Upregulation of tissue inhibitor of metalloproteases-1 (TIMP-1) and procollagen-N-peptidase in hypertension-induced renal damage

Michael Hultström1,2, Sabine Leh1,2,3, Trude Skogstrand2 and Bjarne M. Iversen1,2

1Renal Research Group, Department of Medicine, Haukeland University Hospital, Bergen, Norway, 2Institute of Medicine, University of Bergen, Bergen, Norway and 3Department of Pathology, Haukeland University Hospital, Bergen, Norway

Abstract

Background. Hypertensive renal damage starts in the juxtamedullary cortex (JMC) and gradually extends towards the outer cortex (OC). The intention of the study was to examine if the increase of fibrous tissue in the JMC of the spontaneously hypertensive rat (SHR) is dependent on an increase of collagen synthesis or a decreased collagen breakdown compared to the normotensive control (WKY).

Methods and results. The renal damage was evaluated by light microscopy, and the amount of fibrosis was quantified using Sirius red staining. Real-time RT-PCR was used to quantify mRNA for: collagen-type-1-alpha-1 (colla1), procollagen-n- and -c-proteinase, matrix metalloproteases, MMP-2 and MMP-9, tissue inhibitor of metalloproteases, TIMP-1 and TIMP-2. Western blot was used to quantify the proteins of MMP-2, MMP-9, TIMP-1 and TIMP-2. The relative activities of MMP-2 and MMP-9 were assayed by zymography. The JMC in SHR had an increased amount of collagen as measured by Sirius red and a 15-fold increase in the mRNA for colla1. The gene expression of procollagen-c-proteinase was unchanged while procollagen-n-proteinase was increased in SHR and had the highest expression in the JMC. The mRNA for MMP-2 and MMP-9 showed increased expression in SHR, but not specifically in the JMC. Protein analysis showed increased expression of MMP-2 in SHR and in the JMC. MMP-9 protein was lower in SHR. TIMP-1 was increased in SHR at both mRNA and protein level and more so in the JMC. The mRNA and protein analysis of TIMP-2 showed small differences between SHR and WKY.

Conclusion. An imbalance of collagen metabolism featuring increased synthesis and inhibition of breakdown favours renal interstitial fibrosis in SHR.

Keywords: hypertension; interstitial fibrosis; MMP; procollagen peptidase; TIMP

Introduction

The spontaneously hypertensive rat (SHR) is an experimental model of hypertension and shows increased blood pressure at 4-6 weeks of age. Renal damage occurs gradually and is very mild in a 10-week-old SHR. At this age, they show only slightly increased glomerular capillary pressure ($P_{GC}$) and little glomerulosclerosis [1]. At 40 weeks of age, the juxtamedullary $P_{GC}$ is constantly elevated while the degree of glomerulosclerosis continuously increases with age and is pronounced in 60-70-week-old rats [1]. At 40 weeks of age, significant proteinuria is seen, and it worsens with age in contrast to normotensive Wistar-kyoto rats (WKY), which demonstrate no proteinuria even though this strain exhibits juxtamedullary sclerosis with aging although less than in SHR [1,2].

The morphological changes in juxtamedullary cortex consist of pronounced thickening of the vascular wall of pre-glomerular resistance vessels, mainly the interlobular artery (ILA) but also the afferent arterioles. The interstitium is expanded with infiltration of lymphocytes and deposition of collagen. In addition, hypertrophy and hyperplasia of the cellular components in the glomeruli associated with sclerosis are striking findings in the deep cortex of the hypertensive animals [2,3].

The amount of interstitial fibrosis and glomerular sclerosis is the result of a balance between synthesis and degradation of collagens in the kidney [4]. The total amount of fibrous tissue may be caused by either a change in collagen synthesis or collagen breakdown. This delicate balance is regulated not only by the synthesis of procollagen, but also by its modification into collagen by procollagen-proteinases specific for the n- and c-terminal domain [5], both of which are necessary for collagen fibril formation [6,7]. The breakdown of collagen is affected by matrix metalloproteases (MMP) that in turn are influenced by tissue inhibitors of metalloproteases (TIMP). The process may thereby be modulated at several points, each of which may affect the final outcome of the fibrous process in the kidney.

The mechanisms involved in the development of renal fibrosis are not well studied, and the different models used to study chronic renal failure in hypertension vary in the
pattern in which renal damage progresses. The SHR is a model with late development of chronic renal failure even though hypertension develops at an early age, and it is recognized as a model for essential hypertension, which also usually develops chronic renal failure with ageing. Interstitial fibrosis has been shown to consist of mostly collagen-type-I-alpha-1 (col1a1) while the glomerular and vascular lesions have been found to consist of collagen III and IV [8,9]. In the present study, we have focused on col1a1 as this type of collagen represents a good marker for the increase of interstitial fibrosis [10].

However, while the distribution of fibrosis in the kidney cortex has been previously studied, the intracortical distribution of gene and protein expression has not been investigated. The aim of the present study was to further investigate the regulation of these genes and proteins in different parts of the renal cortex, showing different levels of renal injury.

Material and methods

An expanded description of material and methods can be found in an online supplement.

Animals

A total of 9 WKY and 11 SHR male rats (Møllegaard, Denmark) at the age of 60 weeks were used in the experiments. Three rats were kept in each cage. They were fed standard rat chow and had free access to tap water. Before sacrifice, the systolic blood pressure was measured in all animals and 24 h urine was collected in metabolic cages for quantification of urinary protein excretion. The animals were sacrificed under pentobarbital anaesthesia (120 mg/kg). The experiments were performed in accordance with, and under the approval of the Norwegian State Board for Biological Experiments.

Measurements of systolic blood pressure (sBP)

Systolic BP was measured in unanaesthetized rats using the tail-cuff method (W+S Electronics, Ugo Bazile) after the animals were pre-warmed in a cupboard for 10 min at 35°C.

Urinary protein excretion

Urine was collected for 24 h while the rats were in metabolic cages with free access to food and water. Total urinary protein and albumin concentration was measured by agarose gel electrophoresis on a Hydrasys (Sebia) semiautomated analyzer. Normal values are <20 mg 24 h⁻¹.

Surgical procedures

The abdominal aorta was isolated through a frontal approach, and the kidneys were perfused with ice-cold PBS by cannulating the distal abdominal aorta and ligating the aorta proximal to the renal arteries. Thereafter, the kidneys were removed, decapsulated and weighed.

Tissue preparation

The decapsulated kidneys were cut in transversal slices, which were either put directly in RNA-later, frozen or fixed in 4% buffered formaldehyde. The border between the medulla and cortex was identified by stereomicroscopy, and cortical pieces for mRNA and protein analysis were taken from the outer half (outer cortex) and inner half (juxtamedullary cortex). These pieces were kept in RNA-later at −20°C until use for RNA-isolation. The pieces of frozen tissue were kept at −80°C until use.

Light microscopy

A transversal slice of the left kidney fixed in 4% buffered formaldehyde was embedded in paraffin by standard procedures. Sections were stained with periodic acid-Schiff (PAS) and Lendrum’s Martius scarlet blue (MSB).

Collagen quantification with Sirius red stain and polarization microscopy

Corresponding parts of the kidney tissue containing both inner and outer cortex from all animals were embedded in multitissue paraffin blocks. Collagen content was expressed as percentage detected area.

Quantitative reverse transcriptase PCR

The gene expressions for col1a1, procollagen-c-proteinase, procollagen-n-proteinase, MMP-2, MMP-9, TIMP-1 and TIMP-2 were investigated through real-time RT-PCR. In all quantitative reactions, the same three-step dilution of cDNA standard was used as reference and the results are expressed as the quantity of the relevant mRNA in relation to the quantity of 18S-ribosomal RNA in the same cDNA sample.

Protein extraction

Tissues were lysed in buffer containing 20 mM Tris-HCl, 330 mM Sucrose, pH 7.3, supplemented with one tablet of protease inhibitor cocktail (complete EDTA-free; Roche Diagnostics GMBh, Germany) per 10 ml extracting buffer. After homogenization, samples were centrifuged at 12 000 rpm for 10 min to remove cell debris. Extracted supernatants were stored at −80°C. Protein concentrations were determined using Bradford protein assay reagent.

Western blot analysis

The protein expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 was investigated by western blot analysis by electrophoresis of 60 µg of extracted proteins through 10% SDS-polyacrylamide gel electrophoresis. The bands were visualized by the enhanced chemiluminescence method and quantified using the Image J program. The membrane was then re-probed with anti-actin to adjust for differences in protein loading. Preliminary experiments showed a linear relationship with a good correlation between the amount of loaded protein and measured chemiluminescence for
Zymography

The relative amounts of different proteins are not easily deduced from PCR or western blot because of the unknown relative efficiencies of primers and antibodies. We therefore performed gelatine zymography (Bio-Rad) on the tissue supernatant because findings from this method reveal the intrinsic enzyme activity of MMP-2 and MMP-9.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). The variance between samples from OC and JMC in WKY and SHR for each parameter was tested with ANOVA and the Student–Newman–Keuls test as a post hoc test. $P < 0.05$ was considered statistically significant. Due to the distribution of the results in the histological examination, we have used Mann–Whitney test for comparing between groups and Wilcoxon signed-ranks test within the groups.

Results

In the present study, WKY weighed more than SHR (500 ± 23 g vs 425 ± 10 g, $P < 0.05$) but the kidney weights were not different (1.44 ± 0.07 g vs 1.35 ± 0.03 g). Systolic blood pressure was lower in WKY compared to SHR (135 ± 2 mmHg vs 214 ± 6 mmHg, $P < 0.05$). The urinary protein excretion was 17 ± 1 in WKY while it was 56 ± 7 mg 24 h$^{-1}$ in SHR ($P < 0.05$).

Figure 1A shows a light microscopic overview of transversal slices from WKY rats and SHR where cortex and medulla are clearly visible. Figure 1B and C show representative images of the JMC in WKY and SHR. The most prominent changes were found in the JMC in SHR. There was hypertrophy of the muscular layer in arteries and arterioles. The intima was thickened with prominent endothelial cells, focal infiltration of mononuclear inflammatory cells, insudation of proteinaceous material and pronounced fibroelastosis. Few glomeruli showed a slightly retracted capillary

actin, MMP-2, MMP-9, TIMP-1 and TIMP-2 at the concentrations used in the experiments.
convolute and there were some glomeruli with segmental cellular proliferation, increase of extracellular matrix, prominent podocytes and broad adhesions with Bowman’s capsule. Whereas the glomerular damage was discrete, the tubulointerstitial changes were more prominent but still focal: there was tubular widening and tubular atrophy associated with expanded interstitium with fibrosis and focal infiltrates of mononuclear cells. All these changes were mainly seen in the JMC of SHR, but some foci with tubular atrophy and interstitial fibrosis could also be seen in the OC of SHR. OC and JMC of WKY showed normal arteries, arterioles, tubuli and interstitium. There were singular glomeruli with segmental sclerosis.

The col1a1 mRNA expression was about 15 times higher in the JMC of SHR than in WKY and 2–3 times higher in the JMC when compared to the OC in SHR (Figure 2). The col1a1 mRNA expression was not different in the outer and inner cortex in WKY (Figure 2). Quantification of collagen proteins using image analysis of Sirius red stained sections showed significantly higher levels in the JMC of both strains compared to OC (Figure 3). The high levels of collagen content especially in the JMC in WKY can be explained by pre-existing collagen collars around the large vessels. Collagen content in JMC was significantly higher in SHR compared to WKY reflecting the observed fibrosis in histological sections. Typical pictures with Sirius red staining are shown in Figure 4A–D. The amount of collagen was clearly higher in the JMC of SHR compared to JMC in WKY. A similar pattern was seen under polarized light due to enhancement of its natural birefringency by Sirius red.

The gene expression of procollagen-c-proteinase was not different between groups while procollagen-n-proteinase

![Fig. 3. Percentage interstitial fibrosis in OC and JMC in WKY and SHR quantified by Sirius red staining. *P < 0.05 in comparison with WKY and # with OC in the same strain.](image)

![Fig. 4. Representative images of Sirius-red-stained sections showing inner cortex of (A, C) WKY and (B, D) SHR. Interstitial collagen is red in white light (A, B) and bright under polarized light (C, D) due to enhancement of its natural birefringency by Sirius red. SHR shows focal interstitial fibrosis whereas there is normal collagen content in WKY. Scale bar: 100 µm.](image)
was significantly higher in the JMC of SHR than in the OC and also significantly higher in SHR than in WKY (Figure 5A and B).

The mRNA expression for MMP-2 was twice as high in SHR compared to WKY, with no difference between OC and JMC (Figure 6A). Protein analysis showed, however, a significant increased expression of MMP-2 in the JMC of SHR (Figure 6B), which is exemplified in Figure 6C. In contrast, there seems to be a dissociation between gene expression and protein level for MMP-9 in both WKY and SHR. Protein expression for MMP-9 is significantly reduced in JMC of SHR while mRNA showed higher expression in SHR in both OC and JMC compared to WKY (Figure 7A and B). Interestingly, protein expression is high and mRNA low in both OC and JMC in WKY. The gelatin degradation for active MMP-9 measured by zymography did not show any difference between the two compartments or between SHR and WKY (Figure 7C). The active form of MMP-2 was not observed in the tissue. The latent forms of both MMP-9 and MMP-2 showed a pattern similar to that of active MMP-9 (data not shown).

The mRNA and protein expression for TIMP-1 were expressed three times higher in JMC of SHR compared to JMC of WKY, and these values were significantly higher in JMC compared to OC in SHR. The mRNA for TIMP-1 was higher in OC of SHR compared to WKY, but this was not seen at protein levels (Figure 8A–C). In contrast, small changes were seen in TIMP-2. The mRNA for TIMP-2 was expressed slightly higher in SHR than in WKY and significantly lower in JMC compared to OC in SHR. The same tendency was also seen in OC and JMC of WKY (0.43 ± 0.06 vs 0.31 ± 0.03 TIMP-2 mRNA/18S, \( P = 0.055 \)). Protein analysis of TIMP-2 reveals no differences between WKY and SHR (Figure 9A and B).

**Discussion**

The main result of the present study is that the well-known increase of renal interstitial fibrosis in the juxtamedullary cortex...
cortex of SHR is associated with local increased expression of col1a1 at both protein and mRNA level and increased collagen deposition above this by induction of the procollagen-n-proteinase. This increased deposition of collagen is, however, modified by changed expression of both collagenases and inhibitors of these enzymes. The increased expression of both mRNA and protein TIMP-1 may be of particular importance as it showed a pattern similar to that of col1a1 mRNA in both OC and JMC in SHR.

The tubular and glomerular damage in juxtamedullary cortex of a 70-week-old SHR is associated with high glomerular capillary pressure and leakage of protein into the tubular lumen. Although the glomerular capillary pressure does not increase further after 40 weeks of age in SHR, the degree of glomerulosclerosis, glomerular adherence, focal sclerosis, podocyte damage as well as tubular damage continued to develop [1]. Minor degenerative changes at glomerular and tubular level have been found in the juxtamedullary cortex of old WKY where the glomerular capillary pressure is normal [1].

The mechanisms involved in the damaging of the JMC in hypertension have been investigated in earlier studies conducted in our laboratory. We have found a greater overshoot of renal blood flow (RBF) after a step increase of perfusion pressure in SHR than in WKY and greater in the inner cortex than in the outer cortex in both strains [11]. The difference in the amount of renal damage and fibrosis in the cortex of SHR may be due to an active pressure pulse buffering of the ILA thereby keeping the systemic pressure variations from reaching and damaging the superficial cortex [12]. The successive involvement of larger parts of the cortex may consequently depend on vascular
hypertrophy caused by the increased demands on the ability of the ILA to buffer pressure variation.

The present study shows that the increased collagen deposition in old SHR is associated with increased gene expression of col1a1 as well as one of the necessary enzymes for its deposition, procollagen-n-proteinase; this pattern is reminiscent of that seen in the progression of the functional deficit [11]. It is important to note that the use of Sirius red to quantify fibrous tissue may be hampered by quantitative errors, but it still remains the gold standard for assessing fibrosis [13]. The fact that we see a larger degree of regulation of the procollagen-n-proteinase might be explained by the fact that the procollagen-c-proteinase is more efficient and that it may be procollagen-n-peptidase that is the rate-limiting enzyme [7]. This important role of the regulation of collagen deposition has not been previously shown in hypertensive renal damage as far as we know.

In addition, the expressions of both MMP-2 and MMP-9 mRNA are increased in SHR. The regional distribution of mRNA of MMP-2 and MMP-9 is not closely related to the amount of fibrosis, as the expression of these enzymes is equally high in the OC and the JMC. However, protein expression for MMP-2 tended to be lower in the JMC of SHR, a finding that may suggest a lower degradation of collagens in JMC of SHR. However, the results of the MMP activity assay by zymography did not show any difference between strains or between the parts of the cortex for active MMP-9. The level of active MMP-2 was not measurable. This is in accord with the findings of other investigators in several organs and might indicate that the MMP-9 activity is the quantitatively more important enzyme but might also be a sign of high turnover of MMP-2 in its active form.

Furthermore, mRNA and protein for TIMP-1 showed a high expression in JMC of SHR and demonstrated nearly the same regional distribution as mRNA col1a1. This may indicate a major role in the deposition of col1a1 and development of fibrosis, the hypertensive renal disease of
In contrast, TIMP-2 showed higher mRNA expression in SHR in general while protein activity was not changed.

In the present study, MMP-9 and TIMP-2 show dissociation between mRNA expression and measurable protein levels indicating substantial post-transcriptional regulation. MMP-9 protein synthesis has indeed been shown to be regulated independently of the mRNA expression [14]. On the other hand, little seems to be known about the post-transcriptional regulation of TIMP-2, and the absolute differences between mRNA and protein expression in the present study is not that large considering the large variation of protein levels in WKY (Figure 9B).

MMP-2 and MMP-9 are expressed in a variety of tissues but most importantly in the proximal tubuli, glomeruli and interstitium [15,16]. The increased expression of MMP-2 in SHR indicates that degradation of collagen is taking place and may reduce the total amount of collagen deposition. Recent work has demonstrated that both MMP and TIMP expression are increased in hypertension, and these findings may reflect an abnormal extracellular metabolism in the hypertensive state [17]. MMP-2 and MMP-9 have been shown to be necessary for tubular epithelial mesenchymal transition and may thus stimulate the fibrous process rather than inhibit it [18].

TIMP-1 and TIMP-2 are the two most well-studied inhibitors of metalloproteases and neither of them is specific to any particular MMP [19]. TIMP-1 has been shown to be produced by the interstitial cells in the kidney [20] while TIMP-2 appears to be expressed in glomerular mesangial cells [21]. Our results suggest an important role for TIMP-1 in renal interstitial fibrosis in the SHR. As far as we know, this has not been shown before.

In conclusion, the increased expression of procollagen-

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