# Endothelin-1 upregulates activin receptor-like kinase-1 expression via Gi/RhoA/Sp-1/Rho kinase pathways at transcriptional and post-transcriptional levels in human pulmonary arterial endothelial cells

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# Abstract

Backgrounds

Pulmonary arterial hypertension (PAH) is a disease with poor prognosis that is characterized by pulmonary vasoconstriction and organic stenosis due to abnormal proliferation of pulmonary vascular cells. It has been demonstrated that endothelin (ET)-1 induces pulmonary vasoconstriction through activation of RhoA. Moreover, we previously demonstrated that G*i*, a heterotrimeric G protein, functions upstream of RhoA activation. A gene mutation of activin receptor-like kinase (ACVRL)-1 is recognized in idiopathic or heritable PAH patients. However, little is known about the association between ET-1 and ACVRL-1. In the present study, we investigated the effect of ET-1 on ACVRL-1 expression and aimed to delineate the involvement of the G*i*/RhoA/Rho kinase pathway.

Methods

ET-1 was added to culture medium of human pulmonary arterial endothelial cells (PAECs), and ACVRL-1 expression levels were analyzed using western blotting and quantitative polymerase chain reaction. The promoter activity of ACVRL-1 was evaluated by dual luciferase assay. Before adding ET-1 to the PAECs, pretreatment with pertussis toxin (PTX) or exoenzyme C3 transferase (C3T) was performed for the inhibition of G*i* or RhoA, respectively. Rho kinase was inhibited by Y27632. Active form of RhoA (GTP-RhoA) was assessed by pull-down assay.

Results

ACVRL-1 expression was increased by ET-1 in the PAECs. Pull-down assay revealed that ET-1 rapidly induced a GTP-loading of RhoA. The ET-1-induced RhoA activation was suppressed by pretreatment with PTX or C3T. Further, PTX, C3T, and Y27632 suppressed the ET-1-induced ACVRL-1 expression. The activity of ACVRL-1 promotor and the lifespan of ACVRL-1 mRNA was increased by ET-1. Sp-1, which is one of the transcriptional factors of ACVRL-1, peaked 15 min after adding ET-1 to the PAECs. PTX and C3T prevented the increase of Sp-1 induced by ET-1.

Conclusion

The present study demonstrated that ET-1 increases ACVRL-1 expression at the transcriptional and post-transcriptional levels in human PAECs via the G*i*/RhoA/Rho kinase pathway with involvement of Sp-1.


# Introduction

Pulmonary arterial hypertension (PAH) is a disease with poor prognosis that is characterized by pulmonary vasoconstriction and organic stenosis due to abnormal proliferation of pulmonary artery endothelial cells and smooth muscle cells [1, 2, 3]. It is considered that endothelial dysfunction is associated with these vascular pathologies [4, 5]; however, their detailed molecular mechanisms are still unknown.

Endothelin-1 (ET-1) is a major vasoconstrictor derived from endothelial cells [6]. Today, endothelin receptor agonist is widely used for PAH treatment, and has contributed to the improvement of PAH prognosis. This shows that endothelin plays a crucial role in PAH. It has been demonstrated that ET-1 induces pulmonary vasoconstriction through activation of RhoA, which is a small GTP protein [7]. Many studies have reported that the RhoA/Rho-kinase pathways implicate pulmonary hypertension [8, 9, 10]. Additionally, endothelin receptors are G-protein-coupled receptors [7], and we have previously demonstrated that G*i*, which is a heterotrimeric G protein, functions upstream of RhoA activation [11].

Activin receptor-like kinase-1 (ACVRL-1) is one of the type I cell surface receptors for the transforming growth factor-β (TGF-β) family that is mainly expressed in vascular endothelial cells [12]. A gene mutation of ACVRL-1 is recognized in idiopathic or heritable PAH patients [13]. Although both ET-1 and ACVRL-1 are important molecules for the pathogenesis of PAH, little is known about the association between ET-1 and ACVRL-1.

In the present study, we investigated the effect of ET-1 on ACVRL-1 expression, and aimed to delineate the involvement of the G*i*/RhoA/Rho kinase pathway in pulmonary arterial endothelial cells.

## Methods

# Materials

Most of the reagents used in this study have been described previously [14, 15, 16]. Recombinant human ET-1 was obtained from R&D systems (Minneapolis, MN, USA), and cell permeable exoenzyme C3 transferase (C3T) was purchased from Cytoskeleton, Inc. (Denver, CO, USA). Pertussis toxin (PTX) and actinomycin D were purchased from Merck KGaA (Darmstadt, Germany). Y27632 was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

# Preparation of Endothelial Cells

Human pulmonary arterial endothelial cells (PAECs) were cultured according to the manufacturers' instructions (PromoCell, Heidelberg, Germany) and used for all experiments after 5 to 10 passages.

# Western Blotting

Western blotting was performed as described previously [14, 15, 16]. The lysates of lung tissues were mixed at a ratio of 4:1 with loading buffer (75 mM Tris-HCl, pH 6.8; 10% glycerol; 3% 2-mercaptoethanol, and 2% sodium dodecyl sulfate [SDS]) and heated at 95 °C for 10 minutes. Aliquots containing 20 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred onto polyvinylidene difluoride membranes (Merck KGaA). After incubation with blocking solution at room temperature for 30 minutes, the membranes were incubated for 1 hour at room temperature with a monoclonal antibodies to RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) and ACVRL-1 (Abcam, Cambridge, UK) at a dilution of 1:500, and to β-actin (Santa Cruz Biotechnology) diluted 1:1000 or a rabbit polyclonal antibody to Sp-1 (GeneTex, Inc., Irvine, CA, USA) diluted 1:1000 for immunoblotting. The

signals from immunoreactive bands were visualized by a Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The optical densities of the individual bands were analyzed using Image J 1.48.

# GTP/GDP Exchange of RhoA

GTP-bound active forms of RhoA were assessed using a commercially available assay kit (Cytoskeleton Inc.) according to the manufacturer's instructions [17].

# Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from PAECs using TRIzol reagent (Invitrogen Carlsbad, CA, USA). The RNA was reverse transcribed into first-strand cDNA with a ReverseTra Ace qPCR RT kit (Toyobo Co., Ltd, Osaka, Japan), and the cDNA was subjected to quantitative polymerase chain reaction (qPCR) using a Thunderbird SYBR qPCR Mix (Toyobo) in a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers were designed on the basis of GenBank sequences (ACVRL-1, NM_009612.3 and GAPDH, NM_001289726.1). qPCR reactions were run in duplicates, and the delta-delta Ct method was applied for quantification.

# DNA transfection and luciferase assay

We cloned the firefly luciferase reporter construct ACVRL-1/Luc containing the human putative promoter region of ACVRL-1 (GeneBank: NC_000012.12, position 51906383 to 51907627) into pGL3-Basic vector [18]. pNL1.1.TK [NLuc/TK] was used as a control vector. The constructs were co-transfected into 70% confluent PAECs using a Screenfect A (FUJIFILM Wako Pure Chemical Corporation). Forty-eight hours after transfection, cell lysates were assayed for luciferase activity using a Nano-Glo Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. Cell culture experiments were performed in triplicate.

# Determination of eNOS mRNA stability

To analyze the effect of ET-1 on ACVRL-1 mRNA stability, PAECs were stimulated by ET-1 for 6 hours with or without Y27632, then 5 μg/mL actinomycin D was added. ACVRL-1 mRNA levels were determined by qPCR.

# Statistical analysis

Statistical analyses were performed using ANOVA with Tukey’s post hoc test or Student's t-test where appropriate. A value of $P < 0.05$ was considered significant. Data are expressed as means ± standard errors (SE).

## Results

Western blotting and qPCR demonstrated that protein and mRNA expressions of ACVRL-1 were increased by ET-1 stimulation in the PAECs (Figs. 1A and B). Further, these results indicate that the upregulations of ACVRL-1 were not necessarily dependent on the dose of ET-1.

Pull-down assay revealed that ET-1 rapidly induced a GTP-loading of RhoA, which is active form of RhoA in the PAECs, whereas the levels of RhoA in whole cell lysates were not changed. C3T prevented ET-1-induced RhoA activation (Fig. 2A). Figure 2B shows that the ET-1-induced RhoA activation was suppressed by pretreatment with PTX, suggesting that G*i* is upstream of RhoA activation.

We examined the effects of inhibition of either G*i*, RhoA or Rho kinase on the upregulation of ACVRL-1 expression in ET-1-stimulated PAECs. Western blotting showed that PTX (Fig. 3A), C3T (Fig. 3B) and Y27632 (Fig. 3C) suppressed ET-1-induced ACVRL-1 expression. These results indicate that ET-1 increased the ACVRL-1 expression mediated via the G*i*/RhoA/Rho kinase signaling pathway.

To elucidate whether ET-1-induced ACVRL-1 upregulation occurs at the transcriptional or posttranscriptional level, we evaluated the promoter activity of ACVRL-1 and the stability of mRNA of ACVRL-1. Figure 4A shows that transcriptional activity of the ACVRL-1 promotor was increased by ET-1. In addition, the lifespan of mRNA of ACVRL-1 was sustained in ET-1-stimulated PAECs, and Rho kinase inhibition by Y27632 reversed the stabilization of ACVRL-1 mRNA by ET-1 to the control level (Fig. 4B).

Further, we investigated the role of Sp-1, which is one of the transcriptional factors for ACVRL-1, in ET-1-stimulated PAECs. Figure 5A shows that, after adding ET-1, the level of Sp-1 was increased within 10 min, and peaked after 15 min. Both G*i* inhibition by PTX and RhoA inhibition by C3T prevented the increase of Sp-1 level in response to ET-1 (Fig. 5B, C). These data indicate that rapid RhoA activation via G*i* affects Sp-1 regulation.

# Discussion

We for the first time demonstrated that ET-1 upregulated ACVRL-1 expression at the transcriptional and the post-transcriptional levels via Gi/RhoA/Rho kinase pathway in PEACs. Our results suggest that Sp-1 is associated with the promoter activity of ACVRL-1 via RhoA activation.

In patients with idiopathic PAH, an ET-1 plasma level is correlated with mean pulmonary arterial pressure and pulmonary vascular resistance [19]. Further, it has been reported that ACVRL-1 expression is increased in the PAECs of patients with idiopathic PAH [20]. Taking these clinical findings and our in vitro data together, it is highly possible that elevated ET-1 levels actually increase ACVRL-1 expression in PAH patients. In addition, a previous study has shown that ACVRL-1 deficiency induced the synthesis and release of ET-1 [21]. This phenomenon might have been caused to maintain the levels of ACVRL-1 expression by increasing ET-1 stimulation.

However, in mice models, there have been opposing results of the effect of TGF-β receptors deficiency on PAH. While Jerkic et al. reported that adult ACVRL-1 heterozygous mice spontaneously develop PAH [22],

Gore et al. demonstrated that hypoxia-induced pulmonary hypertension was ameliorated by deficiency of endoglin, which is a TGF-β receptor. Similar to ACVRL-1, gene mutation in endoglin is implicated to PAH [20]. Therefore, the significance of increases in ACVRL-1 due to ET-1 in PAH patients remains unclear, and needs to be resolved in future studies.

Previous studies have shown that whether the modification of RNA expression by RhoA is upregulated or downregulated depends on the type of molecule [23, 24]. Marshall et al. demonstrated that ET-1 upregulated RNA expression of some molecules (e.g. Abra, Srf and Egr2), which were inhibited by C3T in rat cardiomyocytes [23]. On the other hand, Laufs et al. reported that RhoA activation downregulated the expression of endothelial nitric oxide synthase (eNOS) at the post-transcriptional level in endothelial cells. They showed that actin stress fiber reorganization by RhoA activation was associated with post-transcriptional regulation of eNOSmRNA [24].

Murthy et al. reported that Rac1, which is a small GTP protein similar to RhoA, stabilized Sp-1 expression in macrophages [25]. Interestingly, in the current study, ET-1-triggered RhoA activation has been increased the Sp-1 protein within 15 min, whereas the stabilization of Sp-1 by Rac1 was shown to take several hours.

Several studies have reported on the association between ET-1 and PTX-sensitive G protein [26, 27]. However, to date there have been no reports describing the correlation between the regulation of ACVRL-1 by ET-1 and that by G*i*. Further mechanisms of correlation between ET-1/G*i* axis and ACVRL-1 are needed to be elucidated.

# Conclusion

We demonstrated that ET-1 upregulated ACVRL-1 expression in human PAECs at the transcriptional and the post-transcriptional levels. G*i*, RhoA, Rho kinase and Sp-1 are important as the underlying mechanisms of ET-1-induced expression of ACVRL-1 in PAECs.

# Abbreviations

PAH Pulmonary arterial hypertension

ET-1 Endothelin-1

ACVRL-1 Activin receptor-like kinase-1

TGF-β Transforming growth factor-β

C3T Exoenzyme C3 transferase

PTX Pertussis toxin

PAECs Pulmonary endothelial cells

SDS Sodium dodecyl sulfate

qPCR Quantitative polymerase chain reaction

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

# Declarations

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Competing interests

Koichi Sugimoto and Tetsuro Yokokawa belong to a department supported by Actelion Pharmaceuticals Japan. This company was not associated with the contents of this study.

### Funding

None.

### Authors' contributions

Conception: KS and YT. Design of the work: KS and YT. Acquisition of data: KS and TY. Analysis and interpretation of data: KS, TM, SY, TK, and AY. Manuscript writing: KS, and YT. Review and editing: KN and YT. All authors have read and approved the final manuscript.

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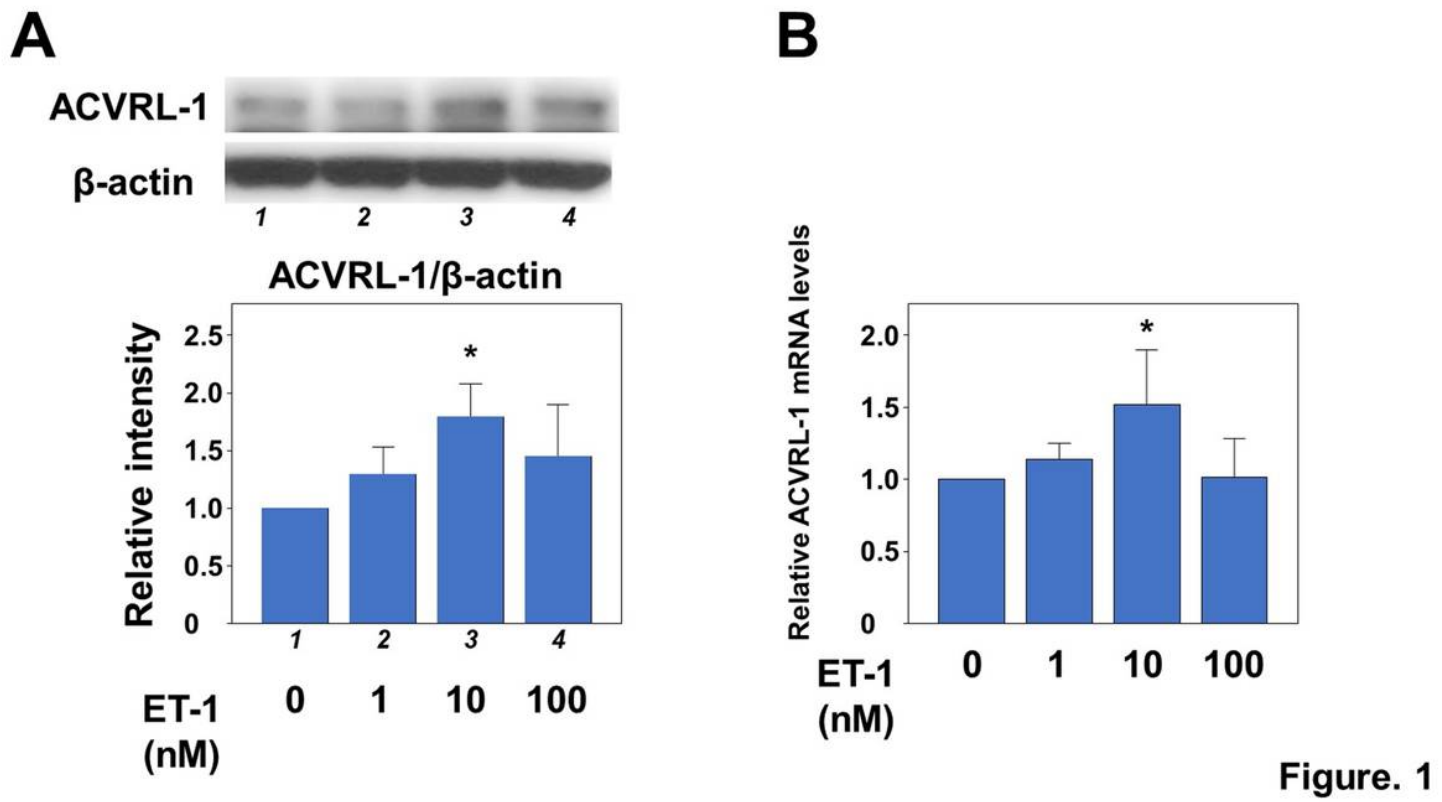
# Figures




## Figure 1

(A) Effects of endothelin (ET)-1 on expression of ACVRL-1 in human pulmonary arterial endothelial cells (PAECs) as determined by western blotting. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (1 nmol/L); lane 3, PAECs + ET-1 (10 nmol/L); lane 4, PAECs + ET-1 (100 nmol/L). PAECs were stimulated by ET-1 at the indicated concentration for 18 hours. Quantitative results of ACVRL-1 were normalized by the levels of β-actin. Bars are means ± SE. of quantitative densitometric analyses from three separate experiments. Representative immunoblots are shown at the top. *P < 0.05 vs control. (B) Effects of endothelin (ET)-1 on RNA expression of ACVRL-1 in human PAECs as determined by qPCR. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (1 nmol/L); lane 3, PAECs + ET-1 (10 nmol/L); lane 4, PAECs + ET-1 (100 nmol/L). PAECs were stimulated by ET-1 at the indicated concentration for 6 hours. Quantitative results of ACVRL-1 were normalized by the levels of GAPDH. Bars are means ± SE. of quantitative analyses from five separate experiments. *P < 0.05 vs control.

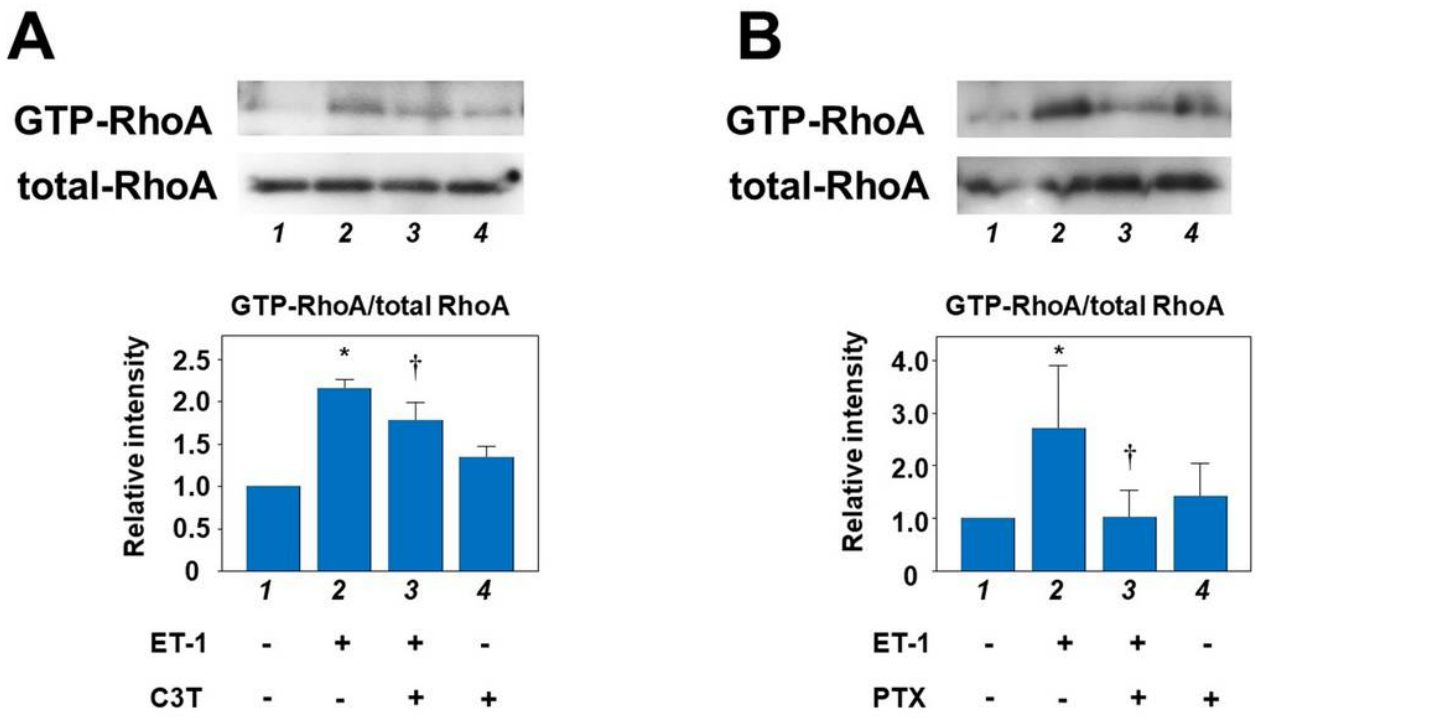



**Figure. 2**

## Figure 2

(A) Levels of GTP-bound active forms of RhoA in cultured human PAECs. PAECs were pretreated with C3T (0.25 μg/mL) overnight before adding ET-1. After 5 minutes of ET-1 stimulation (10 nmol/L), GTP-loadings of RhoA was determined by pull-down assays. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (1 nmol/L); lane 3, PAECs + ET-1 + C3T; lane 4, PAECs + C3T. (B) PAECs were pretreated with 100 ng/mL PTX overnight. RhoA activation was determined by pull-down assay. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + PTX; lane 4, PAECs + PTX. Quantitative results of GTP-RhoA were normalized by total RhoA levels. Bars are mean ± SE. of quantitative densitometric analyses from three separate experiments. Representative immunoblots are shown at the top. *P < 0.05 vs Lane 1, †P < 0.05 vs Lane 2.

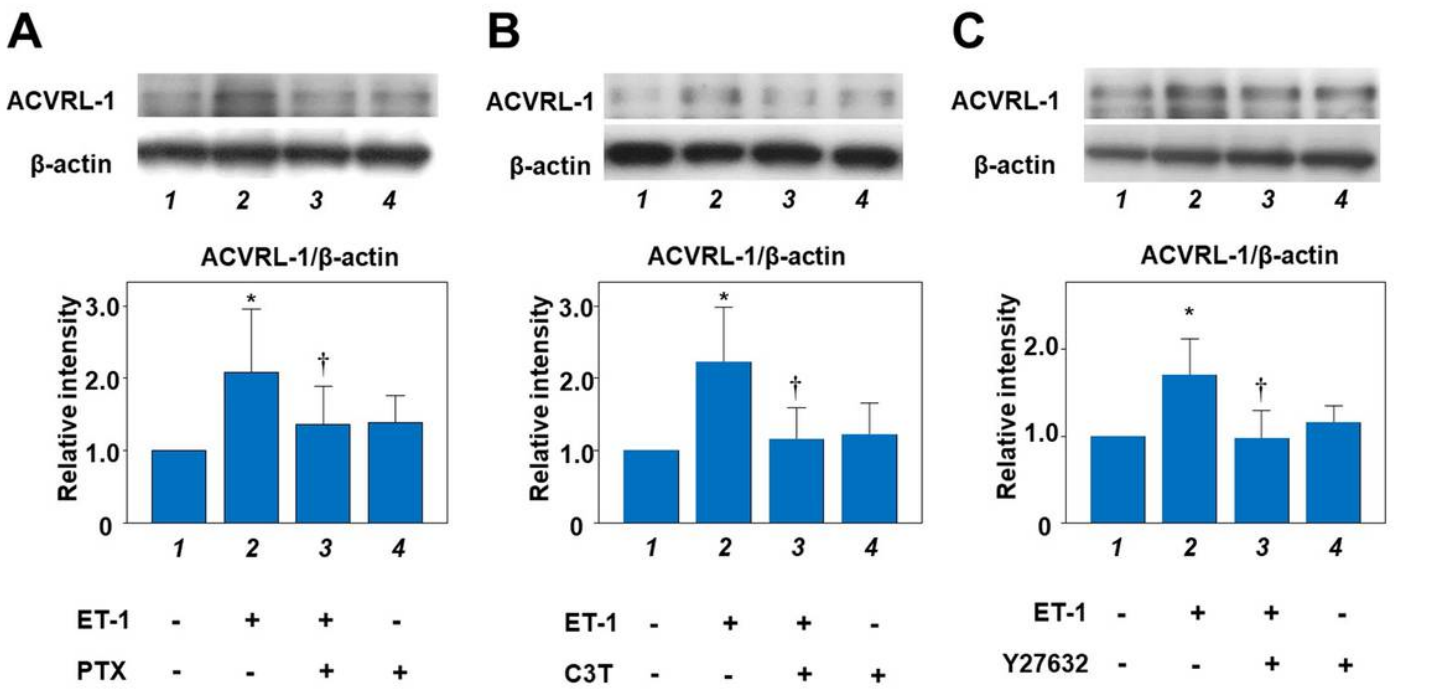



**Figure. 3**

## Figure 3

(A) Effect of Gi inhibition by PTX on ACVRL-1 expression induced by ET-1 in PAECs, as determined by western blotting. After overnight treatment with 100 ng/mL PTX, ET-1 was added to PAECs, and after 18 hours, western blotting of ACVRL-1 was performed. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + PTX; lane 4, PAECs + PTX. (B) Effect of RhoA inhibition by C3T on ACVRL-1 expression induced by ET-1 in PAECs, as determined by western blotting. After overnight treatment with 0.25 μg/mL C3T, ET-1 was added to PAECs, and after 18 hours, western blotting of ACVRL-1 was performed. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + C3T; lane 4, PAECs + C3T. (C) Effect of Y-27632 on ACVRL-1 protein levels in PAECs. Ten μmol/L of Y-27632 were added to PAECs simultaneously with ET-1 stimulation, and ACVRL-1 protein levels were determined by western blotting after 18 hours of culture. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + Y27632; lane 4, PAECs + Y27632. Data are expressed as mean ± SE. of four to five experiments. Representative immunoblots are shown at the top. *P < 0.05 vs Lane 1, †P < 0.05 vs Lane 2.

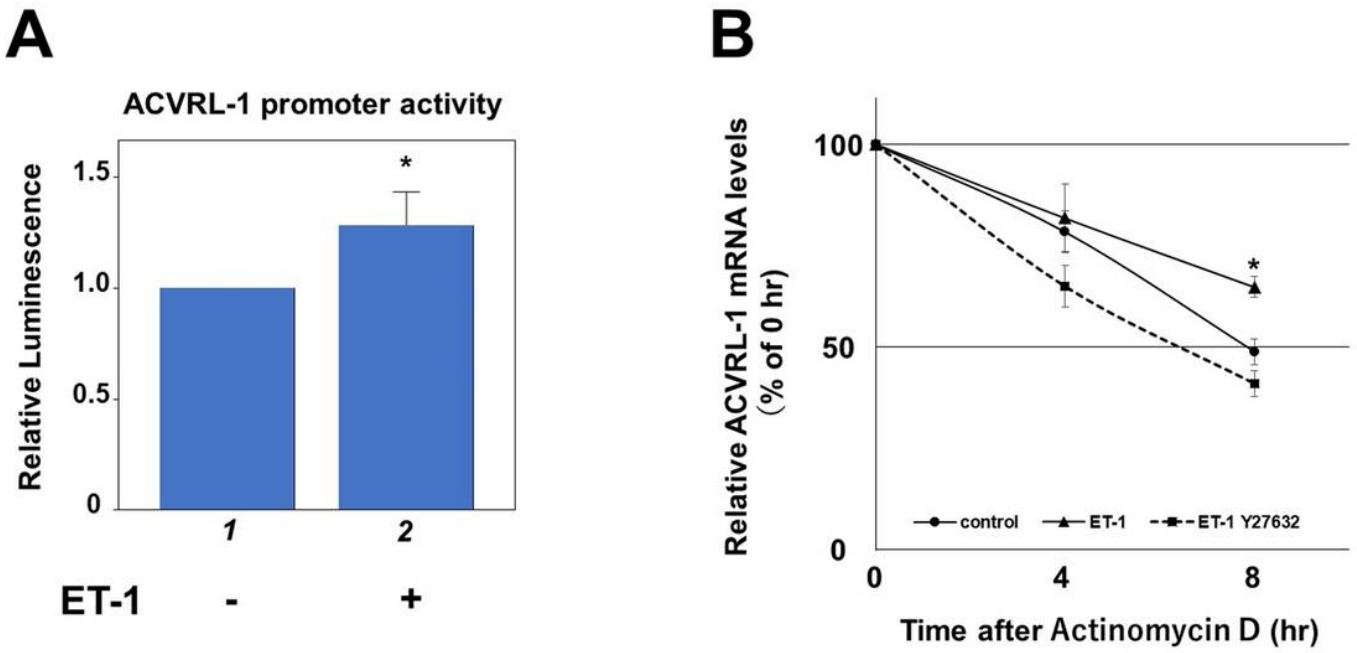



**Figure. 4**

## Figure 4

(A) The promoter activity of the ACVRL-1 gene in PAECs was determined after 6 hours of incubation with or without ET-1. Data are expressed as means ± SE. of five separate experiments. *P < 0.05 vs Lane 1. (B) PAECs were stimulated by ET-1 with or without Rho kinase inhibition by Y27632. After 5 μg/mL actinomycin D addition, ACVRL-1 mRNA levels were determined by qPCR at the indicated time points. Quantitative results of ACVRL-1 were normalized by the levels of GAPDH. Data are expressed as means ± SE. of five separate experiments. *P < 0.05 vs control, PAECs + Y27632.

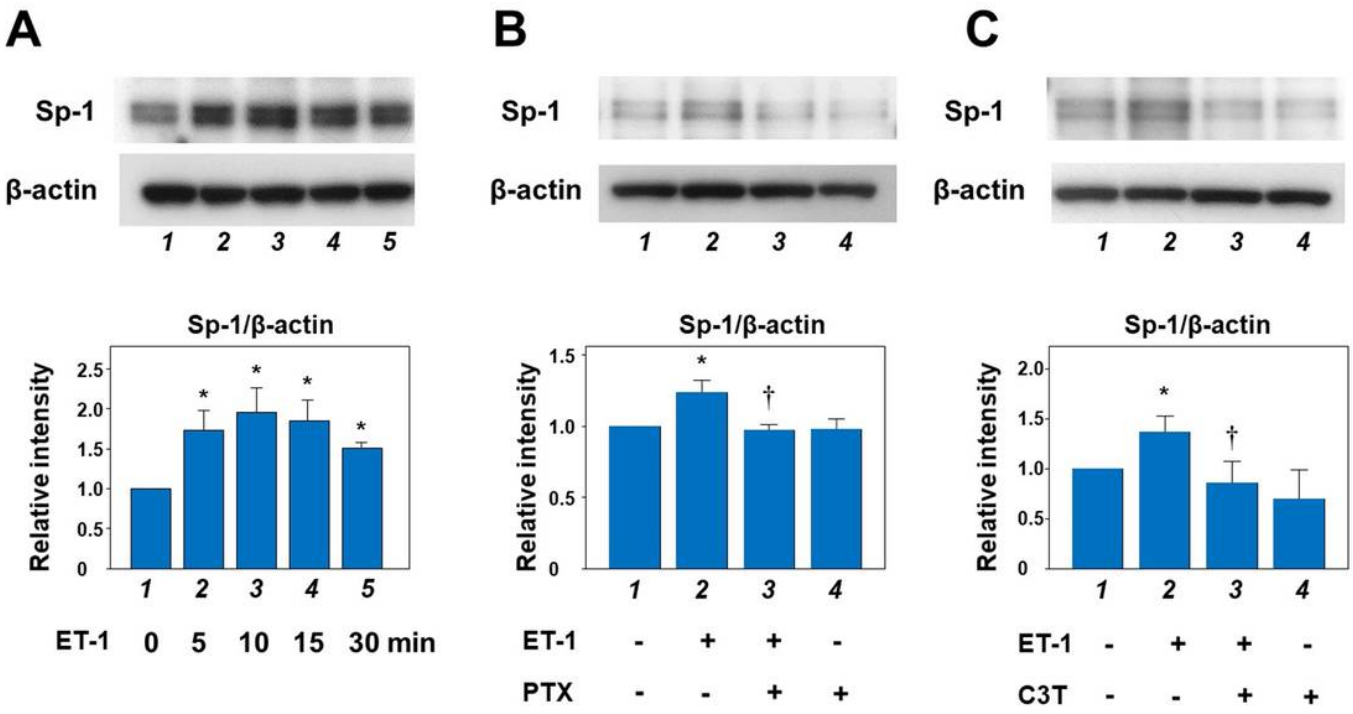



**Figure. 5**

## Figure 5

(A) Levels of Sp-1 protein as determined by western blotting in cultured human PAECs 5 to 30 minutes after adding 10 nmol/L ET-1. (B) Effect of Gi inhibition by PTX on Sp-1 induced by ET-1 in PAECs, as determined by western blotting. After overnight treatment with 100 ng/mL PTX, ET-1 was added to PAECs, and after 30 minutes, western blotting of Sp-1 was performed. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + PTX; lane 4, PAECs + PTX. (C) Effect of RhoA inhibition by C3T on Sp-1 induced by ET-1 in PAECs, as determined by western blotting. After overnight treatment with 0.25 µg/mL C3T, ET-1 was added to PAECs, and after 30 minutes, western blotting of ACVRL-1 was performed. Lane 1, PAECs alone (control); lane 2, PAECs + ET-1 (10 nmol/L); lane 3, PAECs + ET-1 + C3T 0.25 µg/mL; lane 4, PAECs + C3T 0.25 µg/mL. Bars are mean ± SE. of quantitative densitometric analyses from three to five separate experiments. Representative immunoblots are shown at the top. *P < 0.05 vs Lane 1, †P < 0.05 vs Lane 2.