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Impact of Treatment on Myocardial Lysyl Oxidase Expression and Collagen Cross-Linking in Patients With Heart Failure

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Abstract—The aim of this study was to investigate whether torasemide modifies collagen cross-linking in the failing human heart. We analyzed the degree of cross-linking and the expression of the enzyme lysyl oxidase, which regulates cross-linking, in the myocardium of patients with chronic heart failure at baseline and after 8 months of treatment with either torasemide or furosemide in addition to their standard heart failure therapy. Whereas lysyl oxidase protein expression was very scarce in normal hearts, it was highly expressed in failing hearts. Cross-linking was increased ($P<0.001$) in heart failure patients compared with normal hearts. These 2 parameters decreased ($P=0.021$ and $P=0.034$) in torasemide-treated patients and remained unchanged in furosemide-treated patients. In addition, more ($P=0.009$) patients showed normalization of left ventricular chamber stiffness in the torasemide subgroup than in the furosemide subgroup after treatment. Lysyl oxidase expression correlated with cross-linking ($r=0.661$; $P<0.001$), and cross-linking correlated with left ventricular chamber stiffness ($r=0.452$; $P=0.002$) in all patients. These findings show for the first time that lysyl oxidase overexpression is associated with enhanced collagen cross-linking in the failing human heart. In addition, we report that the ability of torasemide to correct both lysyl oxidase overexpression and enhanced collagen cross-linking results in normalization of left ventricular chamber stiffness in patients with heart failure. Lysyl oxidase may thus represent a target for reduction of stiff collagen and improvement of left ventricular mechanical properties in heart failure patients. (***Hypertension*. 2009;53:236-242.**)

Key Words: clinical science ■ collagen ■ extracellular matrix ■ left ventricular chamber stiffness ■ lysyl oxidase ■ torasemide

Myocardial fibrosis may contribute to the increased risk of chronic heart failure (HF) in patients with cardiac diseases.[1] A linkage between fibrosis and left ventricular (LV) dysfunction/failure may be established through different pathways, including increased passive stiffness that impairs diastolic function.[2] Several studies using experimental models of pressure overload have demonstrated that LV chamber stiffness is affected by changes in both myocardial collagen quantity and quality, with the effect of changes in collagen concentration being modified by the degree of cross-linking.[3–6]

Several steps are involved in the process leading to exaggerated collagen deposition and fibrosis. Collagen is synthesized and secreted by fibroblasts and myofibroblasts as a procollagen precursor having amino-terminal and carboxy-terminal propeptides that are cleaved to yield the triple helical monomers of collagen by specific procollagen proteinases.[7] After these proteolytic reactions, the collagen molecules are rapidly and spontaneously assembled into collagen fibrils. Chemical reactions slowly take place within existing collagen fibrils in tissues, leading to formation of covalent bonds between adjacent polypeptide chains and thus making the final collagen fibers less soluble in any solvent and more resistant against proteolytic enzymes. The first step in this reaction sequence is enzymatic: the copper (Cu)-dependent amine oxidase lysyl oxidase (LOX) catalyzes the oxidation of the ε-amino groups in lysine or hydroxylysine residues, resulting in the formation of corresponding aldehydes. Two such aldehydes can then spontaneously react with each other, or one aldehyde can bind to another ε–amino group; both mechanisms produce cross-links connecting 2 polypeptide chains.[8,9]

Previous studies in rats[10,11] and patients[12,13] with HF have shown that whereas treatment with torasemide was associated with a reduction in the amount of histologically proven myocardial fibrosis (as assessed from the measurement of the fraction of myocardial volume occupied by collagen tissue or CVF), treatment with furosemide did not. Thus, we hypothesized that torasemide, but not furosemide, may also modify the quality of collagen (ie,
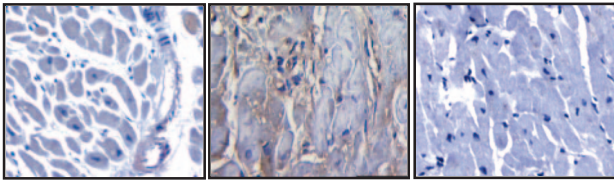
Figure 1. Immunostaining of LOX (in brown) in histological sections of myocardial specimens. The left panel corresponds to 1 control subject and shows very slight staining within some cardiomyocytes and around some small intramural vessels. The middle panel corresponds to 1 patient with chronic HF and shows intense staining located in large areas of interstitial and perivascular fibrosis and within many cardiomyocytes. The right panel shows negative control for the correspondent primary antibody omission. Magnification ×100.

reduction of insoluble collagen and collagen cross-linking) in the myocardium of HF patients. In accordance with this hypothesis, the following end points were defined: (1) to test the effects of torasemide and furosemide, on top of the recommended treatment for HF, on insoluble collagen and collagen cross-linking in patients with HF, (2) to test the effects of torasemide and furosemide on myocardial LOX in these patients, and (3) to test the effects of torasemide and furosemide on LV chamber stiffness in the same patients.

Methods

An expanded version of the methods can be found in an online supplement available at http://www.hypertensionaha.org.

Patients and Study Design

This was a prospective, randomized, parallel group study. Twenty-four white patients with a previous diagnosis of chronic HF were included. After randomization, 12 patients were assigned to 10 to 20 mg torasemide daily (torasemide subgroup) and 12 patients to 20 to 40 mg furosemide daily (furosemide subgroup) for 8 months. Ten patients from each subgroup completed the study. Studies were performed on each patient at enrollment

(baseline) and 8 months after randomization. Septal endomyocardial biopsies were obtained from autopsies of healthy subjects to assess control reference values.

Echocardiographic Assessment

Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained from each patient.

Biochemical Determination

Amino-terminal probrain natriuretic peptide (NT-proBNP) was measured in serum samples by an enzyme-linked immunosorbent assay (Roche Diagnostics).

Histomorphologic and Immunohistochemical Studies

Three transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum. The CVF was determined by quantitative morphometry in sections stained with collagen-specific picro-sirius red, as reported previously.[14]

To distinguish between cross-linked (insoluble) and noncross-linked (soluble) collagen, a colorimetric procedure was used.[15,16] The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. Immunohistochemical analysis for LOX was performed using a mouse monoclonal antibody against LOX (R&D Systems).

Western Blot Studies

Western blot studies were performed as described recently[17] using a specific rabbit polyclonal antibody against LOX (R&D Systems).

Statistical Analysis

Differences between different groups were tested using a Student t test for unpaired data or Mann–Whitney U test. Differences in parameters before and after treatment within each subgroup of patients were tested by a Student t test for paired data or Wilcoxon signed-rank test. Categorical variables were analyzed by the χ^2 Fisher exact test. The correlation between continuously distributed variables was tested by univariate regression analysis and bivariate association. Data are expressed as means±SD and number of patients. A P value <0.05 was considered statistically significant. Analyses were performed with the SPSS 15.0 statistical package.
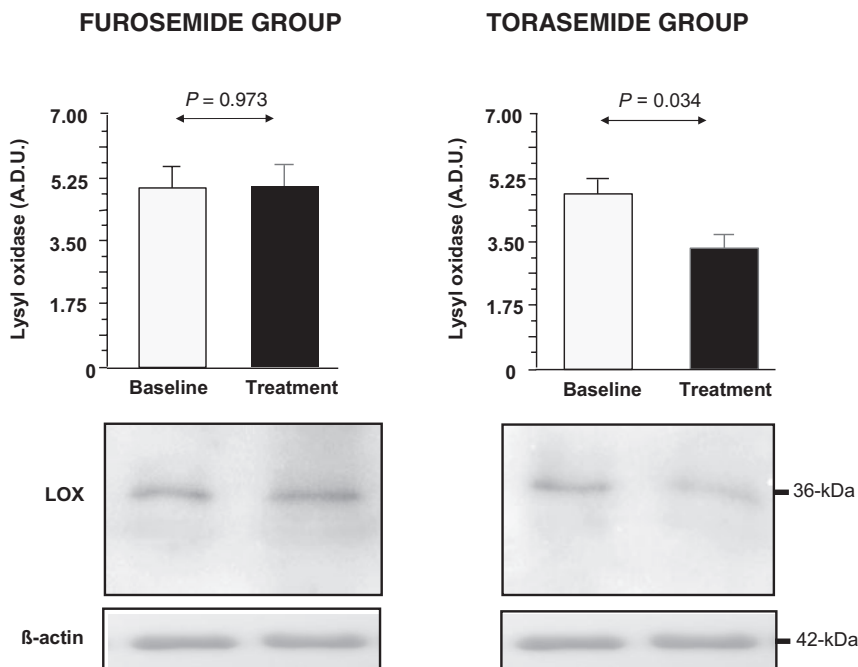

Figure 2. The top panels show histograms of myocardial LOX from patients with chronic HF at baseline and 8 months after randomization to furosemide (left) or to torasemide (right). The bottom panels show representative Western blot autoradiograms of myocardial LOX from 2 patients with chronic HF at baseline and 8 months after randomization to furosemide (left) or to torasemide (right).

Table 1. Types of Collagen and Collagen Cross-Linking in Control Subjects and the Whole Group of Patients With Chronic HF

Parameters	Control Group	HF Group	P
Insoluble collagen, μg/mg	0.95±0.28	8.98±0.98	<0.001
Soluble collagen, μg/mg	0.66±0.17	2.76±0.63	<0.001
Collagen cross-linking	1.43±0.29	3.47±1.03	<0.001

Data are expressed as the mean value±SD. *P* for comparison between the 2 groups.

Results

Expression of Myocardial LOX in Controls and the Whole Group of HF Patients

Figure 1 shows that whereas LOX was almost absent in the myocardium of a control subject, it was highly expressed in fibroblasts, areas of interstitial and perivascular fibrosis, and cardiomyocytes in the myocardium of an HF patient. As shown in Figure 2, one 32-kDa band corresponding to the active form of LOX was identified in myocardial samples from all HF patients. In contrast, the active form of LOX was undetectable in myocardial samples from control subjects.

Types of Collagen and Collagen Cross-Linking in Controls and the Whole Group of HF Patients

As shown in Table 1, the amounts of both insoluble and soluble collagens were significantly higher in HF patients than in control subjects. Whereas insoluble collagen corresponded to 58.7±2.43% of total collagen in control hearts, it represented 76.4±5.88% in failing hearts, a significant difference (*P*<0.001). The degree of collagen cross-linking was

Table 2. Effects of Treatment on Clinical and Biochemical Parameters Assessed in the 2 Subgroups of Patients With Chronic HF

Parameters	Furosemide Subgroup			Torasemide Subgroup			Final P
	Baseline	After TX	P	Baseline	After TX	P	
Age, years	67±10			66±9			
Gender, men/women	8/2			8/2			
Body weight, kg	79.5±12.4	82.4±13.3	0.432	80.7±9.1	82.0±9.2	0.507	0.461
Causes of HF, n							
HHD	9			8			
IHD	1			2			
Types of HF, n							
EF ≥0.40	3			5			
EF <0.40	7			5			
Medications, n							
ACEIs or ARAs	10	10		10	10		
β-Blockers	10	10		10	10		
Digoxin	2	2		3	3		
Diuretics			0.642			0.717	0.002
Furosemide	10	10		0	0		
Torasemide	0	0		10	10		
Hct+amiloride	1	1		0	0		
SBP, mm Hg	137±16	121±13	0.001	144±10	128±7	0.003	0.098
DBP, mm Hg	85±11	73±11	0.004	92±9	75±5	0.001	0.900
LVMI, g/m^2	182±67	173±49	0.289	156±46	154±42	0.750	0.237
RWT	0.36±0.04	0.33±0.03	0.050	0.34±0.09	0.35±0.09	0.200	0.492
LVEDV, mL	173.8±43	168.2±28.0	0.741	166.8±73	162.7±77	0.518	0.244
V_E/V_A	1.23±0.38	1.12±0.47	0.951	1.34±0.53	1.19±0.58	0.208	0.846
IVRT, ms	111.6±25.0	109.5±29	0.518	106.7±15	112.8±23	0.182	0.794
DT, ms	201.6±68.0	198.6±73.0	0.643	199.4±42	219.2±61	0.498	0.531
K_{LV}, mm Hg/mL	0.159±0.091	0.165±0.091	0.570	0.149±0.069	0.127±0.053	0.502	0.287
EF	0.38±0.16	0.38±0.15	0.794	0.39±0.06	0.43±0.08	0.034	0.434
NYHA functional class	2.9±0.56	2.5±0.71	0.168	2.7±0.65	2.2±0.42	0.047	0.264
NT-proBNP, pg/mL	1239±1136	813±503	0.352	1123±706	540±212	0.026	0.043

TX indicates treatment; HHD, hypertensive heart disease; IHD, ischemic heart disease; ACEIs, angiotensin-converting enzyme inhibitors; ARAs, angiotensin type I receptor antagonists; Hct, hydrochlorothiazide; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVMI, LV mass index; RWT, relative wall thickness; LVEDV, LV end-diastolic volume; V_E, maximum early transmitral velocity in diastole; V_A, maximum late transmitral velocity in diastole; IVRT, isovolumic relaxation time; DT, deceleration time; NYHA, New York Heart Association.

Data are expressed as the mean value±SD or number of patients. *P* for comparison of baseline values and values after treatment within each group. Final *P* for comparison of values after treatment between the 2 groups of patients.

Table 3. Effects of Treatment on the Types of Collagen and Collagen Cross-Linking in the 2 Subgroups of Patients With Chronic HF

Parameters	Furosemide Subgroup			Torasemide Subgroup			Final P
	Baseline	After TX	P	Baseline	After TX	P	
Insoluble collagen, μg/mg	9.10±1.26	9.69±0.99	0.012	8.88±0.63	7.50±2.26	0.045	0.028
Soluble collagen, μg/mg	2.71±0.75	2.55±0.67	0.236	2.81±0.51	3.49±1.01	0.046	0.026
Collagen cross-linking	3.66±1.29	4.05±1.14	0.188	3.27±0.71	2.32±1.00	0.021	0.002

TX indicates treatment.

Data are expressed as the mean value±SD. *P* for comparison of baseline values and values after treatment within each group. Final *P* for comparison of values after treatment between the 2 groups of patients.

significantly increased in the myocardium of HF patients compared with control subjects (Table 1).

Effects of Treatment in the 2 Subgroups of HF Patients

Clinical, Echocardiographic, and Biochemical Data
Clinical and echocardiographic characteristics of the 2 subgroups of patients at baseline are presented in Table 2. No significant differences were observed between the subgroups in the etiologies of HF. A depressed ejection fraction (EF; below the normal cutoff value of 0.40) was observed in 70% and 50% of patients from the furosemide subgroup and the torasemide subgroup, respectively. Doppler criteria of diastolic dysfunction were present in 50% and 70% of patients from the furosemide subgroup and the torasemide subgroup, respectively. An abnormally high LV chamber stiffness constant (K_{LV}) value was observed in 60% and 50% of patients from the furosemide subgroup and the torasemide subgroup, respectively. None of these differences reached statistical significance.

Eight months after randomization, patients in the torasemide subgroup and the furosemide subgroup received mean daily dosages of 11.1±0.8 mg and 33.1±3.0 mg of these agents, respectively. Baseline medications other than loop diuretics were maintained unchanged during the treatment period in the 2 subgroups of patients. No adverse effects occurred during the study in either subgroup. The frequency of complications (including hospitalizations and exacerbations of HF) was similar in the 2 subgroups.

Although nonsignificant differences in the values of EF were found after treatment in the 2 subgroups of treatment, the frequency of patients showing normalization of this parameter after treatment was higher (P=0.025) in the

torasemide subgroup than in the furosemide subgroup (80% versus 40%). In addition, although the final values of K_{LV} were similar in the 2 subgroups of patients, the frequency of patients showing normalization of K_{LV} after treatment was greater (P=0.009) in the torasemide subgroup than in the furosemide subgroup (80% versus 0%). As shown in Table 2, the levels of NT-proBNP decreased in the torasemide subgroup but remained unchanged in the furosemide subgroup. After treatment, NT-proBNP levels were lower in torasemide-treated patients than in furosemide-treated patients. Finally, the New York Heart Association functional class decreased in the torasemide subgroup and remained unchanged in the furosemide subgroup (Table 2).

Expression of Myocardial LOX
No significant differences in the expression of LOX protein were found at baseline between the 2 subgroups of patients (Figure 2). Whereas the expression of LOX protein remained unchanged in furosemide-treated patients (5.06±2.57 arbitrary densitometric units [A.D.U.] versus 5.08±2.61 A.D.U.), it decreased (P=0.034) in torasemide-treated patients (4.86±1.78 versus 3.34±1.85 A.D.U.; Figure 2).

Total Collagen, Types of Collagen, and Collagen Cross-Linking
No differences in the baseline values of CVF were observed between the 2 subgroups. Whereas CVF decreased in the torasemide subgroup after treatment (8.29±1.65% versus 4.24±0.74%; P<0.001), it remained unchanged in the furosemide subgroup (7.93±3.05% versus 7.04±3.48%; P=0.549). Final values of CVF were lower (P<0.001) in the torasemide subgroup than in the furosemide subgroup.

The amounts of insoluble and soluble collagen and the degree of cross-linking of the 2 subgroups of patients are
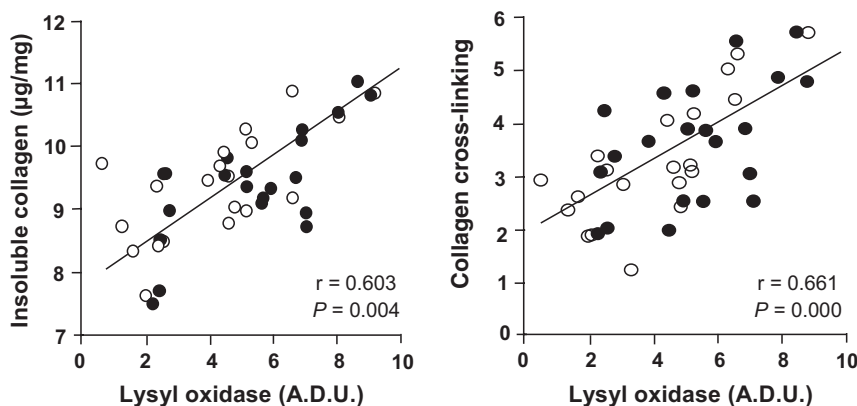

Figure 3. The left panel shows the positive correlation (y=0.246×+8.16) between LOX and insoluble collagen in all patients at baseline (●) and after treatment (○). The right panel shows the positive correlation (y=0.345×+1.911) between LOX and collagen cross-linking in all patients at baseline (black circles) and after treatment (white circles).

Figure 4. The left panel shows the positive correlation ($y=0.032\times+0.050$) between insoluble collagen and K_{LV} in all patients at baseline (●) and after treatment (○). The right panel shows the positive correlation ($y=0.030\times+0.051$) between collagen cross-linking and K_{LV} in all patients at baseline (●) and after treatment (○).
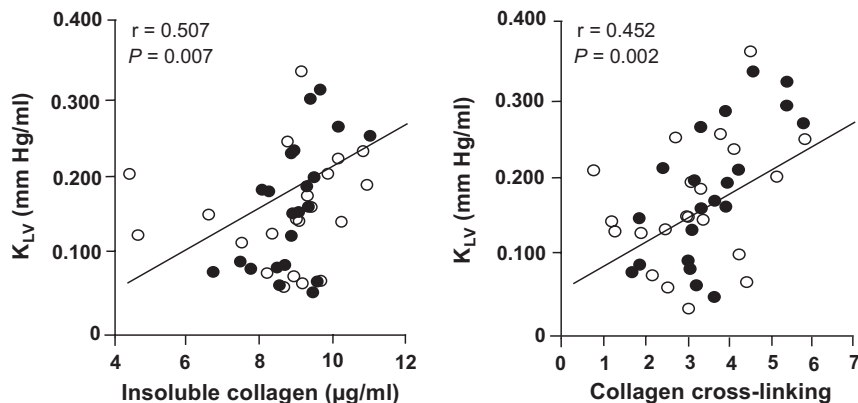
presented in Table 3. No significant differences between the 2 subgroups of patients were found in these parameters at baseline. Whereas insoluble collagen increased in furosemide-treated patients ($P=0.012$), it decreased in torasemide-treated patients ($P=0.045$). In addition, soluble collagen increased ($P=0.046$) in torasemide-treated patients but remained unchanged in furosemide-treated patients. Thus, whereas the degree of collagen cross-linking did not change in furosemide-treated patients, it decreased ($P=0.021$) in torasemide-treated patients.

Association Studies

A positive correlation was found between the expression of LOX protein and the amount of insoluble collagen ($r=0.603$; $P=0.004$) and the degree of cross-linking ($r=0.661$; $P<0.000$) in all patients at baseline and after treatment (Figure 3). These correlations remained significant when we excluded the influence of a number of potential confounding factors (ie, age, body weight, systolic blood pressure, diastolic blood pressure, LV mass index, LV diastolic volume, K_{LV}, and EF) in partial correlation analysis.

The amount of insoluble collagen was positively correlated with K_{LV} ($r=0.507$; $P=0.007$) in all patients at baseline and after treatment (Figure 4). Similarly, the degree of cross-linking was positively correlated with K_{LV} ($r=0.452$; $P=0.002$) in all patients at baseline and after treatment (Figure 4). The 2 correlations remained significant when we excluded the influence of a number of potential confounding factors (ie, age, body weight, systolic blood pressure, diastolic blood pressure, LV mass index, LV diastolic volume, and EF) in partial correlation analysis.

Finally, negative correlations were found between LOX and the deceleration time ($r=-0.579$; $P=0.001$) and between cross-linking and the deceleration time ($r=-0.336$; $P=0.037$) in all patients at baseline and after treatment. These correlations also remained significant after excluding the influence of the above potential confounding factors in partial correlation analysis.

Discussion

The main findings of this study are as follows: (1) the expression of LOX and the degree of collagen cross-linking are abnormally increased in the myocardium of chronic HF patients, (2) both LOX expression and collagen cross-linking

decrease in torasemide-treated chronic HF patients but remain unchanged in furosemide-treated chronic HF patients, and (3) direct correlations exist between LOX and collagen cross-linking and between collagen cross-linking and LV stiffness in chronic HF patients. Collectively, these findings show for the first time that LOX plays a critical role in collagen alterations in the failing human heart.

The Cu-dependent enzyme LOX critically controls the process whereby collagen molecules are assembled and covalently cross-linked to one another, resulting in fibers with increased material stiffness and greater resistance to degradation. Whereas LOX mRNA upregulation has been reported previously in explanted hearts of patients with idiopathic dilated cardiomyopathy,[18] low levels of collagen cross-linking have been reported in the hearts of patients with the same cardiac disease.[19] However, none of these studies explored whether the changes in LOX were simultaneously associated with variations in the formation of insoluble collagen (cross-linked). Here we show that an increase of LOX was associated with an increase of insoluble collagen in the human failing heart, suggesting that upregulation of the enzyme is responsible for excessive collagen cross-linking present in patients from this study. In support of this possibility is our finding that in torasemide-treated patients, the decrease in LOX was associated with the reduction in both collagen cross-linking and the amount of insoluble collagen.

Dyshomeostasis of micronutrients such as Cu and zinc (Zn) is part of the systemic illness that accompanies HF and is simultaneously operative in promoting myocardial remodeling.[20] In particular, the role of Cu in regulating the activity of LOX and Cu/Zn-superoxide dismutase as well as the role of Zn essential to the activity of angiotensin-converting enzyme and matrix metalloproteinases is thought to be of relevance for the regulation of collagen matrix. In this conceptual framework, it is tempting to speculate that because of the different pharmacokinetic properties and pharmacodynamic actions of torasemide and furosemide,[21] these 2 compounds may differentially influence Cu and Zn homeostasis, and this, in turn, may have a different impact on the activity of LOX and other metalloenzymes in the heart.

Of interest, we found that in addition to its localization in the interstitial space and fibroblasts, LOX was also expressed in cardiomyocytes in patients with chronic HF. We have shown recently that cardiomyocytes from chronic HF patients

express procollagen type I carboxy-terminal proteinase (PCP) that regulates the synthesis of mature collagen type I,[13] as well as matrix metalloproteinase-1 that regulates the degradation of collagen fibers.[17] Collectively, these observations suggest that in addition to fibroblasts, cardiomyocytes play a major role in regulating collagen turnover in the failing human heart.

LOX can be regulated at 3 levels: synthesis of LOX precursor by fibroblasts and other fibrogenic cells,[8] extracellular conversion of the precursor into the active enzyme because of the action of PCP,[22,23] and direct stimulation of the activity of the enzyme by cytokines such as transforming growth factor-β.[24] We reported recently that PCP activation was abnormally increased in the myocardium of chronic HF patients and that torasemide, but not furosemide, decreased myocardial PCP activation in these patients.[13] In addition, it has been reported that the expression of transforming growth factor-β is reduced in the myocardium of torasemide-treated rats compared with control rats.[10,11] It is thus tempting to speculate that the reduction in LOX observed after treatment in patients receiving torasemide can be related to the ability of this compound to deactivate PCP or downregulate transforming growth factor-β.

Quantitative assessment of fibrosis in various murine transgenic models, larger animal failure models, and even in humans has not always found correlations between stiffness and collagen content. In other studies, it has been demonstrated that the relative ratio of insoluble to soluble collagen (ie, the degree of cross-linking) is more important. For example, in rats exposed to aortic binding, hypertrophy was accompanied by increased total collagen, yet decreases in insoluble/soluble collagen ratio, and no change in myocardial stiffness.[6] In contrast, spontaneously hypertensive rats had elevated total collagen and higher insoluble/soluble ratio, which correlated with chamber stiffening.[6] Our findings that LV stiffness is correlated with the amount of insoluble collagen and the degree of cross-linking are consistent with the notion that in the failing human heart, the quality of collagen (specifically cross-linking) plays a key role in translating quantity into mechanical stiffness and functional performance of the left ventricle.

This hypothesis receives some support from changes in clinical and biochemical parameters observed in treated patients. In fact, a greater number of patients showed normalization in K_{LV} and EF in the torasemide subgroup than in the furosemide subgroup. In addition, levels of NT-proBNP decreased in torasemide-treated patients but not in furosemide-treated patients. In this regard, it is interesting to remark that myocardial stiffness has been shown to be the most important determinant of the plasma BNP production in patients with HF.[25] Therefore, the ability of torasemide to reduce LOX and collagen cross-linking may contribute to its beneficial clinical impact on HF patients.

Several limitations of the current study must be recognized. First, this was an individually randomized, parallel-group study involving a small number of patients. In addition, it must be recognized that therapy with β-blockers, angiotensin-converting enzyme inhibitors, or angiotensin-receptor antagonists may have influenced the findings. Second, although we did not assess LOX precursor (ie, zymogen) or LOX activity (ie, zymography), the associations found between LOX expression and insoluble collagen and collagen cross-linking suggest that the activity of the enzyme may parallel its expression in the failing human heart. Third, K_{LV} is difficult to measure in clinical practice, even with invasive techniques. However, studies in animals[26] and humans[27] with different hemodynamic conditions have demonstrated that the deceleration time provides an accurate estimate of LV operating stiffness. Finally, it is important to consider the possibility that the different pharmacology of furosemide and torasemide, even in HF patients,[21] may also contribute to the differential effects of the 2 compounds on the cardiac parameters tested in this study.

Perspectives
Findings from this study suggest a role for LOX upregulation in the excess of collagen cross-linking present in patients with chronic HF. In addition, our data suggest that the ability of torasemide to correct both LOX overexpression and enhanced collagen cross-linking may be involved in amelioration of K_{LV} in HF patients treated with this compound. These results support the notion that therapeutic strategies directed not just at reducing the amount of collagen but also at reducing collagen cross-linking should be implemented in the prevention of the adverse impact of myocardial fibrosis on cardiac function in chronic HF patients.

Disclosures
None.

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Online Supplement

Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure

1

Supplemental Methods

Patients and study design

All subjects gave written, informed consent before participating in the study. The investigation conformed to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Institutional Ethics Committee of the Donostia University Hospital.

Between January 2007 and January 2008, 34 consecutive Caucasian patients were screened for this study (Figure S1). All patients were required to have a previous diagnosis of chronic HF by the presence of a least 1 major and 2 minor criteria of the Framingham study during the last 6 months. Six patients with severe clinical conditions other than HF were excluded. Within the remaining 28 patients, 2 patients did not meet the inclusion criteria and 2 patient refused participation in the study. Whereas 85% of the 24 patients finally enrolled in the study had hypertensive heart disease, the remaining 15% showed ischemic heart disease. None of the patients had suffered from previous myocardial infarction.

The study was a prospective, randomized, parallel group, study. Randomization was carried out by our division's pharmacy that computer-generated the randomization sequence dispensed the study medication. All patients were randomly assigned to receive either torasemide or furosemide. After randomization, 12 patients were assigned to torasemide 10 to 20 mg daily (torasemide subgroup) and 12 patients to furosemide 20 to 40 mg daily (furosemide subgroup) for 8 months. Existing recommended salt intake restriction (4 g/day) and concomitant HF medications (i.e., an angiotensin-converting enzyme inhibitor or an angiotensin-receptor antagonist, and a beta-adrenergic blocker) were continued during the study. None of the patients were treated with aldosterone antagonists. During the follow-up 1 patient in the furosemide subgroup died of progressive HF, and 1 patient in the furosemide subgroup and 2 patients in the torasemide subgroup discontinued the medication.

A number of studies, including echocardigraphic evaluation, biochemical determinations and endomyocardial biopsy, were performed in each patient at enrolment (baseline) and 8 months after randomization. The investigators responsible for these studies were not aware of treatment of each patient.

In the population sample of a previous study[1] we measured the effects of either torasemide or furosemide on myocardial fibrosis CVF in HF patients. With the obtained CVF values and considering an α error of 0.05 and a β error of 0.20, we calculated that the minimal sample necessary to observe statistical differences between torasemide-treated patients and furosemide-treated patients was of 9 patients in each group.

A total of 100 autopsies performed at the University Clinic of Navarra were screened between January 2004 and January 2007. Ten hearts (6 men and 4 women, mean age 59 years, range 40 to 68 years) were selected from these cases according to the following criteria: 1) sudden death associated with traumatic injury in the absence of cardiac complications, 2) no medical history or physical and laboratory findings of cardiovascular disease, 3) absence of clinically recognized systemic disorders, 4) absence of atherosclerosis of the major coronary arteries, and 5) normal cardiac weight.

Septal endomyocardial biopsy specimens were taken from these hearts to assess control reference values of myocardial parameters evaluated in this study.

Echocardiographic assessment

Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained in each patient. Left ventricular mass was measured, and left ventricular mass index was calculated by dividing left ventricular mass by body surface area. Ejection fraction (EF) was calculated according to Quinones et al.[2] The following pulsed Doppler measurements were obtained: maximum early transmitral velocity in diastole, maximum late transmitral velocity in diastole, the deceleration time of the early mitral filling wave, and isovolumic relaxation time.

Left ventricular chamber stiffness (K_{LV}) was calculated as the ratio squared according to the following equation:[3] $K_{LV} = (0.07 : deceleration\ time)^2$. The upper normal limit for K_{LV} previously defined in our laboratory for a normotensive population was of 0.151 mm Hg/ml.

Biochemical determination

Amino-terminal pro-brain natriuretic peptide (NT-proBNP) was measured in serum samples by an enzyme-linked immunosorbent assay using a commercial kit (Roche Diagnostics). All determinations were performed by duplicate. The inter- and intra-assay coefficients of variation were 8 and 5%, respectively. The values of NT-proBNP obtained in our laboratory in a control population of 30 apparently healthy donors were 163±8 pg/ml.

Histomorphologic and immunohistochemical studies

Three transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum with a bioptome Cordis 96 cm (7-F) under fluoroscopic guidance after angiographic examination in each patient. Whereas two of the samples were employed for histomorphologic and immunohistochemical studies, the third of the samples was used for Western blot studies.

The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picro-sirius red, as previously reported.[4] All measurements were performed in duplicate by 2 independent observers. The inter- and intra-observer coefficients of variation were <4%.

To distinguish between cross-linked (insoluble) and non-cross-linked (soluble) collagen a colorimetric procedure was employed. First, a fast green-sirius red assay was performed to identify and quantify total collagen.[5] In a second step, a sircol-based assay was performed to obtain and quantify soluble collagen.[6] The amount of insoluble collagen was calculated by subtracting the amount of soluble collagen to the amount of total collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. All measurements were performed in

3

duplicate. The inter- and intra-assay coefficients of variation were 5 and 3%, respectively.

Immunohistochemical analysis for LOX 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). A mouse monoclonal antibody against LOX (R&D Systems) was used as the primary antibody.

Western blot studies

A 5-μg sample of total protein obtained from transvenous endomyocardial biopsies was processed for Western blot as recently described.[7] A specific rabbit polyclonal antibody against LOX (R&D Systems) was used. Bands were detected by peroxidase-conjugated secondary antibodies (Amersham Biosciences) and visualized with the ECL-Plus chemiluminescence system (Amersham Biosciences). Autoradiograms were analyzed using an automatic densitometer (Molecular Imager FX, Bio-Rad). The blots were also probed with a monoclonal ß-actin antibody (Sigma) as a control for loading. Data are expressed as arbitrary densitometric units (A.D.U.) relative to beta-actin expression. All experiments were performed in duplicate. The inter- and intra-assay coefficients of variation were <5%.

Statistical analysis

Differences in cardiac parameters between the control group and the whole group of HF patients, and between the 2 subgroups of HF patients at baseline and after treatment were tested using a Student t test for unpaired data once normality was shown (Shapiro-Wilks test); otherwise, a nonparametric test (Mann-Whitney U test) was used. Differences in parameters before and after treatment within each group of patients were tested by a Student t test for paired data once normality was shown (Shapiro-Wilks test); otherwise, a nonparametric test (Wilcoxon signed-rank test) was used. The Bonferroni correction was applied to the separate univariate tests for protection against Type I error. Categorical variables were analyzed by the chi-square (χ^2) Fisher exact test when necessary. The correlation between continuously distributed variables was tested by univariate regression analysis and bivariate association (Spearman coefficient). Partial correlation coefficients were calculated to assess the independent relationship between the variable of interest (LOX and degree of collagen cross-linking) and K_{LV} after adjustment for relevant covariates previously found to be associated with K_{LV} in univariate regression models: age, body weight, systolic blood pressure, diastolic blood pressure, left ventricular mass index, left ventricular diastolic volume, and EF. Data are expressed as means ± SD, and number of patients. All statistical tests were two-sided with a significance level of 0.05. All statistical tests were performed with the SPSS 15.0 statistical package.

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Supplemental Figures


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                    ┌─────────────────┐
                    │ Assessed for    │
                    │ eligibility     │
                    │ (n=34)          │
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Enrollment

Assessed for eligibility (n=34)

Excluded (n=6)
Not meeting inclusion criteria (n=2)
Refused to participate (n=2)
Other reasons (n=0)

Randomised (n=24)

Allocation

Allocated to furosemide (n=12)
Received allocated furosemide (n=12)
Did not receive allocated furosemide (n=0)

Allocated to torasemide (n=12)
Received allocated toroasemide (n=12)
Did not receive allocated torasemide (n=0)

Follow-up

Lost to follow-up by death (n=1)
Discontinued intervention (n=1)

Lost to follow-up by death (n=0)
Discontinued intervention (n=2)

Analysis
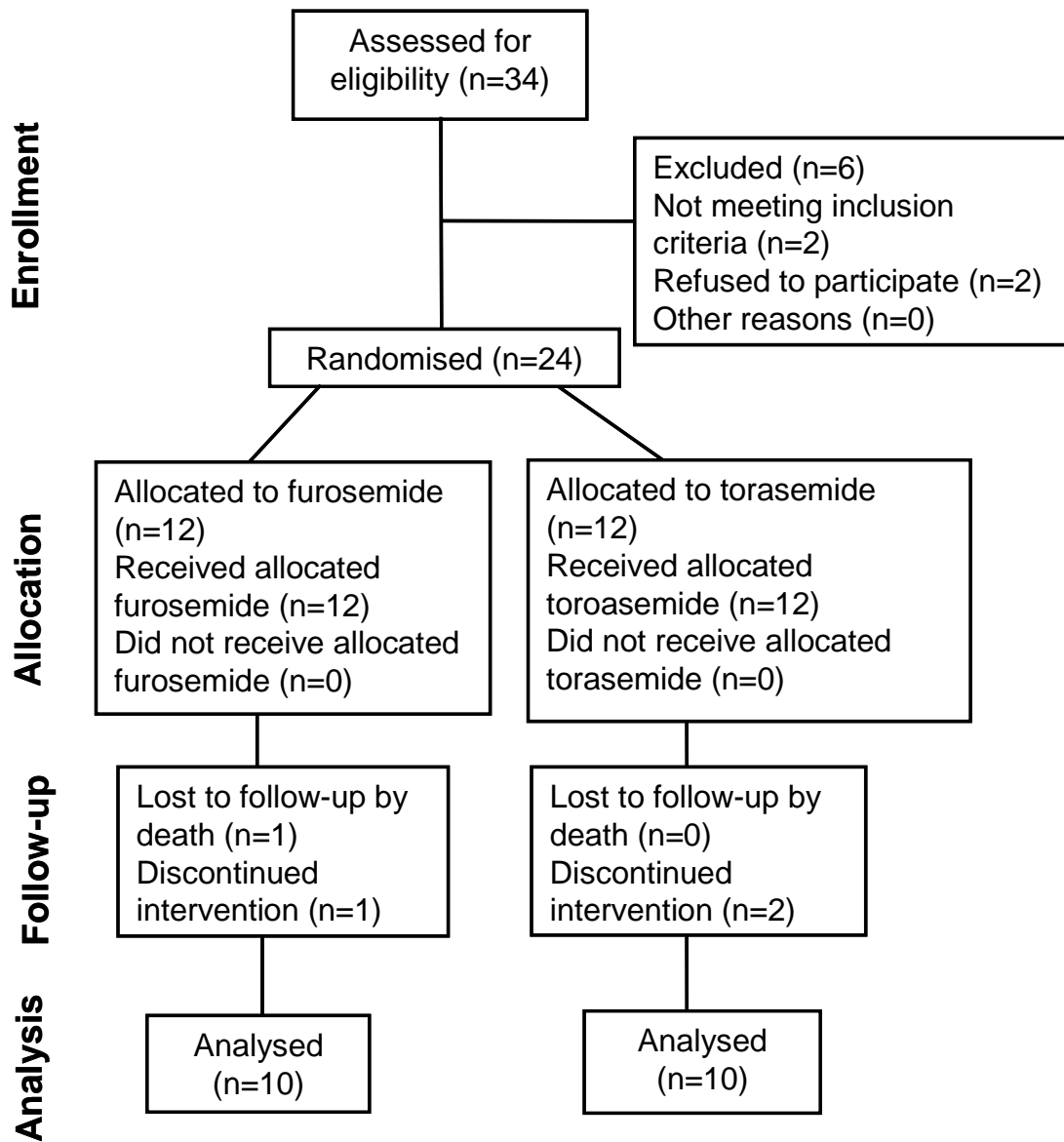
Analysed (n=10)

Analysed (n=10)

Figure S1. Diagram showing the flow of participants through each stage of the study.