Down-Regulation of the Intrarenal Renin-Angiotensin System in the Aging Rat\(^1,2\)

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ABSTRACT

Progressive deterioration of renal function occurs during normal aging. Previous studies on the aging kidney have demonstrated glomerular hemodynamic changes, specifically, glomerular capillary hypertension, as maladaptations that lead to proteinuria and glomerular sclerosis over time. Aging rats treated with angiotensin-converting enzyme inhibition have relatively less proteinuria and sclerosis, suggesting that age-related changes in renal function may be associated with alterations in the intrarenal renin-angiotensin system, which thus may play a major role in the pathogenesis of these maladaptations. To investigate this possibility, renal and systemic renin-angiotensin systems were examined at an early phase of the aging process (3 months) and at a later phase (12 months) in male Sprague-Dawley rats. Although plasma renin and serum angiotensin-converting enzyme concentrations did not differ significantly, the intrarenal system showed down-regulation of renin mRNA and angiotensin-converting enzyme levels with aging, whereas angiotensinogen levels remained stable. The decrease in renin mRNA appeared to precede the fall in plasma renin concentration in the aging process. Additional studies in 15-month-old rats confirmed that, by this time, both basal and stimulated renal renin release rates were impaired in older rats. Thus, both decreased renin synthesis and impaired renin release underlie the fall in plasma renin with normal aging. This decrease may act to lower intrarenal baseline levels of angiotensin II, an adaptation of likely importance in the modulation of intrarenal vascular tone and tubular function in the aging kidney.

Key Words: Kidney, elderly, renin release, angiotensin-converting enzyme

Progressive deterioration of renal structure and function is part of the normal aging process (1,2). In humans, GFR and RPF decline with age to levels about one-half to two-thirds of values in young adults (3,4), in association with increasing glomerular sclerosis (5). By itself, the age-related loss of renal function implies little danger. However, this process is accelerated when functional nephron number is further reduced by acquired renal disease, by acute renal failure or by various systemic diseases with renal involvement. Studies in laboratory rats have implicated hemodynamic mechanisms as contributing to the loss of renal function with aging. With age, whole-kidney GFR and RPF decline (6–8), while proteinuria and albuminuria increase (9–11). Increasing proteinuria may be due, at least in part, to hemodynamic factors (e.g., elevation of glomerular capillary pressure) (10,11). This progressive proteinuria heralds the development of age-related glomerular injury and extensive glomerulosclerosis.

Age-related changes in the activity and responsiveness of the renin-angiotensin system (RAS) are likely to contribute to the functional changes in the aging kidney. Although some studies have examined the circulating RAS in aging, little is known of the intrarenal RAS. For example, aging humans exhibit reduced plasma renin activity (PRA), reductions in plasma active (but not total) renin concentrations, and generally unchanged plasma angiotensinogen levels, changes that are consistent with reduced renin formation, reduced renin release, and/or reduced conversion of active to inactive renin (12,13). In aging rats, the reduced PRA has been associated with a decrease in single-nephron renin content, suggesting decreased formation as one mechanism (14). In elderly humans (15) and animals (10,16), although plasma renin concentration (PRC) is frequently low, blood pressure reduction with angiotensin-converting enzyme (ACE) inhibition suggests a tonic RAS influence on systemic hemodynamics.

This study was designed to explore the regulation of both the circulating and the intrarenal RAS during normal aging in the rat. Thus, renal and systemic RAS were examined in young (3 months) and later (12 to 15
months) aging phases in male Sprague-Dawley rats. We hypothesized that the well-known fall in PRC in aging could result from impaired renin formation and/or release. These studies were designed to explore both renin production and systemic release, in order to look at the mechanism of the age-related fall in PRC.

METHODS

Animals

Two groups of male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used in these studies. All studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the institutional Animal Care and Use Subcommittee. Protocol 1: To examine and determine systemic and intrarenal RAS parameters, rats aged 3 months (young, \( N = 6 \)) or 12 months (older, \( N = 6 \)) were used. They were maintained on a 12-h light/dark cycle with free access to standard chow (Rodent Laboratory Chow 5001; Ralston Purina Co., Richmond, IN) and tap water. Protocol 2: Additional groups of rats aged 3 months (young, \( N = 9 \)) or 14 to 15 months (older, \( N = 9 \)) were maintained under similar conditions and were then used for renin release studies as described below. All rats were placed in metabolic cages, and 24-h urine collections were obtained after a 48-h equilibration period, for measurements of urinary protein and sodium.

Tissue Processing

For mRNA, protein, and PRC studies, rats in Protocol 1 were decapitated. Trunk blood was collected, and kidneys and livers were rapidly removed, snapfrozen in liquid nitrogen, and stored at \(-70^\circ C\) until use.

Gene Expression Studies

Total RNA was extracted from tissues by the guanidium thiocyanate method of Chirgwin et al. (17) and quantitated by absorbance at 260 nm in duplicate. RNA purity was determined by A260/A280 ratios. For quantitative studies, slot blot analysis was performed with serial dilution of total RNA after formaldehyde denaturation. Three concentrations from each sample (2.4, and 8 \( \mu g \)) were blotted in duplicate to nylon filters with a slot blot apparatus (Schleicher and Schuell, Peterborough, NH) and cross-linked by UV light. After prehybridization for 4 h, blots were hybridized overnight in a hybridization buffer to which \( ^{32}P \) cDNA probes were added. Blots were then washed in 0.2 \( \times \) SSC with 0.1% sodium dodecyl sulfate at room temperature for 10 min, then three times at 65°C for 30 min. Blots were exposed to x-ray film (Kodak XAR; Eastman Kodak, Rochester, NY) and subsequently analyzed with a computer scanning program (Desk Scan, Hewlett Packard Version 1.51 and Image. By Wayne Rasband, Research Services Branch, NIMH, Bethesda, MD, Version 1.37). Slopes for relative densitometric values were then calculated and compared. Northern blots were run with 10 \( \mu g \) of total RNA/lane in formaldehyde denaturing gels. After gels were transblotted to nylon filters and the RNA baked at 80°C for 2 h, blots were hybridized to probes of interest.

Probes

The northern or slot blots were hybridized to cDNA full-length rat cDNA probes for angiotensinogen (18) and renin (19). A human ACE cDNA was used for studies of ACE mRNA (20). To control for possible sample variability, blots were also hybridized with a \( \beta \)-actin cDNA probe. Probes were labeled by random priming with \( ^{32}P \) dCTP to an activity of 10\(^8\) dpm/\( \mu g \) of DNA (21).

Immunohistochemical Studies

Kidneys were fixed in 10% formalin, dehydrated, and embedded in paraaffin blocks. Tissue was cut in 7-\( \mu m \) sections. The avidin-biotin ABC method (22) (Vector Laboratories, Burlingame, CA) was used to examine the localization of ACE protein products. The ACE antibody used was a goat anti-rabbit antiserum that reacts with rat (kindly provided by Dr. Richard Soffer) (23). This antiserum stains well in rat tissue at a 1:1,000 dilution.

Functional Studies

Functional studies were performed in Protocol 2 animals to define renal hemodynamic status and also to examine PRC before and after stimulation by hemorrhage. Because the biochemical and mRNA studies in Protocol 1 (see Results below) suggested that renin mRNA levels are reduced before the fall in PRC with aging, these functional studies were performed with slightly older rats. On the day of surgery, rats were anesthetized with Inactin (5-ethyl-2-thiobarbituric acid; 100 mg/kg ip) and placed on a temperature-regulated table. The left femoral artery was catheterized, and a baseline collection of blood was obtained for the measurement of hematocrit (Hct) and inulin and para-aminohippurate (PAH) "blanks." This arterial catheter was used for subsequent periodic blood sampling and for the estimation of the mean arterial pressure (MAP) via an electronic transducer connected to a direct writing recorder. After tracheostomy, venous catheters were inserted for infusions of inulin, PAH, and rat serum. Intravenous infusions of rat serum (6 mL/h) and saline with 10% inulin and 0.8 to 0.9% PAH (1.2 mL/h) were started. The left ureter was catheterized for urine collection. Euvolemia was maintained by infusing isoncotic rat serum at the above rate in a total amount equal to 1% of the body weight, followed by a maintenance infusion rate of 0.42 mL/h (young rats) or 0.72 mL/h (old rats), to maintain the Hct constant. A 1-h equilibration period was observed after the rapid serum infusion was completed. For the calculation of RPF and GFR, timed (20-mm) urine collections were obtained for the determination of flow rates and inulin and PAH concentrations. Blood was obtained simultaneously for measurements of Hct and plasma inulin and PAH concentrations. These measurements permitted the calculation of GFR (inulin clearance) and RPF (PAH clearance) by standard formulas. Renin studies were performed following the above functional studies. For the determination of PRC, 0.3 mL of blood was collected from the femoral artery into chilled glass tubes each containing 4.5 mg of EDTA. These samples were labeled “Pre.” Next, controlled hemorrhage (young, 2 mL; old, 3 mL) was induced over 3 min via the femoral artery catheter, and PRC samples were again collected. These samples were labeled "Post"-hemorrhage. MAP was measured before and after hemorrhage. The left kidney was then removed and weighed. All PRC samples were rapidly centrifuged at 4°C, and the plasma was frozen until analysis.

Analytical Methods

PRC and renal renin concentrations were measured by radioimmunoassay after the incubation of plasma or tissue
homogenate with excess angiotensinogen provided by anephric sheep plasma (24). Angiotensin I (Ang I) levels generated were taken as indicative of the amount of renin present. For plasma, results were expressed as nanograms of Ang I per milliliter of plasma per hour. For tissue renin concentration, the amount of Ang I generated was taken as indicative of the amount of tissue renin present. Total protein was determined in the homogenate by use of the Coomassie brilliant blue dye method of Bradford (Biorad, Burlingame, CA) (25). The tissue renin concentration was thus expressed as nanograms of Ang I per milligram of protein in tissue homogenate per hour. Plasma angiotensinogen levels were estimated after the exhaustive generation of Ang I after the addition of exogenous renin (26). Results were expressed as nanograms of Ang I equivalent per milliliter of plasma per hour. Serum and tissue ACE were determined by fluorometric assay as described by Cushman and Cheung (27), in which levels of His-Leu from Hip-His-Leu substrate are determined and fluorescence is measured at 486 nm with an excitation wavelength of 384 (MFP-66 Fluorescence Spectrophotometer; Perkin Elmer, Norwalk, CT). The fluorescence of phthaldehyde His-Leu was linear from 0.02 to 12 nmol/min. Inulin clearance was calculated from blood and timed urine collections, by the use of a macroanthrone method for inulin measurement (28). PAH clearance was measured by a colorimetric method (29). Urinary total protein content was measured by the use of 3% sulfosalicylic acid precipitation (30). Plasma and urinary sodium content were determined by flame photometry.

**Statistical Analysis**

Statistical analysis was performed by unpaired or paired t test, as appropriate (31). Statistical significance was defined as \( P < 0.05 \). All values are means ± SE.

**RESULTS**

Studies of the circulating RAS in Protocol 1 showed that there was no significant difference in PRC between rats aged 3 months and 12 months (Figure 1a). There also was no significant difference in intrarenal renin protein concentration between the groups (Figure 1b). In contrast, renal renin mRNA was strikingly decreased in older versus young rats (\( P < 0.05 \)).
decreased in the older rats as compared with in the young rats \((P < 0.05)\) (Figure 1c and d).

Plasma angiotensinogen concentrations did not differ between the groups (Figure 2a), nor was there a significant difference in the level of renal angiotensinogen mRNA (Figure 2b). There were no differences in liver angiotensinogen mRNA levels between the age groups (data not shown).

There was no difference in plasma ACE concentration in older rats versus young (Figure 3a). In contrast, renal ACE concentration was significantly lower in the older rats \((P < 0.025)\) (Figure 3b). However, total levels of renal ACE mRNA did not differ (Figure 3c and d). Immunohistochemical studies, performed to examine whether the redistribution of ACE might accompany aging, also found no difference in ACE protein distribution within the kidney (Figure 4).

Table 1 details the cardiovascular and renal function studies in young (3-months-old) and older (14- to 15-months-old) rats from Protocol 2 animals. The older rats had higher values for body weight and kidney weight, although the kidney/body weight ratio did not differ. Both groups were normotensive. The old rats had higher GFR and RPF values, although not when expressed per body weight. Filtration fraction did not differ between the groups. The 24-h urinary protein excretion was higher in the old rats, suggesting the presence of underlying age-related renal disease. Values for urinary sodium excretion were comparable in the two groups.

Studies of the circulating RAS in the Protocol 2 animals showed down-regulation of PRC with age. (This finding, different from that in the 12-month-old rats, is likely because the rats used for this segment were intentionally older than those in Protocol 1.) Prehemorrhage and posthemorrhage values for PRC are depicted in Figure 5. All PRC measurements in both groups of rats were higher than those previously reported in awake rats, presumably reflecting the stimulation of the RAS by the anesthetized state and surgical preparation. Nevertheless, before hemorrhage, renin concentrations were significantly lower in the older rats than in the young rats \((P < 0.02)\). Hemodynamic shock \((\text{MAP} = 66 \pm 6 \text{ mm Hg})\) was then produced, and more profoundly stimulated PRC levels were obtained. Although PRC rose significantly in both groups, values in older rats remained lower \((P < 0.02)\). Both prehemorrhage and posthemorrhage PRC values were significantly lower in the older rats. Furthermore, there was a smaller difference between prehemorrhage and posthemorrhage values in the older than in young rats \((P < 0.05)\). Thus, in the setting of both the modest stimulation of anesthesia and surgery and the profound stimulation of hemorrhage, levels of PRC were lower in the old rats, strongly suggesting the impairment of renal renin release to conventional stimuli.

DISCUSSION

The data presented here demonstrate that the RAS is altered in the aging kidney, with an evident dissociation between plasma and tissue RAS. Although the level of angiotensinogen (substrate) mRNA appears stable, renin mRNA and ACE protein levels are decreased. Thus, by 12 months of age, kidney renin mRNA is down-regulated, and this reduction in tissue renin mRNA precedes the later fall in PRC seen at 15
There were no significant differences in PRC or renal renin concentration in 3-month-old compared with 12-month-old animals. However, by 14 to 15 months, both prehemorrhage and posthemorrhage PRC determinations revealed a significant decrease in circulating renin concentration. Under both conditions, PRC values were lower in the older rats, strongly suggesting impairment of renal renin release. Thus, it would appear that 12 months is a point before the age-related fall in PRC, and the fact that renin mRNA is lower in 12-month-old animals suggests that transcriptional changes in the renin gene likely precede age-related alterations in translational changes into protein and changes in renal renin and in circulating renin concentrations. In this study, the renal renin mRNA content was significantly reduced compared with that in the young rats, at a point (12 months of age) when PRC and renal renin concentration were not yet altered. The maintenance of normal PRC and tissue renin concentrations suggests that the synthesis of renin from available mRNA may be accelerated or that there might be increased peripheral conversion from inactive to active renin in the older rats. An additional possibility is that the observed changes reflect a slower rate of renin protein degradation in the older rats. The combination of low renin mRNA yet normal plasma renin at 12 months might suggest a possible retardation in peripheral plasma renin metabolic clearance. Furthermore, the decrease of renin mRNA could be due to a generalized suppression of renin gene transcription, which might constitute an initial step before the generalized fall in renin expression in the aging process. The regulation of renal renin mRNA has been extensively examined in young but not in old rats. The cause of the decrease in renal renin mRNA in the aging rat is not clear, but it does not appear to be due to differences in sodium intake.
Figure 4. Tissue ACE distribution. (a) ACE distribution in the kidney of a representative older (12 months) rat (magnification, ×100). (b) ACE distribution in a representative young (3 months) rat (magnification, ×100). There was no apparent redistribution in the older rats, as compared with the young.

(as reflected by urinary sodium excretion), because they were similar in the two groups.

Decreased renin mRNA in aging rats is consistent with the reduced formation of intrarenal baseline levels of angiotensin II (Ang II), an adaptation of potential importance in the modulation of intrarenal vascular tone and tubular function in the aging kidney. Decreased Ang II may lead to up-regulation of Ang II receptors, which would explain our earlier findings of an enhanced renal Ang II effect in old rats (32). In that study, exogenous infusion of Ang II induced comparable systemic blood pressure elevation, but greater reductions in GFR and RPF in older rats, suggesting that there is an enhanced renal vascular responsiveness to Ang II in the aging animal. The decrease in circulating renin and the concomitant decrease in Ang II levels, combined with an enhanced renal vascular responsiveness to Ang II (vasoconstriction), suggest up-regulation of Ang II at the receptor level. This increased responsiveness to Ang II may contribute to the enhanced vulnerability of the aging kidney to acute ischemic events. Renal responsiveness to vasodilators in the aging kidney is comparable (32) or impaired (8,10,33) as compared with that in the younger kidney. Thus, enhanced vasoconstrictor action in the absence of augmented vasodilation may render the older kidney susceptible to injury.

There was no change in ACE mRNA in young as compared with old rats. However, there was a significant decrease in ACE protein in the older rats, with no alterations in renal tissue ACE distribution. The regulation of renal ACE is not well studied, but in this study, it did not appear to relate to changes in sodium intake. A possible cause of this diffuse decrease of ACE protein could be tubular injury, tubulointerstitial lesions accompany the aging process (10). In diabetes mellitus, which is also associated with tubular injury, total renal ACE protein is reduced, but not in a diffuse fashion, as was seen in

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a Values are means ± SE. Abbreviations: UprotV, 24-h urinary protein excretion; UNOV, 24-h urinary sodium excretion; NS, not significant (P > 0.05).
aging. In early diabetes, there is redistribution of ACE localization: ACE is less present in the tubule and is more expressed in the glomerulus and vessels (34). In contrast, renal ACE expression is diffusely enhanced in nephritic rats (35). Thus, changes in renal ACE distribution appear to be disease specific, rather than nonspecific adaptations to the presence of renal injury. These disease-associated changes in ACE activity could contribute to localized changes in functions mediated by Ang II: tubular sodium and water flux and glomerular/vascular hemodynamic tone. Comparisons of ACE protein expression in aging and in later diabetes would be of interest, but are not yet available.

In summary, aging Sprague-Dawley rats show a dissociation between changes in plasma and tissue RAS components. In the aging kidney, renin mRNA is down-regulated and appears to precede the fall in PRC. Although substrate levels are stable, renin mRNA and ACE protein levels are decreased. Furthermore, renin release is impaired. Thus, both renin synthesis and renin release are impaired in the older kidney, thereby explaining the fall in PRC with aging. These findings suggest that changes in the intrarenal RAS may act to lower the baseline levels of available Ang II during the aging process, an adaptation of likely importance in modulating intrarenal vascular tone and tubular function in the kidney.

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