Increased myogenic tone precedes structural changes in mild experimental uraemia in the absence of hypertension in rats

Tessa SAVAGE, Aisling C. McMAHON, Adrian M. MULLEN, Cathy A. NOTT*, Susan M. DODD*, Rachel M. TRIBE† and Magdi M. YAQOOB

ABSTRACT

1. Mechanical forces associated with blood flow play important roles in the acute control of vascular tone, the regulation of arterial structure and remodelling and the localization of atherosclerotic plaque. Uraemia is a proatherogenic process and is expected to be associated with impaired vascular reactivity.

2. To study this, 12 male Wistar rats were rendered uraemic by five-sixths nephrectomy and 12 control rats were sham operated simultaneously. After 8 weeks a tail-cuff systolic blood pressure was recorded, blood samples were taken and the animals killed. Isolated femoral arteries were dissected and mounted on a pressure myograph and myogenic tone was assessed over a range of intravascular pressures from 40 to 160 mmHg. Histologically the arteries were comparatively examined for gross morphology, calcification and deposition of collagen.

3. Biochemically the serum urea and creatinine were greater in the uraemic compared with the control rats (urea: $23.5 \pm 6$ mmol/l and $6.8 \pm 0.1$ mmol/l respectively, $P$ not significant; creatinine: $130.7 \pm 13$ mmol/l and $70.3 \pm 5$ mmol/l respectively, $P < 0.01$) but systolic blood pressure was the same in both groups (control, 97$\pm 1$ mmHg; uraemic, 98$\pm 2$ mmHg), compatible with mild uraemia.

4. Myogenic tone was significantly greater in uraemic vessels ($7.3 \pm 1.8\%$ versus $2.3 \pm 0.4\%$ in control, $P = 0.01$). The actual vessel lumen diameter was also smaller in pressurized uraemic vessels compared with control vessels ($471 \pm 30 \mu m$ versus $604 \pm 33 \mu m$, $P < 0.01$) after equilibration in physiological salt solution. However, when incubated in calcium-free physiological salt solution, the passive internal diameter was similar in uraemic vessels ($538 \pm 25 \mu m$ compared with $595 \pm 31 \mu m$ in control). Histologically, there were no differences between the two groups.

5. We conclude that some aspects of vascular reactivity are altered in mild experimental uraemia as shown by a reduced internal lumen diameter and increased myogenic tone. Furthermore, these changes are apparent in the absence of hypertension and precede structural changes.

Keywords: femoral artery, myogenic tone, pressure myography, uraemia.
Abbreviations: ESRD, end-stage renal disease; i.d., internal diameter; LVH, left ventricular hypertrophy; PSS, physiological salt solution.
Correspondence: Mrs T. Savage.
INTRODUCTION

Cardiovascular disease causes over 50% of deaths in end-stage renal disease (ESRD) and even after renal transplantation, accounts for 36% of all deaths in these patients [1]. Several risk factors for cardiovascular disease have been identified as being prevalent in the dialysis population including hypertension, anaemia, lipid abnormalities and glucose intolerance [2,3]. Furthermore, in patients with ESRD left ventricular hypertrophy (LVH) (defined as a left ventricular mass index > 125 g/m²) has independently been found to confer a mortality incidence of about 70% within a 5-year period [4] and is present in 60–70% of these patients [5,6].

Pulse pressure, an indirect measurement of arterial compliance, has been related to the development of LVH in uraemia [7], which suggests that alterations in the structure and/or function of the large arteries may play a role in the development of LVH. London et al. [8] have recently demonstrated that changes such as reduced distensibility and compliance and wall hypertrophy, seen in carotid and femoral arteries of dialysis patients, occur in parallel with changes in left ventricular mass and left ventricular dimensions. Moreover, we have observed using duplex sonography, that even in patients with ESRD without evident cardiac or vascular disease, calcification of the carotid and femoral arteries is common [9], which may contribute to the reduced arterial distensibility seen in these patients.

In our laboratory using the five-sixths nephrectomy model to induce mild experimental uraemia, we have previously demonstrated that left ventricular mass is significantly greater in uraemic rats compared with control rats [10]. However, whether such structural and/or functional changes occur in large arteries in mild experimental uraemia, remains to be determined.

Therefore, in this study we aimed to investigate the pressure–diameter response of isolated femoral arteries to increasing intravascular pressure in mild experimental uraemia and thereby assess myogenic tone and determine its temporal relationship to structural changes.

METHODS

Animals

Male Wistar rats (180–200 g) were housed in the on-site Biological Services Unit individually in holding rooms with a constant temperature of 21 ± 2 °C and humidity of 40 °C. The 24-h day was fixed in a 12-h light/12-h dark cycle. All procedures had prior approval from the Home Office (project licence: 70/3619) and were performed in accordance with the Animals Scientific Procedures Act, 1986.

Induction of uraemia

All animals underwent two surgical procedures in pairs a week apart. Anaesthesia was induced initially by an intramuscular injection into a hind leg of 0.18 ml of Hypnorm\textsuperscript{TM} (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml; Janssen-Cilag Ltd, Saunderton, High Wycombe, Bucks., U.K.) followed by 0.06 ml of diazepam (Phoenix Pharmaceuticals Ltd, Gloucester, U.K.) which was given intraperitoneally. The rat was then shaved in the abdominal area for the first stage of surgery which involved decapsulation and removal of two-thirds of the left kidney for the uraemic group \( (n = 12) \) and decapsulation of the left kidney for the sham-operated control group \( (n = 12) \). The following week the rats were shaved over the area covering the right flank and the uraemic group underwent a total right nephrectomy and the control group was sham operated as described above. For 8 weeks, the rats were pair fed to control for the anorexic tendencies induced by uraemia.

Systolic blood pressure

This was measured on three consecutive days using the tail-cuff method (Programmed Electro-sphygmomanometer PE-300, Nargo Bio-Systems Inc, Houston, TX, U.S.A.) the week before killing. The rats were placed in restrainers with chocolate at the head end which kept them still while systolic blood pressure was recorded on their tails, held stable by the end of the restrainer. The mean of five readings on each day was recorded but for analysis the mean of the third day only was used, as the first 2 days were considered to be a training period.

Anaesthesia

On each study day one rat was studied. The rat was given 0.25 ml of Hypnorm\textsuperscript{TM} intramuscularly into a rear thigh muscle and a further 1.6 ml of diazepam was then given intraperitoneally to induce deep anaesthesia which was tested by lifting the rat’s head and checking that its neck was truly relaxed, and then sharply pinching its claw. If there was no physical reaction it was assumed that the rat was sufficiently anaesthetized to proceed which was usually 5–10 min later. As concurrent experiments were being performed by colleagues on cardiac myocytes, the rat was killed after the opening of the thoracic cavity for rapid removal of the whole heart.

Blood samples

Cardiac venepuncture was performed to obtain blood samples for measurement of urea and electrolytes and a full blood count at the time of killing.

Dissection of the femoral artery

The femoral artery and surrounding muscle was excised from both legs immediately after killing and pinned on to a silastic Petri dish containing cold physiological salt solution (PSS) and left to equilibrate for about 15 min.

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Two segments of the femoral artery (approximately 5 mm) were dissected under a microscope (Stemi SV6, Zeiss, Germany) using microscissors and fine forceps.

**Pressure myography**

The technique employed for studying the femoral artery was perfusion myography which has been described extensively elsewhere [11]. Briefly, the vessel was mounted in the myograph chamber composed of two opposing microglass cannulae (tips ~ 70 μm), and secured at each end with two sutures. The vessel was then perfused with PSS both intraluminally and extraluminally. On either side of the chamber were a series of 3-way taps with windkessels to dampen pulsatile flow and solid state ‘in- line’ pressure transducers. These proximal and distal pressure transducers monitored the respective pressures at each end of the vessel and the mean intraluminal pressure was calculated and maintained by the pressure servo control pump. The internal diameter (i.d.) was measured continuously by the video dimension analyser and the vessel displayed on the video monitor which was connected to a CCD camera positioned on the inverted microscope (× 10).

**Chemicals and solutions**

All other chemicals were purchased from Sigma–Aldrich Company Ltd (Poole, Dorset, U.K.) and all solutions were made up on the day of experimentation. PSS consisted of 5 litres of AnalaR water to which 119 mmol/l NaCl, 4.7 mmol/l KCl 1.17 mmol/l MgSO\(_4\), 25 mmol/l NaHCO\(_3\), 1.18 mmol/l NaH\(_2\)PO\(_4\), 0.026 mmol/l EDTA and 5.5 mmol/l glucose were added and refrigerated until use. To each litre of PSS, 2.5 M CaCl\(_2\) was added immediately before use.

When calcium-free PSS was used, 0.38 g/l of the calcium chelater EGTA was added to the PSS. In potassium-substituted PSS (KPSS), equimolar KCl (mM) replaced NaCl.

**Experimental protocol**

Before the start of the study, five vessels from normal healthy rats of the same age and weight underwent the experimental protocol to ensure that the method of determining myogenic tone in these vessels was reproducible (results not shown).

At the start of the experiment, intraluminal perfusion of the vessel was stopped and the intraluminal pressure was raised to 40 mmHg and left to equilibrate in PSS which constantly perfused the vessel extraluminally at a rate of 19 ml/min for 40 min. The PSS was maintained at 37 °C and gassed continuously with 95% O\(_2\) and 5% CO\(_2\) to yield a pH of 7.4. The viability of the vessel was then tested by a standard procedure as described elsewhere [12], by direct extraluminal application to the perfusion chamber of both L-phenylephrine (1 μmol/l) in KPSS to ensure a rapid vasoconstriction, and acetylcholine (1 μmol/l) to check the function of the endothelium by a subsequent vessel dilation. Criteria for a suitable vessel was dilation to 90% or more of its original diameter. If this did not occur, then the vessel was discarded and another vessel from the same rat was mounted and the procedure repeated. The intraluminal pressure was then raised in 20-mmHg incremental steps up to 160 mmHg. At each step the vessel i.d. was recorded after 10 min. The pressure was then reduced down to 40 mmHg and left to equilibrate in calcium-free PSS for 40 min and then passive diameters were obtained every 10 min at each intravascular pressure as above. Myogenic tone was calculated as follows:

\[
\% \text{ myogenic tone at each intravascular pressure} = \frac{\text{Ca-free PSS i.d.} - \text{PSS i.d.}}{\text{Ca-free PSS i.d.}} \times 100
\]

**Histology**

The second dissected segment of artery was placed in a small bottle of 10% formalin, fixed for 24 h and then subjected to standard paraaffin wax processing. Paraaffin sections (2 μm) were cut from the wax block and dried in an oven set at a temperature of 45 °C overnight. Two standard tinctorial stains were made up in-house and used: an haematoxylin and eosin stain for general morphology to which calcium stains blue; and an Elastic Van Gieson stain to which the elastic lamina stains black, muscle stains yellow and collagen stains red. The slides were examined using light microscopy and a semi-quantitative assessment of calcification and collagen deposition was made by an experienced histopathologist who was blind to the specific grouping of each sample.

**Statistical analysis**

Data are expressed as means ± S.E.M. For each vessel a summary score representing the average myogenic tone for each group was calculated, and the means of these and the other parameters in both the control and uraemic groups were compared using Student’s t-test for paired and unpaired data. Significance was assumed when \( P \leq 0.05. \)

**RESULTS**

**General data**

During the 8-week post-operative period one uraemic rat died from advanced uraemia and during experimentation, data were discarded from one control rat due to the vessel leaking during the experiment. One vessel failed to dilate adequately to acetylcholine and was discarded but successfully replaced by another vessel from the same rat.
Table 1 Baseline measurements of uraemic (n = 11) and control (n = 11) rats
NS = not significant.

<table>
<thead>
<tr>
<th></th>
<th>Uraemic rats</th>
<th>Control rats</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>381 ± 23</td>
<td>385 ± 13</td>
<td>P = NS</td>
</tr>
<tr>
<td>Serum urea (mmol/l)</td>
<td>23.5 ± 6.1</td>
<td>6.8 ± 0.1</td>
<td>P = NS</td>
</tr>
<tr>
<td>Serum creatinine (mmol/l)</td>
<td>130.7 ± 13</td>
<td>70.3 ± 5</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Plasma haemoglobin (g/dl)</td>
<td>11.3 ± 0.6</td>
<td>13.6 ± 0.3</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>98 ± 2</td>
<td>97 ± 1</td>
<td>P = NS</td>
</tr>
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</table>

Figure 1 Lumen internal diameter in uraemic (U) and control (C) femoral arteries in (A) physiological salt solution (PSS) and (B) calcium-free PSS at 40 mmHg (* P < 0.01)

Therefore, the results of this study reflect data collected from 11 uraemic rats and 11 control unpaired rats.

At the time of killing, both the experimental and control rats were of similar weight but the experimental group was mildly uraemic with a 2–3-fold increase serum creatinine and urea and a lower haemoglobin, although this was still within the normal range (Table 1). The systolic blood pressure was the same in both groups and well within the normotensive range (uraemic, 98 ± 2 mmHg, control, 97 ± 1 mmHg, P not significant).

Pressure myography
Both uraemic and control vessels developed tone at 40 mmHg during the equilibration period. The vessel i.d. pressurized at 40 mmHg in PSS was significantly smaller in the uraemic group compared with the controls (471 ± 30 μm versus 604 ± 33 μm, P < 0.01). Moreover a positive correlation between myogenic tone and the severity of uraemia (serum creatinine r = 0.56, serum urea r = 0.58) was observed, indicative of a direct relationship between severity of uraemia and elevation of myogenic tone). In the control group the i.d. remained similar in calcium-free PSS (595 ± 31 μm), but in the uraemic group the vessels dilated significantly (538 ± 25 μm, P < 0.05) (Figure 1).

The mean myogenic tone was greater in uraemic rats than in controls (7.3 ± 1.8% compared with 2.3 ± 0.4%, P = 0.01) and at all pressures (Figure 2). In the control group, myogenic tone fell significantly at 60 mmHg (P < 0.03), reached its nadir at 100 mmHg and thereafter rose steadily but remained below 4%. The pattern of myogenic tone was similar in the uraemic group but the decrease from baseline to 60 mmHg was not significant (P < 0.15), and from 80–160 mmHg there was a persistent rise in myogenic tone which exceeded the baseline level.

Histology
There were no general morphological differences between the uraemic and control unpressurized vessels with respect to size of the lumen and walls. Neither group showed any evidence of calcification, and collagen deposition was similar in both uraemic and control vessels.
DISCUSSION

The results of this study demonstrate that in mild experimental uraemia, myogenic tone is increased in the femoral artery in the absence of hypertension and structural changes. Myogenic tone may be defined as the sustained state of contraction in a muscle without the addition of vasoconstrictor agents or abnormal ion concentrations [13]. The femoral arteries used in this experiment were not preconstricted and were perfused with PSS; therefore we were able to assess myogenic tone. We found very little tone in our control group which is consistent with the observations of others in rats [14,15], in Rhesus monkeys [16], and in rabbits unless in the presence of noradrenaline [17]. However, we did find increased myogenic tone in our uraemic vessels at all intravascular pressures. Myogenic tone has been demonstrated in the femoral arteries of spontaneously hypertensive rats [14,15] even before the onset of hypertension [14] and is increased in the resistance skeletal cremaster artery of the spontaneously hypertensive rat without morphological changes of the vasculature [18]. It may therefore be possible that in our uraemic vessels the mediators of increased myogenic tone may contribute to the development of hypertension in advanced uraemia which occurs in 80–90% of patients approaching ESRD [19].

The fact that systolic blood pressure was the same and normotensive in both the uraemic and control rats was unsurprising, as this specific model of experimental uraemia does not induce high circulating levels of plasma renin and aldosterone which are believed to be associated with the development of hypertension in other models of experimental uraemia [14]. Furthermore, as both uraemic and control rats were identically handled with respect to the measurement of systolic blood pressure, we are confident that the chances of systematic error were less likely and the normotension observed in the uraemic group was genuine. A possible explanation for the increase in myogenic tone but not in systolic blood pressure in the uraemic group, may be consequent to the significantly reduced lumen diameter observed in the uraemic rats. Hecker et al. [21] have reported that in the femoral arteries of rabbits, endothelial release of the vasodilators nitric oxide and prostacyclin was increased 5–7-fold and 11–12-fold respectively by both increasing shear stress by vasoconstriction at a constant flow rate, and by increasing flow rate at a constant diameter [21]. Therefore in the smaller femoral artery lumen of uraemic rats, it is likely that for a given flow rate, there will be increased generation of shear stress with subsequent elevation of nitric oxide and prostacyclin release. The resultant dilatation may delay the development of hypertension seen in advanced uraemia. It is possible that the same phenonmenon is also operative in the elevation of myogenic tone seen in SHRs before the onset of hypertension [13].

In spontaneously hypertensive rats, myogenic tone has been linked to increased calcium influx via L-type voltage-dependent calcium channels [14,15,22]. In concurrent patch-clamping experiments on cardiac myocytes using the same rats, calcium influx via L-type channels was similar in uraemic and control ventricular myocytes, but inactivation of the channels was more rapid in the uraemic myocytes [23]. The role of these channels in uraemic femoral arteries may therefore merit investigation. The current experimental conditions were established in a no-flow state only and as vascular activity in skeletal muscle is modulated by both pressure- and flow-sensitive mechanisms [24], further studies investigating the physiological response to flow are required to assess the relative contribution of both stimuli to vascular reactivity in these vessels.

Histologically, there was no difference between the uraemic and control groups with respect to vessel lumen and wall size. Unfortunately the vessels sent for histology were not additionally fixed at a set pressure of 40 mmHg, so an accurate histological comparison of lumen and wall size with myograph measurements was not possible. However, in vessels pressurized at 40 mmHg on the myograph, the basal lumen diameter of the uraemic vessels was significantly less than that of control arteries. In ESRD, several factors are known to contribute to the aetiology of hypertension including chronic extracellular volume overload [25], excess renin activity [26], overactivity of the sympathetic nervous system [27] and decreased production of nitric oxide due to accumulation of the nitric oxide synthase inhibitor asymmetrical dimethyl-l-arginine [28]. It may be that one or more of these may cause basal arterial constriction before clinical evidence of hypertension becomes apparent.

LVH is present in 60–70% of patients with ESRD [5,6] and is an important prognostic risk factor for cardiovascular death [4]. The aetiology of LVH in this population is multifactorial [2,3] but has been consistently linked to hypertension [29] and vascular changes [7,8]. Although vascular changes such as increased pulse pressure, reduced compliance and wall hypertrophy have been correlated with increased left ventricular mass in ESRD [8], it has proved difficult to assess the relative contributions of structural changes and hypertension as all frequently co-exist in ESRD. In our laboratory, using the same model of experimental uraemia, LVH has been observed in the absence of hypertension [10] and for this reason we did not reassess LVH in this study. It is possible that the functional changes in myogenic tone observed in this study may play a role in the development of LVH in uraemia.

In summary, our study has demonstrated for the first time that in mild experimental uraemia myogenic tone is increased and the lumen diameter of pressurized femoral...
arteries is smaller than in controls. Furthermore, these changes are apparent in the absence of hypertension and precede arterial structural changes.

ACKNOWLEDGEMENTS

This work is dedicated to the late Professor A. E. G. Raine. Portions of this paper were presented at the American Society of Nephrology meeting, San Antonio, Texas in November 1997. We thank Professor Lucilla Poston for generously providing laboratory facilities for learning this technique.

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Received 23 December 1997/20 May 1998; accepted 15 July 1998