Age-related progressive renal fibrosis in rats and its prevention with ACE inhibitors and taurine

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Departments of 1Physiology and 2Medicine, Alcalá University, Madrid; 3Department of Pathology, Granada University, 18012 Granada; and 4Nephrology Section, Hospital Príncipe de Asturias, 28871 Alcalá de Henares, Madrid, Spain

Iglesias-de la Cruz, Carmen, Piedad Ruiz-Torres, Raímundo García del Moral, Manuel Rodríguez-Puyol, and Diego Rodríguez-Puyol. Age-related progressive renal fibrosis in rats and its prevention with ACE inhibitors and taurine. Am. J. Physiol. Renal Physiol. 278: F122–F129, 2000.—Our previous studies demonstrated an increased reactive oxygen species (ROS) production, as well as transforming growth factor-β1 (TGF-β1) expression, in the rat kidney with aging. In the present study, we examined the effect of aging on extracellular matrix (ECM) accumulation and the effects of treatment with angiotensin-converting enzyme inhibitors (captopril and lisinopril) and taurine, an antioxidant amino acid. Age-related increases in types I and IV collagen and fibronectin mRNA expression were found at 24 and 30 mo of age. In contrast, type III collagen only increased in 30-mo-old rats. Captopril-, lisinopril-, and taurine-treated animals showed a statistically significant decrease in ECM protein expression at both ages. Moreover, treatment with taurine reduced the TGF-β1 mRNA levels in 24- and 30-mo-old rats by 40%. Taurine also completely blocked increases in type I collagen expression at both ages. Moreover, treatment with taurine showed a statistically significant decrease in ECM protein expression at both ages. Moreover, treatment with taurine reduced the TGF-β1 mRNA levels in 24- and 30-mo-old rats by 40%. Taurine also completely blocked increases in type I and type IV collagen expression in mesangial cells in response to TGF-β1. Our results demonstrate a protective role from both converting enzyme inhibitors and taurine in the age-related progressive renal sclerosis. In addition, taking into account that taurine is considered as an antioxidant amino acid, present data suggest a role for ROS in age-related progressive renal fibrosis, perhaps through interactions with the TGF-β1 pathway.

transforming growth factor-β1; extracellular matrix proteins; angiotensin-converting enzyme inhibitors; reactive oxygen species; antioxidants

FROM A MORPHOLOGICAL POINT of view, aging is characterized by the development of structural changes, including progressive renal sclerosis with glomerulosclerosis and interstitial fibrosis (9, 16, 21). The biochemical nature of these changes has not been completely defined, and the most detailed approach to the analysis of this problem was the study from Abrass et al. (1). These authors demonstrated by immunohistochemical techniques increased accumulation of different laminin isoforms in the glomerulus, increased fibronectin content, and increased interstitial accumulation of collagen type I (COL I) and III (COL III) in aging rats (1). Surprisingly, no significant changes in collagen type IV (COL IV) expression were detected (1). Therefore, the results of this study suggest that the biochemical nature of the aging-related progressive renal sclerosis differ from other forms of progressive renal disease (1, 8, 14, 31). However, immunofluorescence techniques may lack the necessary sensitivity to detect certain changes in the expression of proteins in renal tissue, and the study by Abrass et al. (1) would need to be supported by additional, more sensitive analyses.

The mechanisms involved in the development of the morphological changes associated with aging have not been definitely elucidated. As transforming growth factor-β1 (TGF-β1) is one of the most relevant cytokines involved in the pathogenesis of some renal diseases characterized by the accumulation of extracellular matrix (ECM) (3, 17, 25, 28), we tested the hypothesis that TGF-β1 is increased in old rats. We demonstrated a significant increase of this cytokine in the kidney in 24-mo-old rats (23). Moreover, as in other experimental conditions (5, 16, 18, 22), angiotensin-converting enzyme inhibitors (ACEI) prevented the increased TGF-β1 mRNA expression observed with age (23). However, although it is generally accepted that the changes in ECM proteins can be a consequence of the changes in TGF-β1 (3, 17, 25, 28), a detailed analysis of this association has not been performed in aging.

Treatment with ACEIs is the most widely used strategy to prevent progressive renal sclerosis in different pathological conditions, including aging (5, 16, 18, 22, 23). However, other alternatives could be useful. A close relationship exists between the increased reactive oxygen species (ROS) synthesis and aging (29). Antioxidant treatment has been proposed to prevent aging-related general disturbances (20). In the kidney, the local synthesis of ROS, at least in experimental animals and cultured cells, seems to increase with age (24), and antioxidant treatment could also prevent the morphological and functional aging-related renal changes.

The present experiments were designed to address some of the problems previously mentioned. First, we tried to perform a more detailed analysis of the changes...
in the ECM proteins in the renal cortex from old rats, by studying the changes in the protein content and the mRNA of these proteins. Second, we demonstrated that ACEIs treatment not only reduces the level of TGF-β1 mRNA expression but also reduces the expression of the mRNAs of different ECM proteins. Finally, we tested the effect of taurine, an amino acid with antioxidant properties (2, 10, 13), on the development of the aging-related accumulation of these proteins.

MATERIALS AND METHODS

Experimental design in vivo. Male Fischer 344 rats were fed a standard laboratory diet (protein 17.2%, fat 2.7%, fiber 3.9%, minerals 4.4%, carbohydrates 59.7%, and calories 3,100 kcal/kg) and provided with water ad libitum. The maximal lifespan of this strain is about 36 mo (30), and the mortality in our 30-mo-old animals was 19%. In the first part of the study (effect of aging), rats were killed at 3, 24, or 30 mo of age (n = 9 at each time point). In the second part of the study (effect of treatments), we divided 21- and 27-mo-old rats into three groups (7 animals per group). One group was treated with captoril (100 mg/kg in drinking water, ~10 mg/kg per day) (15), the second group received lisinopril (28 mg/l in drinking water, ~2.8 mg/kg per day), and the third group was treated with taurine (2% in drinking water). Treatment supplies were changed every 2 days, and treatments never exceeded 3 mo. Seven days before the end of the experimental period, blood pressure (BP) was measured by the tail-cuff method in conscious animals in each group. Urine samples (24 h) were collected and centrifuged to remove contaminants and then stored at −20°C until analysis for protein content. At death, rats were anesthetized with ether, and blood samples were taken from the lower aorta into tubes containing 7.5% EDTA. Plasma was frozen at −20°C until analysis for creatinine content. After perfusion with isotonic saline, kidney cortex was excised and placed immediately in solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) for RNA extraction or in an embedding medium for frozen tissue specimens (OCT compound; Miles, Elk hart, IN) for protein extraction, and these samples were immediately frozen at −80°C.

A group of control rats was used to test the antioxidant ability of taurine. For that purpose, 3-mo-old rats received taurine (2% in drinking water) for 3 mo, and the malondialdehyde (MDA) content in renal cortex was measured as described (24).

Experimental design in vitro. Experiments were performed in cultured human mesangial cells. Cells were cultured under standard conditions, as previously described (6). In short, portions of macroscopically normal, cortical tissue were obtained from a human kidney immediately after nephrectomy. Portions of macroscopically normal, cortical tissue were obtained from a human kidney immediately after nephrectomy (24 h). After perfusion with isotonic saline, kidney cortex was excised and placed immediately in solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) for RNA extraction or in an embedding medium for frozen tissue specimens (OCT compound; Miles, Elk hart, IN) for protein extraction, and these samples were immediately frozen at −80°C.

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equal protein loading. The proteins of the second gel were transferred to a 0.45-µm pore nitrocellulose membrane (Schleicher &amp; Schuell, Dassel, Germany) by the semidy method (Bio-Rad Laboratories, Richmond, CA). Filters were stained with Ponceau S (Sigma) to control for equal transfer. The nitrocellulose membranes were blocked with 10 ml of TTBS buffer (20 mM Tris·HCl, 0.9% NaCl, and 0.05% Tween 20) with 3% BSA overnight at 4°C. The membranes were incubated with the primary antibody, rabbit anti-rat collagen type I (Promega, Madison, WI), diluted in the same buffer for 2 h at room temperature with gentle agitation. Thereafter, the nitrocellulose membrane was washed three times with TTBS with 3% BSA and incubated for 90 min at room temperature with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc specific; Promega) diluted 1:10,000 in TTBS with 3% BSA. After three final washes with fresh TTBS, the membrane was incubated in alkaline phosphatase buffer (10 mM Tris·HCl, 100 mM NaCl, and 5 mM MgCl2, pH 9.5). Secondary antibody bound to the nitrocellulose was detected by incubation with a substrate solution (Sigma), which consisted of nitroblue tetrazolium (330 µg/ml) and 5-bromo-chloro-3-indolyl phosphate (165 µg/ml). The color development was stopped after approximately 5 min by washing the membrane with distilled water.

Statistical analysis. The present experiments were performed on the basis of a paired design. For this purpose, a control rat was selected for each experimental rat (in the case of the different ages, one 3-mo-old rat was assigned to each experimental rat; in the case of the treatments, one 24- or 30-mo-old nontreated rat was assigned to each treated rat). Every sample of these paired rats was run in the same analytical procedure. For the statistical analysis of the Northern blot experiments, the densitometric values of the different ECM proteins were corrected for the densitometric values of GAPDH. In every case, the results shown are the mean ± SE, and they are frequently expressed as percent of their control values. Since samples with n < 10 the normality of the distribution of values is uncertain, nonparametric statistics was used for comparisons. Friedman’s test or Wilcoxon’s test was used. P < 0.05 was considered statistically significant.

RESULTS

Characteristics of old rats. Table 1 shows that urinary protein excretion and body weight in Fischer 344 rats increased progressively with aging. In contrast, no changes were detected in systolic BP or in plasma creatinine levels in rats of different ages. The mean increases of the messages for COL IV and fibronectin were ~1.5- and 3.2-fold, respectively, in kidneys of 24-mo-old rats compared with 3-mo-old rats (Fig. 1). By 30 mo of age, the mean transcript levels rose further to 2.3-fold for COL IV and 5.8-fold for fibronectin (Fig. 1). The mRNAs encoding for interstitial collagens (COL I and COL III) also increased with age. In the case of

![Fig. 1. mRNA expression in kidney cortex of different extracellular matrix (ECM) proteins during aging. A: Northern blots of total RNA from kidney cortex of 3-, 24-, and 30-mo-old animals. B: summary of results at the different ages (collagen type III data are not included, as mRNA expression of this protein in 3- and 24-mo-old animals was minimal). Blots were probed with cDNA encoding α1(IV) collagen (COL IV, circles), fibronectin (FN, squares), α1(I) collagen (COL I, triangles), α1(III) collagen (COL III), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometric analysis of the mRNA expression of the different matrix proteins was corrected with their respective GAPDH. Results are expressed as percent of control rats and represent means ± SE of 9 different animals. *P < 0.05 vs. 3-mo-old rats for every protein considered. **P < 0.01 vs. 3 and 24-mo-old control rats. §P < 0.05 vs. 24-mo-old control rats. ‡P < 0.05 vs. 3-mo-old rats for every protein considered. ¶P < 0.05 vs. 24-mo-old control rats.]

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| Table 1. Age-dependent and treatment-dependent changes in systolic BP, plasma creatinine concentration, urine protein excretion, and body weight in Fischer 344 rats |
|-----------------|----------------|-----------------|-----------------|----------------|
|                | 3 mo           | 24 mo           | 30 mo           |                |
| Systolic BP, mmHg | 144 ± 5.0      | 143 ± 9.0       | 153 ± 5.0       | 141 ± 19       |
| Plasma creatinine, mg/dl | 0.60 ± 0.02   | 0.62 ± 0.04     | 0.65 ± 0.02     | 0.67 ± 0.04    |
| Urine protein excretion, mg/day | 23 ± 3.0      | 310 ± 45*       | 165 ± 23†       | 155 ± 18‡      |
| Body wt, g      | 272 ± 17.4     | 389 ± 11.2*     | 371 ± 16.5*     | 386 ± 19.4*    |

Values are means ± SE; n = 7 in CA and TAU groups; n = 9 in 3-, 24-, and 30-mo-old control rats. C, control; CA, captopril; TAU, taurine. *P < 0.05 vs. nontreated 3-mo-old rats. †P < 0.05 vs. 3- and 24-mo-old control rats. ‡P < 0.05 vs. 24-mo-old control rats. §P < 0.05 vs. 30-mo-old control rats.
COL I, the mean increases were of 1.6-fold and 2.4-fold at 24 and 30 mo, respectively (Fig. 1). COL III did not increase in 24-mo-old rats, but it did in 30-mo-old animals (Fig. 1). To assess whether these changes in the mRNA corresponded to changes in the ECM protein content in renal cortex, the presence of COL I in rats of different ages was tested by Western blot. As shown in the Fig. 2, COL I content increased as a function of age. The densitometric analysis of this increase, performed in three different series of animals, showed mean increases of 42% and 91% in 24- and 30-mo-old rats, respectively.

Response of ECM protein mRNA levels to treatment with ACEIs. Treatment with captopril did not have any effect on the systolic BP or plasma creatinine level, but urinary protein excretion was significantly lower than in nontreated animals of the same age (Table 1). Similar results were observed with lisinopril (data not shown). There appeared to be no harmful effect of the treatments, because total body weight was not affected (Table 1) and none of the animals died during the study. Captopril significantly reduced the mRNA levels of COL IV, fibronectin, and COL I in 24-mo-old rats (Fig. 3), whereas it significantly reduced the mRNA levels of the four ECM proteins analyzed in 30-mo-old animals (Fig. 4). The quantitative analysis of these inhibitions shows that the mean reduction in the mRNA expression of COL IV and COL I ranged between 40–50% irrespective of age, whereas the mean inhibition of the fibronectin and COL III mRNA expression was about 85% (in the case of COL III this inhibition was only observed at 30 mo) (Figs. 3 and 4). When these experiments were performed with another ACEI (lisinopril), the results were similar to those found with captopril. Table 2 summarizes the results of the Northern blots performed for COL IV and COL I normalized for GAPDH mRNA levels, at 24- and 30-mo-old control and lisinopril-treated animals.

Response of ECM protein mRNA levels to treatment with taurine. As in the case of ACEIs, no changes were detected in the general status, systolic BP, and plasma creatinine concentration in the animals receiving taurine with respect to control rats (Table 1). However, urine protein excretion significantly decreased in the taurine-treated rats with respect to their controls (Table 1). As shown in Figs. 5 and 6, the mRNA expressions of COL IV and COL I were significantly reduced by taurine treatment, both in 24- and 30-mo-old rats. In the case of COL IV, the mean reductions were about 60% and 65%, at 24 and 30 mo, respectively (Figs. 5 and 6). Mean decreases of 54% and 58% were detected, after taurine treatment, in the COL I mRNA expression in both groups of old rats, respectively (Figs. 5 and 6). Taurine, administered to control rats for 3 mo, showed a significant antioxidant effect, as it decreased the MDA content in the kidney cortex (control rats 41 ± 5 nmol/g of tissue, n = 4; taurine-treated rats 8 ± 3 nmol/g of tissue, n = 4, P < 0.05 vs. control rats).

Mechanisms of the taurine-dependent reduction in the ECM protein expression. Treatment with taurine significantly reduced the TGF-β1 mRNA levels in the kidney cortex of aged rats. Figure 7 shows that taurine inhibits the expression of the TGF-β1 mRNA in 24- and 30-mo-old rats. Moreover, preincubation of human mesangial cells with taurine prevented the TGF-β1-induced synthesis of COL IV and fibronectin observed in these cells (Fig. 8). As in the case of animals, taurine prevented the hydrogen peroxide-induced increased MDA content in cultured cells (control 0.24 ± 0.02, H2O2 0.34 ± 3, taurine + H2O2 0.23 ± 2; results are expressed as nmol/10^6 cells; n = 4; P < 0.05 for H2O2 vs. the other groups).
DISCUSSION

Together with the report from Abrass et al. (1), the present results constitute a detailed analysis of the changes in ECM proteins in the renal cortex from old rats, although we cannot restrict the changes observed in ECM proteins to the glomerular and tubulointerstitial regions of the kidney. In our study, two normal components of the mesangial matrix, COL IV and fibronectin, and two abnormal components, COL I and COL III, increased with age. The increased expression of the mRNAs of these proteins was progressive, as it was more advanced in 30-mo-old than in 24-mo-old rats. However, in the case of COL III, the increased expression of its mRNA was only detected at 30 mo. The changes in fibronectin, COL I and COL IV were qualitatively comparable with previous descriptions (1). We detected increased COL IV expression in the kidney cortex of aging rats, whereas Abrass et al. (1) did not detect an increase in the protein level by immunohistochemistry. The apparent discrepancy between both works may relate to differences in the sensitivities of both techniques, to changes in the translation or stability of the mRNA or to the turnover of the proteins studied.

ACEI treatment prevented the accumulation of the different ECM proteins studied. These results are comparable to those observed in other pathological conditions, such as diabetes, experimental nephritis, or the reduced renal mass model with progressive kidney disease (5, 16, 18, 22). Lisinopril appeared as effective as captopril in preventing these changes, and a 3-mo treatment was enough to significantly block the increased mRNA expression of ECM proteins, both in 24-

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<th>Age</th>
<th>Control</th>
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<tr>
<td>24 mo</td>
<td>100</td>
<td>33.8 ± 12.6*</td>
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<td>45.9 ± 17.1*</td>
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<td>30 mo</td>
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<td>49.1 ± 10.1*</td>
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<td>34.30 ± 21.2*</td>
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*P < 0.05 vs. control untreated 24- and 30-mo-old rats.
and 30-mo-old rats. We selected two structurally different ACEI to ascertain that the observed effects were the consequence of the blockade of the angiotensin II converting enzyme and not the nonspecific action of a particular molecule.

Considering these results together with the previous description of the decreased expression of the TGF-β1 mRNA induced by the same treatment in old rats (23) and taking into account the well-described properties of this cytokine in the synthesis of ECM matrix proteins (3), the following hypothesis can be proposed for the development of aging-related progressive renal sclerosis. The local renin-angiotensin system could be involved in determining an increase in the local synthesis of TGF-β1 in renal cortex of aging rats. TGF-β1 stimulates the synthesis of ECM proteins, and it probably also blocks their degradation (3), thus inducing the development of the glomerulosclerosis and interstitial fibrosis which characterize the aging process. In this hypothesis, it must not be forgotten that ACEIs also increase kinin synthesis, and a role for these metabolites cannot be ruled out.

The most novel data provided by the present experiments are those related to taurine. This amino acid has been previously used, in other experimental models of disease, to prevent tissue fibrosis. Thus, in pulmonary fibrosis and in streptozotocin-induced diabetes mellitus, taurine supplements prevented the accumulation...
of ECM in lung (11) and kidneys (27). It seems that these effects of taurine are linked to the antioxidant ability of the amino acid (2, 10, 13). In 24- and 30-mo-old rats, the prevention of the increased mRNA levels encoding ECM proteins in the renal cortex was comparable to that observed with ACEI treatment, thus providing an alternative therapeutic approach to the prevention of aging-related progressive renal fibrosis. Moreover, the results of the taurine studies may also provide information of the importance of ROS in the genesis of the accumulation of ECM proteins in aging. If it is accepted that the main biological activity of taurine in our experimental model is as an antioxidant (2, 10, 13) and that the local synthesis of ROS seems to increase with aging (24), then it can be proposed that ROS could trigger the cellular mechanisms to overexpress the mRNA of ECM proteins.

The mechanisms that mediate the taurine effects were evaluated at two levels. First, the changes in the TGF-β1 mRNA expression after taurine treatment were analyzed. As shown in the results, taurine decreased the expression of the TGF-β1 in old rats. This cytokine increases with age in Wistar (23) and in Fischer 344 rats (data not shown). Second, the TGF-β1-stimulated synthesis of ECM proteins in cultured human mesangial cells was evaluated in presence of taurine: the amino acid prevented the effects of TGF-β1 on COL IV and fibronectin synthesis in these cells. A recent report stresses the importance of ROS as mediators of the cellular effects of TGF-β1 (12). Taurine would abrogate the TGF-β1-induced synthesis of ROS, the preceding step in the development of its cellular actions, thus blocking ECM accumulation. In consequence, taurine blocks both the mRNA expression of TGF-β1 as well as the cellular actions of the cytokine, thus providing a useful tool in the prevention of the ECM protein overexpression that characterizes aging.

The consequences from both ACEI and taurine treatments were not only morphological but also functional, as proteinuria decreased in old rats receiving these treatments. Interestingly, the antiproteinuric effect of ACEI and taurine was less marked when started later in life, although their ability for blocking ECM mRNA expressions was maintained. These findings raise important questions about the relationships between proteinuria and ECM accumulation, as the decreased proteinuria can be the cause or the consequence of the ECM changes, but also about the starting time and duration of the selected treatments and the role of ECM protein turnover in the genesis of the detected alterations. Additional experiments are needed to adequately clarify these problems.

In summary, the present results provide a more detailed analysis regarding the biochemical composition of the aging-related progressive renal fibrosis. In addition, they point to taurine as an alternative tool to ACEI for preventing the morphological changes of the kidneys from elderly individuals. Finally, they suggest a role for ROS in the pathophysiology of aging-related progressive renal fibrosis, but direct experimental confirmation is required.

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