Prolonged calcium transients and myocardial remodelling in early experimental uraemia

Aisling C. McMahon¹, Stephen E. Greenwald², Susan M. Dodd², Martin J. Hurst¹ and Anthony E. G. Raine*

¹Anthony Raine Research Laboratories and ²Department of Histopathology and Morbid Anatomy, Barts and The London Queen Mary’s School of Medicine and Dentistry, University of London, London, UK

Abstract

Background. Cardiovascular disease is the most common cause of premature death in patients with end-stage renal disease, possibly due to a specific ‘uraemic cardiomyopathy’. This study was designed to investigate the cardiac changes induced by a moderate impairment of renal function in a model of uraemia.

Methods. Male Wistar rats (n = 11) were rendered uraemic by 5/6 nephrectomy or sham operated (n = 11). After 4 weeks, cardiac dimensions were measured from fixed tissue sections using a digital image analysis technique. In parallel groups of animals, cardiac myocytes were isolated and studied for evidence of functional changes attributable to uraemia. After steady-state field stimulation at 0.5 Hz, intracellular Ca²⁺ handling (using Fura-2) was investigated. Up to 20 consecutive transients were averaged as the extracellular Ca²⁺ was increased.

Results. The 5/6 nephrectomy group had a 75% reduction in glomerular filtration rate, and a 2- to 3-fold increase in serum urea and creatinine compared with sham-operated control animals (P < 0.0001). However, the blood pressure was found to be similar in each group. Histology of the intact hearts (five pairs) showed a significant increase in tissue cross-sectional area (14%; P < 0.04), cross-sectional area of the left ventricle (22%; P < 0.04), and a significant increase in left ventricular wall thickness (15%; P < 0.03). In the single cardiac cell study, under basal conditions (1–2 mM extracellular Ca²⁺) no significant differences in intracellular Ca²⁺ were observed, but in high extracellular Ca²⁺ the uraemic cells were slower to return to diastolic intracellular Ca²⁺ levels (P < 0.05).

Conclusions. The data provide evidence of altered myocardial structure and function in early experimental uraemia. The changes described are consistent with concentric hypertrophy of the left ventricle, which occurs in the absence of hypertension.

Keywords: calcium; cardiomyopathy; hypertrophy; myocyte; rat; uraemia

Introduction

Cardiovascular disease is known to be the most common cause of premature death in patients with end-stage renal disease (ESRD) [1,2]. Data from the European Dialysis and Transplantation Association (EDTA) indicate that over 50% of deaths of patients receiving renal replacement therapy are due to cardiovascular causes [1]. The reasons for this are largely unknown, although a variety of putative factors have been implicated in the pathogenesis of cardiovascular dysfunction. These include many of the common secondary complications of renal disease such as hypertension, anaemia, left ventricular hypertrophy (LVH), hyperparathyroidism, and altered fluid balance. However, there is increasing evidence to suggest the existence of a specific uraemic cardiomyopathy [3,4].

It is clear that renal patients have an increased risk of death from cardiovascular events when compared with the general population. In a large European survey, the incidence of death from myocardial infarction (MI) in Italy and the UK in each population was compared with the rate in each country’s ESRD population [1]. Although the overall rate of death from MI is substantially less in Italy than in the UK, the rate for each renal population remained consistently 16–19 times greater than the baseline incidence. This implies that renal disease amplifies the risk of cardiovascular disease independently of genetic and environmental factors.

Experimental studies support these clinical findings, with evidence of cardiovascular abnormalities clearly...
apparent in models of chronic uraemia. Both structural [5,6] and functional [7,8] changes in the heart have been described following uraemia of short duration. In the present study, as LVH is known to be an independent predictor of survival in ESRD, the changes in blood pressure and cardiac structure after 4 weeks of relatively mild uraemia were investigated. A study of intracellular calcium handling, and the effect of extracellular calcium upon this, was also carried out in single isolated cardiac myocytes.

Materials and methods

Preparation of experimental animals

Male Wistar rats were rendered uraemic by subtotal (5/6) nephrectomy in a two-stage surgical procedure as described previously [8]. Briefly, animals were anaesthetized with Hypnorm, 0.75 ml/kg (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, i.m., Janssen Pharmaceuticals Ltd, Oxford, UK) and diazepam 0.2 ml (10 mg/ml, i.p., Phoenix Pharmaceuticals Ltd, Gloucester, UK) and the left kidney was exposed and decapsulated through a midline abdominal incision. The renal vessels were clamped, and the kidney was substantially resected with removal of the upper and lower poles followed by the lateral aspect, and both the anterior and posterior surfaces of the cortex. One week later the animals were re-anaesthetized in a similar manner, and a total right nephrectomy performed after decapsulation through a flank incision. Weight-matched sham-operated control animals were prepared at the same time by bilateral total right nephrectomy performed after decapsulation and posterior surfaces of the cortex. One week later the sham-operated animal receiving the same amount of standard rat chow consumed by its uraemic partner on the previous day. Tap water was available ad libitum. All animals were studied 28 days after the completion of surgery.

Creatinine clearance measurements

The animals were weighed and placed in metabolic cages for 24 h before being killed to facilitate the collection of urine for creatinine clearance (CrCl) measurements.

Blood pressure

Blood pressure was measured in five pairs of animals immediately before being killed. Under anaesthesia (sodium thiopentone 100 mg/kg by i.p. injection), blood pressure and heart rate were recorded using an automated tail-cuff system (IITC model 229, Linton Instruments, Norfolk, UK). Measurements were repeated a minimum of three times for each animal and only stable, reproducible values included.

Cardiac histology

Hearts were isolated and fixed in 10% formal saline. The specimens were embedded in paraffin wax and cut transversely across the widest point. The largest possible cross section of each heart was stained with haematoxylin and eosin and used for analysis.

Data analysis

The cross-sectional area of each section was measured in arbitrary units using an image analysis system (Magiscan M2, Applied Imaging International, Sunderland, Tyne and Wear, UK). Each slide was illuminated from below and a digital image was captured with a CCTV camera fitted with a macro lens mounted above the section. The total cross-sectional area of the left and right ventricles was calculated by counting the pixels darker than a set grey level threshold. All the background pixels, being transparent, had intensities greater than the threshold level. The cross-sectional area of each lumen was calculated by counting all background pixels surrounded by left ventricular tissue. The region of the section corresponding to the right ventricle was edited out of the image using a light pen and the tissue and lumen areas were re-measured. The difference in area between the original and subsequent measurements made it possible to estimate left and right ventricular tissue areas.

The wall thickness of the left ventricle was also estimated. The outer radius of the left ventricle was calculated from the total area of tissue and lumen, assuming the cross section was circular. Similarly, the inner radius was determined from the lumen area. The difference between these two radii was taken to be the mean left ventricular wall thickness.

Measurement of intracellular calcium transients

The method of ventricular myocyte isolation has been reported in detail previously [8]. Cardiac myocytes were loaded with 5 μM Fura-2/AM and stored in the dark. Rod-shaped striated cells showing no more than two spontaneous contractions per minute were selected for study. Cells were placed in a thermostatically controlled chamber (32°C) placed on the stage of an inverted microscope (Nikon) and superfused with Krebs–Henseleit solution (containing NaCl 119 mM, KCl 4.7 mM, MgSO4 1.2 mM, KH2PO4 1.2 mM, NaHCO3 25 mM, glucose 11.5 mM, ascorbic acid 0.1 mM, CaCl2 1.0 mM; continuously bubbled with 95% O2/5% CO2). Electrical field stimulation was set at 0.5 Hz. Changes in light emitted from a single cell during contraction and relaxation were amplified by a photomultiplier tube. The ratio of emitted light at 510 nm after alternate excitation at 340 and 380 nm was recorded at 5 ms intervals and represents changes in intracellular Ca2+ ([Ca2+]i). The ratio measurements were not quantified due to potential problems associated with the compartmentalization of the dye within the cells.

Initial measurements (Cairn acquisition software) were made over a 50 s period from cells in superfusate containing 1 mM Ca2+ ([Ca2+]s). The [Ca2+]s was then increased to 2 mM, and after a stabilization period of 1 min, further data recorded from the same cell for a similar period. The [Ca2+]s was again doubled to 4 mM and data recorded. Most of the cells studied could not tolerate the superfusate containing greater than 4 mM Ca2+, therefore this was the maximum concentration used. Some cells failed to withstand 4 mM Ca2+ for the time period required, therefore fewer data files were recorded under these conditions than at the lower concentrations studied.

The analysis software used was Datapac II (Run Technologies, CA, USA). From each experimental condition, 20 consecutive transients were used for analysis. Data were filtered using a 30 Hz low pass filter, and averaged to give a single composite transient for each set of conditions. From
the filtered, signal-averaged transient obtained, the following
measurements were taken: (i) time from stimulation to peak
$\left[ \text{Ca}^{2+} \right]_i (T_p)$; (ii) time from peak $\left[ \text{Ca}^{2+} \right]_i$ to 50% restoration of
diastolic $\left[ \text{Ca}^{2+} \right]_i (R_{50})$, and (iii) time from peak $\left[ \text{Ca}^{2+} \right]_i$ to 80%
restoration of diastolic $\left[ \text{Ca}^{2+} \right]_i (R_{80})$. The positions of these
points are shown on a typical averaged transient in Figure 1.

**Statistical analysis**

Data are presented as mean ± SE, where $n$ is number of cells
studied, except where otherwise stated. Student’s $t$-test (two-
tailed) was used to compare the significance of the difference
between the means for normally distributed data. A value of
$P < 0.05$ was considered to be statistically significant.

**Results**

Model of chronic renal failure

Partial nephrectomy ($n = 11$ animals) caused a 3-fold
increase in plasma urea ($6.9 ± 0.3$ control (C) vs
$24.0 ± 3.0$ mmol/l uraemic (U); $P < 0.0001$) and a
2-fold increase in creatinine ($70 ± 0.0$ C vs $120 ±
10$ mmol/l U; $P < 0.0001$) compared with control
animals ($n = 11$; Table 1). Further analysis on a
subset of these animals (five pairs) showed that although plasma Ca and bicarbonate were similar in
each group, the uraemic animals had substantially
lower mean haemoglobin (Hb) and packed cell volume
(PCV) values. Taken together these results confirm the
presence of a moderate biochemical uraemia with
concomitant anaemia in the 5/6 nephrectomy group. In
support of this, these animals were found to have a
highly statistically significant reduction in mean CrCl
of approximately 75% compared with mean control
values ($1.1 ± 0.1$ C vs $0.3 ± 0.1$ ml/min U; $P < 0.0002$).

There was no evidence of hypertension in the
uraemic animals (Table 2). No significant difference
in either systolic or diastolic pressures between control
and uraemic animals was found, and consequently
no difference in mean arterial pressure. Indeed blood
pressures were slightly, although not significantly
lower in the uraemic animals. Heart rate was similar
in the two groups.

**Table 1.** Biochemical profile of control and uraemic animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SE</th>
<th>Uraemic</th>
<th>SE</th>
<th>$P &lt;$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mmol/l)</td>
<td>6.66</td>
<td>0.25</td>
<td>30.5</td>
<td>5.25</td>
<td>0.002</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>53</td>
<td>9</td>
<td>178</td>
<td>29</td>
<td>0.004</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.52</td>
<td>0.09</td>
<td>2.47</td>
<td>0.10</td>
<td>ns</td>
</tr>
<tr>
<td>Bicarbonate (mmol/l)</td>
<td>20.40</td>
<td>1.21</td>
<td>22.20</td>
<td>1.11</td>
<td>ns</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>31.60</td>
<td>0.40</td>
<td>28.60</td>
<td>1.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>14.00</td>
<td>0.64</td>
<td>9.68</td>
<td>0.56</td>
<td>0.002</td>
</tr>
<tr>
<td>PCV</td>
<td>0.40</td>
<td>0.00</td>
<td>0.27</td>
<td>0.02</td>
<td>0.0003</td>
</tr>
<tr>
<td>Urine protein (g/l)</td>
<td>1.08</td>
<td>0.13</td>
<td>3.39</td>
<td>0.98</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SE. For urea and creatinine $n = 11$ in each group. All other values $n = 5$ in each group.
Cardiac histology

Despite the absence of hypertension, there was extensive evidence of cardiac hypertrophy in the uraemic animals compared with controls (five pairs; Table 3). The total tissue cross-sectional area (arbitrary units, AU) was increased by 14% from 77.5 ± 3.3 AU in controls to 88.4 ± 2.7 AU in uraemic animals (P < 0.04). This hypertrophy was particularly pronounced in the left ventricle where there was a 22% increase in tissue area from 46.1 ± 2.2 (C) to 56.1 ± 3.1 (U) AU (P < 0.04). Similarly, the estimated thickness of the left ventricular free wall was increased by 15% (2.3 ± 0.1 C vs 2.7 ± 0.1 U; P < 0.03). There was no change in the areas of the left or right ventricular lumens, and right ventricular tissue areas were also similar. Examples of control and uraemic hearts in cross section are shown in Figure 2.

Ca²⁺ transients

As we have shown previously that uraemia is associated with an impaired contractile response to external Ca, the intracellular handling of Ca was investigated in single ventricular myocytes isolated from six uraemic and six control animals. Increasing the [Ca²⁺]ᵢ from 1 to 4 mM had no measurable effect on the time taken from stimulation to reach peak [Ca²⁺]ᵢ (Tₚ, Figure 3a). In addition, there was no difference in the size of the transients in each group of cells, suggesting that Ca²⁺ release by the sarcoplasmic reticulum per twitch was similar in control and uraemic cells under these conditions. The time to 50% restoration of diastolic [Ca²⁺]ᵢ (R₅₀) is plotted in Figure 3b. There was a tendency for the removal of Ca²⁺ from the cytoplasm in uraemic cells to be slower with increasing [Ca²⁺]ᵢ. At 80% recovery, a significant slowing of the uraemic calcium transient was demonstrable in 4 mM Ca²⁺ (P < 0.05; Figure 3c). This is shown more clearly in Figure 4, in which single averaged Ca²⁺ transients for one control and one uraemic cell are shown. The traces selected were those closest to the mean R₅₀ values in 4 mM Ca²⁺ for each group, and for ease of comparison the peak amplitudes have been normalized to 1.

Discussion

Using an established model of chronic uraemia in the rat, we have demonstrated both structural and functional cardiac abnormalities at tissue and single myocyte levels. These changes were found after exposure to a moderate biochemical uraemia of 4 weeks duration, with no concomitant change in blood pressure.

An increase in left ventricular tissue area of 22% and in wall thickness of 15%, with no change in left ventricular lumen, indicates significant myocardial remodelling and is suggestive of concentric hypertrophy, despite the fact that we found no evidence of hypertension. These changes are of similar magnitude to our previous observations of single myocyte hypertrophy in uraemic animals where there was a small but statistically significant increase in length (9%) and a 15% increase in cell area, which fell just outside statistical significance [8]. Cardiac hypertrophy per se was not measured directly in that study because it is not possible to isolate single myocytes and preserve tissue for histological analysis from the same hearts.

Clinical studies have found LVH to be an independent determinant of survival in ESRD patients [10]; however, in pre-ESRD, LVH is often already present suggesting that significant cardiac changes/remodelling occur early in the progression of the disease and well in advance of any clinical symptoms. Despite these findings, patients with early renal impairment who are otherwise asymptomatic are rarely assessed for cardiovascular dysfunction by echocardiography or other non-invasive techniques. A major difference between the study presented in this paper and the majority of the literature relating to experimental cardiac hypertrophy and failure is therefore the timing. The degree of uraemia was not advanced in these animals, there was no change in blood pressure, and the duration was only 4 weeks. In most other models of heart disease, animals are allowed to develop clinically significant symptoms before cardiac tissue or myocytes are examined. Therefore, the finding of structural abnormalities in this model of early uraemia is of particular significance. It is possible that there was a loss of

Table 3. Histological analysis of cardiac tissue from uraemic and control animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Total tissue</th>
<th>LV tissue</th>
<th>LV lumen</th>
<th>RV tissue</th>
<th>RV lumen</th>
<th>LV thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 5)</td>
<td>77.5 ± 3.3</td>
<td>46.1 ± 2.2</td>
<td>12.7 ± 1.9</td>
<td>13.1 ± 1.1</td>
<td>5.6 ± 0.8</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>U (n = 5)</td>
<td>88.4 ± 2.7</td>
<td>56.1 ± 3.1</td>
<td>12.2 ± 1.0</td>
<td>13.4 ± 1.2</td>
<td>6.7 ± 0.7</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>P &lt;</td>
<td>0.04</td>
<td>0.04</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.03</td>
</tr>
</tbody>
</table>

LV, left ventricular; RV, right ventricular; ns, not significant. All values are in arbitrary units.
nocturnal reduction in blood pressure in the uraemic animals and that this may have contributed to the hypertrophy described. However, without 24-h telemetric recordings it is not possible to exclude a mild hypertension in these animals. Moreover, the structural abnormalities attributed to uraemia in this study differ from structural changes observed in a rat model of chronic anaemia, which was found to induce eccentric hypertrophy due to ventricular volume overload [16]. This was characterized by increases in both left and right ventricular chamber volumes, whereas our uraemic animals developed concentric LVH (i.e. no increase in chamber volume). Nevertheless, the functional consequences of anaemia in association with uraemia should not be overlooked and warrant further investigation.

We have reported previously that 28 days after 5/6 nephrectomy in rats, resulting in a 4-fold increase in plasma urea and creatinine, there was a significant reduction in cardiac output of 21% [7]. This study utilized a standard isolated working heart technique, and also demonstrated that the uraemic hearts displayed a blunted response to increasing \([\text{Ca}^{2+}]_i\) and a tendency towards failure under these conditions. In addition, \(^{31}\text{P-NMR}\) spectroscopy of these isolated hearts revealed a 32% reduction in phosphocreatine and a reduction in phosphocreatine to ATP ratio of 32%, indicating bioenergetic abnormalities.

We subsequently found differences in the contractile properties of single cardiac myocytes isolated from uraemic rats, again 28 days after 5/6 nephrectomy [8]. Although the degree of uraemia induced in this study was relatively mild, causing a 2- to 3-fold increase in plasma urea and creatinine compared with sham-operated control animals, there was some evidence of cellular hypertrophy. Cells from the uraemic animals showed a reduction in contraction amplitude, and also impaired contraction and relaxation velocities when maximally stimulated with calcium. The tendency for the uraemic cardiac myocytes to exhibit impaired contractile function when compared with control cells

![Graphs](image-url)
suggests that Ca\textsuperscript{2+} handling in the cytoplasm may be altered.

In the present study, intracellular Ca\textsuperscript{2+} transients were assessed in both control and uraemic cells, and changes in Ca\textsuperscript{2+} handling under conditions of increasing [Ca\textsuperscript{2+}]\textsubscript{i} described. Increasing the [Ca\textsuperscript{2+}]\textsubscript{i} to supraphysiological levels is a useful method of stimulating cardiac cells as it acts as an inotrope. The results described provide evidence of altered function of uraemic cardiac myocytes under these conditions. The data complement our previous findings where again at physiological [Ca\textsuperscript{2+}]\textsubscript{i} there was no apparent difference between the groups, but at increased [Ca\textsuperscript{2+}]\textsubscript{i}, contraction amplitude, contraction and relaxation velocities were impaired in uraemic cells [8]. Although the \(T_p\) of the transient was not found to be altered in uraemic cells in this study, suggesting that the rate of release of intracellular stores of Ca\textsuperscript{2+} was not different, this finding should be treated with some degree of caution. The sampling period of the system was 5 ms, and the \(T_p\) measurements were 30–35 ms. Thus, only six or seven measurements are made in this time, and it is possible that the peak value might be missed, falling between sample times.

The restoration of [Ca\textsuperscript{2+}]\textsubscript{i} levels following stimulation was shown to be impaired in the uraemic cells (Figure 3). This was not apparent at physiological [Ca\textsuperscript{2+}]\textsubscript{i} concentrations, but became so as [Ca\textsuperscript{2+}]\textsubscript{i} was increased. The changes in calcium handling of uraemic cardiac myocytes are quite distinct from those due to early hypertension in the spontaneously hypertensive rat (SHR), before the transition to overt cardiac failure [17]. Hypertrophied myocytes from the SHR show an increase in the size of the Ca transient and in the amplitude of contraction when externally stimulated. In contrast, although the induction of uraemia caused LVH, the amplitude of the transient was unaltered under these experimental conditions, and we have found previously the contraction amplitude to be reduced [8]. Our current finding of significant abnormalities in intracellular Ca\textsuperscript{2+} kinetics in individual cardiac myocytes after a relatively short exposure to a moderate uraemic insult does not explain the reduction in contraction amplitude. However, taken together the data suggest that other factors governing excitation-contraction coupling are affected. One possibility is that there may be a desensitization of the cardiac myofilaments to Ca\textsuperscript{2+}. Nevertheless, this work provides further evidence supporting the existence of a specific uraemic cardiomyopathy.

Acknowledgements. The authors thank C. A. Nott for the preparation of cardiac sections for histology, and Drs A. F. James, K. T. MacLeod, and M. M. Yaqoob, and Prof. M. P. Feneley for critical comments on the manuscript. This work was supported by a National Kidney Research Fund Project Grant (A. McM.).

References