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ORIGINAL ARTICLE

INVOLVEMENT OF β-ARRESTIN IN ENDOTHELIN RECEPTOR SIGNALING: A POSSIBLE ROLE IN THE PATHOGENESIS OF PULMONARY ARTERIAL HYPERTENSION

Noritomo Narita, Kenji Hanada, Maiko Senoo, Tomo Kato, Natsumi Kudo, Yoshikazu Yokono, Michiko Tsushima, Yuichi Toyama, Masato Narita and Hirofumi Tomita

Abstract **Background:** Endothelin (ET) is a strong vasoconstrictor that plays important roles in the pathogenesis and progression of cardiovascular remodeling. ET receptor (ET-R) antagonists have recently become established as a drug essential for treating pulmonary arterial hypertension (PAH). β-arrestin was originally identified as a regulator of G-protein coupled receptor recycling, but it recently became apparent that β-arrestins act as scaffolds in their own signaling pathway. In this study, we examined the role of β-arrestin in ET-R signaling and explored its possible role in the pathogenesis of PAH.

Methods and Results: The knockdown of β-arrestin1 or β-arrestin2 in human kidney embryonic 293 cells resulted in enhanced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in response to ET. Confocal microscopy showed that, in the absence of stimulation, transiently transfected green fluorescent protein-tagged epidermal growth factor receptors (EGFRs) were located on the plasma membrane, whereas they were internalized in response to ET, as shown by their redistribution into cellular aggregates. Pretreatment with Ro318425 (a protein kinase C inhibitor) or AG1478 (an EGFR antagonist) suppressed ERK1/2 phosphorylation in response to ET.

Conclusions: β-arrestins and EGFR transactivation are involved in ET-R signaling. These new insights may contribute to elucidating further layers in the pathogenesis of PAH.

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Key words: Endothelin; β-arrestin; G-protein coupled receptor; Pulmonary arterial hypertension.

Introduction

Endothelin (ET) was originally extracted from aortic endothelial cells and identified to be a strong vasoconstrictor[1], and to play an important role in the cardiovascular system[2, 3]. ET exerts its biological effects through two types of receptors (ET-Rs), type A and type B, both of which are members of the G-protein coupled receptor (GPCR) superfamily[4]. Type A exerts vasoconstrictive and cardiotonic effects, and type B seems to antagonize type A signaling. Type A is the dominant isoform in vascular smooth muscle cells, where it elevates vascular tone and promotes cell proliferation[4].

β-arrestin, originally identified as a regulator of GPCR recycling, acts as a scaffold in its own signaling pathway and regulates a large network of cellular responses[5]. For instance, β1-adrenergic receptor (β1-AR) couples with the heterotrimeric G-protein, triggering the dissociation of Gαs from Gβγ after catecholamine binding, and this subsequently activates the effector molecule, adenylyl cyclase, that promotes the generation of a second messenger, cyclic AMP (cAMP)[6]. cAMP activates protein kinase A, which exerts cardiotonic and chronotropic effects over a short period; however, chronic activation

Department of Cardiology, Hirosaki University Graduate School of Medicine, Hirosaki, 036-8562 Japan

Correspondence: H. Tomita
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of this pathway results in cardiac dysfunction and pathological remodeling[7]. The G-protein-mediated signaling is rapidly turned off by receptor desensitization, a process that involves phosphorylation of the C-terminal tail of the β1-AR; this is mediated by β-arrestin[7]. In addition to its role in GPCR desensitization, β-arrestin exerts antiapoptotic and cardioprotective effects through transactivation of epidermal growth factor (EGF) receptor (EGFR) via Src, matrix metalloproteinase, heparin-binding EGF-like growth factor (HB-EGF)[8, 9]. Thus, β-arrestin is responsible for a wide diversity of physiological and pathological processes in various GPCRs, as well as playing important roles not only in the cardiovascular system, but also in the endocrine system and in the development of cancer[10, 11].

Pulmonary arterial hypertension (PAH) is characterized by the progressive endothelial dysfunction and increased contractility of small pulmonary arteries, resulting in progressive dyspnea and right heart failure. ET causes vascular contraction, inflammation, fibrosis, and proliferation in vessel walls[4], and it is considered to be a key molecule in the progression of PAH[12]. Recently, it has been reported that ET-R antagonists reduced pulmonary arterial pressure and inhibited the progression of PAH[13, 14]. In clinical practice, ET-R antagonists have become an essential drug for the treatment of PAH[15]. However, the role of β-arrestin in the pathogenesis and progression of PAH has not been examined. In this study, we investigated the involvement of β-arrestin in ET-R signaling.

Methods

Cell culture

Human embryonic kidney (HEK) 293 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified environment with 5% CO^2.

Small interfering RNA knockdown of β-arrestins

HEK 293 cells were seeded into 6-cm dishes. When they reached 60% confluence, they were transfected with human β-arrestin1, β-arrestin2, or non-silencing small interfering RNA (siRNA) using DharmaFECT (Dharmacon, Lafayette, CO, USA), according to the manufacturer's protocol. All the assays were performed 72 h after siRNA transfection.

Extracellular signal-regulated kinase 1/2 phosphorylation assay

HEK 293 cells plated on six-well dishes were serum-starved overnight and stimulated with 100 nM ET (E7764) (Sigma, Ronkonkoma, NY, USA) for 2, 5, and 10 min. After stimulation, the cells were lysed in RIPA lysis buffer (20 mM Tris, pH7.4, 137 mM sodium chloride, 20% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and phosphatase inhibitors) for 1 h at 4 °C and centrifuged for 15 min at 13,200 rpm. Protein concentrations were measured with a Bio-Rad protein assay reagent. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and blocked with Blocking One-P (Nacalai Tesque, Kyoto, Japan) or 5% skimmed milk. The membranes were incubated with antibodies for phosphorylated extracellular signal-regulated kinase (ERK)1/2 (1:1000) (Cell Signaling, Danvers, MA, USA) or total ERK1/2 (1:2000) (Merck Millipore, Burlington, MA, USA) overnight at 4 °C, and then with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. The protein bands were detected by Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare, Chicago, IL, USA), and densitometric analyses were performed using a ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

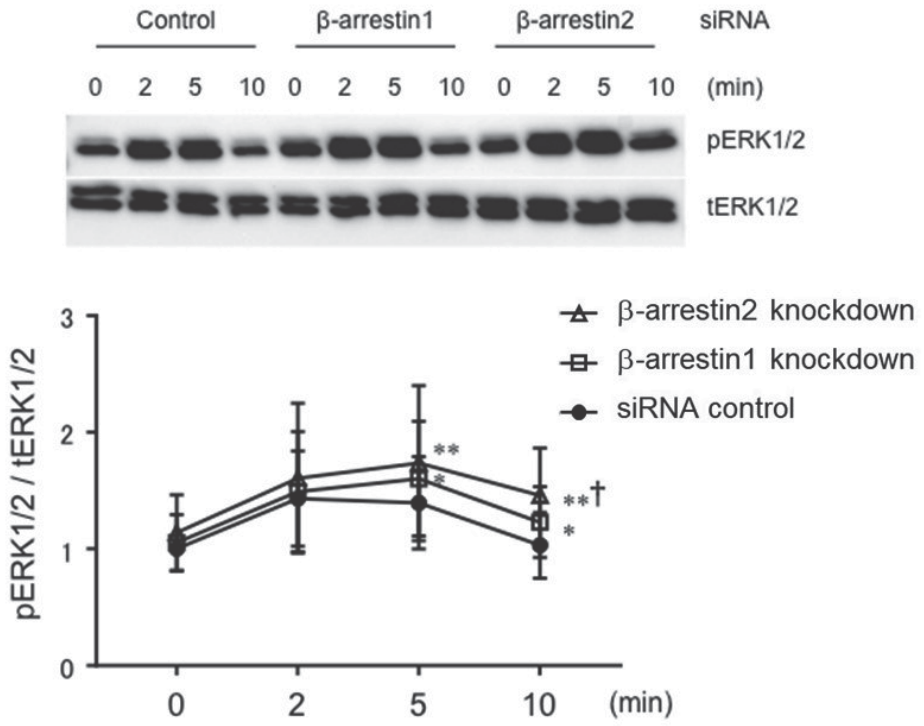

Figure 1. Enhancement by β-arrestin1 or β-arrestin2 knockdown of extracellular signal-regulated kinase (ERK)1/2 phosphorylation induced by endothelin (ET) in human embryonic kidney (HEK) 293 cells. HEK 293 cells were treated with 100 nM ET for 2, 5, or 10 min with or without β-arrestin1 or β-arrestin2 knockdown. Western blotting for phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (tERK1/2) was performed. *p < 0.05 vs. control at the same time point. **p < 0.001 vs. control at the same time point. †p < 0.05 vs. β-arrestin1 knockdown at the same time point. Each n = 10. Error bar indicates standard deviation.

Confocal microscopy

For the EGFR internalization assay, HEK 293 cells were transiently transfected with green fluorescent protein (GFP)-tagged EGFR using lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) and plated on collagen-coated glass bottom cell culture dishes. GFP-tagged EGFR plasmid was kindly provided by Dr. Howard A. Rockman (Duke University, Durham, NC, USA)[8]. After overnight serum starvation, the cells were stimulated with 100 nM ET or 10 ng/mL EGF for 10 min and then fixed with 4% paraformaldehyde. The cells were visualized using a BZ-X Analyzer (Keyence, Osaka, Japan).

Statistics

Data are expressed as mean ± standard deviation. Statistical significance was determined with a two-way analysis of variance (ANOVA) with Tukey's correction for post hoc multiple comparisons, using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered significant.

Results

β-arrestin knockdown enhanced ERK1/2 phosphorylation

ERK1/2 is well known to be involved in the downstream signaling of G-protein- and β-arrestin-dependent pathways. To investigate the role of β-arrestins in those pathways, ERK1/2 phosphorylation assay was performed using HEK 293 cells after the knockdown of β-arrestins. Without β-arrestin knockdown, ERK1/2 phosphorylation was enhanced at 2 and 5 min after

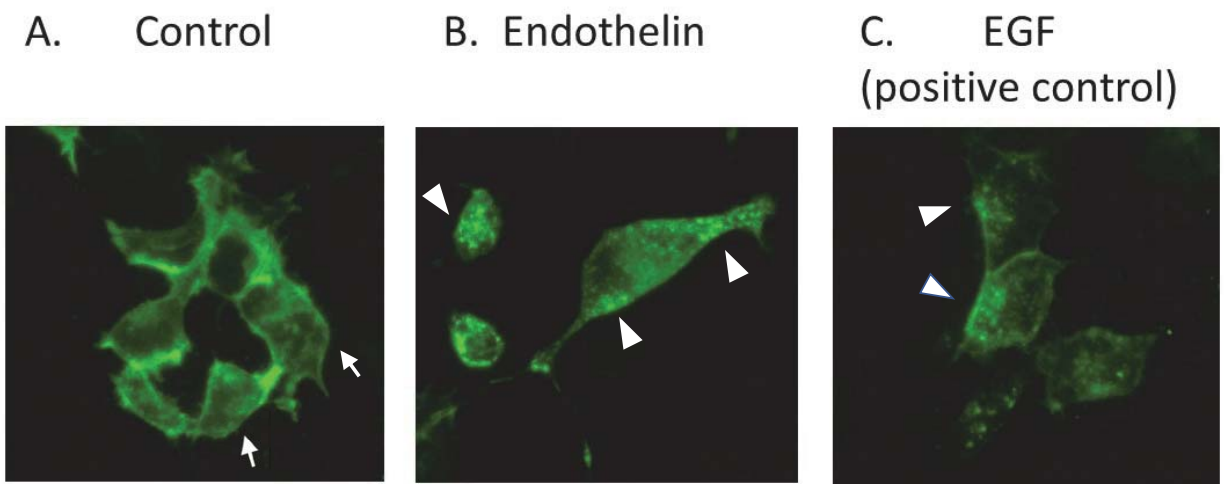

Figure 2. Involvement of epidermal growth factor receptor (EGFR) transactivation in endothelin (ET) receptor signaling. Human embryonic kidney (HEK) 293 cells were transiently transfected with green fluorescent protein (GFP) -tagged EGFR, and then stimulated with 100 nM ET or 10 ng/mL EGF for 10 min. In the absence of stimulation, EGFRs were located on the plasma membrane (A, arrows). Stimulation with ET induced EGFR internalization (B, arrowheads). Stimulation with EGF as a positive control also induced EGFR internalization (C, arrowheads).

ET stimulation at a concentration of 100 nM (both $p < 0.0001$ vs. 0 min) (Figure 1). β-arrestin1 knockdown and β-arrestin2 knockdown both resulted in further enhancement of the ERK1/2 phosphorylation at 5 and 10 min after ET stimulation (β-arrestin1: $p < 0.05$ vs. control at each of the same time points; β-arrestin2: $p < 0.001$ vs. control at each of the same time points). At 10 min after ET stimulation, ERK1/2 phosphorylation was significantly greater with β-arrestin2 knockdown than with β-arrestin1 knockdown ($p < 0.05$).

EGFR transactivation was involved in ET-R signaling

In β-AR, ligand stimulation promotes the shedding of membrane-bound EGF and subsequently transactivates EGFR in a β-arrestin-dependent manner[9]. To examine whether EGFR is involved in ET-R signaling, we transiently transfected HEK 293 cells with GFP-tagged EGFR plasmids and stimulated the cells with ET. In the absence of stimulation, the EGFRs were observed to be located on the plasma membrane. Stimulation with ET at a concentration of 100 nM induced EGFR internalization, as shown by their redistribution into cellular aggregates (Figure 2). This indicated that EGFR transactivation was involved in ET-R signaling.

EGFR and protein kinase C inhibitors suppressed ERK1/2 phosphorylation

To further investigate ET-R signaling, we examined the effect of the EFGR inhibitor AG1478 (Sigma-Aldrich, St Louis, MO, USA) on ERK1/2 phosphorylation in response to ET stimulation. In the control, ERK1/2 phosphorylation was significantly enhanced at 2 and 5 min after stimulation with 100 nM ET (both $p < 0.0001$ vs. 0 min) (Figure 3). Following pretreatment with AG1478, this enhancement was significantly inhibited at 2, 5, and 10 min after ET stimulation ($p < 0.0001$ at 2 min, and $p < 0.01$ at 5 and 10 min after stimulation vs. control at the same time points). These results indicated that EGFR transactivation was involved in ET-R signaling.

Protein kinase C (PKC) is known to be involved in the G-protein-dependent pathway. We therefore examined the effect of the PKC inhibitor Ro318425 (Sigma-Aldrich, St Louis, MO, USA) on ERK1/2 phosphorylation in response to ET stimulation. Pretreatment with Ro318425

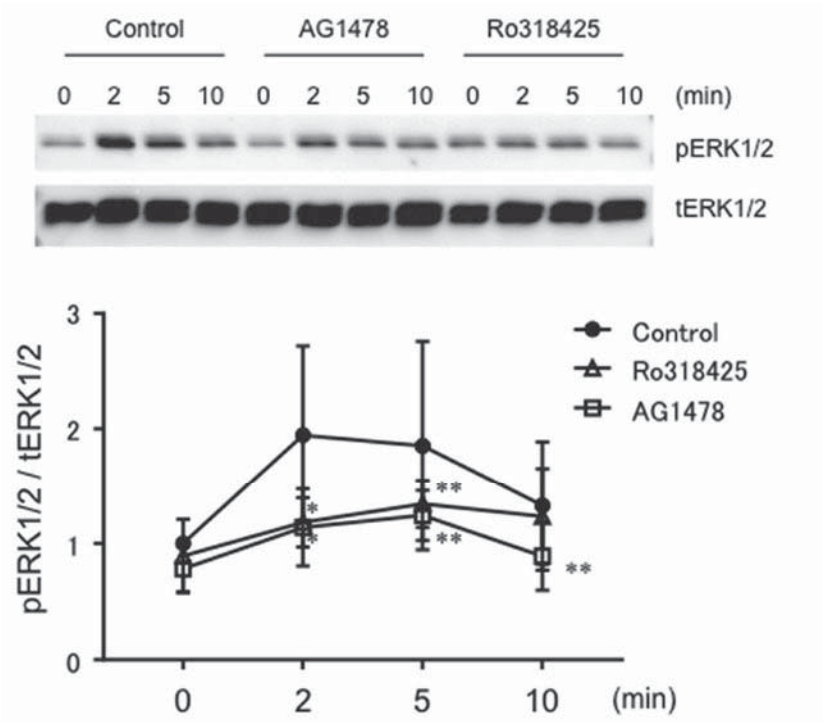

Figure 3. Effects of Ro318425 (a protein kinase C inhibitor) and AG1478 (an epidermal growth factor receptor inhibitor) on ERK1/2 phosphorylation induced by endothelin (ET). Human embryonic kidney (HEK) 293 cells were stimulated with 100 nM ET for 2, 5, and 10 min with or without pretreatment with Ro318425 or AG1478. Western blotting for phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (tERK1/2) was performed. *$p < 0.0001$ vs. control at the same time point. **$p < 0.01$ vs. control at the same time point. Each n = 8. Error bar indicates standard deviation.

significantly inhibited ERK1/2 phosphorylation at 2 and 5 min after ET stimulation ($p < 0.0001$ at 2 min and $p < 0.01$ at 5 min after stimulation vs. control at the same time points) (Figure 3). These results indicated that activation of the G-protein-dependent pathway and consequent PKC activation was also involved in ET-R signaling.

Discussion

The results of this study demonstrated that knockdown of either β-arrestin1 or β-arrestin2 enhanced ERK1/2 phosphorylation in HEK 293 cells in response to ET stimulation, and that EGFR transactivation was involved in ET-R signaling. These results indicated that β-arrestins and EGFR transactivation were involved in ET-R signaling.

ET-R signaling and ERK1/2 activation

The mitogen-activated protein (MAP) kinase cascade is activated by various kinds of GPCRs and receptor tyrosine kinases, and regulates cellular proliferation and differentiation[16]. The activation of ERK, one of the MAP kinases, is a major determinant in the control of a diverse range of cellular processes, including proliferation, survival, differentiation, and motility[17]. This pathway is often upregulated in human tumors, and it is thought that the blockade of ERK signaling would result not only in an antiproliferative effect in tumor cells, but also in antimetastatic and antiangiogenic effects[18]. ERK1/2 is a common downstream signaling of the GPCRs, and involved in G-protein- and β-arrestin-dependent pathways. For instance, ERK1/2 phosphorylation has been shown to be reduced by β-arrestin1 or β-arrestin2 knockdown in HEK 293 cells

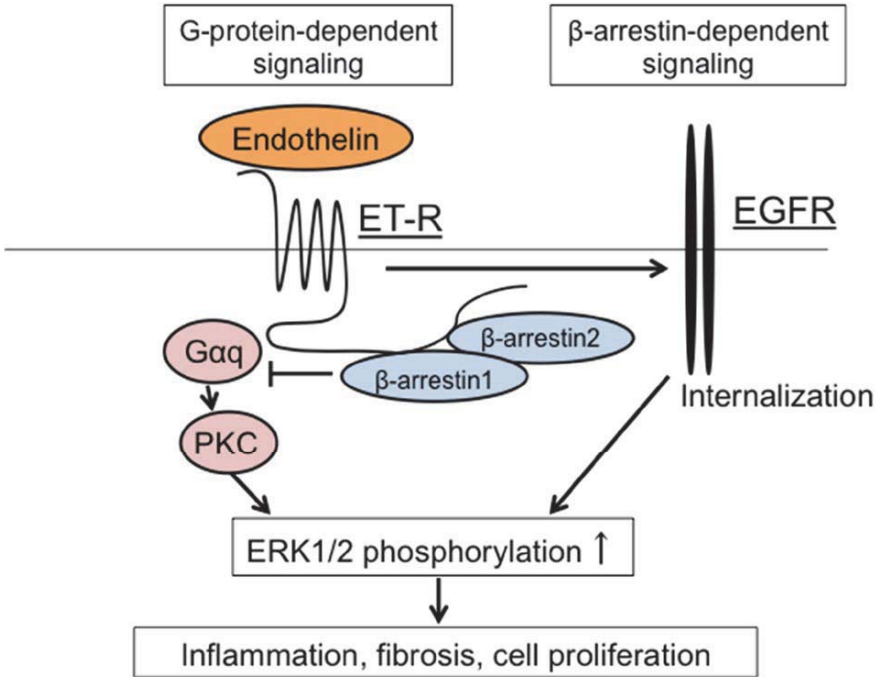

Figure 4. Two possible pathways resulting in extracellular signal-regulated kinase (ERK)1/2 activation. Binding of endothelin (ET) to an ET receptor (ET-R) may activate two types of signaling pathways, the G-protein-dependent signaling and the β-arrestin-dependent signaling pathways. Activated G-protein αq (Gαq) promotes protein kinase C (PKC) activation and subsequently enhances ERK1/2 phosphorylation. Conversely, ET binding to ET-R also transactivates epidermal growth factor receptors (EGFRs). Activated EGFRs are internalized and enhance ERK1/2 phosphorylation. β-arrestin1 and β-arrestin2 may play important roles in desensitizing ET-Rs and inhibiting the further activation of Gαq.

that overexpress β2-AR[19]. Conversely, another study reported that ERK1/2 phosphorylation was increased by β-arrestin1 knockdown but reduced by β-arrestin2 knockdown in angiotensin II type 1 receptor (AT1R)[20, 21]. These findings indicate that the regulation of ERK1/2 phosphorylation by β-arrestin is dependent on the type of GPCR involved. In the present study, we showed that ERK1/2 phosphorylation in HEK 293 cells in response to ET was enhanced by β-arrestin1 or β-arrestin2 knockdown. A possible underlying mechanism for this enhancement is that β-arrestin1 and β-arrrestin2 mediate ET-R desensitization. Downregulation of β-arrestin1 or β-arrestin2 would then impair ET-R phosphorylation and desensitization, resulting in the enhancement of G-protein-dependent signaling and subsequent ERK1/2 phosphorylation. However, a previous study reported that β-arrestin2 knockdown, but not β-arrestin1 knockdown, inhibited ERK1/2 phosphorylation stimulated by ET in vascular smooth muscle cells[22]. Thus, responses to ET stimulation may be dependent on cell type. Although there are two types of ET-R (types A and B), we did not examine the signaling of these types separately. Further studies regarding these important issues are clearly required.

EGFR transactivation is involved in ET-R signaling

EGFR is expressed in various types of cells and is activated by EGF, transforming growth factor-α, and HB-EGF[23]. Activated EGFR causes the activation of MAP kinases and the Akt pathway, which promotes cell proliferation and exerts an antiapoptotic effect[24]. It is well established that β-arrestin mediates EGFR transactivation in β-AR[8] and AT1R[25, 26]; however, the

involvement of EGFR in ET-R signaling has not been fully elucidated. A previous report showed that β-arrestin1 and β-arrestin2 were recruited to ET-R in response to ET, transactivating EGFR via Src and resulting in β-catenin tyrosine phosphorylation in ovarian cancer cells[27]. In the present study, EGFR transactivation in response to ET stimulation was confirmed by two independent methods: western blotting using an EGFR inhibitor and confocal microscopy. EGFR activation has been shown to exert antiapoptotic and cardioprotective effects in β1-AR[9]; however, EGF induces the enhancement of MAP kinase activation and extracellular matrix production, which results in tissue fibrosis[28, 29]. Furthermore, it remains uncertain whether β-arrestins are involved in EGFR transactivation. A possible mechanism for the ET-R signaling incorporating the findings of the present study is summarized in Figure 4.

Possible role of β-arrestin in the pathogenesis of PAH

PAH is defined as elevated mean pulmonary arterial pressure more than 25 mmHg, and is caused by idiopathic or congenital heart diseases, pulmonary diseases, and collagen diseases[13]. ET stimulation results in vascular contraction, inflammation, fibrosis, and proliferation in the vessel wall[4], and it is thought that ET plays a major role in the progression of PAH[12]. PAH is usually progressive and fatal, and until recently there has been no established treatment for it. However, ET-R antagonists have now become available, providing an essential role as one of the three main elements in the treatment of PAH, alongside phosphodiesterase type 5 inhibitors and prostacyclin stimulators. However, despite the advances in medical treatment, the effects of the drugs are limited[13]. In this study, we focused on the role of β-arrestin in ET-R signaling, and showed that β-arrestin1 or β-arrestin2 knockdown enhanced ERK1/2 phosphorylation, which generally enhances the expression of pro-fibrotic genes and induces tissue fibrosis[30, 31]. GPCRs could be a main target for the development of new PAH drugs, and β-arrestin may provide an attractive target[32].

Conclusion

This study demonstrated that β-arrestins and EGFR transactivation are involved in ET-R signaling. Further studies are required to elucidate the role of β-arrestin in ET-R signaling and its possible role in PAH; nevertheless, our results may provide new insights into the pathogenesis of PAH and could potentially contribute to future drug development.

Conflicts of interest

All authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgement

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