Parasympathetic regulation of heart rate in rats after 5/6 nephrectomy is impaired despite functionally intact cardiac vagal innervation

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Abstract

Background. Chronic renal failure is frequently associated with a high risk of sudden cardiac death due to dysfunction of the autonomic nervous system. The pathogenic mechanisms underlying the parasympathetic cardiac dysautonomia are not fully elucidated yet.

Methods. Chronic renal failure was induced in rats by 5/6 nephrectomy. Blood pressure, resting heart rate and plasma levels of creatinine, urea and asymmetric dimethylarginine (ADMA) were measured. To characterize the parasympathetic innervation of the heart, chronotropic responses to atropine, metipranolol and to vagal stimulation in the absence or presence of ADMA were investigated in vivo. In vitro, chronotropic and inotropic effects of carbachol and ADMA and mRNA expression of muscarinic M2 receptors, high affinity choline transporter (CHT1), vesicular acetylcholine transporter (VACHT) and choline acetyltransferase (ChAT) were assessed in the isolated cardiac tissues.

Results. In 5/6 nephrectomy rats, the resting heart rate was significantly higher and the parasympathetic tone, measured as the effect of atropine after administration of metipranolol was significantly lower than in control animals. Plasma ADMA levels were significantly elevated in the uraemic rats and significantly inversely correlated with the effect of atropine on the heart rate. No differences were revealed in the plasma norepinephrine concentrations, negative chronotropic responses to stimulation of the vagus nerves, chronotropic and inotropic responses to carbachol and the relative expression of M2 receptors, CHT1, VACHT and ChAT.

Conclusion. The data suggest that cardioacceleration in chronic renal failure is caused by a diminished cardiac parasympathetic tone in the presence of a functionally intact intrinsic cardiac cholinergic signalling system.

Keywords: asymmetric dimethylarginine; chronic renal failure; parasympathetic innervation; rat heart; subtotal nephrectomy

Introduction

Cardiovascular autonomic dysfunction manifested by abnormal regulation of arterial tone and decreased heart rate variability with predisposition to arrhythmias has been proposed as a factor contributing to the increased incidence of cardiovascular death in patients with end-stage renal disease [1,2]. A number of clinical studies using both invasive and non-invasive techniques have quite consistently documented impaired function of the parasympathetic innervation of the cardiovascular system [3–8]. However, little is known about the pathogenic mechanisms underlying uraemic dysautonomia affecting the parasympathetic branch of the cardiac innervation. The responsiveness to the M1 muscarinic receptor agonist pirenzepine has been found to be unchanged in the uraemic patients as well as the response to the M2 receptor agonist carbachol in left ventricular strips isolated from the rat heart [9]. The density of muscarinic cholinergic receptors in the uraemic rat heart was also found to be unaffected [9]. A single study in uraemic rats reported diminished negative chronotropic response to the vagus nerve stimulation that was attributed to a pre-synaptic action of prostaglandins [10].

The aim of the present study was to further investigate the mechanisms leading to the uraemia-associated dysfunction of the parasympathetic innervation in the rat heart. For this purpose, we performed experiments on rats subjected to renal mass reduction by 5/6 nephrectomy. The tonic influence of parasympathetic innervation on the heart rate was determined at Weeks 1–5, 7 and 10 after subtotal nephrectomy. At Week 10 after 5/6 nephrectomy, the neuronal expression of enzymes and transporters controlling acetylcholine synthesis and release, i.e. choline acetyltransferase (ChAT), choline transporter (CHT1) and vesicular acetylcholine transporter (VACHT), was determined by real-time quantitative PCR in the separated heart atria. Also, mRNA for muscarinic M2 receptors was quantified by the same method in both atria and ventricles. In addition, the
Cardiac parasympathetic innervation in uremic rats

direct effect of the muscarinic receptor agonist carbachol on the isolated spontaneously beating right heart atria and ventricular papillary muscles and the negative chronotropic effect of cervical vagus nerve stimulation were investigated.

Since endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) has been suggested to be a highly significant predictor of sudden cardiac death in uremic patients [11], we measured plasma ADMA levels at Week 7 after subtotal nephrectomy or sham operation. In addition, direct effects of ADMA on the heart rate and its putative interference with the negative chronotropic effect of vagus nerve stimulation were evaluated at Week 10 after the induction of chronic renal failure.

The data show that the parasympathetic tone was significantly decreased in uremic rats and that this change was not associated with disturbances in other parameters studied on both neuronal and effector levels.

Subjects and methods

Animals and experimental design

All experiments were conducted in accordance with the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU) and the relevant Guidelines of the Czech Ministry of Agriculture for Scientific Experimentation on Animals and were approved by the University Committee for Experiments on Laboratory Animals. Wistar 4-month-old male rats (VELAZ, Prague, Czech Republic) were housed individually with free access to food and water. After anaesthesia with sodium pentobarbital (100 mg/l; Sigma Aldrich, Prague, Czech Republic) were housed individually with free access to food and water. After anaesthesia with sodium pentobarbital (100 mg/l kg i.p.; Sigma Aldrich, Prague, Czech Republic), rats underwent surgical and water. After anaesthesia with sodium pentobarbital (100 mg/l kg i.p.; Sigma Aldrich, Prague, Czech Republic) were housed individually with free access to food and water. After anaesthesia with sodium pentobarbital (100 mg/l kg i.p.; Sigma Aldrich, Prague, Czech Republic), rats underwent surgical intervention (n = 17) at Week 7 after subtotal nephrectomy or sham operation. In addition, blood pressure was measured in the beginning of the experiment and then at Weeks 4, 7 and 10 by the tail-cuff method using an RTBP apparatus (Kent Scientific Co., CT, USA). If not stated otherwise, animals were used for further experiments 10 weeks after subtotal nephrectomy or sham operation.

Measurement of the resting heart rate and chronotropic responses to atropine and metipranolol

The heart rate values were obtained from the conscious rats before and then 1–5, 7 and 10 weeks after the surgery. Animals were placed in a small chamber with electrodes in the floor that were connected to an electrocardiograph (EKG Seiva Praktik, Prague, Czech Republic). To estimate the tonic influence of the cardiac parasympathetic innervation on the heart rate, the muscarinic receptor blocker atropine (atropine sulfate, Sigma Aldrich, 4 mg/kg of body weight) was administered subcutaneously after pretreatment with the β-adrenergic receptor antagonist metipranolol (Hoechst-Biotika, Martin, Slovak Republic; 2 mg/kg, s.c.). On the next day, the order of drug administration was reversed to estimate the tonic influence of the sympathetic innervation on β-adrenergic receptors. Antagonist-induced changes were calculated as the differences between pre-drug values and those observed 20 min after the drug administration.

Determination of norepinephrine and ADMA concentrations

At Week 10 after surgery, blood samples were collected on EDTA, centrifuged and drawn aliquots of plasma were stored at −70°C. Norepinephrine concentrations in the plasma were measured using commercial radioluminunoesay kits (IBL Hamburg, FRG). At Week 7 after the induction of chronic renal failure, ADMA concentrations in the plasma were measured using commercial ELISA kits (kit ADMA® ELISA, DLD Diagnostika GmbH, Hamburg, Germany) as previously described [12].

RT-PCR

Total RNA was isolated from left and right atrium and left and right ventricle of subtotally nephrectomized and control animals (n = 10 per group for right atria, n = 4 per group for other heart compartments). Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and RNA was reverse-transcribed using iScript (Bio-Rad, Munich, Germany) for 30 min at 52°C. Real-time quantitative PCR was done in the 1-Cycler (Bio-Rad) using primers and the SYBR Green PCR kit (Bio-Rad). The PCR primers used in the study are listed in Table 1. The PCR conditions were initial denaturation in one cycle of 10 min at 95°C followed by 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. All analyses were done in triplicate. The expression of target genes was normalized with β-actin as a housekeeping gene. The relative expression was calculated by comparison of the received C_T (cycle threshold) values. The PCR products were separated by electrophoresis on a 2.0% Tris-acetate-EDTA agarose gel. Atrial were investigated for expression of all targets, ventricles for M2 receptor expression only. The rationale for this is the absence of intrinsic cardiac ganglia with cholinergic neurons from ventricles, so that neuronal expression of enzymes and transporters involved in acetylcholine synthesis and release (i.e. ChAT, CHT, VACHT) is restricted to atria. Controls run by omission of the RT step or by omission of template were negative.

Inotropic responses to carbachol in vitro

The contractility was studied on the right or left ventricular papillary muscles that were placed into an experimental chamber with an oxygenated Tyrode solution (32°C) of the following composition (in mmol/l): NaCl 137, KCl 4.5, MgCl2 1, CaCl2 2, glucose 10, Hepes 5; pH was adjusted to 7.4 with NaOH. Spontaneous beating frequencies were recorded using the laboratory system Biopac (Biopac Systems, Inc.; Goleta, CA, USA). After a stabilization period, the chronotropic effects of carbachol (10−9 to 10−4 mol/l) or ADMA (10−6 to 10−2 mol/l; N^4,N^6-dimethylarginine dihydrochloride, Sigma Aldrich) were tested.

Stimulation of the vagus nerves

The rats were anaesthetized with urethane 1.5 g/kg, i.p. and artificially ventilated using Pressure Controlled Ventilator (Kent Scientific Co.). The ventilation was adjusted to 90 strokes/min; peak inspiratory pressure 12 cm H2O; inspiration duration 45% of the total breath cycle length. Subcutaneous peripheral limb electrodes were inserted and an electrocardiogram (ECG) was continuously recorded (Biopac Systems) for the entire duration of the experiment. The cervical vagus nerves were isolated and subsequently unilaterally stimulated (ISOSTIM A 320, WPI, Berlin, Germany) by rectangular impulses (amplitude 2 mA, duration 0.5 ms), stimulation frequency 2, 5, 10 and 20 Hz) both before and after decentralization. The sequence of stimulation was (1) right vagus nerve, (2) left vagus nerve, (3) decentralized right vagus nerve and (4) decentralized left vagus nerve. The effect of ADMA on the heart rate prior to and in the course of the vagus nerve stimulation was tested in the same experimental setup, i.e. rats anaesthetized with urethane and artificially ventilated were first subjected to the electrical stimulation of the intact right vagus nerve and then, after a short period of recovery, ADMA in a bolus dose 0.3 mg/kg was administered into the jugular vein and the right vagus nerve was stimulated again.

Chronicotropic responses to carbachol and ADMA in vitro

Chronicotropic responses to carbachol (carbamoylcholine chloride, Sigma Aldrich) were measured on the right atria that were placed into an experimental chamber with an oxygenated Tyrode solution (32°C) of the following composition (in mmol/l): NaCl 137, KCl 4.5, MgCl2 1, CaCl2 2, glucose 10, Hepes 5; pH was adjusted to 7.4 with NaOH. Spontaneous beating frequencies were recorded using the laboratory system Biopac (Biopac Systems, Inc.; Goleta, CA, USA). After a stabilization period, the chronotropic effects of carbachol (10−9 to 10−4 mol/l) or ADMA (10−6 to 10−2 mol/l; N^4,N^6-dimethylarginine dihydrochloride, Sigma Aldrich) were tested.

Intrinsic peripheral limb electrodes were inserted and an electrocardiogram (EKG Seiva Praktik, Prague, Czech Republic) was used (pulse duration 1 ms). Data were recorded using the data acquisition system DiSys (Medisoft International, Prague, Czech Republic). Contraction force (CF) was recorded at a stimulation frequency of 1 Hz and measured in arbitrary units (a.u.). The resting tension was taken as zero. The inotropic effect of carbachol (10−4 mol/l) was measured in the presence of norepinephrine (arterenal hydrochloride, Sigma Aldrich; 10−4 mol/l).

Stimulation of the vagus nerves

The rats were anaesthetized with urethane 1.5 g/kg, i.p. and artificially ventilated using Pressure Controlled Ventilator (Kent Scientific Co.). The ventilation was adjusted to 90 strokes/min; peak inspiratory pressure 12 cm H2O; inspiration duration 45% of the total breath cycle length. Subcutaneous peripheral limb electrodes were inserted and an electrocardiogram (EKG) was continuously recorded (Biopac Systems) for the entire duration of the experiment. The cervical vagus nerves were isolated and subsequently unilaterally stimulated (ISOSTIM A 320, WPI, Berlin, Germany) by rectangular impulses (amplitude 2 mA, duration 0.5 ms, stimulation frequency 2, 5, 10 and 20 Hz) both before and after decentralization. The sequence of stimulation was (1) right vagus nerve, (2) left vagus nerve, (3) decentralized right vagus nerve and (4) decentralized left vagus nerve. The effect of ADMA on the heart rate prior to and in the course of the vagus nerve stimulation was tested in the same experimental setup, i.e. rats anaesthetized with urethane and artificially ventilated were first subjected to the electrical stimulation of the intact right vagus nerve and then, after a short period of recovery, ADMA in a bolus dose 0.3 mg/kg was administered into the jugular vein and the right vagus nerve was stimulated again.
Table 1. Primers used in real time RT-PCR amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer nucleotide sequences</th>
<th>Nucleotides</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2</td>
<td>Forward: CGAGTCTGGTGCAAGGAAGA</td>
<td>698–871</td>
<td>AAB017655</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCATATTGGAGGCCACAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHT1</td>
<td>Forward: CAAGACCAAGGAGGAAAGGAGGAGGAGGAG</td>
<td>1153–1283</td>
<td>AB030947</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCAAACATGCGACTTTGCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V AChT</td>
<td>Forward: GCCACATCGCTCAGCTCAGCTCAGCTCAGCTC</td>
<td>2177–2197</td>
<td>NM_031663</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGTTCATCAAGCAAAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT</td>
<td>Forward: CAACCATCTTCTGGCACTGA</td>
<td>1851–2033</td>
<td>XM_001061520</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAGCAGGCTCCATAGCCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: TCATCACTATCGGCAATGAGCAATGAGCA</td>
<td>821–1029</td>
<td>NM_31144</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCCTTCTGCATCCTGTGCAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M2, muscarinic receptors; CHT1, choline transporter; V AChT, vesicular acetylcholine transporter; ChAT, choline acetyltransferase. All primers were designed according to the cDNA sequence of each gene deposited in the GenBank database.

Table 2. Renal functional parameters

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>n</th>
<th>SNX</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-creatinine (µmol/l)</td>
<td>44.71±0.65</td>
<td>18</td>
<td>127.35±17.11</td>
<td>18</td>
</tr>
<tr>
<td>S-urea (mmol/l)</td>
<td>7.08±0.17</td>
<td>18</td>
<td>24.66±3.05</td>
<td>18</td>
</tr>
<tr>
<td>S-potassium (mmol/l)</td>
<td>5.57±0.09</td>
<td>18</td>
<td>6.26±0.07</td>
<td>18</td>
</tr>
<tr>
<td>Urine volume (ml/24 h)</td>
<td>15.67±1.17</td>
<td>18</td>
<td>39.44±3.15*</td>
<td>18</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>2.58±0.18</td>
<td>6</td>
<td>0.99±0.09*</td>
<td>16</td>
</tr>
<tr>
<td>U-urea (mmol/24 h)</td>
<td>14.66±0.78</td>
<td>18</td>
<td>12.6±0.43</td>
<td>16</td>
</tr>
<tr>
<td>U-Na⁺ (mmol/24 h)</td>
<td>1.44±0.07</td>
<td>6</td>
<td>1.58±0.12</td>
<td>16</td>
</tr>
<tr>
<td>U-K⁺ (mmol/24 h)</td>
<td>3.89±0.17</td>
<td>6</td>
<td>3.43±0.20</td>
<td>16</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>453.5±8.0</td>
<td>30</td>
<td>403.2±7.8*</td>
<td>30</td>
</tr>
<tr>
<td>Remnant kidney mass weight (g)</td>
<td>1.4±0.03</td>
<td>30</td>
<td>2.1±0.01*</td>
<td>30</td>
</tr>
</tbody>
</table>

Comparative data on renal functional parameters in serum (S) and urine (U), body weights and kidney weights of rats 10 weeks after 5/6 nephrectomy (SNX) or sham-operation (SHAM). Values are means ± SEMs. *P < 0.05.

Data analysis

Results are expressed as means ± SEM. Statistical differences were tested by unpaired two-tailed Student’s t-test or by analysis of variance (ANOVA) followed by post hoc Fisher’s least significant difference test, using the software package STATISTICA Cz, version 7 (StatSoft CR, Prague, Czech Republic). Normality of populations and homogeneity of variances were tested before each ANOVA. RT-PCR data were expressed in box plots showing quartiles and were analysed by the non-parametric rank sum test. The results were considered significantly different when P < 0.05.

Results

Effects of renal mass reduction

Table 2 summarizes the data from laboratory tests and general characteristics of rats 10 weeks after 5/6 nephrectomy and of sham-operated controls. Subtotally nephrectomized rats developed severe hypertension from Week 3 onwards (Figure 1).

Resting heart rate and chronotropic responses to atropine and metipranol

The resting heart rate reached significantly higher values in subtotally nephrectomized rats at Weeks 5, 7 and 10 of the experiment (Figure 2A). Cardiac parasympathetic nervous tone was estimated by measuring the chronotropic effect of the muscarinic receptor antagonist atropine that was significantly diminished from Week 4 after 5/6 nephrectomy onwards (Figure 2C). Because of reciprocal interactions between the two branches of the autonomic nervous system, the net effect of atropine was tested after previous administration of metipranol. As shown in Figure 2D, atropine treatment after metipranol administration led to an increase in the heart rate that was significantly smaller in uremic rats from Week 3 after the induction of chronic renal failure. In contrast, the negative chronotropic response to the β-adrenergic receptor antagonist metipranol did not significantly differ between the control and 5/6 nephrectomy rats until the end of the experiment, neither in the absence nor in the presence of atropine (Figure 2E, F). In addition, the ‘intrinsic’ heart rate, i.e. the heart rate after administration of both metipranol and atropine, was significantly depressed in 5/6 nephrectomy rats at Weeks 7 and 10 after the induction of chronic renal failure (Figure 2B).

Norepinephrine and ADMA concentration in the plasma

At Week 10 after the induction of chronic renal failure, norepinephrine concentrations in the plasma were 0.88 ±
Fig. 2. Heart rate after various interventions. The resting heart rate (A), the ‘intrinsic’ heart rate (taken as the value measured after both atropine and metipranolol administration; B), atropine- (ATR; C, D) and metipranolol-induced changes (MP; E, F) in the heart rate and the in sham-operated (n = 7–16) and 5/6 nephrectomy rats (n = 8–16). Values were obtained before subtotal nephrectomy or sham operation (labelled 0 on abscissa) and then 1–5, 7 and 10 weeks thereafter. The effect of atropine (4 mg/kg, s.c.) was examined in the absence (C) and in the presence of metipranolol (D; 2 mg/kg, s.c.) The effect of metipranolol was tested both in the absence (E) and in the presence of atropine (F; 4 mg/kg, s.c.). Data are shown as mean ± SEM. ∗P < 0.05, compared to the respective control value.

0.08 ng/ml and they did not significantly differ from those measured in sham-operated rats (0.74 ± 0.06 ng/ml; P = 0.201).

At Week 7 after surgery, plasma concentrations of ADMA were significantly elevated in the uraemic rats compared to the sham-operated animals, reaching 2.09 ± 0.07 µmol/l and 1.26 ± 0.03 µmol/l, respectively (P < 0.001; n = 5 per group; Figure 3A). Concentrations of ADMA showed significant inverse correlation with the positive chronotropic effect of atropine after metipranolol administration (r = −0.834; P = 0.003; Figure 3B).

**RT-PCR**

Robust expression of the muscarinic receptor M2 was observed in all heart compartments (Figure 4A). ChAT, CHT1 and VACHT mRNA were consistently detected in left atria (Figure 4B), but not in all samples from right atria, corresponding to the predominant localization of cholinergic ganglia on the left atrial side [13]. Neither of these targets showed significant differences in expression in subtotally nephrectomized compared to control animals (Figure 4A, B).
Fig. 3. (A) Concentration of asymmetric dimethylarginine (ADMA) in the plasma of sham-operated (SHAM; n = 5) and subtotally nephrectomized rats (SNX; n = 5) at Week 7 after surgery. *P < 0.001, SNX compared to SHAM. (B) ADMA plasma concentrations significantly inversely correlated with the positive chronotropic effect of atropine (ATR) after metipranolol (MP) administration.

Chronotropic responses to carbachol and ADMA in vitro

The muscarinic agonist carbachol exerted a negative chronotropic effect on the isolated right heart atria that did not significantly differ between the two groups of rats at any concentration of the drug tested (Figure 5A). ADMA slightly, but significantly, decreased the spontaneous beating rate of the heart atria, and this effect was comparable in both uraemic and sham-operated rats (Figure 5B).

Inotropic responses to carbachol in vitro

Carbachol (10⁻⁴ mol/l) decreased the CF of the papillary muscles enhanced by norepinephrine (10⁻⁴ mol/l) at a stimulation frequency of 1 Hz in both experimental groups. There was no difference in CF between sham and subtotally nephrectomized rats in either heart ventricle. CF (normalized to CF in a carbachol-free solution with norepinephrine) was 0.83 ± 0.037 (right ventricle) and 0.45 ± 0.035 (left ventricle) in sham and 0.77 ± 0.053 (right ventricle) and 0.54 ± 0.063 (left ventricle) in subtotally nephrectomized rats (Figure 5C).

Stimulation of the vagus nerves

After anaesthesia, the baseline heart rates were similar in sham and subtotally nephrectomized animals. Vagus nerve stimulation induced a stimulation frequency-dependent decrease in heart rate (Figure 6). The effect was similar for both right and left vagus nerve stimulations, and it was not affected by decentralization of vagus nerves (Figure 6). Administration of ADMA did not affect the baseline heart...
rate, nor did it interfere with the course of the right vagus nerve stimulation.

Discussion

The present study demonstrates significant time-dependent changes in the resting heart rate and cardiac autonomic nervous system regulation in the rat model of chronic renal failure induced by 5/6 renal mass reduction. Subtotally nephrectomized rats displayed the typical signs of renal insufficiency including compromised creatinine clearance, increased serum levels of urea and arterial hypertension. The resting heart rate was elevated in 5/6 nephrectomy rats from Week 5 after renal mass reduction onwards. Although sympathetic overactivity has been repeatedly reported in uraemic patients as well as in various animal models of chronic renal failure [14–17], our results suggest that the increase in the heart rate could be attributed rather to decreased parasympathetic activity. This conclusion is based upon the blunted response to atropine that became apparent at Week 4 after subtotal nephrectomy when only atropine was administered and even at Week 3 when the effect of atropine was measured after previous metipranolol administration. The decrease in the heart rate after β-adrenergic receptor blockade did not differ between the two groups of animals, suggesting either no change in the tonic sympathetic outflow to the heart or establishment of a new balance between increased sympathetic discharge and decreased sensitivity of cardiac β-adrenergic receptors, findings that have been repeatedly reported [9,15,18]. In addition, norepinephrine levels in the plasma that may serve as a rough indicator of the sympathetic nervous system activity [19] did not significantly differ between sham-operated and subtotally nephrectomized rats.

The ‘intrinsic’ heart rate, taken as the value measured after both antagonists’ administration, was significantly reduced in the uraemic animals indicating either the direct damage to sinoatrial cells caused by progressive uraemia or the presence of unspecified circulating substances with negative chronotropic effect. Although multiple alterations of ionic currents, Na-K-ATPase activity and function of calcium-handling proteins have been suggested to be directly implicated in the pathogenesis of uraemic
cardiomyopathy [20–22], the results of studies performed on working cardiomyocytes cannot be easily applied on sinoatrial node cells that have substantially different ionic channels equipment and seem to be less susceptible to various pathological challenges [23,24]. In our experiments, dealing with chronotropic effect of carbachol on the spontaneous beating rate of the isolated right atria, there was no difference in the basal rate between sham-operated and 5/6 nephrectomy rats, which makes direct uraemia-associated lesion to pacemaker cells less probable.

ADMA, an analogue of arginine that functions as an endogenous competitive inhibitor of nitric oxide synthase, emerges as a factor contributing to the increased cardiovascular risk in patients with end-stage renal disease [25]. Numerous studies addressed the putative causal relationship between ADMA levels and increased cardiovascular risk in uraemic patients and mostly, but not uniformly, proposed plasma ADMA levels as a highly significant predictor of both death and cardiovascular events [11,26–28]. In our study, plasma ADMA levels were significantly elevated in uraemic rats, which is in accord with the data previously reported on humans and rats [29,30]. It seemed to be a good candidate for a circulating substance responsible for differences in the ‘intrinsic’ heart rate demonstrated in the present study, since it has been already documented that intravenous administration of ADMA to healthy volunteers significantly reduces heart rate, cardiac output and cardiac output response to upper limb exercise and increases blood pressure and systemic vascular resistance [31]. The ADMA effect on the heart rate was attributed rather to an altered regulation of either endothelial or neuronal nitric oxide synthase in the heart than to a reflex bradycardia caused by blood pressure elevation [31]. Thus, we have also tested the direct action of ADMA on the beating rate of the isolated atria and we have verified the negative chronotropic effect of this guanidino compound in the rat species at physiologically relevant concentration (i.e. 10\(^{-6}\) mol/l). However, even a 10-fold increase in ADMA concentration did not further decrease the beating rate, suggesting that the difference in ADMA plasma levels between 5/6 nephrectomy and sham-operated rats was not responsible for the uraemia-related difference in the ‘intrinsic’ heart rate. Also, comparable magnitude of ADMA action in 5/6 nephrectomy and sham-operated rats makes the implication of ADMA in direct regulation of sinoatrial node activity under uraemic conditions unlikely.

To elucidate the mechanisms responsible for deranged efferent parasympathetic function associated with 5/6 nephrectomy, we addressed both effector and neuronal levels. First, we determined the relative expression of muscarinic receptor M2 in all heart compartments and we did not reveal any change in the respective mRNA quantity due to the renal mass reduction, which is in accord with another study reporting no change in the density of these receptors in the left ventricular membranes [9]. The sensitivity of muscarinic receptors was tested using isolated right atrial

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**Fig. 6.** Stimulation of the vagus nerves. Vagus nerves, right or left, isolated in the cervical region were stimulated in situ or after decentralization by rectangular impulses (amplitude 2 mA, duration 0.5 ms, stimulation frequency 2, 5, 10 and 20 Hz). The sequence of stimulation was (1) right vagus nerve, (2) left vagus nerve, (3) decentralized right vagus nerve and (4) decentralized left vagus nerve. Control rats (SHAM; \(n = 6\); closed symbols); subtotally nephrectomized rats (SNX; \(n = 10\); open symbols).
preparations and ventricular papillary muscles, where no differences were noted in the effects of the muscarinic receptor agonist carbachol between subtotally nephrectomized and sham-operated rats. The effect of uraemia on the tissue responsiveness to cholinomimetic drugs seems to be organ specific, since altered responses to carbachol or acetylcholine were reported in the pancreatic acini [32], oesophagus [33] or various vascular beds [34,35], whereas the carbachol effect on the heart rate remained unchanged both in chronic and acute renal failure [10,36].

Another cause of the diminished effect of atropine in 5/6 nephrectomy rats could reside at the level of acetylcholine turnover and release in the heart. We thus focused our further investigation on mRNA for proteins responsible for acetylcholine synthesis (ChAT, CHT1) and its uptake into the synaptic vesicles (VACht) that serve as reliable markers of the cholinergic neurons [37]. Coexpression of ChAT and VACht in the same cell is a prerequisite for a neuron to be cholinergic [38]. Although both genes share common cholinergic gene locus, their expression can be regulated independently [38,39]. In addition, CHT1 mRNA could be induced by a decrease in ChAT activity thus constituting a compensatory mechanism maintaining adequate cholinergic neurotransmission when the ChAT gene expression is downregulated [40]. Abnormal choline transport has been already documented in red blood cells from patients with chronic renal failure and in cerebral synaptosomes isolated from a rat model of renal failure [41,42]. In our study, the relative expression of mRNA under investigation did not display any changes due to uraemia.

Finally, we decided to verify the reduction in the negative chronotropic effect of the vagus nerve electrical stimulation described by a single group of investigators [10]. We tested separately the responses to right and left vagus nerves stimulation in two settings—with the vagus nerves intact and decentralized in the cervical region—and we were unable to reveal any difference in the negative chronotropic effect of acetylcholine released from the vagus nerves during the stimulation. Interestingly, the baseline heart rates measured in anaesthetized rats before the stimulation of both intact and decentralized vagus nerves did not display any differences among the groups thus indicating that anaesthesia by itself had substantial effect on the heart rate exceeding the tonic influence of the parasympathetic nerves on the heart [43,44].

Since the positive chronotropic effect of atropine after metipranol administration was significantly inversely correlated with plasma ADMA levels, the potential implication of this uraemic toxin in the diminished vagus nerve activity was tested in a separate set of experiments. However, intravenous administration of ADMA prior to vagus nerve stimulation did not affect the baseline heart rate, suggesting that moderate negative chronotropic effect of ADMA demonstrable in vitro was most probably overlapped by the effect of anaesthesia [43,44]. Moreover, intravenous ADMA did not interfere with the negative chronotropic effect induced by vagus nerve stimulation, thus indicating that it did not modify cardiac effects of parasympathetic stimulation on both neuronal and effector levels.

Thus, the present study shows that the uraemia-induced decrease in the effect of atropine on the heart rate reflecting the parasympathetic tone does not seem to arise from defective nerve-effector interactions in the heart. However, it cannot be excluded that the cholinergic outflow to the heart might be depressed at the level of the central nervous system. Uraemic encephalopathy, frequently associated with chronic renal failure, is demonstrated by multiple neurological symptoms including atrophy of cortical and subcortical regions and pontine myelolysis [45,46]. It has also been documented that ADMA is produced in the brain and released into the cerebrospinal fluid [47,48], although its levels in the brain or cerebrospinal fluid in uraemic patients or animal models of chronic renal failure have not been investigated yet. However, some data indicate that other guanidino compounds with nitric oxide synthase inhibitory effect accumulate in the uraemic brain and their levels are significantly correlated to plasma concentrations of urea [49]. Some studies on the role of nitric oxide in the regulation of the central parasympathetic outflow suggest that nitric oxide is involved in the complex regulation of the central parasympathetic outflow resulting in the increased parasympathetic activity of the heart [50].

Taken together, the cardioacceleration associated with chronic renal failure induced by the 5/6 nephrectomy in rats is related rather to a decreased parasympathetic outflow to the heart than sympathetic overactivity. The intrinsic cardiac cholinergic signalling system seems to be intact in the heart of uraemic rats. The impact of renal mass reduction on the parasympathetic neurons in the brainstem and putative implication of ADMA as a nitric oxide synthase inhibitor in the effect of uraemia on the central parasympathetic activity deserves further investigation.

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