Age-dependent renal cortical microvascular loss in female mice

Victor H. Urbieta-Caceres,1 Farhan A. Syed,2 Jing Lin,1 Xiang-Yang Zhu,1 Kyra L. Jordan,1 Caitlin C. Bell,1 Michael D. Bentley,4 Amir Lerman,3 Sudeep Khosla,2 and Lilach O. Lerman1,3

Divisions of 1Nephrology and Hypertension, 2Endocrinology, and 3Cardiovascular Diseases, Mayo Clinic, Rochester; and 4Department of Biological Sciences, Minnesota State University, Mankato, Minnesota

Submitted 9 August 2011; accepted in final form 19 January 2012

Ursieta-Caceres VII, Syed FA, Lin J, Zhu XY, Jordan KL, Bell CC, Bentley MD, Lerman A, Khosla S, Lerman LO. Age-dependent renal cortical microvascular loss in female mice. Am J Physiol Endocrinol Metab 302: E979–E986, 2012. First published February 7, 2012; doi:10.1152/ajpendo.00411.2011.—Renal function and blood flow decline during aging in association with a decrease in the number of intrarenal vessels, but if loss of estrogen contributes to this microvascular, rarefaction remains unclear. We tested the hypothesis that the decreased renal microvascular density with age is aggravated by loss of estrogen. Six-month-old female C57/BL6 mice underwent ovariectomy (Ovx) or sham operation and then were allowed to age to 18–22 mo. Another comparable group was replenished with estrogen after Ovx (Ovx+E), while a 6-mo-old group served as young controls. Kidneys were then dissected for evaluation of microvascular density (by micro-computed tomography) and angiogenic and fibrogenic factors. Cortical density of small microvessels (20–200 μm) was decreased in all aged groups compared with young controls (30.3 ± 5.8 vehicles/mm², P < 0.05), but tended to be lower in sham compared with Ovx and Ovx+E (9.9 ± 1.7 vs. 17.2 ± 4.2 and 18 ± 3.0 vehicles/mm², P = 0.08 and P = 0.02, respectively). Cortical density of larger microvessels (200–500 μm) decreased only in aged sham (P = 0.04 vs. young control), and proangiogenic signaling was attenuated. On the other hand, renal fibrogenic mechanisms were aggravated in aged Ovx compared with aged sham, but blunted in Ovx+E, in association with downregulated transforming growth factor-β signaling and decreased oxidative stress in the kidney. Therefore, aging induced in female mice renal cortical microvascular loss, which was likely not mediated by loss of endogenous estrogen. However, estrogen may play a role in protecting the kidney by decreasing oxidative stress and attenuating mechanisms linked to renal interstitial fibrosis.

kidney; aging; estrogen

AGING IS A PHYSIOLOGICAL PROCESS involving a gradual decline of kidney function and renal blood flow (RBF) (38). Creatinine clearance and glomerular filtration rate correlate inversely with age (17, 31), and the prevalence of chronic kidney disease significantly increases (6, 19). Blood vessels play a key role in the progression of renal damage in aging men and women (23), and loss of kidney tissue in the elderly is closely related to loss of renal vasculature, regardless of sex (15). Histologically, the aging kidney may develop focal and segmental glomerulosclerosis, mesangial matrix expansion, basement membrane thickening, and vascular loss (23), as well as impaired angiogenesis (29). These changes can be observed in both sexes, but women show less functional decline in with age until menopause, when women lose the protective effects of estrogen (16). Indeed, in aging female rats, menopause exacerbates a decrease in renal function, increased renal vascular resistance, and glomerulosclerosis (12), possibly partly attributable to loss of estrogen, which, in humans, renders women more susceptible to renal disease.

Regulation of the renal microcirculation is orchestrated by growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF). Previous studies have indicated that advanced age is associated with reduced expression of growth factors (29, 33) and with impaired angiogenesis (18). In women, aging is also associated with development of menopause and a decline in the protective effects of estrogen, which may be partly mimicked by ovarioectomy (Ovx). Indeed, in female rats loss of estrogen by Ovx exacerbates glomerulosclerosis and cortical tubulointerstitial fibrosis (24) and decreases myocardial and cerebral vascular density (14, 16). However, whether Ovx exacerbates the effects of aging on the kidney microcirculation has not been fully explored.

Methodological limitations have restricted studies of the renal vascular system due to its anatomical complexity. Three-dimensional micro-computed tomography (micro-CT) offers a unique opportunity to study renal microvascular density with high spatial resolution. Therefore, this study was designed to test the hypothesis that decreased renal vascular density in aging female mice would be aggravated by loss of estrogen. To test this hypothesis, we compared microvascular density in young (6-mo-old) mice to those in aged mice that had undergone sham surgery or Ovx at age 6 mo, and to those in Ovx subsequently replenished continuously with estrogen.

METHODS

Study Design

The Mayo Clinic Institutional Animal Care and Use Committee approved this study (A7206). Four groups of female C57/Bl6 mice (n = 10–12 each) were studied. Mice underwent sham or Ovx surgery at the age of 6 mo and were then allowed to age to 18–22 mo. Female mice undergo reproductive senescence and become acyclic by 11–16 mo of age (10, 26), although their estrogen deficiency is moderate and less profound than that observed in postmenopausal women. Nevertheless, to maintain constant estrogen levels throughout the experiment, another group of mice [Ovx + estrogen (E)] was continuously treated, starting immediately after Ovx, with 17β-3 benzoate estradiol (E2) pellets at doses of 10 or 40 μg·kg−1·day−1, based on previous studies showing that these doses span the physiological range (25, 34). Ninety-day estrogen pellets (Innovative Research of America, Sarasota, FL) were implanted near the right shoulder blade and replaced with fresh pellets every 60 days. Since our laboratory recently found that these doses had similar physiological effects (25, 34), Ovx+E data were pooled. The animals were housed in a temperature-controlled room with a daily 12:12-h light-dark schedule, had free access to water, and were pair fed. An additional group of 6-mo-old animals served as young controls.

http://www.ajpendo.org 0193-1849/12 Copyright © 2012 the American Physiological Society
At the completion of the observation period, all mice were euthanized by cervical dislocation, and laparotomy was immediately performed for tissue harvesting. The uteri were carefully dissected and weighed. Both kidneys were dissected and randomly assigned to undergo renal micro-CT imaging or tissue studies.

Micro-CT Preparation and Scanning

A ligature was placed around the aorta proximal to the renal arteries, a cannula inserted distal to it, and both the distal aorta and arterial branches to the liver and bowels were ligated. The aorta was then flushed with a heparinized 0.9% saline solution, and the renal veins cut to allow venous drainage.

When clear perfusate drained through the renal veins, a radio-opaque microfil silicone rubber (MV-122; Flow Tech, Carver, MA) was perfused through the cannula under physiological pressure until it flowed freely from the renal veins. The renal arteries and veins were then ligated, and the kidneys removed. The microfil contrast agent was allowed to polymerize for 24 h, after which each kidney was immersed in 10% formalin and embedded in a synthetic resin (Bioplastic, Wards Natural Science, Rochester, NY).

The kidneys were then scanned with micro-CT at 18-μm resolution, as our laboratory has previously showed (4, 41, 42). Three-dimensional volume images were reconstructed and analyzed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). The spatial density of renal cortical microvessels (20–500 μm) was then determined in 12 equidistant slices in each cortex, as described (4, 41, 42).

Western blotting. In vitro studies were performed to assess angiogenic mechanisms responsible for formation and maintenance of the renal microvasculature, as well as fibrogenic factors that modulate renal remodeling. Standard Western blotting protocols were followed using total homogenates and specific antibodies against VEGF-A (the 189, 165, and 121 isoforms, molecular mass 21 kDa) and its receptors-1 (FLT-1) and -2 (FLK-1) (all 1:200, Santa Cruz Biotechnology), angiomodulin (1:5,000, Abcam), angiotatin (1:5,000, Abcam), Notch-1 (1:500, Novus), transforming growth factor-β (TGF-β), and its downstream effector (total protein and activated form) Smad2/3 (all 1:200, Santa Cruz Biotechnology), b-FGF-2 (the 21- and 23-kDa forms, 1:500, Millipore), plasminogen activator inhibitor (PAI)-1 (BD, 1:2,500), the angiotensin II type 1 receptor (AT1), endothelin-1 (both Santa Cruz, 1:200), protein kinase C (PKC; 1:1,000, Cell Signaling), and nitrotyrosine (Cayman Chemical 1:200). GAPDH was used as loading control.

Histology. To assess development of fibrosis, kidneys samples were embedded in paraffin, 5-μm-thick sections stained with trichrome, and analyzed in 10–15 random fields using a computer-aided image analysis program (MetaMorph, Molecular Devices, Sunnyvale, CA). Values were averaged and expressed as percentage

![Image](http://ajpendo.physiology.org/doi/10.220.33.2)
of staining of total surface area. In situ formation of superoxide anions was also assessed using dihydroethidium immunofluorescence, as previously shown (41). Glomerular density was evaluated as our laboratory recently described in human biopsy material (32). The number of glomeruli per cortical field was manually counted in 10–15 fields of trichrome-stained slides (×10 magnification) and averaged per field. Glomerular score (the number of sclerotic out of 100 glomeruli) was also assessed, as previously published (8).

**Statistical analysis.** Data are reported as means ± SE. ANOVA was used to compare among the groups with a post hoc Student’s unpaired t-test with the Bonferroni correction. Differences were considered significant if \( P < 0.05 \).

**RESULTS**

There was no significant difference in body weight among the four groups (young controls: 24.2 ± 0.8, aged sham: 26.1 ± 1.6, Ovx: 27 ± 1.6, and Ovx+E: 29.0 ± 2.3 g, \( P = 0.23 \)). In contrast, uterine weight was significantly lower in Ovx compared with young control and sham (0.06 ± 0.01 vs. 0.18 ± 0.02 and 0.15 ± 0.02 g, \( P < 0.001 \)), but restored in Ovx+E (0.13 ± 0.01 g, \( P < 0.001 \) vs. Ovx, \( P = 0.08 \) vs. young control).

**Microvascular Density**

Cortical density of small microvessels (diameters 20–200 μm) was significantly decreased in all the aged groups (sham, Ovx, and Ovx+E) compared with young controls (Fig. 1, \( P = 0.03, P = 0.04, \) and \( P = 0.04 \), respectively), but in aged sham was significantly lower than in aged Ovx+E (\( P = 0.02 \)) and tended to be lower compared with aged Ovx (\( P = 0.079 \)). Cortical density of larger microvessels (200–500 μm) was decreased only in aged sham (Fig. 1, bottom right, \( P = 0.04 \) and \( P = 0.001 \) compared with young control and Ovx, respectively). Estrogen supplementation in Ovx+E did not affect cortical microvascular density compared with untreated Ovx.

**Vascular Growth Factors and Fibrogenic Factors**

VEGF expression was significantly lower in aged sham compared with aged Ovx (Fig. 2, \( P = 0.03 \)), while the expression of its receptor FLK-1 significantly decreased in both OVX and sham compared with normal (\( P = 0.03 \) and \( P = 0.01 \), respectively) and was preserved in Ovx+E. However, the
expression of FLT-1, the receptor that sequesters and antagonizes VEGF activity, was downregulated only in Ovx and Ovx+E compared with young control ($P = 0.001$ and $P = 0.04$, respectively), while, in the sham group, it remained similar to that of young control ($P = 0.3$). Notch-1, which inhibits VEGF downstream signaling, slightly decreased only in Ovx compared with young control ($P = 0.06$) and sham ($P = 0.02$). Two FGF-2 bands were detected (molecular masses, 23 and 21 kDa), and both decreased in all aged groups compared with young controls ($P \leq 0.03$, Fig. 2). The expression of the angiogenesis inhibitor angiostatin decreased in Ovx and sham compared with young control ($P = 0.001$ and $P = 0.03$, respectively, Fig. 2), while that of angiomedulin was similar among the groups. The expression of PKC, a downstream mediator of VEGF, was also similar among the groups (Fig. 3).

The expression of the fibrogenic factor TGF-$\beta$ decreased in all groups compared with young control ($P < 0.002$), but in Ovx TGF-$\beta$ was significantly higher than that in both Ovx+E mice and sham (Fig. 3). The activity of its downstream effector phospho-Smad3 (normalized to total protein) showed a decrease only in aged shams. PAI-1 expression was similar among the groups. The expression of the AT1 receptor and endothelin-1, as well as superoxide production (dihydroethidium staining, Fig. 4) and nitrotyrosine expression (Fig. 3), were all lower in Ovx+E compared with Ovx mice.

Trichrome staining showed more extensive regions of interstitial fibrosis in all aging groups compared with young control ($P \leq 0.04$, Fig. 4), but decreased in Ovx+E compared with untreated Ovx. Glomerular density was decreased in Ovx and aged sham animals ($6.5 \pm 0.6$ and $6.9 \pm 0.6$ glomeruli/field, respectively, $P < 0.05$ vs. young controls) compared with young controls ($9.3 \pm 0.5$ glomeruli/field). Estrogen in Ovx+E animals significantly improved glomerular density, although it remained lower than normal ($7.9 \pm 0.4$ glomeruli/field, $P = 0.05$ vs. Ovx, $P < 0.05$ vs. young control). No glomerulosclerosis was observed in young control animals, while some segmental glomerulosclerosis observed in aged and Ovx mice (glomerular score $2.0 \pm 0.4$ and $2.2 \pm 0.5\%$, respectively) tended to decline in Ovx+E ($1.3 \pm 0.2\%$, $P = 0.08$ vs. Ovx), suggesting that estrogen blunts, but may not completely abrogate, glomerulosclerosis in aging mice.

Fig. 3. Renal protein expression (all relative to GAPDH) of the profibrogenic factors transforming growth factor (TGF)-$\beta$ and its mediator phospho-Smad3, plasminogen activator inhibitor (PAI)-1, endothelin (ET)-1, the angiotensin II type 1 (AT1) receptor, protein kinase C (PKC), and nitrotyrosine in young controls, aged shams, and aged Ovx or Ovx+E mice. Top left: representative bands. Top right and bottom: quantitation showing increased profibrogenic signaling in Ovx, which was attenuated by estrogen replacement, as was the expression of nitrotyrosine, the footprint of peroxynitrite, and superoxide presence. Values are means ± SE. *$P < 0.05$ vs. young control. #$P < 0.05$ vs. Ovx+E.
DISCUSSION

The present study shows that aging induced a decrease in the spatial density of both small and large cortical microvessels, associated with inhibition of VEGF and b-FGF signaling. Interestingly, these changes were partly prevented by early Ovx and were not prominently affected by estrogen replenishment. On the other hand, this study shows that aging modified the expression of fibrogenic factors and increased renal interstitial fibrosis that were exacerbated by lack of estrogen in Ovx mice and improved by estrogen replacement. These data, therefore, suggest that aging in female mice leads to largely estrogen-independent cortical microvascular loss and estrogen-dependent renal fibrosis.

Renal function significantly declines with aging, in conjunction with a decrease in RBF and impairment in vasculo-protective mechanism like angiogenesis (30). Previous studies have shown a focal loss of peritubular and glomerular capillaries in aging male rat kidneys (18). However, while estrogen confers relative protection to the women’s kidneys until menopause (27), whether hormonal status in aging affects the kidney microcirculation remained poorly understood.

This study shows that, in female mice, aging leads to marked microvascular loss, which, interestingly, is partly prevented by Ovx. Yet, despite potential effects of estrogen to promote angiogenesis (1), the decrease in its levels in Ovx did not elicit further vascular loss, and neither did estrogen replacement affect renal microvascular density in Ovx. Although not measured in this study, E2 levels were likely lower for a longer duration in Ovx mice, which underwent Ovx at 6 mo of age, than in aged shams. The decrease of uterine weight in Ovx, and its restoration in Ovx+E, demonstrated the effectiveness of our interventions. These observations suggest that loss of estrogen is not a pivotal contributor to vascular loss in aging female mice.

Our findings are underscored by a recent observation of Fortepiani et al. (12) of a greater decline in RBF and renal function, and increase in renal vascular resistance, in aging female rats compared with aged-matched Ovx. In aging spontaneously hypertensive rats, but not in Ovx, this was associated

**Fig. 4.** Representative staining (blue, ×20; left) and quantitation (right) of renal trichrome (top) and dihydroethidium (DHE; bottom) in young controls (A), aged shams (B), and aged Ovx (C) or Ovx+E (D) mice. Renal fibrosis and oxidative stress in Ovx decreased after estrogen. Values are means ± SE. *P < 0.05 vs. young control. #P < 0.05 vs. Ovx+E. †P < 0.05 vs. aged sham.
with an increase in serum testosterone, the source of which was likely the postmenopausal ovary, which is an androgen-secreting organ (2). Hence, we cannot rule out a possible role of androgen removal in the Ovx model.

The aging-related decline in renal cortex microvascular density in this study was associated with altered angiogenic signaling. The expression of the prominent angiogenic factor VEGF may decrease in aging in males (29) and contribute to microvascular loss. Interestingly, we observed that VEGF expression was lower in aged sham compared with Ovx, suggesting that the decrease was not secondary to loss of estrogen alone. Speculatively, unopposed androgen might have driven microvascular loss in aging. On the other hand, the FLK-1 receptor, which mediates the angiogenic effect of VEGF, decreased in both Ovx and sham compared with normal animals, while the expression of FLT-1, the receptor that sequesters and inhibits VEGF activity, was downregulated in Ovx and Ovx+E, but not in sham. Similarly, the expression of Notch-1, a negative downstream regulator of VEGF (22), decreased in Ovx compared with aged shams, possibly as an additional compensatory mechanism to prevent microvascular loss. The failure to downregulate FLT-1 and Notch-1 in aged shams in the face of decreased VEGF and Flk-1 expression may, therefore, blunt effective VEGF signaling in this group.

The expression of the endogenous anti-angiogenic factor angiostatin was decreased in both Ovx and sham, suggesting a compensatory mechanism that was relatively preserved in aging. The attenuated angiogenic activity was also supported by decreased expression of b-FGF in all aged mice, which may have contributed to microvascular rarefaction. However, the expression of the downstream VEGF mediator PKC and its binding protein angiomodulin was not different among the groups, suggesting that only parts of VEGF signaling pathway are regulated by aging and E2 (Fig. 2).

The loss of microvascular density and VEGF expression could also be due to nephron loss, ultimately leading to loss of microvasculature. However, unlike microvascular density, we found that glomerular density declined and glomerular score increased in Ovx and aged sham animals compared with young controls and improved (although not fully restored) by estrogen, possibly consequent to attenuated renal fibrosis. Nevertheless, aging- and Ovx-induced glomerulosclerosis might lead to microvascular loss. Furthermore, glomerular density did not necessarily parallel VEGF expression, which was downregulated only in aged shams, while glomerular density was also decreased in Ovx. Thus decreased VEGF expression is unlikely to be only due to loss of glomeruli.

Renal interstitial fibrosis is an important mechanism by which aging decreases kidney function (15, 35). We observed that renal interstitial fibrosis significantly increased in all groups compared with young controls, but was reduced in estrogen-treated Ovx animals, suggesting that estrogen may attenuate profibrotic mechanisms. TGF-β is a key factor in renal fibrogenesis (37), yet both TGF-β and its signaling mediator Smad2/3 were downregulated in aged females compared with young controls. These findings imply relative preservation of TGF-β signaling in Ovx compared with aging, but no additional effect of estrogen. However, the effects of aging and Ovx on TGF-β seem to be inconsistent, possibly due to different study conditions, such as special aging rat models (39), mouse strains (9), or in vitro settings (7). In a pertinent study, Lane et al. (20) observed decreased TGF-β1 mRNA in normal rats during aging from 10 to 22 wk old, underscoring our findings in mice. On the other hand, they observed further decreased TGF-β1 mRNA in aged Ovx rats. Our results show a similar trend, with the exception of increased TGF-β expression in aged Ovx compared with aged control. This differential effect of Ovx might be attributed to the different age at which Ovx was performed (2 wk vs. 6 mo of age), species, or other experimental conditions.

Nevertheless, we observed that TGF-β and Smad3 expression was higher in Ovx compared with sham, and TGF-β expression decreased in estrogen-treated Ovx mice, implicating TGF-β signaling in renal fibrosis in Ovx. In our aged sham mice, but no other group, decreased TGF-β was accompanied with decreased p-Smad3 expression. The mechanisms that sustained Smad2/3 phosphorylation in aged Ovx mice, despite decreased TGF-β expression, can be speculated. Smad signaling can be activated via TGF-β-independent pathways, such as angiotensin II (40) or inflammatory cytokines (21). Moreover, the androgen-androgen receptor system directly associates with Smad3 (5) and negatively regulates its signaling (36). Given that the postmenopausal ovary is an androgen-secreting organ (2), Ovx might remove this inhibitory effect and allow some Smad phosphorylation, although TGF-β expression decreased. Yet we cannot rule out the possibility that Smad signaling was activated by alternative, TGF-β-independent pathways in Ovx, perhaps as a compensatory mechanism to the downregulated protein expression of TGF-β. Both TGF-β and PAI-1 can be induced by angiotensin II, reactive oxygen species, cytokines, and growth factors and can, in turn, promote renal tubulointerstitial fibrosis (3). The downregulation of TGF-β in this

Fig. 5. A diagram showing the pathways stipulated to be involved in the effects of aging and Ovx on the female mouse kidney. Aging impaired expressions of growth factors (VEGF, b-FGF), leading to renal microvascular rarefaction. Ovx induced oxidative stress and upregulated fibrogenic factors that subsequently induced renal fibrosis. Aging and Ovx seem to have dual effects on TGF-β expression, depending on the model and experimental conditions, as indicated by the parallel stimulatory (solid arrow) and inhibitory (dashed line) symbols. Estrogen replacement attenuated renal fibrosis by ameliorating renal oxidative stress and fibrosis in aged mice, but had minimal effects on angiogenesis.
study in the Ovx+E group links renal fibrogenic mechanisms to estrogen deficiency in Ovx mice.

Importantly, renal fibrosis might potentially be secondary to oxidative-stress and renin-angiotensin system activity, both of which are activated in aging females (28). However, in our study, in situ superoxide production was markedly elevated only in aged Ovx, and estrogen supplementation decreased both superoxide production and expression of nitrotyrosine (the footprint of peroxynitrite), suggesting a decrease in oxidative stress. Estrogen supplementation also downregulated the AT1-receptor expression, linking augmented oxidative stress and the renin-angiotensin system to prolonged estrogen deprivation, but not necessarily to aging per se. In the recently published Rancho Bernardo Study, a 10-yr follow-up showed that, in postmenopausal women, estrogen use improved blood pressure and decreased urine albumin-to-creatinine ratio, without affecting in glomerular filtration rate (13). These observations suggest that continuous estrogen use attenuates albuminuria, which may be mediated by improved blood pressure control or possibly by decreased renal fibrosis, as observed in the present study.

Limitations

Our study was limited by the unavailability of measures of renal function, blood pressure, and systemic markers. It is possible that an increase in blood pressure contributed to renal microvascular loss, although, in aging female rats, renal injury seems to be dissociated from elevated blood pressure (11, 12). In addition, estrogen replacement using subcutaneous pellets cannot fully mimic the physiological environment, such as normal fluctuations over the course of the estrus cycle. However, the dose that we used effectively prevented bone loss in female mice (25, 34), suggesting that it is physiologically efficacious. Further studies will be needed to dissect the role of TGF-β signaling pathways in Ovx and aging. In summary, we observed that aging elicited a significant decrease in renal cortical microvascular density and increased interstitial fibrosis in female mice. Microvascular loss was partly prevented by early Ovx, suggesting, in aging, that the postmenopausal ovary may contribute to evolving renal injury. Microvascular loss might also be partly responsible for the renal interstitial fibrosis observed in aged shams. Conversely, while cortical microvascular density was relatively preserved in Ovx mice, activation of profibrogenic mechanisms, including TGF-β signaling, endothelin-1, and oxidative stress, were greater in Ovx than in aged sham and attenuated by estrogen replacement, as was AT1 expression, suggesting a role for estrogen in ameliorating renal oxidative stress and fibrosis in aged mice (Fig. 5). Further studies are needed to establish the role of microvascular loss as a determinant of renal deterioration during aging and if enhancement of it architecture might improve renal function.

ACKNOWLEDGMENTS

Present address of F. A. Syed: Abbott Laboratories, Worcester, MA.

GRANTS

This study was partly supported by grant numbers DK-73608, DK-77013, HL-77131, AG-028936, and HL-085307 from the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


