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ARTICLE

Endothelin-1 induces lysyl oxidase expression in pulmonary artery smooth muscle cells[1]

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Abstract: The increase in thickening of the arterial wall of pulmonary arterial hypertension (PAH) includes cellular proliferation as well as matrix deposition and interrupted internal elastic lamina (IEL) consisting of a thick homogeneous sheet of elastin. Little is, although, known about the detail of IEL formation in PAH. Endothelin-1 is overexpressed in pulmonary arterioles of PAH. We aimed to examine the expression of genes contributing to IEL formation in pulmonary artery smooth muscle cells (PASMCs) especially focused on lysyl oxidase (LOx), an exreacellular matrix enzyme that catalyzes the cross-linking of collagens or elastin. We quantified mRNA expressions of genes contributing to IEL formation including LOx in PASMCs using real-time quantitative polymerase chain reaction. We stimulated human PASMCs with endothelin-1 with prostacyclin or trapidil. Endothelin-1 significantly increased LOx expression. Prostacyclin and trapidil restored endothelin-1-induced LOx expression to the basal level. Endothelin-1 increased LOx expression strongly in PASMCs from PAH patients compared to those from controls. Trapidil reduced LOx expression only in PASMCs from PAH patients. Overexpressed endothelin-1 in PAH patients can increase expression of LOx and agitate cross-linking of elastin and collagen, resulting in ectopic deposition of these in the vascular media.

Key words: elastin, lysyl oxidase, endothelin, pulmonary artery smooth muscle cells, pulmonary hypertension.

Résumé : L'augmentation de l'épaississement de la paroi artérielle dans l'hypertension artérielle pulmonaire (HAP) comprend la prolifération des cellules, ainsi que le dépôt de matrice avec des interruptions dans la limitante élastique interne (LEI) constituée d'une couche d'élastine épaisse et homogène. Mais on sait peu de choses des détails de la formation de la LEI dans l'HAP. En cas d'HAP, l'endothéline 1 est surexprimée dans les artérioles pulmonaires. Nous visions à examiner l'expression de gènes contribuant à la formation de la LEI dans les cellules musculaires lisses d'artère pulmonaire (CMLAP), en nous centrant particulièrement sur la lysyl oxydase (LOx), une enzyme de la matrice extracellulaire qui catalyse la réticulation du collagène ou de l'élastine. À l'aide de la technique de PCR quantitatif en temps réel, nous avons quantifié l'expression de l'ARNm de gènes participant à la formation de la LEI, y compris l'enzyme LOx, dans les CMLAP. Nous avons stimulé des CMLAP humains avec de l'endothéline 1, et avec de la prostacycline ou du trapidil. L'endothéline entraînait une augmentation marquée de l'expression de la LOx. Avec la prostacycline et le trapidil, l'expression de la LOx engendrée par l'endothéline se rétablissait aux valeurs du début de l'expérience. L'endothéline 1 a entraîné une plus forte augmentation de l'expression de la LOx dans les CMLAP des patients atteints d'HAP que chez les témoins. Le trapidil a entraîné une diminution de l'expression de la LOx uniquement dans les CMLAP des patients atteints d'HAP. En conclusion, la surexpression de l'endothéline 1 chez les patients atteints d'HAP peut entraîner une augmentation de l'expression de la LOx et troubler la réticulation de l'élastine et du collagène, ce qui se traduit par leur dépôt ectopique dans la média vasculaire.

Mots-clés : élastine, lysyl oxydase, endothéline, cellules musculaires lisses d'artère pulmonaire, hypertension pulmonaire.

Introduction

Pulmonary arterial hypertension (PAH) is characterized by the progressive pulmonary vascular obstruction, although the precise cause remains unclear. The pathologic thickening of the arterial wall is considered to include cellular proliferation as well as matrix deposition (Pietra et al. 2004) and interrupted internal elastic lamina (Aiello and Canzian 2009). Internal elastic lamina (IEL) consists of a thick homogeneous sheet of elastin. The proliferating cells are phenotypically smooth muscle cells migrated from the medial layer through gaps in the interrupted IEL. The increased thickness with fewer gaps of the IEL may act as a barrier that prevents smooth muscle cell migration in patients with pulmonary hypertension without intimal proliferative lesions (Aiello et al. 2003). It is also reported that fragmentation of the IEL was

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Table 1. The primers for QRTPCR.

mRNA	Abbreviation	Sense (5′ to 3′)	Antisense (5′ to 3′)
TaqMan Probe System			
Bone morphogenetic protein 1	BMP1	accctgggcagctacaagt	tgaggaatccgccacaag
Elastin		cagctaaatacggtgctgctg	aatccgaagccaggtcttg
Fibrillin 2		gacagcagatcctgccaag	ggcaggttattacatgttccaga
Fibulin 5		ctgccctccaggctacatc	cctgtgctcacattcgttga
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc
Integrin alpha V	IntegrinαV	aagctgagctcatcgtttcc	gcacaggaaagtcttgctaagg
Integrin beta 3	Integrinβ3	cgctaaatttgaggaagaacg	gaaggtagacgtggcctcttt
Hypoxanthine phosphoribosyltransferase 1	HPRT1	ctttgctttccttggtcagg	acacttcgtggggtcctttt
Lysyl oxidase	LOx	ggatacggcactggctactt	gacgcctggatgtagtaggg

associated with endothelial abnormalities and clinical evidence of pulmonary hypertension (Rabinovitch 1998).

A hypothetical model for elastic fiber assembly has been assumed that includes pivotal roles for elastin, lysyl oxidase (LOx), fibulins, and microfibrils (comprised mainly of fibrillin). Elastin (tropoelastin) is transported to the plasma membrane, where it is organized into small aggregates and cross-linked by LOx. Propeptide of proLOx is cleaved by bone morphogenetic protein 1 (BMP1) producing mature LOx. The aggregates are then transferred to extracellular microfibrils, which interact with the cell through integrins. Fibulin 4 and (or) fibulin 5 assist the transfer of elastin aggregates to the microfibril and facilitate elastin to coalesce into larger structures. The elastin aggregates are further cross-linked by LOx to form the complete elastic fiber. (Wagenseil and Mecham 2007). It has been reported that increased elastase activity degrades IEL in the hypertensive pulmonary artery (Todorovich-Hunter et al. 1992; Kim et al. 2011), although little is reported about the detail of IEL formation in PAH (Tojais et al. 2017).

Endothelin-1 (ET-1) is overexpressed in remodeled pulmonary arterioles of PAH patients and concerned to play an important role in pathogenesis of the disease (Galiè et al. 2013). ET-1 enhances secretion of collagen type I by human skin fibroblasts (Xu et al. 1998), yet the link between ET-1 and elastin formation is largely unknown.

We assume a hypothesis that disorganized formation of IEL is involved in the pathogenesis of PAH. Hence, we aimed to examine the expression of genes contributing to IEL formation in pulmonary artery smooth muscle cells (PASMCs) and endothelial cells (PAECs) especially focused on LOx, which has emerged as a player in cardiovascular diseases (Rodríguez et al. 2008).

Materials and methods

This study was approved by the local Institutional Ethics Committee at Erasmus University Hospital, Brussels, Belgium.

Lung tissue sampling study population

Lung tissue and pulmonary arteries were sampled at lung transplantation in seven patients with idiopathic PAH (IPAH) and during lobectomy or pneumectomy for a localized lung cancer in 12 control patients. The control patients underwent transthoracic echocardiography preoperatively to rule out pulmonary hypertension, and lung specimens were sampled at a distance from tumor areas. None of the patients had BMPR2 or activin-like kinase type 1 mutations.

Culture of human PASMCs

Human PASMCs were cultured from explants of pulmonary arteries in 10% fetal calf serum (FCS) in Dulbecco's modified Eagle medium (DMEM), as previously described (Dewachter et al. 2006). The PASMCs were incubated in normoxic conditions with 5% CO_2 for several weeks and transferred into new cell culture flasks. For some experiments, we used purchased human PASMCs and PAECs (Lonza, Allendale, New Jersey, USA). Cells were used between passages 3 and 6.

RNA extraction, cDNA preparation, and real-time quantitative polymerase chain reaction (QRTPCR)

Total RNA was extracted from cells using RNeasy Mini kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's instructions. RNA concentration was determined by standard spectrophotometric techniques. First-strand cDNA synthesis was performed with the SuperScript II Reverse Transcriptase system (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions.

For the QRTPCR experiments using the TaqMan Probe System, sense and antisense primers were designed using the Roche Universal Probe Library Assay Design Center website for the genes of elastin, fibrillin 2, LOx, BMP1, fibulin 5, integrin αv, integrin β3, hypoxanthine phosphoribosyltransferase 1 (HPRT1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences (Table 1).

For each sample, an amplification reaction was performed in duplicate with Fast Start Essential DNA Probe Master (Roche Diagnostics, Mannheim, Germany), specific primers, TaqMan UPL probe, and diluted-template cDNA. Signal detection and analysis of the results were performed using LightCycler 96 (Roche Diagnostics). Relative quantification was achieved with the comparative 2-ΔΔ Ct method by normalization with HPRT1 or GAPDH RNA (Dewachter et al. 2006).

In vitro stimulation with ET-1

The cells were subjected to synchronization by serum deprivation in 0% FCS–DMEM for 48 h before collection (Dewachter et al. 2009; Maruyama et al. 2015). The cells were treated with ET-1 (0.1 or 1 μmol/L) (Sigma–Aldrich, St. Louis, Missouri, USA), prostacyclin (10 ng/mL), or trapidil (500 μg/mL) and incubated at 37 °C for 24 h (ET-1) or 5 h (prostacyclin and trapidil). Samples were collected in lysis buffer (Qiagen SA) and stored at −80 °C until measurement of mRNA expression.

Statistical analysis

Effects of ET-1, prostacyclin, and trapidil treatment were analyzed using Microsoft Excel and add-in software Statcel2 (OMS Publishing, Saitama, Japan). Mann–Whitney's *U* test was used for comparison. Values of $P < 0.05$ were accepted as significant.

Results

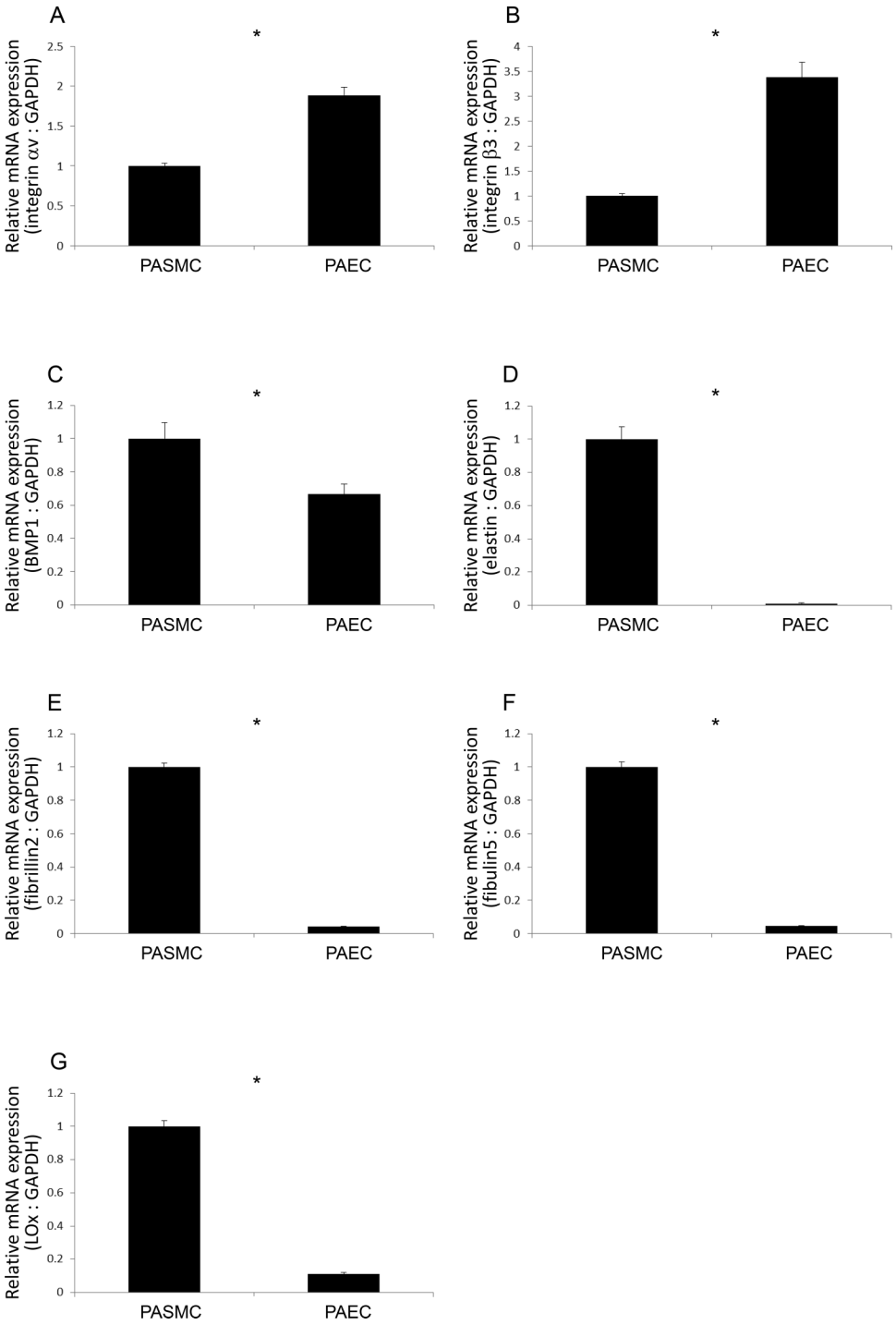
Gene expressions contributing to IEL formation in PASMCs and PAECs

We ascertained the different expression patterns of genes contributing to IEL formation in PASMCs and PAECs. As shown in Fig. 1, integrin αv and integrin β3 were expressed more predominantly in PAECs than in PASMCs, and BMP1 was expressed in both PAECs and PASMCs. In contrast, elastin, fibrillin 2, fibulin 5, and LOx were exclusively expressed in PASMCs, while they were barely expressed in PAECs.

Effects of ET-1 on LOx expression

To evaluate the effects of ET-1, PASMCs were stimulated with ET-1 (0.1 μmol/L) for 24 h. Integrin αv, integrin β3, BMP1, elastin,

Fig. 1. Gene expressions contributing to IEL formation in PASMCs and PAECs in basal level. (A) Integrin αv, (B) integrin β3, (C) BMP1, (D) elastin, (E) fibrillin 2, (F) fibulin 5, and (G) LOx in purchased PASMCs (left) and purchased PAECs (right) were quantified by QRTPCR. Data are indicated as mean + SEM ($n = 4$). *$P < 0.05$ using Mann–Whitney's U test.

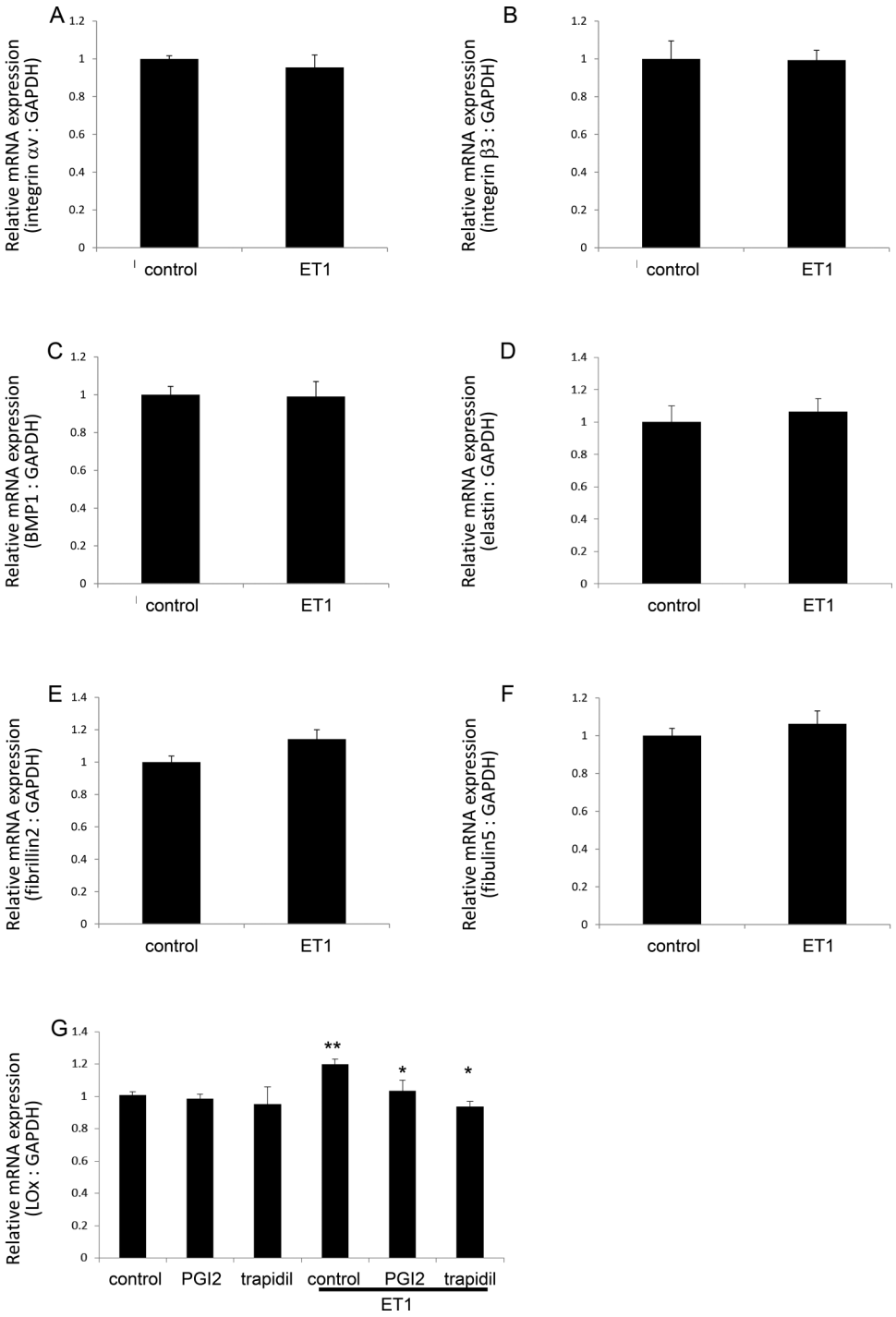


fibrillin 2, and fibulin 5 remained at a similar level after ET-1 stimulation (Figs. 2A–2F). On the other hand, ET-1 significantly increased LOx expression. We evaluated the suppressive effect of prostacyclin and trapidil, which are reported to counteract the mitogenic activity in vascular smooth muscle cells (Li et al. 2004; Bönisch et al. 1998). Either prostacyclin (10 ng/mL) or trapidil (500 μg/mL) for 5 h restored LOx expression induced by ET-1 to the basal level (Fig. 2G). Neither prostacyclin nor trapidil alone affected the LOx expression.

Gene expressions in PASMCs from controls and IPAH patients by QRTPCR

We evaluated the expression of the genes contributing to IEL formation in PASMCs from IPAH patients. As shown in Fig. 3, LOx,

Fig. 2. Relative gene expressions of (A) integrin αv, (B) integrin β3, (C) BMP1, (D) elastin, (E) fibrillin 2, and (F) fibulin 5 in purchased PASMCs were quantified by QRTPCR after ET-1 stimulation (0.1 μmol/L) for 24 h. (G) LOx expression in ET-1-stimulated PASMCs treated with prostacyclin (PGI2) (10 ng/mL) or trapidil (500 μg/mL) for 5 h. Data are indicated as mean + SEM ($n = 4$). *$P < 0.05$ compared with ET-1-stimulated control, **$P < 0.01$ compared with baseline control without ET-1-stimulation, respectively, using Mann–Whitney's U test.



elastin, fibrillin 2, fibulin 5, integrin αv, integrin β3, and BMP1 were expressed in PASMCs from both PAH and control at similar levels (statistically not significant).

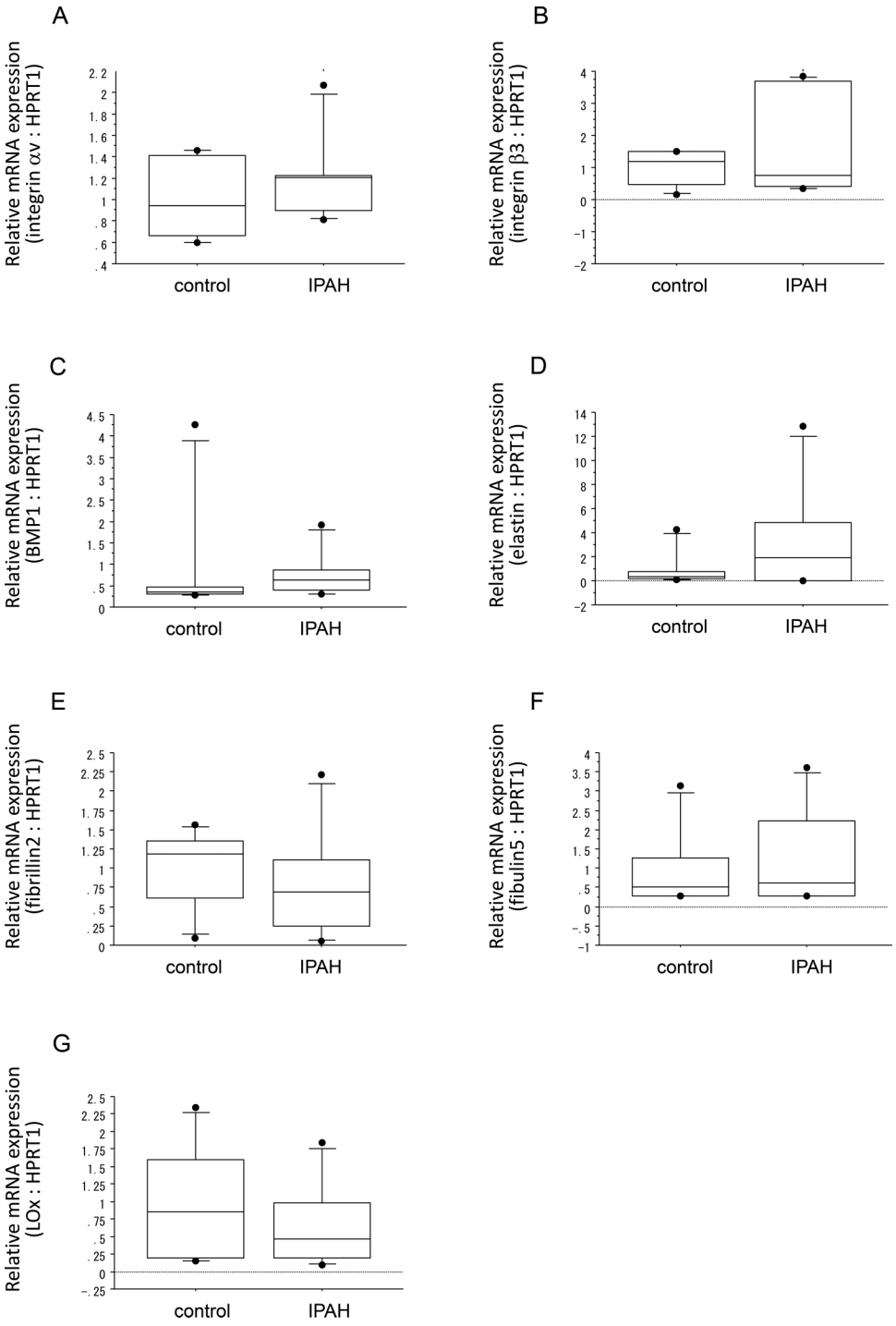
Distinctive response in LOx expression in PASMCs from IPAH patients

We evaluated the response of PASMCs primary cultured from IPAH patients to stimulations (Fig. 4). ET-1 (1 μmol/L) for 24 h increased LOx expression in PASMCs from both control (Fig. 4A) and IPAH patients (Fig. 4B), although induction of the expression reached a statistically significant level only in the latter cells. Trapidil at a dose of 500 μg/mL suppressed LOx expression in PASMCs from IPAH patients (Fig. 4D), while it did not affect it in those from controls (Fig. 4C).

Discussion

This is the first report demonstrating increased LOx expression in PASMCs by stimulation with ET-1 as well as the restoration by

Fig. 3. Box plots presenting (A) integrin αv, (B) integrin β3, (C) BMP1, (D) elastin, (E) fibrillin 2, (F) fibulin 5, (G) and LOx in primary-cultured PASMCs from control (left: $n = 6$) and from IPAH (right: $n = 6$) in baseline without stimulation. Statistically not significant.

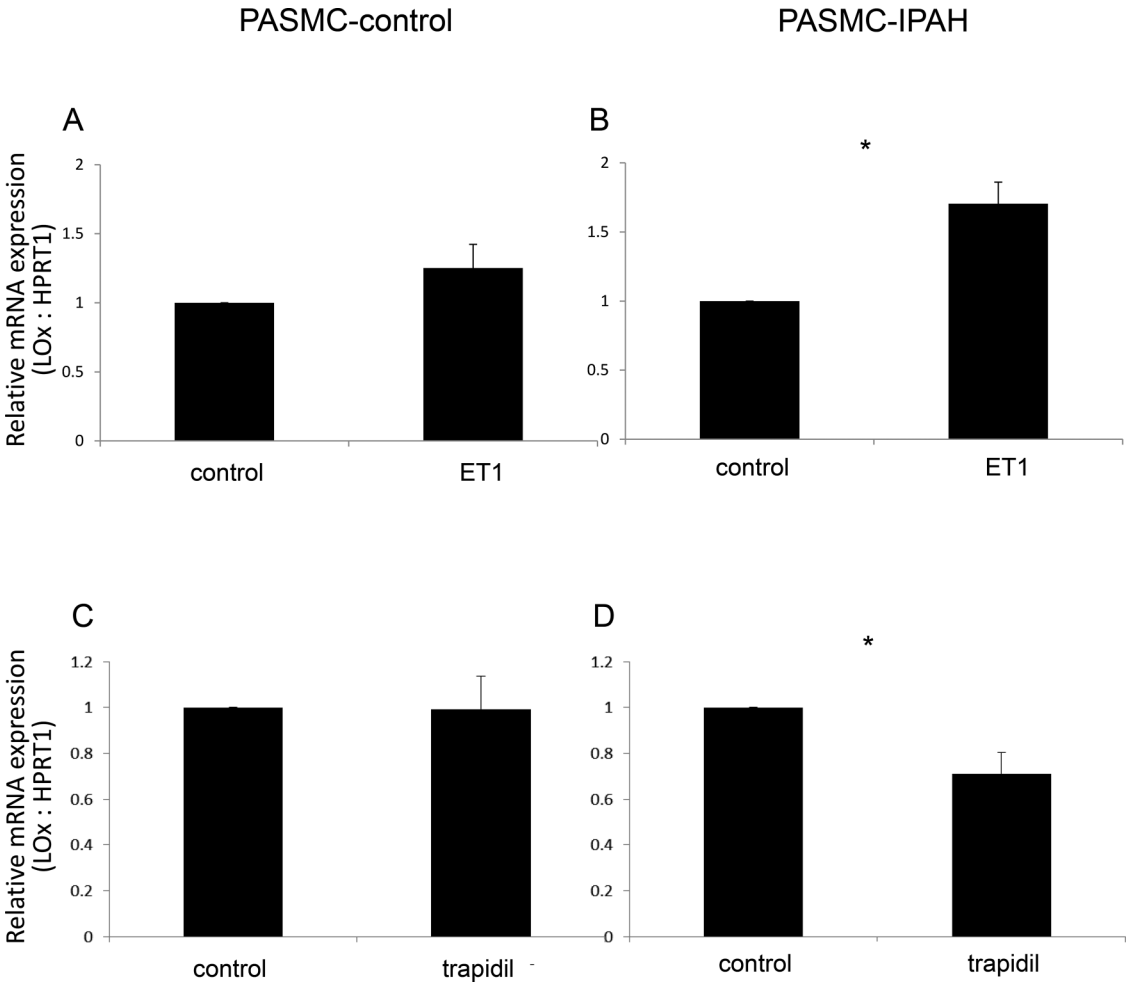


prostacyclin and trapidil. We are also the first to present that, in PASMCs from IPAH patients, LOx expression was significantly induced by ET-1 and was suppressed by trapidil.

Degenerated elastic lamina and matrix deposition are observed in remodeled pulmonary arteries from PAH patients (Pietra et al. 2004). Alteration of the extracellular matrix (ECM) and elastin fragmentation is also found to play an important role in vascular complications such as plaque destabilization and rupture (Duca et al. 2016).

LOx is an ECM enzyme that catalyzes the cross-linking of collagens or elastin in the extracellular compartment (Payne et al. 2007) including internal elastic lamina. LOx has been found to inhibit proliferation of vascular smooth muscle cells (Hurtado et al. 2008). On the other hand, LOx is a potent chemoattractant for both vascular smooth muscle cells and fibroblasts (Lucero et al. 2008) and actively participates in ECM deposition in cardiovascular diseases (Rodríguez et al. 2008). It was reported that LOx expression was elevated in lungs of patients with IPAH, and it played

Fig. 4. LOx expression in PASMCs from IPAH patients responding to ET-1 and trapidil. Treatment with ET-1 (1 μmol/L) for 24 h to PASMCs from (A) controls (n = 8) and (B) IPAH patients (n = 4). Treatment with trapidil (500 μg/mL) for 5 h to PASMCs from (C) controls (n = 10) and (D) IPAH patients (n = 4). Results are expressed as relative fold increase over the mRNA expression of the corresponding baseline control and indicated as mean + SEM. *P < 0.05 using Mann–Whitney's U test.



a causal role in experimental pulmonary hypertension (Nave et al. 2014). Among patients with systemic sclerosis, patients who had a moderate to high systolic pulmonary artery pressure had a higher serum LOx level (Vadasz et al. 2019).

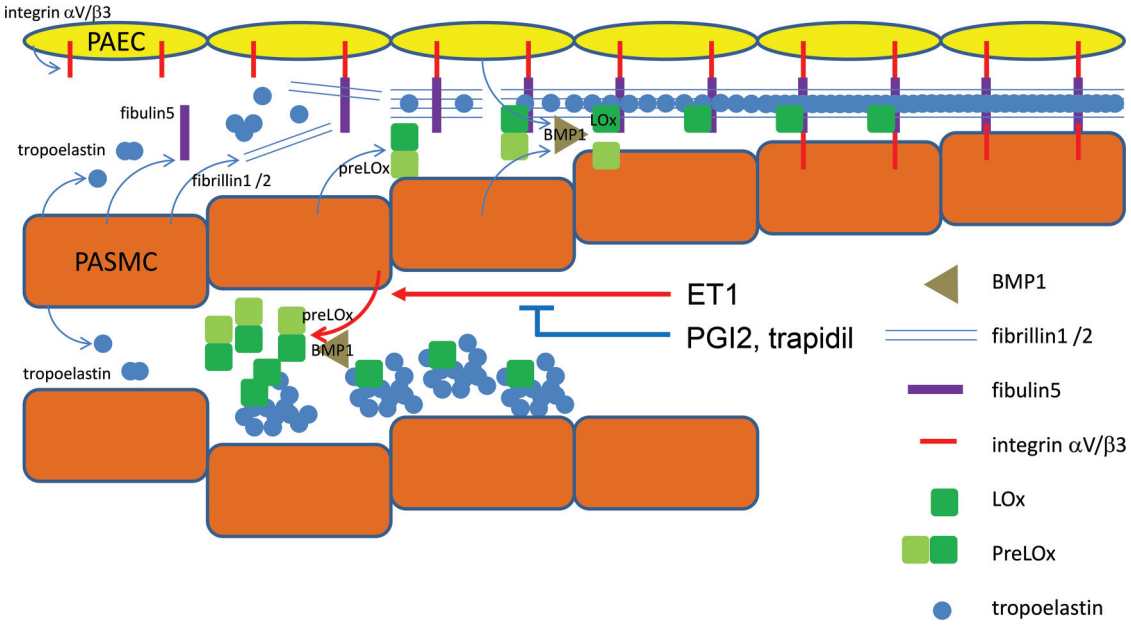
Trapidil (triazolopyrimidine) is a clinical drug used to treat coronary artery diseases as an antiplatelet drug and vasodilator. Trapidil has been reported to exert its antimitogenic effects on vascular smooth muscle cells by direct activation of PKA in a cyclic adenosine monophosphate (cAMP) independent manner (Bönisch et al. 1998). On the other hand, prostacyclin signaling modulates the phenotype of human vascular smooth muscle cells via cAMP-dependent PKA activation (Fetalvero et al. 2006). Activated PKA phosphorylates and translocates cAMP response element-binding protein (CREB) into the nucleus (Delghandi et al. 2005). CREB potentiates its transcription activity by recruitment of several transcription coactivators, such as CBP and p300, thereby controlling expression of target genes. Treprostinil, a prostacyclin analogue, inhibits proliferation and collagen deposition by fibroblasts through activating cAMP (Lambers et al. 2018). cAMP is reported to upregulate the expression of LOx in rat aorta smooth muscle cells (Ravid et al. 1999). On the contrary, both prostacyclin and trapidil restored LOx expression induced by ET-1 and did not affect the expression in control PASMCs in the present report (Figs. 2G and 4C). Further research is required concerning this discrepancy and molecules in the PKA–CREB pathway.

Our QRTPCR experiment showing the different expression patterns of IEL-related genes in PASMCs and PAECs (Fig. 1) reveals that, among PAECs and PASMCs, structural elements of IEL such as elastin, fibrillins, and LOx are secreted exclusively from PASMCs and that the elastin sheet constituted by these molecules will be anchored between PAECs and PASMCs by integrins expressed predominantly on the surface of PAECs. BMP1 from both cell types cleaves and activates preLOx to mature LOx. These might help to illustrate the mechanism by which the elastic lamina exists in the appropriate location, i.e., between the intima and media (Fig. 5).

In the present report, baseline LOx expression level in vitro did not differ statistically between primary-cultured PASMCs from IPAH patients and those from controls (Fig. 3G). Expression of LOx was, however, induced significantly by ET-1 in PASMCs from IPAH patients (Fig. 4B). Considering that ET-1 is upregulated in pulmonary arteries of pulmonary hypertension (Giaid et al. 1993), our findings are compatible with the recent report that LOx expression is elevated in lungs of IPAH patients (Nave et al. 2014).

We report here that ET-1 induced LOx expression significantly and trapidil decreased LOx expression in PASMCs from IPAH patients compared to those from the control. Increased expression of LOx presumably agitates cross-linking of elastin and collagen and ectopic deposition of these in the media as well as disorganized elastic lamina formation, resulting in the remodeling of the

Fig. 5. Schematic of elastin lamina generation. Elastin (tropoelastin) is transported to the plasma membrane of PASMCs, where it is organized into small aggregates and cross-linked by LOx. Propeptide of proLOx is cleaved by bone morphogenetic protein-1 (BMP-1) producing mature LOx. The aggregates are then transferred to extracellular microfibrils, which interact with the cell through integrins expressed predominantly in PAECs. Fibulin 4 and (or) fibulin 5 assist the transfer of elastin aggregates to the microfibril and facilitate elastin to coalesce into larger structures. The elastin aggregates are further cross-linked by LOx to form the complete elastic fiber. Increased expression of LOx might agitate cross-linking of elastin and collagen and ectopic deposition of them in the media. Prostacyclin (PGI2) and trapidil restore the upregulated LOx expression. [Colour online.]



pulmonary artery in PAH patients. Prostacyclin is one of the strongest clinical tools to treat PAH not only by vasodilation but also by an antiremodeling effect, although the detailed mechanism remains unclear. According to our present data, restoring upregulated LOx expression participates in its therapeutic effect. Distinctive LOx expression patterns in PASMCs from IPAH patients in response to ET-1 and trapidil (Fig. 4) suggest impairments in the PKA–CREB pathway in IPAH.

In conclusion, LOx expression in PASMCs is increased by ET-1 and restored by prostacyclin and trapidil. ET-1 increased LOx expression strongly in PASMCs from PAH patients, suggesting agitated cross-linking of elastin and collagen and ectopic deposition of these in the vascular media.

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