Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodelling in hypertension in rats

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Abstract

**Objective:** To assess whether the variable impact of quantitative changes in myocardial collagen on left ventricular (LV) diastolic myocardial stiffness (myocardial $k$) and remodelling (increased volume intercept of diastolic pressure–volume relations) in LV hypertrophy (LVH) is associated with alterations in myocardial collagen cross-linking. **Methods:** We evaluated myocardial collagen content (hydroxyproline concentrations [HPRO]) and the degree of myocardial collagen cross-linking (solubility to cyanogen bromide digestion) in 14–15- and 21–22-month-old spontaneously hypertensive rats (SHRs), and in aortic-banded rats with pressure overload hypertrophy (POH). **Results:** In rats with POH and in SHRs irrespective of age, increases in myocardial [HPRO] were noted. However, hypertensive rats differed in the material and geometric properties of the myocardium, and in qualitative aspects of fibrosis. In 14–15-month-old SHRs myocardial $k$ (determined from diastolic stress–strain relations) and insoluble (cross-linked) [HPRO] were increased, but no LV remodelling or increases in myocardial soluble (non-cross-linked) [HPRO] were noted. In rats with POH, LV remodelling and increases in soluble myocardial [HPRO] occurred, but no increase in $k$ or insoluble myocardial [HPRO] were observed. In 21–22-month-old SHRs, increases in $k$, soluble and insoluble myocardial [HPRO], as well as LV remodelling occurred. **Conclusions:** Collagen cross-linking may determine the diverse relation that exists between increases in myocardial collagen concentrations and either myocardial stiffness as well as LV dilatation in LVH, albeit an effect that is modulated by collagen quality.

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1. Introduction

Myocardial fibrosis is a potentially important deter-

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and chamber remodelling, a number of discrepant results remain unexplained. Normal myocardial stiffness [4] and modest increases in myocardial stiffness [5] are both associated with increments in collagen concentrations; pharmacological-induced improvements in stiffness may occur without decreases in collagen concentrations [6]; and decreases in collagen concentrations do not necessarily reduce stiffness [7]. Moreover, increments in myocardial collagen concentrations may occur without chamber remodelling [8]; tissue fibrosis is not a necessary prerequisite for chamber remodelling [8–10]; and reverse remodelling is associated with increases [11–13] in collagen concentrations. Therefore, the contribution of enhanced myocardial collagen concentrations toward diastolic dysfunction and chamber remodelling is not established.

Recent data suggest that increments in myocardial collagen cross-linking contribute to enhanced stiffness [6], and decreases in cross-linking to chamber remodelling [9]. We therefore hypothesized that the diverse relationship that exists between the degree of myocardial fibrosis and both stiffness and chamber remodelling, could in-part be explained through a modulating influence of alterations in myocardial collagen cross-linking on the impact of fibrosis on cardiac diastolic characteristics. In order to examine this hypothesis we evaluated myocardial interstitial changes in rat models of hypertensive heart disease with varying functional abnormalities, including increased stiffness without remodelling, remodelling without increased stiffness, and both remodelling and increased stiffness.

2. Methods

The present study was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC approval numbers; 2000/40/5, 98/28/4, and 97/44/5).

2.1. Animal models

To produce a model of POH in rats associated with LV dilatation, but no increase in myocardial stiffness, a suprarenal abdominal aortic stenosis was created in 130–170 g male Sprague–Dawley rats as previously described [14]. In the present study a 21 gauge needle was used as a guide to determine the internal diameter of the stenotic lesion. In this model of POH we have previously shown little effect on myocardial stiffness [5] and in a pilot study designed to assess the effects of a wider diameter aortic band (21 as opposed to a 22 gauge needle used to determine the internal diameter of the stenotic lesion), although LV dilatation was noted on echocardiography, no changes in myocardial stiffness were noted. Sham operations were performed in a separate group of rats (n = 11). Sham-operated rats and rats with POH were assessed for haemodynamic changes and myocardial collagen characteristics 7.5–8 months after surgery. Of the rats operated on to produce POH, 37 survived the immediate post-operative period, and 19 died prior to the collection of haemodynamic data. To assess myocardial collagen changes in a model of increased myocardial stiffness without LV dilatation, 14–15-month-old spontaneously hypertensive rats (SHRs, n = 14) and age-matched Wistar Kyoto (WKY) controls (n = 11) were employed [6]. Moreover, to assess myocardial collagen changes in a model of increased myocardial stiffness with concomitant LV dilatation, 21–22-month-old SHRs (n = 10) and age-matched WKY controls (n = 12) were employed [15]. In the 21–22-month-old SHRs, six animals had clinical evidence of heart failure (either pleuro-pericardial effusions, and/or calcified left atrial thrombi).

2.2. LV cavity size and geometry determined in vivo

In rats with POH and in sham-operated controls, LV internal dimensions were determined at regular intervals throughout the study in anaesthetized animals using echocardiography as previously described [14]. In this study all data were acquired using a model 2500 Hewlett-Packard echocardiograph with a 7.5 MHz transducer.

In SHRs with concentric LV geometry (14–15-month-old rats), the leading edge of the posterior wall endocardial surface was difficult to identify with accuracy using echocardiographic techniques, as the papillary muscle obscured the surface. Hence, LV geometry and dimensions were determined at controlled filling pressures in anaesthetized, ventilated, open-chest SHRs and WKY rats using alternative techniques developed in our laboratory that have previously been described and validated elsewhere [6,16,17]. Briefly, LV end diastolic (LVED) short axis external diameters were measured using piezoelectric ultrasonic transducers, and LVED pressures (LVEDP) determined from a fluid-filled catheter inserted through the apex of the LV. The catheter-pressure transducer combination used for this study had an amplitude–frequency response uniform to 10 Hz. Measurements of LVED external diameters and LVEDP were obtained over a range of LVEDP values from 1 to 10 mmHg by manipulating blood volume using an iso-oncotic solution. LVED radius (r) and wall thickness (h) were determined from previously described formulae [16]. LVED relative wall thickness was determined from LVED h-to-r ratios determined over a range of LVEDP values. Statistical comparisons of LVEDr and LVED relative wall thickness values were made on LVEDr and LVEDh/r intercepts of the LVEDP–LVEDr and LVEDP–LVEDh/r relations, respectively (LVEDr0 and LVEDh/r0).

2.3. Isolated, perfused heart preparations

Following the collection of haemodynamic data in vivo,
hearts were excised and LV diastolic pressures determined over a range of filling volumes in isolated, perfused heart preparations as previously described [9]. Briefly, hearts were perfused retrogradely at a constant flow (12 ml g$^{-1}$ wet heart weight) with 37 °C physiological saline solution, and paced at 360 beats min$^{-1}$ with platinum electrodes attached to the left atrium and the apex of the heart. LV diastolic pressures were determined by use of a water-filled balloon-tipped catheter. LV pressures were determined at as many multiple small increments in volume as were practically possible to improve on the accuracy of curve fitting during later analysis. LV remodelling was assessed from the volume intercept ($V_0$) of the LV diastolic pressure–volume ($P$–$V$) relation [9]. Myocardial stiffness (myocardial $k$) was determined from the slope of diastolic stress–strain relations using previously described formulae [18].

2.4. Myocardial collagen

Samples of LV tissue were weighed and stored at −70 °C for tissue analysis. Myocardial hydroxyproline concentration ([HPRO]) was assessed using the method of Stegemann and Stalder after acid (HCl) hydrolysis [19]. Myocardial collagen was also extracted and digested with cyanogen bromide (CNBr) [5]. The CNBr digested collagen sample was subjected to acid hydrolysis and [HPRO] determination. The amounts of non-cross-linked (soluble) and cross-linked (insoluble) collagen in the myocardium were determined as previously described based on the solubility of myocardial collagen to CNBr digestion [6,9].

2.5. Analysis

Regression analysis was used to determine the lines of best fit for the cardiac function relations. The stress–strain relations were linearized for statistical comparisons. Linear regression analysis and Pearson’s correlation coefficient were used to assess the relationship between myocardial stiffness and total collagen concentration as well as each of the fractions. Differences in LV weight, internal dimensions, geometry, performance, and myocardial collagen biochemical analysis between SHR and WKY groups were assessed by a one-factor analysis of variance (ANOVA) followed by a Tukey post hoc test and between POH and SHAM groups by an unpaired Welch $t$-test. As data obtained in 14–15- and 21–22-month-old WKY rats were shown to be similar, data for all WKY rats were pooled for graphic representations. All values in the text are represented as mean±S.E.M.

3. Results

3.1. LV weight

Aortic-banded rats and SHR at both 14–15 and at 21–22 months of age had increases in LV weight as compared to their respective age-matched controls (Table 1).

3.2. LV cavity size, remodelling, and geometry

Consistent with LV dilatation, rats with POH developed an increase in LV internal diameters as compared to sham-operated controls throughout the treatment period of the study (data for the final echocardiographic assessment are given in Fig. 1, upper left panel). In keeping with LV remodelling, rats with POH had a right shift in the LV diastolic $P$–$V$ relation and an increase in the LV volume intercept of this relation (Fig. 1, lower and upper right panels).

SHRs at 14–15 months of age had normal LVEDP–LVED internal radius values (LVEDr) is illustrated in the upper left panel of Fig. 2) and a normal volume intercept of the LV diastolic $P$–$V$ relation (inset of Fig. 3) as compared to age-matched WKY controls. Consequently, the increase in LV weight noted in 14–15-month-old SHRs (Table 1) translated into an increase in relative wall thickness as determined at controlled filling pressures (Fig. 2, upper right and lower panels). However, in keeping with the development of LV remodelling, 21–22-month-old SHRs developed an increase in LVED internal dimensions as determined at controlled filling pressures (upper left panel of Fig. 2), a right shift in the LV diastolic $P$–$V$ relation (Fig. 3) and an increased volume intercept of the LV diastolic $P$–$V$ relation (inset of Fig. 3). Thus, even though 21–22-month-old SHRs had marked increases in LV weight (Table 1), LVED relative wall thickness was

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<td><strong>Effect of aortic-bandings and spontaneous hypertension on left ventricular (LV) and body (BW) weight</strong></td>
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<tr>
<th>POH, SHAM, SHR</th>
<th>14–15 months (n=14)</th>
<th>21–22 months (n=10)</th>
<th>14–15 months (n=11)</th>
<th>21–22 months (n=12)</th>
</tr>
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<tr>
<td><strong>LV weight (g)</strong></td>
<td>1.72±0.04**</td>
<td>1.31±0.05</td>
<td>1.11±0.04*</td>
<td>1.34±0.04**†</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>681±15</td>
<td>682±23</td>
<td>363±9</td>
<td>351±12*</td>
</tr>
<tr>
<td><strong>LV/BW×10$^{-3}$</strong></td>
<td>2.53±0.07*</td>
<td>2.07±0.07</td>
<td>3.12±0.10*</td>
<td>3.85±0.18**†</td>
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POH, Pressure overload hypertrophy; SHAM, sham-operated controls; SHR, spontaneously hypertensive rats; WKY, normotensive Wistar Kyoto control; LV, left ventricle; BW, body weight. * $P<0.01$, ** $P<0.001$ versus SHAM or age-matched WKY; † $P<0.001$ versus 14–15-month-old SHRs.
similar to WKY controls and reduced in comparison to 14–15-month-old SHRs (lower panel and upper right panel of Fig. 2), an effect that can only be attributed to LV remodelling.

3.3. Myocardial stiffness

Myocardial stiffness was unaltered in rats with POH, but was enhanced in both 14–15- and 21–22-month-old SHRs (Fig. 4).

3.4. Myocardial collagen

Rats with POH, as well as SHRs at 14–15 and 21–22 months of age developed an increase in myocardial [HPRO] with the greatest increase being in 21–22-month-old SHRs (Fig. 5). However, because of a decrease in % myocardial collagen soluble to CNBr digestion (SHRs=33±2, WKY=41±2, P<0.05), an increase in insoluble (cross-linked collagen) myocardial collagen concentrations, without a significant increment in the concentration of the soluble portion (non-cross-linked collagen), and hence an increase in the ratio of insoluble to soluble collagen occurred in SHR at 14–15 months of age (Fig. 5). In contrast, rats with POH had an increase in % myocardial collagen soluble to CNBr digestion (POH=39±7, SHAM=23±3, P<0.05), and hence an enhanced soluble myocardial collagen concentration, without a significant increase in the concentration of the insoluble portion (Fig. 5). Consequently the ratio of insoluble to soluble collagen was decreased in concordance with the increase in the percentage of collagen soluble to CNBr digestion.
3.5. Myocardial stiffness and myocardial collagen

In SHR rats with POH and LV remodelling without increases in myocardial stiffness, myocardial non-cross-linked, but not cross-linked collagen concentrations were enhanced. With advanced myocardial fibrosis in SHR rats, where concomitant LV remodelling and an increase in myocardial stiffness was noted, increments in both cross-linked and non-cross-linked myocardial collagen concentrations were observed.

The variable effect of increases in myocardial collagen concentrations on material properties may be attributed to alterations in either collagen phenotypes, or cross-linked characteristics. Although type I collagen is thought to be a stiffer phenotype, only modest changes in myocardial stiffness may follow increases in type I-to-III myocardial collagen ratios in hypertensive heart disease [5, 9]. Moreover, type I-to-III myocardial collagen ratios are decreased in older compared to younger SHR rats [16], when myocardial stiffness is increased [15, 20]. Consequently, alterations in myocardial collagen phenotypic ratios cannot explain disparate effects of fibrosis on material properties.

4. Discussion

In the present study SHR rats with increases in myocardial stiffness and without evidence of LV remodelling had increments in myocardial cross-linked collagen, but not non-cross-linked collagen concentrations. In contrast, in
In contrast, an augmented myocardial collagen cross-linking and enhanced concentrations of collagen in the cross-linked form accompany increments in myocardial stiffness [6]. In this regard, in the present study hypertensive rats without significant increases in myocardial cross-linked collagen concentrations (aortic-banded rats) failed to exhibit changes in myocardial material properties, whereas hypertensive rats with increases in myocardial cross-linked collagen concentrations (14–15- and 21–22-month-old SHRs) were noted to have an augmented stiffness. Although we have previously shown the importance of collagen cross-linking in mediating myocardial stiffness in younger SHRs [6], we have provided no data to show that increases in total, but not cross-linked myocardial collagen concentrations fail to translate into a stiffer myocardium in POH, or that myocardial cross-linked but not non-cross-linked collagen concentrations parallel stiffness in older SHRs.

Although part of the reason for the enhanced myocardial cross-linked collagen concentrations in 14–15-month-old SHRs was because of an augmented cross-linking (as evidenced by the decreases in collagen soluble to CNBr digestion), the same mechanism does not explain increases in myocardial cross-linked collagen concentrations in 21– 22-month-old SHRs. In the latter group, collagen solubility was enhanced which would tend to reduce cross-linked collagen concentrations. Hence increases in myocardial cross-linked collagen concentrations in older SHRs are attributed to an augmented synthesis [21], and not to alterations in cross-linked properties. Importantly, increments in myocardial cross-linked collagen concentrations in older SHRs also cannot be attributed to reduced collagen degradation, as an increased collagenase activity has been shown to occur in SHRs at this age [20].

The role of myocardial fibrosis as a determinant of chamber remodelling is controversial. The interstitial changes that are apparently more closely related to the development of chamber remodelling seem to be an increased activity of matrix metalloproteinases (MMPs) [8] and a decrease in collagen cross-linking [9,22] (which is likely to increase the susceptibility of myocardial collagen to MMP-induced degradation). These changes are thought to lead to breaks or tears in the myocardial collagen matrix and subsequently to produce myocyte slippage. Indeed, in the present study in rats with LV remodelling (aortic-banded rats and 21–22-month-old SHRs) increases in myocardial non-cross-linked collagen concentrations were noted. Although we have previously shown that LV remodelling accompanies increments in myocardial non-cross-linked collagen concentrations in cardiac hypertrophy [9], the present study provides the first data to show how these changes simultaneously impact on myocardial material properties as well as on LV remodelling. Our data indicate that LV remodelling without increments in myocardial stiffness accompanies increases in myocardial collagen concentrations if the collagen that accumulates is predominantly of the non-cross-linked phenotype. In contrast, if both cross-linked and non-cross-linked collagen accumulates, both LV remodelling and enhanced myocardial stiffness occur.

Importantly, in the present study, increases in non-cross-linked myocardial collagen concentrations in rats with
POH and 21–22-month-old SHRs were mediated by changes in collagen cross-linking (as evidenced by the enhanced solubility to CNBr digestion) as well as through an accumulation of myocardial collagen. These data support our previous proposal that myocardial fibrosis could contribute to LV remodelling through increases in myocardial collagen susceptible to the effects of MMPs (non-cross-linked phenotype) [9]. Indeed, pharmacological agents known to inhibit the synthesis of myocardial collagen are capable of attenuating the progression of LV remodelling [23] and to contribute to reverse LV remodelling [24] in human heart failure. Moreover, in SHRs the use of an antihypertensive agent that decreases myocardial collagen concentrations (including non-cross-linked collagen concentrations) has been shown to prevent the development of LV remodelling, despite being ineffective at regressing LV hypertrophy [16], and reverse remodelling in human heart failure (following the use of an LV assist device) is associated with an enhanced collagen cross-linking [22].

A possible limitation of this study is the use of indirect methods to determine collagen concentrations and the amount of collagen cross-linking. However both of these methods are well established techniques [19,25]. Furthermore with respect to alterations in cross-linking in association with LV remodeling, similar findings to ours have been observed in other studies [10,11].
been reported by others [3, 22, 26] using alternative indirect techniques to determine collagen content and the extent of collagen cross-linking.

In summary, the data in the present study suggest that the cross-linked characteristics of collagen influence whether increments in myocardial collagen concentration impact on stiffness and chamber remodelling in hypertensive heart disease. These data provide a potential explanation for the variable effects of fibrosis on myocardial material properties and chamber remodelling. Hence,
Fig. 6. Correlations between myocardial stiffness (myocardial $\kappa$) and myocardial collagen characteristics in spontaneously hypertensive rats (SHRs) and their normotensive Wistar Kyoto controls (WKY). Correlation coefficients ($r$) and their $P$-values are shown for SHRs and WKY rats. Correlation coefficients and their $P$-values for aortic-banded rats with pressure overload hypertrophy (POH) and their sham-operated controls (SHAM) are included for comparison. For other abbreviations refer to Fig. 5.

Increases in myocardial collagen concentration may play a role in both myocardial stiffness and dilatation, however these effects are modified by alterations in collagen quality. Therefore, pharmacological agents used in cardiac disease should target both the quantity and the quality of myocardial collagen.

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