Effect of Renal Denervation on Neurohumoral Activation Triggering Atrial Fibrillation in Obstructive Sleep Apnea

Dominik Linz,* Mathias Hohl,* Alexander Nickel, Felix Mahfoud, Michael Wagner, Sebastian Ewen, Ulrich Schotten, Christoph Maack, Klaus Wirth,† Michael Böhm†

Abstract—Obstructive sleep apnea is characterized by repetitive collapses of the upper airway, negative thoracic pressure periods, and intermittent hypoxia, stimulating the autonomic nervous system. The increased sympathetic drive during obstructive sleep apnea results in postapneic blood pressure rises and neurohumoral activation potentially involved in the initiation and progression to permanent atrial fibrillation (AF). In a pig model mimicking obstructive sleep apnea, we studied the effects of repetitive obstructive respiratory events for 4 hours on the occurrence of spontaneous AF episodes, postapneic blood pressure rises, and neurohumoral activation. In addition, renal sympathetic denervation was performed to investigate the impact of the sympathetic nervous system. Repetitive obstructive respiratory events caused pronounced postapneic blood pressure rises, prolonged duration of spontaneous AF episodes triggered by spontaneous atrial beats, and increased plasma renin activity and aldosterone concentrations. This was associated with increased nicotinamide adenine dinucleotide phosphate-oxidase activity, reduced antioxidative capacity, and elevated expression of connective tissue growth factor, a redox-sensitive mediator of fibrosis. Renal sympathetic denervation inhibited postapneic blood pressure rises and decreased plasma renin activity and aldosterone concentrations. The occurrence and duration of spontaneous AF were reduced comparable with a combined pharmacological blockade of angiotensin receptor and β-adrenoceptor. Increased atrial oxidative stress, together with the activation of profibrotic pathways and intermittent hypoxia, was not attenuated after renal sympathetic denervation. Repetitive obstructive respiratory events triggered spontaneous AF, increased atrial oxidative stress, and activated profibrotic pathways in the atrium. Renal sympathetic denervation reduced spontaneous AF and postapneic blood pressure rises by combined reduction of sympathetic drive and components of the circulating renin–angiotensin system. However, the generation of atrial oxidative stress was not modulated. (Hypertension. 2013;62:00-00.) ● Online Data Supplement

Key Words: atrial fibrillation • autonomic nervous system • oxidative stress • sleep apnea, obstructive

Obstructive sleep apnea (OSA) is associated with a high prevalence of cardiovascular diseases such as hypertension and atrial fibrillation (AF).1,2 OSA is characterized by repetitive total collapses of the upper airway, leading to intermittent hypoxia, forced inspiration-induced negative tracheal and thoracic pressure (NTP), postapneic blood pressure rises,3 and increased sympathetic activation.4 Stimulation of vascular and cardiac α- and β-adrenoceptors by the neurotransmitters of the sympathetic nervous system leads to neurohumoral activation and increased reactive oxygen species (ROS) generation via nicotinamide adenine dinucleotide phosphate-oxidase (NAPDH-oxidase)5 and mitochondria.6 Furthermore, elevated NAPDH-oxidase activity is associated with permanent AF in mice and humans7,8 and, in particular, with an early event in the natural history of postoperative AF.9 Therefore, increased sympathetic activation may play an important role for the initiation and progression of AF in OSA.

Modulation of the autonomic nervous system by renal sympathetic denervation (RDN) has been shown to be effective in reducing both renal norepinephrine spillover and muscle sympathetic nerve activity.10–12 In a pig model mimicking OSA, 1-time applied NTP during 2 minutes of obstructive respiratory events increased inducibility of AF by premature electric stimuli,13,14 which could be reduced by β-adrenoceptor blockade and RDN.15 However, the role of sympathetic activation for spontaneous AF episodes and the reasons for the progression to permanent AF in OSA...
remain unknown. In this study, we extended the protocol and performed repetitive obstructive respiratory events with applied NTP maneuvers during 4 hours of mimicking conditions described in patients with OSA. By treating pigs with RDN, the role of sympathetic activation for spontaneous AF episodes, postapneic blood pressure rises, systemic neurohumoral activation, and atrial oxidative stress as a potential initiator for the progression to permanent AF was specifically addressed.

Methods

For detailed methods, please refer to the online-only Data Supplement. 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 (National Institutes of Health Publication No. 85-23, revised 1996).

Experimental Design of a Model for OSA

In chest-closed male castrated pigs (25–30 kg) of the German Landrace (anesthetized with 20% urethane (0.8 mL/kg IV load, 0.4 mL/kg per hour maintenance) and 4% α-chloralose (0.4 mL/kg IV load, 0.1 mL/kg per hour maintenance), a tracheotomy was performed for tracheal occlusion and for application of NTP at −80 mbar by a negative pressure device.1,2,3,4 The experimental design is depicted in Figure S1 in the online-only Data Supplement. Group 1 (n=8) served as a control (Ctr). In group 2 (n=24), repetitive NTP maneuvers (4 NTP maneuvers/h, 2 minutes) were conducted for 4 hours (NTP). In group 3 (n=26), a similar protocol with repetitive NTP maneuvers was conducted 1.5 hours after RDN (NTP+RDN). In 5 animals, 3 hours of repetitive NTP maneuvers in the presence of irbesartan (1 mg/kg bolus followed by 0.3 mg/kg per hour IV) were followed by 1 hour of repetitive NTP maneuvers after a bolus of atenolol (3 mg/kg; NTP+atenolol). In 3 additional animals, 1 hour of repetitive NTP maneuvers in the presence of atenolol (3 mg/kg bolus followed by 0.6 mg/kg per hour IV) was followed by 3 hours of repetitive NTP maneuvers after irbesartan (1 mg/kg bolus followed by 0.3 mg/kg per hour IV; NTP+atenolol+irbesartan). After 4 hours, all animals were euthanized, blood samples were taken, and cardiac tissue was frozen for further biochemical analysis in Ctr (n=5), NTP (n=6), and NTP+RDN (n=7).

Electrophysiological Examinations

Spontaneous premature atrial beats and AF episodes during tracheal occlusion with applied NTP were registered by monophasic action potentials (MAP) recordings from the endocardium of the right atrium.13–15

Atrial Gene and Protein Expression

Gene Expression Quantification

RNA was prepared from tissue using posqGold TriFast extraction reagent per manufacturer’s protocol. For cDNA preparation, 2 μg of RNA was digested with DNAse (Peqlab, Germany) and reverse transcribed using the HighCap cDNA RT Kit (Applied Biosystems, Germany) according to the manufacture’s protocol. TaqMan polymerase chain reaction was conducted in a StepOne plus thermocycler using Gene Expression Assay with connective tissue growth factor (CTGF; Ss 03392397_m1) and GAPDH (Ss03375435_u1) as probes (Applied Biosystems, Germany). The ΔΔCt was used for statistical analysis and 2−ΔΔCt for data presentation.

Western Blot Analysis

Protein was separated via SDS-PAGE and transferred to nitrocellulose membrane (Whatman, Germany). Membranes were washed and exposed to the following primary antibodies: anti-CTGF (sc-14939), anti-11β-hydroxysteroid-dehydrogenase-2 (11β-HSD2; sc-365529), anti–mineralocorticoid receptor (MR; ab27774), anti–peroxiredoxin-3 (Prx-3; LF-PA0004), and anti–GAPDH (MAB374). Secondary antibodies were incubated for 60 minutes at room temperature. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Germany). Autoradiographs were quantified using ImageQuant–TM software (Molecular Dynamics, Germany). Data are presented as intensity optical density.

Quantification of Oxidative Stress in Atrial Tissue

NADPH-oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay using a scintillation counter. Reduced glutathione (GSH) and its oxidized form (GSSG) were quantified by a modified method of Ellman. The GSH content was calculated as the difference between total glutathione and the GSSG content.

Components of the Renin–Angiotensin Aldosterone System and Blood Gases

Plasma renin activity, plasma aldosterone, and blood gases (Paco2, Pco2, pH, O2 saturation) were determined.

Figure 1. Occurrence of spontaneous atrial premature beats during repetitive negative tracheal and thoracic pressure (NTP) maneuvers. A, Number of spontaneous atrial premature beats per minute of NTP maneuver. B, Percentage of pigs with spontaneous atrial fibrillation (AF) episodes within 4 hours of experimental period, and C, respective duration of spontaneous AF episodes in control (Ctr) animals, animals with repetitive NTP maneuvers (NTP), and NTP maneuvers after renal sympathetic denervation (RDN; NTP+RDN). D, Representative registration of atrial monophasic action potentials with spontaneous atrial AF episode induced by spontaneous atrial premature beats during an NTP maneuver.
Statistics
Data are presented as mean±SEM. For comparisons of single repeated-measures only, an unpaired or paired Student t test was used. For multiple repeated-measures comparisons with the same baseline, repeated-measures 2-way ANOVA was used, followed by Dunnet test to compare individual mean differences if ANOVA was significant. A P<0.05 was considered significant. For all statistical calculations, software SAS 8 was used.

Results

Effect of RDN on Spontaneous AF During Repetitive Obstructive Respiratory Events
In control animals, a low number of spontaneous atrial premature beats appeared during the 4 hours of experimental period. Repetitive NTP maneuvers significantly increased the number of spontaneous atrial premature beats (P=0.0001 versus Ctr). This increase in spontaneous atrial premature beats was significantly attenuated by RDN (P=0.0001 versus NTP; Figure 1A). Spontaneous atrial premature beats regularly triggered spontaneous AF episodes during the experimental period (Figure 1B). In control animals, no spontaneous AF episode was observed. In 80% of all animals with repetitive NTP maneuvers, ≥1 AF episode occurred (P=0.0001 versus Ctr). In contrast, spontaneous AF was registered in 23% of animals after RDN. Spontaneous AF episodes were significantly shorter in RDN compared with NTP (P=0.0061 versus NTP; Figure 1C). Figure 1D shows an original MAP recording of a representative episode of AF triggered by spontaneous atrial premature beats. Importantly, in 5 control animals, 6 NTP animals, and 7 RDN animals without MAP catheters advanced into the atrium, comparable occurrence of spontaneous premature atrial beats and AF episodes was observed, excluding mechanical induction.

Effect of RDN on Postapneic Blood Pressure Rises, Atrial Pressure, and Changes in Blood Gases
Repetitive NTP maneuvers resulted in pronounced postapneic blood pressure rises, which were reduced by ≈55% after RDN (systolic: 97±2 to 173±14 mm Hg in NTP versus 92±3 to 125±8 mm Hg in NTP+RDN; P=0.0322; Figure 2). Importantly, NTP-induced changes in blood gases (Table S1) and atrial pressure (Table S2) were not modified by RDN.

Effect of RDN on Neurohumoral Activation
Repetitive NTP maneuvers resulted in a pronounced increase in plasma renin activity (P<0.0001 versus Ctr) and aldosterone plasma concentrations (P<0.0001 versus Ctr) at the end of 4 hours of repetitive NTP maneuvers (Figure 3A and 3B). Angiotensin II (AngII) atrial tissue concentration

Figure 2. Blood pressure during repetitive negative tracheal and thoracic pressure (NTP) maneuvers. A, Blood pressure registration in control (Ctr) animals and (B) in animals with repetitive NTP maneuvers (NTP) without and (C) with renal sympathetic denervation (RDN; NTP+RDN).
was significantly increased by repetitive NTP maneuvers (P=0.02 versus Ctr; Figure 3C). Aldosterone atrial tissue concentration and MR expression remained unchanged. However, there was a trend toward elevated 11β-HSD2, which may enhance aldosterone bioavailability at the MR (P=0.082 versus Ctr; Figure S2A–S2C). RDN significantly attenuated NTP-induced increase in circulating renin activity (P=0.002 versus NTP) and aldosterone plasma concentrations (P=0.016 versus NTP), whereas atrial tissue levels of AngII, aldosterone, 11β-HSD2, and MR protein expression were not affected by RDN.

Effect of RDN on Atrial Oxidative Stress

One important source for ROS induced by repetitive obstructive respiratory events is the NADPH-oxidase, which produces superoxide (O2•−). O2•− is transformed to hydrogen peroxide (H2O2) by superoxide dismutase, whereas H2O2 is eliminated by peroxiredoxin and glutathione peroxidase (Figure 4A). Because increased formation of ROS requires increased detoxification by antioxidative systems, the redox state of glutathione and peroxiredoxin serves to report the levels of actual tissue oxidative stress. Repetitive NTP maneuvers increased basal (P=0.063 versus Ctr) and PMA (phorbol-12-myristate-13 acetate)-stimulated NADPH-oxidase activity (12.6±0.8 RLU [relative-light-units]/s per milligram versus 15.9±1.0 RLU/s per milligram; P=0.031; Figure 4B). This was associated with an elevated oxidation of H2O2-detoxifying enzyme peroxiredoxin toward Prx-SO3 (P=0.051 versus Ctr; Figure 4C). The levels of GSH were unchanged in all groups (Figure 4D), whereas the levels of GSSG were significantly increased by repetitive NTP maneuvers (P=0.033 versus Ctr; Figure 4E). Consequently, the significantly decreased GSH/GSSG ratio (P=0.035 versus Ctr) indicates an oxidized redox state and, thus, an elevation of oxidative stress in NTP (Figure 4F). RDN did neither attenuate enhanced NADPH-oxidase activity, nor Prx-SO3 levels, nor the redox state of glutathione (Figure 4D–4F).

Effect of RDN on Activation of Profibrotic Pathways

Gene expression of the CTGF, an important and redox-sensitive inducer of fibrosis, was substantially elevated compared with controls after repetitive NTP maneuvers (1.2±0.3 versus 9.9±3.6; P=0.035; Figure 5A), whereas there was no elevation in CTGF protein expression (Figure 5B). RDN did not consistently attenuate mRNA or protein expression of CTGF.

Effect of Irbesartan and Atenolol on Spontaneous AF and Postapneic Blood Pressure Rises

To elucidate the role of the renin–angiotensin aldosterone system (RAAS) and the sympathetic nervous system on occurrence of spontaneous AF and postapneic blood pressure rises, we treated animals with the angiotensin receptor blocker irbesartan, the β-adrenoceptor blocker atenolol, or a combined administration with both. Both irbesartan and atenolol significantly reduced the occurrence of atrial premature beats (NTP: 6.2±1.3/min versus NTP+irbesartan: 2.7±0.3/min [P=0.021 versus NTP] versus NTP+atenolol: 4.3±0.3/min [P=0.042 versus NTP]) and AF duration [NTP: 58.2±8 s versus NTP+irbesartan: 40.0±4 s (P=0.051 versus NTP) versus NTP+atenolol: 38.0±3 s (P=0.036 versus NTP)]. The combination of atenolol and irbesartan resulted in a further inhibition of the occurrence of atrial premature beats (NTP+irbesartan+atenolol: 1.2±0.5/min; P=0.0078 versus NTP) and AF duration (NTP+irbesartan+atenolol: 22.0±4 s; P=0.001 versus NTP), independent of the order of administration. The combined effect was comparable with RDN (NS versus 1.0±0.1 atrial premature beats/min NTP and 15±2 s AF duration in RDN+NTP). Postapneic blood pressure rises were not modulated by irbesartan or atenolol alone and only slightly inhibited by irbesartan plus atenolol (systolic blood pressure—NTP: 110±2 to 171±10 mm Hg versus NTP+irbesartan+atenolol: 107±3 to 155±5 mm Hg [P=0.052 versus NTP]).

Discussion

In this pig model mimicking conditions described in patients with OSA, 4 hours of repetitive obstructive respiratory events in healthy animals caused pronounced postapneic blood pressure rises, increased the number of spontaneous atrial beats regularly triggering spontaneous AF episodes, and initiated neurohumoral activation. RDN reduced plasma renin activity and aldosterone concentrations, which was accompanied by a reduction in postapneic blood pressure rises, spontaneous atrial premature beats, and duration of spontaneous AF episodes. This antiarhythmic effect was comparable with the combination pharmacological β-adrenoceptor (atenolol) and angiotensin receptor blockade (irbesartan) but superior to atenolol or irbesartan alone. In atrial tissue, repetitive obstructive respiratory events led to increased NADPH-oxidase activity, which was associated with decreased antioxidative capacity and elevation of the redox-sensitive profibrotic factor.
Obstructive Respiratory Events Increase Spontaneous Premature Atrial Beats to Trigger AF

NTP during obstructive respiratory events\(^\text{15}\) and consequent atrial distension\(^\text{16}\) lead to shortening of the atrial refractory period, thus increasing the vulnerable phase, which results in stabilization of re-entry circuits perpetuating AF.\(^\text{17}\) NTP-induced electric remodeling was mediated by combined sympathovagal activation because shortening of atrial refractoriness could be modulated by atropine, \(\beta\)-adrenoceptor blockade, and RDN.\(^\text{13–16}\) In the present study, repetitive obstructive respiratory events during 4 hours significantly increased the occurrence of spontaneous premature atrial beats, which regularly resulted in AF episodes, thereby representing a relevant trigger for AF in OSA.

RAAS Activation as a Trigger for the Progression to Permanent AF

In this study, repetitive obstructive respiratory events led to pronounced postapneic blood pressure rises, together with increased components of the circulating RAAS and local atrial AngII. These data are in agreement with previous studies on atrial remodeling in the context of OSA\(^\text{11,18–20}\) but also other pathophysiological conditions.\(^\text{21–24}\) In our experiments, a blockade of the angiotensin receptor by irbesartan significantly reduced the occurrence of spontaneous premature atrial beats and AF duration, suggesting that activation of the circulating RAAS may at least partly mediate the initiation and perpetuation of AF during obstructive respiratory events. In accordance, long-term RAAS inhibition has been shown to avoid structural atrial remodeling and AF under several pathophysiological conditions.\(^\text{21,22}\)

Unchanged atrial aldosterone levels or atrial MR expression in our study is in agreement with previous studies investigating the effect of permanent aldosterone infusion in rats\(^\text{25}\) and patients with AF,\(^\text{24}\) where no changes in atrial MR expression were observed. Importantly, we showed a trend toward increased expression of 11\(\beta\)-HSD2, which tightly regulates MR binding. 11\(\beta\)-HSD2 selectively metabolizes intracellular glucocorticoids, which under normal conditions can be found in higher concentrations than aldosterone, to receptor-inactive metabolites, thus allowing MR occupancy by aldosterone and

CTGF. This increase in atrial oxidative stress and the induction of profibrotic pathways were not attenuated by RDN.

![Diagram of superoxide anion detoxification](https://example.com/diagram.png)
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Atrial tissue fibrosis impairs electrophysiological cell-to-cell coupling and induces conduction disturbances. In our model, were shown to upregulate NADPH-oxidase activity.28 Occurring in our model, were shown to upregulate NADPH-oxidase activity.28

Activation of profibrotic genes. A, mRNA expression of connective tissue growth factor (CTGF) and (B) Western blot analysis of CTGF protein expression in control (Ctr) animals, animals with repetitive negative tracheal and thoracic pressure (NTP) maneuvers (NTP), and NTP maneuvers after renal sympathetic denervation (RDN; NTP+RDN). mRNA expression was analyzed by TaqMan polymerase chain reaction and normalized to GAPDH. Protein expression was normalized against GAPDH and expressed in intensity optical density (IOD; *P<0.05).

Role of Atrial Oxidative Stress During Repetitive Obstructive Respiratory Events

Previous studies suggested that oxidative stress may play a vital role in patients with OSA-related AF. Here, already 4 hours of repetitive obstructive respiratory events resulted in local atrial oxidative stress, with decreased antioxidative capacity indexed by the redox state of the nonenzymatic antioxidants glutathione and peroxiredoxin. A reason could be the systemic and local activation of the RAAS, because in cell culture experiments and animal models, elevated levels of circulating AngII promoted oxidative stress. An increased atrial NADPH-oxidase activity is in agreement with the observation that NADPH-oxidase plays an important role, especially in the early and transient increases in ROS in the setting of postoperative AF in patients. Importantly, besides AngII, also hypoxia, elevated sympathetic tone, and pronounced changes in atrial pressure, as occurring in our model, were shown to upregulate NADPH-oxidase activity.

Potential Impact of Repetitive Obstructive Respiratory Events on Atrial Remodeling

Activation of the systemic and local atrial RAAS with accompanying atrial oxidative stress may result in atrial tissue fibrosis, potentially creating an arrhythmogenic structural substrate for AF if it endures for a longer time. Atrial tissue fibrosis impairs electrophysiological cell-to-cell coupling and induces conduction disturbances. In this acute model mimicking OSA, increased atrial gene expression of CTGF was observed, which is a potent profibrotic factor in the atrial remodeling process in various pathophysiological conditions, including AF. In fact, electric field stimulation of isolated atrial myocytes induced paracrine secretion of AngII and ROS, which in turn induced expression of CTGF. Similar effects have been observed in a dog model with atrial tachypacing. In addition, in a rat model of OSA, repetitive obstructive respiratory events during 4 weeks resulted in structural atrial remodeling characterized by increased fibrosis formation and cellular hypertrophy.

Effect of RDN on Atrial Arrhythmias, RAAS Activation, and Atrial Oxidative Stress

Previously, we have shown that RDN ameliorated the hypoxia/NTP-induced shortening of atrial effective refractory period and inhibited artificially induced episodes of AF during 1 single obstructive respiratory event. In the current study, the role of neurohumoral changes induced by RDN for postapneic blood pressure rises and spontaneous AF episodes during repetitive obstructive respiratory events was investigated. RDN significantly blunted an increase in circulating RAAS components. This was associated with a clear reduction in postapneic blood pressure rises and occurrence of spontaneous AF episodes by inhibiting spontaneous atrial premature beats representing the trigger for AF in OSA. In addition, RDN significantly reduced the duration of spontaneous AF episodes. RDN might display its antihypertensive and antiarrhythmic effects by a combined inhibition of the sympathetic system and the circulating RAAS, both highly upregulated during obstructive respiratory events. In line with this hypothesis, inhibition of postapneic blood pressure rises, premature atrial beats, and AF duration after combined angiotensin receptor plus β-adrenoceptor blockade was comparable with the effects of RDN. In contrast, angiotensin receptor or β-adrenoceptor blockade alone was not effective.

The effect of RDN on local atrial neurohumoral activation, however, seems to be more complex. Despite unchanged atrial aldosterone levels and MR expression, the increase in atrial AngII tissue concentration and 11β-HSD2 expression, as well as the decrease in atrial antioxidative capacity (GSH/GSSG ratio), was not completely inhibited by RDN. In line, an increase in atrial CTGF gene expression, as a possible downstream target of increased oxidative stress, was not prevented by RDN. Importantly, RDN did not modulate changes in blood gases and atrial pressure during obstructive respiratory events with applied NTP at −80 mbar in our pig model of OSA. The main source for ROS during hypoxia/reoxygenation is the mitochondrion, because deletion of mitochondrial, but not cytosolic, superoxide dismutase aggravates ischemia/reperfusion injury in the heart. Recently, crosstalk between NADPH-oxidase and mitochondrial ROS production was uncovered. It may be speculated that sympathetic inhibition by RDN attenuated neuroendocrine-mediated ROS production, whereas the fraction of ROS contributed by mitochondria in response to hypoxia/reoxygenation was not affected. This may explain why the level of Prx-SO₃ was hardly affected by RDN. In this context, it is interesting to note that the development of left ventricular hypertrophy and fibrosis in response to AngII infusion was prevented by a mitochondrially targeted drug or catalase expression, whereas targeting cytosolic...
oxidative stress was not effective. More long-term studies in OSA models will have to clarify whether RDN is effective also in preventing structural remodeling and, thus, the development of AF in the long run.

Limitations
We developed an in vivo animal model comprising conditions described in patients with OSA, such as upper airway occlusion, negative intrathoracic pressure, hypoxia, and hypercapnia. Four hours of repetitive OSA maneuvers were sufficient to trigger spontaneous AF episodes combined with activation of RAAS, ROS, and profibrotic pathways potentially involved in the initiation of a substrate for AF. However, the effects of RDN could only be investigated within 4 hours because longer anesthesia of the animals resulted in hemodynamic instabilities (own unpublished observation). The long-term impact of RDN on the development of an atrial substrate in OSA has to be explored in further studies. Herein, we performed combined surgical and chemical renal nerve ablation, which might be more complete compared with the catheter-based approach. However, it has been shown that norepinephrine reduction in the kidney was similar with catheter-based RDN and surgical RDN.35

Conclusions and Perspectives
We used pigs in sinus rhythm and applied NTP, together with intermittent hypoxia during repetitive tracheal occlusions for 4 hours, to elucidate the underlying mechanisms, leading to spontaneous initiation and progression of AF. Here, we identified spontaneous atrial premature beats as potent triggers for AF. In addition, we observed an activation of the RAAS associated with increased atrial oxidative stress and induction of profibrotic pathways, representing potential mediators for the progress to permanent AF. Modulation of the sympathetic nervous system by RDN reduced plasma renin activity and plasma aldosterone levels, reduced postapneic blood pressure rises, inhibited spontaneous atrial premature beats, and reduced the duration and occurrence of spontaneous AF episodes. RDN likely shows its antihypertensive and antiarrhythmic effects rather by a combined than single inhibition of either the sympathetic nervous system or the circulating RAAS, because only pharmacological blockade of both (angiotensin receptors and β-adrenoceptors) displayed effects comparable with RDN. Because RDN led only to a partial, but not complete, amelioration of oxidative stress induced by NTP and to a near-complete reduction in plasma renin activity and aldosterone concentration, it can be concluded that atrial oxidative stress is not only mediated by neurohumoral mechanisms but also through other neurotransmitter-independent mechanisms such as intermittent hypoxia/reoxygenation cycles and repeated occurrence of pronounced changes in atrial and intrathoracic pressure. Whether RDN may show antiarrhythmic effects in patients with OSA deserves further clinical studies.

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We thank Jeannette Zimolong and Kathrin Weber for their excellent technical assistance.

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Disclosures
None.

References
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Effect of renal denervation on neurohumoral activation triggering atrial fibrillation in obstructive sleep apnea.

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Experimental Model for OSA

In 66 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. We applied NTP at -80 mbar during tracheal occlusion corresponding to NTPs found in patients with OSA. Blood pressure was measured by a TIP-catheter (Millar PC 350; Millar Instruments, Houston, Texas, USA) in the femoral artery. Right atrial pressure changes were investigated by a catheter that was advanced via the femoral vein (Gould, P23 series pressure transducer, Hato Rey, Puerto Rico). Bipolar body surface ECG was recorded using subcutaneous needle electrodes in the classical lead II arrangement.

In 26 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 RDN was performed in 40 additional pigs serving as controls.

Electrophysiological Examinations

Local atrial action potentials 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, Seelheim, 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. Determination of the diastolic pacing threshold (0.5-1 mV) before each tracheal occlusion maneuver revealed no significant changes during the experimental period. A particular effort had to be made to obtain atrial MAPs of sufficient quality. The catheter was left at one location, confirmed by a regular and stable baseline and amplitude of the MAPs during NTP-procedures. Right atrial MAP duration was evaluated from 70% repolarization during regular pacing (BCL=300ms). When atrial MAP-signals showed an irregular rapid activation (cycle length <200 ms, duration >5 seconds), AF was diagnosed. AF-duration was determined. AF-episodes induced by a spontaneous premature atrial beat during tracheal occlusion with applied NTP were separately registered. To exclude mechanical induction of spontaneous premature atrial beats and spontaneous AF-episodes by the MAP-catheter, no MAP-catheter was advanced into the atrium in all pigs used for tissue sample collection (5 animals of group 1, 6 animals of group 2 and 7 animals of group 3).

Western Blot Analysis

Pig atrial tissue was homogenized in 5 volumes of homogenization buffer (in mmol/L): Na$_2$EDTA 5.0, NaF 25.0, sucrose 300.0, PMSF 1.0, benzamidine 1.0 and KH$_2$PO$_4$ 30.0 (pH 7.0) containing complete protease inhibitors (Roche, Germany) and phosphatase inhibitors (Roche, Germany). The homogenate was centrifuged at 16000 g for 20 min. 50 μg of protein was denatured at 95°C for 5 min and separated on either 8% or 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (0.2 μm pore size, Whatman Protron, Dassel, Germany). Membranes were washed in Tris-buffered saline containing 1% Tween 20 (TBS-T) blocked in TBS-T containing 5% non-fat dry milk for 120 min at room temperature and exposed to one of the following primary antibodies (dilution 1:10000). Anti-CTGF (sc-14939, Santa Cruz Biotechnology), Anti-11β-HSD2 (sc-365529, Santa Cruz Biotechnology), Anti-Mineralocorticoid Receptor (ab2774, abcam), Anti-Peroxiredoxin-SO3
(LF-PA0004, AbFrontier), Anti-GAPDH (MAB374, Millipore). Secondary antibodies goat anti-rabbit (Sigma-Aldrich, Deisenhofen, Germany; 1:2500), goat anti-mouse (170-6516 Bio-Rad, Germany; 1:2500) and rabbit anti-goat (172-1034, Bio Rad, Germany; 1:5000) were incubated for 60 min at room temperature. Proteins were visualized by enhanced chemiluminescence according to the manufacturer’s guidelines (Amersham Pharmacia Biotech, Freiburg, Germany). Autoradiographs were quantified by imaging densitometry and analyzed by the „ImageQuant-TM“ b Software (Image Quant, Molecular Dynamics, Krefeld, Germany). Data are presented as intensity optical density (IOD).

Reverse Transcription
RNA was prepared from pig atria tissue using peqGold TriFast (PeqLab, Erlangen, Germany) extraction reagent per manufacture’s protocol. For cDNA preparation 2µg of RNA was digested with DNase (Peqlab) than reverse transcribed using the HighCap cDNA RT Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacture’s protocol.

Taq-Man PCR
Taq-Man PCR was conducted in a StepOne plus thermocycler using TaqMan Gene Expression Assay Master Mix (Applied Biosystems, Germany). Signals were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase controls. No template controls were used to monitor for contaminating amplifications. The ∆Ct was used for statistical analysis and 2^−∆∆Ct for data presentation. Primers used to amplify the transcripts were as follows (purchased by Applied Biosystems): CTGF (Ss 03392397_m1), GAPDH (Ss03375435_u1).

NADPH-Oxidase Activity Assay
Activity of nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) was measured as previously described 4 by a lucigenin-enhanced chemoluminescence assay in a buffer B containing (in mmol/L) phosphate 50 (pH 7.0), EGTA 1, protease inhibitors (Complete, Roche, Germany), sucrose 150, lucigenin 0.005, and NADPH 0.1 as substrate. Left atrial tissue was homogenized in ice-cold buffer B lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/mL and 250µl aliquots were measured in a scintillation counter (Lumat LB 9501, Berthold, Germany) over 10 min in 10s intervals. For stimulation, 250 µL aliquots were incubated with PMA (phorbol 12-myristate 13 acetat) for 10 minutes at 37°C and measured.

Determination of Glutathione Level
Sample preparation: Small pieces of frozen atria were cut off and minced in 300µl of buffer (pH 7.4), containing 106.1 mM K₂HPO₄,18.7 mM KH₂PO₄, 6.3 mM EDTA (Sigma, Taufkirchen, Germany) with an electrical homogenizer MICCRA D-1 (Art, Müllheim, Germany). For GSSG-measurement 3 mM of 1-Methyl-2-vinylpyridine (Sigma, Taufkirchen, Germany) was added for derivatization of glutathione before homogenization. After centrifugation (3500 rpm, 10 min, 4°C), the protein content in supernatant fraction was determined by the method of Lowry. Determination of glutathione levels: GSH and GSSG were quantified as described previously with (minor) modifications: 50µg and 500µg were used for quantification of GSH and GSSG, respectively, without using sulfosalicylic acid due to a better signal to noise-ratio. The GSH content was calculated by the difference of total glutathione and the GSSG content and the results were expressed as nmol/mg protein according to the rate determination of calibration curves 5.

Components of the Renin Angiotensin Aldosterone System and Blood Gases
Plasma renin activity was measured according to Peters et al. 6 Plasma aldosterone was determined with a commercial radioimmunoassay (Aldosterone Coat-A-Count RIA 100T, Siemens Healthcare Diagnostics). Aldosterone (RIA-Kit, Coat-A-Count, Aldosterone, Diagnostic Products Corp) and angiotensin II (RIA-Kit, Nichols Institute Diagnostika GmbH) tissue concentrations were determined. Quantification of the latter was performed after
separation through high-performance liquid chromatography. Blood gas analyses (pO₂, pCO₂, pH, O₂-saturation) were performed directly before and at 2 minutes of each tracheal occlusion with applied NTP.

References


**Experimental Design Flow Chart:** Control Group (Ctr): 8 animals with tracheotomy and sham RDN-procedure served as a control. NTP Group (NTP): In 24 animals, repetitive NTP-maneuvers (4 NTP-maneuvers/hour, á 2 min) were conducted over 4 hours at 1.5 hours after a sham RDN-procedure. NTP + RDN Group (NTP + RDN): In 26 animals, repetitive NTP-maneuvers were conducted over 4 hours beginning at 1.5 hours after RDN. The animals were sacrificed after 4 hours of repetitive NTP-maneuvers, blood samples were taken and atrial tissue was harvested. In five animals 3 hours of repetitive NTP-maneuvers in the presence of the irbesartan were followed by 1 hour of repetitive NTP-maneuvers after a bolus of atenolol (NTP+ Irbesartan+ Atenolol). In three animals 1 hour of repetitive NTP-maneuvers in the presence of the atenolol was followed by 3 hours of repetitive NTP-maneuvers in the additional presence of irbesartan (NTP+ Atenolol+ Irbesartan).
Online Supplement Figure S2

RAAS-Signaling Components: Effect of repetitive NTP-maneuvers on (A) atrial aldosterone concentration in control (Ctr) animals, animals with repetitive NTP-maneuvers (NTP) and NTP-maneuvers after RDN (NTP + RDN). (B) Western blot analysis of mineralocorticoid-receptor protein expression and (C) 11β-hydroxysteroid-dehydrogenase 2 (11β-HSD2) in Ctr, NTP and NTP + RDN animals.
Table S1:

Blood Gases in Control (Ctr), NTP and NTP + RDN Pigs with Repetitive Obstructive Apneas.

<table>
<thead>
<tr>
<th>Blood Gas Analysis at</th>
<th>Ctr</th>
<th>NTP</th>
<th>NTP + RDN</th>
</tr>
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<tbody>
<tr>
<td>pH</td>
<td>Baseline 7.427±0.032</td>
<td>7.422±0.071</td>
<td>7.442±0.183</td>
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<tr>
<td></td>
<td>2 min NTP 7.426±0.042</td>
<td>7.234±0.144</td>
<td>7.293±0.192</td>
</tr>
<tr>
<td>pO₂ (mmHg) Baseline</td>
<td>170.9±6.9</td>
<td>185.4±7.2</td>
<td>152.2±5.9</td>
</tr>
<tr>
<td></td>
<td>2 min NTP 185.5±8.7</td>
<td>45.1±10.4</td>
<td>48.4±9.5</td>
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<tr>
<td>pCO₂ (mmHg) Baseline</td>
<td>38.3±1.6</td>
<td>37.6±0.9</td>
<td>41.2±1.2</td>
</tr>
<tr>
<td></td>
<td>2 min NTP 56.4±4.9</td>
<td>65.0±3.8</td>
<td>62.1±5.2</td>
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<tr>
<td>SO₂ (%) Baseline</td>
<td>99.4±0.7</td>
<td>99.5±0.2</td>
<td>98.8±1.5</td>
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<td></td>
<td>2 min NTP 99.4±0.3</td>
<td>45.9±5.2</td>
<td>52.2±4.9</td>
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</table>

n=5 per group

Table S2:

Atrial Pressure [mmHg]

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-apneic</th>
<th>Intra-apneic At 2 min</th>
<th>Post-apneic At maximal BP</th>
<th>Recovery After 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr</td>
<td>4.3±0.3</td>
<td>4.8±0.4</td>
<td>4.5±0.5</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>NTP</td>
<td>4.8±0.5</td>
<td>-20.3±4.1</td>
<td>8.4±0.3</td>
<td>4.2±0.9</td>
</tr>
<tr>
<td>NTP + RDN</td>
<td>4.4±0.4</td>
<td>-19.2±5.1</td>
<td>6.3±1.7</td>
<td>4.5±1.1</td>
</tr>
</tbody>
</table>

n=5 per group