Myocardial lysyl oxidase regulation of cardiac remodeling in a murine model of diet-induced metabolic syndrome

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Zibadi S, Vazquez R, Moore D, Larson DF, Watson RR. Myocardial lysyl oxidase regulation of cardiac remodeling in a murine model of diet-induced metabolic syndrome. Am J Physiol Heart Circ Physiol 297: H976–H982, 2009. First published July 10, 2009; doi:10.1152/ajpheart.00398.2009.—Metabolic syndrome (MetS) represents an increased risk of cardiovascular disease. Although its individual components adversely affect cardiac structure and function, the extent to which multiple components of MetS affect the cardiac extracellular matrix (ECM) has not been well characterized. Lysyl oxidase (LOX) is one of the cardiac ECM-modifying enzymes that catalyze the formation of collagen cross-linking. Our objective was to define the effect of diet-induced MetS on the LOX enzyme. MetS was induced in male C57BL/6 mice by administering a high-fat, high-simple carbohydrate diet for 6 mo. Gene expression was determined by real-time PCR. The cardiac protein expression and enzymatic activity of LOX were measured. The severity of fibrosis was assessed by histology and hydroxyproline assay. Cardiac diastolic function was assessed by in vivo analysis of the pressure-volume relationship. LOX, matrix metalloproteinases, and their tissue inhibitors were assessed by in vivo analysis of the pressure-volume relationship. LOX, matrix metalloproteinases, and their tissue inhibitors were analyzed, and of these three, LOX was most significantly changed in the MetS mice. Despite the blunted gene expression of LOX isoforms, MetS mice demonstrated a significant upregulation of bone morphogenetic protein-1. Correspondingly, there was an increase in the ratio of protein expression of mature to proenzyme LOX by 25.9%, enhanced LOX activity by 50.0%, and increased cardiac cross-linked collagen compared with the controls. This fibrotic response coincided with a marked increase in end-diastolic pressure, increased left ventricular stiffness, and impaired diastolic filling pattern. Our data signify that diet-induced MetS alters the remodeling enzymes, mainly LOX, thereby altering ECM structure by increasing the amount of cross-linking and inducing diastolic dysfunction.

THE METABOLIC SYNDROME (MetS) represents a clustering of cardiovascular risk factors affecting about 22% of the adult population and over 40% of those aged 50 and older in industrialized countries (12). Its key features include abdominal obesity, insulin resistance, hypertension, and dyslipidemia. These factors act synergistically to increase the risk of adverse cardiovascular events and are associated with high cardiovascular morbidity and mortality (20, 28). Arterial hypertension leads to myocardial fibrosis and hypertrophy, resulting in diastolic dysfunction (18). Furthermore, insulin resistance, obesity, and atherosclerosis contribute to the development of left ventricular remodeling and diastolic dysfunction (8, 19, 32). Recent studies support the association between MetS and diastolic dysfunction (9, 15). The coexistence of systolic and diastolic dysfunction in MetS patients has also been observed (5, 37).

Diastolic dysfunction, an abnormality seen early in MetS (9, 15), is associated with excessive and stiffened cardiac extracellular matrix (ECM) and myocardial fibrosis, resulting in impaired ventricular relaxation and diastolic filling pattern. Interstitial fibrosis is characterized by an increased deposition of ECM components due to production overwhelming the degradation. Among the ECM-modifying enzymes, lysyl oxidases (LOXs) are central to ECM alternation, since these enzymes catalyze a key step in the cross-linking of collagen and elastin. The formation of collagen cross-links contributes to increased ventricular stiffness and reduced compliance by stabilizing ECM against enzymatic degradation (3).

Although myocardial fibrosis and diastolic dysfunction have been reported in MetS, characteristic changes in the expression and activity of LOX remained unclear. Hence, the present study focuses on the effects of diet-induced MetS on cardiac ECM remodeling, especially LOX and cross-linked collagen.

MATERIALS AND METHODS

Animal model. This study protocol was approved by the Animal Review Committee at The University of Arizona. The procedures in the “Guidelines for the Care and Use of Laboratory Animals and Principles of Laboratory Animal Care” were followed in this study. Male C57BL/6 mice were purchased from Jackson (Bar Harbor, ME) at 4 wk of age and maintained under standard conditions. Mice (n = 8/group) were fed for 6 mo either a control diet (7% fat, 20% protein, 59.2% carbohydrate, F5371, BioServ, Frenchtown, NJ) or a high-fat, high-simple carbohydrate (HFHSC) diet (35% fat, 20% protein, 35.4% simple carbohydrate, BioServ, F5366) that included 35% of calories from lard oil, which is rich in saturated and monounsaturated fatty acids. Fatty acid analysis revealed that the HFHSC diet contained 36.9 mg ω-6 fatty acids/g diet, in a ratio of 102.6:1 to ω-3 fatty acids (Table 1). Mice were fed ad libitum. Food intake and body weight were measured at baseline and on a monthly basis. After 6 mo of treatment, all of the mice were evaluated with the transthoracic echocardiogram and the Millar conductance catheter system and tissues were harvested for genome and structural characterization.

Metabolic assessment. Mice were fasted for 6 h before blood draws. Arterial blood (0.8 ml) was obtained by direct cardiac puncture under anesthesia. Serum concentrations of total cholesterol and triglyceride were determined by enzymatic colorimetric methods, performed by the Department of Pathology laboratory at The University of Arizona. Glucose was measured in blood using an Accu-Chek Comfort Curve Kit from Roche Diagnostics (Indianapolis, IN). Serum insulin level was measured with an ELISA kit from Linco Research (St. Charles, MO).

Blood pressure measurement. Systolic blood pressure in unanesthetized mice was measured by the noninvasive tail-cuff method using a XBP1000 computerized mouse-tail blood pressure system (Kent Scientific, Torrington, CT). Systolic blood pressure values are an average of three consecutive measurements.
Table 1. Fatty acid composition of diets

<table>
<thead>
<tr>
<th>Fatty acid composition</th>
<th>Diet</th>
<th>HFHSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total saturated</td>
<td>28.5</td>
<td>142.3</td>
</tr>
<tr>
<td>Total monounsaturated</td>
<td>35.7</td>
<td>178.5</td>
</tr>
<tr>
<td>Total PUFAs</td>
<td>7.5</td>
<td>37.3</td>
</tr>
<tr>
<td>Total ω-6 PUFAs</td>
<td>7.4</td>
<td>36.9</td>
</tr>
<tr>
<td>Total ω-3 PUFAs</td>
<td>0.07</td>
<td>0.36</td>
</tr>
<tr>
<td>w-6/w-3 PUFAs</td>
<td>102.6</td>
<td>102.6</td>
</tr>
</tbody>
</table>

Fatty acid content is given in milligram per gram diet. HFHSC, high-fat, high simple carbohydrate; PUFAs, polyunsaturated fatty acids.

**Microsurgical methods and in vivo hemodynamics measurements.** The Millar conductance catheter system was used as has been described previously by our laboratory (39, 42). All of the mice were anesthetized with urethane in saline (1,000 mg/kg ip) and a chloralos in propylene glycol (50 mg/kg ip) and ventilated. The external jugular vein was cannulated for volume administration. The apical portion of the heart and the inferior vena cava were exposed through a substernal-transverse incision. A high-fidelity 1.4-Fr transducer catheter (Millar Instruments, Houston, Texas) was inserted into the left ventricle through the apex. Pressure-volume (P-V) loops were acquired, and hemodynamic parameters were computed using PVAN software, as reported previously (42).

**RNA extraction and real-time polymerase chain reaction.** Candidate gene expression in cardiac tissue was measured using real-time PCR as described in detail elsewhere (42). Briefly, purified RNA was converted into cDNA using the Superscript protocol (Invitrogen, Carlsbad, CA). Diluted cDNA was used as the template to quantify the relative content of mRNA, using QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) and the Rotor-Gene RG-6000 with a 72-well rotor (Corbett Research, Brisbane, Australia). The candidate gene threshold cycle numbers were normalized by the respective β-actin (BC040513) gene. The level of expressions was expressed as a fold change in the expression of candidate genes relative to the control mice gene expression. Custom primers were designed using the Primer3 and synthesized by Integrated DNA Technologies. The following candidate genes have been investigated: matrix metalloproteinase (MMP) genes, pro-MMP-2 (NM_008610) and pro-MMP-9 (NM_013599); tissue inhibitor of MMP (TIMP) genes; TIMP-2 (NM_011594.3) and TIMP-4 (NM_008639); LOXs genes, LOX (NM_010728) and LOXL-3 (NM_013586); and BMP-1 (NM_009755.2).

**Western blot analysis of LOX.** Cardiac tissues were homogenized in cold CellLytic mammalian tissue lysis/extraction reagent (Sigma-Aldrich) and quantified using the Bio-Rad protein assay. Twenty micrograms of proteins were applied onto a NuPage 10% Bis-Tris Gel (Invitrogen), separated at 200 V for 50 min using NuPAGE MOPS SDS running buffer (Invitrogen), and subsequently transferred onto polyvinylidene difluoride membranes. The membranes were incubated in a blocking buffer containing 5% BSA (Sigma-Aldrich) in TBS with 0.1% Tween-20 (TBS-T) at room temperature for 1 h. The membranes were probed with rabbit polyclonal anti-LOX (1:32,000, Novus Biological) in 1× TBS-T overnight at 4°C and washed three times in TBS-T. The corresponding anti-rabbit secondary horseradish peroxidase-conjugated antibody was from Calbiochem. The reactions were developed with ECL Western blotting detection reagents (GE Healthcare). The images were obtained by exposure to X-ray films, and the band densities were analyzed by Quantity One 4.6.2, Basic (Bio-Rad). For normalization, β-actin antibody was used as a loading control.

**Determination of LOX activity.** LOX activity was measured by a high-sensitive fluorescent assay modified from the report of Palama-kumbura and Trackman (30). Briefly, 25 mg of cardiac tissue were homogenized in 300 μl of CellLytic (Sigma) at −80°C and reconsti-tuted to 1 ml with LOX buffer [1.2 mol/l urea and 50 mmol/l sodium borate (pH 8.2)] and then centrifuged at 12,000 g for 10 min. After the collection of the supernatant, LOX enzymatic activity was detected by the production of hydrogen peroxide from alkyl monoamine substrate and detected with fluorescent resorufin produced by horseradish peroxidase-catalyzed oxidation of N-acetyl-3,7-dihydroxyphe-noxazine (Amplex red) at wavelengths 563 and 587 nm. Parallel unknown samples were prepared by adding 500 μmol/l β-aminopropionitrile fumarate to completely inhibit the activity of LOX. The emission intensity difference was converted into the amount of hydrogen peroxide produced by the action of LOX when compared with the fluorescence of a nanomoles hydrogen peroxide standard plot.

**Determination of MMP activity.** Gelatin zymography assay was performed according to the method described by Zibadi et al. (42). Briefly, 10 mg of cardiac tissue were homogenized in 1 ml of zymograph extraction buffer, and the extraction supernatants were applied to precast 10% polyacrylamide gel Zymogram (Novex). After activation and development, the bands were quantified with image analysis software (Bio-Rad GS-800). Determination of myocardial total and cross-linked collagen. Hydroxyproline is an amino acid found exclusively in connective tissues and is used as a means to quantify collagen concentration. For measurement of total myocardial hydroxyproline, dried left ventricular samples were hydrolyzed in 6 N HCl for 72 h at 120°C followed by neutralization with 2.5 M NaOH. Hydroxyproline levels were then quantified by comparison with a standard colorimetric curve of transhydroxyproline (Sigma) according to the methods of Stegeman and Stadler (35). Collagen cross-linking was determined using cyanogen bromide digestion according to Woodiwiss et al. (38), which is a modification of that originally described by Mukherjee and Sen (26). Dried left ventricular samples were homogenized in PBS and then centrifuged at 4,000 g for 10 min. The resulting pellet was then washed in 2% SDS three times to remove noncollagenous proteins, each time followed by centrifugation at 4,000 g for 10 min. The 2% SDS was removed by three successive washes in PBS followed by two washes in acetone. After the second acetone wash, the resulting pellet was air dried and resuspended in 20 mg/ml cyanogen bromide in 70% formic acid and incubated at 25°C for 24 h. After incubation, the suspension was centrifuged at 4,000 g for 20 min. The resulting supernatant was dried using a speed vacuum followed by the measurement of hydroxyproline as described above. Percent cross-linking was determined by comparing the total hydroxyproline with cyanogen bromide-soluble hydroxyproline. The data were expressed as microgram of collagen per milligram of dry heart weight, assuming that collagen contains an average of 13.5% hydroxyproline.

**Cardiac tissue histology.** Cardiac fibrosis was evaluated by standard Picosirius red-staining procedures, performed by the Department of Pathology laboratory at The University of Arizona.

**Statistics.** The data are expressed as means ± SE. Statistical analyses were performed with SPSS software (SPSS, Chicago, IL). Unpaired Student’s t-test (two tailed) was used for the comparison of two groups when the data met the assumptions of the t-tests. Comparable nonparametric test (Mann-Whitney test) was substituted when tests for normality and equal variance failed. A value of P <0.05 was used as a criterion for statistical significance.

**RESULTS**

**Metabolic profile.** Table 2 summarizes the metabolic characteristics of the mice fed the MetS-inducing diet. The treatment with the HFHSC diet was associated with a significant increase in fasting serum levels of total cholesterol, triglyceride, and glucose by 59.7, 58.6, and 72.3%, respectively, compared with those of the controls. The HFHSC diet resulted in a significant fourfold increase in insulin concentration compared with that of controls (P <0.05). Moreover, there was a marked
increase in tail systolic blood pressure as well as body weight in HFHSC-fed mice ($P < 0.0001$). Thus the HFHSC-fed mice developed a spectrum of biochemical and metabolic abnormalities that are hallmarks of MetS.

**Hemodynamic parameters and diastolic function.** With the use of a conductance catheter P-V loop analysis, the end-diastolic volume was found to be significantly reduced, whereas there was a marked increase in the end-diastolic pressure in HFHSC-fed mice ($P < 0.05$, Fig. 1, A and B). Figure 1C shows a steeper slope of the end-diastolic P-V relationship ($P < 0.05$) in the HFHSC group (increased by 71.6%, $P < 0.01$). This change suggests an increased ventricular stiffness and reduced compliance. Correspondingly, there is a repressed left ventricular filling rate ($dV/dt_{max}$, $P < 0.01$), a characteristic of an abnormal filling phase of the cardiac cycle (Fig. 1D). These changes were accompanied by a consistent leftward and upward shift of the left ventricle P-V loop (Fig. 1E).

**Cardiac LOX gene and protein expression and activity.** Cardiac LOX and LOXL-3 mRNA levels remained unchanged in HFHSC-fed mice compared with the controls. However, there was a significant increase in gene expression of bone morphogenetic protein-1 (BMP-1; 1.8-fold, $P < 0.05$), which process pro-LOX, yielding the mature enzyme (35) (Fig. 2A). Western blot analysis revealed two immunoreactive bands corresponding to the proenzyme (50 kDa) and the mature (30 kDa) forms of LOX. As shown in Fig. 2B, the ratio of protein expression of mature LOX to pro-LOX was $25.9\%$ greater in HFHSC-fed mice ($P < 0.05$), suggestive of the increased activation of the latent pool of pro-LOX by BMP-1. Correspondingly, HFHSC-fed mice displayed a $50.0\%$ increase in LOX enzymatic activity compared with controls ($P < 0.05$, Fig. 2C).

**Cardiac MMPs gene expression and activity.** There was a nonsignificant trend toward a lower gene expression ratio of MMPs to TIMPs in HFHSC-fed mice compared with controls (Fig. 3A). Notably, HFHSC-fed mice exhibited significantly
lower MMP-2 (62 kDa) activity when compared with the controls ($P < 0.01$), supporting the reduced collagen degradation rate and therefore the resultant increase in ventricular stiffness. A similar decreasing trend in pro-MMP-2 activity was detected, which was not significantly different compared with that of the controls (Fig. 3, B and C).

Myocardial total and cross-linked collagen characteristics. The fibrotic response to diet-induced MetS was assessed by histology and hydroxyproline assay. The Picosirius red staining of cardiac sections revealed marked fibrosis in the myocardium of HFHSC-fed mice (Fig. 4A). Figure 4B demonstrates the ventricular collagen content measured by hydroxyproline assay. Total cardiac collagen content increased by 44.9% in HFHSC-fed mice compared with the controls ($P < 0.05$). The fibrillar collagen cross-linking in the HFHSC-fed mice was 68.1% more than that in controls ($P < 0.05$). These findings coincided with a moderate decrease in MMP-2 activity and a marked increase in LOX activity, resulting in a reduction of collagen degradation and a cumulative increase in the total and cross-linked collagen. An alteration in cross-linking in HFHSC mice explains the changes in diastolic function.

DISCUSSION

The relationship between MetS and cardiovascular disease has been unequivocally established clinically and in experimental models (5, 37). Despite these observations, the role of...
ECM-modifying enzyme LOX in MetS-associated cardiac ECM remodeling remains unclear. The C57BL/6 mouse strain has been used as a model for studies of diet-induced obesity, diabetes (27), atherosclerosis (21, 33), and hypertension (24). Although the diets used to induce these individual disorders are all high in fat, the amount of fat and the fatty acid composition differ significantly between diets. In the current study we show that treatment with a HFHSC diet for 6 mo in male C57BL/6 mice induced hyperinsulinemia, hyperglycemia, dyslipidemia, and hypertension, which resemble the metabolic disorders seen in patients with MetS. The present study used this model to investigate the impact of diet-induced MetS on ECM components and diastolic function.

Myocardial ECM contains fibrillar collagen that ensures the structural and functional integrity of the adjoining cardiomyocytes. Cardiac ECM is not a static structure rather a dynamic entity crucial to myocardial adaptation to pathological stress. A disruption of ECM homeostasis results in maladaptive cardiac remodeling, diastolic dysfunction, and finally heart failure. We demonstrated the induction of cardiac interstitial fibrosis in male mice after 6 mo of treatment with a HFHSC diet, as evident by the increased collagen deposition and the degree of cross-linking. This observation was in agreement with earlier data (1), where treating female rats with a high-fat diet for 8 wk was associated with extensive myocardial perivascular fibrosis. Our data indicated that the fibrotic response was not associated with significant changes in the expression of profibrotic genes. However, diet-induced MetS was associated with moderately suppressed MMP-2 activity and a significantly increased LOX activity and a subsequent increase in cardiac fibrillar collagen and the extent of cross-linking, resulting in pathological fibrosis. Notably, cardiac function evaluated by P-V loop analysis showed an impaired diastolic filling and diastolic dysfunction.

One of the key enzymes that contributes to the perturbation of ECM homeostasis is the LOX enzyme, which was affected by diet-induced MetS (Fig. 2). The dysregulation of LOX could underlie the onset and progression of fibrotic pathologies (34). LOX is a novel copper-containing amine oxidase that initiates the covalent cross-linking of collagen and elastin in ECM. LOX is known to be synthesized as a preproenzyme, secreted as a 50-kDa N-glycosylated proenzyme. It is then proteolytically cleaved by BMP-1 to the 30-kDa catalytically active mature enzyme (34). Recent molecular cloning has revealed the existence of a LOX family consisting of LOX and four LOX-like proteins, with a complex tissue-specific expression pattern and a great variation in mRNA levels. Among three LOX and LOX-like proteins highly expressed in the cardiovascular system (LOX, LOXL-2 and -3), LOX is the most abundant form in the heart and the only one that uses collagen as a substrate (25, 30). LOX-L-2 has been shown to be abundant in the early stage of cardiac development in the fetal heart (25), whereas LOXL-3 mRNA expression is almost exclusively restricted to the adult aorta (25). Our data demonstrated that despite the blunted gene expression of LOX isoforms, there was an increase in the ratio of protein expression of mature to proenzyme form of LOX and its enzymatic function. Thus diet-induced MetS was associated with nonsignificant changes in the mRNA levels of LOX, which could be due to the epigenetic silencing of LOX transcription (6). However, there was a significant increase in BMP-1 gene expression, which explains the increased proteolytic processing of the latent pool of pro-LOX and, therefore, the increase in the enzymatic activity in the HFHSC-fed mice, despite the unchanged mRNA level. However, BMP-1 itself undergoes posttranslational modification including cleavage of prodomain and glycosylation to convert to the functional proteinase (14). Factors that regulate BMP-1 gene expression and its posttranslational modification have not been clearly defined in the literature. We also demonstrated that increased LOX activity parallels the increased fibrillar collagen cross-linking and ventricular stiffness in mice with diet-induced MetS (P <0.05). Moreover, we showed that LOX enzymatic activity positively correlates with the slope of the end-diastolic P-V relationship (B), an index of ventricular stiffness (R² = 0.78, Fig. 2D). Increased collagen cross-linking has also been shown to correlate with the left ventricular stiffness (2, 40).

It is unclear whether the HFHSC diet used to induce MetS can provoke a direct effect on cardiac LOX. However, individual components of MetS, such as hypertension (40) and diabetes (22), are associated with an increased LOX-mediated cross-linking. MetS is often associated with excessive activity of angiotensin II (10). Angiotensin II increases the levels of LOX mRNA and secreted enzyme activity in rat vascular smooth muscle cells (16). Transforming growth factor-β (TGF-β), which is overexpressed in MetS (4), also increases LOX expression and activity (29). Interestingly, TGF-β concomitantly upregulates the production of fibronectin (17), which is involved in LOX proteolytic activation (11), a mechanism that could participate in the induction of LOX by TGF-β. Furthermore, serum levels of several members of the fibroblast growth factor family, another regulator of LOX expression (7), are increased in MetS (13, 41).

Left ventricular hypertensive remodeling has polymorphic characteristics, including interstitial fibrosis, which adversely
increases myocardial stiffness, leading to an impairment of diastolic function (18). Furthermore, hypertensive remodeling is associated with an increased LOX enzymatic activity and a LOX-mediated cross-linking (40). Therefore, the observed interstitial fibrosis and diastolic dysfunction in diet-induced MetS might be partially attributable to hypertension. The major limitation of this pilot study was the absence of a comparison group with antihypertensive therapy to elucidate the contribution of hypertension to the observed effects. As to the current knowledge, it is not clear to what extent this cardiac ECM remodeling can be caused by hypertension or other individual components of MetS. Another weakness of this study was the lack of intermediate time points. Because of our focus on LOX enzymatic pathway and the limited number of cardiac tissues available, we were only able to evaluate MMP and TIMP gene expressions and the enzymatic activity of MMPs, which only showed a moderate suppression of MMP-2 activity. Moreover, we faced difficulty in obtaining reliable transthoracic echocardiographic measurements of the overweight HFHSC-fed mice, possibly due to an increased pericardial and abdominal adipose tissue. Therefore, we focused only on in vivo P-V loop analysis. Furthermore, the effect of anesthetics on catecholamine overflow could have resulted in vascular depression and a fall in blood pressure, as observed in P-V loops obtained from both groups.

We also studied the effect of diet-induced MetS on MMP, another ECM-modifying enzyme. ECM degradation is regulated by MMPs and their endogenous inhibitors TIMPs. Clinical studies and experimental models of cardiac fibrosis have reported alterations in the balance between the MMPs and TIMPs, especially at intermediate time points in the progression to cardiac failure (23). In the present study, there was a nonsignificant trend toward a lower ratio of MMP to TIMP gene expressions in the left ventricular tissue of HFHSC-fed mice. The zymogram revealed that the proteolytic activity of MMP-2 was moderately suppressed in diet-induced MetS (Fig. 3), suggesting that ECM degradation was diminished. The classical model for the cell surface activation of pro-MMP-2 is through the formation of a trimolecular complex comprising membrane type-1 MMP, TIMP-2, and pro-MMP-2 (36). As TIMP-2 mRNA level remained unchanged, the observed decreased activity of MMP-2 may have arisen from the reduced processing of pro-MMP-2 by membrane type-1 MMP.

In summary, we demonstrated that diet-induced MetS is associated with an increased left ventricular collagen cross-linking and interstitial fibrosis, and diastolic dysfunction. We suggest that increased proteolytic processing of LOX and its activity may be a determinant in the pathophysiology of MetS-associated ECM remodeling. LOX may thus represent a target for the reduction of stiff collagen and an improvement of the left ventricular mechanical properties in patients with MetS. However, for a deeper understanding of the role of LOX in MetS-induced ECM remodeling, further structural-functional studies using other models of MetS are warranted.

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