Original Article

Effects of Early and Late Chronic Pressure Overload on Extracellular Matrix Remodeling

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The left ventricle (LV) remodels with age and in response to pressure overload. While aging and pressure overload are superimposed in the clinical context, the structural and functional consequences of the individual processes are not well-understood. Accordingly, the objective of this study was to compare the effects of both early and late chronic hypertension on extracellular matrix (ECM) remodeling. The following groups of Dahl rats were studied: 1) young salt-resistant (control, $n=6$); 2) young salt-sensitive (early phase of chronic hypertension, $n=6$); 3) middle-aged salt-resistant (aging, $n=5$); and 4) middle-aged salt-sensitive (late phase of chronic hypertension, $n=6$). We measured LV mass (LVM) and body weight (BW) and immunoblotted a panel of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and ECM proteins. Total collagen increased, several MMPs decreased, and TIMP-1 increased in the early phase of hypertension, consistent with fibrosis. Active MMP-8 decreased from $8,010 \pm 81$ U in young salt-resistant to $5,260 \pm 313$ U in young salt-sensitive ($p < 0.05$) rats. During the late phase, chronic hypertension decreased total collagen levels and increased MMP-8 and MMP-14 (all $p < 0.05$). Based on good-fit modeling analysis, MMP-14 (45 kDa) correlated positively with changes in LVM/BW during the early phase. In conclusion, this is the first study to evaluate MMP levels during both early and late chronic phases of hypertension. Our results highlight that ECM remodeling in response to pressure overload is a dynamic process involving excessive ECM accumulation and degradation. (*Hypertens Res* 2008; 31: 1225–1231)

Key Words: matrix metalloproteinases, tissue inhibitor of metalloproteinase, aging, hypertension, hypertrophy

Introduction

Hypertension is a leading cause of congestive heart failure in the United States (1). In response to pressure overload, the initial response of the myocardium is hypertrophic, with cardiac myocyte growth occurring in a concentric manner to reduce wall stress and preserve function of the left ventricle (LV). Prolonged pressure overload can induce further structural changes, which can impair diastolic function and in time

lead to heart failure. Myocyte hypertrophy and fibrosis resulting in increased LV mass (LVM) are prominent features during the early phase of pressure overload. The mechanisms that mediate the transition from compensated hypertrophic growth to heart failure, however, are poorly understood. During the later phases of chronic pressure overload, the myocardium is also subjected to changes that normally occur as a result of the aging process. Differentiating between events that occur during aging and pressure overload, vs. those events that occur during aging alone, will increase our understanding of the

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This study was supported by grants GM072928 (H.B.D.), AG20256 (C.H.-L.), and HL75360 (M.L.L.).

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Received November 27, 2007; Accepted in revised form January 28, 2008.

Table 1. LV Necropsy and Biochemical Analysis

	Young salt resistant	Young salt sensitive	Middle-aged salt resistant	Middle-aged salt sensitive
LVM (mg)	537±11	697±6*	700±18*	1,257±31 ^{†,‡}
Body weight (g)	254±5	282±4	372±8*	373±19 [†]
LVM/BW (mg/g)	2.12±0.04	2.48±0.03	1.88±0.04	3.43±0.26 ^{†,‡}
Protein (% LVM)	7.19±0.40	10.44±0.46*	7.23±0.86	4.81±0.84 [†]
Collagen (% LVM)	1.48±0.13	2.28±0.10*	1.18±0.06	0.81±0.18 [†]

Data are presented as mean±SEM. Sample sizes are young salt resistant ($n=6$), young salt sensitive ($n=6$), middle-aged salt resistant ($n=5$), and middle-aged salt sensitive ($n=6$). * $p<0.05$ compared with young salt resistant group. [†] $p<0.05$ compared with young salt sensitive group. [‡] $p<0.05$ compared with middle-aged salt resistant group. LVM, left ventricle mass; BW, body weight.

mechanisms involved during the late phase of chronic hypertension.

The extracellular matrix (ECM) serves as a structural entity to support myocyte shape and alignment, as well as overall myocardial architecture. As such, changes to the ECM have been causally associated with changes in LV function (2). Matrix metalloproteinases (MMPs) are a family of 25 zinc-dependent enzymes that regulate ECM turnover. MMPs are regulated by 4 endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). While changes in MMP-9 and TIMP-1 have been investigated in acute models of hypertension (3), whether other MMPs/TIMPs are altered during the early phase of chronic hypertension and whether an altered balance of MMPs and TIMPs persists into the late phase remains unclear.

The Dahl salt-sensitive rat is a model of chronic hypertension (4). Impairments in renal function initiate volume and pressure overload, which induces LV hypertrophy and can transition to heart failure. Dahl salt-sensitive rats fed a low salt diet are already hypertensive when first measured at 3 months of age (5, 6). Because this model has not been characterized in terms of LV ECM remodeling, the purpose of the present study was to evaluate ECM mechanisms during the initial phase and during the transition between LV hypertrophy to heart failure. We evaluated LV MMP, TIMP, collagen, and fibronectin profiles following the early or late phases of chronic hypertension.

Methods

Animal Experiment

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Dahl salt-sensitive rats were used to model chronic hypertension, and Dahl salt-resistant rats were used as normotensive controls. In this model, a diet supplemented with 8% salt

is often used to induce immediate hypertension and a rapid progression to congestive heart failure (7). We have previously shown that Dahl salt-sensitive rats fed a low salt diet develop chronic hypertension as they age (5). Mean arterial pressures increases steadily from 120 mmHg to 160 mmHg as they age from 3 to 12 months (5). Dahl salt-resistant rats have a normal blood pressure of approximately 100 mmHg that does not increase with age (unpublished observations, manuscript in preparation). Thus, Dahl salt-sensitive rats fed a low salt diet are an excellent model of the slow progression of chronic hypertension and heart failure typically observed in the aging population. Female Dahl rats ($n=23$) were weaned on a low salt (0.05% NaCl) diet, and divided into four groups: 1) young salt-resistant rats at age 4.0 ± 0.0 months ($n=6$); 2) young salt-sensitive rats at age 4.0 ± 0.0 months (early phase of chronic hypertension, $n=6$); 3) middle-aged salt-resistant rats at age 13.0 ± 0.0 months ($n=5$); and 4) middle-aged salt-sensitive rats at age 15.0 ± 0.3 months (late phase of chronic hypertension, $n=6$). Dahl rats were sacrificed as previously described (5, 8). The LV was removed, weighed, and immediately snap-frozen.

Protein Extraction and Total Collagen Content

A slice from the mid myocardium of each LV was weighed, homogenized with 2.5 mL of extraction buffer (Reagent 4, which contains 7 mol/L urea, 2 mol/L thiourea, and the detergent amidosulfofetaine-14; Sigma, St. Louis, USA), and incubated at 30°C for 15 min to extract total protein. Total protein concentrations were determined by Bradford Assay (BioRad, Hercules, USA). Because of the high urea content, protein extracts were diluted 1:40 to ensure compatibility with the Bradford assay. All samples (5 µg) were run on a 26-well 4–12% Bis-Tris polyacrylamide gel (BioRad) and stained with Coomassie blue to verify the accuracy of the Bradford assay protein concentration measurements.

Total collagen content was determined in protein extracts using the microplate picrosirius red assay (9, 10). Equal amounts of myocardial extracts (10 µg total protein) were added to triplicate wells of a 96-well microtiter plate. The samples were dried in the incubator and stained for 1 h with

Fig. 1. Representative MMP-14 immunoblot. *A*: Immunoblot with an MMP-14 antibody which recognizes multiple MMP-14 forms: pro-MMP-14, active MMP-14 and MMP-14 fragments. +: positive control. *B*: Pro-MMP-14 (65 kDa) levels increased in the young salt-sensitive group compared to the young salt-resistant group, and decreased in the middle-aged salt-sensitive group compared to the young salt-sensitive group. *C*: Active MMP-14 (54 kDa) levels decreased in the young salt-sensitive and middle-aged salt-resistant group compared to the young salt-resistant group, and increased in the middle-aged salt-sensitive group compared to the young salt-sensitive and middle-aged salt-resistant groups. *D*: Soluble MMP-14 (45 kDa) levels increased in the middle-aged salt-sensitive group compared to the young salt-sensitive and middle-aged salt-resistant groups. *E*: MMP-14 (40 kDa) levels also increased in the middle-aged salt-sensitive group compared to the young salt-sensitive and middle-aged salt-resistant groups. All densitometric data are plotted as arbitrary units. * $p < 0.05$ compared with the young salt-resistant group, † $p < 0.05$ compared with the young salt-sensitive group, # $p < 0.05$ compared with the middle-aged salt-resistant group.

100 μ L of 0.1% picosirius red in saturated picric acid (w/v). The dye was solubilized in 100 μ L of 0.1 mol/L NaOH, and the plates were read by spectrophotometry at an absorbance of 540 nm. Vitrogen 100 purified collagen (Collagen Biomaterials, Palo Alto, USA) was used as a positive control and to generate a standard curve.

Immunoblotting

Total protein (10 μ g) was loaded onto 26-well 4–12% Bis-Tris or 3–8% Tris-acetate polyacrylamide gels (BioRad). A liver tumor homogenate (10 μ g) was also loaded as a positive control. Protein was then transferred from the gel to a nitrocellulose membrane. Actin was blotted, to confirm equal

Fig. 2. MMP immunoblotting results. Data are presented as mean $\pm$ SEM arbitrary units. Sample sizes are young salt-resistant ($n=6$), young salt-sensitive ($n=6$), middle-aged salt-resistant ($n=5$), and middle-aged salt-sensitive ($n=6$). * $p < 0.05$ compared with the young salt-resistant group, † $p < 0.05$ compared with the young salt-sensitive group, # $p < 0.05$ compared with the middle-aged salt-resistant group.

loading of samples, using an anti-actin antibody (Sigma) at a 1:10,000 dilution. The densitometry for actin was $11,812 \pm 114$ U for young salt-resistant, $11,784 \pm 123$ U for young salt-sensitive, $11,742 \pm 105$ U for middle-aged salt-resistant, and $11,752 \pm 174$ U for middle-aged salt-sensitive rats ($p=0.982$).

The following primary antibodies were then used for immunoblotting: MMP-2, MMP-3, MMP-12, MMP-13, MMP-14, TIMP-1, TIMP-2, TIMP-4, fibronectin (Chemicon, Temecula, USA); MMP-7 and MMP-8 (Calbiochem, San Diego, USA); TIMP-3, collagen III and collagen IV (Accurate Chemical and Scientific Corp., Westbury, USA) and MMP-9 and collagen I (Sigma). All primary antibodies were used at a 1:2,000 dilution. A goat anti-rabbit secondary antibody (Vector, Burlingame, USA) was used at 1:5,000. Chemiluminescence (Pico Substrate Chemiluminescence kit; Pierce, Rockford, USA) was used for detection. Films were scanned into the 4,000 mm imager (Kodak, Rochester, USA), and Molecular Imaging software (Kodak) was used to determine densitometry values, expressed as arbitrary units.

Statistical Analyses

Data are presented as the mean $\pm$ SEM. ANOVA with Bonfer-

roni correction (Stata, College Station, USA) was used to evaluate changes among the four groups. Values of $p < 0.05$ were considered statistically significant. For the analysis, four pair-wise comparisons were evaluated: 1) the young salt-resistant vs. the young salt-sensitive group to determine differences during the early stage of hypertension; 2) the young salt-resistant vs. the middle-aged salt-resistant group to determine differences with aging; 3) the middle-aged salt-resistant vs. the middle-aged salt-sensitive group to determine differences during the late phase of chronic hypertension; and 4) the young salt-sensitive vs. the middle-aged salt-sensitive group to determine differences during the late phase of chronic hypertension superimposed on aging.

Good-fit regression modeling was performed to evaluate relationships between changes in the LVM/BW and changes in MMPs within each group. We fitted the data of LVM/BW (denoted by Y) to the data of 16 MMP variables (denoted by X), namely, MMP-2 (72 kDa), MMP-3 (57 kDa), ..., and MMP-14 (40 kDa), using the software packages Minitab and Excel. We fit Y and functions of Y to each X and functions of X . Fitting Y to various subsets of X variables was also considered. We set Y_j as the Y variable for the j -th group, for $j=1, 2, 3$, and 4. Large values of the coefficient of determination (r^2) and small p -values were both used to assess the models.

Fig. 3. Immunoblotting results for TIMPs (top), collagen I (middle), and fibronectin (bottom). Data are presented as mean $\pm$ SEM arbitrary units. Sample sizes are young salt-resistant ($n=6$), young salt-sensitive ($n=6$), middle-aged salt-resistant ($n=5$), and middle-aged salt-sensitive ($n=6$). * $p < 0.05$ compared with the young salt-resistant group, † $p < 0.05$ compared with the young salt-sensitive group, # $p < 0.05$ compared with the middle-aged salt-resistant group.

Results

LV Necropsy and Biochemical Analyses

As shown in Table 1, LVM increased during the early phase of hypertension, during aging, and during the late phase of hypertension. When corrected for increases in body weight, the LVM/BW ratio remained significantly elevated in the late phase chronic hypertensive group. This data indicates that later stages of chronic hypertension induce hypertrophy above that normally seen with aging. Total protein and collagen levels, as a percent of LVM, both increased in the early phase, suggesting increased protein synthesis and fibrosis during the initial phase. Interestingly, protein and collagen levels were decreased during the late phase, suggesting that global fibrosis is not maintained with late stage chronic

hypertension. Whether regional focal (reparative) fibrosis increased was not examined. The decrease in collagen, combined with the increase in LVM, suggests increased dilation in the late phase hypertension group.

Immunoblotting

The immunoblot for MMP-14 is shown as a representative in Fig. 1. The following four bands were analyzed by densitometry: 65 kDa and 54 kDa bands, which represent the pro and active forms of MMP-14, respectively, and 45 kDa and 40 kDa bands, which are degradation products. The 54 kDa active MMP-14 band was differentially expressed among the groups. In the young salt-resistant vs. young salt-sensitive groups (early phase of hypertension) and the young salt-resistant vs. middle-aged salt-resistant groups (aging), active MMP-14 decreased. In contrast, the middle-aged salt-resis-

tant vs. middle-aged salt-sensitive groups (late phase of chronic hypertension) and young salt-sensitive vs. middle-aged salt-sensitive groups (late phase of chronic hypertension superimposed on aging groups) showed increased active MMP-14 levels. Densitometry values for differentially expressed MMPs are shown in Fig. 2, and densitometry values for TIMPs, collagen I, and fibronectin are shown in Fig. 3. MMP-2, TIMP-2, and collagen IV levels were not changed between groups.

In the early phase hypertension comparison (young salt-resistant vs. young salt-sensitive groups), several ECM components decreased. TIMP-3 levels changed only during the initial hypertension phase, when TIMP-3 levels decreased in the young salt-sensitive group. In the aging comparison (young salt-resistant vs. middle-aged salt-resistant groups), there was a similar pattern of decreased ECM levels. While net MMP levels decreased between the young salt-resistant and young salt-sensitive groups (early phase hypertension), net MMP levels increased in the late phase chronic hypertension comparison (middle-age salt-resistant vs. middle-age salt-sensitive groups). Pro MMP-8 was changed only with late phase chronic hypertension, suggesting that MMP-8 may play a dominant role in long term pressure overload. Degraded collagen I (the 25 kDa product) and full length collagen III both decreased only with the late phase of chronic hypertension, consistent with picrosirius red assay results.

The late phase chronic hypertension superimposed on aging comparison (young salt-sensitive vs. middle-aged salt-sensitive groups) did not show a consistent pattern of either increasing or decreasing ECM components, indicative of a mixed pattern. Active MMP-3 and active MMP-12 were only changed in the late phase of chronic hypertension superimposed on aging, with both being decreased in the middle-aged salt-sensitive group. These results indicate a temporal shift in MMP/TIMP expression that correlates with the increase in LVM and net loss of total collagen.

Effects of MMPs on the Ratio of the LVM/BW

We evaluated the relationship between MMPs and changes in the LVM/BW ratio. Based on Shapiro-Wilks' test for the normality data (11), the LVM/BW for each group had a normal distribution. We compared the means (and variances) of the four distributions using samples of LVM/BW data and found that the means (and variances) differed significantly among the groups. From this, we conclude that the four samples are from different normal distributions. Because of this difference, correlation analyses were separately performed to demonstrate whether there was a linear or quadratic relationship between Y (LVM/BW) and each of the 16 X (MMP) variables. To determine whether any of the X variables explained the change in LVM/BW ratios, we performed good-fit modeling. The only good-fit models were: Y_2 (early phase hypertension) = $1,770.9 - 1.019 X_{15} + 0.00019556 (X_{15})^2 - 0.00000001 (X_{15})^3$; and Y_3 (aging) = $-483.8 + 0.1636 X_{10}$

$- 0.000018076 (X_{10})^2 + 0.00000000065 (X_{10})^3$. X_{15} was MMP-14 (45 kDa) and X_{10} was MMP-12 (45 kDa). Based on these results, the early phase of hypertension showed correlations between LVM/BW and degraded MMP-14 (45 kDa), while aging showed correlations between LVM/BW and active MMP-12 (45 kDa).

Discussion

The goal of this study was to compare the effects of early and late phases of chronic hypertension on MMP, TIMP, collagen, and fibronectin profiles. The most significant findings of this study were that the initial stage of hypertension and aging showed similar ECM profiles (decreased MMPs and increased fibrosis). In contrast, late phase chronic hypertension was characterized by increased MMPs and decreased fibrosis. Because the late phase of chronic hypertension is naturally superimposed on aging, the net effect is a loss of collagen and increase in the collagenases MMP-8 and MMP-14. This study provides the most complete evaluation of ECM remodeling in the Dahl salt-sensitive rat model of chronic hypertension and provides novel insight into ECM mechanisms involved in the LV hypertrophy that occurs with hypertension and aging. No other study that we are aware of has examined the consequence of 4 and 15 month pressure overload on LV ECM profiles in rats.

ECM levels were altered, as evidenced by the increase in collagen content for rats in the early phase of hypertension and aging along with the decreased collagen levels seen during the late phase of chronic hypertension superimposed on aging. Accompanied by the changes in collagen content were changes in particular MMP and TIMP levels, which suggests that the ECM degradation patterns parallel the flux in MMPs and TIMPs. The general trend of decreased MMPs suggests that ECM turnover may occur at a decelerated rate during the early phase. MMPs, particularly gelatinases, regulate myocardial fibrosis by stimulating both collagen degradation and synthesis (12). MMPs have been shown to generate numerous bioactive peptides that influence ECM levels, and collagen degradation by MMPs has been shown to generate collagen peptides that stimulate collagen synthesis (13). TIMP-3 only changed in the early stage of hypertension, suggesting that its decrease has a role in the early response to pressure overload. Fedak *et al.* have previously shown that TIMP-3 null mice develop dilated cardiomyopathy at 21 months of age, suggesting a role for TIMP-3 in maintaining ECM homeostasis and normal cardiac function (14). Because this study was performed on female rats, future studies that evaluate effects on male rats are warranted, in order to determine whether there are gender-related differences in the ECM response.

Similar to the early phase, aging showed a profile of decreased MMPs. Pro and active MMP-9 levels changed only in the aging or the late phase chronic hypertension and aging groups, suggesting that MMP-9 is highly influenced by aging. It is important to remember that aging, in this paper, refers to

the transition from young to middle-aged, and therefore cannot be extrapolated to studies dealing with old and/or senescent groups. Consistent with the results from this study, we have previously reported that, for CB6F1 mice, the transition from young to middle-aged is also accompanied by a net decrease in MMPs (15).

The ECM profile displayed in the late phase of chronic hypertension was nearly opposite from that seen in the aging and early phase hypertension comparisons. The concomitant increases in both MMPs and TIMPs may indicate abnormal ECM turnover at the post-translational level (16–18). MMP-8 and MMP-14 are both collagenases and have previously been associated with aging and/or ECM remodeling, although neither has been examined in chronic hypertension (15, 19). We did not evaluate MMP-1 in this study, because adult rodents do not express the MMP-1 gene (20). MMP-1 is likely to be very relevant in the clinical setting, however, since Ishikawa *et al.* have demonstrated, in human hypertensive patients with left ventricular hypertrophy, that plasma MMP-1 levels significantly correlate with both the pulse pressure and the mean blood pressure (21).

In conclusion, early and late chronic pressure overload induces distinct ECM phenotypes reflective of changes in particular MMPs and TIMPs. While MMPs are predominantly attenuated during the initial pressure overload, the increase in MMPs during the late phase of chronic pressure overload provides a rationale for evaluating the effects of therapeutic regulation of particular MMPs and TIMPs during later stages.

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