Collagen Cross-Linking But Not Collagen Amount Associates With Elevated Filling Pressures in Hypertensive Patients With Stage C Heart Failure

Potential Role of Lysyl Oxidase

Begoña López, Ramón Querejeta, Arantxa González, Mariano Larman, Javier Díez

Abstract—We investigated whether the quality of myocardial collagen associates with elevated left-sided filling pressures in 38 hypertensive patients with stage C chronic heart failure. Filling pressures were assessed invasively measuring pulmonary capillary wedge pressure. Left ventricular chamber stiffness constant was calculated from the deceleration time of the early mitral filling wave. The fraction of myocardial volume occupied by total collagen tissue and collagen type I fibers was assessed histomorphologically. The degree of collagen cross-linking (CCL), which determines the formation of insoluble stiff collagen, was assessed by colorimetric and enzymatic procedures. The expression of lysyl oxidase (LOX), which regulates CCL, was assessed by Western blot. Compared with patients with normal pulmonary capillary wedge pressure (≤12 mm Hg; n = 16), patients with elevated pulmonary capillary wedge pressure (>12 mm Hg; n = 22) exhibited increases of left ventricular chamber stiffness constant, fraction of myocardial volume occupied by total collagen tissue, fraction of myocardial volume occupied by collagen type I fibers, CCL, insoluble stiff collagen, and LOX. Pulmonary capillary wedge pressure was correlated with left ventricular chamber stiffness constant (r = 0.639; P < 0.001), insoluble stiff collagen (r = 0.474; P < 0.005), CCL (r = 0.625; P < 0.001), and LOX (r = 0.410; P < 0.05) in all of the patients but not with fraction of myocardial volume occupied by total collagen tissue or fraction of myocardial volume occupied by collagen type I fibers. In addition, CCL was correlated with insoluble stiff collagen (r = 0.612; P < 0.005), LOX (r = 0.538; P < 0.01), left ventricular chamber stiffness constant (r = 0.535; P < 0.005), peak filling rate (r = −0.343; P < 0.05), ejection fraction (r = −0.430; P < 0.01), and amino-terminal propeptide of brain natriuretic peptide (r = 0.421; P < 0.05) in all of the patients. These associations were independent of confounding factors. These findings indicate that, in hypertensive patients with stage C heart failure, it is only the quality of collagen (i.e., degree of cross-linking) that associates with elevated filling pressures. It is suggested that LOX-mediated excessive CCL facilitates the increase in left ventricular stiffness with the resulting elevation of filling pressures in these patients.

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Key Words: collagen • filling pressures • heart failure

Arterial hypertension has been long considered as one of the most common etiologic conditions predisposing to heart failure (HF).1 Furthermore, a recent systematic review shows that HF development remains a major problem in treated hypertensive patients.2 It has been shown that myocardial fibrosis, a common finding in postmortem studies and endomyocardial biopsies of patients with hypertensive heart disease (HHD),3 is associated with the increase of left ventricular (LV) chamber stiffness4,5 and the development of clinically overt HF in these patients.6,7 It has been proposed that the increase in LV stiffness can contribute to compromised diastolic function and elevate left-sided filling pressures (FPs) in hypertensive patients.8

The definition of myocardial fibrosis is based on the quantification of an excess of collagen fiber deposition, as assessed by staining techniques. However, it must be considered that collagen-dependent LV chamber stiffness is influenced not only by the amount of collagen fibers but also by their qualitative properties (i.e., the degree of collagen cross-linking [CCL] among collagen fibrils that determines the insolubility, stiffness, and resistance to degradation of the resulting fibers; the relative proportions of collagen types I and III fibers; and the diameter of the collagen fibers and their spatial alignment).9 In fact, as demonstrated in different experimental models of pressure overload, although LV chamber stiffness is affected by changes in both collagen...
quantity and quality, the effects of changes in collagen quantity, particularly the abundance of collagen type I, are modified by collagen quality, in particular the degree of CCL.10–12 Therefore, a more complete understanding of the quantitative and qualitative variations of myocardial collagen matrix and their impact on LV function is critical as we strive to develop specific and effective therapies for HF. In this conceptual framework, this study was designed to investigate whether it is the quantity (as assessed by the amounts of total or collagen type I fibers) or the quality (as assessed by the degree of CCL and availability of insoluble collagen [insCol]) that associates with FPs in hypertensive patients with HHD and chronic stage C HF. In addition, the expression of lysyl oxidase (LOX), which regulates CCL,13 and the enzymes procollagen type I carboxy-terminal proteinase (PCP) and furin, which participate in the activation of LOX,14 was also assessed. Finally, a number of biomarkers related to collagen were measured to investigate whether any association exists between their circulating levels and parameters assessing the quantity and quality of myocardial collagen.

Methods

Subjects

All of the subjects gave written informed consent to participate in the study, and the institutional review committee approved the study protocol. The study conformed to the principles of the Helsinki Declaration. The population consisted of 38 hypertensive patients with a previous clinical diagnosis of chronic stage C HF based on the presence of ≥2 major and 2 minor Framingham criteria.14 The included patients were in New York Heart Association functional classes II to IV. Table S1 (available in the online-only Data Supplement) shows the characteristics of these patients. (For further details see the Expanded Methods section in the online-only Data Supplement).

Three transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum from each patient during the cardiac catheterization procedure. Septal endomyocardial biopsies were obtained from autopsies of 10 age- and sex-matched persons found to have died of cardiovascular disease and with no LV hypertrophy.

Noninvasive Cardiac Studies

Two-dimensional echocardiographic-Doppler and pulsed-Doppler imaging was performed in all of the patients. (For further details see the Expanded Methods section in the online-only Data Supplement).

Nuclear Studies

To rule out microvascular ischemia, single photon emission computed tomography was performed in all of the patients. In addition, peak filling rate (PFR) and time to PFR were calculated.15

Invasive Cardiac Studies

After significant coronary artery disease (≥50% stenosis in a major epicardial coronary artery) was discarded, pulmonary capillary wedge pressure (PCWP) was measured. Values of PCWP >12 mm Hg were considered as abnormally elevated.16

Biochemical Determinations

Plasma amino-terminal propeptide of brain natriuretic peptide and aldosterone were measured using commercial kits. Circulating biomarkers related to collagen were measured in all of the patients using commercial ELISA kits. Procollagen type I carboxy-terminal pro-

Histomorphological Studies

The area of myocardium occupied by total collagen tissue or collagen volume fraction (CVF) was determined by quantitative morphometry in sections stained with collagen-specific picro-sirius red, as reported previously.17 To distinguish between cross-linked (insCol) and noncross-linked collagen (soluble collagen), colormetric and enzymatic procedures were used, as described previously.18 The concentration of each form of collagen was related to the total amount of protein. The degree of CCL was calculated as the ratio between the insCol and noncross-linked collagen.

Immunohistochemical analysis of collagen type I was performed on formalin-fixed and paraffin-embedded sections. Immunohistochemical staining was performed by the avidin peroxidase-labeled dextran polymer method. Positive staining was visualized with DAB Plus (Boehringer Mannheim Corp), and tissues were counterstained with Harris hematoxylin (Sigma). The area of myocardium with positive staining for collagen type I (C1/VF) was analyzed by quantitative morphometry, as described previously.6

Molecular Studies

The protein expression of LOX, PCP, and furin was analyzed in myocardial samples from 23 patients. A 25-μg sample of total protein obtained from each biopsy was processed for Western blot, as described previously.19 Data are expressed as arbitrary densitometric units relative to β-actin expression. (For further details see the Expanded Methods section in the online-only Data Supplement).

Statistical Analysis

To analyze the differences in histomorphological, biochemical, and molecular parameters between controls and patients and between patients with either increased or normal PCWP, a Student t test for unpaired data was used once normality was demonstrated; otherwise, the Mann-Whitney U test was performed. Categorical variables were analyzed by the χ2 test or Fisher exact test when necessary. The correlation between continuously distributed variables was tested by correlation coefficients and univariate regression analysis. The influence of confounding factors on correlations was excluded by partial correlation analysis for quantitative parameters. Values are expressed as mean±SEM and categorical variables as numbers and percentages. A value of P<0.05 was considered statistically significant.

Results

Clinical, Echocardiographic, and Biochemical Characteristics

The clinical characteristics of the 2 groups of patients are shown in Table S1 (available in the online-only Data Supplement). No significant differences in the assessed parameters were found between the 2 groups of patients.

Table 1 shows the echocardiographic parameters assessed in the 2 groups of patients. The values of LV mass index (LVMII), LV end-diastolic diameter (LVEDD), and LV end-diastolic volume (LVEDV) were higher in patients with elevated PCWP than in patients with normal PCWP. Patients with elevated PCWP exhibited lower values of LV ejection fraction (LVEF), deceleration time, and PFR than patients...
with normal PCWP. The value of LV chamber stiffness constant was increased in patients with elevated PCWP compared with patients with normal PCWP. Although patients with elevated PCWP exhibited higher values of amino-terminal propeptide of brain natriuretic peptide than patients with normal PCWP, plasma aldosterone levels were similar in the 2 groups of patients (Table S1).

### Histomorphological and Molecular Myocardial Parameters

Compared with control subjects, the whole group of patients exhibited higher values of CVF and CIVF (Table 2). Interestingly, a strong direct correlation \((r=0.969; P<0.001)\) was found between CVF and CIVF in all of the HF patients (Figure S1). The amounts of insCol and noncross-linked collagen were increased by 9-fold and 4-fold, respectively, in patients with elevated PCWP compared with patients with normal PCWP (21233\(\pm\)1463 versus 29234\(\pm\)1808 arbitrary densitometric units; \(P<0.001\)). The expression of LOX was enhanced in patients with elevated PCWP compared with patients with normal PCWP (5.61\(\pm\)0.42 versus 4.34\(\pm\)0.55 arbitrary densitometric units; \(P<0.001\); Figure 2). PCP expression was reduced in patients with elevated PCWP compared with patients with normal PCWP (21233\(\pm\)1463 versus 29234\(\pm\)1808 arbitrary densitometric units; \(P<0.001\)). The expression of furin did tend to be higher \((P=0.05)\) in patients with elevated PCWP than in patients with normal PCWP (0.41\(\pm\)0.08 versus 0.24\(\pm\)0.11 arbitrary densitometric units).

### Analysis of Associations

Correlations among collagen-related histomorphological and molecular parameters and the main parameters assessing LV function are presented in Table S2. In summary, the degree of CCL was higher in patients with elevated PCWP than in patients with normal PCWP (3.63\(\pm\)0.23 versus 2.88\(\pm\)0.15, \(P<0.01\); Figure 1).

The expression of LOX was enhanced in patients with elevated PCWP compared with patients with normal PCWP (5.61\(\pm\)0.42 versus 4.34\(\pm\)0.55 arbitrary densitometric units; \(P<0.001\); Figure 2). PCP expression was reduced in patients with elevated PCWP compared with patients with normal PCWP (21233\(\pm\)1463 versus 29234\(\pm\)1808 arbitrary densitometric units; \(P<0.001\)). The expression of furin did tend to be higher \((P=0.05)\) in patients with elevated PCWP than in patients with normal PCWP (0.41\(\pm\)0.08 versus 0.24\(\pm\)0.11 arbitrary densitometric units).

### Figure 1

Degree of collagen cross-linking in the myocardium of heart failure patients with either normal or elevated pulmonary capillary wedge pressure (PCWP).

### Figure 2

Protein expression of the enzyme lysyl oxidase (LOX) in heart failure patients with either normal or elevated pulmonary capillary wedge pressure (PCWP).
function are presented in Table 3. CCL was directly correlated with PCWP (Figure 3A) and inversely correlated with PFR (Figure 3B) and LVEF (Figure 3C). In addition, insCol and LOX were directly correlated with PCWP in all of the patients. Finally, LOX was inversely associated with LVEF in all of the patients. Of interest, PCWP was directly associated with LV chamber stiffness constant (r = 0.639; P < 0.001) in all of the patients. All of these correlations remained significant when we excluded the influence of a number of potential confounding factors (ie, age, sex, LVMI, LVEDD, and LVEDV) in partial correlation analysis. No correlations were found between CVF and CIVF with LV functional parameters in this study.

CCL was correlated with insCol (r = 0.778; P < 0.001) and LOX (r = 0.538; P < 0.01) in all and 23 patients, respectively. In addition, CCL was correlated with LV chamber stiffness constant (r = 0.535; P < 0.005; Figure 4A) and amino-terminal propeptide of brain natriuretic peptide (r = 0.421; P < 0.05; Figure 4B) in all of the patients. These correlations remained significant when we excluded the influence of a number of potential confounding factors (ie, age, sex, LVMI, LVEDD, and LVEDV) in partial correlation analysis.

Finally, a direct correlation was found between LOX and furin (r = 0.599; P < 0.05) in all of the patients. This correlation remained significant when we excluded the influence of a number of potential confounding factors (ie, age, sex, LVMI, LVEDD, and LVEDV) in partial correlation analysis.

Serum CITP was inversely associated with insCol (r = −0.451; P < 0.01), this association being independent of a number of potential confounding factors (ie, age, sex, LVMI, LVEDD, LVEDV, estimated glomerular filtration rate, MMP-1, and TIMP-1) in partial correlation analysis. No further associations were found between circulating biomarkers of collagen type I metabolism and insCol or CCL. In addition, serum procollagen type I carboxy-terminal propeptide was directly correlated with both CVF (r = 0.735; P < 0.001) and CIVF (r = 0.689; P < 0.001) in all of the patients, with these associations being independent of a number of potential confounding factors (ie, age, sex, LVMI, LVEDD, and LVEDV) in partial correlation analysis. No correlations were found between procollagen type III amino-terminal propeptide and the quantity (ie, CVF) and quality (ie, insCol and collagen cross-linking) of myocardial collagen.

Discussion

The 3 main findings of this study are as follows: (1) FPs are associated with the quality (ie, the degree of cross-linking) but not with the quantity of collagen present in the myocardium of patients with HHD and stage C HF; (2) the degree of collagen cross-linking was associated with impairment of LV diastolic and systolic function; and (3) the degree of cross-linking is associated with the expression of the enzyme LOX in the myocardium of these patients. An increase in myocardial stiffness translates into a reduced compliance of the left ventricle and the resulting increase in FPs. Other than variations in intrinsic cardiomyocyte stiffness, changes in the collagen matrix also influence myocardial stiffness. In particular, Badenhorst et al demonstrated in experimental models of pressure overload that myocardial and LV chamber stiffness are affected by changes in both collagen quantity and quality (ie, cross-linking), with the effects of changes in

Table 3. Correlations of Collagen-Related Histomorphological and Molecular Parameters and Left Ventricular Function Parameters in Hypertensive Patients With Heart Failure

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CCL P r</th>
<th>InsCol P r</th>
<th>CVF P r</th>
<th>CVF P r</th>
<th>LOX P r</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCWP, mm Hg</td>
<td>&lt;0.001 0.625</td>
<td>&lt;0.005 0.474</td>
<td>NS 0.230</td>
<td>NS 0.235</td>
<td>&lt;0.05 0.410</td>
</tr>
<tr>
<td>PFR, edv/s</td>
<td>&lt;0.05 −0.343</td>
<td>NS −0.266</td>
<td>NS −0.261</td>
<td>NS −0.293</td>
<td>NS 0.086</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>&lt;0.01 −0.430</td>
<td>NS −0.274</td>
<td>NS −0.083</td>
<td>NS −0.084</td>
<td>&lt;0.05 −0.418</td>
</tr>
</tbody>
</table>

CCL indicates collagen cross-linking; InsCol, insoluble collagen; CVF, total collagen volume fraction; LOX, lysyl oxidase; PCWP, pulmonary capillary wedge pressure; PFR, peak filling rate; LVEF, left ventricular ejection fraction; NS, nonsignificant.

Figure 3. A, Direct correlation (y = 4.65x + 0.42) between the degree of collagen cross-linking (CCL) and pulmonary capillary wedge pressure (PCWP) in all of the patients. B, Inverse correlation (y = −0.24x + 2.23) between the degree of collagen cross-linking and peak filling rate (PFR) in all of the patients. C, Inverse correlation (y = −8.07x + 73.72) between the degree of collagen cross-linking and left ventricular ejection fraction (LVEF) in all of the patients.
collagen content being modified by collagen cross-linking. In accordance with these data, we found that it is the degree of CCL and the abundance of insCol but not the amount of total or type I collagen fibers that associate with LV chamber stiffness (ie, LV chamber stiffness constant) and FPs (ie, PCWP) in patients with HHD and stage C HF. Thus, CCL emerges as an important determinant of the function of the left ventricle in these patients.

In this regard, an association has been reported between an excess of CCL and diastolic dysfunction in patients with HF and with preserved LVEF of different etiologies. On the other hand, a reduction in CCL has been found to be associated with improved systolic function in patients with HF, with reduced LVEF submitted to support with LV assist devices. Therefore, our observation that the increase of HF, with reduced LVEF submitted to support with LV assist associated with improved systolic function in patients with other hand, a reduction in CCL has been found to be inversely correlated with insCol, this correlation being independent of MMP-1 and TIMP-1. Taking into account that collagen cross-linking determines collagen insolubility and that the qualitative alterations of the myocardial collagen matrix have a functional impact in patients with stage C HF of hypertensive origin. This is further reinforced by the finding that CCL is also associated with the amino-terminal propeptide of brain natriuretic peptide, an index of the severity of HF, in these patients.

Cross-linking is a process whereby collagen fibrils are covalently linked to one another resulting in insoluble fibers with increased material stiffness and resistance to degradation by MMPs. There are 2 major groups of collagen cross-links, those derived from advanced glycation end product–mediated glycation of lysine and hydroxylysine residues and those initiated by the enzyme LOX that catalyzes the oxidation of peptidyl lysine side chains of fibrillar collagens, resulting in the formation of corresponding allysine aldehydes. Associations of enhanced myocardial LOX expression with increased CCL and LV stiffness have been reported in animals with genetic or induced hypertension, as well as in hypertensive patients without and with HF. Therefore, our finding that LOX is associated with CCL in patients from this study suggests that the altered regulation of this enzyme may play a role in collagen-dependent disturbances of LV function in stage C HF of hypertensive origin.

LOX is secreted from fibrogenic cells as a 50-kDa proenzyme that appears to have little or no enzymatic activity and is processed in the extracellular environment to produce the 32-kDa catalytically active enzyme and a 18-kDa propeptide. Previous evidence suggests that the processing of the pro-LOX precursor into the LOX active enzyme is mainly accomplished by the metalloproteinase PCP and to a lesser degree by the serine protease furin. Here we report that LOX is associated with furin but not with PCP in the myocardium of patients with HHD and HF, thus suggesting that the regulation of this convertase is altered in HF hypertensive patients with elevated PCWP. Interestingly, it has been reported that microRNA-24 regulates protein and mRNA levels of furin in cardiac fibroblasts. Furthermore, microRNA-24 downregulation has been found to be associated with fibrosis in hypertrophic hearts. Further studies are required to elucidate the potential contribution of furin in collagen alterations of HF patients.

In addition, regarding the potential usefulness of circulating biomarkers of collagen metabolism to evaluate the quality of myocardial collagen, CITP levels were found to be inversely correlated with insCol, this correlation being independent of MMP-1 and TIMP-1. Taking into account that collagen cross-linking determines collagen insolubility and that insCol seems to be resistant to degradation by MMPs, it can be assumed that the more insCol is formed the less collagen fibers will be degraded, thus contributing to fibrosis. On the other hand, the above association would suggest that serum CITP may constitute a biomarker of the process of collagen cross-linking in the myocardium. Nevertheless, we are aware that further studies are necessary to definitively test this hypothesis.

Several limitations need to be acknowledged. First, this was a study involving a relatively small number of patients, but because of the nature of the goals under investigation, it was adequately powered. Second, it must be recognized that therapy may have confounded the findings and their interpretation. Nevertheless, none of the patients were treated with the loop diuretic torsemide that has been shown to reduce myocardial LOX and CCL in stage C HF patients. Third, we performed biopsies of the right side of the interventricular septum to assess the characteristics of collagen tissue. However, as we have shown previously, in terms of deposition of collagen fibers, the septum is representative of the free wall.

**Perspectives**

Data here presented suggest that a shift in treatment strategies for HF of hypertensive origin directed more specifically at affecting the process of collagen cross-linking rather than reducing the content of collagen may be warranted in the attenuation of the adverse functional impact of myocardial...
collagen matrix alterations. In this conceptual framework, although some recent clinical data suggest that pharmacological interference with advanced glycation end product–mediated cross-linking is not associated with beneficial clinical effect in HF patients,15 other findings suggest that inhibition of LOX-mediated cross-linking results in functional and clinical benefits in these patients.18 Therefore, a deeper knowledge of the underlying mechanisms involved in LOX dysregulation in the failing human heart are required to generate both diagnostic and therapeutic strategies targeting the enzyme to identify and interfere with, respectively, its detrimental actions in HF patients.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is New?**
- FPs are associated with the quality (ie, the degree of CCL) but not with the quantity of collagen present in the myocardium of patients with HHD and stage C HF.

**What Is Relevant?**
- The association of LOX with CCL in patients from this study suggest that disregulation of this enzyme may play a role in disturbances of LV function in stage C HF of hypertensive origin, thus pointing to LOX as a potential target for novel therapeutic strategies in HF.

**Summary**

Data here presented point to an excess of CCL as responsible for alterations of LV compliance and function in patients with HF of hypertensive origin. Because of the potential involvement of LOX in this collagen alteration, the possibility to develop either genetic or biochemical and imaging markers of myocardial LOX expression and/or activity could help to explore its contribution to the diagnostic and prognostic handling of patients with HHD and likely other cardiac diseases also evolving with HF. In this setting, circulating CITP emerges as a potential biomarker of the amount of myocardial insoluble collagen. This would represent the first biomarker of the type of myocardial collagen that, because of its physicochemical properties, more negatively impacts on LV function. Finally, a deeper knowledge of LOX structure/function, as well as of the underlying mechanisms involved in myocardial LOX disregulation, may help to generate effective therapeutic strategies targeting the enzyme to prevent the qualitative alterations of the myocardial collagen matrix in HF patients.
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