Atrial fibrillation down-regulates renal neutral endopeptidase expression and induces profibrotic pathways in the kidney

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Aims Recent studies suggest that atrial fibrillation (AF) substantially influences microvascular flow in ventricular myocardium. This process may contribute to the occurrence of heart failure in AF. In general, development of heart failure and renal dysfunction go hand-in-hand causing systemic fluid overload and oedema. So far, it is unknown whether AF itself influences renal function. The aim of the present study was to determine the impact of AF on renal gene expression in a closed chest rapid atrial pacing model.

Methods and results A total of 14 pigs were studied. In five pigs, rapid atrial pacing (AT) was performed for 7 h (600 bpm); in five additional animals, rapid atrial pacing was performed in the presence of irbesartan infusion (irbesartan group). Four pigs were instrumented without interventions (sham). After the pacing period, renal expression of collagen I\alpha_1 and I\alpha_3, transforming growth factor-\beta (TGF-\beta), neutral endopeptidase (NEP; the main enzyme involved in natriuretic protein metabolism), and atrial natriuretic peptide (ANP) were determined by RT–PCR and immunoblot analysis. Functional in vitro experiments were performed using HEK-293 kidney cells. Renal mRNA expression of NEP was substantially down-regulated during AT (AT: 12.7 ± 9.3% vs. sham: 100 ± 43.4%; P < 0.01). Results at the mRNA level were confirmed at the protein level. Irbesartan therapy did not prevent down-regulation of NEP. In contrast, TGF-\beta_1 mRNA expression was up-regulated (AT: 208.5 ± 79.3% vs. sham: 100 ± 34.6% P < 0.05). Collagen and angiotensin II type 1 receptor (AT1R) expression were not significantly altered by AT. HEK-293 cells were used to determine the potential humoral factors involved in down-regulation of NEP. Application of aldosterone, ANP, asymmetric dimethylarginine, and angiotensin peptides failed to cause down-regulation of renal NEP expression in vitro.

Conclusion AT reduces NEP expression and stimulates TGF-\beta_1 signalling in the kidneys. Thus, even brief episodes of AT affect renal gene expression, which may account for structural renal changes and alterations of renal function in the long term.

KEYWORDS
Angiotensin; Arrhythmia; Fibrillation; Kidney; Oxidative stress; Pacing; Peptidase

Introduction
Atrial fibrillation (AF) induces significant electrophysiological and structural changes in the myocardium, which may contribute to an adverse prognosis.1–7 The mechanisms, however, as to how AF may contribute to an increased mortality have not been fully elucidated so far. Studies suggest that AF substantially influences contractility of and micro-vascular flow in ventricles.8–10 This process may contribute to the occurrence of heart failure in AF. In general, development of heart failure and renal dysfunction go hand-in-hand.11,12 Importantly, kidney function appears to be one of the most important independent predictors for long-term mortality in patients with cardiovascular diseases.11,12 So far, it is unknown whether AF itself influences renal function.

The aim of our animal study was to determine if brief episodes of atrial tachyarrhythmia simulated by rapid atrial
pacing affect renal gene expression, which may alter kidney function in the long term. Neutral endopeptidase (NEP, also called CD10) is expressed as a typical brush-border enzyme in renal tubulus cells.\textsuperscript{13,16} Renal NEP degrades natriuretic peptides such as atrial natriuretic peptide (ANP) and brain-derived natriuretic peptide. Systemic levels of both peptides are increased during AF.\textsuperscript{15–17} Natriuretic peptides have antihypertrophic and antiﬁbrotic properties, and therefore, inhibition of NEP activity or loss-of-enzyme function appears protective for the kidney.\textsuperscript{18,19} In contrast, transforming growth factor-β (TGF-β1) induces organ fibrosis, which is a ﬁnal common pathway of organ failure in various diseases.\textsuperscript{20} Thus, we hypothesized that AF affects renal NEP and TGF-β1 expression, which in turn alters amounts and composition of extracellular matrix by changing the expression of pro-collagens. Local and systemic concentrations of angiotensin peptides,\textsuperscript{21} aldosterone,\textsuperscript{22,23} ANP,\textsuperscript{15,16} and asymmetric dimethylarginine (ADMA)\textsuperscript{24,25} are consistently increased in AF and are generally regarded as major promoters of AF-associated pathomechanisms. Therefore, the impact of these agents on renal NEP expression was assessed \textit{in vitro} using the NEP-expressing human embryonal kidney cell line, HEK-293.

Materials and methods

Rapid atrial pacing model

A total of 14 pigs (24 ± 3 kg) were studied. The Institutional Animal Care and Use Committee of the University of Magdeburg approved the animal experiments. Pigs were premedicated and intubated as previously described.\textsuperscript{21} Anaesthesia was maintained with 2% isoflurane and supplemental oxygen given via the endotracheal tube. Oxygen saturation was continuously monitored and kept above 90% throughout the procedure. An arterial sheath (6F) was surgically introduced into the left carotid artery. Sheaths (7F and 8F) were also introduced into the left jugular vein and the left femoral vein. After complete instrumentation, 1000 U of heparin was given intravenously and repeated every hour. Two quadrupolar diagnostic electrode catheters were used for rapid atrial pacing in the high right atrium. Rapid atrial pacing (AT) was performed in five animals (pacing group) at a rate of 600 bpm (twice diastolic threshold) for 7 h. In five additional animals, rapid atrial pacing was performed in the presence of ibersartan infusion (1 mg/kg bolus followed by 0.3 mg/kg/h i.v.; ibersartan group). Four pigs were instrumented without further interventions (sham). I.v. fluids were administered to maintain mean arterial blood pressure at 70 mmHg and central venous pressure at 5 ± 1 mmHg in all animals during the study period. After the 7 h pacing period, the left kidney was exposed and the inferior pole of the kidney was resected. Tissue samples were immediately frozen in liquid nitrogen.

RNA isolation and reverse transcription

Total RNA was prepared from ~250 mg of renal tissue by using the innuPREP RNA mini kit (Analytik Jena, Jena, Germany) following the recommended protocol. One microgram of total RNA was transcribed into cDNA using AMV reverse transcriptase (Promega, Mannheim, Germany) as described previously.\textsuperscript{22}

Quantitative PCR

The iCycler (BioRad, Munich, Germany) was used for quantitative PCR. All samples were analysed in triplicate. A 25 μL reaction mixture consisted of 1 × SensiMix (Quantace, UK), 0.5 μL of SYBR-Green I (Quantace, UK), 1 μL cDNA, and 0.3 μmol/L of the specific primers indicated in Table 1.

Initial denaturation at 95°C for 10 min was followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. Quantities of GAPDH mRNA were used to normalize cDNA contents. The fluorescence intensity of the double-strand specific SYBR-Green I, reflecting the amount of the PCR product, was read in real time at the end of each elongation step. Then, amounts of specific initial template mRNA were calculated by determining the time point at which the linear increase of the sample PCR product started, relative to the corresponding points of a standard curve; these are given as arbitrary units.

Western blotting

Frozen tissue samples from all animals were pulverized in liquid nitrogen and subsequently homogenized in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 10% glycerol, 10 mM K2HPO4, 0.5% NP-40), containing 1 × protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), 1 mM sodium vanadate, 0.5% deoxycholate, 0.1 mM PMSF, 20 mM NaF, and 20 mM glycerol-2-phosphate (all from Sigma, Heidelberg, Germany). Tissue homogenates were centrifuged at 15 000 rpm for 15 min, and the resulting supernatant was stored at −20°C until further use.

HEK-293 cells were cultured in six-well plates (Nunc, Heidelberg, Germany) at a density of 2 × 10\textsuperscript{4} cells in 2 mL RPMI/10% FCS with the additions of either 5 nM aldosterone, 4 μM ADMA, angiotensins II, III, or IV (5 μM), or 5 nM ANP for 24 h. Cells were then washed twice in PBS and directly lysed by adding 300 μL of the lysis buffer described above.

Extracted proteins (30 μg per lane) were separated by 10% PAGE, followed by transfer to nitrocellulose membranes (Schleicher and Schuell BioScience, Dassel, Germany). Membranes were blocked with Roti-Block (Roti, Karlsruhe, Germany), and then incubated with the primary monoclonal antibodies against CD10/C24 (Ab-2, NeoMarkers, diluted 1:1000 in TBS), TGF-β1 (clone TBZ1, diluted 1:500 in TBS, Acris antibodies, Hidenhausen, Germany). Anti-mouse horseradish-peroxidase-conjugated antibodies (diluted 1:2000 in PBS; Cell Signalling, Frankfurt/Main, Germany) were applied after washing the blots three times in PBS. For chemiluminescence detection, the SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, USA) was used. To compare the different groups, densitometric quantification was performed only on equally processed blots exposed on the same X-ray film.
Histological evaluation

The kidneys were removed and fixed in 2.5% glutaraldehyde and OsO4 for Durcupan embedded semi-thin (500 nm) sections, which were stained with 0.1% Toluidine Blue and examined with a Zeiss Axiophot microscope.

Statistical analysis

Continuous variables are presented as $\bar{x} \pm SD$. Comparison between groups was done using the ANOVA analysis.

Results

Renal expression of neutral endopeptidase and atrial natriuretic peptide

Rapid atrial pacing caused a substantial loss of the renal expression of NEP at both mRNA and protein levels. As shown in Figure 1A, 7 h of pacing decreased NEP mRNA amounts to $30.4 \pm 7.7\%$ of sham ($100 \pm 24.8\%$; $P < 0.01$). Pacing in the presence of irbesartan did not prevent this loss of NEP mRNA expression ($20.8 \pm 5.2\%$). At the protein level, pacing reduced basic amounts of NEP ($39.7 \pm 9.4\%$) when compared with sham ($100 \pm 10.7\%; P < 0.01$) (Figure 1B). As observed at the mRNA level, irbesartan was not capable of maintaining NEP levels ($50.6 \pm 9.4\%$).

ANP is a well-established substrate of NEP. RT–PCR analyses revealed that the renal amount of ANP mRNA was significantly reduced in response to AT ($P < 0.05$). As shown in Figure 1C, mRNA amounts of ANP were substantially down-regulated in response to pacing. In the presence of irbesartan, there was a weak tendency towards less severe reduction in ANP mRNA levels. However, changes in ANP-mRNA expression (compared with sham and pacing group) did not reach statistical significance.

Renal expression of transforming growth factor-β and collagens 1α1 and 1α3

In contrast to the changes in the expression of NEP, we observed a pronounced induction of renal expression of TGF-β1 in response to acute atrial pacing. Amounts of TGF-β1 mRNA were increased to $248 \pm 64.5\%$ ($P < 0.05$) compared with sham-treated animals ($100 \pm 17.3\%$). Irbesartan showed a clear tendency to prevent this pacing-dependent induction of TGF-β1 mRNA ($102.1 \pm 23.7\%; P = 0.06$) (Figure 2).

AT is known to promote atrial fibrosis and probably also contributes to fibrotic tissue alterations elsewhere in the body. The elevated renal expression of TGF-β1 might well locally induce pro-fibrotic gene expression. However, we did not observe significant changes in the renal amounts of collagens 1α1 and 1α3 mRNA in response to pacing, neither in the presence nor absence of irbesartan, respectively (Figure 3).

Renal histomorphology after atrial pacing

Histomorphological changes were observed in the two treatment groups. Semi-thin kidney sections from all animals showed segmental attachment of glomerular tufts to Bowman’s capsule in both pacing groups. In these groups, the Bowman’s capsules appeared disintegrated in a number of glomeruli. Furthermore, proximal tubules showed vacuoles and protein casts. None of these changes were observed in sham animals (Figure 4).
Regulation of neutral endopeptidase mRNA expression in HEK-293 cells

We used the HEK-293 cell line to identify possible humoral factors that might be responsible for the observed down-regulation of renal NEP expression in response to AT in vivo. Aldosterone, angiotensins II, III, and IV, as well as ADMA all failed to decrease NEP mRNA amounts. In contrast, 24 h of culture in the presence of these compounds led to a significant increase of NEP mRNA in HEK-293 cells (Figure 5).

Discussion

To the best of our knowledge, this study is the first to demonstrate that AF influences gene expression of the kidneys. Renal NEP (CD10) expression is massively down-regulated during AF, whereas the expression of TGF-β1 is up-regulated. Notably, treatment with an angiotensin II receptor antagonist did not prevent changes in the expression of NEP or TGF-β1.

Atrial fibrillation is known to affect gene and protein expression in the heart. Recent studies have clearly shown elevated levels of angiotensin II and its active metabolites angiotensin III and angiotensin IV [Ang(2–8) and Ang(3–8), respectively], aldosterone, ANP, and ADMA (inhibitor of endothelial nitric oxide) during AF. Thus, humoral factors appear to be of particular interest to understand the pathophysiological impact of AF on non-cardiac/renal tissue. Therefore, we assessed the effect of these particular humoral factors on NEP expression in HEK-293 cells in vitro. However, none of these substances (humoral factors) caused a down-regulation of renal NEP expression in vitro. Thus, other not yet identified humoral factors and/or non-humoral mechanisms such as alterations in renal microvascular flow appear to induce the down-regulation of renal NEP during AF. In particular, alterations in renal blood flow and the switch from continuous flow into pulsatile flow and/or the occurrence of oxidative stress within renal tissue might be possible mechanisms. An imbalance between increased afferent glomerular arteriolar resistance induced by endogenous vasoconstrictors (catecholamines, etc.) and efferent arteriolar resistance is known to induce renal dysfunction. The integrity of brush-border microvilli is rapidly disturbed by oxygen or ATP depletion, and therefore, loss of brush-border enzymes is an early sign of renal damage. NEP is expressed in brush-border membranes. These structures are very sensitive to oxidative stress and ischaemia. Oxidative injury and normotensive ischaemic renal injury may cause loss of brush-border microvilli and damage of tubular cells leading to loss of renal NEP expression. Atrial fibrillation is known to induce oxidative stress with subsequent activation of redox-sensitive signalling and gene expression at the tissue level. It could be hypothesized that these processes may also occur in the kidney. However, we have not determined specific markers for oxidative stress in renal tissue in the present study. During rapid pacing,
mean arterial blood pressure levels remained at ~70 mmHg and central venous pressure at 5 mmHg in all animals. This indicates that the observed renal changes are not due to frank hypotension or changes in blood volume. Nevertheless, we have not analysed the impact of AF in awake non-sedated animals, and therefore, the present data should be extrapolated with caution. Whether down-regulation of renal NEP contributes to the increase in systemic ANP levels during long-lasting AF remains unknown. Interestingly, local ANP gene expression in the kidneys was reduced during AT. Nevertheless, loss of natriuretic peptide-degrading enzymes in the kidneys has clearly been shown to be renal-protective. The process might be activated to counterbalance the increased renal expression of TGF-β1 during AF. TGF-β1 induces interstitial fibrosis (up-regulation of collagen synthesis) via activation of the connective tissue growth factor. The duration of rapid atrial pacing in the present study was rather short. Thus, despite up-regulation of TGF-β1 after 7 h of rapid pacing, a significant up-regulation of collagen could not be demonstrated. Longer pacing durations might be necessary to show an increase in renal amounts of collagen.

Clinical studies have demonstrated an association between renal function and AF. In general, impaired renal function and dialysis are risk factors for the occurrence of AF. Interestingly, the occurrence of AF after kidney transplantation independently predicted death (adjusted hazard ratio 3.2; 95% CI 2.9–3.6) and graft loss. Thus, AF itself can affect renal function to a clinically relevant extent, which is also supported by the present study. Further studies are warranted to elucidate whether AF does increase the decline of the glomerular filtration rate (GFR) in long-term. An AF-induced decline of GFR might also effect renal excretion (pharmacokinetics) of drugs including anti-arrhythmic agents, and thereby, AF might contribute to increased drug levels and potentially toxic effects of various pharmacological substances. In addition, decline in renal NEP expression might contribute to increased ANP levels in the systemic circulation by reduced peptide degradation.

Conclusions

Brief episodes of rapid atrial pacing affect renal gene expression patterns, which may account for structural renal changes and alterations of renal function long term. Further studies are warranted to assess the clinical relevance of the present experimental findings.

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References

Renal molecular biology in AF


