Role of lysyl oxidase in myocardial fibrosis: from basic science to clinical aspects

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López B, González A, Hermida N, 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 299: H1–H9, 2010. First published May 14, 2010; doi:10.1152/ajpheart.00335.2010.—Because of its dynamic nature, the composition and structure of the myocardial collagen network can be reversibly modified to adapt to transient cardiac injuries. In response to persistent injury, however, irreversible, maladaptive changes of the network occur leading to fibrosis, mostly characterized by the excessive interstitial and perivascular deposition of collagen types I and III fibers. It is now becoming apparent that myocardial fibrosis directly contributes to adverse myocardial remodeling and the resulting alterations of left ventricular (LV) anatomy and function present in the major types of cardiac diseases. The enzyme lysyl oxidase (LOX) is a copper-dependent extracellular enzyme that catalyzes lysine-derived cross-links in collagen and elastin. LOX-mediated cross-linking of collagen types I and III fibrils leads to the formation of stiff collagen types I and III fibers and their subsequent tissue deposition. Evidence from experimental and clinical studies shows that the excess of LOX is associated with an increased collagen cross-linking and stiffness. It is thus conceivable that LOX upregulation and/or overactivity could underlie myocardial fibrosis and altered LV mechanics and contribute to the compromise of LV function in cardiac diseases. This review will consider the molecular aspects related to the regulation and actions of LOX, namely, in the context of collagen synthesis. In addition, it will address the information related to the role of myocardial LOX in heart failure and the potential benefits of controlling its expression and function.

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The Formation of Collagen Fibers

Collagens are synthesized in precursor form, procollagen chains, with NH₂- and COOH-terminal propeptide extensions within the fibroblasts and the myofibroblasts (65). Depending on the procollagen type, chains can be either identical [e.g., 3 α₁ (III)-chains as in procollagen type III] or different [e.g., 2 α₁ (I)-chains and 1 α₂(I)-chain as in procollagen type I]. Newly synthesized procollagen chains associate into trimers, leading to the nucleation and folding of the triple helical region in a zipper-like manner from the COOH to the NH₂ terminus (14). Once formed, procollagen molecules are translocated to the Golgi apparatus, packaged into vesicles that will fuse with the cell membrane to extrude their content to the extracellular space.

Following or during the secretion of procollagen types I and III, terminal propeptides are removed by zinc (Zn)-dependent metalloproteinases [e.g., procollagen NH₂ and COOH proteinases (PNP and PCP, respectively)], thereby triggering spontaneous self-assembly of mature collagen molecules into fibrils (55). The removal of COOH-terminal propeptide is more efficient than the removal of NH₂-terminal propeptide (28) and is absolutely required for normal fibril assembly and collagen deposition (45). In contrast, a certain degree of normal retention of COOH-terminal propeptide occurs in a tissue-specific manner and retained procollagen NH₂ propeptide does not appear to inhibit fibril assembly (45).

Fibrillar collagen types I and III must be cross-linked to form the final collagen types I and III fibers that are highly resistant against proteolytic enzymes and exhibit the physical properties of tensile strength. There are two major groups of cross-links: those initiated by the enzyme LOX and those derived from nonenzymatically glycated lysine and hydroxyllysine residues (56). It is now recognized that the matricellular protein thrombospondin-4 binds to fibrillar collagens, thus facilitating the process of cross-linking and the organization of the final collagen fiber (49). Once formed, a preexisting network consisting of fibronectin and α₁β₁ and α₃β₁-integrins has been shown to have the efficient deposition of collagen types I and III fibers to form the extracellular collagen network (79).

The LOX Enzyme

Molecular aspects. LOX is a copper (Cu)-dependent amine oxidase that belongs to a family of LOX proteins (Table 1). The mature form of LOX is derived from a precursor that is encoded by a conserved, single-copy gene localized to human chromosome 5q23 (22). Besides the gene encoding LOX, new LOX-like genes have been identified and cloned, suggesting the existence of a family consisting of LOX and four LOX-like proteins (LOXL-1, -2, -3, and -4) with a complex tissue-specific expression pattern and a great variation in mRNA levels (46). Among the three LOX and LOX-like proteins highly expressed in the cardiovascular system (LOX, LOXL-1 and -3), LOX is the most abundant form in the heart and the only one that uses collagen as a substrate. LOXL-2 has been shown to be abundant in the early stage of cardiac development in the fetal heart, whereas LOXL-3 mRNA expression is almost exclusively restricted to the adult aorta.

Regulation. LOX is synthesized as a proenzy me that after posttranslational modifications in the endoplasmic reticulum and Golgi apparatus is secreted to the extracellular space where it is processed to form the mature active enzyme. Therefore, LOX can be regulated at three levels: synthesis of LOX precursor by fibroblasts and myofibroblasts, extracellular conversion of the precursor into the mature enzyme, and direct stimulation of the activity of the enzyme (Fig. 1). The LOX gene is a well-established transcriptional target of hypoxia-inducible factor-1α (15, 21), and the activation of hypoxia-inducible factor-1α signaling is associated with fibrogenesis by increasing the expression of the LOX gene and other collagen matrix modifying genes (25). Recently, it has been reported that advanced glycation end products (AGEs) induce the binding of transcription factors, such as nuclear factor-κB and activator protein-1, on the LOX promoter (51), suggesting a possible involvement of AGEs in LOX gene regulation that may account for the documented colocalization of increase in LOX mRNA and protein levels with enhanced AGEs in some pathological conditions. LOX mRNA expression can also be regulated at the posttranscriptional level by humoral factors. For instance, transforming growth factor-β (TGF-β) has been shown to stabilize LOX mRNA in rat (4) and human (61) fibroblasts, thus leading to an enhanced synthesis of the LOX precursor. On the contrary, the prostanoid prostaglandin E₂ decreases the basal level of LOX mRNA and prevents the LOX precursor increase induced by TGF-β in fibroblasts (4, 61).

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 30-kDa catalytically active enzyme and a 18-kDa propeptide. This processing is mainly accomplished by the Zn-dependent PCP (54, 75) and to a lesser degree by the mammalian Tolloid-like-1 protein (74) and aminopeptidase B (35). PCP, a

Table 1. Characteristics of the different members of the LOX family

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Human Chromosome</th>
<th>mRNA and Protein Size</th>
<th>Highest mRNA Levels and Adult Tissue Distribution</th>
<th>Similarity to LOX, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX</td>
<td>5</td>
<td>4.8 kb 417 aa</td>
<td>Lung, skeletal muscle, kidney, heart</td>
<td>100</td>
</tr>
<tr>
<td>LOXL-1</td>
<td>15</td>
<td>2.4 kb 574 aa</td>
<td>Lung, heart, spleen, skeletal muscle, pancreas</td>
<td>85</td>
</tr>
<tr>
<td>LOXL-2</td>
<td>8</td>
<td>4.0 kb 774 aa</td>
<td>Lung, thymus, skin, testis, ovary</td>
<td>58</td>
</tr>
<tr>
<td>LOXL-3</td>
<td>2</td>
<td>3.3 kb 753 aa</td>
<td>Heart, uterus, testis, ovary</td>
<td>65</td>
</tr>
<tr>
<td>LOXL-4</td>
<td>10</td>
<td>3.5 kb 756 aa</td>
<td>Skeletal muscle, testis, pancreas</td>
<td>62</td>
</tr>
</tbody>
</table>

LOX, lysyl oxidase; LOXL, LOX-like; aa, amino acids.
member of the astacin family of enzymes, is a product of the Bmp1 gene (i.e., bone morphogenic protein-1) (33) that is highly expressed in the myocardium (26). Recent data suggest that the ECM glycoprotein fibronectin facilitates LOX processing by bringing PCP into close proximity or by altering the conformation of LOX proenzyme to make the cleavage site more accessible (16).

Recent data suggest that increased reactive oxygen species of mitochondrial origin stimulate LOX-dependent collagen cross-linking activity in cultured human fibroblasts (41). Although the increase in LOX activity was associated with enhanced mRNA and protein expression, a direct influence of reactive oxygen species on the active form of the enzyme has been proposed (41). Finally, homocysteine thiolactone, which occurs in mammalian systems as a metabolic by-product of methyl transfer from S-adenosylhomocysteine, is able to directly inhibit LOX activity through a direct covalent interaction with the enzyme carbonyl cofactor lysine tyrosylquinone (LTQ) (37).

Functions. LOX has been traditionally known for one function, the extracellular catalysis of lysine-derived cross-links in fibrillar collagens (and elastin). In fact, LOX catalyzes the oxidation of peptidyl lysine side chains of fibrillar collagens, resulting in the formation of corresponding allysine aldehydes (57, 64). These aldehydes can spontaneously form condensation products with each other or with a lysine residue, thereby cross-linking two fibrillar collagen proteins. The condensation products undergo further nonenzymatical rearrangements, resulting in the formation of stable end products like pyridoline or deoxypyridoline, allowing covalent cross-linking of insoluble collagen fibers in the ECM. Although from in vitro findings it has been proposed that the presence of cross-links determines the resistance of collagen fibers to their degradation by metalloproteinases, the hypothesis has yet to be verified in vivo (57).

It has been proposed that the oxidation process catalyzed by LOX occurs through a ping-pong kinetic mechanism (a two substrate reaction with no ternary complex formation) that involves the formation of a Schiff base with the enzyme cofactor LTQ followed by α-proton abstraction and reduction of the carbonyl cofactor (40). The formed imine intermediate is then hydrolyzed releasing the corresponding aldehyde. Finally, the reduced enzyme is reoxidized in the presence of molecular oxygen restoring the LTQ group and producing hydrogen peroxide and ammonia as by-products (53).

Recent reports have demonstrated novel roles for LOX, including its involvement in motility and migration in fibroblasts (50) and regulation of cell adhesion (20). In addition, LOX expression and activity have been observed in the cytoplasm and nucleus (29) and implicated in cell signaling and transcriptional gene regulation, as evidenced by the activation of the collagen III α1-promoter through LOX-induced binding of Ku antigen (18). Even more recent are the findings showing that the proregions of LOX, other than the catalytic domain, can bind to proteins, as has been reported for elastin (71).

Role of LOX in Myocardial Fibrosis

From the above considerations, it appears that LOX acts as a biological control point in the process of fiber-forming stiff collagen (73). In addition, the induction of LOX seems to be a general feature observed in a variety of fibrotic processes in different organs and tissues (42). Therefore, it can be hypothesized that the increased expression and/or activity of LOX can be critically involved in the increase of myocardial stiffness and the derangement of LV function that are associated with myocardial fibrosis in certain conditions characterized by both increased mechanical load and humoral injury of the myocardium (Fig. 2). A number of findings support this possibility.

Experimental findings. It has been reported that compared with normotensive Wistar-Kyoto rats, spontaneously hypertensive rats (SHRs) with LV hypertrophy (LVH) exhibited significant increases in the myocardial expression of LOX mRNA and protein (both the precursor and the active form) and collagen cross-linking and deposition (24) (Fig. 3). In addition, myocardial TGF-β-dependent signaling (as assessed by the
expression of phosphorylated Smad2, and 38- and 42-kDa connective tissue growth factor isoforms) was also significantly higher in SHRs than in Wistar-Kyoto rats (24). Of interest, all these histomorphological and molecular alterations were not found in the hearts of SHRs treated with a synthetic peptide (P144) that blocks the binding of TGF-β to its type III receptor or β-glycan (24), suggesting a role for this cytokine in LOX upregulation and overactivity present in this model of hypertensive cardiac fibrosis.

BALB/c mice and C57 wild-type (WT) mice were treated with 50 mg/l of N\textsubscript{G}-nitro-L-arginine methyl ester in their drinking water for 30 days (81). Blood pressure increased by 30% in the two strains of mice. However, LV stiffness significantly increased in the BALB/c mice and did not change in the C57 WT mice (81). The total myocardial collagen content, the degree of collagen cross-linking, and the activity of LOX significantly increased in the BALB/c mice and did not change in the C57 WT mice (81). These findings suggest that LOX-mediated collagen cross-linking is related to fibrosis and LV stiffness independently of blood pressure.

In the LV myocardium of C57BL/6 mice with metabolic syndrome induced by administering a high-fat, high-simple carbohydrate diet for 6 mo, there was an increase in the ratio of mature to proenzyme LOX, enhanced LOX activity, increased cross-linked collagen, and interstitial fibrotic tissue compared with the controls (82). This fibrotic response coincided with a marked increase in end-diastolic pressure, increased LV stiffness, and an impaired diastolic filling pattern (82). Therefore, LOX may be involved in the pathophysiology of metabolic syndrome-associated myocardial remodeling that underlies alterations of the LV mechanical properties and function in this syndrome.

Although rats (34) and mice (13) with organ Cu deficiency due to dietary Cu restriction exhibit concentric LVH, myocardial fibrosis and LV diastolic and systolic dysfunction, decreased papillary muscle passive stiffness, dynamic stiffness, and tensile strength have been reported in Cu-deficient rats (23). On the other hand, dietary Cu restriction has been shown to induce myocardial TGF-β overexpression and fibrosis in rats (12). Finally, mice with pressure overload secondary to aortic constriction and chronic HF exhibit myocardial fibrosis and increased LOX activity that are prevented in animals treated with a Cu supplement (27). Therefore, although contradictory data have been reported, the available evidence points to the possibility that myocardial LOX overactivity may contribute to Cu deficiency-induced cardiac disease.

An increased collagen content and cross-linking and a marked upregulation of LOX were observed in the remaining viable free wall of rats with surgically produced moderate to large transmural infarcts in the LV free wall (43). Therefore,
LOX may also play a role in the adverse structural remodeling occurring remote to myocardial infarction, which contributes to HF of ischemic origin.

**Clinical findings.** It has been reported that the expression of the active form of LOX is abnormally increased in the fibrotic myocardium of patients with hypertensive heart disease (HHD) and chronic HF (39). LOX was highly expressed in areas of interstitial and perivascular fibrosis, as well as in fibroblasts and cardiomyocytes in the myocardium of these patients (Fig. 3). Of interest, significant positive correlations were observed between LOX and collagen cross-linking and between collagen cross-linking and LV stiffness in patients with HHD and chronic HF (39). Furthermore, significant inverse correlations were found between LOX and the deceleration time and between collagen cross-linking and the deceleration time in these patients (39). Thus an upregulation of the enzyme can be involved in the altered mechanical properties and diastolic dysfunction of the hypertensive left ventricle via excessive collagen cross-linking. This possibility adds further support to the notion that in the failing heart, the quality of collagen (specifically cross-linking) plays a key role in translating quantity (e.g., fibrosis) into derangements of functional performance of the left ventricle (5). Interestingly, a relative excess of PCP, as assessed by the increase of PCP active form with respect to PCP zymogen, has been found in the myocardium of patients with HHD and chronic HF (38). It is thus tempting to speculate that the increase in LOX seen in these patients can be related to a PCP-mediated increased conversion of its precursor into the active enzyme.

An association of LOX with the degree of collagen cross-linking and the amount of insoluble collagen in the fibrotic myocardium of patients with aortic valve stenosis has been reported very recently in preliminary form (76). In addition, a direct association was also found between PCP and LOX in these patients (76). Of interest, collagen cross-linking was associated with LV stiffness (76). Albeit preliminary, these data suggest that the upregulation of the PCP-LOX axis may be involved in myocardial fibrosis and increased LV chamber stiffness present in patients with aortic stenosis.

It has been shown that LOX mRNA and protein expression were increased in the myocardium of patients with dilated cardiomyopathy (DCM) and end-stage HF compared with normal hearts (63). Of interest, the excess of LOX was associated with both an increased TGF-β expression and collagen content in DCM hearts (63). Since similar findings have been reported in mice with dystrophin-deficient cardiomyopathy also characterized by severe LV dilatation and failure (67), it can be suggested that LOX may be involved in the development of the fibrotic lesion that accounts in end-stage DCM.

**Emerging aspects.** There are seven single nucleotide polymorphism sites in the LOX coding region, including C225G, G409C, G473A, C476A, G816A, T924G, and A1135G. The G473A causes a change of an Arg at residue 158 to Gln (LOX Arg158Gln) and has the highest polymorphic frequency (31). The Arg158Gln is near the PCP cleavage site of the LOX precursor between Gly168 and Asp169 (72). As a result, LOX Arg158Gln could affect PCP cleaving efficiency and reduce the availability of the LOX active form. Recently, an association has been described between the G473A polymorphism and oral submucous fibrosis (a lesion characterized by fibrosis of the oral submucosa and the progressive tissue rigidity) in South Asian older males chewing areca nuts (62). Therefore, the possibility that LOX-dependent cardiac fibrosis is genetically determined requires further investigation.

**Therapeutic Modulation of LOX in Cardiac Diseases**

Despite that myocardial fibrosis is recognized as a lesion with a major detrimental impact on cardiac function, relatively few therapies directly target the mechanisms of this significant disease modifier. In this regard, strategies that act on the enzymatic steps of collagen metabolism present clear-cut therapeutic opportunities to modulate myocardial fibrosis. In particular, in the last few years, LOX has emerged as an attractive pharmacological target because of its involvement in myocardial fibrosis (60) (Table 2).

<table>
<thead>
<tr>
<th>Compounds tested at the preclinical level</th>
<th>TGF-β, transforming growth factor-β; AGE, advanced glycation end product.</th>
</tr>
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<tbody>
<tr>
<td>P144 (TGF-β type III receptor β-glycan-derived synthetic peptide)</td>
<td></td>
</tr>
<tr>
<td>Alagebrium (AGE-mediated cross-link breaker)</td>
<td></td>
</tr>
<tr>
<td>Hydroxamates (procollagen C proteinase inhibitor)</td>
<td></td>
</tr>
<tr>
<td>Compounds tested at the clinical level</td>
<td></td>
</tr>
<tr>
<td>Torasemide (loop diuretic)</td>
<td></td>
</tr>
<tr>
<td>Alagebrium (AGE-mediated cross-link breaker)</td>
<td></td>
</tr>
<tr>
<td>Succinyl hydroxamate UK-383367 (procollagen C proteinase inhibitor)</td>
<td></td>
</tr>
</tbody>
</table>

Experimental data. Although some drugs interfering with TGF-β expression (i.e., pirfenidone and tranilast) have shown promising antifibrotic effects in animal models of hypertension (30, 44), no evidence has been provided yet that this is due to the reduced expression of LOX. In contrast, the chronic in vivo blockade of the TGF-β type III receptor β-glycan with the peptide P144 has been shown to be associated with the reduced expression of LOX mRNA and the precursor and active form of LOX protein, as well as with the decrease in collagen cross-linking and deposition in the myocardium of treated SHRs (24). Interestingly, the administration of P144 was not accompanied by either toxic or immunological alterations, suggesting that β-glycan-derived peptides may represent a new strategy to downregulate LOX and prevent myocardial fibrosis in conditions such as hypertension.

AGEs can contribute to increased myocardial stiffness and altered LV function through the nonenzymatic glycation of lysine and hydroxylysine residues in collagen types I and III molecules (83). In addition, as mentioned previously, AGEs may induce the LOX gene and protein expression (51), thus also facilitating LOX-dependent collagen cross-linking. Under this perspective, the results of recent studies clearly demonstrated that ALT-711 or alagebrium, a breaker of AGE-related protein cross-links, ameliorated the adverse cardiac changes associated with diabetes and hypertension. In fact, whereas in diabetic animals, ALT-711 improved LV function, decreased myocardial collagen content, and improved its solubility by reducing its cross-linking (6), in older spontaneously hypertensive rats, it reduced LV mass and collagen content (68). Whether alagebrium acts directly on glycated collagen cross-links or whether its activity depends on the blockade of the ability of AGEs to induce LOX remains to be determined.

The blockade of LOX extracellular processing by PCP could be an alternative approach to act on the enzyme. Chemical
inhibitors of PCP activity that are nontoxic to cells in culture have been developed (9). These inhibitors are hydroxamate derivatives of diamino acid (10), glutamic acid (58), and succinic acid (2) that bind specifically to the Zn atom in the active site of PCP and thereby inhibit enzyme activity. Interestingly, some succinyl hydroxamates have shown anti-infective properties in either a cell-based model of collagen deposition (fibroplasia model) or in a rabbit model of cutaneous scarring (2).

Direct LOX inhibitors include primary amines that react with the carbonyl group of the active site of the enzyme. Therefore, LOX is irreversibly inhibited by aminonitriles such as β-aminopropionitrile (BAPN), postulated as an active site-directed inhibitor that forms a covalent adduct with the enzyme (69). It has been shown that treatment with BAPN decreases collagen cross-linking and myocardial stiffness in the left ventricle of normal adult pigs (32). Vicinal diamines (a certain kind of halogenated alky alamines), hydralazines, and aminoalkylaziridines with strict structural requirements are also inhibitors of LOX (17, 47, 48). Taurine, niacin, heparin, and 2-mercaptopyridine-N-oxide have been also reported as LOX inhibitors in vitro (1) and in vivo (3). Finally, a number of homocysteine thiolactones analogs also inhibit LOX activity via their interaction with the LTQ carbonyl cofactor (37). The major limitation of all these compounds is their in vivo toxicity. For instance, the administration of BAPN to animal models causes lathyrism, a disorder characterized by an extensive damage of connective tissues (66). Taking this into account, the development of cardiac-targeted LOX direct inhibitors could prove to be more efficient.

Clinical observations. López et al. (39) have reported that the myocardial expression of the active form of LOX, collagen cross-linking, and the amount of fibrosis, as well as LV stiffness, decreased in patients with HHD and HF after 8 mo of treatment with the loop diuretic torasemide in addition to standard HF therapy (39) (Fig. 4). On the other hand, furosemide failed to produce these changes (39). Interestingly, it has been reported that torasemide, but not furosemide, reduced the relative excess of PCP in the myocardium of patients with HHD and HF (38) (Fig. 4). In addition, it is interesting to remark that whereas torasemide possesses a Zn-binding region, furosemide does not (8). Therefore, torasemide can behave as the hydroxamic acid derivatives that inhibit PCP activity by binding to the Zn atom in the active site of the enzyme. Thus, although the LOX precursor was not analyzed in the study by López et al. (39), the possibility exists that the reduction in LOX observed after treatment with torasemide can be related to the ability of this compound to blockade PCP activity and blunt the conversion of the LOX precursor in its mature form.

Alternatively, it has been reported that TGF-β expression is reduced in the myocardium of torasemide-treated rats compared with control rats (77, 78). Therefore, although LOX mRNA was not assessed by López et al. (39), the possibility also exists that torasemide can interfere with a TGF-β-dependent LOX upregulation in the myocardium of patients with HHD and HF. Finally, the possibility that prostaglandin E2 may be involved in the ability of torasemide, but not furosemide, to reduce LOX expression is not probable, taking into account that the two compounds similarly stimulate the prostanoid in humans (11).

The cardiac effects of the AGE-cross-link breaker alagebrum have been investigated in small and open label studies performed in patients with diastolic dysfunction. In one study, alagebrum reduced LV mass and improved diastolic function in elderly patients with diastolic HF (35). In another study, alagebrum improved diastolic function and LV geometric remodeling in patients with systolic HF, although these results have been only published as an abstract (70), and they are pending to be confirmed in a larger ongoing study (80). As mentioned above, it would be of interest to ascertain whether LOX is or is not directly involved in these effects of alagebrum in humans.

Based on the available experimental information on the antifibrotic effects of the PCP inhibitors succinyl hydroxamates, further study is needed on UK-383367 as a candidate for evaluation in clinical studies as a topically applied, dermal anti-scarring agent (2).

Conclusions and Perspectives

Progress has been made in the last years in developing an integrated understanding of extracellular collagen biosynthesis.
and deposition that includes analyses of extracellular enzymatic-driven processes. In the context of understanding the regulation of myocardial collagen deposition, additional information is still needed regarding the role of LOX in different biological contexts. In addition, further studies are needed regarding the contribution of LOX to perturbations of collagen cross-linking and deposition present in cardiac diseases evolving with myocardial fibrosis. 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 cardiac diseases. Finally, a deeper knowledge of LOX structure/function as well as the underlying mechanisms involved in myocardial LOX dysregulation is also required to generate effective therapeutic strategies targeting the enzyme to prevent the consequences of its excessive profibrotic activity in HF patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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