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Lysyl oxidase propeptide inhibits smooth muscle cell signaling and proliferation

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Abstract

Lysyl oxidase is required for the normal biosynthesis and maturation of collagen and elastin. It is expressed by vascular smooth muscle cells, and its increased expression has been previously found in atherosclerosis and in models of balloon angioplasty. The lysyl oxidase propeptide (LOX-PP) has more recently been found to have biological activity as a tumor suppressor, and it inhibits Erk1/2 Map kinase activation. We reasoned that LOX-PP may have functions in normal non-transformed cells. We, therefore, investigated its effects on smooth muscle cells, focusing on important biological processes mediated by Erk1/2-dependent signaling pathways including proliferation and matrix metalloproteinase-9 (MMP-9) expression. In addition, we investigated whether evidence for accumulation of LOX-PP could be found in vivo in a femoral artery injury model. Recombinant LOX-PP was expressed and purified, and was found to inhibit primary rat aorta smooth muscle cell proliferation and DNA synthesis by more than 50%. TNF-α-stimulated MMP-9 expression and Erk1/2 activation were both significantly inhibited by LOX-PP. Immunohistochemistry studies carried out with affinity purified anti-LOX-PP antibody showed that LOX-PP epitopes were expressed at elevated levels in vascular lesions of injured arteries. These novel data suggest that LOX-PP may provide a feedback control mechanism that serves to inhibit properties associated with the development of vascular pathology.

Keywords: Lysyl oxidase; Lysyl oxidase propeptide; Cell proliferation; Matrix metalloproteinase-9; Map kinase; Cell signaling; Extracellular matrix; Vascular smooth muscle cells; Atherosclerosis; Restenosis; Western blots; Immunohistochemistry

Tumor necrosis factor-α (TNF-α) contributes to the development of atherosclerotic lesions [15]. Important among its many activities, TNF-α promotes the proliferation of smooth muscle cells (SMCs) [15] and it stimulates matrix metalloproteinase (MMP) production [6]. Thinning of the fibrous cap by MMPs is undesirable due to the release of sequestered factors that leads to thrombosis [15]. TNF-α regulation of MMP-9 is of particular importance in atherosclerotic lesion development and in fibrous cap thinning [3,20]. Thus, up-regulation of MMP-9 expression by TNF-α can promote development of atherosclerotic lesions by multiple mechanisms that include stimulation of SMC proliferation and MMP-9 production.

Lysyl oxidase is required for the maturation of collagen and elastin precursors in the biosynthesis of a functional extracellular matrix [13]. Lysyl oxidase catalyzes the oxidative deamination of lysine residues in elastin precursors and of lysine and hydroxylysine residues in fibrillar collagen precursors to form peptidyl-aldehydes. Formation of these aldehydes by lysyl oxidase is the final enzymatic step required for the formation of essential lysine-derived

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cross-linkages [13]. It is now understood that lysyl oxidase enzymes are encoded by a five-member gene family: lysyl oxidase itself (LOX), and lysyl oxidase-like proteins (LOXL1–LOXL4) [5]. These proteins all have a conserved C-terminal domain that encodes the catalytic enzyme region, and an N-terminal propeptide region. The LOX propeptide (LOX-PP) is unique in structure and has little sequence identity to LOXL1 and no sequence similarity to the other LOXL proteins [5]. LOX-PP contains the tumor suppressor functionality of LOX and it inhibits ras-dependent signaling, including Erk1/2 Map kinase activation [10,24]. LOX is expressed by vascular SMCs, and LOX deficient mice die at parturition and exhibit major cardiovascular defects [18]. Lysyl oxidase enzyme activity and expression are increased in atherosclerosis, and in models of restenosis [2,12,22].

Due to the presence of LOX in vascular tissues and smooth muscle cells, we asked whether LOX-PP might have biological activities in vascular SMCs. Because LOX-PP is an effective inhibitor of Erk1/2 Map kinases [19,28], and this Map kinase mediates TNF-α stimulated SMC proliferation and MMP-9 expression [20], we determined whether LOX-PP inhibits vascular SMC proliferation and TNF-α stimulated MMP-9 expression, and whether LOX-PP directly inhibits the MEK/Erk enzymes. Finally, we determined whether LOX-PP is detectable in vivo in femoral arteries that had been subjected to guidewire injury and in sham controls. Findings implicate a novel role for LOX-PP to provide a feedback mechanism to control vascular SMC proliferation and MMP production.

Materials and methods

Cell culture. Primary rat aorta smooth muscle cells (SMC) were isolated from 2-day-old rats [23], and were cultured in Dulbecco's modified Eagles medium containing 10% fetal bovine serum, 250 U/ml penicillin, 250 μg/ml streptomycin, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate. Cells were utilized after passage 1 [21,29], as indicated.

Recombinant lysyl oxidase propeptide. The full length rat LOX-PP (residues 22–162) containing the osteonectin (BM40) signal peptide replacing the natural LOX signal peptide was cloned into a tetracycline inducible plasmid to produce LOXPP/pcDNA4/TO/myc-His. The construct itself was confirmed by DNA sequencing. Stable transfectants were generated by Lipofectamine 2000 mediated transfection of TREX 293 cells (Invitrogen), and selected with 5 μg/ml blasticidin and 150 μg/ml zeocin. Resistant cells were induced with 1 μg/ml doxycycline for 48 h, and media containing LOX-PP was purified on a nickel affinity column (BioRad Bio-Scale Mini Profinity IMAC Cartridge) essentially as described [9], followed by further purification by ultrafiltration. Urea was removed by exhaustive dialysis against water or PBS. Nanospray LC/MS/MS mass spectroscopy of tryptic digests confirmed that rat LOX-PP is produced. SDS–PAGE stained with Coomassie Blue R250 revealed a single band with an apparent molecular size of 35 kDa that is fully consistent with our published studies [7]. In selected experiments LOX-PP expressed in bacteria was utilized [9].

Cell growth assay. SMC were grown in 12-well plates to 60% visual confluence and then placed in media containing 0.2% serum for 24 h. Cells were treated with fresh 0.2% serum-containing medium in the presence or absence of LOX-PP (10 μg/ml) alone, or vehicle alone (control). Cells were collected after 48 h by treatment with trypsin/EDTA, centrifugation at 1000*g*. Cells were suspended in 50 μl DMEM and stained with 0.2% trypan blue, and viable cells were counted using a hemocytometer. Data are from three independent cultures per group per time point.

DNA synthesis assay. Cells were grown in 12-well plates and grown for 4 days to near visual confluence in DMEM containing 10% serum, and then cultured in serum free medium containing 0.1% bovine serum albumin (BSA) for 24 h. Cells were treated with LOX-PP for 2.5 h before TNF-α (20 ng/ml) addition for 24 h. Cells were labeled with 2μCi/ml [^{3}H]thymidine for 18 h. Experiments were terminated by washing the cells with ice-cold PBS, precipitation with 10% TCA, and extraction of the DNA with 0.2 M NaOH, 0.1% SDS. Radioactivity was measured by liquid scintillation counting.

RNA isolation and real time PCR. Total RNA was isolated from SMC cultures and purified using RNeasy mini-RNA purification kits (Qiagen). Intact RNA samples (1 μg) were reverse-transcribed using random primers and (Applied Biosystems Reverse Transcription kit). Aliquots were then utilized in qPCR reactions with TaqMan probes (Applied Biosystems) for MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in an Applied Biosystems GeneAmp Prism 7500 System. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method and mRNA levels were normalized to GAPDH mRNA [1].

Erk1/2 map kinase phosphorylation assay. SMC were grown to near confluence and then placed in serum-free medium containing 0.1% BSA for 24 h. TNF-α (20 ng/ml) was then added with or without LOX-PP or vehicle in serum-free medium. Cells were lysed with ice-cold buffer: 50 mM Tris—Cl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, PMSF 0.5 mM, DTT 1 mM, PNPP 10 mM, NaF 10 mM, β-glycerophosphate 10 mM, protease inhibitor, and phosphatase inhibitor. Samples were then subjected to 10% SDS–PAGE and Western blotting for phosphorylated Erk1/2 and for total Erk1/2 with primary antibodies from Cell Signaling [1]. Signals were quantitated using a digital densitometry system (Versadoc; BioRad).

MEK/Erk2 activity assays. The ability of LOX-PP to directly inhibit enzymes in the Erk1/2 Map kinase pathway was measured. Recombinant purified and active MEK1, MEK2, and inactive Erk2 enzymes were purchased from Upstate Biotechnology. MEK1 and MEK2 activity assays were performed according to the kinase assay protocols provided by Upstate in incubations done in the presence or absence of LOX-PP. In stage 1, MEK1, or MEK2, respectively, were incubated with inactive Erk2 in the presence or absence of LOX-PP in a final volume of 25 μl. Aliquots of Stage I reactions are then assayed for the amount of active Erk2 resulting from MEK phosphorylation and activation of Erk2, using [γ^{32}P]ATP and myelin basic protein as substrate. In some experiments, LOX-PP was added to Stage II reactions to determine if LOX-PP can inhibit Erk2 activity directly.

Femoral artery injury and immunohistochemistry. Eleven-week-old mice were subjected to guidewire-induced femoral artery injury or sham surgery [11,25]. After 14 days, animals were sacrificed, femoral arteries were fixed and embedded in paraffin [11]. Block biopsies were sectioned (6 μm) and subjected to immunohistochemistry with anti-LOX-PP antibody. Sections were deparaffinized and antigen retrieval was performed by microwave heating of sections in 10 mM sodium citrate, pH 6. LOX-PP antibody was raised against a synthetic peptide corresponding to murine lysyl oxidase residues 78–115, and affinity purified against using an antigen affinity column. Antibody staining was performed with 1 μg/ml LOX-PP primary antibody according to methods previously utilized [27]. Sections were counterstained with hematoxylin. No immunostaining was observed in sections stained with non-immune IgG instead of primary LOX-PP antibody.

Results

LOX-PP inhibits SMC proliferation

Pro-LOX, mature LOX, and therefore LOX-PP are expressed in vascular smooth muscle cells [26]. Here, we