Renal failure induces telomere shortening in the rat heart

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ABSTRACT

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INTRODUCTION Renal failure increases cardiovascular risk and aggravates cardiac remodelling induced by myocardial infarction (MI). Telomeres are the sequences of DNA forming the end of chromosomes. Shortening of telomere length in the heart has been associated with ageing, dysfunction of cardiac myocytes and left ventricular failure. We investigated whether moderate and severe renal failure (1) shortens cardiac telomere length and (2) causes excess cardiac telomeric shortening in hearts after an MI.

METHODS Male Wistar rats (n=53) were subjected to none, mild or severe renal failure induced by sham, unilateral (UNX) or 5/6th nephrectomy (5/6NX). In half of the rats, MI was induced at week 2 by ligation of the left coronary artery. Urinary protein excretion, serum creatinine and systolic blood pressure (SBP) were measured shortly before autopsy. Focal glomerulosclerosis was assessed by histological analysis. Heart weight was measured and DNA was isolated from non-infarcted cardiac tissue. Telomere length was assessed by real-time PCR.

RESULTS Proteinuria and FGS remained unchanged in UNX and MI compared to control, but strongly increased in 5/6NX, UNX + MI and 5/6NX + MI. Serum creatinine levels at the end of the study were increased 4-fold in 5/6NX and 10-fold in 5/6NX + MI. Heart weight was increased in both 5/6NX groups and UNX + MI, but not UNX and MI, compared to control. 5/6NX and groups with combined renal ablation and MI showed a similar reduction in telomere length as found after MI, resulting in an approximate 20% reduction compared to control. Consequently, no excess telomere shortening was observed in hearts from rats with renal ablation and an MI.

CONCLUSION We found that severe renal failure, but not mild renal failure, leads to hyperthrophy and shortening of telomeres in the heart.
INTRODUCTION
Cardiovascular complications frequently occur in renal failure patients. These complications may be the consequence of systemic changes induced by renal failure, such as hypertension, anaemia, electrolyte and metabolic disturbances or (coronary) atherosclerosis. However, recent research suggests that renal failure also causes unbeneficial changes of the heart independently from these systemic changes. For example, experimental renal failure has been shown to reduce tolerance of the heart to ischemia, independently from possible confounders, such as hypertension, sympathetic activation or salt retention. This resulted in increased size of myocardial infarction following coronary ligation. Furthermore, experimental renal failure impaired cardiac function and architecture of the healthy heart, demonstrated by a lowered cardiac output and cardiac remodelling as evidenced by left ventricular hypertrophy, interstitial fibrosis and a decreased capillary density.

The process of cardiac remodelling, which leads to impairment of cardiac function, shows similarities with features of cardiac ageing. Recent investigations suggest that in general ageing and impairment of cellular function are associated with shortening of telomeres. Telomeres form the end of chromosomes and consist of specific base pair repeats. Functional telomeres protect the end of chromosomes, thereby preventing loss of genetic information. Telomere length is determined by the balance between loss of DNA base pairs due to erosion or incomplete DNA replication after every cell cycle and the elongation of the telomere by the enzyme telomerase. Telomerase consists of multiple compounds, among which the RNA template TERC and reverse transcriptase TERT. Damage to telomeres or excessive shortening of telomeres lead to genomic instability or activation of cellular damage-responses such as cellular senescence and apoptosis.

Telomere length in the heart is associated with cardiac functioning. In patients with heart failure, mean telomere length was decreased compared to healthy, age-matched controls. Moreover, in vitro data showed overexpression of TERT to suppress telomeric shortening and prevent apoptosis of cardiomyocytes after ischemic injury. Also, increased telomerase activity to enhance telomere length was observed in dogs with progressive heart failure. Furthermore, in vivo data of a study in a mouse model revealed that overexpression of TERT resulted in hyperplasia with subsequent hypertrophy of cardiomyocytes, without development of cardiac fibrosis or loss of cardiac function. Finally, telomeric shortening occurred in telomerase knock-out mice and was associated with left ventricular failure and unbeneficial morphological changes of the heart, i.e. left ventricular dilation.

However, it is not yet known whether cardiac remodelling due to renal function loss is associated with telomeric shortening. In this study, we investigated whether renal failure causes telomeric shortening in the heart and, if so, whether it causes excess telomeric shortening in the already failing heart.

To determine this, we assessed telomere length by means of real time PCR on DNA from cardiac tissue of rats with mild or severe renal failure following renal ablation, with myocardial infarction and in the combination.

MATERIALS AND METHODS
Experimental animals
Male Wistar rats (250-275 g) were housed under standard conditions. During the study, rats received a normal salt diet (0.3% NaCl) and had free access to food and water. Animal experiments were approved by the institutional animal ethical committee.
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Surgical procedures
To induce mild renal failure, rats were subjected to unilateral nephrectomy under anaesthesia with 2.0% isoflurane in O₂/N₂O (1:2). Laparotomy was performed, with subsequent removal of the right kidney after careful separation of the kidney from adrenal gland and surrounding tissue. For severe renal failure, 5/6th nephrectomy was performed on the rats under anaesthesia with 2.0% isoflurane in O₂/N₂O (1:2). The right kidney was removed as described above, with additional interruption of two third of the blood supply of the left kidney, achieved by ligation of the proximal branch of the renal artery. If, after visual inspection, less than two third of the renal perfusion appeared to be cut off, additional smaller branches of the renal artery were ligated or coagulated.
To induce heart failure, rats were subjected to myocardial infarction. Briefly, thoracotomy was performed under anaesthesia with 2.0% isoflurane in O₂. Myocardial infarction (MI) was induced by ligating the left ascending coronary artery. In groups with mild or severe renal failure, additional MI was performed 2 weeks after renal ablation. Sham operations for renal ablation and MI were performed on the control group.
Animals with severe renal failure were sacrificed under 2.5% isoflurane anesthesia in O₂/N₂O (1:2) 12 weeks after renal ablation with measurement of hemodynamic parameters and collection of the heart. Animals with mild or no renal failure were sacrificed 16 weeks after renal ablation or sham operation, following the same procedure as in animals with severe renal failure.

Measurement of systolic blood pressure and renal function parameters
Systolic blood pressure was measured by tail cuff plethysmography (IITC Life Science, Woodland Hills, CA, USA) in awake and restrained animals. Rat tails were heated by placing the tails under a lamp (250W) for 5 to 10 minutes. Elevated temperature was maintained by a 150W lamp. The tail cuff was inflated to a pressure of 200 mmHg and subsequently slowly deflated, while measuring systolic blood pressure of the rats.
Proteinuria was measured in samples of 24-hours urine collected with metabolic cages. For determination of total urinary protein the trichloroacetic acid (TCA) method (Nephelometer Analyzer II, Dade Behring, Marburg, Germany) was used. Plasma creatinine levels were measured using the photometric determination according to the Jaffe method (Ecoline Mega, DiaSys Diagnostic Systems GmbH, Holzheim, Germany).
Kidneys were fixed in formaline for 48 hours and subsequently embedded in paraffin. For analysis of focal glomerulosclerosis (FGS), sections of 3 µm were stained with periodic acid Schiff (PAS). The degree of FGS was assessed in 50 glomeruli by scoring semi-quantitatively on a scale of 0 to 4 originally described by Raij et al. FGS was scored positively when mesangial matrix expansion and adhesion to Bowman's capsule was present in the same quadrant. When 25% of the glomerulus was affected, a score of 1+ was adjudged, 50% was scored as 2+, 75% as 3+ and 100% as 4+. Overall FGS score is expressed as arbitrary units (AU) with a maximum of 200. An examiner blinded for the groups evaluated all sections.

DNA isolation and real time PCR
Genomic DNA was isolated from frozen cardiac tissue according to the manufacturer's instruction (QIAamp DNA Mini Kit, Qiagen). In groups that underwent MI, tissue was taken from the non-infarcted region and corresponding areas were used for the other groups. Briefly, cardiac tissue was cut into
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small pieces and incubated overnight at 56°C in a mixture of lysis buffer and proteinase. Buffer for precipitation was added and this mixture was incubated at 70°C for 10 minutes, followed by centrifugation. Subsequently, 96% ethanol was added to the supernatant and the sample was pipetted on a column and washed twice with washing buffers. Distilled water was used for elution of the genomic DNA. DNA concentration was measured with a spectrophotometer. Real time PCR on telomeres was performed using the method of Cawthon et al.16 in which mismatches in primers exclude the possibility of primer-dimer formation without affecting the hybridization of the primers to the DNA. Real time PCR was performed according to the thermal profile of incubation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 15 seconds and 54°C for 2 minutes. Each reaction consisted of a mixture of real time PCR reagents with a total volume of 50µL, containing 10µL of DNA dissolved in distilled water. Primers used for amplification of telomeres were Tel-1: 5′ggt ttt gag ggt gag ggt gag ggt gag ggt gag ggt gag ggt gag ggt gag-3′ and Tel-2: 5′-tcc cta cta tcc cta tcc cta tcc cta tcc cta-3′.

Quantities of telomeric DNA were normalized to the quantities of the genomic AT1-receptor, also assessed by real time PCR. The thermal profile of this real time PCR consisted of incubation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Primers were: AT1rat-f: 5′-acg tgt tct cag cat cga ccg cta cc-3′ and AT1rat-r: 5′-aga atg ata agg aaa ggg aac aag aag ccc-3′ (Biogen, Malden, The Netherlands).

Statistical analysis
All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by a LSD post hoc test to identify the groups that were different from each other P< 0.05 were considered statistically significant.

Table 1. General and cardiac characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UNX</th>
<th>S/6NX</th>
<th>MI</th>
<th>UNX+MI</th>
<th>S/6NX+MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>465 ± 11</td>
<td>485 ± 16</td>
<td>488 ± 9</td>
<td>472 ± 12</td>
<td>524 ± 18</td>
<td>423 ± 26bc</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132 ± 3</td>
<td>123 ± 3</td>
<td>174 ± 7abc</td>
<td>118 ± 6</td>
<td>137 ± 4</td>
<td>160 ± 7abc</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17 ± 2</td>
<td>19 ± 3</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>326 ± 12</td>
<td>336 ± 5</td>
<td>352 ± 16</td>
<td>352 ± 34</td>
<td>329 ± 7</td>
<td>371 ± 15</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.31 ± 0.05</td>
<td>1.36 ± 0.04</td>
<td>1.57 ± 0.03</td>
<td>1.48 ± 0.06</td>
<td>1.57 ± 0.05</td>
<td>1.85 ± 0.12abcd</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM. UNX, unilateral nephrectomy; S/6NX, 5/6th nephrectomy; MI, myocardial infarction; SBP, systolic blood pressure. #: P< 0.05 versus Control, #: versus UNX, #: versus S/6NX, #: versus MI, #: versus UNX + MI.
Table 2. Renal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UNX</th>
<th>5/6NX</th>
<th>MI</th>
<th>UNX+MI</th>
<th>5/6NX+MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>19 ± 3</td>
<td>22 ± 6</td>
<td>256 ± 36&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>15 ± 5</td>
<td>154 ± 58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>215 ± 45&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>FGS (%)</td>
<td>4 ± 1</td>
<td>5 ± 1</td>
<td>36 ± 4&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>3 ± 1</td>
<td>19 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>43 ± 3&lt;sup&gt;abde&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/L)</td>
<td>18 ± 2</td>
<td>20 ± 2</td>
<td>88 ± 13</td>
<td>29 ± 2</td>
<td>24 ± 1</td>
<td>193 ± 78&lt;sup&gt;abde&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM. UNX, unilateral nephrectomy; 5/6NX, 5/6<sup>th</sup> nephrectomy; MI, myocardial infarction; FGS, focal glomerulosclerosis. <sup>a</sup>: P< 0.05 versus Control; <sup>b</sup>: P< 0.05 versus all other groups.

RESULTS

General characteristics
No mortality was observed in sham or renal ablation groups. MI and UNX + MI resulted in an acute mortality of 20% and 25%, respectively. A higher mortality was observed in 5/6NX + MI, this group showed a 47% overall mortality. Body weight was lower in 5/6NX + MI compared to UNX, 5/6NX and UNX + MI.

Cardiac characteristics
Left ventricular infarct size was not significantly different in the three groups that underwent MI. and no significant differences in heart rate were observed between the groups (table 1). Heart weight was increased in UNX + MI, 5/6NX and 5/6NX + MI compared to control. Heart weight in 5/6NX + MI was even higher compared to the UNX group and the MI group. Systolic blood pressure was increased in the 5/6NX and 5/6NX + MI group compared to all other groups.

Renal characteristics
To evaluate renal impairment after UNX and 5/6NX, we measured plasma creatinine, proteinuria, and focal glomerulosclerosis (FGS), as shown in table 2. Proteinuria was significantly higher in UNX + MI, 5/6NX and 5/6NX + MI compared to control. Furthermore, FGS was increased in UNX + MI, 5/6NX and 5/6NX + MI compared to control. Plasma creatinine was increased in 5/6NX + MI compared to all other groups.

Telomere length
Mean telomere length was decreased by about 20% in rats that underwent MI compared to control (figure 1). In the groups that underwent renal ablation, mean telomere length was decreased in 5/6NX to a similar extent as observed in MI. In contrast, telomere length was not affected in the UNX group. In groups with combined renal ablation and MI, telomere length was reduced to a similar extent as found in groups with either 5/6NX or MI (figure 1).
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**DISCUSSION**

In this study, we found that severe renal failure in the rat led to an increase in heart weight and a shortening of cardiac telomeres, while such changes were absent in animals with mild renal function loss. The cardiac changes observed in severe renal impairment were similar in degree as observed in rats that underwent myocardial infarction. In addition, the combination of severe renal failure and myocardial infarction led to an excess increase of heart weight, but not to an excess shortening of telomeres.

Renal failure is known to cause alterations in heart function and morphology. Other studies have revealed that severe renal failure leads to left ventricle hypertrophy, increased interstitial fibrosis and a decreased capillary density. On the functional level, severe renal failure decreased cardiac output and ischemia tolerance. In our study, we observed severe renal failure to cause a more distinct deterioration of the cardiac condition than mild renal failure. A possible explanation is the more pronounced rise in systolic blood pressure in severe renal failure compared to mild renal failure.

Previous studies have already revealed a role of telomeres in coronary diseases. For example, heart failure patients showed a decreased cardiac telomere length compared to age matched controls. Furthermore, patients with coronary artery disease showed a decreased telomere length in the endothelial cells from coronary arteries compared to age matched control. Telomeres are excessively shortened at atherosclerotic sites. In TERC-null mice, which are unable to express telomerase, accelerated shortening of telomeres and upregulation of the pro-apoptotic protein p53 were observed, which subsequently led to increased apoptosis of myocytes, pathological cardiac remodelling, including ventricular dysfunction with thinning of the myocardium, and eventually sudden cardiac death.

There are strong indications that oxidative stress plays an essential role in the process of telomeric shortening. Oxidative stress occurs when the formation of ROS exceeds the activity of antioxidant mechanisms. Cultured human fibroblasts exposed to mild oxidative stress clearly showed accelerated telomeric shortening, while treatment with an antioxidant minimized telomeric shortening under oxidative stress. Furthermore, overexpression of extracellular superoxide dismutase, an antioxidant
gene, inhibited telomeric shortening in cultured human fibroblasts under both normal cell culture conditions and oxidative stress\textsuperscript{21}. In addition, patients with mitochondrial diseases leading to increased production of reactive oxygen species showed a decreased telomere length of leukocytes compared to healthy, age matched controls\textsuperscript{22}. Together, it is most likely that increased cardiac oxidative stress is responsible for the observed telomeric shortening.

Also in severe renal failure, increased oxidative stress is a likely candidate to convey the cardiac telomeric shortening. Several studies reported increased oxidative stress in animals or humans with chronic renal failure. It was shown in rats that oxidative stress increased after 5/6\textsuperscript{th} nephrectomy and was ameliorated by antioxidant treatment\textsuperscript{23}. Furthermore, it was shown that patients with moderate to severe chronic renal failure, who did not receive renal replacement therapy, had increased levels of oxidative stress markers\textsuperscript{24}. Moreover, increased oxidative damage to DNA was observed in patients with chronic uremia\textsuperscript{6}. These findings might explain the observed telomeric shortening in both severe renal failure groups in our study. Increased oxidative stress in renal failure is believed to be caused by increased circulating levels of angiotensin II, which effectuates upregulation of NADPH oxidase, a major source of production of ROS.

Increased oxidative stress is also observed in the heart after myocardial infarction, and is probably the cause of telomeric shortening in the heart. Increased production of ROS has been reported in post-MI failing hearts due to mitochondrial dysfunction\textsuperscript{25}. Furthermore, ROS are generated by reperfusion during early heart failure in post-MI hearts\textsuperscript{25,26}. Moreover, \textit{de novo} production of angiotensin II in the heart after myocardial infarction represents another source of oxidative stress in the post-MI heart\textsuperscript{27}. To summarize, oxidative stress is likely to play an essential role in the process of telomeric shortening both after MI and in severe renal failure. Oxidative stress is believed to be responsible for other cardiac changes in severe renal failure, such as a decreased capillary density\textsuperscript{28}.

Our study encounters some limitations. First, we determined telomere length from non-infarcted heart tissue. Thus, the determined telomere length does not only reflect mean telomere length of myocytes, but of all cells in heart tissue, including fibroblasts, vascular smooth muscle cells and endothelial cells. However, the number of these cells, and therefore their contribution to total DNA content, is very small compared to the number of myocytes in the heart. Therefore, we postulated that the determined telomere length predominantly reflects mean telomere length of myocytes. Secondly, the severe renal failure groups were sacrificed at week 12 because of the deteriorating clinical condition of the rats, whereas the other groups were sacrificed 4 weeks later. Therefore, we cannot be certain that telomere length would not have decreased more in the severe renal groups if they had been followed for a longer time span. However, if this were true, we merely underestimate the effect of severe renal function loss compared to mild renal function loss.

**CONCLUSION**

We found that severe renal failure, but not mild renal failure, leads to hyperthrophy and telomeric shortening in the heart. These findings might contribute to an explanation for the increased cardiac morbidity and mortality in subjects with severe renal failure.
REFERENCES


