breathing. Atropine given after amiodarone completely inhibited NTP-induced AERP-shortening. The combined blockade of I_{Kr} and I_{Kur}/I_{to} by sotalol plus AVE0118, however, attenuated NTP-induced AERP-shortening and AF-inducibility independent of the order of administration.

Conclusions: The atrial proarrhythmic effect of NTP simulating obstructive apneas is difficult to inhibit by class III antiarrhythmic drugs. Neither amiodarone nor blockade of I_{Kr} or I_{Kur}/I_{to} attenuated NTP-induced AERP-shortening. However, the combined blockade of I_{Kur}/I_{to} and I_{Kr} suppressed NTP-induced AERP-shortening.

Introduction

Patients with atrial fibrillation (AF) show a high prevalence of obstructive sleep apnea (OSA), ranging from 32 to 49%¹⁻³. Patients with untreated OSA have a higher recurrence of AF after cardioversion and after pulmonary vein antrum isolation⁴⁻⁷. Previously, we described atrial electrophysiological changes in a pig model for obstructive apneas⁸. Increased susceptibility to AF was caused by a pronounced shortening of the atrial effective refractory period (AERP) and monophasic action potentials (MAP) in the pig right atrium as a response to applied negative tracheal pressure (NTP). Negative tracheal pressure occurs in patients with OSA due to diaphragm contraction during upper airway obstruction and results in a negative intrathoracic pressure and increased transmural pressure gradients⁹⁻¹¹. Vagal activity was identified as the major mechanism involved in NTP-induced AERP-shortening in this model, since NTP-induced AERP-shortening, NTP-induced MAP-shortening and AF-inducibility were completely prevented by atropine. Acetylcholine dependent potassium channels are discussed to be one of the most relevant components by which vagal tone induces AERP-shortening in the atrium¹². The aim of this study was to investigate the effect of the blockade of different potassium channels on NTP-induced AERP-shortening, NTP-induced MAP-shortening and AF-inducibility during obstructive events. We tested the effect of the blockade of the early activated potassium currents I_{Kur} (ultrarapid delayed rectifier potassium current) and I_{to} (transient outward current) by AVE0118¹³ compared with the blockade of the late activated potassium currents I_{Kr} (the rapid component of delayed rectifier potassium current) by sotalol¹⁴ on AERP, MAP and AF-inducibility during normal breathing and during tracheal obstruction with applied NTP. Since AERP-shortening induced by NTP is vagally mediated involving acetylcholine sensitive potassium channels (K_{ACh})

we tested the antiarrhythmic effects of the multichannel blocker amiodarone, which has been shown to block K_{ACh} -channels in therapeutical concentrations¹⁵.

Methods

All animal studies conform with the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

21 closed chest male castrated pigs (25–30 kg) of the German Landrace were anesthetized with 20% urethane (0.8 ml/kg i.v. load, 0.4 ml/kg/h maintainance) and 4% alpha-chloralose (0.4 ml/kg i.v. load, 0.1 ml/kg/h maintainance. The pigs were instrumented as described previously ⁸. Briefly, a tracheotomy was performed to place an endotracheal tube. This tube was used for tracheal occlusion and to apply different levels of negative tracheal pressures by a negative pressure device. The Mueller maneuver, forced inspiration against airway obstruction, is used in the clinical setting to simulate conditions, particularly negative thoracic pressure, occurring during obstructive sleep apnea. As a modification of this maneuver, we applied NTPs at -100mbar.

Blood pressure was monitored by a catheter with a pressure transducer at the tip (Millar PC 350; Millar Instruments, Houston, Texas, USA) in the femoral artery. Blood gas analysis (pO_2 , pCO_2 , pH, O_2 -saturation) was performed before and at the end of each NTP-maneuver. Blood gases at baseline and during tracheal occlusion with applied NTP were not influenced by the different drugs (data not shown). The cervical vagi were dissected and bilateral vagotomy was performed in some of the pigs.

Electrophysiological measurements

Atrial effective refractory period (AERP) at a basic cycle length of 300ms was measured before, during, and after the NTP-maneuvers as described previously¹⁶. Briefly, atrial responses to the pacing procedure were visualized by monophasic action potentials (MAP) from the endocardium of the right atrium via a MAP pacing catheter (combined MAP- & stimulation catheter, 7F, Föhr Medical Instruments GMBH, Seeheim, Germany). The catheter was inserted via a femoral vein. The tip of the catheter was advanced to the lateral right atrium to record a stable and sharp MAP-signal. This catheter was left at one location to ensure AERP determination at a fixed anatomic site throughout the experiment. A train of 5 basic stimuli (S1, pulse duration 1 ms) at twice diastolic pacing threshold was followed by an extrastimulus (S2) starting about 30 ms below the expected AERP with a 5-ms increment (UHS 20, universal heart stimulator; Biotronik, Berlin, Germany). The shortest coupling interval able to elicit a propagated atrial response minus 3 ms was taken as the AERP. The AERP-measurement frequently induced episodes of AF during NTP-maneuvers (AF-inducibility). Additionally, inducible AF-duration was determined.

For analysis of right atrial MAP-signals, MAP electrode and position of the electrode were the same as used for AERP measurements (see above). A particular effort had to be made to obtain atrial MAPs of sufficient quality. MAP-signals were just used for analysis, if a regular baseline and amplitude of the MAPs could be maintained for at least 30 sec and if the shape of the MAP was stable during NTP-procedures. Right atrial MAP duration (MAP) was evaluated from 70% repolarization during regular pacing (BCL=300ms).

Drugs

Amiodarone (3mg/kg ("low dose") and 5 mg/kg ("high dose"), synthesized at Sanofi-Aventis, Frankfurt, Germany), AVE0118 (3mg/kg, synthesized at Sanofi-Aventis, Frankfurt, Germany), atropine (0.02 mg/kg) and sotalol (1 mg/kg, d,l-sotalol synthesized by Bristol-Myers Squibb, New York, US) were dissolved in 0.5 ml DMSO and 2.5 ml of polyethyleneglycol (PEG) 400 (Riedel-de Haen, Seelze, Germany). All solutions were injected over 5 minutes. NTP-runs were performed 10 minutes after the end of the infusion. As a control, vehicle as a control was injected 15 min before the second baseline NTP-run in all animals.

Study design and timing sequence

The study design is shown in figure 1. A complete negative tracheal pressure (NTP) run included atrial effective refractory period (AERP) measurements at baseline and at every 30 sec during a 2 min period of either simple tracheal occlusion at the end of expiration or 2 min of NTP of -70 or -100mbar and at 5 and 15 min during the recovery period between the different NTP-maneuvers of 15 min. Recovery of the AERP was shown by AERP measurement in the recovery period between the NTP-maneuvers. After the first run, vehicle was given to all animals and a second complete run was performed. After those two complete runs at baseline the different drugs were administered. In group 1 (n=5), low dose amiodarone (3mg/kg) was followed by atropine. In group 2 (n=4), high dose amiodarone (5mg/g) was followed by bilateral vagotomy. In group 3 (n=6), sotalol was given first followed by AVE0118. In group 4 (n=6), AVE0118 was given first followed by sotalol. Recovery of the AERP and the maintenance of the AERP-prolonging effect of the first drug during normal breathing were shown by an additional AERP measurement in the recovery period between the NTP-maneuvers.

Figure. 1: Flow chart of the electrophysiological protocol (EP-protocol): (A) A complete EP-protocol included AERP-measurement, MAP-recording and AF-inducibility during normal breathing and every 30 sec during 2 min of tracheal occlusion with applied NTP at -100mbar with a recovery period of 15min between the different NTP maneuvers. **(B)** The experimental design is shown.

Statistic analysis

Data are presented as mean±SEM. For comparisons of single repeated measures only, a paired Student's t-test was used. For multiple comparisons with the same baseline, repeated-measures ANOVA was used, followed by Dunnet's test to compare individual mean differences if ANOVA was significant. A p value of <5% was considered significant.

Results

Electrophysiological effects of amiodarone, AVE0118, and sotalol during normal breathing

Figure 2 shows the separate and combined effects of low dose amiodarone and atropine (A), of high dose amiodarone and vagotomy (B) and of AVE0118 and sotalol (C and D) on the atrial refractory period during normal breathing. Low dose amiodarone, high dose amiodarone, AVE0118 and sotalol alone increased the AERP by 7.2 ± 3.2 ms, 11.1 ± 2.1 ms, 10.8 ± 1.7 ms and 25.8 ± 4.2 ms, respectively. The AERP-prolonging effect of amiodarone, AVE0118 and sotalol during normal breathing at baseline and at the end of recovery period was unchanged within 30 minutes. The combination of AVE0118 and sotalol prolonged the AERP by 45.8 ± 3.3 ms ($p<0.01$ vs. the sum of separate AERP-prolongation by sotalol and AVE0118) indicating that the class III effects of AVE0118 and sotalol are synergistic in the pig atrium. AVE0118 (3 mg/kg) did not prolong the QTc interval, (QTc was 413 ± 15 ms before and 403 ± 02 ms 10 min after injection). Sotalol (1mg/kg) showed the expected prolongation of the QTc-interval (25.1% increase; from 426 ± 5 ms to 533 ± 12 ms; $p<0.001$), amiodarone did not induce a significant increase in QTc (411 ± 8 ms before and 420 ± 5 , n.s.) in pigs.

Figure 2: Effect of low dose amiodarone followed by atropine **(A)**, high dose amiodarone followed by bilateral vagotomy **(B)**, AVE0118 followed by sotalol **(C)** and sotalol followed by AVE0118 **(D)** on AERP during normal breathing (* $p < 0.01$ vs. respective baseline, + $p < 0.01$ vs. after AVE0118 (C), and vs. after sotalol (D)).

Effect of amiodarone, AVE0118 and sotalol on NTP-induced AERP- and MAP-shortening

Tracheal occlusion without applied NTP did not change the AERP, neither before nor after drug administration. In contrast, the application of NTP induced a pronounced AERP-shortening (from 147.8 ± 7.9 ms to 105.1 ± 10.6 ms, $p < 0.001$) and MAP-shortening at 70% of repolarization (from 145.2 ± 3.8 ms vs. 101.9 ± 2.9 ms, $p < 0.001$). In 3 control animals, 4 repetitive full electrophysiological protocols including NTP-manuevers were conducted over a time frame similar to those of the treated animals. NTP-induced changes in electrophysiology and recovery at each repetition over time were constant⁸.

In figure 3, the effect of different class III-AADs and their combination on NTP-induced AERP-shortening is shown. Neither low dose nor high dose amiodarone attenuated NTP-induced AERP-shortening. However, both, atropine and bilateral vagotomy applied after the administration of amiodarone completely inhibited NTP-induced AERP-shortening (155.0 ± 4.2 ms to 152.0 ± 9.8 ms after atropine vs. 168.1 ± 5.1 ms to 94.6 ± 10.5 ms after low dose amiodarone, $p < 0.01$) and NTP-induced MAP-shortening at 70% of repolarization (159.2 ± 3.2 ms to 152.3 ± 4.9 ms after atropine vs. 165.3 ± 2.7 ms to 97.6 ± 6.9 ms after low dose amiodarone, $p < 0.01$). The $I_{K_{ur}}$ -blocker AVE0118 and the I_{K_r} -blocker sotalol alone failed to inhibit NTP-induced AERP-shortening (160.2 ± 9.5 ms to 112.2 ± 9.1 ms vs. 147.8 ± 7.9 ms to 105.1 ± 10.6 ms before AVE0118 (n.s.) and 185.3 ± 7.8 ms to 131.2 ± 19.3 ms vs. 155.3 ± 4.2 ms to 92.8 ± 7.0 ms before sotalol (n.s.), respectively) and NTP-induced MAP-shortening (166.6 ± 7.5 ms to 107.1 ± 8.9 ms vs. 146.7 ± 6.5 ms to 98.5 ± 9.8 ms before AVE0118 (n.s.) and 182.5 ± 6.5 ms to 127.1 ± 17.6 ms vs. 152.2 ± 3.7 ms to 97.7 ± 6.0 ms before sotalol (n.s.), respectively). In contrast the combination of AVE0118 and sotalol

significantly reduced NTP-induced AERP-shortening (204.5 ± 5.3 ms to 174.5 ± 8.3 ms after the combination of sotalol and AVE0118, $p < 0.001$ vs. respective NTP-induced AERP-shortening at baseline before drug administration) and NTP-induced MAP-shortening (199.8 ± 6.3 ms to 180.4 ± 6.9 ms after the combination of sotalol and AVE0118, $p < 0.001$ vs. respective NTP-induced MAP-shortening at baseline before drug administration) independent of the order of administration.

Figure 3: Effect of low dose amiodarone followed by atropine **(A)**, high dose amiodarone followed by bilateral vagotomy **(B)**, AVE0118 followed by sotalol **(C)** and sotalol followed by AVE0118 **(D)** on AERP during tracheal occlusion with applied NTP at -100 mbar (* $p < 0.001$ vs. respective baseline, + $p < 0.01$ vs. NTP-induced AERP-shortening before drug administration).

Figure 4 shows changes in AERP (left) during tracheal occlusion without (A) and with (B) applied NTP and MAP-recordings (right) during the respective AERP-measurements. The combination of sotalol and AVE0118 (C) suppressed NTP-induced AERP-shortening and reduced AF-inducibility.

Figure 4: Changes in atrial electrophysiology during 2 min of tracheal occlusion without (A) or with applied negative tracheal pressure (NTP) at -100 mbar before (B) and after sotalol plus AVE0118 (C). A respective individual example of changes in AERP is shown (left) and corresponding MAP signals during representative AERP-measurements at the end of 2 minutes of tracheal occlusion is shown (right).

Effect of amiodarone, AVE0118 and sotalol on NTP-Induced AF-Inducibility and AF-duration

AF was not inducible by a single premature stimulus during normal breathing. NTP-induced AERP-shortening was associated with increased inducibility of AF by a single premature stimulus during the AERP-measurement from 0% during normal breathing to 87.7% during tracheal occlusion with applied NTP ($p<0.00001$). In figure 5, the effect of different class III-AADs and their combination on NTP-induced AF-susceptibility is shown. Neither low dose nor high dose amiodarone suppressed NTP-induced AF-inducibility significantly. Atropine applied after low dose amiodarone administration and bilateral vagotomy performed after high dose amiodarone completely inhibited NTP-induced AF-inducibility. AVE0118 and sotalol alone did not reduce NTP-induced AF-inducibility. In contrast the combination of sotalol and AVE0118 significantly reduced AF-inducibility during NTP-maneuvers (15% vs. 87.7% at baseline before drug administration, $p<0.001$).

Inducible AF-duration was just mildly reduced by amiodarone (89 ± 8 s vs. 122 ± 11 s at baseline, n.s.), sotalol (75 ± 10 s vs. 119 ± 10 s at baseline, $p<0.05$) and AVE0118 (59 ± 8 vs. 130 ± 12 s at baseline, $p<0.01$). However, the combination of sotalol plus AVE0118 (15 ± 8 s vs. 125 ± 11 s at baseline, $p<0.01$) caused a pronounced reduction in inducible AF-duration.

Figure 5: Percent of tracheal occlusions with applied NTP at -100 mbar with inducible AF and effect of low dose amiodarone followed by atropine (group 1), high dose amiodarone followed by bilateral vagotomy (group 2), AVE0118 followed by sotalol (group 3) and sotalol followed by AVE0118 (group 4). Tracheal occlusion without applied NTP for 2 minutes did not cause AF-inducibility in the pig right atrium. (* $p < 0.001$ vs. respective baseline)

Discussion

In a pig model simulating the effect of obstructive apneas on the atrium we showed that the combined blockade of the early activated potassium currents I_{Kur}/I_{to} (AVE0118) and the late activated potassium current I_{Kr} (sotalol) could attenuate NTP-induced AERP-shortening, NTP-induced MAP-shortening and AF-inducibility. By contrast, the I_{Kur}/I_{to} - or the I_{Kr} -blocker alone was not effective. Surprisingly, amiodarone, which blocks multiple potassium currents including the acetylcholine dependent potassium current I_{KACH} , did not inhibit NTP-induced AERP-shortening or MAP-shortening although increased vagal tone was clearly identified to mediate electrophysiological changes.

Pig model with applied negative tracheal pressure (NTP) simulating obstructive apneas

Previously we have shown that AF-inducibility in the right atrium was highly increased by application of NTP in a pig-model simulating obstructive apneas⁸. NTP maneuvers induced pronounced AERP-shortening, MAP-shortening and AF-inducibility. Release of NTP resulted in a prompt cardioversion and AERP-shortening and MAP-shortening were reversible. In contrast, hypoxia and hypercapnia alone during tracheal occlusion without applied NTP did not induce electrophysiological changes in the atrium. Increased vagal tone was identified to mediate electrophysiological changes in this model, as suggested by the fact, that AERP-shortening, MAP-shortening and enhancement of AF-inducibility were completely prevented by atropine. Acetylcholine stimulates muscarinic M-receptors and activates the I_{KACH} current in the atria, which is normally small or absent. Increased activity of inward I_{KACH} causes shortening of the atrial action potential and thus promotes AF¹⁵.

I_{KACH} -blockade

It has been reported that some class III antiarrhythmic drugs inhibit I_{KACH} -currents. Two mechanisms by which these antiarrhythmic drugs inhibit I_{KACH} have been proposed: drugs like sotalol¹⁸ block the muscarinic receptors and others like amiodarone^{19, 20} inhibit I_{KACH} by depressing the function of the I_{KACH} channel itself. Studies demonstrated that amiodarone effectively suppressed the shortening of atrial effective refractory period and the wavelength for reentry during vagal stimulation, thereby terminating and preventing cholinergic atrial fibrillation¹⁵. Both sotalol and amiodarone are multichannel blocking antiarrhythmic drugs. Blockade of the I_{KACH} -current is considered to be responsible for the superior efficacy of amiodarone compared to other more selective I_{Kr} -blockers like e.g. dofetilide. Additionally, also AVE0118 blocks the I_{KACH} at higher dosages¹³. Amiodarone, sotalol and AVE0118 did not sufficiently inhibit NTP-induced AERP-shortening or MAP-shortening. However, atropine administration and bilateral vagotomy after amiodarone were effective in inhibiting AERP-shortening indicating that AERP-shortening was still mediated by vagal activation. The major component of I_{KACH} arises from the muscarinic receptor (mAChR) activation. Recent evidence suggests that other mAChR subtypes are present in the heart and that these couple to distinct potassium currents.²¹⁻²³ Whether amiodarone blocks all of those channels is unknown. Possibly, amiodarone does not block I_{KACH} channels and sotalol does not block the muscarinic receptors by 100% in the concentrations used in our study. Incomplete I_{KACH} -blockade or muscarinic receptor blockade during pronounced increase in ACh-release during NTP-maneuvers might fail to attenuate the AERP-shortening. We tested the effect of a lower and a higher dosage of amiodarone¹⁵. AERP was prolonged by both dosages of amiodarone during normal breathing, but AERP-shortening could not be prevented. In pilot experiments, higher dosages of amiodarone (up to 10 mg/kg) failed to inhibit NTP-induced AERP-shortening, caused

prolongation of QRS-duration possibly due to an increased sodium-channel blocking effect and resulted in relevant hemodynamic changes (data not shown). Therefore we did not systematically investigate higher dosages of amiodarone in our study.

Additionally it is important to consider the duration of increased vagal tone in our model. The atria of our pigs were exposed to an increased vagal tone for only two minutes. In previous studies, vagal tone was increased by a long term infusion of carbachol or acetylcholine or by chronic stimulation of vagal nerve. Long term vagal stimulation might create a substrate which differs significantly from the acute vagal stimulation by NTP. Another explanation may be the stretch sensitivity of K_{ACh} -channels. Increased transmural pressure gradients during NTP-maneuvers result in atrial stretch. Atrial stretch has been shown to modulate $I_{K_{ACh}}$ -channel activity probably via a direct mechanical effect on the channel protein ²⁴. This modulation of channel protein structure might influence the effective binding of amiodarone.

I_{Kur}/I_{to} -blocker and I_{Kr} -blocker

During normal breathing, the AERP-prolonging effect of I_{Kur}/I_{to} -blockers was lower compared to I_{Kr} -blockers. This is in line with previous findings that the AERP-prolonging effects of I_{Kur} -blockers are lower in the right pig atrium compared to the left pig atrium. The opposite effect profile applies to I_{Kr} -blockers ¹⁷.

The most obvious atrial electrophysiological change during NTP-maneuvers was the AERP-shortening and MAP-shortening caused by a pronounced vagal activation and thereby an enhanced ACh-dependent potassium current ⁸. The strong action potential shortening may depress the plateau of the action potential ¹². The relevance of different potassium currents for the repolarization of the action potential is directly dependent on the AP-duration. During shorter atrial action potentials, the relative importance of the early activating potassium currents such as the I_{to} and the I_{Kur} for

repolarization is increased and the late activated potassium currents such as I_{Kr} are relegated from an active participation to a passive role (repolarization reserve)²⁵. In AF-induced electrical remodeling, the efficacy of I_{Kr} -blockade by sotalol is diminished^{26,27} and blockade of I_{Kur} by AVE0118 exerted an enhanced class III effect²⁸⁻³⁰. However, NTP-induced AERP-shortening differs from AF-induced AERP-shortening (electrical remodeling). In AF-induced electrical remodeling, vagal activation seems to play a minor role, as atropine failed to suppress the atrial tachypacing induced AERP-shortening in the goat³¹. During NTP-induced AERP-shortening caused by an increased vagal activation not only the late activated potassium channels I_{Kr} but also the early activated potassium currents I_{to} and I_{Kur} may lose relevance for repolarization due to a strong activation of the I_{KACH} . Indeed, the blockade of I_{Kur}/I_{to} by AVE0118 failed to attenuate NTP induced AERP shortening in our study. This finding is in line with a study by Pandit et al. who demonstrated in vagal-AF in pigs and in a mathematic model that I_{Kur} is an ineffective anti-arrhythmic drug target in vagal AF.³² Interestingly, in our experiments the combination of a blocker of the early activated potassium currents I_{Kur}/I_{to} (AVE0118) plus a blocker of the late activated potassium current I_{Kr} (sotalol) significantly reduced NTP-induced AERP-shortening and NTP-induced MAP-shortening independent of the order of administration. Consequently the premature beats within the AERP-measurements only induced very short episodes of atrial tachycardia. During NTP-induced AERP-shortening caused by a strong activation of I_{KACH} , the I_{Kur}/I_{to} and I_{KACH} blocker AVE0118 might prolong the early repolarisation and thereby the plateau of the action potential. This restores a condition of repolarisation under which the late activated potassium current I_{Kr} regains relevance for repolarisation. A synergistic effect of I_{Kur} and I_{Kr} -blockers has already been shown in electrical remodelled goat atria²⁹ where the combination of AVE0118 and sotalol showed a larger effect than the sum of their separate effects.

Besides its pronounced class III effect, sotalol also shows some beta-adrenergic blocking effect. However, atenolol and propranolol failed to inhibit NTP-induced AERP-shortening in pilot experiments indicating that sympathetic activation does not seem to play a relevant role in this pig model simulating obstructive events.

Limitations

We applied a premature beat to induce AF. In some animals, we observed that AF was induced by spontaneous atrial premature beats, but this spontaneous induction of AF was too rare for a systematic evaluation. A further limitation is that our investigations were made at a single fixed anatomical position in the right atrium. AERP-measurement in the left atrium and the investigation of inhomogeneity in conduction, conduction velocity or spatial distribution of refractoriness in the atrium would have required thoracotomy. In pigs with thoracotomy application of NTP would not be possible. Additionally, we just investigated the acute effect of amiodarone in our model. The chronic administration of amiodarone might have shown a different effect. We tested the dosages as described elsewhere^{13, 16, 17}. Due to limitations in solubility of AVE0118 and pronounced QT-prolongation by sotalol we did not further increase the doses tested in this study.

Conclusion

In a pig model of obstructive apneas we showed that the combination of a blocker of the early activated potassium current I_{Kur}/I_{to} (AVE0118) and a blocker of the late activated potassium current I_{Kr} (sotalol) could reduce NTP-induced AERP-shortening and MAP-shortening, AF-inducibility and AF-duration. By contrast, the I_{Kr} - or I_{Kur}/I_{to} -blocker alone was not effective. Surprisingly, amiodarone, a known blocker of the

acetylcholine dependent potassium channel did not prevent NTP-induced AERP-shortening, although increased vagal tone was clearly identified to mediate electrophysiological changes in this model. Our findings suggest that the atrial proarrhythmic effects of obstructive apneas are difficult to inhibit by current antiarrhythmic drugs and that the combined blockade of the early (I_{Kur}/I_{to}) and the late activated potassium current (I_{Kr}) may be a promising strategy to treat AF in patients with OSA. Our data could at least partly explain how AF patients with OSA could become refractory to current class III antiarrhythmic drugs and may therefore be susceptible for progression to permanent AF.

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Chapter 5

Renal Sympathetic Denervation Suppresses Postapneic Blood Pressure Rises And Atrial Fibrillation in a Model For Sleep Apnea.

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Abstract

Background: The aim of this study was to identify the relative impact of adrenergic and cholinergic activity on atrial fibrillation (AF)-inducibility and blood pressure in a model for obstructive sleep apnea (OSA). OSA is associated with sympathovagal dysbalance, AF and postapneic blood pressure rises. Renal denervation (RDN) reduces renal efferent and possibly also afferent sympathetic activity and blood pressure in resistant hypertension.

Methods: The effects of RDN compared to beta-blockade by atenolol on atrial electrophysiological changes, AF-inducibility and blood pressure during obstructive events and on shortening of atrial effective refractory period (AERP) induced by high-frequency stimulation of ganglionated plexi were investigated in 20 anesthetized pigs.

Results: Tracheal occlusion with applied negative tracheal pressure (NTP, at -80 mbar) induced pronounced AERP-shortening and increased AF-inducibility in all pigs. RDN but not atenolol reduced NTP-induced AF-inducibility (20% vs. 100% at baseline, $p=0.0001$) and attenuated NTP-induced AERP-shortening more than atenolol (27 ± 5 ms vs. 43 ± 3 ms after atenolol, $p=0.0272$). Administration of atropine after RDN or atenolol completely inhibited NTP-induced AERP-shortening. AERP-shortening induced by high-frequency stimulation of ganglionated plexi was not influenced by RDN, suggesting that changes in sensitivity of ganglionated plexi do not play a role in the antiarrhythmic effect of RDN. Postapneic blood pressure rise was inhibited by RDN and not modified by atenolol.

Conclusions: We showed that vagally mediated NTP-induced AERP-shortening is modulated by RDN or atenolol, which emphasizes the importance of autonomic dysbalance in OSA associated AF. Renal denervation displays antiarrhythmic effects by reducing NTP-induced AERP-shortening and inhibits postapneic blood pressure rises associated with obstructive events.

Introduction

Patients with obstructive sleep apnea (OSA) show a high prevalence of atrial fibrillation (AF) ¹⁻³ and OSA is considered an etiologic factor in the development of hypertension ⁴ and in the progress of resistant hypertension ⁵. Severe bradycardia and atrioventricular conduction disturbances together with post-apneic blood pressure rises during the arousal are frequently seen in OSA and suggest sympathovagal activation ^{6,7}. While enhanced vagal tone is known to induce shortening of the atrial effective refractory period (AERP), increased sympathetic tone may increase spontaneous triggered activity both of which, when simultaneously occurring, could induce and maintain AF ⁸. Additionally, repetitive postapneic blood pressure surges may lead to atrial structural changes and thus an arrhythmogenic substrate for AF. Previously, we showed that negative tracheal pressure (NTP) during obstructive respiratory events leads to pronounced shortening of the AERP thereby perpetuating AF ⁹. These electrophysiological changes were mainly mediated by increased vagal tone since they were completely inhibited by atropine or bilateral vagotomy. However, less is known about the relative impact of adrenergic and cholinergic activity on AF inducibility and maintenance in OSA.

We hypothesized that modulation of the sympathetic nervous system might reduce AF-susceptibility and postapneic blood pressure rises in OSA. Renal denervation is a new therapeutic approach to reduce sympathetic activity, blood pressure and apnea–hypopnea index (OSA severity) in resistant hypertension ¹⁰⁻¹³. However, the effect of RDN on AF-inducibility and blood pressure during and after obstructive events is unknown. We tested the effect of denervation of the afferent and efferent renal sympathetic nerves previously shown to reduce renal and whole body sympathetic

activity¹⁰⁻¹², and compared it to beta-receptor blockade by atenolol on atrial electrophysiological changes and postapneic blood pressure rises in a pig model for OSA. To check whether RDN displays its antiarrhythmic effects by a modulation of sensitivity of the intrinsic cardiac nervous system, we investigated the influence of RDN on sensitivity of right atria to stimulation of autonomic ganglionated plexi.

Methods

All animal studies were performed in accordance with the guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental model for OSA

In 15 chest-closed male castrated pigs (25–30 kg) of the German Landrace (anesthetized with 20% urethane (0.8 ml/kg i.v. load, 0.4 ml/kg/h maintenance) and 4% alpha-chloralose (0.4 ml/kg i.v. load, 0.1 ml/kg/h maintenance), a tracheotomy was performed to place an endotracheal tube. This tube was used for tracheal occlusion and to apply different levels of negative tracheal pressures (NTP) by a negative pressure device^{9,14}. The Mueller maneuver (forced inspiration against airway obstruction after deep expiration generating a negative pressure of about 40 mmHg) is used in the clinical setting to simulate conditions, particularly negative thoracic pressure, during obstructive sleep apnea¹⁵. As a modification of this maneuver, we applied NTP at -80 mbar during tracheal occlusion corresponding to NTPs found in patients with OSA^{6,7,15,16}.

Blood pressure was measured by a TIP-catheter (Millar PC 350; Millar Instruments, Houston, Texas, USA) in the femoral artery. Bipolar body surface ECG was recorded using subcutaneous needle electrodes in the classical lead II arrangement. The ECGs were analyzed concerning heart rate and conduction times according to the AHA/ACCF/HRS Recommendations for the Standardization and Interpretation of the Electrocardiogram before and after RDN or atenolol. The QT interval was corrected using the Bazett formula ($QT_c = QT / \sqrt{RR}$). Blood gas analyses (pO_2 , pCO_2 , pH, O_2 -saturation) were performed directly before and at 2 minutes of each tracheal occlusion with applied NTP. In seven animals, both kidneys were approached through bilateral retroperitoneal flank incisions. Both kidneys were surgically denervated by cutting all visible nerves in the area of the renal hilus and by stripping approximately 1 cm of the adventitia from the renal artery. The area was then moistened with a 20% phenol/ethanol solution for 10-15 min. Left renal flow was measured with a doppler flow probe (transit time flowmeter module system from Transonic Systems Inc.; Germany) positioned on the blood vessels. Following RDN, the animals were allowed to re-equilibrate for 1.5 hours. Significant reduction of the reproducible post-apneic BP-rise and the absence (<5% change) of a decrease in renal blood flow induced by tracheal occlusion with applied NTP were taken as evidence of the completeness of RDN. Sham

surgical procedure with kidney exposition without renal denervation was performed in three additional pigs serving as a time control. Results for vehicle-time controls for atenolol and atropin have been published in ^{9,14}. Vehicle application did not change NTP-induced electrophysiological parameters or hemodynamics within 4 hours.

Five additional pigs (35-41 kg) were anesthetized with pentobarbital ¹⁷ and a right thoracotomy at the 4th intercostal space provided access to the right lung and atria through a pericardiotomy. At the caudal end of the sinus node between the right

superior and inferior pulmonary vein, a plaque electrode containing 2 electrodes was sutured on the fat pad (anterior right ganglionated plexi (GP)). For electrical GP-stimulation, the following stimulation parameters were used: frequency: 20 Hz, stimulus duration: 0.1 ms, voltage: 0.1-5 volt. GP-stimulation, which slowed heart rate by approximately 50% at baseline, was applied before and 1.5 h after bilateral RDN.

Electrophysiological examinations

During normal breathing, atrial effective refractory period (AERP) was measured at a basic cycle length (BCL) of 400, 300 and 240 ms before and after RDN or atenolol. Additionally, AERP measurements at a representative basic cycle length (BCL) of 300 ms and AF-inducibility were performed before, during, and after tracheal occlusion. An extended description of electrophysiological examinations is available as an online Data Supplement (please see <http://hyper.ahajournals.org>).

Drugs

Atenolol (3mg/kg) and atropine was dissolved in 0.5 ml dimethyl sulfoxide (DMSO) and 2.5 ml of polyethyleneglycol (PEG) 400. All solutions were injected over 5 minutes. Vehicle as a control was injected 15 min before the second baseline NTP-run in all animals of group 1. Atenolol was used in a concentration which resulted in a maximal heart rate reduction and AERP-prolonging effect in previous studies^{9,14}.

Experimental design

The electrophysiological study protocol (EP) and the experimental design are depicted in figure 1. AERP-measurements were performed and AF-inducibility was investigated during normal breathing and at every 30 seconds during 2 minutes of tracheal occlusion with applied NTP at -80 mbar. Recovery of the AERP was shown by AERP-measurement at 15 min between the different tracheal occlusion

maneuvers. After the first run, both kidneys were approached through bilateral retroperitoneal flank incisions in group 1 and vehicle was given to all animals of group 2. Bilateral RDN was performed in group 1 and atenolol was given to all animals of group 2. Two additional runs of the electrophysiological study protocol were performed in exactly the same manner 15 min and 1.5 hours after RDN and 15 min after atenolol. In group 3, AERP-shortening induced by high frequency stimulation of GPs was investigated at baseline and 1.5 hours after RDN. Sham surgical procedure was performed in three additional pigs serving as a time control.

Statistics

Data are presented as mean $\pm$ SEM. For comparisons of single repeated measures only, a paired Student's t-test was used. For multiple repeated measures comparisons with the same baseline, repeated-measures two-way ANOVA was used, followed by Dunnet's test to compare individual mean differences if ANOVA was significant. A p value below 0.05 was considered significant. For all statistical calculations software Everstat V5 based on SAS 8 was used.

Figure 1: Experimental Design Flow Chart: (A) A complete electrophysiological study protocol (EP-study, EP) included atrial effective refractory period (AERP)- and atrial fibrillation (AF)-inducibility-measurement during normal breathing and every 30 seconds during 2 minutes of tracheal occlusion with applied negative tracheal pressure (NTP) at -80 mbar. **(B)** Experimental design. Group 1: Effect of bilateral renal denervation (RDN) on NTP-induced AERP-shortening and AF-inducibility. Group 2: Effect of atenolol on NTP-induced AERP-shortening and AF-inducibility. Group 3: Effect of bilateral RDN on AERP-shortening induced by high frequently stimulation of ganglionated plexi (GP).

Results

Electrophysiological effects of RDN and atenolol during normal breathing

Figure 2 shows the effects of bilateral RDN (A) and atenolol (B) on AERP during normal breathing. Atenolol increased the AERP by 27.2 ± 3.2 ms at 300 ms BCL ($p=0.0001$). RDN did not influence AERP significantly during normal breathing. During normal breathing, PQ-interval was prolonged and heart rate was reduced by RDN and atenolol. P-wave duration, QRS-duration and cQT-time were not modified by RDN or atenolol. (figure S1, please see the online Data Supplement).

Figure 2: Changes in Atrial Effective Refractory Period During Normal Breathing: Effect of renal denervation (RDN) (**A**) and atenolol (3mg/kg) (**B**) on atrial effective refractory period (AERP) at different basic cycle lengths (BCL: 400 ms, 300 ms and 240 ms) (filled symbols: baseline; white symbols: after treatment).

Effect of RDN and atenolol on NTP-induced AERP-shortening

Tracheal occlusion without applied NTP did not change the AERP, neither before nor after RDN or atenolol. In contrast, the application of NTP during tracheal occlusion induced a pronounced AERP-shortening (from 167.8 ± 7.9 ms to 105.2 ± 10.6 ms, $p=0.0001$). In 3 control animals, repetitive full electrophysiological study protocols including NTP-maneuvers and sham operation were conducted over the time frame similar to those of the treated animals. NTP-induced changes in electrophysiology and recovery at each repetition over time were constant. In figure 3, the effect of RDN and atenolol followed by atropine on NTP-induced AERP-shortening during tracheal occlusion is shown.

Figure 3: Changes in Atrial Effective Refractory Period During Tracheal Occlusion: Changes in atrial effective refractory period (AERP) (dotted line, left ordinate) during tracheal occlusion with applied negative tracheal pressure (NTP) at -80 mbar for 120 seconds (continuous line, right ordinate) before **(A)** and after renal denervation (RDN) or atenolol **(B)** followed by atropine **(C)**. Representative time curves of a pig of group 1 (RDN followed by atropine, white squares) and a pig of group 2 (atenolol followed by atropine, black diamonds) are shown.

RDN reduced NTP-induced AERP-shortening (AERP-shortening: 27 ± 5 ms after RDN vs. 43 ± 3 ms after atenolol, $p=0.0272$) (figure 4 A) and NTP-induced MAP-shortening at 70% of repolarisation (MAP-shortening: 22 ± 2.1 ms after RDN vs. 47 ± 7.2 ms after atenolol, $p=0.0024$) (figure 4 B) more than atenolol. However, atropine applied after the administration of atenolol or RDN completely inhibited NTP-induced AERP-shortening and MAP-shortening.

Figure 4: Electrophysiological Changes During Tracheal Occlusion: (A) Effect of renal denervation (RDN) and atenolol followed by atropine on changes in atrial effective refractory period (AERP) induced by tracheal occlusion with applied negative tracheal pressure (NTP) at -80 mbar (ordinate). (B) Representative atrial multiple action potential (MAP) recordings during AERP-measurements before and after RDN or atenolol. (C) Percent of tracheal occlusions with inducible atrial fibrillation (AF) (ordinate) and effect of RDN and atenolol followed by atropine.

Effect of RDN and atenolol on NTP-induced AF-inducibility

AF was not inducible by a single premature stimulus during the AERP-measurement procedure during normal breathing. NTP-induced AERP-shortening and MAP-shortening were associated with increased inducibility of AF by a single premature stimulus during the AERP-measurement from 0% during normal breathing to 100% during tracheal occlusion with applied NTP ($p=0.0001$). In figure 4 C, the effect of RDN and atenolol on NTP-induced AF-inducibility is shown. RDN inhibited NTP-induced AF-inducibility. Atenolol did not reduce NTP-induced AF-inducibility significantly but inducible AF-duration was shortened (27 s vs. 122 s at baseline, $p=0.0037$). After administration of atropine following RDN or atenolol no episode of AF was inducible.

Effect of RDN on sensitivity of right atria to stimulation of autonomic ganglionated plexi

RDN did not influence AERP-shortening induced by GP-stimulation as a result of programmed electrical stimulation (AERP-shortening: 51 ± 4 ms vs. 56 ± 5 ms after RDN, $p=0.2915$).

Effect of RDN and atenolol on postapneic blood pressure rises and blood gases

Postapneic changes in blood pressure (BP) after bilateral RDN or atenolol are shown in figure 5. RDN abolished post-apneic BP surge. Suppression of the post-apneic BP rise was not observed after atenolol. NTP-induced changes in blood gases were not modified by RDN or atenolol (table S1, please see the online Data Supplement).

Figure 5: Postapneic Blood Pressure Rise: Original registration of postapneic blood pressure changes in a pig before (control) and 1.5 hours after renal denervation (RDN) **(A)**. Postapneic blood pressure changes (ordinate) before (control) **(B)** and 1.5 hours after RDN **(C)** and 15 minutes after atenolol **(D)**.

Discussion

In this study we showed that postapneic blood pressure rises were abolished by RDN but not by atenolol. Arrhythmogenic electrophysiological changes during tracheal occlusion with applied NTP, mainly characterized by a pronounced shortening of AERP, can be modified by RDN and atenolol. However, AF-inducibility was inhibited by RDN only and not by atenolol. AERP-shortening induced by high-frequency stimulation of ganglionated plexi (GP) was not modified by RDN.

Several observations suggest that the autonomic nervous system plays an important role in both the initiation and the maintenance of AF in humans. Vagal nerve stimulation and acetylcholine infusion shortens the AERP⁸. By contrast, activation of sympathetic activity showed variable effects on AERP^{8,18,19}. Interestingly, studies in lone AF patients and in animal models of both intermittent rapid atrial pacing and congestive heart failure have indicated that AF onset is associated with simultaneous sympathovagal activation rather than with an increase in vagal or sympathetic drive alone²⁰⁻²³. Previously we have shown increased AF-susceptibility and AF-duration caused by pronounced AERP-shortening in a pig model for OSA^{9,14}. Negative thoracic pressure (NTP) during breathing attempts against the obstructed upper airways was identified to be a trigger for AERP-shortening. AERP-shortening was mediated by pronounced vagal activation since it was completely inhibited by atropine and bilateral vagotomy. However, increased negative thoracic pressure, hypoxia and hypercapnia during obstructive apnea are associated with severe bradycardia and atrioventricular block together with post-apneic blood pressure rises suggesting simultaneous sympathovagal activation^{6,7}. Direct recordings of muscle sympathetic nerve activity showed increased sympathetic activation during apneic

episodes in OSA patients ¹⁶ or in apnea divers ²⁴. Sympathetic activity may increase profoundly during the application of negative tracheal pressure resulting in the pronounced postapneic blood pressure rise.

The sympathetic nervous system can be modulated by blockade of beta-receptors and RDN in humans ¹⁰⁻¹². NTP-induced AERP- and MAP-shortening were significantly reduced by RDN and atenolol. This indicates that sympathetic modulation affects NTP-associated electrophysiological changes. Atropine after RDN or atenolol completely inhibited NTP-induced AERP- and MAP-shortening, showing that vagal activation beyond sympathetic activation is one significant determinant of NTP-induced shortening of atrial refractoriness.

The suppression of vagally mediated AERP- and MAP-shortening during obstructive events by RDN or atenolol indicates an interaction between the acetylcholine (ACh)-induced AERP-shortening and the reduced activation of the sympathetic nervous system. Consistently, Sharifov et al. ²⁵ showed that ACh-mediated AF was facilitated by isoproterenol, which decreased the threshold of ACh concentration for AF induction and increased AF duration. Patterson et al. ²⁶ showed that the synergistic action of both the sympathetic and the parasympathetic neurotransmitter are required to initiate rapid ectopic discharges in superfused pulmonary vein preparations. Indeed, we observed that AF was induced by spontaneous atrial premature beats during tracheal occlusion in some animals, which may represent the occurrence of ectopic discharges triggering AF. However, this spontaneous induction of AF was too rare for a systematic evaluation.

RDN but not atenolol reduced NTP-induced AF-inducibility. Catheter-based denervation of the kidneys by ablation of the efferent sympathetic and afferent somatic fibers has been shown to be effective in reducing both renal and whole-body norepinephrine spillover and muscle sympathetic nerve activity ¹⁰⁻¹². Altering the signals from the kidney to the hypothalamus is expected to impact down-stream organ systems including the vasculature and the heart ²⁷. Catheter-based RDN in patients with resistant hypertension effectively reduced blood pressure ¹⁰⁻¹², improved insulin resistance ²⁸ and sleep apnea severity ¹³. Interestingly, NTP-induced post-apneic blood pressure surges were attenuated by RDN suggesting a sympatho-inhibitory effect. However, similar effects have not been reported for beta-blocker treatment clearly indicating a different mechanism of action. Possibly, RDN might additionally modify alpha- adrenoceptors pathways which are not influenced by atenolol. Interestingly, activation of the intrinsic cardiac nervous system results in atrial arrhythmias that involve intrinsic cardiac neuronal alpha-adrenoceptors ²⁹.

RDN resulted in a more pronounced inhibition of NTP-induced AERP-shortening compared to atenolol, which might explain the superior antiarrhythmic effect of RDN compared to atenolol. Changes in blood gases during tracheal occlusion or p-wave duration as a marker of atrial conduction were neither modified by RDN nor by atenolol suggesting an antiarrhythmic effect independent of changes in blood gases or atrial conduction. RDN and atenolol prolonged PQ-interval and reduced heart rate which may represent the modulation of sympathetic activity. First supportive data for potential antiarrhythmic effect of acute RDN has recently been published by Ukena et al. ³⁰. They reported reduced ventricular tachyarrhythmias in patients with electrical storm and congestive heart failure after catheter-based RDN. However, data from clinical studies powered to detect these effects are lacking.

Importantly, long-term OSA has been shown to be associated with significant atrial remodeling characterized by atrial enlargement, reduction in voltage, site-specific and widespread conduction abnormalities, and longer sinus node recovery in humans ³¹. Apart the described antiarrhythmic electrophysiological effects of RDN, RDN might be able to attenuate the development of an atrial proarrhythmic substrate by inhibiting postapneic blood pressure surges as shown in this study. Additionally, RDN has been proven to reduce OSA severity in OSA-patients ¹³.

We tested, whether parts of the antiarrhythmic effects of RDN can be attributed to a modulation of the sensitivity of the intracardiac autonomic nervous system containing ganglionated plexi (GPs). GPs may modulate the interactions between extrinsic and intrinsic cardiac autonomic nervous system ³² and contain efferent cholinergic and adrenergic neurons influencing the atrial myocardium. GP-ablation combined with a pulmonary vein ablation procedure has been shown to further improve success rates of reversion in paroxysmal AF-patients ³³. In a dog model for central sleep apnea GP-ablation inhibited AF-inducibility ³⁴. In this study, AERP-shortening induced by high-frequency stimulation of GPs was not modified by RDN. This suggests that changes in sensitivity of GPs do not play a role in the antiarrhythmic effect of RDN.

Limitations

Spontaneous induction of AF was too rare for a systematic evaluation. Therefore we applied a premature beat during tracheal occlusion with applied NTP to induce AF. AERP-measurement in the left atrium and the investigation of the spatial distribution of refractoriness, of inhomogeneity in conduction and conduction velocity in the

atrium would have required thoracotomy. In pigs with thoracotomy application of NTP would not be possible. Additionally, catheter based renal nerve ablation in humans is possibly not complete, as indicated by a norepinephrine spillover reduction by only about 50%¹⁰. We performed complete surgical renal nerve ablation in this study. The precise measurement of sympathetic activity and GP-activation during and after obstructive events deserves further studies.

Conclusions and Perspectives

RDN reduced postapneic blood pressure rises, which may attenuate OSA-associated structural atrial arrhythmogenic remodeling. Additionally, vagally mediated NTP-induced AERP- and MAP-shortening is modulated by RDN or atenolol, which emphasizes the importance of autonomic dysbalance in OSA associated AF. RDN but not atenolol reduced AF-inducibility. RDN displays antiarrhythmic effects independent of a modulation of sensitivity of intracardiac ganglionated plexi, suggesting that ganglionated plexi do not play a relevant role in the antiarrhythmic effect of RDN. Modulation of the autonomic nervous system by RDN might be useful to reduce atrial arrhythmogenesis and deserves to be tested in clinical studies and in AF-models for structural and/or electrical atrial remodelling.

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Chapter 6

Zooming in on a Focus: High Density Mapping of the Interaction between Ectopy and Re-entry during Atrial Fibrillation

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Abstract

Background: The contribution of ectopic focal discharges to the perpetuation of atrial fibrillation (AF) is debated. The interaction of ectopic focal discharges with re-entry mechanisms during AF is unknown and criteria for the identification of ectopy during AF have not been developed.

Methods: In open chest experiments in goats (n=6), stationary ectopic focal discharges were elicited by topical application of aconitine (ACO) on the epicardial surface. AF was induced by burst stimulation of the atria. We performed high density endo-epicardial mapping to investigate the interaction of focal tachycardia induced by the topical epicardial application of ACO with activation pattern during AF.

Results: The topical application of ACO-crystals on the left atrium in the middle of the mapping area induced rapid focal discharges with radial spread of activation exactly at the place of ACO-application (ACO cycle length (CL): 242 ± 15 ms). At the site of earliest activation local electrograms did not show R-waves. At the same site, atrial effective refractory period (BCL=200ms: 135 ± 2 ms vs. 135 ± 4 ms, n.s.) was unchanged. During overdrive stimulation local conduction velocity (CV) was also not affected by the topical application of aconitine (80 ± 3 cm/s vs. 80 ± 3 cm/s, n.s.). However, episodes of burst-induced AF lasted longer than before aconitine application (470 ± 85 sec. vs. 26 ± 19 sec., $p < 0.01$) while AFCL was unchanged (133 ± 5 ms vs. 134 ± 5 ms, n.s.). During AF the rate of breakthrough was more than 10-fold higher (1.14 ± 0.14 per cycle vs. 0.08 ± 0.03 per cycle before aconitine, $p < 0.01$). However, breakthroughs occurred wide-spread throughout the mapping area, remote from the site of ACO-application. The mean of epicardial coupling intervals of breakthroughs and the mean of AFCL did not differ significantly (131 ± 3 ms vs. 131 ± 5 ms, n.s.). More than 80% of all local electrograms at the site of earliest activation of breakthroughs showed R-waves, but only 44% of them could be explained by

transmural conduction. Flecainide inhibited ACO-induced rapid focal discharges at the site of ACO-application and reduced inducibility of AF.

Conclusions: The existence of a single localized site with high propensity to ectopy causes a pronounced increase in AF stability and in the breakthrough rate during AF despite the fact that the cycle length of the ectopic tachycardia is far slower than the AFCL during AF. However, breakthroughs related to localized ectopy may not be stationary, but wide-spread, do not show prematurity in coupling interval, and in most cases do show R-waves in the electrograms. Thus, identification of ectopic focal discharges during AF remains a challenge.

Introduction

The mechanisms of initiation and perpetuation of atrial fibrillation (AF) are still unclear. A variety of re-entry mechanisms have been proposed to contribute to the perpetuation of AF^{1,2} including multiple wavelets, meandering or stationary re-entry circuits or rotors.³⁻⁷ While these models consider continuously conducted excitation as the source of local atrial activation, other experiments suggested that also ectopic discharges alone could contribute to AF perpetuation.^{8,9} Interestingly, epicardial mapping studies demonstrated that in patients with persistent AF the rate of activations showing radial spread of activation (breakthroughs) was 2–3 fold higher than in patients with acutely induced AF.^{2,10} However radial spread of activation from a localized site during AF does not allow direct conclusion regarding the mechanism of these activations. Breakthroughs might either be explained by transmural conduction of fibrillation waves or by ectopic focal discharges.^{2,11,12} Ectopic focal discharges might be caused by changes in atrial calcium-handling as observed AF patients and animal models for AF.¹³⁻¹⁵

Flecainide has been shown to open ryanodine receptors thereby reducing calcium-spark mass.¹⁶ Therefore, flecainide might be effective to suppress ectopic focal discharges during AF.

In this study, we used a model of focal AF suggested by Scherf et al..⁸ We applied aconitine on the left atrial epicardium in goats to induce stationary ectopic focal discharges. The objectives of this study were: 1. to study the complex interaction between localized focal discharges and fibrillation waves during AF, 2. to identify criteria for detection of ectopic focal discharges during AF, and 3. to study the effect of flecainide on AF maintained by ectopic focal discharges.

Methods

All experimental procedures were approved by the local ethical committee for animal experiments of Maastricht University.

Goat Model: Open chest experiments

In this study, 6 goats were anesthetized with sufentanyl (6 mg/kg/h), midazolam (0.8 mg/kg/h) and pancuronium (0.3 mg/kg/h). After left-sided thoracotomy, a high-density endo-epicardial mapping device was positioned in the left atrium as described previously.^{10,11} The device was carefully closed to make contact with the endocardial bundle network and the epicardial surface. When satisfactory signals were present (sharp deflections in the absence of injury currents) the distance between the endocardial and the epicardial part of the device was fixated with a screw (2.5 – 3.0 mm). A silver plate in the thoracic cavity served as indifferent electrode. Unipolar signals were recorded during AF and during atrial pacing using a custom-made 256-channel mapping amplifier (filtering bandwidth 0.1-400 Hz, sampling rate 1 kHz, A/D resolution 16 bits). A train of 10 basic stimuli (S1, pulse duration 1 ms, BCL 200 ms) at twice diastolic pacing threshold was followed by an extra-stimulus (S2) starting about 30 ms below the expected atrial effective refractory period (AERP) with a 2-ms increment. The shortest coupling interval able to elicit a propagated atrial response was taken as the AERP. AF was induced by atrial burst pacing.

After baseline measurements of refractory period, conduction velocity during atrial pacing and AF duration, aconitine (0.5 mg) was applied at the centre of the mapping area of the same animal that had previously received burst pacing for AF induction. Epicardial aconitine application in the middle of the mapping area caused a regular and stable atrial tachycardia (CL: 200-230 ms) after ~10min. 20-30min after aconitine application, AERP was determined in the middle of the mapping area and at two

opposing sites outside the mapping area. Thereafter, AF was induced by atrial burst pacing.

Analysis of fibrillation electrograms

AF-electrogram files of 4 seconds were analyzed. Local activation times were identified as points of maximum negative dV/dt in each unipolar electrogram. Median AF cycle length (AFCL) was calculated using all fibrillation intervals of all electrodes. Conduction times were determined by calculating the difference in activation time between each electrode and its neighbors.

Identification of individual fibrillation waves followed an algorithm described earlier.^{11,12} Waves were classified as:

- a) Peripheral waves if their earliest activation time point was at the border of the mapping area or
- b) Breakthrough if their earliest activation time point was within the mapping area.

The starting points of all breakthroughs were then tested for their plausibility to result from transmural conduction using a previously published algorithm.¹² For this purpose the closest contralateral activation time points within an area of 5x5 electrodes (8.0 x 8.0 mm) in the opposing layer of the atrial wall were determined. Breakthroughs were considered to be due to transmural conduction of existing fibrillation waves if the calculated conduction velocity between the contralateral source and the site of breakthrough was between 100-20 cm/s, questionable if it was between 20-5 cm/s and not explainable by transmural conduction if no preceding activity within this given time range was found.^{11,12} Additionally, the presence of a unipolar R-wave (>1 mV) at the breakthrough sites was determined. Electrograms with an unstable baseline or with large superimposed ventricular far-field complexes

were excluded. The degree of prematurity of “focal” fibrillation waves was determined by comparing their coupling interval with the average AF cycle length (AFCL) at the site of origin.¹⁷

Quantification of endo-epicardial dissociation

Electrical dissociation of between the epicardial layer and the endocardial bundle network was determined based on differences in activation time or direction of propagation of fibrillation waves between the two layers as described previously.^{11,12} The endo-epicardial dissociation was expressed as percentage of the number and percentage of the surface area of dissociated and non-dissociated fibrillation waves.

Flecainide experiments

Flecainide (0.2mg/kg/min, i.v.) was infused 60min after aconitine-application. Endpoints of the cardioversion experiments were cardioversion to sinus rhythm, ventricular arrhythmias, QRS prolongation of >100%. Measurements of AF-inducibility and AERP-measurement were repeated after cardioversion to sinus rhythm.

Statistical analysis

All data are expressed as mean±SEM. Significance of differences in means between baseline and after aconitine application was calculated using a one way ANOVA with Newman-Keuls post-test and a paired Student t-test for significance of differences in means between the endocardial and the epicardial layer in the same specimen. P values <0.05 were considered significant.

Results

Atrial tachycardia induced by epicardial application of aconitine

Approximately 10min after topical application of aconitine in the middle of the mapping area a regular and stable atrial tachycardia (CL: 200-230 ms) occurred (Fig. 1). The earliest atrial activation time of each atrial tachycardia was localized in the middle of the epicardial mapping area at the site of aconitine application. The tachycardias were characterized by homogeneous, radial spread of activation. Electrograms at the site of earliest activation showed S-wave morphology. The earliest atrial activation time in the endocardial mapping area was registered with a transmural time delay of 6.6 ± 0.2 ms. However, epicardial aconitine-application did not induce AF in our model.

Figure 1: Isochronal map of atrial electrical activation pattern from simultaneous endo-epicardial recordings of aconitine-induced atrial tachycardia (time between isochrones 5ms). Point of earliest activation is on the epicardial plane in the middle of the mapping area at the site of aconitine application followed by a homogenous radial spread of activation. The earliest atrial activation time in the endocardial mapping area was registered with a transmural time delay of 6.6 ms.

Effect of aconitine on refractory period and conduction velocity

The effect of aconitine on refractoriness and conduction velocity is summarized in figure 2. Atrial regular pacing (BCL 200ms) from 2 different directions did not reveal any changes in atrial local conduction velocity by aconitine (Fig. 2A). Epicardial aconitine application did not change AERP at a BCL of 200ms, neither at the site of aconitine application nor in its surrounding in the left atrium (Fig. 2B).

Figure 2: Effect of aconitine on the substrate for AF. (A) Atrial local conduction velocity (BCL: 200 ms) before and after aconitine. **(B)** AERP (BCL: 200 ms) at the site of aconitine application and in its surrounding in the left atrium.

AF before and after epicardial aconitine application

Figure 3 shows the influence of aconitine on fibrillatory process during AF. Burst-pacing induced AF-episodes after aconitine application were significantly longer compared to AF-episodes before aconitine application (Fig. 3A). AFCL was not changed by aconitine (Fig. 3B). The number and the surface area of fibrillation waves showing endo-epicardial dissociation was not altered significantly by aconitine (Fig. 3C). After aconitine application the rate of breakthroughs was 10-fold higher than before aconitine application (Fig. 3D).

Figure 3: Influence of aconitine on fibrillatory process during AF: (A) Inducible AF-duration, (B) AF-cycle length and (C) number and the surface of dissociated or non-dissociated waves and (D) the breakthrough rate/cycle before and after aconitine are shown.

Characterization of breakthroughs

While the site of earliest activation during the aconitine induced atrial tachycardia occurred exactly at the site of aconite application, after induction of AF the breakthroughs were spread around this site (mean distance from site of aconitine application: 4.7 ± 1.1 mm) (Fig. 4). The degree of prematurity breakthroughs was determined by comparing their coupling interval preceding the breakthroughs with the average AFCL at the site of origin. Epicardial coupling intervals preceding the breakthroughs at the site of earliest activation did not differ significantly from the average AFCL at this site (Fig. 5A). Additionally, a high percentage (82%) (Fig. 5B) of unipolar atrial electrograms at the site of earliest activation did show R-waves (>1 mV). Representative EGs are shown in 3C. However, based on the assumption of physiological endo-epicardial conduction velocities (between 100-20 cm/s) only 44% of all breakthroughs could be explained by transmural activation (breakthrough) from preceding contralateral waves. The remaining 56% could not be explained by transmural activation.

Figure 4: Local distribution of new appearing waves (breakthroughs,) during AF at baseline (at the top), during atrial tachycardia induced by aconitine application in the middle of the mapping area (second row) and during AF after epicardial aconitine-application (at the bottom) of all individual goats. Size of the stars ( ) represents the number of breakthroughs at one location.

Figure 5: (A) Epicardial coupling intervals of earliest activation at the site of breakthroughs compared to AFCL of the left atrium. (B) Percentage of unipolar atrial electrograms at the site of earliest activation with or without R-waves. (C) Representative electrograms at the site of earliest activation.

Effect of flecainide

Flecainide completely inhibited the focal atrial tachycardia induced by aconitine and prolonged atrial cycle length (Fig. 6A). Burst-pacing induced AF-episodes after aconitine application were shortened from 470 s to 22 s after flecainide (Fig. 6B). AERP was prolonged (Fig. 6C) and local conduction velocity during regular pacing (BCL 200 ms) was reduced (53 cm/s vs. 79 cm/s before flecainide, $p<0.01$) by flecainide.

Figure 6: Effect of flecainide:

- (A) Atrial-cycle length,
 - (B) inducible AF-duration and
 - (C) AERP (BCL: 200 ms)
- at baseline, after aconitine and after flecainide.

Discussion

Epicardial aconitine application elicited a regular and rapid atrial tachycardia originating from the site of aconitine application with a rapid radial spread of activation. Aconitine application did not influence AERP or local conduction velocity. However, despite the lack of changes in refractoriness and conduction velocity, the duration of AF-episodes induced by burst stimulation was longer and the number of breakthroughs increased by a factor 10 after aconitine application. The degree of endo-epicardial dissociation did not change after aconitine application. Breakthroughs were not stationary but wide-spread around the site of aconitine application, lacked prematurity, and most of them showed an R-wave morphology in the electrograms. Flecainide suppressed aconitine-induced atrial tachycardia and shortened the duration of AF episodes.

Potential mechanisms of AF

After decades of basic and clinical research, the mechanisms of initiation and perpetuation of atrial fibrillation are still not fully understood.¹ Different types of reentry like multiple wavelets, rotors, or their combination have been described as perpetuators of AF.³⁻⁷ In the 1990s, Haissaguerre et al. introduced the concept of “focal activity” as a trigger and perpetuator of paroxysmal forms of AF.¹⁸ In a group of 45 patients with AF they could demonstrate that the paroxysms were induced by focal activity (bursts) mainly originating from the myocardial sleeves in the pulmonary veins. The local nature of these depolarizations is suggestive for ectopic focal discharges in the pulmonary veins as the source of the fibrillatory process. Patterson et al. demonstrated that bursts from the pulmonary veins in patients often are initiated by atrial tachycardia followed by a pause. Endocardial mapping of the

pulmonary veins revealed focal spread of activation of pulmonary vein firing.¹⁹ However, radial spread of activation from a localized site during AF does not allow direct determination of the mechanism of an arrhythmia. Therefore, in this study, we investigated the complex interaction between stationary ectopic focal discharges induced by aconitine and fibrillation waves. Furthermore, we tested the validity of traditionally accepted criteria to identify ectopic focal discharges during AF.

Aconitine produces AF maintained by ectopic focal discharges

The first in-vivo model of ectopic focal discharges triggering atrial tachyarrhythmias goes back to experiments of Scherf et al. In 1949, they applied aconitine to atria of dogs and induced thereby AF.⁸ In this study, we used a modification of this model. Local epicardial application of aconitine on the left atrium of goats caused a rapid regular atrial tachycardia with a rapid radial spread of activation originating from the site of aconitine-application. Unipolar local electrograms at the site of earliest activation did show S-wave morphology demonstrating that ectopic focal discharges at this site generate this tachycardia. To investigate the effect of stationary localized ectopic focal discharges induced by aconitine on the fibrillatory process during AF, we induced AF by atrial burst pacing after aconitine application. AF-episodes were much longer than during the baseline measurement before aconitine application. Also, aconitine caused a strong increase in breakthrough rate during AF. Longer AF episodes after aconitine application might either be caused by disseminated ectopic discharges induced by aconitine or by changes in the substrate for AF e.g. by changes in refractoriness or atrial conduction.^{1,20-23} Additionally, increased endo-epicardial dissociation during AF after aconitine application might increase the likelihood for transmural conduction, which could increase the number of breakthroughs.^{11,12} Endo-epicardial mapping studies revealed no changes in

refractoriness, atrial conduction or endo-epicardial dissociation during AF after aconitine application suggesting an unchanged substrate for AF. To test for the plausibility of transmural conduction versus ectopic focal discharges as sources of breakthroughs, we used high-resolution simultaneous endo-epicardial in-vivo mapping.^{11,12} Only 44% of all breakthroughs could be explained by transmural conduction. Together with the fact that aconitine increased the rate of breakthrough by factor 10 by enhancing the propensity to ectopic focal discharges these data suggest that the far majority of breakthroughs during AF after aconitine application is caused by ectopic focal discharges. Furthermore, in this model the strong increase of AF stability is due to ectopic focal discharges induced by aconitine but not due to changes in refractoriness or conduction velocity.

A single and low frequency site of ectopy is sufficient to increase AF stability

A very remarkable finding in this study is that if only one site shows an enhanced propensity to ectopy AF duration may become much longer. This even holds true if – as it is the case here – the cycle length of this ectopy is actually longer than the cycle length during AF. The presence of ectopic focal discharges during AF has indirectly been postulated by many studies on alterations of calcium (Ca^{2+}) handling during AF.¹ Very recently we could directly demonstrate the existence of ectopic focal discharges during AF by high density simultaneous endo-epicardial mapping in goats with persistent AF. This study demonstrated that 13% of all breakthroughs are likely due to ectopic focal discharges (not more than ~1.5% of all fibrillation waves). It remained unclear however what the quantitative contribution of ectopic focal discharges to the perpetuation of AF actually is. The present study demonstrates that a single site of ectopy might already be sufficient to very significantly prolong duration of AF episodes.

Traditional ectopy criteria fail to detect ectopic focal discharges during AF

Subsequently, we studied whether the electrophysiological behavior of the breakthroughs observed in this study – largely caused by ectopic focal discharges – would meet the traditional criteria for ectopic activity.¹⁷ We first tested for the presence of unipolar R-wave at the breakthrough sites. In the case of ectopic focal discharges, a unipolar electrogram recorded at the site of ectopy should show S-wave morphology, whereas in the case of transmural conduction of pre-existing fibrillation waves an R-wave is expected. Secondly, the degree of prematurity of “focal” fibrillation waves was determined to identify a rapidly firing focus during AF. Finally, we tested local stationarity as a criterion, which would be particularly applicable in the case of this study because we knew where the site of maximal ectopic propensity was localized. To our surprise, breakthroughs related to localized ectopy induced by aconitine were not stationary, but wide-spread around the site of aconitine application. They did not show prematurity in coupling interval and the majority of them did show R-waves. This indicates that traditionally used parameters to identify ectopic focal discharges as a source of breakthroughs during AF failed to identify aconitine-induced “focal” breakthroughs during AF. Several factors might contribute to this unexpected finding. Ectopic discharges occurring at the surface of the atrium in the middle of the mapping area should generate electrograms with typical configuration for focal discharges (S-wave). This morphology was clearly visible in the sustained atrial tachycardia induced by aconitine before induction of AF. During AF, however, the gradient of ectopic propensity surrounding the crystal interacts with the heterogeneity in regaining excitability. This complex interaction of the sequence of recovery and the spatial gradient in ectopic propensity might have caused ectopic focal discharges occurring not just at the very site of aconitine application but also more remote from it. This includes the possibility that ectopic

focal discharges may occur in deeper layers of the atrial wall so that the resulting fibrillation waves propagates a small distance before in encounters the surface of the atrium. This small distance might be sufficient to produce local source currents generating an R-wave. Finally, the fact that there is no prematurity of the activations leading to breakthroughs caused by ectopic focal discharges might be explained by the short excitable gap in this model and also by the fact that as soon as a fibrillation wave originated by a very early ectopic focal discharge starts to propagate the prematurity will get lost very quickly because of the low conduction velocity in an area with low excitability. Thus, our data imply that identification of an ectopic focal discharges during the fibrillatory process of AF based on electrogram morphology, stationarity, and prematurity might be less reliable than previously believed.

Effect of flecainide on focal AF

To further support the hypothesis that the occurrence of focal breakthroughs may contribute to the stabilization of AF after aconitine application, we investigated the effect of inhibition of aconitine-induced ectopic discharges on AF-duration. At the molecular level, aconitine binds to sodium channels and prolongs their open state favoring entry of a large quantity of sodium into the cytosol, which may result in Ca^{2+} -overload and triggered activity.^{13,14,24-26} Sodium channel blockade has been shown to inhibit AF in the presence of aconitine.²⁷ Additionally, the sodium blocker flecainide blocks open ryanodine receptors, increases the frequency of spontaneous Ca^{2+} release which reduces the Ca^{2+} -spark mass.¹⁶ This reduced spark mass has been shown to lower the likelihood for proarrhythmic Ca^{2+} waves. In our study, flecainide completely inhibited the occurrence of spontaneous atrial tachycardia after aconitine and inducible AF-episodes were significantly shortened. This generally suggests an important role of focal discharges for increased breakthrough rate and longer AF-

episodes after aconitine-application. However, in our study flecainide also elicited additional effects, like AERP-prolongation and conduction-velocity. Several different mechanisms have already been proposed to be responsible for the antiarrhythmic effect of flecainide. Wijffels et al. suggested that the antiarrhythmic effect of flecainide was due to a widening of the excitable gap during AF.²⁸ In the goat model of AF, they demonstrated that the 'wavelength' of fibrillation waves (atrial refractory period x conduction velocity) was shortened by flecainide, which in itself would favor the persistence of AF. However, they found that cardioversion of AF by a variety of antiarrhythmic compounds (including flecainide) always went along with widening of the temporal excitable gap (difference between AF cycle length and atrial refractoriness during AF) despite very different effects on refractory period and conduction velocity. A related mechanism was suggested by Kneller et al. in computer modeling studies. They demonstrated that because flecainide reduces conduction velocity preferentially at sites of wave turning fibrillation waves are more likely to collide with boundaries of the available substrate.²⁹ In goats with 6 months of AF, Eckstein et al. showed that flecainide partially resynchronized electrical activity between the thin subepicardial layer and the endocardial bundle network, reduced transmural conduction thus the breakthrough rate.³⁰ Inhibition of focal discharges by flecainide as described in our study might be an additional mechanism, which might contribute to the potent antiarrhythmic effect of flecainide.

Summary and conclusion

The existence of a single localized site with high propensity to ectopy induced by aconitine causes a strong increase in AF stability. While the breakthrough rate strongly increases other parameters of the AF substrate remain unchanged supporting the hypothesis that the breakthroughs are due to ectopic focal discharges

and that they significantly contribute to the high stability of AF. Breakthroughs related to localized ectopy during AF may not be stationary, but wide-spread, may lack prematurity in coupling interval, and may show R-waves. This suggests a complex interaction between ectopy and re-entry during the fibrillatory process. Identification of ectopic focal discharges during AF remains a challenge.

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Chapter 7:

General Discussion

General Discussion

Understanding the mechanisms involved in the initiation and progression of AF is important to optimize treatment of AF. The present study represents an integrative approach to assess arrhythmogenic mechanisms for the development and progression of AF under different pathophysiological conditions. The focus of this study lies on the characterization of the substrate for AF in diabetes and obstructive sleep apnea, both associated with increased recurrences of AF and failure of pharmacological and catheter based ablation therapy. Subsequently, targeted intervention of newly identified pathophysiological mechanisms was tested on the induction and progression of AF. Importantly, atrial arrhythmogenic substrate is not just characterized by structural changes and alterations in atrial refractoriness but also by ectopic focal discharges inducing and perpetuating AF. Up to very recently, clear parameters to reliably identify ectopic discharges during AF were lacking complicating the development of targeted intervention. High-density endo-epicardial mapping was deployed to characterize the complex interaction between ectopic focal discharges and reentry mechanisms during AF¹ but this technique has not been developed so far for clinical routine practice.

Development of an atrial substrate for AF in diabetes

Diabetes is a strong independent risk factor for AF and stroke.²⁻⁴ Impaired LA-emptying function might underlie reduced exercise tolerance and enhanced risk for strokes in diabetic patients.⁵⁻⁷ The mechanisms leading to the development of an arrhythmogenic atrial substrate and impaired LA-emptying function in diabetes are unknown.

In this thesis, long-term diabetic conditions lead to a structural arrhythmogenic remodeling associated with local conduction disturbances and impairment of atrial emptying function. The activity of the protease CathepsinA was increased in atrial tissue. CathepsinA has been shown to be upregulated in the heart under several pathophysiological conditions and peptide hormones like angiotensin I and bradykinin have been described as substrates for CathepsinA in vitro.^{8,9} Inhibition of atrial CathepsinA-activity by a new CathepsinA inhibitor⁸ attenuated the development of arrhythmogenic substrate in diabetic rats. CathepsinA inhibition was associated with a reduction of left atrial bradykinin consumption, which may explain its anti-remodeling effect. Pharmacological CathepsinA-inhibition might provide a new promising atrial up-stream therapy strategy in diabetes (Chapter 2).

Arrhythmogenic mechanisms involved in atrial arrhythmogenesis in obstructive sleep apnea

Patients with obstructive sleep apnea (OSA) show a high prevalence of atrial fibrillation (AF) ranging from 32 to 49% and patients with severe OSA have a 4-fold increased risk for AF.¹⁰⁻¹² OSA was the strongest predictor of recurrent AF following catheter ablation¹³ and patients with untreated OSA have a higher risk of recurrence of AF after successful cardioversion.¹⁴ Additionally, OSA is associated with failure of pharmacological antiarrhythmic treatment.¹⁵ The finding that appropriate treatment of OSA with continuous positive airway pressure (CPAP) is associated with lower recurrence of AF suggests a causal association of OSA inducing AF.¹⁶ However, the mechanisms causing AF in OSA are not completely understood. Obstructive apneas are caused by collapse of the upper airway during sleep resulting in repetitive forced inspiration against the obstructed upper airway. Correspondingly substantial negative changes in intratracheal pressure down to -100 mbar were observed in OSA

patients.¹² Besides, obstructive respiratory events are associated with hypoxia and hypercapnia.

In this thesis, applied negative tracheal pressure (NTP) during tracheal obstruction in obstructive sleep apnea was identified as the main contributor to arrhythmogenic electrophysiological changes during obstructive respiratory events. NTP led to pronounced shortening of atrial effective refractory. This was mediated by increased vagal activation as indicated by complete prevention by atropin and vagotomy. By contrast, hypoxia and hypercapnia without applied NTP did not result in shortening of atrial refractoriness (Chapter 3). Selective blockade of single atrial potassium channels by antiarrhythmic drugs currently used in the clinic (sotalol, amiodarone) failed to inhibit the arrhythmogenic electrophysiological changes. Just the combined blockade of several potassium channels resulted in a sufficient antiarrhythmic effect, supporting the need for multichannel blockers (Chapter 4). This suggests that obstructive respiratory events create a highly arrhythmogenic substrate in the atrium that is difficult to suppress using available AADs and differs significantly from other conditions for which AADs have shown sufficient efficacy.^{17,18} This is in line with a recent clinical study, which showed that patients with severe OSA respond less likely to antiarrhythmic drug therapy for AF than those with milder forms of OSA. Interestingly, non-responders to AADs had higher apnea-hypopnea indexes than responders. Minimum oxygen saturation did not differ between the two groups.¹⁵ In line with our study, this study suggests that not hypoxia but the obstructive events seem to be important for AF recurrence, limiting AAD efficacy in patients with OSA.¹⁸ In our pig model for obstructive sleep apnea, we showed antiarrhythmic effects of modulation of sympathetic system by renal sympathetic denervation (Chapter 5). Interestingly, renal sympathetic denervation displayed a substantially more

pronounced antiarrhythmic effect in this pig model for sleep apnea compared to clinically used antiarrhythmic drugs like sotalol or amiodarone. Renal denervation might represent a promising antiarrhythmic treatment strategy, particularly in patients with obstructive sleep apnea, which has been shown to be associated with failure of pharmacological antiarrhythmic treatment.¹⁵

In a small study in humans, the atrial antiarrhythmic effects of RDN combined with circumferential pulmonary vein isolation was investigated.¹⁹ Patients who received both procedures showed significant reductions in average systolic and diastolic blood pressure, whereas those in the circumferential pulmonary vein isolation-only group did not show any significant improvement in blood pressure. Moreover, at one-year follow-up, 69% of patients who received both procedures no longer had AF, compared to only 29% of those in the circumferential pulmonary vein isolation-only group. In a case report, even RDN without circumferential pulmonary vein isolation reduced blood pressure and attenuated paroxysmal AF-episodes, which were symptomatic and drug-resistant before RDN.²⁰ Whether renal denervation can also display antiarrhythmic effects in normotensive patients in the clinical setting needs to be investigated in future studies.

Identification of ectopic discharges during AF

Besides structural changes and alterations in atrial refractoriness, also ectopic focal discharges are discussed to play a relevant role for the induction and perpetuation of AF. Epicardial mapping studies in patients with acute and persistent AF revealed a 2–3 fold increase in wavefronts with radial spread of activation appearing in the middle of the mapping area, which cannot be explained by propagation in the epicardial plane.²¹ Origin of these waves could either be ectopic focal discharges triggered by delayed after-depolarisations or waves originating from the opposing layer

of the atrial wall encountering excitable tissue at the site of breakthrough.^{1,22-23} Simultaneous epi-endocardial mapping of atrial activation in goats revealed that the majority of breakthrough events could be explained by transmural conduction from the contralateral wall.^{1,23} Ectopic discharges might be caused by changes in atrial calcium-handling as observed in both, AF-patients and AF animal models.²⁴⁻²⁶ However, simultaneous endo-epicardial high density mapping can at the present time not be implemented in clinical routine of AF ablation. Although parameters to reliably identify ectopic discharges during AF are highly desirable they are currently not available. Also, the precise interaction between ectopic focal discharges and reentry mechanisms in AF are unknown.

In this thesis, ectopic focal discharges were induced by epicardial application of aconitine, which increases the propensity to focal discharges (chapter 6). Subsequently, the interaction between AF and ectopic focal discharges was characterized. Epicardial application of aconitine resulted in focal discharges not strictly limited to the site of aconitine application. AF-episodes after aconitine application lasted longer and were associated with increased occurrence of breakthroughs independent of changes in atrial refractoriness and atrial conduction. Simultaneous epi-endocardial mapping revealed that just a minority of breakthroughs could be explained by transmural conduction. Therefore the majority of breakthroughs must have been caused by ectopic focal discharges induced by aconitine. However, traditionally used parameters to characterize focal discharges during AF failed to identify breakthroughs after aconitine application, suggesting a complex interaction between focal discharges and the fibrillatory process. Hove Madsen reported increased incidences of spontaneous calcium release events ("sparks") in patients with AF.²⁵ Sparks can trigger intracellular calcium waves, which then can trigger delayed after-depolarisations and ectopic focal discharges.

Knollmann reported on the effect of flecainide on sparks and could demonstrate that flecainide blocks ryanodine receptor calcium release channels in the open state and thereby reduces the spark mass.^{27,28} In our study, flecainide suppressed focal discharges induced by aconitine and reduced AF-duration supporting the concept that focal discharges play an important role in maintaining AF and that its inhibition (as shown by flecainide) results in an antiarrhythmic effect. Valid criteria to identify focal discharges during AF would largely facilitate the development of new treatment strategies targeting focal discharges (Chapter 6). Unfortunately, our study demonstrates that traditional criteria for ectopy are insufficient to identify focal discharges during AF.

Conclusions

The arrhythmogenic substrate differs significantly dependent on the underlying pathophysiological conditions. Diabetes and OSA are both associated with an arrhythmogenic substrate for AF, which differs from other conditions like lone-AF, congestive heart failure or hypertension. As treatment of AF should depend on the underlying arrhythmogenic mechanisms, we tested the antiarrhythmic effects of interventions targeting the proposed mechanisms.

As a result of this thesis, we conclude the following:

1. Inhibition of the protease CathepsinA, which was highly upregulated in the atrium of diabetic rats attenuated the development of an arrhythmogenic atrial substrate. This suggests that CathepsinA inhibition might represent a promising target for antiarrhythmic upstream treatment in diabetes.

2. Pronounced shortening in atrial refractoriness during obstructive respiratory events was associated with increased AF-inducibility in a pig model for obstructive sleep apnea. Importantly, antiarrhythmic drugs currently used in the clinic failed to inhibit the arrhythmogenic electrophysiological changes. Modification of the sympatovagal system by renal sympathetic denervation showed potent antiarrhythmic effects and provides a promising strategy to treat vagally mediated AF in OSA.
3. In a goat model for AF maintained by aconitine, we demonstrated a complex interaction between focal discharges and the fibrillatory process. Identification of focal discharges during AF is challenging, as traditional parameters failed to clearly discriminate between focal discharges and transmural conduction as the source of breakthroughs. Reduction of focal discharges by flecainide showed pronounced antiarrhythmic effects.

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Summary

Diabetes and obstructive sleep apnea are associated with increased recurrence of atrial fibrillation (AF) and failure of pharmacological and catheter based ablation therapy. The present study was conducted to characterize the atrial substrate for AF in animal models for diabetes and obstructive sleep apnea and to identify new potential antiarrhythmic therapy strategies.

In Zucker diabetic fatty rats, a rat model for type 2 diabetes, we identified the development of an arrhythmogenic substrate mainly characterized by structural alterations leading to increased AF-susceptibility and impaired atrial emptying function. In atrial tissue, the enzyme CathepsinA, involved in angiotensin and bradykinin metabolism, was highly upregulated. Pharmacological inhibition of CathepsinA by SAR1 attenuated the development of a functional and arrhythmogenic atrial substrate representing a promising target for atrial upstream therapy in diabetes (Chapter 2).

To identify mechanisms involved in the increased AF-susceptibility in obstructive sleep apnea, we developed a pig model for OSA. In anesthetized pigs, applied negative tracheal pressure mimicking obstructive respiratory events lead to pronounced shortening of atrial effective refractory mediated by increased vagal activation (Chapter 3). Selective blockade of single atrial potassium channels by antiarrhythmic drugs currently used in the clinic (sotalol, amiodarone) failed to inhibit the arrhythmogenic electrophysiological changes. Just the combined blockade of several potassium channels resulted in a sufficient antiarrhythmic effect, supporting

the need for multichannel blockers (Chapter 4). Interestingly, also modulation of autonomic nervous system by renal sympathetic denervation reduced OSA associated shortening in refractoriness. Therefore, renal sympathetic denervation might provide a promising tool to treat AF in obstructive sleep apnea (Chapter 5).

The interaction between ectopy and reentry mechanisms during AF is unclear. In a goat model, the existence of a single localized site with high propensity to ectopy induced by aconitine caused a strong increase in AF stability. While the breakthrough rate strongly increases other parameters of the AF substrate remain unchanged supporting the hypothesis that the breakthroughs are due to ectopic focal discharges and that they significantly contribute to the high stability of AF. However, breakthroughs related to localized ectopy induced by aconitine were not stationary, but wide-spread around the site of aconitine application. This suggests a complex interaction between ectopy and re-entry during the fibrillatory process. Identification of ectopic focal discharges during AF remains a challenge. New and valid mapping criteria are necessary to identify focal discharges during AF, which may be important for the development of new treatment strategies targeting focal discharges (Chapter 6).

Samenvatting

Diabetes en het obstructief slaapapneusyndroom worden beide geassocieerd met een toename in het heroptreden van atriumfibrilleren (AF) en met het falen van medicamenteuze behandeling en catheterablatie. De huidige studie werd uitgevoerd om het atriale substraat van AF te karakteriseren in diermodellen van diabetes en van het obstructief slaapapneusyndroom, en om nieuwe potentiële anti-aritmische therapieën te identificeren.

In Zucker diabetische ratten, een diermodel voor type 2 diabetes, hebben we de ontwikkeling van een aritmogeen substraat bestudeerd. Deze ontwikkeling werd voornamelijk gekarakteriseerd door structurele veranderingen die leidden tot een verhoogde gevoeligheid voor AF en tot een verminderde atriale lediging (pomp-functie). In atriaal weefsel was de functie van het enzyme CathepsinA, dat deel uitmaakt van het angiotensine en bradykinine metabolisme, sterk opgewaardeerd. Medicamenteuze inhibitie van CathepsinA door SAR1 versterkte de ontwikkeling van een functioneel en aritmogeen atriaal substraat. Als zodanig vertegenwoordigt CathepsinA een veelbelovend aangrijppunt voor atriale 'upstream therapy' in de behandeling van diabetes (Hoofdstuk 2).

Om de mechanismes vast te leggen die betrokken zijn in de toegenomen gevoeligheid voor AF in het obstructief slaapapneusyndroom hebben we een varkensmodel voor obstructief slaapapneu ontwikkeld. In verdoofde varkens werd negatieve tracheale druk toegepast om de obstructieve ademhalingsepisodes na te bootsen. Dit leidde tot een uitgesproken verkorting van de atriale effectieve refractaire periode, door een toename in vagale activatie (Hoofdstuk 3). Het selectief blokkeren van geïsoleerde atriale kaliumkanalen door anti-aritmische medicijnen die gebruikt worden in de huidige klinische praktijk (zoals sotalol en amiodarone) was

niet in staat om de aritmogene elektrofysiologische veranderingen te inhiberen. Enkel het simultaan blokkeren van meerdere kaliumkanalen leidde tot een afdoende anti-aritmisch effect. Dit feit onderstreept de nood aan multikanaal blokkers (Hoofdstuk 4). Een interessante bevinding was dat ook het moduleren van het autonome zenuwstelsel door renale denervatie de verkorting van de refractaire periode, geassocieerd met obstructieve slaapapneu, bewerkstelligde. Hierdoor zou renale sympathische denervatie ook een veelbelovende therapie kunnen zijn om AF te behandelen in patiënten met obstructief slaapapneu (Hoofdstuk 5).

De interactie tussen ectopie- en 'reentry'-mechanismes gedurende AF is nog niet uitgeklaard. In een geit-model leidde de aanwezigheid van een enkele site met een hoge neiging tot aconitine-geïnduceerde ectopie tot een sterke toename in AF-stabiliteit. Tevens observeerden we een sterke toename in het aantal 'breakthroughs' terwijl de overige parameters van het AF-substraat onveranderd bleven. Dit ondersteunt de hypothese dat het optreden van 'breakthroughs' een gevolg is van ectopische focale elektrische ontladingen en dat deze 'breakthroughs' gevoelig bijdragen tot de hoge stabiliteit van AF. Echter, 'breakthroughs' gerelateerd aan de aconitine-geïnduceerde plaatselijke ectopie waren niet stationair, maar traden op in een wijdverspreid gebied rondom de applicatieplaats van aconitine. Deze vaststelling veronderstelt een complexe interactie tussen ectopie en 'reentry' tijdens het fibrillatieproces. Het vastleggen van ectopische focale elektrische ontladingen blijft een uitdaging. Nieuwe en gevalideerde mappingcriteria zijn noodzakelijk om ectopische focale elektrische ontladingen tijdens AF te identificeren. Deze nieuwe mappingcriteria zijn mogelijk een belangrijk onderdeel voor de ontwikkeling van nieuwe behandelingsstrategieën die focale elektrische ontladingen aanpakken (Hoofdstuk 6).

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Curriculum Vitae

26.12.1982	Born in Giessen/Germany
2002	Abitur (High School Diploma)
2002 – 2009	Medicine degree in Mainz, Germany
2009	State examination in medicine (MD)
02.09.2009	Conferral of doctorate (Dr. med.)
01.08.09 – 30.10.10	Research Fellowship (Cardiovascular Research Institute Maastricht)
Since 1.11.10	Resident of cardiology (University Homburg/Saar)

Awards:

2011	Traveller award, Deutschen Gesellschaft für Kardiologie
2011	Young Investigator Award, Deutsche Hochdruckliga
2011	Poster Prize, Deutschen Hochdruckliga
2012	Innovationspreis der Deutschen Hochschulmedizin
2012	Traveller award, European Society of Cardiology,
2012	Young Investigator Award, Deutsche Hochdruckliga
2012	Poster Prize, Deutschen Hochdruckliga
2013	Traveller award, EHRA EUROPACE
2013	Best Poster, ESC-Congress

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