Protein restriction in lactation confers nephroprotective effects in the male rat and is associated with increased antioxidant expression

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1Department of Clinical Biochemistry, University of Cambridge, Addenbrookes Hospital, Cambridge, United Kingdom; and 2Deparments of Nephrology and Hypertension and 3Pathology, University Medical Centre, Utrecht, The Netherlands

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Tarry-Adkins JL, Joles JA, Chen J-H, Martin-Gronert MS, van der Giezen DM, Goldschmeding R, Hales CN, Ozanne SE. Protein restriction in lactation confers nephroprotective effects in the male rat and is associated with increased antioxidant expression. Am J Physiol Regul Integr Comp Physiol 293: R1259–R1266, 2007. First published June 20, 2007; doi:10.1152/ajpregu.00231.2007.—Telomere shortening has been implicated in the aging process and various age-associated disorders, including renal disease. Moreover, oxidative stress has been identified as an initiator of accelerated telomere shortening. We have shown previously that maternal protein restriction during lactation leads to reduced renal telomere shortening, reduced albuminuria, and increased longevity in rats. Here we address the hypothesis that maternal protein restriction during lactation is nephroprotective and associated with increased expression of antioxidant enzymes and decreased age-dependent renal telomere shortening. Newborn rats were suckled by a dam fed either a control (20% protein) or low-protein (8% protein) diet. All animals were weaned onto standard chow. Offspring that had been suckled by protein-restricted mothers had reduced albuminuria, N-acetyl-glucosaminidase, and urinary aldosterone excretion. These animals also did not show significant age-dependent renal telomere shortening and hence had significantly longer telomeres at 12 mo of age. This lack of renal telomere shortening was associated with increased levels of the antioxidant enzymes manganese superoxide dismutase, glutathione peroxidase, and glutathione reductase. These findings suggest that beneficial effects of slow growth during lactation are associated with increased antioxidant capacity and prevention of age-dependent telomere shortening in the kidney.

renal disease; albuminuria; telomeres; antioxidant enzymes

PREVALENCE OF CHRONIC renal disease (CRD) and resultant end stage renal failure is threatening to reach epidemic proportions over the next decade (30) with CRD rates growing at 8% per annum (1). Many strategies have been shown to prevent progression of renal disease, including the control of proteinuria (1). In cases of renal disease, postnatal protein restriction has been demonstrated to be effective in nephropathogenesis in both adult rats (4, 20) and humans (14). However, the possible renoprotective effects of protein restriction during specific time periods, such as lactation, are not clear. In our laboratory, a rat model of protein restriction during lactation, whereby rat pups born to dams fed a 20% protein diet were cross-fostered to 8% protein-fed mothers until weaning, produced offspring with a normal birth weight that grew slowly during lactation (22). When weaned onto standard laboratory chow, these animals had significantly increased longevity (22) and reduced albuminuria throughout life (33) compared with the control offspring of mothers fed a 20% protein diet during pregnancy and lactation.

Beneficial effects of reduced nutrition and, consequently, slow growth during lactation have been demonstrated in human studies. These have primarily been observed in studies comparing the long-term effects of formula feeding compared with breast-feeding. The latter, in general, is associated with a lower plane of nutrition and, consequently, slower growth during suckling. Studies have also shown that formula-fed infants are more overweight later in childhood than breast-fed infants (18). Studies of premature babies randomly fed infant feeding formula or breast milk have also demonstrated that those that were breast-fed had reduced atherosclerotic risk factors later in childhood (38). Moreover, slower growth during the first 2 wk of life has been suggested to be particularly beneficial in this low-birth-weight population (39).

The mechanisms by which nutrition and growth during the suckling period impact on long-term health and longevity is unknown. However, our previous studies have suggested that effects on the rate of telomere shortening may play a role (22, 23). Telomeres have been implicated in the aging process for a number of years (2), and it has been suggested that telomere shortening may be linked to age-associated diseases, including kidney dysfunction (17). Oxidative stress has been implicated in accelerated telomere shortening, as oxidative damage is less well repaired in telomeric DNA than elsewhere in the chromosome (36, 46). Three major antioxidant enzymes in the oxidative stress defense network are manganese superoxide dismutase (MnSOD), glutathione peroxidase (Gpx), and glutathione reductase (GR). MnSOD is located predominately in the mitochondria and is essential for life as demonstrated by the neonatal lethality of MnSOD-deficient mice (27). In addition, mice expressing 50% normal levels of MnSOD show increased susceptibility to oxidative stress and severe mitochondrial dysfunction due to increased reactive oxidative species (27). However, the association between MnSOD, increased oxidative stress, and lifespan has been not shown in all studies (44). Moreover, MnSOD overexpression has been shown to be protective in cisplatin-induced renal injury (9). Gpx is particularly important in end stage renal disease with significant reduction of this antioxidant enzyme reported in patients with diabetic nephropathy and chronic renal failure and in those undergoing hemodialysis (7, 13, 26). GR-deficient mice sub-
Animals

All of the procedures involving animals were conducted under the British Animals (Scientific Procedures) Act (1986) under license (PIL80/8594). Wistar rat dams were placed on an ad libitum standard laboratory chow diet (20% protein) and water until pregnancy was confirmed through observation of vaginal plugs. When pregnancy was confirmed, dams were fed either a 20% normal protein isocaloric diet (control) or an isocaloric low-protein (LP) (8%) diet. Both of these diets were purchased from Arie Blok, Woerden, The Netherlands, and the detailed dietary composition has been previously described (40). Cross-fostering techniques were then used to generate protein-restricted offspring during lactation. The postnatal protein restriction (PLP) group was offspring of rat dams fed the control diet during pregnancy and then nursed by rat dams fed an LP (8%) diet. The control group was control-fed offspring that were suckled by the same control-fed rat dams. To prevent any stress or rejection to the animals involved in cross-fostering, the pups were transferred with some of their own bedding. No pup rejection or stress was observed following this procedure. All animals were weaned onto a standard diet containing 20% protein (SDS, Witton, UK) from 21 days of age and remained on it until the end of the study. Mean litter weights were recorded between days 3 and 21.

Because we have recently documented accelerated telomere shortening and more albuminuria in male than in female rats (42), the present study was performed in males. One male from each litter was maintained until 3 mo of age and then killed. Immediately after death, the kidneys were removed. The left kidney was visually dissected into cortex and medulla. Tissue was snap frozen in liquid nitrogen and stored at −80°C until required for analysis. A second male from each litter was killed at 12 mo of age when blood, left kidney (cortex and medulla), and the whole right kidney was collected for histology. Twenty-four hour urine samples were also taken at 1, 3, 5, 7, and 9, and 12 mo of age. During urine collection, rats were housed in metabolic cages with free access to chow and water for 24 h.

Telomere Length Measurement

Nonsheared, high-molecular size DNA (average size 97 kb) was isolated from the cortex and medulla by using a commercial kit for DNA extraction using the protocol provided by the manufacturer (Qiagen). DNA quantity and integrity were determined using a spectrophotometer (GeneQuant; Pharmacia Biotech). DNA (1.2 μg) was digested with HinfI and RsaI restriction enzymes (6, 42). The digested DNA was then separated using pulsed-field gel electrophoresis (6, 42). After electrophoresis, the gels were checked for nonspecific degradation of the undigested DNA and complete digestion of the digested DNA by staining with ethidium bromide. The gels were visualized using an Alpha Imager UV light source (Alpha Innotech) and photographed with a P/N Polaroid film. The separated DNA fragments were transferred by Southern blot analysis onto nylon-positive membranes (Roche Diagnostics), and telomeric repeat length was determined using a slightly modified commercial method of chemiluminescent detection (42). The telomeric signals were analyzed using Adobe Photoshop and MacBas computer software. Molecular weight markers used per gel were a midrange pulsed-field gel marker (New England Biolabs), and dihydrogen (low-range) molecular weight marker (Roche Diagnostics). Standard undigested and digested genomic control DNA was run on each gel to demonstrate the efficiency of the digestion and to minimize any intergel differences. Gels were run in duplicate to produce average %telomere length values. Each gel was accepted on the criteria that the %telomere length of the control DNA in any of the four telomeric regions analyzed (1.3–4.2 kb, 4.2–8.6 kb, 8.6–48.5 kb, and 48.5–145 kb) was <1.5 SD from the mean.

Telomere length was measured as described previously, whereby the percentage of intensity (%telomere length) of the telomeric signal was determined in four molecular size regions as defined by molecular weight markers. Specific grid squares were placed around the telomeric smear according to the previously detailed molecular weight regions. %Telomere length (expressed as %photo-stimulated luminescence) was measured (6, 42).

Antioxidant Enzyme Quantification

Western blot analysis was used to determine expression levels of MnSOD, Gpx-1, and GR in the cortex and medulla of the same animals. Protein was extracted by hand homogenization of 1 mg of renal tissue in 1 ml TK lysis buffer [50 mM HEPES, 150 mM NaCl, 2% Triton X-100, 100 mM Na Orthovianadate, 500 mM NaF, 100 mM Na3P04·12H20, 500 mM EDTA (pH8)], and the resultant supernatant was then assayed by using copper sulfate and bicinchoninic acid (Sigma-Aldrich, St. Louis, MO). Protein (20 μg) was loaded onto 10% polyacrylamide gels, electrophoresed, and transferred to polyvinylidene difluoride membrane (42). MnSOD was detected using anti-MnSOD (type II)-specific rabbit IgG (Abcam), Gpx-1 was detected using anti-Gpx-1-specific rabbit IgG (Abcam), and an anti-GR-specific rabbit IgG (Abcam) was used to measure GR. To check linearity and reproducibility, a standardization gel consisting of one sample (S1–20 μg protein), 50% of the same sample S1 (50%), and a further 20 μg protein from a different sample (S2) were loaded five times onto one gel. Each gel was then loaded with 20 μg protein per sample plus the same S1, S1 (50%), and S2 samples. Gels were accepted on the criterion that the ratios of S1/S1 (50%) and S1/S2 were less than the mean integrated density value (arbitrary units) of the standardization gel ± 1.5 SD.

Urine and Serum Analysis

Albuminuria was measured by using a rat-specific enzyme-linked immunoassay (IDS; Spi-Bio). Results were expressed as milligram per day. NAG activity was measured by using a colorimetric method (PPR Diagnostics). Results were expressed as micromole per day. Urine and serum aldosterone were measured by using a goat anti-mouse IgG immunoassay kit (Cayman Chemicals). Results were expressed as nanograms per day and picograms per day, respectively. Urine and plasma creatinine were measured by the Jaffe method (Sigma, St. Louis, MO).

Histological Assessment

The formalin-fixed right kidneys from 12-mo-old rats were embedded in paraffin blocks and microtomed into 4-μm-thick sections, stained with PAS and blindly analyzed by an independent histopathologist. Fifty glomeruli were scored per rat for proliferation, matrix expansion, sclerosis, and adhesion in quadrants with 0 indicating no
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change and 4 indicating a change in all quadrants. A score of 1 or 2 was regarded as partial focal glomerulosclerosis (FGS) and a score of 3 or 4 as total FGS. Incidence of FGS (partial or total) is expressed as a percentage.

Statistical Analysis

Telomere length, MnSOD levels, and aldosterone excretion were analyzed by using a factorial two-way ANOVA with maternal diet and age as the independent variables, followed by Duncan’s post hoc testing where appropriate. Albuminuria and NAG excretion were analyzed using a repeated-measures two-way ANOVA with maternal diet and age as the independent variables. Student-Newman-Keuls post hoc testing was used for the albumin and NAG excretion data. All unpaired data (histological assessment) were analyzed using Student’s t-test. All data were represented as mean ± SE, unless stated otherwise. Albuminuria and NAG excretion were represented as log-transformed data. In all cases, a P value < 0.05 was considered statistically significant.

RESULTS

Physical Characteristics

Average litter weights and sizes and body weights. No significant difference in litter weight at birth or litter size was seen between groups (data not shown). The PLP group, which had birth weights similar to the control animals, became significantly (P < 0.001) lighter than the control group by day 7 and remained smaller throughout lactation (Table 1). These differences remained with the PLP animals being significantly (P < 0.001) lighter than controls until the end of the study (Fig. 1).

Kidney weights. The PLP animals showed significantly (P < 0.001) reduced absolute kidney weights at both 3 and 12 mo of age. However, relative kidney weights remained similar between groups at both ages (Table 2).

Urine and Serum Analysis

Albuminuria. The PLP animals were significantly less albuminuric compared with the control group at 5, 7, 9, and 12 mo of age (P < 0.05 to P < 0.01) (Fig. 2A). Urinary albumin excretion significantly (P < 0.001) increased with age in both groups.

Urinary NAG excretion. Significantly (P < 0.001) reduced levels of urinary NAG were found in PLP compared with control at 1 mo of age (Fig. 2B). An initial significant (P < 0.001) rise in urinary NAG excretion was observed between 1 and 3 mo of age in both groups.

Serum aldosterone concentration and urinary aldosterone excretion. There was a significant effect of maternal diet on urinary aldosterone (P < 0.05) with levels being reduced in the PLP group (Fig. 3A). There was also a significant effect of age (P < 0.001) with levels increasing with age in both groups. There was a significant interaction between maternal diet and age (P = 0.041) with urinary aldosterone increasing more with age in the control animals. At both 3 and 12 mo of age no significant difference in serum aldosterone was observed between groups (Fig. 3B), and no significant difference in serum aldosterone was seen between 3 and 12 mo of age.

Creatinine parameters. At 12 mo of age there were no significant differences in creatinine excretion (corrected for body weight) (18 ± 4 μmol·l·100 g body wt<sup>-1</sup>·24 h<sup>-1</sup> vs. 25 ± 2 μmol·l·100 g body wt<sup>-1</sup>·24 h<sup>-1</sup>), plasma creatinine (28 ± 3 μmol/l vs. 31 ± 2 μmol/l) or creatinine clearance (971 ± 228 μl·100 g body wt<sup>-1</sup>·min<sup>-1</sup> vs. 1,174 ± 75 μl·100 g body wt<sup>-1</sup>·min<sup>-1</sup>) between control and PLP groups, respectively.

Telomere Length Data

Effect of maternal diet. At 3 mo of age, no significant difference in kidney cortical telomere length was seen between any groups. However, by 12 mo, significantly (P < 0.05) fewer short telomeres (1.3- to 4.2-kb size range) were observed in the kidney cortex of control animals at 12 mo compared with 3 mo. In contrast, no significant difference in telomere length was observed in the medulla region at 3 or 12 mo of age (Fig. 4B).

Effect of age. Significantly (P < 0.05) more short (1.3–4.2 kb; 21.6 ± 2.2% vs. 15.8 ± 2.1%) and significantly (P < 0.01) fewer long (48.5–145 kb; 17.9 ± 1.5% vs. 24.2 ± 1.5%) telomeres were found in the kidney cortex of control animals at 12 mo compared with 3 mo. In contrast, no significant difference in telomere length was observed in the cortical region of the PLP group at 12 mo compared with 3 mo (48.5–145 kb; 20.4 ± 1.3% vs. 23.5 ± 2.8%) and (1.3–4.2 kb; 14.9 ± 1.9% vs. 11.3 ± 2.3%) 12 mo compared with 3 mo (Fig. 4A). In the medulla, no significant difference in telomere length was observed at 12 mo compared with 3 mo (Fig. 4B).

Table 1. Effect of lactation protein restriction on preweaning body weights of male rats

<table>
<thead>
<tr>
<th>Birth</th>
<th>3 Days</th>
<th>7 Days</th>
<th>14 Days</th>
<th>21 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.6±0.1</td>
<td>7.7±0.1</td>
<td>17.0±0.3</td>
<td>35.4±0.7</td>
</tr>
<tr>
<td>PLP</td>
<td>7.2±0.3</td>
<td>7.4±0.3</td>
<td>11.3±0.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>17.4±1.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SE in grams of body weight; n =15 per group (1 pup per litter). PLP, postnatal protein restriction. Data were collected at birth, 3, 7, 14, and 21 days of age. *P < 0.001 vs. control.

Table 2. Effect of lactation protein restriction on absolute (g) and relative kidney weights at 3 and 12 mo of age

<table>
<thead>
<tr>
<th>Absolute Kidney Weight, 3 mo</th>
<th>Relative Kidney Weight, 3 mo</th>
<th>Absolute Kidney Weight, 12 mo</th>
<th>Relative Kidney Weight, 12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6±0.03</td>
<td>0.40±0.01</td>
<td>2.1±0.07</td>
</tr>
<tr>
<td>PLP</td>
<td>1.2±0.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.37±0.01</td>
<td>1.6±0.07&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SE; n =12 rats per group. *P < 0.001 (vs. control).
Antioxidant Enzyme Data

All protein expression data are expressed as a percentage of the mean value of 3-mo cortex or medulla samples as appropriate.

MnSOD. No significant difference in MnSOD protein expression levels were observed at 3 mo of age in either cortex or medulla regions between the two groups. However, by 12 mo, significantly \( (P < 0.05) \) more MnSOD protein was detected in the cortex of the PLP group compared with controls (Fig. 5A). In the medulla, at 12 mo of age no significant difference in MnSOD levels was observed (data not shown). There was no effect of age on MnSOD expression.

Gpx-1 and GR. At 3 mo, significantly \( (P < 0.01) \) increased Gpx-1 and GR protein expression levels were observed in the cortex of the PLP group compared with controls; however, by 12 mo, levels of Gpx-1 and GR were similar between groups (Figs. 5, B and C). In the medulla region, no significant difference at either age was seen between control and PLP groups (data not shown). There was a significant interaction between maternal diet and age with a significant upregulation of Gpx-1 and GR in the control group between 3 and 12 mo; however, Gpx-1 and GR levels in the PLP group remained stable between ages.

Histology of the Renal Cortex

Scoring for FGS revealed no statistical differences in either partial \( (11.5 \pm 2.0\% \text{ vs. } 8.5 \pm 1.1\%) \) or total FGS \( (9.5 \pm 2.3\% \text{ vs. } 4.9 \pm 1.0\%) \) between control and PLP groups, respectively. Representative sections are shown in Fig. 6. Histological analysis was not performed on the renal medulla due to sample insufficiency.

DISCUSSION

The aim of this study was to investigate nephroprotective effects of protein restriction during lactation in male Wistar rats and to explore associated mechanisms. The kidney is a major organ of interest in the rat, as it has been reported that the most common cause of death among laboratory rats is renal failure (21). We have previously shown that male rats that were protein restricted during lactation lived significantly longer than controls (22) and had significantly reduced albuminuria over aging (33). We explored the potential mechanisms of this...
by examining telomere length, renal function, and antioxidative stress enzymes.

Consistent with previous findings, PLP offspring were of similar birth weight to controls but grew slowly during lactation so that they were significantly smaller than the controls by day 7. These body weight trajectories were maintained throughout the study. In absolute terms, the PLP group demonstrated significantly smaller kidneys at both 3 and 12 mo; however, there was no significant change in renal weight when expressed relative to body weight.

No effect of maternal diet was seen in renal telomere length at 3 mo of age. By 12 mo of age, significantly fewer short (1.3–4.2 kb) cortical telomeres were observed in the PLP group compared with controls. Significant difference in the smallest telomere length may be particularly relevant, as it has been shown that the shortest telomere, not the average telomere length, is critical for cell viability and chromosome stability in telomerase null mice (19). The significant effect of maternal diet on telomere length was only observed in the cortex region of the kidney and not in the medulla. This interesting regional disparity suggests that cortical telomeres may be more susceptible to the effects of maternal diet than those in the medulla. This interesting regional disparity suggests that cortical telomeres may be more susceptible to the effects of maternal diet than those in the medulla.

Telomere shortening with age has also been shown to be greater in the kidney cortex than the medulla in both humans (29) and rats (42). This may be linked to differences in mitochondrial density (28) and, consequently, oxidative damage between the two regions.

It is well known that oxidative stress can accelerate telomere shortening (24, 46), and, as the kidney is one of the most metabolically active of all tissues (43), we measured levels of three major antioxidant enzymes in both renal regions at 3 and 12 mo of age. MnSOD is a mitochondrial antioxidant enzyme responsible for the dismutation of $O_2^-$ into $H_2O_2$. Gpx-1 and GR are integral components of the thioredoxin system (43). At 3 mo, significant upregulation of cortical Gpx-1 was seen in the PLP group, suggesting that the PLP group could be more efficient in catalyzing $H_2O_2$ into $H_2O$ and $O_2$. Moreover, significant upregulation of cortical GR in the PLP group...
It is established that aldosterone plays a critical role in many forms of renal disease (10), and oxidative stress is thought to play a key role in the development of aldosterone-induced renal injury. The reduced urinary aldosterone at both ages in PLP animals may be again indicative of these animals being subjected to less renal injury and oxidative stress. Because serum aldosterone was not significantly different between groups at either 3 or 12 mo of age, differences in urinary aldosterone excretion could be due to local synthesis of aldosterone in the kidney. Thus it is possible that the reduction of aldosterone excretion in the PLP group is related to renal protection. Overall renal function, as measured by plasma creatinine and creatinine clearance revealed no significant effect of maternal diet. We believe this is due to hyperfiltration of the nonsclerotic glomeruli in the control group, which is known to enhance albuminuria (41) and eventually lead to renal dysfunction. Indeed, the male Wistar rat strain was shown to develop slowly evolving glomerular injury by 19 mo of age (3), suggesting that at a later time point stronger differences in renal injury and kidney function may have become apparent.

In summary, these data suggest that the increased longevity of the postnatal growth-restricted offspring may be related to the absence of significant age-associated reduction of long cortical telomeres that were present in the control animals. This may partially be explained by the significant upregulation at 12 mo of cortical MnSOD that may act as a protective mechanism to preserve cortical renal telomere length in the older PLP animals. Increased levels of Gpx-1 and GR in the PLP group at 3 mo suggest that protein restriction during the suckling period
exerts a mild oxidative stress to which Gpx-1 and GR are upregulated as a protective mechanism, akin to the idea of the hormesis hypothesis, recently proposed to explain the protective effects of caloric restriction (34, 37). Reduced levels of urinary aldosterone in the PLP group may also indicate that the PLP animals experience less renal damage with age as aldosterone has been demonstrated to play a key role in structural renal injury, extracellular matrix accumulation, and renal fibrosis (5, 25). In addition, aldosterone has been shown to stimulate oxidative stress (5) and an aldosterone antagonist has also been shown to increase expression of MnSOD, copper-zinc SOD, and catalase antioxidant enzymes, suggesting oxidative stress protection (32). It is also known that aldosterone can induce renal glomerular dysfunction leading to albuminuria (15). Throughout aging, the PLP animals have reduced albuminuria and NAG excretions compared with the control group, which may be reflective of the PLP animals experiencing less renal damage in terms of less glomerular and proximal renal tubule damage. The renoprotective effect of lowering protein intake in adult rats in the course of renal injury is well known (4, 20). However, such pronounced effects when restricted to the lactation period are a novel finding.

We therefore propose as a working hypothesis that reduced albuminuria and lower NAG excretion is reflective of reduced renal injury in terms of reduced glomerular and proximal renal tubule damage, suggesting that renoprotection may be present in the PLP group. The upregulation of MnSOD, Gpx-1, and GR antioxidant enzymes may also suggest that the PLP group confers oxidative stress protection. Moreover, the upregulation of antioxidant enzymes and fewer renal cortex telomeres may explain the observed increased longevity of rats, which grew slowly during the lactation period.

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GRANTS

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