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Cardiorenal Fibrosis and Dysfunction in Aging: Imbalance in Mediators and Regulators of Collagen

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Abstract

Cardiorenal fibrosis is a biological process that increases with age and contributes to dysfunction of the heart and kidney. While numerous circulating and tissue hormones, cytokines and enzymes have been identified in the development of cardiorenal fibrosis, several reports have suggested that the anti-fibrotic natriuretic peptide system (NPS), pro-fibrotic renin-angiotensin-aldosterone system (RAAS), transforming growth factor-beta 1 (TGF-β1), matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) are fundamental regulators and mediators of this process. However, the simultaneous assessment of these components in the development of age-mediated cardiorenal fibrotic remodeling is not completely understood. Thus, we assessed cardiorenal structure and function, the circulating NPS and RAAS and the cardiorenal tissue gene expression of collagen (Col) I, Col III, TGF-β1, MMP-9 and TIMP-1 in 2 and 20 month old Fischer rats. Our studies determined that aging was characterized by an increase in cardiorenal fibrosis that was accompanied with cardiorenal dysfunction. These alterations were associated with lower circulating atrial and C-type natriuretic peptides and higher angiotensin II and aldosterone levels in the aged rats. Moreover, we observed a decrease in Col I and III, an increase in TIMP-1 and no change in MMP-9 mRNA expressions in the aged heart and kidney, while TGF-β1 expression increased only in the aged kidney. We conclude that the age-mediated alterations in these fibrotic regulator and mediator profiles favors collagen accumulation due to an imbalance between the NPS and RAAS as well as a decline in the degradative pathway, thus suggesting a therapeutic opportunity to target these components.

Graphical Abstract

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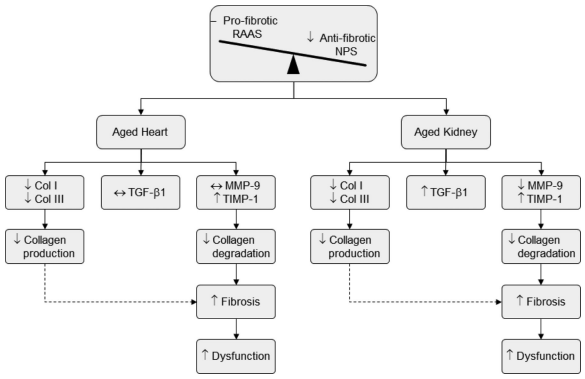

Keywords

fibrosis; cardiorenal; natriuretic peptides; renin-angiotensin-aldosterone system; gene expression

1. INTRODUCTION

The heart and kidney are hormonally linked via the natriuretic peptide system (NPS), where the cardiac and reno-endothelium derived atrial natriuretic peptide (ANP) and c-type natriuretic peptide (CNP) respectively, mediates the inhibition of cardiorenal fibrosis and adverse remodeling through the particulate guanylyl cyclase receptors [1, 2]. The NPS is counter-regulated by the renin-angiotensin-aldosterone system (RAAS) of which angiotensin II (ANG II) via the angiotensin receptor 1 (AT1) and aldosterone through the mineralocorticoid receptor contribute to adverse cardiac and renal remodeling and fibrosis [3]. Notably, combined fibrosis of the heart and kidney are hallmarks of aging [4, 5] and indeed in the extreme, contribute to both heart failure and chronic kidney disease. The interstitium of the heart and kidneys are dynamic structures that are reflective of a continuous process of synthesis and degradation of extracellular matrix (ECM) proteins including collagen and of which is influenced by circulating neurohumoral factors [6]. Indeed, an emerging view is that fibrosis of the heart and kidney involves simultaneous remodeling of both organs leading to chronic cardiorenal disease. Furthermore, the important relationship between the NPS and RAAS in the setting of age-related cardiorenal fibrosis is not clearly understood.

To date, ANP and CNP have been shown to inhibit DNA synthesis and fibroblast proliferation [7-9]. Whereas activated RAAS as well as transforming growth factor-beta 1(TGF-β1) markedly stimulate collagen deposition and the formation of cardiac and renal fibrosis [10, 11]. Moreover, the fibroblast is a key regulator collagen turnover by a balancing of collagen synthesis and degradation [12]. This regulatory process involves the interaction between the matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) [13-15], of which MMP-9 and TIMP-1 in particular, is altered in models of cardiac and renal disease [16-22]. While fibrosis remains the hallmark of cardiorenal aging and contributes to cardiorenal impairment, the mechanisms for accentuated collagen deposition in the aged heart and kidney remain poorly defined, in part,

due to the lack of simultaneous assessment of myocardial and renal fibrosis in the same setting.

Here we wanted to confirm and extend investigations on age-mediated changes in the key regulators and mediators of fibrosis and dysfunction in both the heart and kidney. We utilized an experimental rat model of aging to assess the balance between the anti-fibrotic NPS and pro-fibrotic systems RAAS and TGF-β1 and its relationship with left ventricular (LV), renal cortical and renal medullary fibrosis as well as cardiorenal structure and function. We also defined the gene expressions of collagen (Col) I, Col III, MMP-9 and TIMP-1 in the heart and kidney. We hypothesized that aging would be associated with: 1) a relative deficiency of plasma ANP and CNP as well as an activation of circulating ANG II and aldosterone; 2) parallel increases in LV, cortical and medullary fibrosis coupled with impaired cardiorenal function and, 3) changes in the collagen degrading pathway due to an imbalance in the expression of MMP-9 and TIMP-1. Our study supports the concept that an imbalance between key regulators and mediators of fibrosis, such as the NPS, RAAS and TGF-β1 pathway, characterizes cardiorenal aging and represents a therapeutic opportunity.

2. METHODS

2.1 Animals

Studies were performed in 2- and 20-month old male Fischer rats (Harlan Laboratories; n=10 per age group). The Fischer (F344) rat is a widely utilized animal model for aging studies and was developed by the National Institute of Aging[23]. This inbred rat strain closely mimics many characteristics of CV and renal aging seen in humans and also exhibits a lower tumor rate than non-inbred rat strains[24-30]. This experimental study was performed with approval of the Mayo Clinic Institutional Animal Care and Use Committee and in accordance with the Animal Welfare Act.

2.2. Echocardiography

As routinely carried out in our laboratory, standard 2-dimensional echocardiography was performed on lightly anesthetized (1.5% isoflurane in oxygen) rats using the Vivid 7 ultrasound system (GE medical Systems) and 10S transducer with ECG monitoring. M-mode images and 2-dimensional parasternal short axis images were recorded for off-line analyses of left ventricular structure and function using EchoPAC software (EchoPAC PC BTO 9.0.0, GE Healthcare) are previously described in detail [29]. Diastolic wall strain (DWS), as an index of diastolic stiffness based on the linear elastic theory, was calculated with the following equation: (systolic posterior wall thickness – diastolic posterior wall thickness) / systolic posterior wall thickness [31, 32].

2.3. Renal Function

Rats were place in metabolic cages with free access to food and water and allowed to acclimatize for 24 hours prior to collection. A 24-hour urine collection was performed on ice after acclimatization for urinary protein excretion assessment as previously described [28]. Glomerular filtration rate (GFR) was also assessed by inulin clearance on anesthetized (2.0-2.5% isoflurane in oxygen) rats as described previously [28].

2.4. Blood Pressure and Plasma Collection

Blood pressure (BP) and plasma collection was performed as previously described [29].

2.5. LV and Renal Tissue Collection

The hearts and kidneys were removed from anesthetized (2.0-2.5% isoflurane in oxygen) rats and the LV, renal cortex and medulla were carefully dissected. A cross-section of the LV and kidney containing both cortex and medulla were preserved in 10% formalin for histological analysis of fibrosis. The remaining LV, renal cortex and medulla were quickly snap frozen in liquid nitrogen.

2.6. Neurohumoral Analysis

Plasma ANP [33], CNP [29], ANG II [34] and aldosterone [35] were determined by commercially available radioimmunoassays as described in prior studies.

2.7. Histological Analysis for Cardiorenal Fibrosis

Cross-sections of paraffin-embedded LV and renal tissue (4 μm) were stained with picrosirius red and quantified as previously described [28, 29].

2.8. Quantitative mRNA Expression

As previously described [36], real-time polymerase chain reaction (RT-PCR) was used to quantify Col I, Col III, TGF-β1, MMP-9, TIMP-1 and 18S gene expression levels. Briefly, total RNA was extracted from ~30 mg cardiac or renal cortical or medullary tissues using Qiagen RNeasy kit (Qiagen, Hilden, Germany). Then the cDNA was reverse transcribed and triplicate cDNA aliquots were amplified using sequence-specific primers (Geneworks, Adelaide, SA, Australia) with TagMan fluorogenic probe for TGF-β1 and 18S (Applied Biosystems) or SYBR Green detection for Col I, Col III, MMP-9 and TIMP-1 (Applied Biosystems) using an ABI prism 7900HT sequence Detection System (Applied Biosystems). The primer pairs were designed using Primer Express 2.0 software (Applied Biosystems) based on published sequences (http://www.ncbi.nlm.nih.gov). 18S rRNA was used as an endogenous control in all experiments and used to standardized quantitation for the expression of each gene.

2.9. Statistical Analyses

Our results are expressed as mean ± SE. Student unpaired *t-tests* were employed for single comparisons between age groups. All statistical analyses were performed using Graphpad Prism 6 software. Statistical significance was accepted as $P<0.05$.

3. RESULTS

3.1. Age and Cardiorenal Structure and Function

Cardiorenal structure and function as well as BP are reported in Table 1. As expected, there were significant increases in body, left ventricular, total kidney weights with aging. The aged rats exhibited significantly larger LV cavity dimension, elevated urinary protein excretion as well as significantly lower ejection fraction, diastolic wall strain and GFR.

Analysis of anesthetized rats *in vivo* revealed a significantly higher systolic and diastolic BP, with no change in heart rate (data not shown) in the aged rats.

3.2. Age on Circulating NPS and RAAS

Table 2 reports circulating ANP, CNP, angiotensin II and aldosterone with aging. Specifically, the aged rats had significantly lower plasma levels of ANP and CNP, whereas plasma levels of ANG II and aldosterone were significantly higher compared to the young rats.

3.3. Age and Cardiorenal Fibrosis and Fibrosis-Related Gene Expression

The magnitude of fibrosis in the LV, renal cortex and medulla (Figure 1A, B and C, respectively) was significantly increased in the aged rats compared to the young rats. As type I and III collagens are the major fibrillar collagens responsible for fibrosis in normal and diseased heart and kidneys, we examined the effect of age on collagen synthesis regulated at the transcriptional level by RT-PCR. As illustrated in Figure 2, the expression of mRNA for LV Col I and III significantly decreased with age. While in the kidney there was a strong trend for a reduction in Col I mRNA expression in the renal cortex and medulla, whereas there was significant reduction in Col III mRNA in the renal cortex and trended to decrease in the renal medulla. Aging did not change levels of TGF-β1 mRNA expression in the LV, although there was a significant increase in TGF-β1 transcript levels in the renal cortex and medulla.

3.4. Age and Cardiorenal Gene Expression of Regulators of Collagen Turnover

As illustrated in the Figure 3, mRNA expression of MMP-9 were not altered in the LV, however was significantly lower in the renal cortex and trended to be lower in the renal medulla in the aged rats. TIMP-1 gene expression in the LV, renal cortex and medulla significantly increased with aging. Moreover, an essential component to ECM degradation is reflected by the MMP-9/TIMP-1 ratio and this important ratio is reported in Table 3. The data presented here suggest that net cardiorenal MMP activity, based on the MMP-9/TIMP-1 ratio, was significantly lower in the LV, renal cortex and medulla, thus suggesting a decrease in collagen degradation.

4. DISCUSSION

Our study defines the parallel increase in fibrosis and associated cardiorenal dysfunction in the heart and kidney, together with key circulating and tissue modulators of fibrogenesis in an experimental rat model of aging. Specifically, we found an important shift in the balance between the anti-fibrotic NPS, specifically ANP and CNP and the pro-fibrotic RAAS and TGF-β1 pathway that favors fibrogenesis. We also report that age mediated cardiorenal fibrosis is correlated with an overall decrease in collagen degradation, rather than an increase in collagen production, as the gene expression of TIMP-1 increased, while Col I, Col III and MMP-9/TIMP-1 ratio decreased. Together, these data suggest that altered collagen turnover at the gene level is modulated by a number of pathways that are associated with age-mediated cardiorenal fibrosis and dysfunction.

Fibrosis of the heart and kidney is a hallmark of aging [4, 5], however few investigations have simultaneously investigated key systems that contribute to this phenotype in both the heart and kidney. Here we confirm previous reports by our group and others [28, 29, 37, 38] that age-mediated cardiorenal fibrosis increases with aging in the rat. Interestingly, Col I and Col III mRNA transcripts decrease in the aging LV, renal cortex and medulla, thus suggesting the increase in fibrosis in the aged heart and kidney is not likely due to increase in collagen production. Indeed, the observation that BP is increased with age could support an important load dependent mechanism in the formation of fibrosis in the LV.

The RAAS, TGF-β1 and NPS are fundamental pathways in implicated in cardiorenal fibrogenesis due to disease and aging, with the RAAS and TGF-β1 promoting organ fibrosis through fibroblast proliferation, collagen synthesis and accumulation and the NPS counteracting these fibrotic processes [3, 39]. In the present investigation, we found a significant increase in the circulating ANG II and aldosterone in the aged rat. *In vitro* studies have demonstrated that ANG II or aldosterone increase collagen gene expression [40], stimulate TGF-β1 production in fibroblasts [41, 42] and induced fibroblast proliferation [43]. Indeed, as studies has suggested that TGF-β1 is key factor that contributes to the formation of cardiac and renal fibrosis in various disease states [44, 45], we also quantified the gene expression of TGF-β1 in the aging heart and kidney. We found that TGF-β1 mRNA remained unchanged between the young and aged LV, however was significantly up regulated in both the aged renal cortex and medulla. Our data differs, in part from previous studies, as elevated plasma ANG II and aldosterone seen the aged rats was not associated with increased Col I or III gene expression in the LV or kidney. However, the elevation in ANG II and aldosterone may have contributed to the increase in TGF-β1 gene expression seen the kidney and is consistent with previous studies demonstrating the RAAS effects on TGF-β1 production and renal fibrosis [11]. Notably, recent studies have reported that TGF-β1 can suppress collagen production through the induction of a negative regulator of collagen transcription such as CUX1 [46]. Thus, additional studies are needed to define the unique regulatory role and interaction of TGF-β1 and the RAAS on cardiorenal fibrogenesis in aging.

The relationship between the RAAS and NPS continues to evolve and in setting of aging, remains to fully be understood. Here, we confirmed our previous study [29] that the aged rat is characterized by a significant decrease of plasma CNP. Interestingly, we also observed a significant decline in circulating ANP in the aged rats. This decrease in ANP could be, in part, responsible for elevated ANG II and aldosterone as well as cardiorenal fibrosis. Specifically, ANP has been demonstrated to directly inhibit aldosterone production [47], aldosterone-stimulated nuclear translocation of the mineralocorticoid receptor [48] and renin release [49], as well as counteract the effects of ANG II [50] and suppress organ fibrosis through various mechanisms [7, 8, 51, 52]. While our work and by others [53], in part, supports the idea that ANP may be decreased with older age, review of the human and experimental literature, albeit limited, is generally in contrast this finding and support an elevation of ANP with aging [54, 55]. It is tempting to speculate that differences in rat strain, age groups or concomitant disease seen in older humans such as hypertension, chronic kidney disease or atrial fibrillation could account for this discrepancy. Although the

overall deficiency of ANP and CNP may have a multifactorial role in the development of cardiorenal fibrosis seen in this experimental aging model, further studies in humans from children to the elderly, especially without concomitant disease, is warranted.

Our findings underscore the importance of collagen degradation in the setting of cardiorenal aging. Importantly organ fibrosis is regulated, in part, by a group of enzymes called MMPs, which degrade ECM proteins, and their endogenous inhibitors, TIMPs [13-15]. Given the decline in both Col I and III mRNA levels in the aging heart and kidney seen here, it is plausible that a reduction collagen turnover at the transcription level during cardiorenal aging may be a function of a decline in collagen degradation. Here, we found no change in MMP-9 gene expression in the LV, however there was decrease in MMP-9 mRNA expression the renal cortex and medulla, suggesting that there is an impairment in the regulation of collagen degradation. Our postulation is further supported by the fact that the gene expression of cardiac and renal TIMP-1, which has high specificity for MMP-9 [56], was significantly increased. As there was no increase in MMP-9 mRNA levels coupled with elevated levels of TIMP-1, this balance shift is suggestive of an impaired proteolytic process to degrade collagen in the aged heart and kidney. Our data are consistent with this notion as the cardiorenal MMP-9/TIMP-1 ratios were significantly lower in the aged rats compared to the young rats. This is consistent, in part, with the elegant study by Bonnema *et al.* [57], who reported changes in plasma MMPs, TIMPs and its ratio with human aging are in favor of decreased ECM degradative capacity. Thus the aged heart and kidney may be characterized as an environment with the inability to breakdown collagen resulting in fibrosis, which may be in contrast to processes that are initiated by disease and result in an increase in fibrosis.

There are several limitations to the current study and we should be cautious in interpreting the current data. Additional investigations are needed to evaluate the cardiorenal tissue expression of the NPS (i.e. atrial and ventricular levels) and RAAS including their receptors, as well as a detailed assessment of the MMP and TIMP protein and activity levels including and beyond MMP-9 and TIMP-1. Moreover, comprehensive studies are needed in higher species as well as multiple age groups in order to have a more complete assessment of these mediators and regulators of collagen turnover and fibrosis that gradually occur during the aging process. Despite these limitations, this investigation does provide additional insights into myocardial and renal fibrosis due to aging in a parallel process.

5. CONCLUSION

Our findings in the aging Fischer rat demonstrates that cardiorenal fibrosis is characterized by an imbalance between the anti-fibrotic NPS and pro-fibrotic RAAS/TGF-β1 pathways, as well as a shift in the MMP-9 and TIMP-1 profiles, that favor a decline in collagen degradation, rather than increased production. These age-mediated changes in the cardiorenal ECM may, in part, contribute cardiorenal dysfunction including diastolic impairment and proteinuria. This study lays the foundation for further investigations in the aged heart and kidney, to define whether enhancing collagen degradation by augmenting the NPS, inhibiting the RAAS and/or other novel molecular pathways including direct

manipulation of TGF-β1, the MMPs or TIMPs will delay fibrosis and reduce the risk for cardiorenal dysfunction.

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Highlights

- Experimental aging is associated with a parallel increase in cardiorenal fibrosis
- An imbalance between the NPS, RAAS and TGF-β1 pathways favors fibrogenesis
- Cardiorenal fibrosis is correlated with a decline in collagen degradation with aging

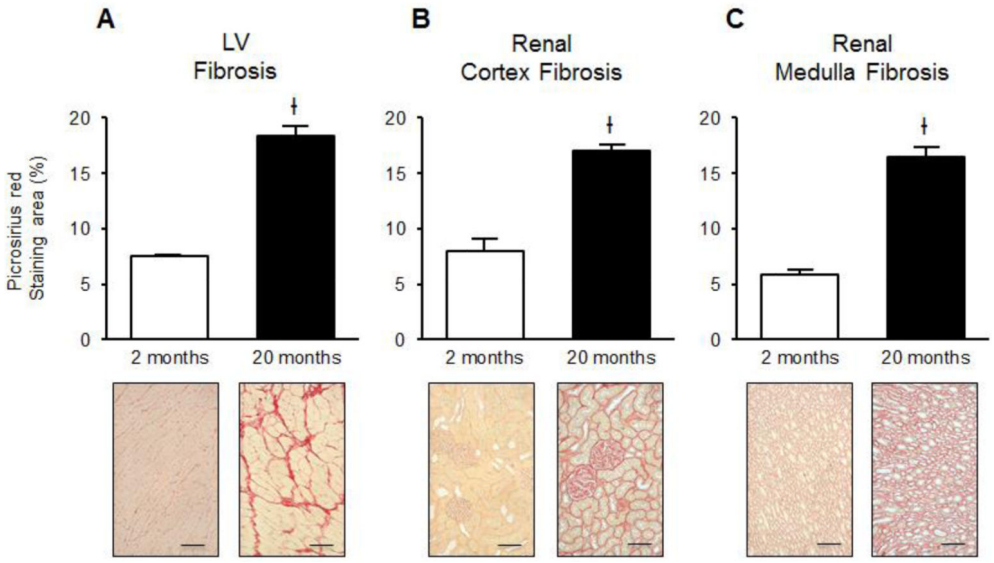

Figure 1.

Effects of aging on LV (A), renal cortical (B) and medullary (C) fibrosis as determined by picrosirius red staining in 2 and 20 month old Fischer rats at 20x objective magnification. Ł P<0.001 vs. 2 months. Black scale bar = 100 μm.

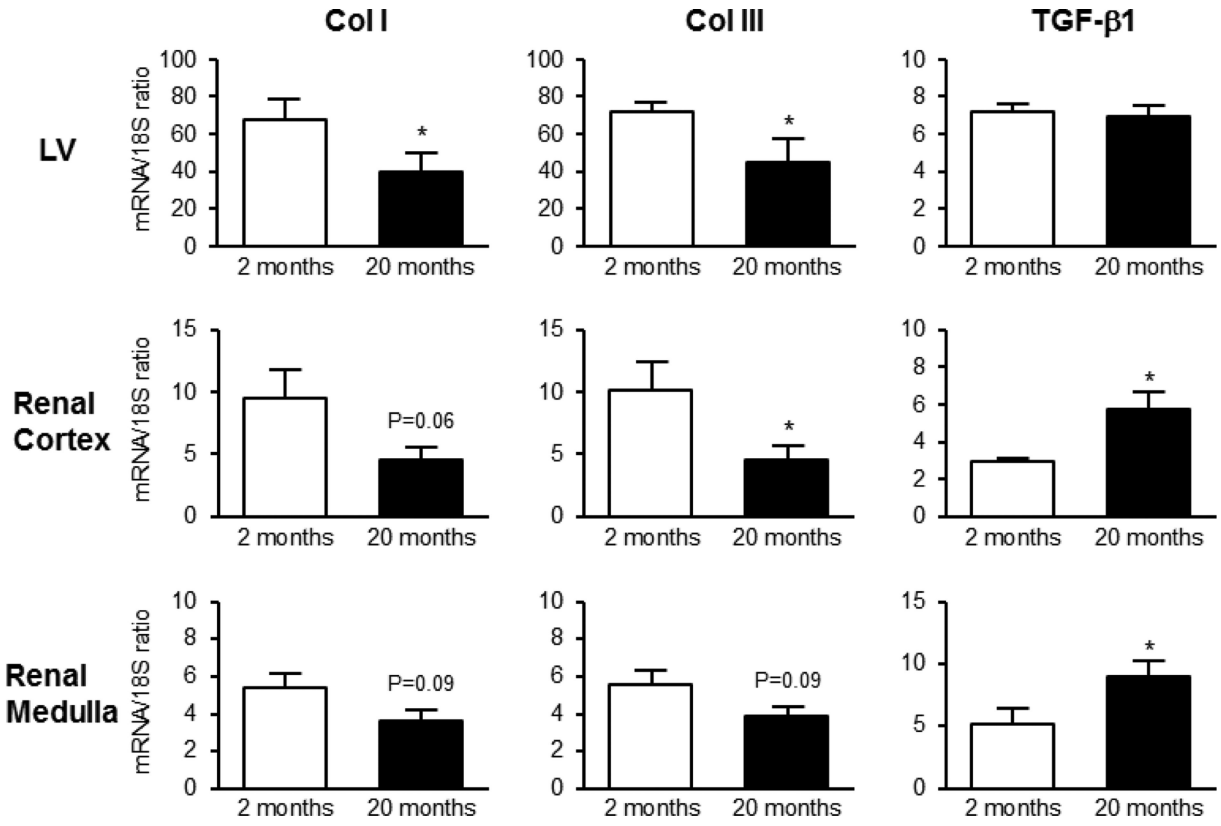

Figure 2.

mRNA expression of pro-fibrotic genes Col I, Col III and TGF-β1 (first, second and third column, respectively) in the LV (upper row), renal cortex (middle row) and medulla (lower row) between 2 and 20 month old Fischer rats. * P<0.05 vs. 2 months.

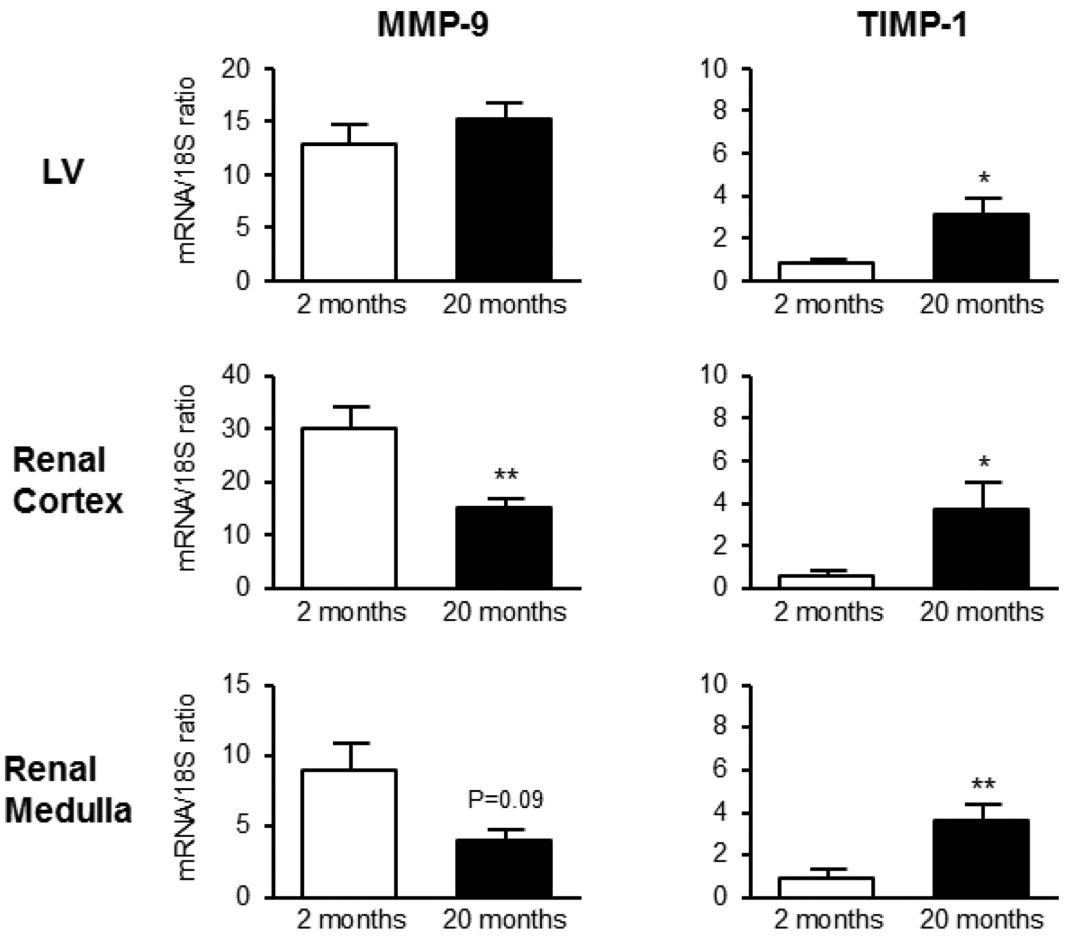

Figure 3.

mRNA expression of regulators of collagen turnover genes MMP-9 and TIMP-1 (first and second column, respectively) in the LV (upper row), renal cortex (middle row) and medulla (lower row) between 2 and 20 month old Fischer rats. * P<0.05 or ** P<0.01 vs. 2 months.

Table 1

Cardiorenal structure and function and blood pressure between 2 and 20 months old Fischer rats

Parameter	2 months	20 months
Body Weight (g)	210 ± 3	449 ± 6†
Cardiac Structure and Function		
LV Weight (mg)	470 ± 7	812±22†
LVEDD (mm)	6.74 ± 0.10	7.41 ± 0.08†
LVESD (mm)	3.28 ± 0.10	4.35 ± 0.09†
EF (%)	88 ± 1	79 ± 1†
Diastolic Wall Strain	0.39 ± 0.01	0.26 ± 0.03†
Renal Structure and Function		
Total Kidney Weights (mg)	1623 ± 35	2729 ± 56†
GFR (ml/min/kg)	3.95 ± 0.23	2.52 ± 0.33**
Protein Excretion Rate (μg/min)	5.6± 0.4	15.6 ± 3.6†
Blood Pressure		
Systolic BP (mmHg)	101 ± 2	116 ± 3**
Diastolic BP (mmHg)	91 ± 2	102 ± 4*

Values are mean ± SE.

*P<0.05

**P<0.01 or

†P<0.001 vs. 2 months. LV = left ventricular; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; EF = ejective fraction; GFR = glomerular filtration rate; BP = blood pressure

Table 2

Plasma natriuretic peptides, angiotensin II and aldosterone between 2 and 20 month old Fischer rats

Parameter	2 months	20 months
ANP (pg/ml)	27 ± 3	14 ± 1**
CNP (pg/ml)	34 ± 4	10 ± 1**
ANG II (pg/ml)	11 ± 3	20 ± 3*
Aldosterone (ng/dl)	18 ± 3	40 ± 6**

Values are mean ± SE.

* P<0.05 or

** P<0.01 vs. 2 months. ANP = atrial natriuretic peptide; CNP = c-type natriuretic peptide; ANG II = angiotensin II

Table 3

MMP-9 to TIMP-1 ratio in the left ventricle, renal cortex and medulla between 2 and 20 month old Fischer rats

Parameter	2 months	20 months
LV MMP-9/TIMP-1	17.01 ± 3.92	7.10 ± 1.14*
Renal Cortex MMP-9/TIMP-1	58.69 ± 8.13	6.07 ± 0.98**
Renal Medulla MMP-9/TIMP-1	21.68 ± 9.85	1.18± 0.10*

Values are mean ± SE.

* P<0.05 or

** P<0.01 vs. 2 months. MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase