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Short communication

# Lysyl oxidase resolves inflammation by reducing monocyte chemoattractant protein-1 in abdominal aortic aneurysm

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ABSTRACT

Lysyl oxidase (LOX) is an enzyme critical for the stability of extracellular matrix and also known to have diverse biological functions. Little is known, however, about the role of LOX in regulating inflammation. Here we demonstrate that LOX suppresses secretion of monocyte chemoattractant protein-1 (MCP-1) in cultured vascular smooth muscle cells. Furthermore, enhancement of LOX activity reduces MCP-1 in a mouse model of abdominal aortic aneurysm (AAA), thereby preventing macrophage infiltration and AAA progression. These findings suggest that LOX has a novel function in resolving inflammation by reducing MCP-1 in AAA.



## 1. Introduction

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that catalyzes the crosslinking of collagens and elastin, and is thus critical for the stability of the extracellular matrix (ECM). Many lines of evidence have shown that LOX has diverse biological functions, including regulation of gene transcription, cell migration, adhesion and transformation [1–3]. Little is known, however, regarding the role of LOX in regulating inflammation.

Previously we found that inflammatory signalling through c-Jun N-terminal kinase (JNK) inhibits LOX activity in vascular smooth muscle cells (VSMCs), and demonstrated that JNK inhibition induced regression of experimental abdominal aortic aneurysms (AAA) [4] that are characterized by chronic inflammation and ECM degradation [5]. We further showed that overexpression of LOX prevents AAA expansion in mice [4]. In addition, inhibition of LOX by β-aminopropionitrile fumarate (BAPN) in rat elastase-induced AAA frequently results in aortic dissection, which is another manifestation of ECM degradation [6]. Together these findings indicate that LOX plays an important role in stabilizing the ECM in AAA. In the present study, we investigated another possibility that LOX might regulate not only ECM stability but also inflammatory responses in cultured VSMCs and in the mouse model of AAA.

## 2. Materials and methods

### 2.1. Adenoviral vectors

Adenoviral vectors encoding LOX with a C-terminal HA epitope tag (Ad-LOX), β-galactosidase (lacZ) gene with a nuclear localizing signal (Ad-LacZ), constitutively active MKK7 (Ad-caMKK7), and wild type JNK1 (Ad-JNK1) were prepared as previously described [4,7,8].

### 2.2. Cell culture experiments

VSMCs were obtained from the aortae of 8-week-old male Wistar rats as described previously [9]. All experiments were performed using cells at passage 3. Confluent VSMCs were serum-starved for 24 h and then treated with 200 μM β-aminopropionitrile fumarate (BAPN, Sigma) for inhibition of LOX activity. For overexpression of LOX, VSMCs were transfected with Ad-LOX for 24 h prior to serum starvation. Ad-LacZ was used at the same multiplicity of infection as a control. For specific activation of the JNK pathway, VSMCs were cotransfected with Ad-caMKK7 and Ad-JNK1.

### 2.3. Cytokine array

The conditioned media was analyzed with the cytokine array membrane (Rat Cytokine Antibody Array, Ray Biotech), according to the manufacturer's instructions.

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### 2.4. ELISA analysis

The concentration of monocyte chemoattractant protein-1 (MCP-1) in the conditioned media was quantified by a sandwich enzyme immunoassay technique using the rat MCP-1 ELISA Kit (Pierce), according to the manufacturer's instructions.

### 2.5. LOX activity assay

The cell lysates were subjected to the fluorometric assay for LOX enzyme activity as previously described [4,10].

### 2.6. Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from VSMCs using RNeasy (Qiagen). mRNA was quantified by the LightCycler Instrument (Roche Applied Science) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and QuantiTect Primer Assay (Qiagen). MCP-1 transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

### 2.7. Animal experiments

AAA was induced in mice by periaortic application of 0.5 M $CaCl_2$ as previously described [4,11]. We performed adenoviral gene transfer with 20 μl of Ad-LOX ($n=8$) or Ad-LacZ ($n=5$) at the same titer ($1.5\times10^9$ PFU/ml) 3 weeks after $CaCl_2$ treatment as previously described [4]. After an additional 3 weeks, mice were sacrificed and perfusion-fixed with 3.7% formaldehyde/PBS for histological analyses. For immunostaining of MCP-1, mice were sacrificed 3 days after adenoviral gene transfer and excised aortae were frozen with Tissue-Tek OCT compound (Sakura Finetek). The animal experimental protocols were approved by the Animal Review Board of Yamaguchi University School of Medicine.

### 2.8. Histological analysis

Paraffin-embedded sections were stained with hematoxylin–eosin (HE) and elastica van-Gieson (EVG) stains for histologic analysis and antibodies to appropriate antigens were used for immunohistochemistry as previously described [4,12]. Phosphorylated JNK (P-JNK) was detected with anti-phospho-specific JNK antibody (Promega). MCP-1 was detected with an anti-mouse MCP-1 antibody (R&D Systems) using frozen sections. These proteins were visualized by an avidin–biotin complex technique. Macrophages were detected with an anti-mouse Mac-3 antibody (BD Biosciences) and visualized by indirect immunofluorescence staining with the Alexa Fluor 488 conjugated antibody (Molecular Probes). TO-PRO-3 (Molecular Probes) was used for nuclear staining. The number of macrophages was determined by counting Mac-3-positive cells in four high-power fields (HPFs, 143 μm × 143 μm) per mouse.

### 2.9. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using student's unpaired *t*-test or analysis of variance (ANOVA), in which the post-test comparison was performed by a method of Bonferroni.

## 3. Results

### 3.1. Array analysis of cytokines after overexpression of LOX in VSMCs

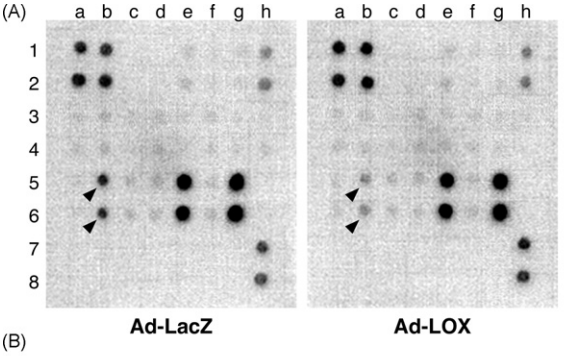
To explore the possibility that LOX directly regulates inflammatory molecules, we transfected rat aortic VSMCs with an adenovirus encoding full-length LOX (Ad-LOX) and used the conditioned media to perform a comprehensive analysis of 19 cytokines and chemokines using the cytokine antibody array. The results showed that secretion of MCP-1 was decreased in the VSMCs transfected with Ad-LOX compared to those with the control Ad-LacZ (Fig. 1). The protein levels of other cytokines remained unchanged after enhancement of LOX activity in VSMCs. Therefore, we focused on MCP-1 as a candidate molecule that links LOX activity with inflammation.

### 3.2. Role of LOX activity in regulation of MCP-1 secretion in vitro

To demonstrate that LOX regulates MCP-1 secretion in VSMCs, LOX activity was enhanced by transfection with Ad-LOX or inhibited by treatment with BAPN (200 μM), a chemical inhibitor of LOX. Treatment of VSMCs with BAPN reduced LOX activity (48 ± 12% reduction) and significantly increased MCP-1 secretion (1.8 ± 0.3 fold, $p<0.01$ compared to Control, Fig. 2A). Enhancement of LOX activity by Ad-LOX markedly reduced secretion of MCP-1 (63 ± 9% reduction, $p<0.01$ compared to Ad-LacZ, Fig. 2A) as well as mRNA level of MCP-1 (40 ± 16% reduction, $p<0.01$ compared to Ad-LacZ). These data clearly indicate that LOX activity downregulates MCP-1 in VSMCs.

We next examined whether activation of the JNK pathway, an inflammatory signalling pathway, affects the role of LOX activity in regulation of MCP-1 secretion. To specifically activate the JNK

(A)




(B)

| | a | b | c | d | e | f | g | h |
|---|---|---|---|---|---|---|---|---|
| 1 | Pos | Pos | Neg | Neg | CINC-2 | CINC-3 | CNTF | Fractalkine |
| 2 | Pos | Pos | Neg | Neg | CINC-2 | CINC-3 | CNTF | Fractalkine |
| 3 | GM-CSF | IFN-γ | IL-1α | IL-1β | IL-4 | IL-6 | IL-10 | LIX |
| 4 | GM-CSF | IFN-γ | IL-1α | IL-1β | IL-4 | IL-6 | IL-10 | LIX |
| 5 | Leptin | MCP-1 | MIP-3α | β-NGF | TIMP-1 | TNF-α | VEGF | BLANK |
| 6 | Leptin | MCP-1 | MIP-3α | β-NGF | TIMP-1 | TNF-α | VEGF | BLANK |
| 7 | BLANK | BLANK | BLANK | BLANK | BLANK | BLANK | BLANK | Pos |
| 8 | BLANK | BLANK | BLANK | BLANK | BLANK | BLANK | BLANK | Pos |

**Fig. 1.** Array analysis of cytokines after overexpression of LOX in VSMCs. Cultured VSMCs were transfected with Ad-LOX or Ad-LacZ. The conditioned media was analyzed with the cytokine array. (A) Representative images of array membranes. Arrowheads indicate the signals for MCP-1. (B) The position of cytokines and controls on the membrane. Pos: positive control, Neg: negative control, CINC: cytokine-induced neutrophil chemoattractants, CNTF: ciliary neutrophil factor, GM-CSF: granulocyte macrophage-colony stimulating factor, IFN: interferon, IL: interleukin, LIX: lipopolysaccharide-induced CXC chemokine, MIP: macrophage inflammatory protein, NGF: nerve growth factor, TIMP: tissue inhibitor of metalloproteinase, TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor.