Matrix Metalloproteinase Inhibition Improves Cardiac Dysfunction and Remodeling in 2-Kidney, 1-Clip Hypertension

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ABSTRACT

Background: Enhanced cardiac matrix metalloproteinase activity (MMPs) has been associated with ventricular remodeling and cardiac dysfunction. It is unknown whether MMPs contribute to systolic/diastolic dysfunction and compensatory remodeling in 2-kidney, 1-clip (2K1C) hypertensive rats. To test this hypothesis, we used 2K1C rats after 2 weeks of surgery treated or not with a nonspecific inhibitor of MMPs (doxycycline).

Methods and Results: We found that blood pressure and $\frac{\Delta dP}{\Delta t}$ increased in 2K1C rats compared with sham groups, and these parameters were attenuated by doxycycline treatment ($P < .05$). Doxycycline also reversed cardiac hypertrophy observed in 2K1C rats ($P < .05$). Hypertensive rats showed increased MMP-2 levels in zymograms and in the tissue by immunofluorescence ($P < .05$) compared with sham groups. Increased total gelatinolytic activity was observed in untreated 2K1C rats when compared with sham groups ($P < .05$). Doxycycline decreased total gelatinolytic activity in 2K1C rats to control levels ($P < .05$).

Conclusion: An imbalance in gelatinolytic activity, with increased MMP-2 levels and activity underlies the development of morphological and functional alterations found in the compensatory hypertrophy observed in 2K1C hearts. Because function and structure were restored by doxycycline, the inhibition of MMPs or their modulation may provide beneficial effects for therapeutic intervention in cardiac hypertrophy. (J Cardiac Fail 2010;16:599–608)

Key Words: MMP-2, cardiac hypertrophy and hypertension.

Left ventricular hypertrophy (LVH) is an adaptation of the heart to sustained arterial hypertension. The structural remodeling of the myocardium is probably a major contributory factor to the increased morbidity and mortality rates that are associated with pressure overload cardiac hypertrophy.

The stroma of the heart maintains the structure of the myocardium thus contributing to ventricular function through the transmission of myocyte-generated force to atrial and ventricular chambers and to relengthening of myocytes during diastole. Chronic hypertension induces progressive accumulation of interstitial collagen fibers during LVH, which parallels an increase in heart weight. It has been pointed out that this fibrotic response leads to progressive abnormalities of diastolic ventricular filling and relaxation, and ultimately to systolic dysfunction. These alterations may increase the risk for myocardial infarction, ventricular arrhythmias, congestive heart failure, and sudden death. An improved understanding of the pathophysiology of hypertensive cardiac hypertrophy may lead to better therapeutic targets.

Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent endopeptidases that are important for extracellular matrix (ECM) degradation. Their activity leads to ECM turnover (synthesis and degradation) that result in matrix remodeling. Increased MMPs, particularly MMP-2, have provided important mechanistic insights associated with the pathophysiology of cardiac dysfunction and remodeling associated or not with increased...
afterload. For example, increased MMP-2 levels were implicated in diabetic cardiac dysfunction and remodelin, myocardial infarction, ischemia-reperfusion injury, and hypertensive cardiomyopathy. Indeed, in addition to its participation in ECM remodeling, enhanced MMP-2 activity may cause cardiac dysfunction, which possibly results from proteolytic degradation of intracardiomycyte targets, including troponin I and myosin light chain. Further supporting this notion, a recent study showed that MMP-2 expression directly mediates ventricular remodeling and dysfunction, independently of any superimposed injury. Moreover, increased circulating MMP-2 levels were reported in clinical hypertension, heart failure, and LVH.

Experimental renovascular hypertension (2-kidney, 1-clip [2K1C] hypertension) is typically associated with pronounced cardiac hypertrophy 6 to 12 weeks after the surgery. Although MMP-2 has been shown to play an important role in the vascular alterations found after 8 weeks of hypertension in this model, no previous study has examined whether increased MMP levels, especially MMP-2, play a role in the compensatory LVH of 2K1C hypertension. Because there is strong evidence that increased cardiac MMP-2 activity causes cardiac remodeling and dysfunction, we hypothesized that MMP-2 expression and activity increase during the development of compensatory hypertrophy in 2K1C rats, thus leading to cardiac dysfunction, which could be prevented by inhibiting MMPs with doxycycline.

Nonspecific MMPs inhibition with doxycycline produced beneficial effects in animal models associated with decompensated heart failure, such as ischemia/reperfusion and myocardial infarction. However, the potential therapeutic benefit of doxycycline in compensatory cardiac remodeling has not been established yet. Because doxycycline is a Food and Drug Administration—approved drug, further studies on the effects of this inhibitor on LVH in animals are necessary. Here, we show that doxycycline protects against functional and morphological cardiac changes associated with the compensatory remodeling found in 2K1C hypertension.

Materials and Methods

Experimental Animals and Protocol

Experimental protocols followed standards and policies of the University of Sao Paulo’s Animal Care and Use Committee. The animals were handled according to the guiding principles published by the National Institutes of Health. Renovascular hypertension was induced in rats as previously described. Briefly, male Wistar rats (180 g) were anesthetized using ketamine (100 mg/kg intraperitoneally)/xylazine (100 mg·kg·g intraperitoneally) and, after a midline laparotomy, a silver clip with an internal diameter of 0.20 mm was placed around the left renal artery. Sham-operated rats underwent the same surgical procedure, except for the placement of the renal artery clip. The rats were maintained on a 12-hour light/dark cycle at a room temperature (22 to 25°C) with free access to standard rat chow and water.

The animals (10 per group) were randomly assigned to 1 of 4 experimental groups as follows: 1) 2K1C hypertension group that received water (2K1C); 2) 2K1C hypertension group that received doxycycline (2K1C + doxy); 3) sham-operated group that received water (sham); 4) sham-operated group that received doxycycline (sham + doxy). Doxycycline was given by gavage (30 mg·kg·day, by mouth). Treatment with doxycycline was started 2 weeks after 2K1C hypertension was induced and maintained for additional eight weeks. Tail systolic blood pressure (SBP) was assessed weekly by tail-cuff plethysmography, and rats were considered to be hypertensive when SBP was higher than 160 mm Hg 2 weeks after the surgery.

Assessment of Left Ventricular Function

Eight rats of each group were anesthetized with tribromoethanol (250 mg/kg, intraperitoneally) after 8 weeks of treatment with doxycycline. Polyethylene catheters (Becton Dickinson and Co, Sparks, MD) were inserted into the right femoral artery for the direct measurement of arterial pressure. Another polyethylene catheter was inserted into the right carotid artery, and was carefully introduced into the left ventricle for measurement of left ventricular pressures. The arterial and ventricular catheters were connected to pressure transducers (P23Gb; Statham, Hato Rey, PR) and the signals were properly amplified. Arterial and left ventricular pressures were sampled continuously (1 kHz) on an IBM/PC equipped with an analog to digital interface (DI-220; Dataga Instruments, Akron, OH). A computer program (Advanced CODAS; Dataga Instruments) was used to analyze the data and extract beat-by-beat time series of heart rate (HR) and mean arterial pressure (MAP). The first derivative of left ventricular pressure (dp/dt) was calculated and values of the maximum rate of isovolumic pressure development (+dp/dtmax) and the maximum rate of isovolumic pressure decay (−dp/dtmax) were used as indices of contractility and relaxation, respectively. At the end of the experiments, the hearts were harvested and washed in saline and the left ventricle was frozen and stored at −80°C until used for biochemical determinations.

Harvesting and Preparation of the Hearts

After 8 weeks of treatment the animals (6 to 8 per group) were weighed, anesthetized, and their thoracic cavity was opened to expose the still beating heart. The hearts were rapidly removed, rinsed in ice cold 0.9% saline solution, blotted, weighed, and fixed as a whole in phosphate-buffered 10% formalin (pH 7.3) for histological study. Both ventricles from each heart were isolated and cut into 2 fragments by a mid-ventricular coronal section. Each block was serially cut in the same direction and 4-μm thick sections were stained with hematoxylin and eosin. The left ventricular wall and septum thickness, the area corresponding to the left ventricular chamber were determined in the first 3 histological sections of each block. Morphometric analyses were carried out with Image J software (http://rsb.info.nih.gov/ij). Average myocyte diameter was determined from 20 measurements in each ventricle, which were made at a magnification of ×400 in longitudinally oriented myofibers. Myocyte diameter was obtained in the region of the nucleus of those cells in which the nuclear envelope was sharply defined at both ends, which corresponds to the nucleus length and to its position at an equal distance from the sides of the myocytes. A skilled observer blinded to the treatment groups made the measurements with the Leica Qwin software (Leica Imaging Systems Ltd, Cambridge, UK) in conjunction with a Leica microscope (Leica DMR, Leica...
Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis Gelatin Zymography for Determination of MMP-2 and MMP-9 Levels

In the present study, gelatin zymography of MMP-2 and MMP-9 from left ventricle samples was performed as previously described. These MMPs are separated by molecular weight after gel electrophoresis under denaturing conditions. Thereafter, the enzymes are refolded and the proteolytic activity of each form is visualized in the stained zymograms.

Briefly, frozen left ventricle samples (10 per group) were homogenized in extraction buffer (300 μL for each 0.1 g of left ventricle sample) containing 10 mM CaCl$_2$, 50 mM Tris-HCl pH 7.4, 0.1% Brij, 0.15 M NaCl, 1 mM Phe (1,10 orto phenanthro-lene), 1 mM phenylmethanesulphonylfluoride, and 1 mM N-ethylmaleimide. The samples were placed on ice within a refrigerator for 20 hours and then centrifuged at 10,000 g for 15 minutes. The protein content was measured using the Bradford method. Thereafter, the samples were diluted 1:1 with sample buffer (final concentration: 2% sodium dodecyl sulfate, 125 mM Tris-HCl; pH 6.8, 10% glycerol, and 0.001% bromophenol blue), and subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis co-polymerized with gelatin (0.1%) as the substrate. After electrophoresis was completed, the gel was incubated for 1 hour at room temperature in a 2% Triton X-100 solution, and incubated at 37°C for 16 hours in Tris-HCl buffer, pH 7.4, containing 10 mmol/L CaCl$_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250, and then destained with 30% Sh%

**Table 1. Heart Rate, Heart Weight, and Body Weight**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>Sham + Doxy</th>
<th>2K1C</th>
<th>2K1C + Doxy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>328.2 ± 16.7</td>
<td>345.3 ± 18.1</td>
<td>363.3 ± 19.2</td>
<td>329.0 ± 11.4</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.49 ± 0.05</td>
<td>1.55 ± 0.07</td>
<td>1.72 ± 0.20*</td>
<td>1.56 ± 0.07</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>533.1 ± 18.0</td>
<td>534.0 ± 27.1</td>
<td>446.0 ± 23.1</td>
<td>512.3 ± 23.7</td>
</tr>
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2K1C, 2-kidney, 1 clip, doxy, doxycycline. Values are mean SEM (n = 7). *P < .05 vs. other groups.

Fig. 1. Doxycycline attenuated the blood pressure increase. Noninvasive (A) and invasive blood pressure analysis (B, C, D). The 2-kidney, 1-clip (2K1C) group shows increased blood pressure compared with basal values of the other groups (*P < .05). 2K1C rats treated with doxycycline show increased blood pressure when compared with basal values (#P < .05). Mean ± SEM (n = 8–10).
methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using a Kodak Electrophoresis Documentation and Analysis System 290 (Kodak, Rochester, NY). Gelatinolytic activities were normalized with regards to an internal standard (fetal bovine serum) to allow integral analysis and comparison. The forms of MMP-2 were identified as bands at 72 and 64 KDa.

In Situ Zymography and Immunohistochemistry to Assess MMP-2 Expression

MMPs activities in situ were measured in frozen left ventricles (5 per group) using DQ Gelatin (E12055, Molecular Probes, OR) as a fluorogenic substrate. Briefly, left ventricles samples were embedded in Tissuetek and cut into 5-μm sections with a cryostat. Sample sections were incubated with 1.0 μg/mL DQ gelatin in Tris-CaCl2 buffer (50 mM Tris, 10 mM CaCl2, 1 μM ZnCl2) in dark humidified chambers for 1 hour. The sections were examined with fluorescent microscopy (Leica Imaging Systems Ltd., Cambridge, England) and the image was captured at X400. Proteolytic activity was detected as bright green fluorescence, which indicates substrate breakdown, and was evaluated by using ImageJ Program (National Institutes of Health).

To evaluate MMP-2 expression, 5-μm tissue sections were incubated with mouse anti-MMP-2 monoclonal antibody (MAB3308, Chemicon, USA), for 1 hour in a dark humidified chamber (at a 1:1000 dilution). Red fluorescence was visualized by adding a rhodamine-conjugated anti-mouse secondary antibody (1:200) (AP160P, Chemicon) for 1 hour. To confirm the specificity of antibodies, the primary antibody was omitted and substituted by phosphate-buffered saline + 1% BSA. Rhodamine did not bind nonspecifically to the tissue sections. MMP-2 expression was detected as bright red fluorescence, and was evaluated using the ImageJ software.

Statistical Analysis

The results are expressed as means ± SEM. Between-group comparisons were assessed by 2-way analysis of variance or by 1-way analysis of variance followed by the Tukey test. A probability value < .05 was considered significant.

Results

Doxycycline Attenuates 2K1C-induced Hypertension

Similar baseline SBP were found in the study groups (Fig. 1A), and hypertension was observed 2 weeks after surgery in 2K1C groups. Although SBP increased in untreated 2K1C rats, treatment with doxycycline attenuated these increases by 47 mm Hg in 2K1C + doxy group (P < .05 vs. untreated 2K1C; Fig. 1A). These findings were confirmed by invasive measurement of arterial blood pressure at the end of treatment period. We found lower systolic (approximately 30 mm Hg), diastolic, and mean arterial blood pressure in 2K1C + doxy rats when compared with untreated 2K1C group (P < .05, Fig. 1B, C, and D, respectively). We found no significant differences in body weight and heart rate among the study groups (Table 1).

Treatment with Doxycycline Ameliorates the Cardiac Dysfunction and Remodeling Induced by 2K1C Hypertension

We found increased maximum ascending (approximately 29% higher) and declining (approximately 17% higher) rates of left ventricular pressure (+dP/dtmax and -dP/dtmax, respectively) in the 2K1C group compared with sham groups. These alterations were improved approximately 50% by the treatment with doxycycline (both P < .05; Fig. 2).

We found evidence for concentric remodeling in the hearts from 2K1C hypertensive rats at the end of the study period. The heart weight/body weight ratio was approximately 25% higher in the 2K1C group compared with the sham groups (P < .05; Fig. 3A, 3B). Doxycycline prevented this increase in heart weight/body weight ratio (P < .05; Fig. 3A, 3B). In parallel with the alterations in heart weight/body weight ratio, 2K1C hypertension induced 30% to 38% increases in left ventricle and septum thickness (both P < .05; Fig. 3C, 3E) and a 23% decrease in the left
ventricle chamber area ($P < .05$; Fig. 3D). Treatment with doxycycline reversed these alterations (Fig. 3C, 3D, 3E).

The left ventricle alterations were associated with increased myocyte diameter in the 2K1C group ($P < .05$, Fig. 4). Doxycycline prevented the increases in myocyte diameter found in hypertensive rats ($P < .05$, Fig. 4).

**Fig. 3.** Effects of doxycycline in cardiac morphological alterations induced by hypertension. (A) Representative photomicrographs of transverse heart sections for left ventricle area determination. (B) Body weight/heart weight ratio, left ventricle wall thickness, left ventricular chamber area, and septum wall thickness (panels C, D, and E, respectively). Values are expressed as means ± S.E.M. * $P < .05$ vs. other groups (n = 8).

**Doxycycline Inhibits 2K1C Hypertension-induced Upregulation of MMP Activity and MMP-2 Expression in the Left Ventricle**

Gelatin zymograms were used to assess MMP levels in left ventricles extracts. Figure 4A shows a representative zymogram of left ventricles depicting 2 bands corresponding...
to 2 MMP-2 forms (64 KDa and 72 KDa) and no bands corresponding to MMP-9. Left ventricles from 2K1C rats showed higher levels of the 72 KDa MMP-2 form (and total MMP-2) compared with those from sham or sham + doxy animals (both $P < .05$; Fig. 5B, 5C, 5D), thus indicating that 2K1C hypertension increased MMP-2 concentrations by 27% in the left ventricle. Treatment with doxycycline was apparently not associated with significant differences in MMP-2 levels in 2K1C + doxy group compared with the 2K1C group (Fig. 5B, 5C, 5D). However, it should be clear that gelatin zymography separates MMPs from bound inhibitors, therefore the apparently increased MMP-2 levels in 2K1C + doxy group probably does not reflect increased MMP-2 activity in vivo because doxycycline inhibits this enzyme.

We assessed the gelatinolytic activity in the hearts by in situ zymography, which provides a measure of total proteolytic activity. We found a 32% higher gelatinolytic activity in the hearts of 2K1C hypertensive rats compared with those found in the sham groups ($P < .05$; Fig. 6A, 6B). Treatment with doxycycline decreased the gelatinolytic

**Fig. 4.** Doxycycline reverses the increased diameter of myocytes induced by hypertension. (A) Representative photomicrographs of sections from left ventricle myocytes (original magnification ×400). (B) Values of minor diameter of myocytes. Data are shown as mean ± SEM. * $P < .05$ vs. other groups (n = 6).
activity in hypertensive rats to similar levels as compared with those found in the sham groups, as revealed by lower gelatinolytic activity in 2K1C + doxy group compared with the 2K1C group (P < .05; Fig. 6A, 6B).

To further support the zymogram findings implicating MMP-2 as a major player in the increased gelatinolytic activity in the hearts from hypertensive rats, we examined MMP-2 expression by immunohistochemistry. These additional results confirmed that 2K1C hypertension increases MMP-2 levels in the left ventricle, and showed that treatment with doxycycline tended to decrease these levels (Fig. 6A, 6C).

**Discussion**

The identification of mechanisms involved in cardiac hypertrophy is of major importance. Although many studies have suggested that increased MMP-2 activity is implicated in cardiac remodeling, no previous study has examined the involvement of MMP-2 in cardiac remodeling of 2K1C hypertensive rats. This is the first study reporting increased MMP-2 activity and expression at the protein level associated with systolic/diastolic dysfunction and marked LVH in 2K1C rats.

The present study shows that a known MMP inhibitor (doxycycline) normalized total gelatinolytic activity, attenuated the increases in blood pressure, and prevented both functional and structural cardiac alterations caused by hypertension. These findings are in line with those recently reported by our group, which showed that doxycycline completely prevented the aortic alterations associated with increased MMP-2 levels in 2K1C animals, even though blood pressure did not completely return to normal levels. Together, these findings suggest that MMPs (possibly MMP-2) play a key role in cardiovascular remodeling induced by 2K1C hypertension.

Renovascular hypertension upregulates the renin-angiotensin system, and increased angiotensin II levels induces the transition of cardiac fibroblasts to myofibroblasts that, in turn, produce increased amounts of MMPs. Angiotensin II also activates NAD(P)H oxidase and increased amounts of reactive oxygen species are produced in 2K1C hypertension. This is of major relevance because oxidative stress strongly activates MMPs. Therefore, it is highly probable that doxycycline may have produced the beneficial effects reported here as a result of MMP inhibition.

Although other MMPs may also be involved, we focused on MMP-2 in the present study because this MMP is the...
most widely implicated MMP in cardiac remodeling and dysfunction.\textsuperscript{16,26,27} Indeed, attenuated myocyte hypertrophy has been shown in MMP-2 knockout mice subjected to aortic banding.\textsuperscript{53} Moreover, increased MMP-2 activity was shown to impair cardiac contractility in animal models of ischemia/reperfusion or diabetes mellitus. These conditions may lead to proteolysis of sarcomere proteins, thus explaining the cardiac dysfunction.\textsuperscript{20,22,54} In line with the findings reported here, increased MMP-2 levels were shown during the compensatory phase of cardiac remodeling in spontaneously hypertensive rats and in hypertensive Dahl salt-sensitive rats.\textsuperscript{23,55} Interestingly, augmented cardiac contractility was shown both in the present study and during the compensatory phase of the previous studies.\textsuperscript{3,55} Together, these results are consistent with the notion that MMP-2 may be implicated in transition of compensatory to maladaptive remodeling.\textsuperscript{56} Our results confirm that MMP-2 may play a central role in cardiac remodeling, and that MMP inhibition may mitigate the damage caused by excessive proteolysis from upregulated MMP-2 activity and maybe prevent the evolution of this condition to heart failure.

Compensatory remodeling secondary to hypertension is an adaptive response that may progress to maladaptive remodeling, which includes cardiomyocyte hypertrophy, increased left ventricular and septal wall thickness, and reduction in left ventricle chamber, thus affecting cardiac function,\textsuperscript{48} as we found in the present study. Doxycycline, however, reversed these alterations, although this drug has not normalized the arterial blood pressure. These results suggest that MMPs (especially MMP-2) may be effective pharmacological targets in the prevention of cardiovascular alterations caused by hypertension.

Although doxycycline is not a selective MMP-2 inhibitor, growing evidence indicates that MMP-2 plays a major role in cardiac dysfunction.\textsuperscript{16,18,26} In addition to modifying the extracellular matrix, MMP-2 augments cellular migration and may also act within cardiomyocytes, thus leading to degradation of structural and contractile proteins.\textsuperscript{16,18,26} Structural alterations associated with upregulated MMP-2 may ultimately result in decreased cardiac contractility and heart failure.\textsuperscript{16}

Doxycycline is a pharmacological tool widely used to examine the involvement of MMPs in many disease...
This MMPs inhibitor (30 mg·kg·day orally) reduced myocardial infarct size and improved systolic dysfunction in animals. Conversely, doxycycline (100 mg·kg·day; subcutaneously) did not prevent left ventricular remodeling and impaired compensatory hypertrophy after myocardial infarction. Moreover, doxycycline (160 mg·kg·day; orally) accelerated LVH to heart failure progression after thoracic aorta constriction. \(^{40}\) These discrepancies between studies may be due to differences in the doses used in different studies. Although doxycycline was effective at low doses (<30 mg·kg·day), higher doses (160 g·kg·day) produced no effects or deleterious effects. Consistent with this suggestion, we found that doxycycline (30 mg·kg·day orally) ameliorated cardiac function and reversed LVH. These findings reinforce the need of more dose-response studies to better evaluate the effects produced by doxycycline.

Doxycycline may act as an oxygen radical scavenger, and may also produce anti-inflammatory and anti-apoptotic effects.\(^{59–61}\) Increased formation of reactive oxygen species, apoptosis, and inflammation are involved in hypertension\(^{50,62,63}\) and may lead to cardiac dysfunction. Therefore, additional mechanisms may explain the positive effects produced by doxycycline in the present study.

In conclusion, this study suggests that MMPs are involved in the development of structural and functional alterations present in compensatory hypertrophy induced by 2K1C hypertension. These alterations were prevented by doxycycline, even though this drug produced only partial antihypertensive effects, thus suggesting that MMPs inhibition may have protective effects against the cardiac alterations of hypertension.

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**Disclosures**

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