Chronic AT\textsubscript{1} Blockade Stimulates Extracellular Collagen Type I Degradation and Reverses Myocardial Fibrosis in Spontaneously Hypertensive Rats

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Abstract—It has been suggested that left ventricular fibrosis in spontaneously hypertensive rats (SHR) is the result of both exaggerated collagen synthesis and insufficient collagen degradation. We have shown previously that chronic treatment with the angiotensin II type 1 receptor antagonist losartan results in diminished synthesis of collagen type I molecules and reversal of myocardial fibrosis in SHR. This study was designed to investigate whether losartan also affects the extracellular degradation of collagen type I fibers in the left ventricle of SHR. The study was performed in 30-week-old normotensive Wistar-Kyoto rats (WKY), untreated SHR, and SHR treated with orally administered losartan (20 mg/kg per day) for 14 weeks before they were killed. Ventricular collagenase activity was determined by degradation of [\textsuperscript{14}C]collagen with tissue extracts. Ventricular expression of tissue inhibitor of metalloproteinases 1 (TIMP-1) mRNA was analyzed by Northern blot. A histomorphometric study of the left ventricle was performed in all rats. Compared with WKY, SHR exhibited left ventricular hypertrophy, increased (\textit{P} \leq 0.05) blood pressure, left ventricular collagen volume fraction and TIMP-1 mRNA, and diminished (\textit{P} \leq 0.05) collagenase activity. After the treatment period, blood pressure was higher (\textit{P} \leq 0.05) in losartan-treated SHR than in WKY, and no significant differences were noted in the remaining parameters between the 2 strains of rats. Compared with untreated SHR, treated SHR showed no left ventricular hypertrophy, diminished (\textit{P} \leq 0.05) blood pressure, left ventricular collagen volume fraction and TIMP-1 mRNA, and increased (\textit{P} \leq 0.05) collagenase activity. These results suggest that the transcription of the TIMP-1 gene is upregulated in the hypertrophied and fibrotic left ventricle of adult SHR. Upregulation of TIMP-1 may account for diminished collagenase activity in the myocardium of those rats. Chronic angiotensin II type 1 receptor blockade with losartan resulted in inhibition of TIMP-1 expression and stimulation of collagenase activity in the left ventricle of SHR. It is proposed that angiotensin II may facilitate myocardial fibrosis in SHR by depressing the collagenase-mediated extracellular degradation of collagen fibers. (Hypertension. 2000;35:1197-1202.)

Key Words: angiotensin - collagen - collagenases - losartan - rats, inbred SHR - tissue inhibitor of metalloproteinases

Myocardial fibrosis is known to occur in humans with left ventricular hypertrophy (LVH) associated with hypertension\textsuperscript{1} and contributes critically to the overall risk associated with LVH.\textsuperscript{2} This fibrosis consists of an increase in the interstitial and perivascular content of fibrillar collagen type I and type III.\textsuperscript{3} Evidence suggests that the myocardial synthesis of collagen type I fibers is increased in adult rats with genetic hypertension (ie, spontaneously hypertensive rats [SHR]).\textsuperscript{4-9} Chronic blockade of the angiotensin II type 1 (AT\textsubscript{1}) receptor with losartan resulted in the normalization of collagen synthesis and reversal of left ventricular fibrosis in SHR, which suggests a role for angiotensin II in increased collagen synthesis in that model.\textsuperscript{6}

The excess of ventricular collagen in patients with hypertensive LVH may be a result of both exaggerated collagen synthesis and inadequate collagen degradation.\textsuperscript{10,11} The rate-limiting step in the extracellular degradation of collagen is the catalytic cleavage by interstitial matrix metalloproteinases (MMPs).\textsuperscript{12} Nine MMPs have been identified, cloned, and sequenced, and these are divided into 3 groups (collagenase, stromelysin, and gelatinase) that are based broadly on substrate preferences. Interstitial collagenase (MMP-1) accounts for the degradation of up to 40% of newly synthesized collagen type I and type III in different tissues.\textsuperscript{13} The MMP activity is regulated by a family of naturally occurring tissue inhibitors of metalloproteinases (TIMPs).\textsuperscript{12,14} Four members of this family have been identified: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Unlike the other TIMPs, TIMP-1 is synthesized by most types of connective tissue cells, acts against all members of the MMPs family of enzymes, and is
highly inducible by cytokines, hormones, and growth factors.15,16 Recent data suggest that the balance between MMPs and TIMPs is critical in several disease states characterized by organ fibrosis, such as idiopathic pulmonary fibrosis17 and liver cirrhosis.18

A collagenase/TIMP-1 system has been identified and characterized in the rat myocardium.19 To assess this system in experimental genetic hypertension, we measured the proteinase activity of collagenase and the messenger (mRNA) expression of TIMP-1 in 30-week-old SHR with established LHV and myocardial fibrosis. To assess collagen type I metabolism, serum levels of the carboxy-terminal propeptide of procollagen type I (PIP) and the pyridoline cross-linked telopeptide domain of collagen type I (CITP) were determined as markers of collagen type I synthesis and degradation, respectively.20 To evaluate whether chronic AT1 blockade influences the myocardial collagenase/TIMP-1 system, all determinations were repeated in 16-week-old SHR that were treated for 14 weeks with losartan.

Methods

Study Design

The investigation conforms to the Guide for the Care and Use of Laboratory Animals.21 Sixteen-week-old male Wistar-Kyoto rats (WKY) (n = 18) and 16-week-old SHR (n = 18) were observed in our colony for an additional 14 weeks and were killed at the age of 30 weeks. In addition, 16-week-old SHR (n = 18) were treated with oral losartan (20 mg · kg body wt-1 · day-1) for 14 weeks (the SHR-L group) and were then killed. The drug was dissolved in drinking water, and the concentration was adjusted for the daily water intake and body weight to obtain an average daily dose of 20 mg · kg body weight-1 · day-1. All rats were housed in individual cages with free access to standard rat chow and tap water in a quiet room with constant temperature (20° to 22°C) and humidity (50% to 60%). The rats were provided by Harlan UK Limited (Bicester, UK).

Systolic blood pressure and diastolic blood pressure were measured in all rats every 2 weeks by the standard tail-cuff method by means of an LE 5007 Pressure Computer (Letica Scientific Instruments).

Preparation of Tissue Samples

Before the rats were killed, they were weighed and anesthetized with sodium thiopental (30 mg/kg IP). Nine rats in each group were killed by decapitation, and blood was obtained to conduct biochemical analysis of serum peptides. The heart was removed intact and was weighed. The left ventricle was then dissected and weighed. Left ventricular index was calculated by dividing the left ventricle weight by the body weight of each animal.

The hearts of the remaining 9 rats in each group were fixed by retrograde perfusion according to Richer et al22 with some modifications as we previously described.23 Each heart was excised and was cut perpendicular to the apex-to-base axis into 3 pieces. Two of these pieces were immediately frozen in liquid nitrogen and were stored at −80°C for later mRNA and proteinase analyses. The remaining piece of the heart was postfixed by immersion in buffered 4% paraformaldehyde for 5 hours, was dehydrated, and was embedded in paraffin.

Extraction of mRNA

Total RNA from frozen ventricular tissue samples was extracted according to the method of Chomczynski and Sacchi24 by means of Ultraspec RNA reagent (Biotecx Laboratories, Inc). The RNA concentration was determined spectrophotometrically at 260 nm, and ethidium bromide-stained agarose gels were used to check its integrity. The mRNA was isolated from total RNA with the Oligotex mRNA kit (Qiagen), and the concentration was then determined spectrophotometrically. Aliquots were stored at −80°C.

Northern Blot Analysis

The mRNA (2 μg) was separated in a 1% denaturing formaldehyde agarose gel, was blotted on nylon membranes by overnight capillary blotting, and was fixed by ultraviolet irradiation. Blots were prehybridized in 5× standard saline citrate; 50% formamide; 5× Denhardt’s solution; 50 mMol/L sodium phosphate, pH 6.5; 0.1% sodium dodecyl sulfate; and 100 μMol/L salmon sperm DNA at 42°C. Hybridization was performed in 50% formamide solution at 42°C for 16 hours. cDNA probe was labeled with [3P]dCTP by the random primer DNA labeling kit, Amersham Iberica.

The concentration of the labeled probe in the hybridization solution was 1 × 106 counts · min-1 · μl-1. The membrane was washed and was exposed to Kodak X-Omat AR film between 2 intensifying screens at −70°C. The relative density of each band was determined via laser densitometry. Standardization was performed by hybridization of the same membrane by means of a cDNA probe for GAPDH (ATCC). The resulting density of TIMP-1 band was expressed relative to the density of the GAPDH band for the correction of the difference in mRNA loading and in transfer to the nylon membrane.

The probe used for the quantification of the expression of mRNA of the rat TIMP-1 was a 0.8-kb fragment from the 3′ untranslated region of the rat TIMP-1 cDNA.

Collagenase Assay

Collagenase activity was determined by degradation of [14C]collagen (10 mCi · mmol-1 · liter ) with left ventricular myocardial extracts (approximately 25 mg) according to Moore and Spilburg.25 The procedure included previous activation of myocardial procollagenase with trypsin (Sigma Chemical Co) as previously reported.26 Activation was terminated by addition of 1 μl of 10 μMol/L soybean trypsin inhibitor (Sigma).14 C-labeled collagen in the supernatants of the processed samples was counted with a scintillation counter. In control experiments, we observed no liberation of soluble 14C counts, which indicated that neither trypsin nor trypsin-soybean trypsin inhibitor complex was able to degrade [14C]collagen. Collagenase activity was measured as released counts per minute and was calculated as micrograms of degraded [14C]collagen per milliliter per 30 minutes per milligram of protein; the protein content of each dish was determined according to the method of Lowry et al.27

Serum Peptides

Serum samples to determine PIP and CITP values were taken at the time of blood collection and were stored at −40°C until manipulation. The 2 peptides were determined by specific radioimmunoassays by means of specific antisera (Orion Diagnostica) as described previously.3

Histomorphological Study

For histomorphological analysis, coronal sections (4 μm thick) of the left ventricle were obtained from the paraformaldehyde-fixed paraffin embedded specimens. The equator was selected as being representative of the whole ventricle. Each section was stained with Sirius red F3BA. The collagen volume fraction (CVF) was determined as a percentage by quantitative morphometry with an automated image analysis system (Visilog 4.1.5, Noesis) as previously described.28 For qualitative assessment of fibrosis, Sirius red F3BA stained sections were viewed through a polarized-light microscope.

Immunohistochemical Study

For immunohistochemical study, the avidin-biotin complex method was used as described previously.4 The primary antibody used was collagen type I (Biogenex) at a dilution of 1:50. A semiquantitative scale was developed to measure the amount of interstitial and perivascular collagen type I seen at low power (×10). The amount of collagen type I was graded on a scale of 0 to 3+; 0 represented the

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absence of collagen type I; 1+, mild deposits; 2+, moderate deposits; and 3+, severe deposits.

Statistical Analysis

Results were presented as mean±SEM computed from the average measurements obtained from each group of rats. Differences among the 3 groups of rats were tested by 1-way analysis of variance after normality had been demonstrated (Shapiro-Wilks test); otherwise, a nonparametric test (Kruskal-Wallis) was used. If significant differences were obtained by analysis of variance, subsequent multiple comparisons were made with Scheffé’s test, or the contrast coefficient matrix test (Levene’s test) was applied when the variances were not homogeneous. After the Kruskal-Wallis test, we used the Mann-Whitney U test to check differences between the 2 groups. For nonquantitative data, a χ² method (Pearson) was used to analyze the differences among the 3 groups of animals. A value of \( P < 0.05 \) was considered statistically significant.

Results

Blood Pressure and Left Ventricular Hypertrophy

At the conclusion of the experiment, both systolic and diastolic blood pressures were increased \( (P < 0.05) \) in SHR when compared with those in WKY and those in the SHR-L group (Table 1). Thus mean arterial pressure was higher \( (P < 0.05) \) in SHR than in WKY and treated SHR (Table 1). The mean arterial pressure in 14 treated SHR was above the upper limit seen in WKY (156 mm Hg). In the remaining rats in the SHR-L group, mean arterial pressure was within the limits measured in WKY. Overall, the mean arterial pressure measured at the end of the experiment was higher \( (P < 0.05) \) in the SHR-L group than in the WKY group (Table 1).

SHR had LVH when expressed as the increase \( (P < 0.05) \) in left ventricular weight normalized to body weight (left ventricular index) (Table 1). The left ventricular index in the SHR-L group was close to the value in the WKY and lower \( (P < 0.05) \) than that determined in SHR (Table 1). None of the rats in the SHR-L group exhibited LVH after treatment.

Left Ventricular Fibrosis

As shown in Figure 1, Sirius red F3BA stained the interstitial myocardium in WKY very slightly. In contrast, large areas of interstitial and perivascular accumulation of collagen (yellow) in SHR can be seen as large strands that are irregular in size and shape. In contrast, very small amounts of interstitial collagen are seen in SHR treated with losartan and in WKY.

| TABLE 1. Blood Pressure and Parameters of Cardiac Hypertrophy, Fibrosis, and Collagen Type I–Derived Peptides in Normotensive Wistar-Kyoto Rats and Spontaneously Hypertensive Rats |
|-----------------|---------|--------|
| Parameter       | WKY     | SHR    | SHR-L  |
| Systolic blood pressure, mm Hg | 164±3   | 230±3*  | 186±5† |
| Diastolic blood pressure, mm Hg | 125±3   | 190±3*  | 145±5† |
| Mean arterial pressure, mm Hg | 136±3   | 201±4*  | 165±5† |
| Left ventricular index, mg/g | 2.85±0.07 | 3.22±0.12* | 2.78±0.05 |
| Collagen volume fraction, % | 3.38±0.18 | 5.39±0.91* | 3.34±0.21 |
| PIP, µg/L | 8.93±1.04 | 12.69±0.95* | 9.00±0.56 |
| CITP, µg/L | 3.77±0.13 | 4.00±0.27 | 4.15±0.30 |
| CITP/PIP, ×100 | 42±2   | 31±3*  | 46±4   |

Values are expressed as mean±SEM. WKY indicates Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; SHR-L spontaneously hypertensive rats treated with oral losartan; PIP, carboxy-terminal propeptide of procollagen type I; and CITP, pyridoline cross-linked telopeptide domain of collagen type I. *\( P < 0.05 \) vs WKY rats and SHR-L; †\( P < 0.05 \) vs WKY rats.
group, CVF was within the limits measured in WKY. Microscopic examination revealed that both perivascular and interstitial deposition of collagen were diminished in the SHR-L group when compared with the levels of deposition in SHR.

Although more animals exhibited low grades of deposition of collagen type I in the WKY group, more animals exhibited high grades in the SHR group (Table 2). After administration of losartan, the distribution of treated SHR was displaced to values seen in WKY (Table 1). The $\chi^2$ (Pearson) method was used to detect the statistical significance of differences among groups in the distribution of frequencies.

Expression of the TIMP-1 Gene

Representative autoradiograms of the Northern blots of the expression of TIMP-1 mRNA are presented in Figure 2. Untreated SHR demonstrated increased TIMP-1 mRNA in comparison with that of WKY. The expression of TIMP-1 mRNA was lesser in SHR-L than in SHR.

The relative amount of ventricular TIMP-1 mRNA was increased ($P<0.05$) in SHR compared with that in WKY ($0.25 \pm 0.02$ versus $0.12 \pm 0.01$) (Figure 2). TIMP-1 mRNA levels were lower ($P<0.05$) in the SHR-L ($0.17 \pm 0.02$) than in SHR (Figure 3). Although TIMP-1 mRNA levels did tend to be higher in the SHR-L than in WKY, the difference was not statistically significant.

Collagenase Activity

Collagenase activity was decreased ($P<0.05$) in SHR compared with that in WKY ($1.99 \pm 0.33$ versus $2.98 \pm 0.35 \mu g$ degraded collagen/mL/30 minutes/mg protein) (Figure 3). Chronic administration of losartan resulted in enhanced ($P<0.05$) collagenase activity in treated SHR ($3.15 \pm 0.25 \mu g$ degraded collagen/mL/30 minutes/mg protein) (Figure 3). No differences were observed in collagenase activity in WKY and SHR-L.

Collagen Type I Metabolism

The serum concentration of PIP was higher ($P<0.05$) in SHR than in WKY (Table 1). Administration of losartan was associated with the diminution ($P<0.05$) of PIP in treated SHR (Table 1).

No significant differences in CITP values were observed among the 3 groups of rats (Table 1). Because a balance exists between the synthesis and the degradation of collagen type I in physiological conditions,$^{13}$ the CITP/PIP ratio was calculated to assess indirectly the degree of coupling in those 2 processes. As shown in Table 1, the ratio was diminished ($P<0.05$) in SHR compared with that in WKY. The administration of losartan was associated with enhancement ($P<0.05$) of the ratio in treated SHR (Table 1).

Discussion

The excess of ventricular collagen seen in animals and humans with hypertensive LVH has been proposed to be a result of both exaggerated collagen synthesis and inadequate collagen degradation.$^{10,11}$ We previously reported that adult SHR with LVH and left ventricular fibrosis exhibited the biochemical features of increased extracellular synthesis of collagen type I fibril-forming molecules.$^6$ We have thus investigated whether the extracellular degradation of collagen type I fibers is depressed in the left ventricle of adult SHR.

Two findings of this study suggest that alterations in the collagenase/TIMP-1 system may account for a diminished
degradation of collagen type I fibers in the ventricular myocardium of SHR. In fact, although the activity of collagenase is abnormally diminished, expression of the TIMP-1 gene is abnormally increased in the hypertrophied and fibrotic left ventricle of adult SHR.

Collagenase is a Zn$^{2+}$-dependent and Ca$^{2+}$-dependent proteinase that initially cleaves collagen and promotes an unfolding of the triple helix of the collagen peptide chains so that other collagenolytic enzymes such as gelatinase A and B or MMP-2 and MMP-9 and nonspecific proteolytic enzymes such as trypsin can further digest collagen fibrils. However, TIMP-1 is a multifunctional protein secreted by most connective tissue cells and macrophages that blocks the activation of collagenase from both its latent form and its catalytic activity. Therefore, although we have not determined TIMP-1 at the protein level, our findings suggest that an excess of this factor may depress collagenase activity in the myocardium of adult SHR. This alteration, in combination with the increased synthesis of collagen type I molecules, may facilitate the exaggerated deposition of collagen type I fibers seen in the left ventricle of these rats. The presence of normal CITP levels in association with increased PIP levels observed in SHR support this possibility.

A number of cytokines and growth factors regulate the expression of TIMP-1. The available evidence suggests that there is a molecular mechanism for regulation of the expression of TIMP-1 at the transcriptional level in most tissues, including the myocardium. In this regard, transforming growth factor β (TGF-β) has been shown to stimulate the transcription of TIMP-1. Increased TGF-β gene expression and activity have recently been found in the left ventricle of adult SHR. Further studies are required to establish whether an excess of TGF-β accounts for the upregulation of TIMP-1 in SHR from the current study.

Another finding of this study is that collagenase activity and TIMP-1 gene expression were normalized in SHR treated with losartan. These findings would suggest that treatment with losartan restored collagenolytic activity in the left ventricle of treated SHR. Thus the antifibrotic effect of losartan in SHR seems to be due not only to its ability to diminish the synthesis of collagen type I molecules but also to its capacity to stimulate the degradation of collagen type I fibers. This is supported by the tendency to increased CITP despite the decrease in PIP observed in treated SHR.

The chronic administration of losartan was not associated with complete normalization of blood pressure in treated SHR. Furthermore, analysis of the individual data shows that the intensity of TIMP-1 and collagenase changes was independent of the antihypertensive efficacy of the drug. Thus a nonhemodynamic mechanism can be involved in the ability of losartan to inhibit TIMP-1 gene expression and stimulate collagenase activity in SHR. Angiotensin II was found to up-regulate TIMP-1 gene expression in rat heart endothelial cells, and this effect was blocked by losartan. Thus it can be proposed that chronic AT$_1$, blockade results in down-regulation of TIMP-1 in the ventricular myocardium of SHR. On the other hand, angiotensin II mediates the formation of TGF-β in cardiac cells through an AT$_1$-mediated mechanism. It has been reported that chronic administration of losartan resulted in diminished expression of TGF-β in SHR. Thus the possibility also exists that losartan diminishes angiotensin II-dependent TGF-β formation, which in turn restores the equilibrium between collagenase and TIMP-1 in the left ventricle of SHR.

Finally, administration of losartan resulted in complete reversal of LVH in treated SHR, despite a noncomplete control of blood pressure. This observation and the previous finding that myocardial angiotensin II concentrations are elevated in SHR compared with those in WKY, further support the theory that in addition to pressure overload, nonhemodynamic factors (ie, angiotensin II) participate in the left ventricular growth present in this genetic model of hypertension.

In conclusion, our data suggest that regulation of the collagenase/TIMP-1 system is altered in the hypertrophied and fibrotic left ventricle of adult untreated SHR so that extracellular collagen type I degradation is depressed in these rats. Our findings also suggest that angiotensin II may facilitate myocardial fibrosis in SHR by up-regulating TIMP-1 and decreasing collagenolytic activity. Thus the cardio reparative properties of losartan in SHR may be due in part to its ability to normalize the collagenase/TIMP-1 system in the left ventricle of these rats.

### References


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