Prostaglandin E₂ Inhibits Elastogenesis in the Ductus Arteriosus via EP4 Signaling

Running title: Yokoyama et al.; PGE₂ inhibits elastogenesis via EP4 signaling

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Abstract

Background—Elastic fiber formation begins in mid-gestation and increases dramatically during the last trimester in the great arteries, providing elasticity and thus preventing vascular wall structure collapse. However, the ductus arteriosus (DA), a fetal bypass artery between the aorta and pulmonary artery, exhibits lower levels of elastic fiber formation, which promotes vascular collapse and subsequent closure of the DA after birth. The molecular mechanisms for this inhibited elastogenesis in the DA, which is necessary for the establishment of adult circulation, remain largely unknown.

Methods and Results—Stimulation of the prostaglandin E₂ (PGE₂) receptor EP4 significantly inhibited elastogenesis and decreased lysyl oxidase (LOX) protein, which catalyzes elastin cross-links in DA smooth muscle cells (SMCs), but not in aortic SMCs. Aortic SMCs expressed much less EP4 than DASMCs. Adenovirus-mediated overexpression of LOX restored the EP4-mediated inhibition of elastogenesis in DASMCs. In EP4-knockout mice, electron microscopic examination showed that the DA acquired an elastic phenotype that was similar to the neighboring aorta. More importantly, human DA and aorta tissues from seven patients showed a negative correlation between elastic fiber formation and EP4 expression, as well as between EP4 and LOX expression. The PGE₂-EP4-c-Src-PLC signalling pathway most likely promoted the lysosomal degradation of LOX.

Conclusions—Our data suggest that PGE₂ signaling inhibits elastogenesis in the DA, but not in the aorta, through degrading the LOX protein. Elastogenesis is spatially regulated by PGE₂-EP4 signaling in the DA.

Key words: prostaglandins; elasticity; signal transduction; pediatrics; muscle, smooth
Introduction

Elastic fibers are the largest structures in the extracellular matrix (ECM). Beginning with the onset of pulsatile blood flow in the developing aorta and pulmonary artery, smooth muscle cells (SMCs) in the vessel wall produce a complex ECM that ultimately defines the mechanical properties that are critical for proper function of the neonatal and adult vascular system. As such, hemodynamics and mechanical stress are considered to be the main regulators in the formation of the vascular elastic fiber system during development.

The ductus arteriosus (DA) and its connecting elastic arteries, i.e., the descending aorta and the main pulmonary trunk, are exposed to essentially the same mechanical forces and hemodynamics. However, since 1914, it has been widely recognized in multiple species that the DA exhibits sparse elastic fibers in the middle layer compared to adjacent elastic arteries, as well as disassembly and fragmentation of the internal elastic lamina. In the human fetal aorta, newly synthesized un-cross-linked elastin appears at 23 weeks of gestational age to be unevenly distributed on the surface of microfibrils, where it forms continuous strips of variable width. However, the DA exhibits fewer elastic fibers than the aorta. This decreased elastogenesis is the hallmark of the vascular remodeling of the DA in humans and a variety of other species. It has been suggested that this muscular phenotype of the DA allows it to collapse easily at birth when prostaglandin E2 (PGE2) is withdrawn and blood flow between the aorta and the pulmonary artery is reduced, thereby permitting immediate postnatal closure of the DA. Conversely, it is known that abnormalities of elastic fibers and elastic lamina are primarily responsible for the persistence of the DA in some human cases. These abnormalities likely prevent intimal cushion formation and make it difficult to collapse the arterial wall. Therefore, it is important to understand the molecular mechanisms of how elastogenesis is regulated in the DA. Although
Hinek et al. have demonstrated that truncated 52-kDa tropoelastin and the reduction of elastin binding protein negatively regulates elastic fiber formation in the DA\textsuperscript{12,13}, the mechanisms for impaired elastogenesis in the DA wall are not yet fully understood, despite nearly a century of research\textsuperscript{8}.

During mid- to late gestation, fetuses are exposed to abundant PGE\textsubscript{2} that is released from the placenta\textsuperscript{14} in accordance with the time course of impaired elastic fiber formation in the DA. The biological effects of PGE\textsubscript{2} depend on the prostanoid EP receptor subtypes EP\textsubscript{1} through EP\textsubscript{4}\textsuperscript{15}. Among the EP subtypes, EP\textsubscript{4} is highly expressed in the DA of multiple species, including mice, rats, and humans, and regulates the DA muscular tone\textsuperscript{16-19}. In addition to the DA muscular contraction, remodeling of the ECM during the fetal and neonatal period is necessary to complete the anatomical closure of the DA\textsuperscript{16,20,21}. Our previous studies have demonstrated the role of PGE\textsubscript{2}-EP\textsubscript{4} signaling in DA remodeling, in which EP\textsubscript{4} stimulation promotes intimal thickening, which is characteristic of the remodeling of the DA, in a hyaluronan-dependent and -independent manner\textsuperscript{16,21-23}.

In this context, we hypothesized that PGE\textsubscript{2} inhibits elastogenesis in the DA through PGE\textsubscript{2}-EP\textsubscript{4} signaling. In the present study, we examined the molecular mechanisms of the inhibitory regulation of elastogenesis in human DA tissues and rodent DASMCs. We demonstrated that activation of EP\textsubscript{4} promoted degradation of the mature lysyl oxidase (LOX) protein, a cross-linking enzyme for elastic fibers, only in the DA (and not in the aorta), leading to poor elastogenesis.

Methods

Expanded methods are described in the Online Data Supplement.
Animals and tissues

We used Wistar rat fetuses from timed-pregnant mothers (SLC Inc., Hamamatsu, Japan). Pooled tissues of the DA, aorta, and pulmonary arteries were obtained from rats on day 21 of gestation (n > 60). Generation and phenotypes of EP4-knockout mice have been described previously17. All mice were C57BL/6 background littermates from heterozygote crosses. All animal studies were approved by the institutional animal care and use committees of Yokohama City University and Waseda University.

Human tissues of the DA

Human DA tissues were obtained from Yokohama City University Hospital and Kanagawa Children’s Medical Center at the time of corrective operations. Detailed patient information is summarized in Supplemental Table 1. The study was approved by the human subject committees at both Yokohama City University and Kanagawa Children’s Medical Center. All samples were obtained after receiving written informed parental consent.

Tissue staining and immunohistochemistry

Elastic fiber formation was evaluated by Elastica van Gieson staining. Immunohistochemical analysis was performed as previously described16. A color extraction method using BIOREVO bz-9000 and associated software (KEYENCE, Osaka, Japan) was performed to quantify elastic fiber formation and expression of EP4 and LOX. Three serial paraffin-embedded sections per each patient were subjected to elastica staining and immunohistochemistry. More than 19 fields in the smooth muscle layer of the DA and aorta were examined in each slide. The area stained dark purple indicated elastic fibers and diaminobenzidine (DAB)-stained colors, EP4- or LOX-positive areas, were extracted from matched area and counted using the software. Correlations of elastic fiber formation and EP4 and LOX expression were examined using more than
independent 19 fields within one patient. We examined sections from a total of 7 patients, and the correlation coefficient and p-value of each patient are shown in Supplemental Table 2.

**Immunocytochemistry**

Vascular SMCs were plated on glass coverslips in 10% FBS in DMEM. The culture medium was then changed to 10% FBS in DMEM/F-12 alone, PGE₂, AE1-329, sulprostone, butaprost, or BAPN (day 1). Each drug was added on day 4. To examine the effect of silencing EP4 on elastic fiber formation, reverse transfection of DASMCs with EP4-targeted siRNA was performed according to the manufacturer’s instructions on days 1 and 4, and treated with AE1-329 on days 2 and 5. To examine the effect of overexpression of LOX or EP4, the cells were infected with adenoviruses at 10 multiplicities of infection on days 1 and 4. AE1-329 was added to the cells on days 2 and 5. All cells were fixed in 10% buffered formalin on day 7. The fixed cells were stained with anti-elastin antibody as previously described. All images were taken using a Nikon TE2000 (Nikon Instruments Inc, Tokyo, Japan) and processed under the same settings.

**Quantitative measurement of insoluble elastin**

Newly synthesized insoluble elastin was measure as previously described. Briefly, DASMCs were subconfluently plated on 60-mm dishes. Three days after plating, 20 Ci [³H]valine was added to each dish (day 0). AE1-329 (1 M) or PBS was added on days 0 and 4. The cells were harvested in 0.1M acetic acid on ice on day 7. The cells were boiled in 0.1N NaOH for 1 h. The insoluble pellets were boiled with 5.7N HCl for 1 h. The radioactivity was measured with a scintillation counter.

**Statistical analysis**

Data are shown as the mean ± SEM of independent experiments. The Mann-Whitney U-test, Kruskal-Walis test, and Pearson correlation coefficient were used to determine the statistical
significance of the data. A value of $p < 0.05$ was considered significant.

Results

**EP4 signaling inhibits elastogenesis in the ductus arteriosus (DA) in vivo**

In the late gestation period, the DA exhibits disassembly and fragmentation of the internal elastic lamina and sparse elastic fibers in the middle layer compared to its two connecting arteries, the aorta and the pulmonary artery (Figure 1A), despite the fact that they are exposed to essentially the same hemodynamics. The expression of rat EP4 is greater in the DA than in the aorta and the pulmonary artery on the 21st day of gestation (day 21) (Figure 1B)\(^{16}\). We examined the association between the expression of EP4 mRNA and elastogenesis in developing mouse fetuses (Figure 1C). In day 12.5 mice, organized elastic fibers were not observed in either the DA or the aorta, whereas *in situ* hybridization analysis revealed that the expression of EP4 mRNA was clearly higher in the DA than in the aorta or the pulmonary artery. In day 16.5 and day 18.5 mice, the formation of elastic fibers was observed more clearly in the aorta than in the DA. In these developing stages, obvious abundant expression of EP4 mRNA was observed in the DA, but not in the aorta. To examine the effect of EP4 on elastogenesis *in vivo*, we examined elastic fiber formation in the DA of EP4\(^{-/-}\) mice. In EP4\(^{-/-}\) mice, which die postnatally as a result of persistent patent DA (PDA)\(^{17,25}\), we found that the DA acquired an elastic phenotype that was similar to that of the neighboring aorta, as determined by elastica staining (Figure 1D-E), a color extraction method of elastica staining (Figure 1F), and electron microscopic examination (Figure 1G-H).

**Human vascular tissues show a negative correlation between elastic fibers and EP4 expression**
We also investigated the relationship between elastic fiber formation and EP4 expression in surgical samples from 7 patients with coarctation of the aorta who underwent surgical repair of aortic narrowing (Figure 2A, Supplemental Table 1). In concurrence with the findings in rodents, there was less elastic fiber formation in the DA than in the normal aorta, and the cells stained with anti-EP4 antibody were far more abundant in the DA (Figure 2B). Indeed, statistical analysis revealed that the correlation was significant between the amount of EP4 expression and the degree of inhibited elastic fiber formation (Figure 2C, Supplemental Table 2). Thus, elastogenesis is inhibited when EP4 is abundant. Taken together, these in vivo data suggest that EP4 plays a primary role in the inhibition of elastogenesis of the DA in humans and rodents.

**EP4 signaling inhibits elastogenesis in DASMCs**

To clarify the role of EP4 in elastogenesis in detail, we evaluated the elastic fiber assemblies in rat DASMCs using an in vitro system, as reported on previously. In the control group, DASMCs developed an abundant meshwork of elastic fibers (Figure 3A). In the presence of PGE2 or the EP4 agonist ONO-AE1-329, however, DASMCs developed a poor meshwork of elastic fibers. Neither the EP1/3 agonist sulprostone nor the EP2 agonist butaprost had any effect on elastic fiber development. LOX is a cross-linking enzyme that forms insoluble mature elastic fibers. Its specific small molecule inhibitor β-aminopropionitrile fumarate (BAPN) impaired elastic fiber formation (Figure 3A). To quantify the amount of mature (i.e., cross-linked) elastic fibers inhibited by EP4 stimulation, we metabolically labeled newly synthesized elastin with [3H]valine, and measured the incorporation of [3H]valine in the NaOH-insoluble fraction of these cells, which reflects the amount of newly synthesized mature elastic fibers. As shown in Figure 3B, in DASMCs, we detected a significant decrease in the incorporation of [3H]-valine into the insoluble fraction when ONO-AE1-329 was added to the medium (Figure 3B). When the
expression of EP4 mRNA was decreased by 89% by RNA interference, DASMCs developed elastic fiber formation even in the presence of ONO-AE1-329 (Figure 3C). To further confirm the existence of EP4-mediated impaired elastic fiber formation, we used rat aortic SMCs, which express much less EP4 than DASMCs (Figure 3D). When EP4 was forcibly expressed in aortic SMCs by EP4 gene transfer, elastogenesis was markedly impaired by ONO-AE1-329, whereas ONO-AE1-329 did not attenuate elastic fiber formation in the LacZ control (Figure 3E). These in vitro results indicate that PGE2-EP4 stimulation is responsible for the impaired elastogenesis of the DA.

**EP4 signaling inhibits elastic fiber formation by decreasing the LOX protein**

In the process of elastic fiber assembly, soluble elastin precursors (tropoelastin) are deposited on microfibrils\(^1,26\). They are then cross-linked by LOX, which confers elastic properties to elastic fibers\(^27\). Inactivation of the LOX gene is known to cause structural alterations in the arterial walls, leading to cardiovascular abnormalities\(^28\). In this context, we investigated the expression of the LOX protein in human surgical samples. In contrast to EP4, there were significantly fewer cells stained with anti-LOX antibody in the DA (Figure 4A). When elastic fiber formation and the expression of EP4 and LOX were quantified, LOX expression was positively correlated with elastic fiber formation, whereas it was negatively correlated with EP4 expression (Figure 4B, Supplemental Table 2). Interestingly, elastic fiber formation and EP4- or LOX-positive cells in the transitional zone appeared intermediate between the DA and aorta. We think that this finding supports previous reports that suggested that the coarctative ridge, a narrowed pathological segment in the aorta, is formed by mixed tissues from the native aorta and migrated tissues of DA origin\(^29,30\).

Next, we examined the effect of EP4 stimulation on LOX protein expression. We found
that the amount of mature LOX form was significantly decreased in the culture media of DASMCs and DASMC lysates in the presence of PGE2 and ONO-AE1-329 (Figure 4C-D). The effects of ONO-AE1-329 were dose- and time-dependent (Figure 4E-F). Other EP isoform-specific agonists had little effect. Interestingly, stimulation of EP4 did not change the expression levels of tropoelastin and fibrillin-1 proteins, which are the main components of elastic fibers (Supplemental Figure 1A-D). In the next LOX detection, we used whole cell lysate containing both intracellular and extracellular LOX protein. Although these EP4-mediated effects were not detected in aortic SMCs (ASMCs), the EP4 agonist significantly decreased the expression of the LOX protein in ASMCs when EP4 expression was induced using the adenovirus (Figure 4G-H).

When LOX expression was induced using the adenovirus (Supplemental Figure 2A-B), elastogenesis was largely restored in the ONO-AE1-329-treated DASMCs (Figure 4I). Thus, the reduction in LOX played a primary role in the EP4-mediated impairment of elastogenesis. The expression levels of matrix metalloproteinases (MMPs) and their activity were not altered in the DASMCs by EP4 stimulation (Supplemental Figure 3A-B). Nor was there any difference in MMP2 activity between rat tissues of the DA and aorta (Supplemental Figure 3C), suggesting that EP4 signaling plays a role in inhibiting elastogenesis, but not in promoting elastolysis in the DA.

The c-Src-PLC signal pathway plays a role in the PGE2-EP4-induced reduction in LOX protein

Next, we examined the downstream signal pathway responsible for the EP4-mediated reduction in LOX protein expression levels. Although our previous studies have demonstrated that cAMP and its downstream pathways play a primary role in EP4-mediated DA remodeling16, 22, 23, the cAMP-protein kinase A (PKA) or cAMP-exchange protein activated by the cAMP (Epac)
pathway did not play a role in the EP4-mediated reduction in LOX protein (Figure 5A-B).

Instead, we found that the EP4-induced reduction in LOX protein was restored by the phospholipase C (PLC) inhibitor U73122 (Figure 5C-E), but not by Gβγ, protein kinase C, or phosphoinositide 3-kinase (PI3K) inhibitors (gallein, bisindolylmaleimide I, LY294002) (Figure 5C-D). Furthermore, the PLC activator m-3M3FBS significantly decreased the expression levels of LOX protein in DASMCs (Figure 5F). Because several recent studies have demonstrated that PGE2 promotes cancer cell migration via the EP4-c-Src signal pathway31, 32 and that c-Src plays a critical role in the phosphorylation of PLCγ in several cell types33, 34, we hypothesized that the c-Src-PLCγ signal pathway may be involved. We found that ONO-AE1-329 significantly increased PLCγ1 phosphorylation (Figure 5G). In contrast, the Src-family kinase inhibitor PP2 significantly decreased PLCγ1 phosphorylation (Figure 5H-I) and restored the reduction in LOX protein induced by ONO-AE1-329 (Figure 5J-K). These results support our hypothesis that the c-Src-PLCγ signal pathway plays a primary role in the PGE2-EP4-induced reduction in LOX protein.

**EP4 signaling promotes LOX degradation in lysosomes**

Although ONO-AE1-329 decreased the expression of LOX protein in DASMCs, we found that ONO-AE1-329 did not decrease the mRNA expression of LOX (Figure 6A). Active LOX is synthesized as a 50 kDa inactive LOX proenzyme (pro-LOX), which is secreted into the extracellular space. Pro-LOX is then processed by proteolysis into a functional 32 kDa enzyme LOX and an 18 kDa propeptide27, 35. Using a pro-LOX-specific antibody, we found that the pro-LOX protein itself was not decreased by ONO-AE1-329 (Figure 6B-C), indicating that LOX was decreased post-translationally. BMP1 is a major protease that cleaves pro-LOX in the extracellular space27. However, ONO-AE1-329 did not change the expression of BMP1 mRNA.
or protein in DASMCs (Supplemental Figure 4A-B). Instead, we found that lysosomal degradation inhibitors, such as NH₄Cl and bafilomycin, eliminated the EP4-induced reduction in LOX protein (Figure 6D-E). These lysosomal degradation inhibitors also restored the PLC-mediated reduction in LOX protein (Figure 6F-G). Furthermore, we found that the clathrin-mediated endocytosis inhibitors chlorpromazine (CPZ) and phenylarsine oxide (PAO) similarly restored the EP4-induced reduction in LOX protein (Figure 6H-J). Administration of CPZ also restored the PLC-induced reduction in LOX protein (Figure 6K-L). In comparison, the caveolar endocytosis inhibitor methyl-beta-cyclodextrin (MβCD), the macropinocytosis inhibitor ethylisopropylamiloride (EIPA), and the proteosomal inhibitor MG132 showed little or no effect on LOX protein reduction (Supplemental Figure 5A-C). These data suggest that PGE₂-EP₄-PLC stimulation promotes the degradation of the LOX protein in lysosome through clathrin-mediated endocytosis.

Discussion

Although it is widely recognized in multiple species that the DA exhibits sparse elastic fibers in the middle layer and disassembly and fragmentation of the internal elastic lamina, the molecular mechanism for these has not yet been identified. The current study demonstrated a novel role of PGE₂ in spatially regulating elastogenesis by LOX protein degradation via the EP4-c-Src-PLCγ signal pathway in the DA, which contributes to the transition from fetal to neonatal circulation. Previous studies have demonstrated that abnormalities of elastic fibers are primarily responsible for PDA in some human cases¹⁰,¹¹. According to the Gittenberger-de Groot group’s observation, there are several types of abnormal elastogenesis that can cause PDA. The following two types are of particular importance: 1) thickened subendothelial elastic lamina with sparse or slightly

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increased elastic fibers in the media, and 2) aortification of the ductal wall\textsuperscript{10}. PDA with aortification of the ductal wall in EP4-knockout mice resembles the latter phenotype. These abnormalities of elastic fibers are likely to prohibit intimal cushion formation and make it difficult to collapse the arterial wall. This suggests that the control of elastogenesis is clinically important. Pharmacological treatment for PDA, such as indomethacin after birth, may have an adverse effect on the inhibition of elastic fiber formation in the DA, especially in premature infants. This should be further investigated in a future study.

The EP4 receptor is highly expressed in the DA compared to the adjacent arteries\textsuperscript{16}; it is coupled to G\textsubscript{as} and increases intracellular cAMP formation. The roles of EP4-cAMP signaling have been well studied in the DA. We and others have demonstrated that EP4 signaling induces vasodilation and hyaluronan-mediated vascular remodeling of the DA through cAMP-dependent PKA\textsuperscript{16,21,23} and that it promotes the migration of DASMCs and subsequent intimal thickening through Epac\textsuperscript{22}. Additional downstream signaling pathways of EP4 have been demonstrated in other cell types. EP4 utilizes G\textsubscript{ai} and PI3K, which are generally activated by G\textsubscript{b}\textsubscript{y}\textsubscript{36}. In addition to these well-known signaling pathways of EP4, our findings revealed c-Src-PLC\textgamma signaling as a novel EP4 downstream pathway, as well as the inhibitory role played by EP4 signaling in elastogenesis. This EP4-signaling pathway was found to be independent of cAMP signaling, including PKA and Epac, and G\textsubscript{b}\textsubscript{y} and PI3K. Studies using colorectal and lung cancer cells have suggested that \beta-arrestin1 bound to EP4 activates c-Src\textsuperscript{31,32}. In the DA, however, we did not identify an association between EP4-c-Src signaling and \beta-arrestin1 using \beta-arrestin1-targeted siRNA (data not shown). Ma et al. clearly demonstrated that G\textsubscript{as} and G\textsubscript{ai} proteins directly stimulate the kinase activity of c-Src\textsuperscript{37}. Since the EP4 receptor is coupled to G\textsubscript{as} and G\textsubscript{ai}, direct association between these G proteins and c-Src may activate its downstream signaling in EP4-
mediated degradation of the LOX protein. This possibility should be validated in a future study.

LOX is a copper-dependent amine oxidase that catalyzes the cross-linking of elastin and collagen and ensures the stability of the ECM\textsuperscript{38}. Since LOX is the isoform responsible for 80% of the LOX activity in aortic SMCs\textsuperscript{39}, it is essential to the maintenance of the tensile and elastic features of the vascular system\textsuperscript{38}. LOX is synthesized as a pre-protein. After signal peptide hydrolysis, enzyme glycosylation, cooper incorporation, and lysine tyrosylquinone generation, the enzyme is released into the extracellular space. Then, BMP-1 processes LOX, yielding the mature LOX form and its pro-peptide\textsuperscript{27}. The mechanisms of the transcriptional regulation of LOX have been extensively studied. Interferon-\(\gamma\) (IFN-\(\gamma\)), transforming growth factor-beta (TGF-\(\beta\)), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and angiotensin II induce LOX gene expression via the interferon regulatory factor 1 (IRF-1) transcriptional factor in multiple tissues, including blood vessels\textsuperscript{40, 41}. On the other hand, atherogenic concentrations of low-density lipoprotein and TNF\(\alpha\) reduce LOX mRNA\textsuperscript{42, 43}. Song et al. have also shown that IFN-\(\gamma\) inhibits LOX gene expression through binding to the antagonistic transcriptional factor, IRF-2, in vascular SMCs\textsuperscript{44}.

In contrast to our understanding of these transcriptional regulations of LOX, little is known regarding LOX protein metabolism. In the present study, we demonstrated for the first time that the PGE\textsubscript{2}-EP\textsubscript{4} signal promoted lysosomal degradation of the LOX protein. Recently, one study that used lysosomal inhibitors and Vps18-deficient mice demonstrated that LOX protein was degraded through lysosomes in Purkinje cells\textsuperscript{45}. However, the detailed molecular mechanisms triggering the degradation of LOX protein have not been reported and should be examined in future studies. Once LOX is cleaved from the proenzyme, it acts as a highly reactive enzyme. The mature LOX form catalyzes an oxidative deamination of lysine and hydroxylysine
residues to peptidyl α-aminoadipic-δ-semialdehydes. These highly reactive semialdehydes can spontaneously condense to form intra- and intermolecular covalent cross-linkages. Elastic fiber formation must be highly regulated to ensure the integrity of vascular and other tissues. Therefore, in addition to transcriptional regulation, the existence of protein regulation of LOX that we demonstrated in this study is physiologically reasonable.

The Rabinovitch group has extensively studied the molecular mechanisms of the sparse elastic fiber formation in the medial layer of the DA. Their studies have demonstrated that LOX activity does not differ between the lamb DA, aorta, and pulmonary artery. Our study demonstrated that the LOX protein was dramatically decreased by EP4 signaling in rodents and humans, suggesting that LOX activity is decreased in these DAs. Currently, we do not have a clear explanation for the apparent inconsistency in terms of LOX expression and activity. Further research is required to determine the species difference in LOX protein metabolism and activity. The Rabinovitch group also demonstrated that there is decreased insolubilization of elastin in the DA that is associated with the truncated 52-kDa tropoelastin that lacks the C-terminal, which is unrelated to heightened elastolytic activity. Similarly, our results showed that MMP-2 activity does not differ between the DA and the aorta, suggesting that impaired elastogenesis rather than enhanced elastolytic activity provides a muscular arterial property to the DA.

The present study demonstrated that LOX expression is important during development. However, LOX expression is known to be markedly responsive to a variety of pathological states, including wound repair, aging, and tumorigenesis. In particular, strong evidence exists regarding the involvement of a reduction in LOX activity in the pathogenesis of vascular diseases characterized by destructive remodeling of the arterial wall. Previous reports demonstrated that aortic aneurysm and coronary dissections were related to a disturbance in
LOX expression in animal models and humans\(^47,48\). Therefore, the regulation of LOX expression is considered an attractive therapeutic target. In this study, it should be noted that there seems to be a threshold value for EP4 expression to induce a decrease in elastic fibers and LOX (Figures 2C and 4B). In our previous report, analyses of human aortic aneurysmal tissues demonstrated that EP4 expression is greater in aneurysmal legions than that in non-diseased areas\(^49\). Further studies are required to investigate whether EP4-mediated LOX regulation plays a role in pathological conditions.

Taken together, these findings suggest that PGE\(_2\)-EP4 signaling inhibits elastogenesis in the DA by degrading LOX protein. The PGE\(_2\)-EP4-mediated LOX protein regulation via a previously unrecognized signaling pathway may also provide the basis for therapeutic strategies that target vascular elastogenesis.

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Conflict of Interest Disclosures: None.

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**Figure Legends:**

**Figure 1.** EP4 signaling attenuated elastic fiber formation *in vivo.* (A) Elastica van Gieson stain (elastica stain) of rat fetus on day 21 of gestation (e21). PA: pulmonary artery. (B) Expression of EP4 mRNA of the rat DA, aorta, and pulmonary artery (PA) on day 21 of gestation. n = 6. (C) Developmental changes in elastic fiber formation and EP4 mRNA by *in situ* hybridization in mouse fetus on days 12.5 (e12.5), 16.5 (e16.5), and 18.5 (e18.5) of gestation. Expression of EP4 mRNA was higher in the DA than in the aorta and pulmonary artery. Conversely, elastic fiber formation is sparser in the DA than in the other arteries. (D-E and G-H) Elastica stain and electron microscopic images of wild-type and EP4−/− mice on day 18.5 of gestation. Elastic fiber formation was restored in the DA of EP4−/− mice. (F) Quantification of the elastic fiber formation of D and E using a color extraction method. n = 8. *p < 0.05, **p < 0.01. Scale bars: 200 μm (A); 100 μm (upper and lower panels of C); 50 μm (D, E); 20 μm (middle panels of C); 5 μm (G, H).

**Figure 2.** Human vascular tissues show negative correlation between elastic fibers and EP4 expression. (A) A representative image of the elastica stain of a human neonatal vessel. (B) Elastica stain and immunohistochemistry for EP4 in human neonatal vessels. (C) Representative
results of quantification of elastic fiber formation and EP4 protein expression using a color extraction method. Values are shown in arbitrary units. Scale bars: 200 μm (A); 50 μm (B).

**Figure 3.** EP4 signaling attenuated elastic fiber formation *in vitro.* (A and C) Immunostaining for elastin (red) and DNA (blue) of DASMCs treated with each drug indicated and/or EP4-targeted siRNA (EP4 si). AE1-329: ONO-AE1-329, CTRL: control, Neg si: negative control siRNA. Pharmacological activation of EP4 by ONO-AE1-329 attenuated elastic fiber formation. (B) Incorporation of [3H]valine was quantified in DASMCs treated with or without AE1-329. n=8, ***p < 0.001. (D) Expression of EP4 mRNA in DASMCs, ASMCs, and pulmonary SMCs (PASMCs). n = 6, **p < 0.01. (E) Immunostaining for elastin (red) and DNA (blue) of the EP4- or LacZ-overexpressing ASMCs treated with or without AE1-329. Activation of EP4 did not affect elastic fiber formation in LacZ-overexpressing ASMCs, whereas it decreased elastic fiber formation in EP4-overexpressing ASMCs. Each drug was used at 1 μM. Scale bars: 20 μm.

**Figure 4.** EP4 signaling attenuated elastic fiber formation via decreased LOX expression. (A) Representative images of immunohistochemistry for LOX in human neonatal vessels. (B) Representative results of quantification of elastic fiber formation, LOX, and EP4 protein expression using a color extraction method. Values are shown in arbitrary units. (C) Western blotting for LOX in supernatant (sup.) and lysate of SMCs treated for 72 h. (D) Quantification of C. n = 4, *p < 0.05 vs. CTRL. Numbers on the bars indicate p-values. Administration of PGE2 or the EP4 agonist AE1-329 decreased the mature LOX form in both supernatant and lysate of DASMCs but not in ASMCs. (E) Dose-dependent effects of AE1-329 (24 h incubation) on LOX protein in DASMCs. (F) Time-dependent reduction in LOX protein in DASMCs. (G) Protein
expression of LOX was decreased in EP4-overexpressing ASMCs (Adv.EP4) treated with AE-329 for 24 h. (H) Quantification of G. n = 4, *p < 0.05 vs. CTRL. (I) Immunostaining for elastin (red) and DNA (blue) of the LOX- or LacZ-overexpressing DASMCs treated with or without AE1-329. Each drug was used at 1 μM. Scale bars: 50 μm (A); 20 μm (I).

Figure 5. EP4 signaling decreased LOX protein via the c-Src-PLCγ pathway in DASMCs. (A) LOX protein expression in DASMCs treated with non-selective (Br-cAMP, 50 μM), PKA-selective (Bnz-cAMP, 50 μM) or Epac-selective (Me-cAMP, 50 μM) cAMP analogs for 24 h. The AE1-329 was administered in the presence or absence of the PKA inhibitor PKI (10 μM). (B) Quantification of A. n = 4–6, *p < 0.05, **p < 0.01 vs. CTRL. cAMP signaling did not affect the EP4-mediated reduction of LOX protein. (C) LOX protein expression in DASMCs treated with gallein (Gβγ inhibitor, 10 μM), U73122 (PLC inhibitor, 10 μM), bis (bisindolylmaleimide: PKC inhibitor, 10 μM), or LY294002 (PI3K inhibitor, 1 μM) in the presence of AE1-329. (D) Quantification of C. n = 4–6, *p < 0.05, **p < 0.01 vs. CTRL. Inhibition of PLC eliminated the EP4-mediated reduction of LOX protein. (E) Dose-dependent effect of U73122 in DASMCs treated with AE1-329. (F) Dose-dependent inhibitory effect of m-3M3FBS on LOX protein expression. (G and H) Phosphorylation of PLCγ in DASMCs treated with PP2 (Src inhibitor) and/or AE1-329. (I) Quantification of H. n = 4–6, *p < 0.05. Inhibition of Src attenuated the EP4-mediated phosphorylation of PLCγ. (J) PP2 attenuated the EP4-mediated reduction of LOX protein. (K) Quantification of J. n = 4–6. *p < 0.05. Whole cell lysate was used for LOX detection. AE1-329 was used at 1 μM.

Figure 6. EP4 signaling decreased LOX protein through lysosomal degradation. (A and B)
Expression of LOX mRNA, pro-LOX, and LOX protein in DASMCs treated with AE1-329. mRNA of LOX and pro-LOX protein were not decreased by EP4 agonist in DASMCs. (C) Quantification of B. n = 4. (D and F) Administration of lysosomal inhibitors (NH₄Cl or bafilomycin) for 24 h restored the AE1-329- or m-3M3FBS (0.5 μM)-induced reduction of LOX protein. (E and G) Quantification of D and F, respectively. n = 6. (H and I) Administration of clathrin-mediated endocytosis inhibitors (CPZ or PAO) for 24 h restored the AE1-329-induced reduction of LOX protein. (J) Quantification of H and I. n = 4–6. (K) Administration of CPZ for 24 h restored the m-3M3FBS (0.5 M)-induced reduction of LOX protein. (L) Quantification of K. *p < 0.05, **p < 0.01. ***p < 0.001. Whole cell lysate was used for LOX detection. AE1-329 was used at 1 μM.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Prostaglandin E₂ Inhibits Elastogenesis in the Ductus Arteriosus via EP4 Signaling
Utako Yokoyama, Susumu Minamisawa, Aki Shioda, Ryo Ishiwata, Mei-Hua Jin, Munetaka Masuda, Toshihide Asou, Yukihiho Sugimoto, Hiroki Aoki, Tomoyuki Nakamura and Yoshihiro Ishikawa

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents

8-p-Methoxyphenylthion-2-Omethyl-cAMP (pMe-cAMP) and N6-benzoyl-adenosine-cAMP (Bnz-cAMP) were purchased from BioLog Life Science Institute (Bremen, Germany) and Sigma (St. Louis, MO), respectively. PGE$_2$, sulprostone, butaprost, gallein, BAPN, bisindolylmaleimide (bis), U73122, U0126, LY294002, PAO, EIPA, and 8-Bromo-cAMP (Br-cAMP) were purchased from Sigma-Aldrich (St. Louis, MO). CPZ, MβCD, MG132, and NH$_4$Cl were obtained from Wako (Osaka, Japan). The PKA inhibitor (14–22), bafilomycin A1, PP2, and m-3M3FBS were obtained from Calbiochem (Darmstadt, Germany). ONO-AE1-329 was kindly provided by ONO Pharmaceutical Company (Osaka, Japan). Antibodies for LOX and pro-LOX for immunoblotting were obtained from Abcam (Cambridge, UK) and Novus Biological (Littleton, CO), respectively. Anti-LOX antibody for immunohistochemistry and anti-BMP-1 were obtained from US Biological (Swampscott, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-elastin and anti-EP4 antibodies were obtained from Elastin Products Company (Owensville, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Anti-PLC$_\gamma$ and anti-phosphorylated PLC$_\gamma$ antibodies were obtained from Cell Signaling (Beverly, MA). Anti-MMP-2 and anti-MMP-9 antibodies were from R&D Systems (Minneapolis, MN). Anti-fibrillin-1 antibody was kindly
provided from Dr. Nakamura (Kansai University, Japan).

**Isolation and culture of rat smooth muscle cells (SMCs)**

Vascular SMCs were obtained from the DA and aorta of Wistar rat fetuses on the 21st day of gestation (SLC Inc.) as previously described. Using the same protocol, pulmonary SMCs were isolated from the branch extralobular pulmonary arteries from Wistar rats on the 21st day of gestation. SMCs were used at passages 4 to 6.

**Immunoblot analysis**

Proteins from whole cells were analyzed by immunoblotting as previously described.

**Adenovirus construction**

Adenovirus of EP4 was kindly provided from Dr. Y. Kobayashi (Matsumoto Dental University, Japan). A control adenovirus vector with LacZ was used at the same multiplicity of infection.

**RNA interference (siRNA)**

Double-stranded siRNAs to the selected regions of EP4 (stealth RNAi RSS331316) and the negative siRNA purchased from Invitrogen (San Diego, CA). According to the manufacturer’s instructions, cells were transfected with siRNA (300 pmol), using Lipofectamin RNAiMAX (Invitrogen).

**Quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)**

Isolation of total RNA and generation of cDNA were performed and RT-PCR analysis was done
as previously described. The primers were designed based on the rat nucleotide sequences of EP4 (5’-CTC GTG GTG CGA GTG TTC AT-3’ and 5’-AAG CAA TTC TGA TGG CCT GC-3’) and BMP-1 (5’-CAT CTC CAT CGG CAA GAA C-3’ and 5’-CTC GAC TTC CTG AAC TTC CAT C-3’). Each primer set was designed between multiple exons. The abundance of each gene was determined relative to the 18S transcript.

Electron microscopy

Electron microscopic analysis for elastic fiber formation was performed as previously described.

Gelatin zymography

MMP activity was examined by gelatin zymography as previously described.

In situ hybridization

Expression of EP4 mRNA in mice fetuses on day 12.5, 16.5, and 18.5 of gestation was evaluated by in situ hybridization. A 543 bp DNA fragment corresponding to nucleotide positions 1373 to 1915 of mouse EP4 cDNA (Gen-Bank NM_008965) was cloned into pGEMT-Easy vector (Promega, Madison, WI) and used for the generation of sense and antisense RNA probes. Digoxigenin-labeled RNA probes were prepared with DIG RNA Labeling Mix (Roche, Basel, Switzerland). Hybridization was performed with probes at concentrations of 300 ng/ml in the Probe Diluent-1 (Genostaff, Tokyo, Japan) at 60°C for 16 h. After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti-DIG AP conjugate (Roche) diluted 1:1000 with TBST for 2 hr at room temperature (RT). Coloring reactions were
performed with NBT/BCIP solution overnight and then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), and mounted with CC/Mount (DBS).
### Supplemental Table 1.

#### Summary of patient profile

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<th>Age at Operation</th>
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<tr>
<td>7</td>
<td>1 month</td>
<td>hypoLV, CoA, VSD</td>
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</table>

CoA: Coarctation of the Aorta, VSD: Ventricular Septum Defect,

TGA: Transposition of the Great Arteries, hypoLV: hypoplastic Left Ventricule.
Supplemental table 2.

Correlation between elastic fiber formation and expression of EP4 and LOX

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</table>

r: correlation coefficient; n: number of sampling points. *, p < 0.05; **, p < 0.01; ***, p < 0.001
Supplemental Figure 1

A

fibrillin-1

CTRL  PGE2  AE1-329

B

Relative expression of fibrillin-1

CTRL  PGE2  AE1-329

p = 0.17

C

tropoelastin

CTRL  PGE2  AE1-329

GAPDH

D

Relative expression of tropoelastin

CTRL  PGE2  AE1-329

p = 0.87
Supplemental Figure 2

A

<table>
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<td>Adv.LOX</td>
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</table>

B

Relative protein expression of LOX

- $p = 0.34$
- *
Supplemental Figure Legends

Supplemental Figure 1

EP4 signaling did not affect protein expression of tropoelastin and fibrillin-1.

(A) Protein expression of fibrillin-1 in culture medium of DASMCs treated with either PBS, PGE₂ (1 μM), or AE-329 (1 μM) for 72 h. (B) Quantification of (A), n = 4. (C) Protein expression of tropoelastin in whole cell lysate of DASMCs treated with either PBS, PGE₂ (1 μM), or AE-329 (1 μM) for 72 h. (D) Quantification of (C), n = 4.

Supplemental Figure 2

Overexpression of LOX protein in DASMCs transfected with Adv.LOX.

(A) Protein expression of LOX in culture medium of DASMCs transfected Adv.LacZ or Adv.LOX in the presence or absence of AE-329 (1 μM). The time-course of transfection and drug administration was same as Figure 4I. (B) Quantification of (A), n = 4, *p < 0.05.

Supplemental Figure 3

EP4 signaling did not affect expression or activation of MMP-2 or -9 in DASMCs.

(A) Protein expression of MMP-2 and-9 in DASMCs treated with or without AE1-329 (1 μM) for 72 h. (B) Gelatin zymography of DASMCs treated with 1 μM of PGE2 or each EP agonist. (C) Gelatin zymography of the rat DA and aorta on the 21st day of gestation.
Supplemental Figure 4

EP4 signaling did not change BMP-1 expression in DASMCs.

(A) Expression of BMP-1 mRNA in DASMCs treated with or without AE1-329 (1 μM) for 24 h. n = 4. (B) Representative image of protein expression of BMP-1 in DASMCs treated with or without AE1-329 (1 μM) for 72 h.

Supplemental Figure 5

LOX degradation was associated with caveolar endocytosis, macropinocytosis, and proteosome.

(A) Representative figures of protein expression of LOX in whole cell lysate of DASMCs treated with MbCD, EIPA, or MG132 in the presence of AE1-329 (1 μM).
References


