Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity

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Tarry-Adkins, Jane L., Susan E. Ozanne, Anthony Norden, Hanane Cherif, and C. Nicholas Hales. Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. Am J Physiol Renal Physiol 290: F509–F516, 2006. First published September 27, 2005; doi:10.1152/ajprenal.00215.2005.—It is well documented that females live longer than males and more renal damage occurs in males. However, the underlying mechanisms are not fully understood. The aim of this study was to define aging effects on albuminuria and kidney telomere length from male and female rats and to determine mechanisms, which may explain any observed differences. Cellular senescence is known to play a major role in nephropathology, and as such, a range of senescence markers were compared in male and female renal tissue. Oxidative stress has been shown to accelerate telomere shortening and elicit cellular growth arrest. Thus major antioxidants, MnSOD, glutathione peroxidase I, and glutathione reductase, were also evaluated. Urinary albumin excretion increased with age in both sexes, but the increase was greater in males than females. In the cortex and medulla of both male and female rats, age-related telomere shortening occurred, the effect being more pronounced in males than in females. The cortical region had more short telomeres than the medulla in both genders. p53 And p21 expression over time significantly increased in males, but not in females. MnSOD expression was elevated in female vs. male cortex. Gpx1 and glutathione reductase levels were increased in the older female cortex compared with males. Our findings indicate that a reduction in oxidative damage protection may be responsible for accelerated telomere shortening over time, resulting in increased cellular senescence, loss of renal function, and death in male rats.

telomeres; senescence

THE HUMAN AGING PROCESS IS associated with a decline in renal function (6). However, there is a marked gender disparity in the severity of renal dysfunction associated with aging. Men suffer from a more rapid progression of chronic renal disease than women (28), and men with chronic renal disease of various etiologies show a more rapid decline in renal function with time than do women (23). Urinary albumin excretion, an index of renal damage (24), is significantly elevated in human males compared with females (30).

In our studies of factors relating to longevity in rats, we observed that male rats die younger than females (10) and noted that male rats most frequently die of renal failure (9). Therefore, we focused on changes in the kidney as a possible cause of this difference in longevity. We showed that rat kidney telomeres, the functional ends of chromosomes consisting of G-rich sequences of TTAGGG single-stranded DNA, shorten with age (5, 14). Moreover, we demonstrated that male rats have shorter renal telomeres than females, a disparity which increases with age (5). We speculated that age-related telomere shortening may be an important factor for determining loss of renal function with age and hence, in the male, longevity (14). Studies using human renal cortex and medulla have also shown that telomeres shorten with age and that the shortening is most prominent in the cortical region (20). Oxidative damage can accelerate telomere shortening (34), and female rats have higher mitochondrial antioxidant gene expression and lower oxidative damage than males (4).

The aim of this study was therefore to investigate mechanisms, which may provide an insight into differences in telomere length observed between male and female rats, through analysis of senescent marker expression profiles and antioxidant enzymes, as well as comparison of renal dysfunction end points, such as albuminuria. In addition, telomere length, senescent proteins, and antioxidant enzymes were compared between cortical and medullary regions in these animals.

MATERIALS AND METHODS

Animals

All the procedures involving animals were conducted under the British Animals (Scientific Procedures) Act (1986) and in accordance with the Ethical Review Committee of The University of Cambridge. Male and female Wistar rats used in this study had free access to standard laboratory chow and water and were housed individually at 22°C on a controlled 12:12-h light-dark cycle. Three age groups of male and female animals, from mothers fed a standard laboratory chow, were studied: 3, 7, and 15 mo (n = 6 per group). At each time point, the males and females used were littermates.

The animals from each group were placed in metabolic cages for 24-h urine collections approximately 3 days before being killed at the relevant time point (3, 7, or 15 mo). The urine samples were snap-frozen in liquid nitrogen and stored at −80°C until required for urinalysis. Immediately after the animals were killed, the kidneys were removed and visually dissected into cortex and medulla regions. The tissue was snap-frozen in liquid nitrogen and stored at −80°C until required for DNA extraction and analysis.

Telomere Detection

Nonsheared, high-molecular-size DNA (average size 97 kb) was isolated from the cortex and medulla tissue samples based on a commercial method of DNA extraction (Qiagen) (5). DNA quantity and integrity were determined spectrophotometrically (GeneQuant; Pharmacia Biotech). DNA (1.2 μg) was digested with HinfI and RsaI restriction enzymes (16.6 U/μg DNA, Roche Diagnostics) for 2 h at...
37°C. The digested DNA was then separated using Pulsed Field Gel Electrophoresis (PFGE) (Chef-DR III; Bio-Rad, Hercules, CA) for 9 h at 6 V/cm, with a switching time of 1–30 s.

Controls used in each gel were a midrange Pulsed Field Gel (PFGE) marker (New England Biolabs), the same undigested and digested DNA sample and a dioxygenin (DIG; low range) molecular weight marker. At each time point analyzed, cortex and medulla from the same animal and male and female samples were compared with each other. Each gel was run in duplicate to produce average percent telomere length values. To minimize any intergel differences, the control DNA samples were loaded onto each gel. Gels were accepted on the basis that the percent telomere length of the control DNA in any of the four telomeric regions analyzed was <1.5 SD from the mean.

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 P/N Polaroid film.

The separated DNA fragments were transferred by Southern blotting onto a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) as described elsewhere (5). The transferred DNA was then cross-linked onto the nylon membrane using a UV Stratallinker (TM 2400; Stratagene).

Telomeric repeat length was determined using a modified commercial method of chemiluminescent detection (Roche Diagnostics). The blotted DNA fragments were hybridized to a 5′-TTAGGG-3′ DIG-labeled probe specific for telomeric repeats. Hybridization was carried out in a 42°C water bath for 3 h, and blots were washed according to the manufacturer’s recommendations. The telomere probe was visualized by chemiluminescence following incubation with an alkaline phosphatase-linked DIG-specific antibody. The addition of a highly sensitive alkaline phosphatase substrate produced a chemiluminescent signal. The chemiluminescent signal was detected on Hyperfilm ECL (Amersham Pharmacia, Amersham, UK). The telomere signals were analyzed using Adobe Photoshop and MacBas computer software.

**Telomere Length Analysis and Quantification**

Telomere length was measured using our novel method of analysis whereby the percentage 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 following molecular weights: 112–48.5, 48.5–8.6, 8.6–4.2, and 4.2–1.3 kb. Percent telomere length, expressed as Photo Stimulated Luminescence (PSL), was measured as previously (5).

**Senescent Marker and Antioxidant Quantification**

Western blot analysis was used to determine expression levels of p53, p21\(^{WAF-1}\), MnSOD (manganese superoxide dismutase), glutathione peroxidase 1 (Gxp 1), and glutathione reductase in the cortex and medulla of the same samples and from different animals. Optimal protein-loading experiments showed that glutathione reductase required 15 μg protein; all the other antibodies needed 30 μg protein. All protein was loaded onto 10% polyacrylamide gels, electrophoresed for ~4 h at 150 V, and transferred to Immobilon-P membrane (Millipore). p53 was detected using anti-human/mouse/rat polyclonal p53-specific goat IgG (R&D Systems), an anti-mouse monoclonal p21\(^{WAF-1}\) antibody (Biosource) was used for p21 \(^{WAF-1}\) detection, and anti-MnSOD (type II)-specific rabbit IgG (Upstate Biochemical) was used for MnSOD detection. Anti-Gxp1 and anti-glutathione reductase-specific rabbit IgGs were used for detection of Gxp 1 and glutathione reductase (Ab-Cam). To check linearity and reproducibility, a standardization gel, consisting of one sample (S1–30 μg protein), 50% of the same sample S1 (50%), and a further 30 μg protein from a different sample (S2), were loaded five times onto one gel. Protein concentrations for glutathione reductase were 15 μg for S1 and 7.5 μg for S2. Each gel was then loaded with 30 μg protein per sample plus the same S1, S1 (50%), and S2 samples, and 20 μl mcf-7 cell lysate (Santa Cruz) as a positive control for the p53 blots. Gels were accepted on the criterion that the ratios of S1/S1 (50%) and S1/S2 were less than the mean initial distribution volume of the standardization gel ± 1.5 SD.

**Urinalysis**

Albuminuria was measured using a rat-specific enzyme-linked immunosassay (IDS-Spi-Bio) in urine from the same animals that underwent telomere length analysis. Results were expressed as milligrams per 24 hours.

**Statistical Analysis**

Telomere length, senescent marker, and antioxidant data were analyzed using a three-way ANOVA with gender, age, and region (cortex and medulla) as the independent variables, followed by Duncan’s post hoc test. The data were represented as means ± SE. Albuminuria data were analyzed using the Kruskal-Wallis nonparametric test. The data were expressed as median values ± interquartile ranges. For all data sets, a P value <0.05 was considered statistically significant.

**RESULTS**

**Albuminuria Data**

In both male and female rats, 24-h albuminuria levels increased with age (Fig. 1). In the males, at 7 mo, the 24-h urinary albumin excretion increased significantly compared with 3 mo (P < 0.05). Further significant increases in albuminuria were observed between 7 and 15 mo in the male animals (P < 0.001). Female rats showed a similar pattern of increased albuminuria with age; however, the increase was shallower than observed in the male animals with no significant increase seen between 7 and 15 mo of age. Additionally, by 15

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**Fig. 1.** Effect of gender on 24-h albuminuria in 3-, 7-, and 15-mo (m) rats (n = 6). Urinary albumin excretion per 24 h was quantified as described in MATERIALS AND METHODS. Results are expressed as medians ± interquartile ranges. Effect of age: *P < 0.05, ***P < 0.001 vs. 7 mo. Effects of gender: P < 0.05 at 15 mo.
mo, the male animals were significantly more albuminuric compared with the females (*P < 0.05).

Telomere Length Data

Effect of age. In both male and female rats, cortex and medulla telomeres decreased in length with age (Fig. 2, A-D). At 7 and 15 mo, male rats had significantly more short (4.2–1.3 kb) telomeres compared with 3 mo (*P < 0.001). Further shortening was observed between 7 and 15 mo, in both cortical (*P < 0.001) and medullary (*P < 0.01) regions of the kidney. Although female rats exhibited significant renal telomere shortening with age, the effect was less pronounced than in the males; this was most evident in the cortex between 7 and 15 mo, whereby a significant reduction (*P < 0.05) of long (112.5–48.5 kb) telomeres was seen in males compared with females.

Effect of gender. Male rats had more short cortical telomeres (4.2–1.3 kb) compared with the female animals at 3 mo (*P < 0.01), 7 mo (*P < 0.001), and 15 mo (*P < 0.001). Similar observations were made in the medulla, but the differences only reached statistical significance at 7 mo (*P < 0.05) and 15 mo (*P < 0.001) of age. The greatest difference in telomere length between males and females was observed at 15 mo of age. Males at 15 mo showed substantially more short (4.2–1.3 kb) telomeres than females in both cortex (24.9 ± 1.6 compared with 18.8 ± 0.6%; *P < 0.001) and medulla (20.7 ± 1.2 compared with 14.8 ± 0.7%; *P < 0.01).

Effect of region. The cortex had more short telomeres (4.2-to 1.3-kb range) compared with the medulla in all age groups studied in both genders. At 3 mo, the results were males (12.6 ± 1.1 compared with 7.8 ± 0.8%; *P < 0.01), females (8.5 ± 1.0 compared with 5.8 ± 0.8%; *P < 0.05). At 7 mo, the results were males (18.9 ± 0.4 compared with 13.1 ± 0.9%; *P < 0.01), females (14.0 ± 0.6 compared with 10.2 ± 0.9%; *P < 0.01). At 15 mo, the results were males (24.9 ± 1.6 compared with 20.6 ± 1.2%; *P < 0.01) and females (18.8 ± 0.6 compared with 14.8 ± 0.7%; *P < 0.01).

p53 Expression Data

As the cortical values of p53 expression in male and female rats at 3 mo of age were similar, expression values were normalized to the 3-mo male cortical mean value for the male data set, and the female data set was normalized to the 3-mo female cortical mean value. In both renal regions, no significant difference in p53 expression was observed between 3 and 7 mo in either gender. However, between 7 and 15 mo, a striking gender disparity was observed in both cortex and medulla. In the males, significant (*P < 0.001) elevation in p53 expression was observed between 7 and 15 mo, whereas no significant difference was observed in the females between these ages (Fig. 3, A-D). No significant difference in p53 expression was observed between cortex and medulla regions at any time point, in either gender.

p21 Data

As the cortical values of p21 expression in male and female rats at 3 mo of age were similar, expression values...
were normalized to the 3-mo male cortical mean value for the male data set, and the female data set was normalized to the 3-mo female cortical mean value. In male rats, there was no difference in p21 expression between 3 and 7 mo; however, between 7 and 15 mo, p21 expression was significantly (P < 0.05) elevated. Conversely, p21 expression in the females did not significantly increase over time (Fig. 4, A-D).

Fig. 3. Effect of age on p53 expression profile in the cortex of (A) male and (B) female rats and the medulla of (C) male and (D) female rats (n = 6). Protein was extracted and p53 expression levels were determined by Western blot analysis as described in MATERIALS AND METHODS. Results are expressed as means ± SE. IDV, initial distribution volume. ***P < 0.001 vs. 7-mo animals.

Fig. 4. Effect of age on p21 expression in cortical and medullary regions from male and female rats (n = 6). Protein was extracted and p21 expression levels were determined by Western blot analysis as described in MATERIALS AND METHODS. Results are expressed as means ± SE. Comparisons are 7 vs. 15 mo (*P < 0.05).
Antioxidant Enzyme Data

There was no significant change in MnSOD, Gxp1, or glutathione reductase expression with age. However, in the cortex region, significantly increased levels of MnSOD were found in the female animals compared with males at all time points studied ($P < 0.05$ at 3 and 7 mo, $P < 0.01$ at 15 mo; Fig. 5, A-C). MnSOD levels were also significantly elevated in the female cortex samples compared with medulla in 3 and 15 mo of age ($P < 0.05$; Fig. 5, A-C). There was statistically no effect of gender on Gxp1 protein expression levels in either cortex or medulla at any time point; however, at 15 mo, there was a borderline significant ($P < 0.07$) increase in Gxp1 expression in the cortex of females compared with males. At all three ages studied, Gxp1 levels were markedly (between 50 and 71%) elevated in the cortex compared with the medulla, in both genders ($P < 0.001$; Fig. 6, A-C). Significantly ($P < 0.05$) increased levels of glutathione reductase expression were ob-

DISCUSSION

The aim of this study was to investigate the potential mechanisms that could contribute to the known differences in longevity between male and female rats, focusing on the role of renal dysfunction.

The results of our current study first confirm previous data showing that male rats are markedly more albuminuric than female animals, the disparity becoming evident after puberty.
We observed that the gender disparity became most apparent between 7 and 15 mo of age. Between these ages, 24-h albuminuria concentrations increased more than threefold in the males; however, the females demonstrated no significant increase between 7 and 15 mo.

Telomeres have been implicated in the aging process for a number of years (1) and it has been suggested that telomere shortening maybe linked to age-associated diseases, including kidney dysfunction (9). Therefore, we analyzed renal telomere length in the cortex and medulla of the same animals to establish possible reason for the striking gender dimorphism observed in 24-h albuminuria. We demonstrated that both cortex and medulla of male and female rat kidneys underwent significant age-related telomere shortening and that this shortening was more pronounced in the male animals compared with females. Additionally, significantly more short renal telomeres were observed in males at all ages studied. This paralleled our previous finding that whole kidney telomeres in male and female rats shortened with age (5, 14). Moreover, it raised the possibility that the increased prevalence of critically short renal telomeres in male rats may indeed be associated with their increased 24-h albuminuria concentrations. However, the parallel in accelerated renal telomere shortening in the male rat with increased urinary albumin excretion rates does not demonstrate cause and effect. Therefore, we sought to measure senescent marker profiles in the same tissue, which may act as a mechanistic link between telomere length and albuminuria.

We found that expression of p53, the major growth-arrest protein linked to telomere shortening, was markedly increased in old male rats, in both cortex and medulla, compared with younger animals. However, no significant increase was observed over time in the female animals in either renal region. The age-related elevation of p53 in the male rats is consistent with the accelerated renal telomere shortening and raised albuminuria levels. Indeed, it is known that critically short telomeres can induce telomere structure alterations, resulting in stabilization and upregulation of p53 activity. p53 Can then induce growth arrest via the activation of the cyclin-dependent kinase inhibitor p21 (29). Consistent with this, the expression profile of p21 was very similar to that of p53. In 15-mo-old male rats in both cortex and medulla, there was an increase in p21 compared with younger animals. Again, consistent with the p53 data, no significant change in p21 expression was found in the females. To our knowledge, this is the first time that gender differences in age-related senescence marker proteins have been shown in the rat.

It is well known that DNA oxidative damage can accelerate telomere shortening and contribute to the aging process (16). Therefore, we compared expression levels of abundant antioxidants MnSOD and Gxp I and glutathione reductase in the cortex and medulla of the same animals. At all time points studied, levels of MnSOD were higher in the female compared with the male cortex. Moreover, Gxp I and glutathione reductase levels at 15 mo of age were increased in the female compared with male cortex; however, no significant gender disparity was observed in the medulla in any enzyme. These data suggest that male rats have reduced antioxidant protection, which is consistent with a study showing stronger antioxidant defense in female rat kidney, heart, and liver (15). Decreased antioxidant levels in males may be partly responsible for acceleration of age-related kidney telomere shortening. Indeed, glutathione has recently been shown to increase telomerase activity in vitro (3). This telomere shortening may lead to upregulation of p53 and p21 senescent proteins as both oxidative and replicative induced telomere shortening can lead to cell senescence (25, 32, 33). Renal cell senescence may ultimately lead to increased albuminuria, renal dysfunction, and premature death in male rats.

It must be noted that sex hormones can influence p53 and p21 activity and protein levels independently of oxidative stress. In vitro studies using cancer cell lines have shown that estrogens can increase p53 levels via an indirect mechanism; however, direct effects of estrogen on p53 activity are unclear.
Conversely, androgens have been associated with inhibition of p53 protein abundance or activity in prostate cells (reviewed in Ref. 27). Moreover, estrogen-exposed cancer cells caused induction of p21 as well as p53 (35). It has been demonstrated that ovarian hormones are protective in the development of renal injury (8, 26). Estrogens in vivo are known to act indirectly as potent antioxidants through upregulation of antioxidant genes, including GPx and MnSOD, via the MAP kinase and NF-κB pathways (31). It is therefore possible that indirect antioxidant properties of the female sex hormones also contribute to the preservation of telomere length in the female rat, resulting in increased longevity. Furthermore, estrogens have been shown to protect telomeres from shortening by activating telomerase through the tHERT telomerase catalytic subunit (17, 21). Conversely, testosterone has been shown to confer no antioxidant properties (22), and the presence of androgens rather than the absence of estrogens may promote glomerular injury in the male rat (2).

We also demonstrated that the rate of telomere shortening with age seemed to differ, dependent on the cell population involved. We observed that age-dependent telomere shortening in the renal cortex of the rat was more accelerated than that in the medulla region, in both male and female animals. This is the first time that this observation has been demonstrated in the rat. Our finding reflects the human situation whereby cortex telomeres shorten over time more rapidly than medulla (20). In aerobic organisms, the mitochondrial electron transport chain is thought to be the major site of production of reactive oxygen species (ROS), which accumulate over time (12, 13). However, this idea has been recently challenged (11). Mammalian proximal renal tubules contain an abundance of large mitochondria dependent on oxidative rather than anaerobic metabolism (18). In contrast, the cells within the inner medulla are known to have greatly reduced mitochondrial content and several enzymes involved in oxidative metabolism are downregulated significantly (7). Thus cortical cells may be more liable to generate ROS via oxidative metabolism than the more anaerobic medullary cells. Any increased ROS production in cortical cells could explain the observation that the cortical telomeres shorten more quickly over time than the medullary telomeres. No significant difference in p53 or p21 activity was observed between the cortex and medulla regions of either the male or female rats at any age studied. It may be possible that the smaller but significant differences seen in telomere length between cortex and medulla regions were not sufficient to induce upregulation of p53 and p21 levels in the cortex.

Significant increases in MnSOD levels were observed in the cortex compared with medulla, in females, at all time points analyzed; however, there was no difference in males. Moreover, higher levels of Gpx1 and glutathione reductase were found in the cortex compared with medulla in both genders. This finding is consistent with these enzymes being expressed in the mitochondria. Moreover, these data may suggest that the cortex needs increased levels of MnSOD, Gpx1, and glutathione reductase to act as a protection against high levels of oxidative stress generated in this region.

In summary, we show that the increased albuminuria observed in male rats compared with females is accompanied by accelerated kidney telomere shortening, increased expression of the senescence markers p53 and p21, and reduced MnSOD, Gpx1, and glutathione reductase. We therefore propose as a working hypothesis that decreased antioxidant capacity may induce accelerated telomere shortening, which leads to renal cell senescence with reduced renal cell function, albuminuria, renal failure and, eventually, reduced longevity of male animals.

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References


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