Osteopontin-mediated myocardial fibrosis in heart failure: a role for lysyl oxidase?

Begoña López[1], Arantxa González[1], Diana Lindner[2,3], Dirk Westermann[2,3], Susana Ravassa[1], Javier Beaumont[1], Idoia Gallego[1], Amaia Zudaire[1], Cristina Brugnolaro[1], Ramón Querejeta[4], Mariano Larman[4], Carsten Tschöpe[2], and Javier Díez[1,5]*

[1]Division of Cardiovascular Sciences, Centre for Applied Medical Research, University of Navarra, Pamplona, Spain; [2]Department of Cardiology and Pneumology, Campus Benjamin Franklin, Charité-Universitaetsmedizin Berlin, Berlin, Germany; [3]Department of General and Interventional Cardiology, University Heart Centre Hamburg, Hamburg, Germany; [4]Division of Cardiology, Donostia University Hospital, San Sebastián, Spain; and [5]Department of Cardiology and Cardiac Surgery, University of Navarra Clinic, University of Navarra, Pamplona, Spain

Aims	We investigated whether the pro-fibrotic matricellular protein osteopontin (OPN) is associated with the enzymes involved in the extracellular synthesis of fibril-forming collagen type I (i.e. procollagen C-proteinase, PCP) and its cross-linking to form insoluble fibrils (i.e. lysyl oxidase, LOX) in heart failure (HF) of hypertensive origin.
Methods and results	OPN, PCP, and LOX were assessed by histochemical and molecular methods in the myocardium of 21 patients with hypertensive heart disease (HHD) and HF. Whereas the myocardial expression of OPN was very scarce in control hearts ($n = 10$), it was highly expressed in HF patients ($P < 0.0001$). OPN was directly correlated with LOX ($r = 0.460$, $P = 0.041$), insoluble collagen ($r = 0.534$, $P = 0.015$), pulmonary capillary wedge pressure ($r = 0.558$; $P = 0.009$), and left-ventricular (LV) chamber stiffness ($r = 0.458$, $P = 0.037$), and inversely correlated with LV ejection fraction ($r = -0.513$, $P = 0.017$) in all patients. OPN did not correlate with PCP and other parameters assessing collagen synthesis by fibroblasts or degradation by matrix metalloproteinases. *In vitro* studies showed that OPN significantly ($P < 0.05$) increases the expression and activity of LOX in human cardiac and dermal fibroblasts.
Conclusion	An excess of OPN is associated with increased LOX and insoluble collagen, as well as with LV stiffness and systolic dysfunction in patients with HHD and HF. In addition, OPN up-regulates LOX in human fibroblasts. It is suggested that the OPN−LOX axis might facilitate the formation of insoluble collagen (i.e. stiff and resistant to degradation) and the subsequent alteration in LV mechanical properties and function in patients with HHD and HF.
Keywords	Fibrosis • Heart failure • Lysyl oxidase • Osteopontin

1. Introduction

Myocardial fibrosis may contribute to the development and progression of chronic heart failure (HF) in patients with hypertensive heart disease (HHD).[1] A linkage between fibrosis and left-ventricular (LV) dysfunction/failure may be established through different pathways, including increased LV chamber passive stiffness that primarily impairs diastolic function, but when severe, also compromises systolic performance.[2] Studies using experimental models of HF,[3–5] as well as clinical studies performed in patients with HF[6,7] have demonstrated that the effects of myocardial fibrosis on LV mechanics and function depend critically on both the amount of mature fibril-forming collagen type I and its insolubility, that determines its stiffness and resistance to degradation by matrix metalloproteinases (MMPs). Of interest, increased expression of the enzyme responsible for the synthesis of mature collagen type I (i.e. procollagen C-proteinase or PCP) and the enzyme responsible for the formation of cross-linked insoluble collagen (i.e. lysyl oxidase or LOX) have been reported in the myocardium of hypertensive patients with HF.[6,8,9]

The available evidence in experimental models of pressure overload[10–12] and in patients with cardiac diseases other than HHD[12–15] supports that an excess of the matricellular protein osteopontin (OPN) is involved in myocardial remodelling. Although OPN has been shown to promote fibroblast differentiation to myofibroblasts[16] and to inhibit MMPs involved in collagen degradation,[17] no data are available on its ability to influence the synthesis of collagen type I molecules and

the process of cross-linking to form collagen type I fibres. Therefore, this study was mainly designed to explore whether OPN is associated with PCP and LOX in myocardial samples from patients with HHD having HF. In addition, the direct effect of OPN on the expression and activity of LOX was assessed in cultured human fibroblasts. Finally, we also evaluated whether circulating OPN can be a biomarker of myocardial OPN and fibrosis in patients with HHD and HF.

2. Methods

2.1 Subjects

All 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 hypertensive population consisted of 21 patients with systolic blood pressure (SBP) and diastolic blood pressure (DBP) of >139 and/or 89 mmHg, respectively, or with SBP and DBP values below 140 and/or 90 mmHg under antihypertensive treatment. All patients underwent appropriate clinical and laboratory evaluation to exclude secondary hypertension. All patients exhibited HHD as indicated by the presence of LV hypertrophy (LVH) in the echocardiogram. Other cardiac diseases associated with LVH, as well as coronary artery disease were excluded after complete medical examination, which included a diagnostic cardiac catheterization. All patients had a previous clinical diagnosis of chronic HF based on the presence of at least one major and two minor Framingham criteria.[18] *Table 1* shows the clinical characteristics of these patients.

Three transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum from each patient during the cardiac catheterization procedure. Two of the biopsy samples were used for molecular studies and the remaining sample for the histomorphological and immunohistochemical procedures.

Septal endomyocardial biopsies were obtained from autopsies of 10 age- and gender-matched healthy subjects (mean age, 59.40 ± 3.27 years; seven male/three female) to assess control reference values of the histomorphological and molecular parameters. An additional group of 29 age- and gender-matched healthy subjects (mean age, 58.95 ± 1.71 years; 22 male/7 female; body mass index, 25.32 ± 0.63 kg/m^2; SBP, 116 ± 3 mmHg; DBP, 76 ± 2 mmHg) were used as controls for biochemical studies.

2.2 Echocardiographic study

Two-dimensional echocardiographic imaging, targeted M-mode recordings, and Doppler ultrasound measurements were obtained in each patient as previously described.[19] The presence of LVH was established when LV mass index (LVMI) was >111 g/m^2 for men and >106 g/m^2 in women.[20] The LV end diastolic volume (LVEDV) was calculated using the Teichholz formula.[21] LV ejection fraction (LVEF) was calculated according to Quinones *et al.*[22]

The following pulsed-Doppler measurements were obtained: maximal early transmitral velocity in diastole (V_E), maximal late transmitral velocity in diastole (V_A), isovolumic relaxation time (IVRT), and deceleration time (DT). DT was calculated as the time from the peak of the E-wave to the zero-velocity intercept of the regression line of the E-wave velocity deceleration profile. LV chamber stiffness (K_{LV}) was calculated as the ratio squared according to the following equation: $K_{LV} = (0.07/DT)^2$ mmHg/mL.[23]

2.3 Invasive cardiac studies

After significant coronary artery disease (≥50% stenosis in major epicardial coronary arteries) was discarded, pulmonary capillary wedge pressure (PCWP) was measured during the cardiac catheterization procedure.

Table 1 Clinical, echocardiographic, and biochemical characteristics in hypertensive patients with heart failure

Parameters	Mean ± SD	95% CI
Age, years	64.4 ± 12.07	58.89–69.87
Male/female, *n*	16/5	
Body mass index, kg/m^2	29.2 ± 3.07	27.75–30.54
NYHA functional class, *n*/%		
I	0/0	
II	5/24	
III	13/62	
IV	3/14	
Blood pressure, mmHg		
Systolic	152.14 ± 16.78	144.51–159.78
Diastolic	90.95 ± 9.57	86.59–95.31
Heart rate, beats/min	73.09 ± 17.57	65.09–81.09
Medications, %		
Loop diuretics	100	
Beta-blockers	100	
ACE-Is or ARBs	100	
Digoxin	29	
LVMI, g/m^2	158.76 ± 43.55	138.94–178.58
Relative wall thickness, mm	0.35 ± 0.08	0.32–0.39
LVEDD, mm	57.02 ± 9.08	52.89–61.16
LVESD, mm	41.77 ± 11.47	36.55–46.99
LVEDV, mL	165.58 ± 62.85	136.97–194.19
LVESV, mL	85.92 ± 54.18	61.25–110.58
LVEF, %	46.62 ± 18.61	39.47–53.77
V_E/V_A	1.56 ± 0.99	0.93–2.19
DT, ms	195.76 ± 64.91	165.84–225.68
K_{LV}, mmHg/mL	0.175 ± 0.10	0.128–0.222
IVRT, ms	101.75 ± 18.71	92.99–110.51
PCWP, mmHg	16.62 ± 1.49	13.51–19.73
Serum NT-proBNP, fmol/mL	512.17 ± 282.67	361.81–663.06
Plasma aldosterone, pg/mL	136.88 ± 138.18	68.17–205.60

SD, standard deviation of the mean; CI, confidence interval; NYHA, New York Heart Association; ACE-Is, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; LVMI, left-ventricular mass index; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; LVEF, LV ejection fraction; V_E, maximum early transmitral flow velocity in diastole; V_A, maximum late transmitral flow velocity in diastole; DT, deceleration time; K_{LV}, LV chamber stiffness; IVRT, isovolumic relaxation time; PCWP, pulmonary capillary wedge pressure; NT-proBNP, amino-terminal propeptide of brain natriuretic peptide.

2.4 Biochemical determinations

Venous blood samples were obtained from the left-antecubital vein in healthy controls, and from the left-antecubital vein and the coronary sinus in patients during the cardiac catheterization procedure and stored at −40°C for further simultaneous processing.

OPN was measured in plasma by ELISA (Assay Designs). The inter- and intra-assay coefficients of variation were 9.2 and 4.0%, respectively. Amino-terminal propeptide of brain natriuretic peptide (NT-proBNP) was measured in plasma samples by ELISA (Biomedica Gruppe). The inter- and intra-assay variation coefficients were 8 and 5%, respectively. Plasma aldosterone was measured by radioimmunoassay using a commercial kit (Biovendor). The inter- and intra-assay variation coefficients were 9 and 7%, respectively.

2.5 Histomorphological and immunohistochemical studies

The area of myocardium occupied by collagen tissue or collagen volume fraction (CVF) was determined by quantitative morphometry in sections stained with collagen-specific picro-sirius red, as previously reported.[24] The amount of insoluble collagen was assessed using colorimetric and enzymatic procedures as previously described.[6]

Immunohistochemical analysis for OPN, α-smooth muscle actin, and collagen type I was performed on formalin-fixed and paraffin-embedded sections. Immunohistochemical staining was performed by the avidin peroxidase-labelled dextran polymer method. Positive staining was visualized with DAB Plus (Boehringer Mannheim Corp.), and tissues were counterstained with Harris haematoxylin (Sigma). Mouse monoclonal antibodies against OPN (Abcam; dilution 1:500), α-smooth muscle actin (Sigma; dilution 1:2000), and collagen type I (Biogenesis; dilution 1:10) were used as primary antibodies.

A semiquantitative scale was developed to assess OPN expression. The myocardial surface area with positive staining for collagen type I was analysed by quantitative morphometry, as previously described.[24]

2.6 Molecular studies

A 25 μg sample of total protein obtained from each biopsy was processed for western blot for OPN, PCP, LOX, MMP-1, MMP-2, MMP-9, and TIMP-1 as previously described.[25] Specific rabbit polyclonal antibodies against OPN (Abcam; dilution 1:2500), PCP (R&D Systems; dilution 1:500), LOX (R&D Systems; dilution 1:10000), MMP-1 (Oncogene; dilution 1:2000), MMP-2 (Neomarkers; dilution 1:1000), MMP-9 (Neomarkers; dilution 1:100), and TIMP-1 (Chemicon; dilution 1:200) were used. Bands were detected by peroxidase-conjugated secondary antibodies (Amersham Biosciences) and visualized with the ECL-Plus chemiluminescence system (Amersham Biosciences). Autoradiograms were analysed using an automatic densitometer (Molecular Imager FX, Bio-Rad). The blots were also probed with a monoclonal β-actin antibody (Sigma) as a control for loading. Data are expressed as arbitrary densitometric units (A.D.U.) relative to β-actin expression.

mRNA levels of the α_1 chain of procollagen type I were analysed in myocardial samples by real-time quantitative RT−PCR as previously described.[26]

Reverse transcription was performed with 200 μg of total RNA by using Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed with an 7900 HT Fast Real-time PCR system according to the manufacturer's recommendations (Applied Biosystems) by using specific TaqMan MGB fluorescent probes for human α_1 chain of procollagen type I (Hs00176329), and a specific TaqMan MGB fluorescent probe for human constitutive 18S ribosomal RNA as endogenous control. Data were analysed as arbitrary units (A.U.) relative to 18S ribosomal RNA.

2.7 *In vitro* study

Adult human dermal fibroblasts (HDFa line; GIBCO) and primary cell cultures of human cardiac fibroblasts were used. These primary cell cultures were obtained by outgrowth from endomyocardial biopsies from the right side of the ventricular septum of patients with dilated cardiomyopathy (LVEF< 30%), and shown to express specific fibroblasts markers (i.e. vimentin and P4HB) as previously described.[27,28]

Dose−response stimulation curves were performed with HDFa fibroblasts and subsequently a concentration of OPN of 125 ng/mL was selected as the lower concentration increasing pro-LOX protein expression when compared with unstimulated cells (*Figure 1*). Cells were left to expand until they reached 80% confluency. Cells were then starved in reduced-serum medium for 24 h, prior to OPN (Peprotech) or vehicle stimulation for another 24 h.

LOX mRNA and the precursor of LOX protein (i.e. pro-LOX) levels were analysed in cell lysates using the methodology described earlier. A 10 μg sample of total protein obtained from cell lysates was used for the determination of pro-LOX protein using LOX antibody (R&D System) at a dilution of 1:500. For the measurement of LOX mRNA, the specific TaqMan MGB fluorescent probe for human LOX mRNA (Hs00942480_m1) was employed.

LOX activity was measured with a commercially available fluorimetric assay (AAT Bioquest) which utilizes a proprietary LOX substrate that releases hydrogen peroxide using the Amplilite[TM] ADHP substrate in HRP-coupled reactions. The procedure used followed the manufacturer's indications and the measured activity was corrected by total protein amount. Data are expressed as fold-change when compared with control conditions.
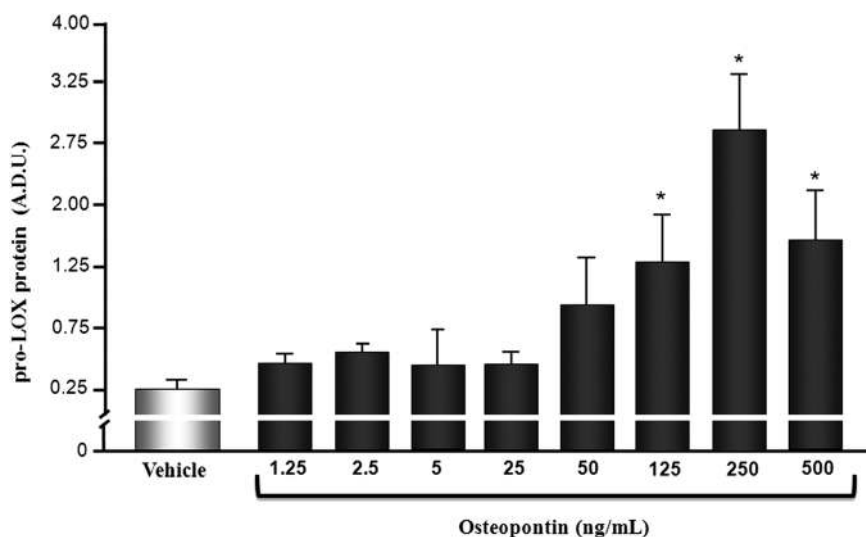

Figure 1 Dose−response curve for osteopontin-mediated lysyl oxidase stimulation in human dermal fibroblasts. Pro-lysyl oxidase protein levels determined in HDFa fibroblasts incubated with either vehicle or different doses of osteopontin for 24 h. *P < 0.05 when compared with vehicle. A.D.U., arbitrary densitometric units.

Connective tissue growth factor (CTGF) silencing was performed using lipofectamine 2000 (Invitrogen) and 10 pmol/mL of siRNA (Silencer Select siRNA, Ambion) in HDFa fibroblasts. CTGF mRNA levels were analysed with the specific TaqMan MGB fluorescent probe for human CTGF mRNA (Hs01026927_g1). Time−response curves for CTGF inhibition were performed finding a 58% inhibition after 12 h, 75% inhibition after 24 h, 77% inhibition after 36 h, 88% inhibition after 48 h, and 86% inhibition after 72 h. Accordingly, cells were placed in reduced-serum medium and transfected with either CTGF siRNA or a scramble siRNA; after 24 h, cells were stimulated with OPN (125 ng/mL) or vehicle and harvested 24 h later.

Human cardiac fibroblasts were stimulated with OPN (250 ng/mL) or kept in control conditions for 24 h in order to study the differentiation of fibroblasts to myofibroblasts as assessed by α-smooth muscle actin expression. Immunocytochemical detection of α-smooth muscle actin was performed in 4% paraformaldehyde fixed cells incubated with a monoclonal mouse antibody against human α-smooth muscle actin (Dako) at a 1:100 dilution. Subsequently cells were exposed to a biotinylated anti-mouse antibody and Cy3-conjugated streptavidin (Jackson ImmunoResearch). Nuclei were stained with Dapi (Invitrogen). The expression of α-smooth muscle actin in cardiac fibroblasts cells was below 5% in baseline conditions. Stimulation with OPN (250 ng/mL) for 24 h did not increase the fraction of cells positive for α-smooth muscle actin.

2.8 Statistical analysis

To analyse the differences in histomorphological, molecular, and biochemical parameters between controls and patients, and in LOX activity and expression between cells incubated with or without OPN, a Student's *t*-test for unpaired data was used once normality was demonstrated; otherwise the Mann−Whitney *U* test was performed. Categorical variables were analysed by the χ^2 test or Fisher's 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 ± SD with 95% confidence intervals (CI) and categorical variables as numbers and percentages. A value of two-sided $P < 0.05$ was considered statistically significant.

3. Results

3.1 Assessment of myocardial fibrosis

The histomorphological, immunohistochemical, and biochemical parameters related to collagen assessed in the myocardium of controls and patients are presented in *Table 2*. All these parameters were significantly increased in patients when compared with controls. Of interest, the fraction of total collagen corresponding to insoluble collagen was increased ($P < 0.0001$) in patients [77.12 ± 5.23% (95% CI: 74.59−79.65)] compared with controls [58.08 ± 9.52% (95% CI: 43.55−75.39)] (see Supplementary material online, *Figure S1*).

PCP expression was not increased in HF patients compared with controls [2.50 ± 0.61 A.D.U. (95% CI: 2.16−2.84) vs. 2.29 ± 0.39 A.D.U. (95% CI: 2.11−2.49); $P = 0.210$] (see Supplementary material online, *Figure S2*). 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 (see Supplementary material online, *Figure S2*).

No correlations were found between myocardial PCP and parameters assessing myocardial collagen or left-ventricular function in patients from this study. Myocardial LOX was directly correlated with insoluble collagen ($r = 0.805$, $P = 0.00017$), PCWP ($r = 0.563$, $P = 0.010$), and K_{LV} ($r = 0.459$, $P = 0.025$), and inversely correlated with the LVEF ($r = -0.580$, $P = 0.015$) in all patients. In addition, a direct

Table 2 Parameters related to myocardial collagen matrix in control subjects and hypertensive patients with heart failure

Parameters	Controls (*n* = 10)	Patients (*n* = 21)	*P*-value
Insoluble collagen, µg/mg	0.95 ± 0.74	8.99 ± 0.87	<0.0001
[95% CI]	[0.43−1.46]	[8.55−9.42]	
Soluble collagen, µg/mg	0.66 ± 0.53	2.76 ± 0.57	<0.0001
[95% CI]	[0.29−1.04]	[2.39−2.96]	
Collagen volume fraction, %	1.95 ± 0.22	7.71 ± 2.73.	<0.0001
[95% CI]	[1.79−2.11]	[6.37−8.76]	
Collagen type I volume fraction, %	2.03 ± 0.22	7.63 ± 2.36	<0.0001
[95% CI]	[1.88−2.19]	[6.56−8.71]	

SD, standard deviation; CI, confidence interval.

correlation was found between insoluble collagen and PCWP ($r = 0.569$, $P = 0.009$) and K_{LV} ($r = 0.526$, $P = 0.017$) in all patients. These correlations remained significant when we excluded the influence of a number of potential confounding factors (i.e. age, gender, systolic and diastolic blood pressure, LV mass and dimensions) in partial correlation analysis.

3.2 Assessment of myocardial OPN

Although 100% control subjects exhibited absent (50%) or mild (50%) immunostaining for OPN, 100% patients exhibited moderate-to-severe immunostaining, this difference being significant ($P < 0.0001$). *Figure 2* shows that although OPN was almost absent in the myocardium of a control subject, it was highly expressed in the areas of interstitial and perivascular fibrosis, as well as in some cardiomyocytes in the myocardium of a patient with HHD and HF. Of interest, OPN was localized in fibrotic foci where fibroblasts and myofibroblats were present in the failing myocardium (*Figure 2*).

OPN was identified in myocardial samples from all patients with HHD and HF by western blot. As shown in *Figure 3*, direct correlations were found between OPN and LOX ($r = 0.460$, $P = 0.041$) and insoluble collagen ($r = 0.534$, $P = 0.015$) in the myocardium of all patients. These correlations remained significant when we excluded the influence of a number of potential confounding factors (i.e. age, gender, systolic and diastolic blood pressure, LV mass and dimensions) in partial correlation analysis.

In addition, myocardial OPN was directly correlated with collagen type I ($r = 0.600$, $P = 0.004$) and CVF ($r = 0.566$, $P = 0.007$) (*Figure 3*), the latter correlation being dependent on age. No significant correlations were found between myocardial OPN and soluble collagen, α_1 chain of procollagen type I mRNA, PCP, MMP-1, MMP-2, MMP-9, and TIMP-1 in all patients.

Myocardial OPN was directly correlated with PCWP ($r = 0.558$, $P = 0.009$; *Figure 4*) and K_{LV} ($r = 0.458$, $P = 0.037$; *Figure 4*), and inversely correlated with LVEF ($r = -0.513$, $P = 0.017$; *Figure 4*) and plasma aldosterone ($r = -0.432$, $P = 0.043$) in all patients. These correlations were not influenced by age, gender, body mass index, systolic and diastolic blood pressure, LV mass and dimensions.
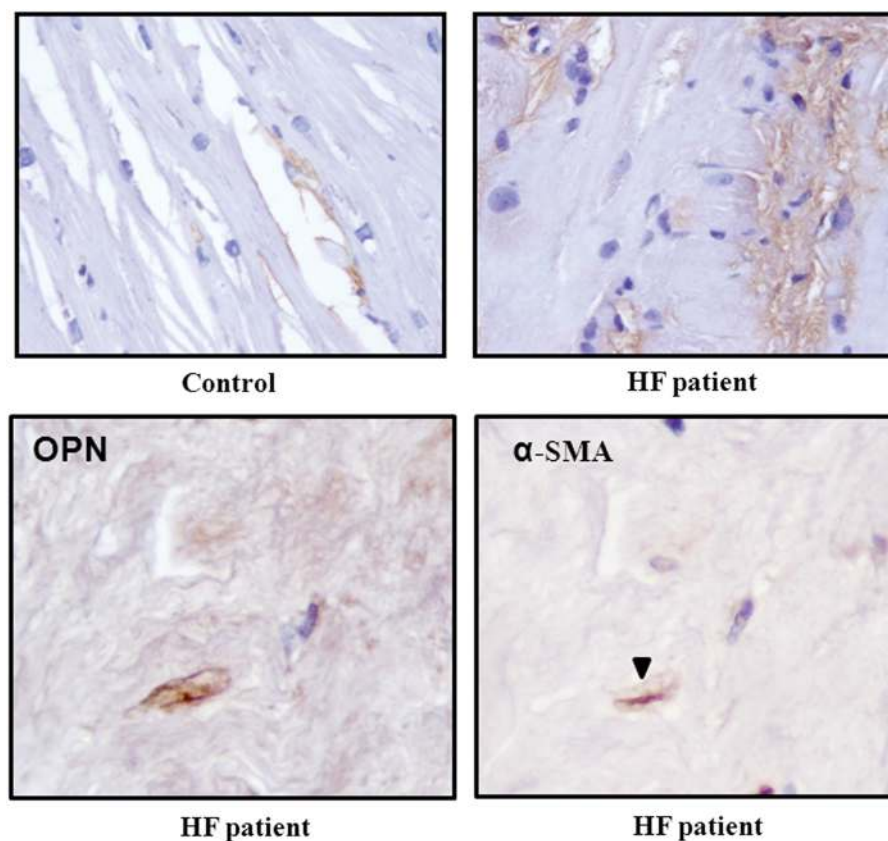
Figure 2 Expression of osteopontin (in brown) in histological sections of myocardial specimens. Upper panels: the left panel corresponds to one control subject and shows very slight staining within the interstitial space. The right panel corresponds to one patient with heart failure and shows intense staining located in large areas of interstitial and perivascular fibrosis and some cardiomyocytes. Lower panels: co-localization of myocardial osteopontin and myofibroblasts in fibrotic foci. The left panel corresponds to osteopontin staining (brown) in a fibrotic foci in the myocardium of one patient with heart failure. The right panel shows a myofibroblast (brown, arrowhead), positive for α-smooth muscle actin (α-SMA), in the same region. (Magnification ×40.)

3.3 Assessment of plasma OPN

Plasma concentration of OPN measured in blood from the left antecubital vein was increased in patients with HHD and HF compared with control subjects [27.48 ± 8.03 ng/mL (95% CI: 11.36−30.76) vs. 20.57 ± 7.36 ng/mL (95% CI: 15.16−50.64); $P = 0.006$]. However, no differences were found in plasma OPN measured in blood from the coronary sinus [27.81 ± 7.56 ng/mL (95% CI: 24.37−31.25)] and the left antecubital vein in patients with HHD and HF.

No significant correlations were found between myocardial OPN and plasma OPN measured in blood from either the coronary sinus or the left antecubital vein in patients with HHD and HF. In addition, plasma OPN was not correlated with echocardiographic and biochemical parameters in these patients.

3.4 *In vitro* data

Pro-LOX protein expression increased in a dose-dependent manner upon OPN stimulation in HDFa fibroblasts (*Figure 1*). In both freshly isolated human cardiac fibroblasts and HDFa, pro-LOX protein levels were also increased in OPN-incubated fibroblasts (125 ng/mL) when compared with control cells ($P = 0.029$ and $P = 0.011$, respectively; *Figure 5*). Similarly, LOX activity was increased in both fibroblast types ($P = 0.046$ for human cardiac fibroblasts and $P = 0.004$ for HDFa)

exposed to OPN as compared with control cells (*Figure 5*). Interestingly, although 125 ng/mL of OPN induced an increase ($P = 0.003$) in LOX mRNA levels in HDFa fibroblasts [2.50 ± 0.06 A.U. (95% CI: 1.75−3.25) vs. 1.00 ± 0.06 A.U. (95% CI: 0.25−1.75)] it did not change ($P = 0.201$) LOX mRNA levels in human cardiac fibroblasts [0.85 ± 0.13 A.U. (95% CI: 0.273−1.408) vs. 1.00 ± 0.06 A.U. (95% CI: 0.697−1.302)].

Although CTGF silencing had no effect on LOX activity in baseline conditions, it blunted ($P = 0.002$) OPN-induced increase in LOX activity (*Figure 6*). On the other hand, CTGF silencing induced a decrease ($P = 0.001$) in LOX mRNA expression in baseline conditions, and it prevented OPN-induced increase in LOX mRNA expression ($P = 0.007$) (*Figure 6*).

4. Discussion

The main findings of this study are as follows: (i) an excess of myocardial OPN is associated with increased LOX, insoluble collagen, and collagen type I deposition in HF patients with HHD; (ii) myocardial OPN is associated with LV filling pressures, stiffness, and systolic dysfunction in these patients; and (iii) OPN up-regulates LOX activity and expression in human cardiac and dermal fibroblasts. Although the association of OPN with LOX does not necessarily represent a causative relation, it
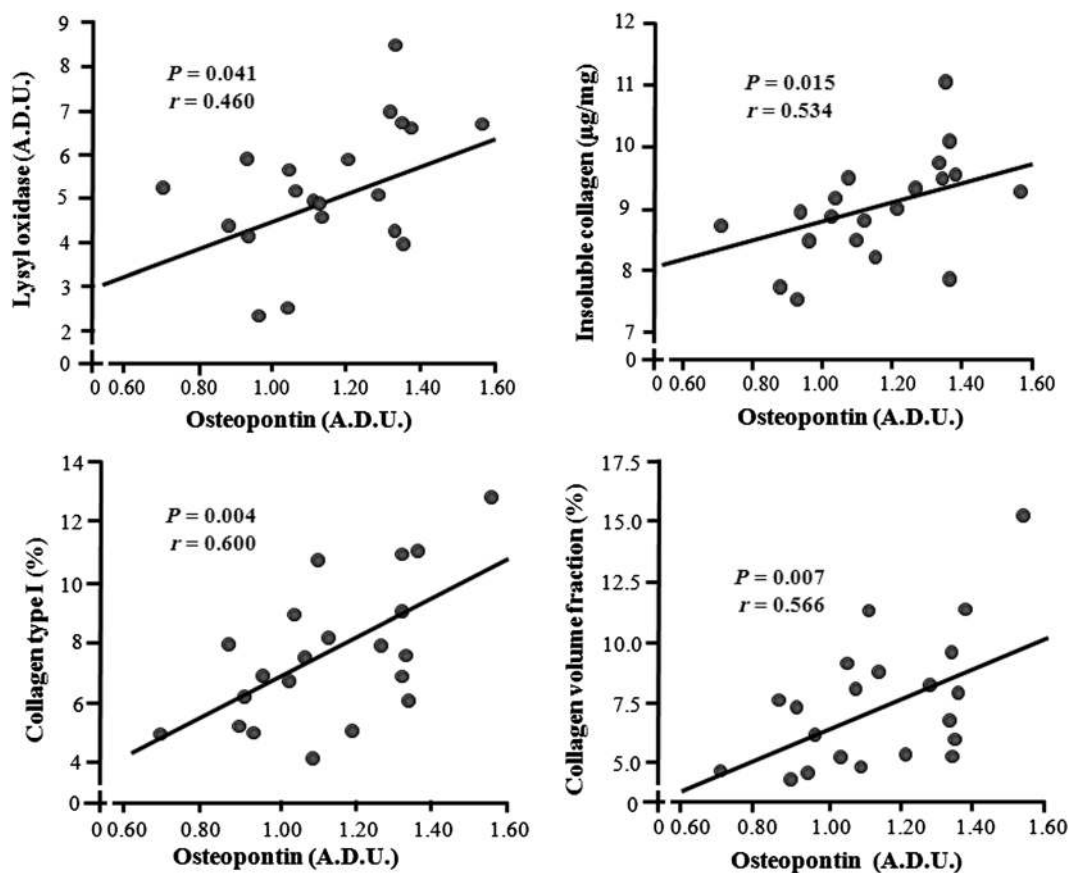
Figure 3 Associations of osteopontin with collagen metabolism and deposition. The left upper panel shows the direct correlation ($y = 3.285x + 1.487$) between osteopontin and lysyl oxidase in patients with heart failure (HF). The right upper panel shows the direct correlation ($y = 2.112x + 6.566$) between osteopontin and insoluble collagen in HF patients. The left lower panel shows the direct correlation ($y = 6.557x + 0.131$) between osteopontin and collagen type I in HF patients. The right lower panel shows the direct correlation ($y = 7.164x - 0.680$) between osteopontin and collagen volume fraction in HF patients. A.D.U., arbitrary densitometric units.

provides a new pro-fibrotic mechanism in patients with HF of hypertensive origin.

The finding that myocardial OPN was abnormally increased in HF patients with HHD expands previous findings in HF patients with ischaemic heart disease, dilated cardiomyopathy, and hypertrophic cardiomyopathy,[12–14] thus providing for the first time evidence that myocardial OPN is up-regulated in the human pressure-overloaded failing myocardium, as it does in animal models of HF secondary to arterial hypertension or aortic banding.[11]

Although in a murine model of mechanical pressure overload by aortic banding, the increase of OPN did not seem to play a significant role in the development of myocardial fibrosis,[29] other studies have shown that angiotensin-[30–32] and aldosterone-induced[33,34] OPN expression mediates the cardiac fibrotic effect of these two hormones. Similarly, myocardial fibrosis induced by interleukin-18 (IL-18) in rodents has been shown to be associated with enhanced expression of OPN.[10] Since all the patients included in the current study were under treatment with either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, the contribution of angiotensin II to the increase in myocardial OPN seen in patients with HHD and HF remains to be determined. Although plasma OPN has been shown to be associated with plasma aldosterone in patients with dilated

cardiomyopathy,[35] no correlation was found between these two parameters in the present study, thus making it unlikely for this hormone to be a major factor involved in the increase in OPN. Moreover, an inverse correlation was found between plasma aldosterone and myocardial OPN in these patients. However, it has to be taken into account that cardiac aldosterone may be increased in HF without changes in systemic plasma aldosterone levels.[36] Therefore, a role for cardiac aldosterone inducing OPN expression cannot be excluded. On the other hand, the potential contribution of IL-18 to the excess of OPN seen in the failing human hypertensive heart deserves further studies.

Fibrosis compromises tissue stiffness and adversely affects myocardial viscoelasticity, leading to LV diastolic and systolic dysfunction.[37] Myocardial stiffness is affected not just by the amount of myocardial collagen, but also by its quality (i.e. the insolubility of collagen fibrils and the amount of collagen type I fibres).[1] Therefore, the strong associations found here between OPN and insoluble collagen, collagen type I, PCWP, and LVEF support a role for OPN in alterations of the myocardial collagen matrix that result in the deterioration of the compliance and function of the left ventricle in patients with HHD and HF. This possibility is supported by the findings that cardiac induction of OPN in rats submitted to pressure overload is accompanied by myocardial fibrosis, increased LV chamber stiffness, and reduced LVEF.[10]
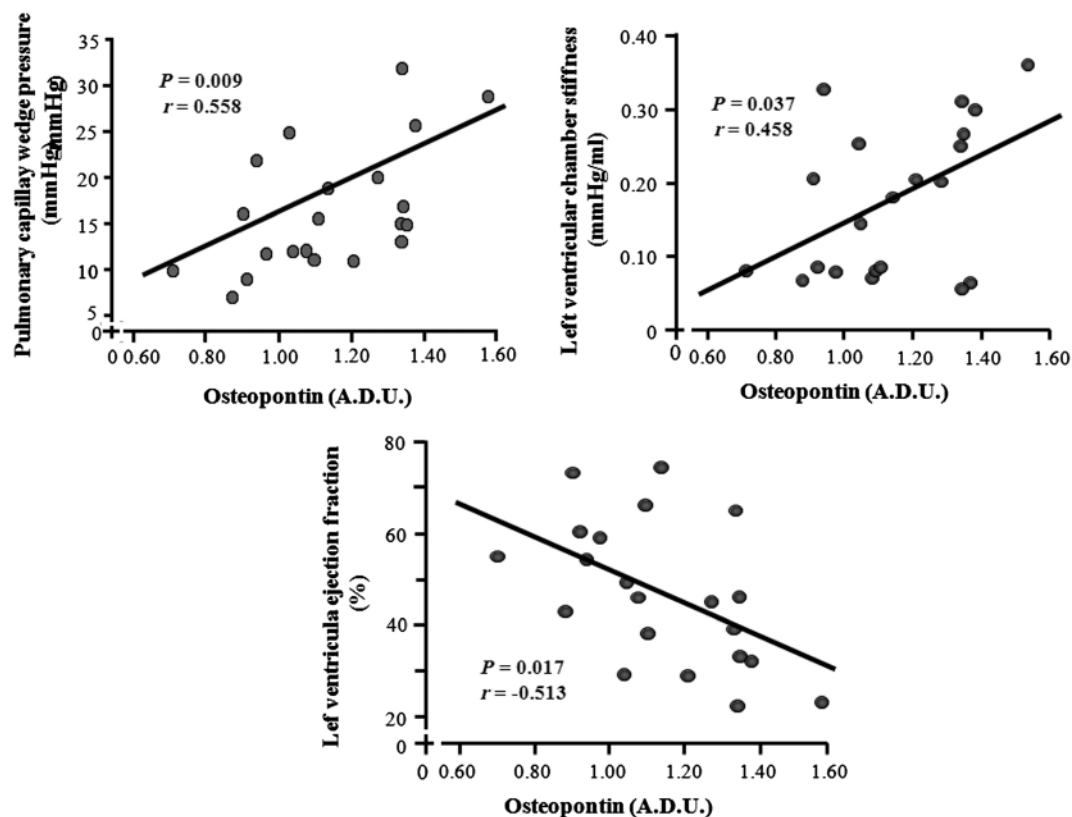
Figure 4 Associations of osteopontin with left-ventricular parameters. The left upper panel shows the direct correlation ($y = 0.220x - 0.077$) between osteopontin and pulmonary capillary wedge pressure (PCWP) ($y = 17.657x - 3.586$) in patients with heart failure (HF). The right upper panel shows the direct correlation ($y = 0.220x - 0.077$) between osteopontin and left-ventricular chamber stiffness constant in HF patients. The lower panel shows the inverse correlation ($y = -37.326x + 89.322$) between osteopontin and left-ventricular ejection fraction in HF patients. A.D.U, arbitrary densitometric units.

Although some experimental findings suggested that OPN might facilitate myocardial fibrosis through the differentiation and activity of myofibroblasts[16] and the inhibition of MMP-mediated degradation of collagen fibres,[17] no associations of OPN with parameters assessing these two aspects were found in this study. However, we report data suggesting a potential novel mechanism of OPN-mediated fibrosis in the human failing hypertensive myocardium: stimulation of LOX-dependent cross-linking of collagen type I fibrils to form collagen fibres. This possibility is further supported by our *in vitro* observation that OPN increases LOX activity and stimulates the expression of the LOX precursor in human cardiac and dermal fibroblasts. Of interest, the effect of OPN on LOX mRNA expression differs between freshly isolated cardiac fibroblasts and HDFa; this different behaviour might be related to either the different origin of the fibroblasts or to the process of immortalization and proliferation properties of the cell line.

Our *in vivo* and *in vitro* findings suggest that OPN can be a new factor involved in the regulation of LOX, as are hypoxia-inducible factor-1α, advanced glycation end product-dependent transcription factors, and transforming growth factor-β.[38] Interestingly, although OPN increased LOX activity and protein expression in both dermal and cardiac fibroblasts it only increased LOX mRNA levels in dermal fibroblasts, suggesting that although translational mechanisms can be involved in OPN-mediated LOX up-regulation in the two cell subtypes, transcriptional mechanisms may also play a role in the dermal subtype. Since

OPN stimulates the CTGF expression in cardiac fibroblasts[16] and that CTGF regulates LOX expression in these cells,[39] the possibility exists that OPN-induced LOX up-regulation is mediated by CTGF in the myocardium of HF patients from this study. This is supported by our *in vitro* data showing that CTGF silencing blunted OPN-induced increase in LOX activity and expression.

Finally, we have found that plasma OPN is abnormally increased in patients with HF of hypertensive origin. This goes along with recent findings in patients with HF secondary to either ischaemic heart disease or dilated cardiomyopathy.[15,40,41] However, in contrast to data by Tamura *et al.*[42] in patients with myocardial infarction, our data do not support that OPN is released from the heart into the coronary circulation (i.e. we did not found a gradient between plasma OPN measured in blood from coronary sinus and blood from the antecubital vein). Furthermore, plasma OPN was not associated with myocardial OPN or with LV morphological and functional parameters. Therefore, our findings do not support a role for plasma OPN as a cardiac biomarker of myocardial OPN in patients with HHD and HF pointing to a major contribution by extracardiac sources (e.g. inflammatory circulating cells, other organs) to circulating OPN in this condition.

4.1 Limitations

Several limitations need to be acknowledged. First, this was a study involving a relatively small number of patients, but because of the nature
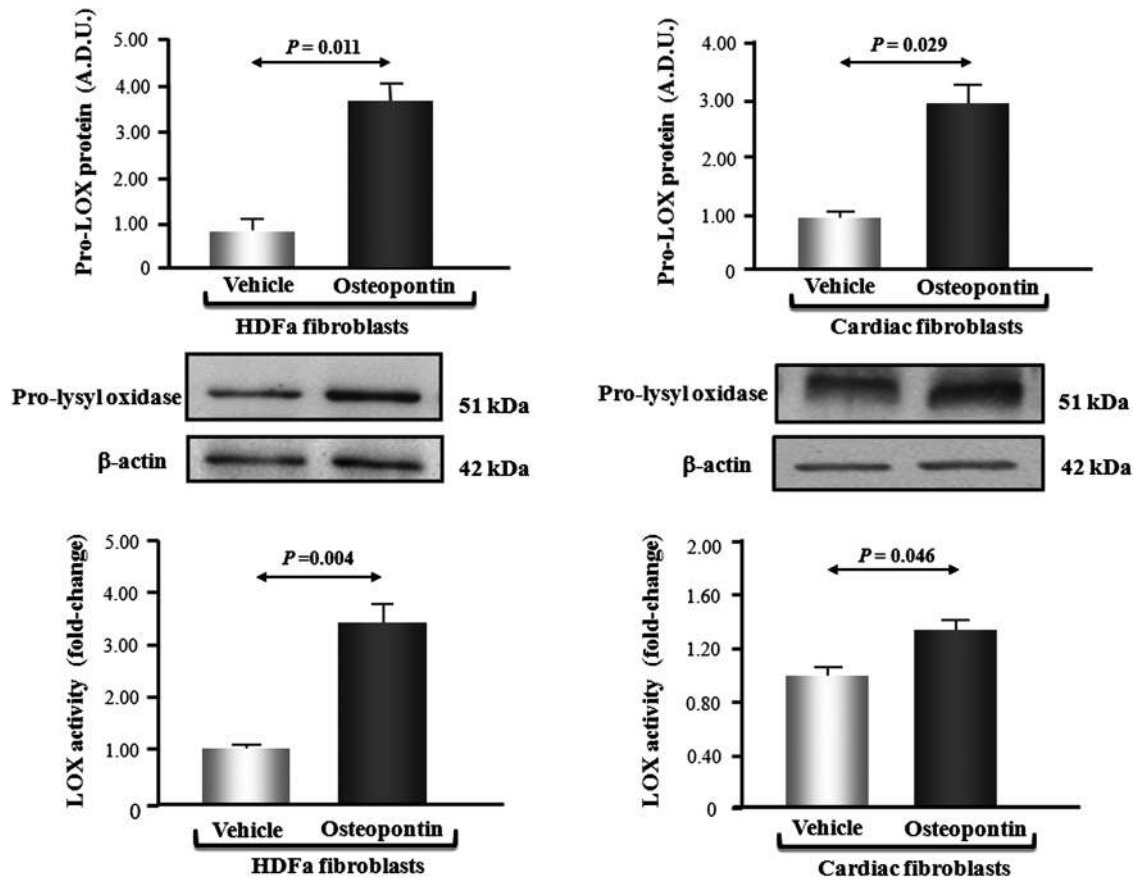
Figure 5 Osteopontin-mediated regulation of lysyl oxidase in human fibroblasts. The upper panels show pro-LOX protein levels determined in HDFa fibroblasts (left panel) and freshly isolated human cardiac fibroblasts (right panel) incubated with either vehicle or 125 ng/mL osteopontin for 24 h. The lower panels show lysyl oxidase (LOX) activity determined in human dermal adult fibroblasts (HDFa) (left panel) and freshly isolated human cardiac fibroblasts (right panel) incubated with either vehicle or 125 ng/mL osteopontin for 24 h. Data are presented as fold change vs. unstimulated cells.
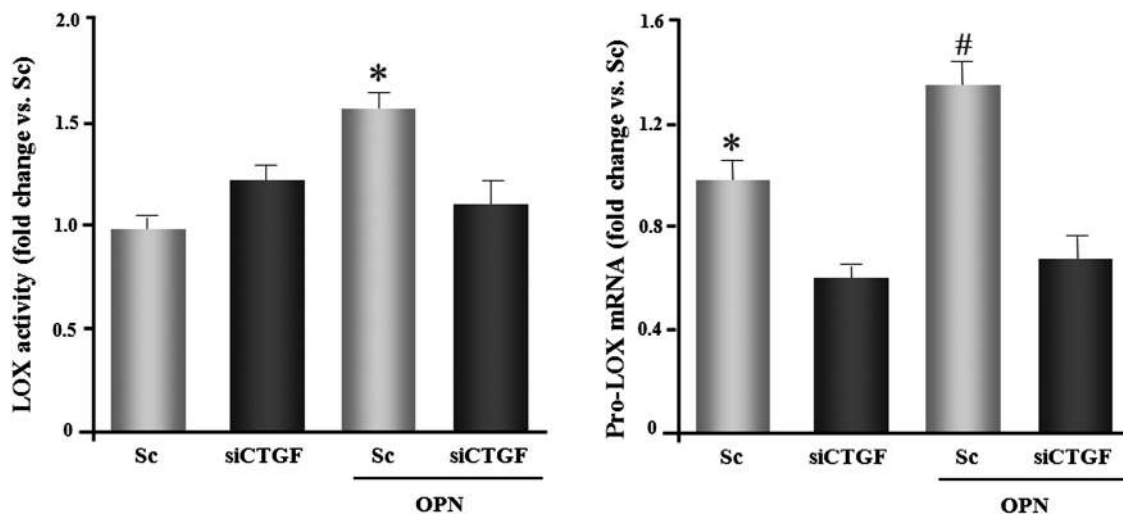

Figure 6 CTGF involvement in osteopontin-mediated effects in human fibroblasts. The left panel shows LOX activity in the presence or absence of CTGF silencing. The right panel shows pro-LOX mRNA expression in the presence or absence of CTGF silencing. Data are presented as fold change vs. unstimulated cells. *$P < 0.05$ vs. all other conditions; #$P < 0.05$ vs. siCTGF and OPN + siCTGF. Sc, scramble siRNA; siCTGF, siRNA for CTGF; OPN, osteopontin.

of the goals under investigation, this design is appropriate. Secondly, since our patient sample comprised patients with HHD in whom other cardiac conditions had been excluded, the results may not be generalized to HF patients with cardiac diseases different from HHD. Third, we performed biopsies of the interventricular septum to assess the fibrotic effect of LV pressure loading. However, as we have shown previously,[25] in terms of myocardial fibrosis, the septum is representative of the free wall. Finally, collagen cross-linking formed from glycation may also affect collagen insolubility, therefore the increase of insoluble collagen detected in the current study cannot be exclusively attributed to an excess of LOX.

4.2 Conclusions

In conclusion, we show that OPN is associated with LOX and insoluble collagen in the myocardium of patients with HHD and HF. Furthermore, OPN is also associated with LV filling pressures, stiffness, and systolic dysfunction in these patients. Since we found that OPN up-regulates LOX activity and expression in human cardiac and dermal fibroblasts, the possibility emerges that this matricellular protein is involved in fibrosis through the control of LOX-mediated cross-linking of collagen type I, thus leading to a stiff and highly resistant to degradation collagen. In this conceptual framework, the OPN−LOX axis can be a novel target to treat myocardial fibrosis and its detrimental impact on LV function in patients with HF of hypertensive origin.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

References

1. Brower GL, Gardner JD, Forman MF, Murray DB, Voloshenyuk T, Levick SP et al. The relationship between myocardial extracellular matrix remodeling and ventricular function. *Eur J Cardiothorac Surg* 2006;**30**:604–610.
2. Burlew BS, Weber KT. Cardiac fibrosis as a cause of diastolic dysfunction. *Herz* 2002;**27**:92–98.
3. Bing OHL, Fanburg BL, Brooks WW, Matsushita S. The effect of the lathyrogen β−amino proprionitrile (BAPN) on the mechanical properties of experimentally hypertrophied rat cardiac muscle. *Circ Res* 1978;**43**:632–637.
4. Norton GR, Tsotetsi J, Trifunovic B, Hartford C, Candy GP, Woodiwiss AJ. Myocardial stiffness is attributed to alterations in cross-linked collagen rather than total collagen or phenotypes in spontaneously hypertensive rats. *Circulation* 1997;**96**:1991–1998.
5. Badenhorst D, Maseko M, Tsotetsi OJ, Naidoo A, Brooksbank R, Norton GR et al. Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodeling in hypertension in rats. *Cardiovasc Res* 2003;**57**:632–641.
6. López B, Querejeta R, González A, Beaumont J, Larman M, Díez J. Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure. *Hypertension* 2009;**53**:236–242.
7. Kasner M, Westermann D, López B, Gaub R, Escher F, Kühl U et al. Diastolic tissue Doppler indexes correlate with the degree of collagen expression and cross-linking in heart failure and normal ejection fraction. *J Am Coll Cardiol* 2011;**57**:977–985.
8. López B, González A, Beaumont J, Querejeta R, Larman M, Díez J. Identification of a potential cardiac antifibrotic mechanism of torasemide in patients with chronic heart failure. *J Am Coll Cardiol* 2007;**50**:859–867.
9. López B, Querejeta R, González A, Larman M, Díez J. 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. *Hypertension* 2012;**60**:677–683.
10. Yu Q, Vazquez R, Khojeini EV, Patel C, Venkataramani R, Larson DF. IL-18 induction of osteopontin mediates cardiac fibrosis and diastolic dysfunction in mice. *Am J Physiol Heart Circ Physiol* 2009;**297**:H76–H85.
11. Singh K, Sirokman G, Communal C, Robinson KG, Conrad CH, Brooks WW et al. Myocardial osteopontin expression coincides with the development of heart failure. *Hypertension* 1999;**33**:663–670.
12. Graf K, Do YS, Ashizawa N, Meehan WP, Giachelli CM, Marboe CC et al. Myocardial osteopontin expression is associated with left ventricular hypertrophy. *Circulation* 1997;**96**:3063–3071.
13. Stawowy P, Blaschke F, Pfautsch P, Goetze S, Lippek F, Wollert-Wulf B et al. Increased myocardial expression of osteopontin in patients with advanced heart failure. *Eur J Heart Fail* 2002;**4**:139–146.
14. Satoh M, Nakamura M, Akatsu T, Shimoda Y, Segawa I, Hiramori K. Myocardial osteopontin expression is associated with collagen fibrillogenesis in human dilated cardiomyopathy. *Eur J Heart Fail* 2005;**7**:755–762.
15. Schipper ME, Scheenstra MR, van Kuik J, van Wichen DF, van der Weide P, Dullens HF et al. Osteopontin: a potential biomarker for heart failure and reverse remodeling after left ventricular assist device support. *J Heart Lung Transplant* 2011;**30**:805–810.
16. Lenga Y, Koh A, Perera AS, McCulloch CA, Sodek J, Zohar R. Osteopontin expression is required for myofibroblast differentiation. *Circ Res* 2008;**102**:319–327.
17. Krishnamurthy P, Peterson JT, Subramanian V, Singh M, Singh K. Inhibition of matrix metalloproteinases improves left ventricular function in mice lacking osteopontin after myocardial infarction. *Mol Cell Biochem* 2009;**322**:53–62.
18. Ho KL, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure. The Framingham Study. *J Am Coll Cardiol* 1993;**22**(Suppl. A):6A–13A.
19. Querejeta R, Varo N, López B, Larman M, Artiñano E, Etayo JC et al. Serum carboxy-terminal propeptide of procollagen type I is a marker of myocardial fibrosis in hypertensive heart disease. *Circulation* 2000;**101**:1729–1735.
20. Ganau A, Devereux RB, Roman MJ, de Simone G, Pickering TG, Saba PS et al. Patterns of left ventricular hypertrophy and geometric remodeling in essential hypertension. *J Am Coll Cardiol* 1992;**19**:1550–1558.
21. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence of absence of asynergy. *Am J Cardiol* 1976;**37**:7–11.
22. Quinones MA, Pickering E, Alexander JK. Percentage of shortening of the echocardiographic left ventricular dimension: its use in determining ejection fraction and stroke volume. *Chest* 1978;**74**:59–65.
23. Little WC, Ohno M, Kitzman DW, Thomas JD, Cheng CP. Determination of left ventricular chamber stiffness from the time for deceleration of early left ventricular filling. *Circulation* 1995;**92**:1933–1939.
24. Querejeta R, López B, González A, Sánchez E, Larman M, Martínez Ubago JL et al. Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. *Circulation* 2004;**110**:1263–1268.
25. López B, González A, Querejeta R, Larman M, Díez J. Alterations of the pattern of collagen deposition may contribute to the deterioration of systolic function in hypertensive patients with heart failure. *J Am Coll Cardiol* 2006;**48**:89–96.
26. Goikoetxea MJ, Beaumont FJ, González A, López B, Querejeta R, Larman M et al. Altered cardiac expression of peroxisome proliferator-activated receptor-isoforms in patients with hypertensive heart disease. *Cardiovasc Res* 2006;**69**:899–907.
27. Lindner D, Zietsch C, Becher PM, Schulze K, Schultheiss HP, Tschöpe C et al. Differential expression of matrix metalloproteases in human fibroblasts with different origins. *Biochem Res Int* 2012;**2012**:875742.
28. Westermann D, Lindner D, Kasner M, Zietsch C, Savvatis K, Escher F et al. Cardiac inflammation contributes to changes in the extracellular matrix in patients with heart failure and normal ejection fraction. *Circ Heart Fail* 2011;**4**:44–52.
29. Xie Z, Singh M, Singh K. Osteopontin modulates myocardial hypertrophy in response to chronic pressure overload in mice. *Hypertension* 2004;**44**:826–831.
30. Collins AR, Schnee J, Wang W, Kim S, Fishbein MC, Bruemmer D et al. Osteopontin modulates angiotensin II-induced fibrosis in the intact murine heart. *J Am Coll Cardiol* 2004;**43**:1698–1705.
31. Kupfahl C, Pink D, Friedrich K, Zurbrügg HR, Neuss M, Warnecke C et al. Angiotensin II directly increases transforming growth factor beta 1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. *Cardiovasc Res* 2000;**46**:463–475.
32. Matsui Y, Jia N, Okamoto H, Kon S, Onozuka H, Akino M et al. Role of osteopontin in cardiac fibrosis and remodeling in angiotensin II-induced cardiac hypertrophy. *Hypertension* 2004;**43**:1195–1201.
33. Rocha R, Rudolph AE, Frierdich GE, Nachowiak DA, Kekec BK, Blomme EA et al. Aldosterone induces a vascular inflammatory phenotype in the rat heart. *Am J Physiol Heart Circ Physiol* 2002;**285**:H1802–H1810.

34. Nagata K, Obata K, Xu J, Ichihara S, Noda A, Kimata H *et al.* Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and failure in low-aldosterone hypertensive rats. *Hypertension* 2006;**47**:656–664.

35. Del Ry S, Giannessi D, Maltinti M, Cabiati M, Prontera C, Iervasi A *et al.* Increased plasma levels of osteopontin are associated with activation of the renin-aldosterone system and with myocardial and coronary microvascular damage in dilated cardiomyopathy. *Cytokine* 2010;**49**:325–330.

36. Ohtani T, Ohta M, Yamamoto K, Mano T, Sakata Y, Nishio M *et al.* Elevated cardiac tissue level of aldosterone and mineralocorticoid receptor in diastolic heart failure: Beneficial effects of mineralocorticoid receptor blocker. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**:R946–R954.

37. Burlew BS, Weber KT. Connective tissue and the heart. Functional significance and regulatory mechanisms. *Cardiol Clin* 2000;**18**:435–442.

38. López B, González A, Hermida F, Valencia F, de Teresa E, Díez J. Role of lysyl oxidase in myocardial fibrosis: from basic science to clinical aspects. *Am J Physiol Heart Circ Physiol* 2010;**299**:H1–H9.

39. Adam O, Theobald K, Lavall D, Grube M, Kroemer HK, Ameling S *et al.* Increased lysyl oxidase expression and collagen cross-linking during atrial fibrillation. *J Mol Cell Cardiol* 2011;**50**:678–685.

40. Rosenberg M, Zugck C, Nelles M, Juenger C, Frank D, Remppis A *et al.* Osteopontin, a new prognostic biomarker in patients with chronic heart failure. *Circ Heart Fail* 2008;**1**:43–49.

41. Soejima H, Irie A, Fukunaga T, Oe Y, Kojima S, Kaikita K *et al.* Osteopontin expression of circulating T cells and plasma osteopontin levels are increased in relation to severity of heart failure. *Circ J* 2007;**71**:1879–1884.

42. Tamura A, Shingai M, Aso N, Hazuku T, Nasu M. Osteopontin is released from the heart into the coronary circulation in patients with a previous anterior wall myocardial infarction. *Circ J* 2003;**67**:742–744.