

The Glucagon-Like Peptide-1 Receptor Agonist Liraglutide Improves Hypoxia-Induced Pulmonary Hypertension in Mice Partly via Normalization of Reduced ET_B Receptor Expression

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Summary

The glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide is an incretin hormone mimetic used in the treatment of diabetes. However, the effects of liraglutide on pulmonary hypertension (PH) and pulmonary endothelin (ET) system are unknown. Eight-week-old C57BL6/J mice were injected liraglutide or vehicle for 5 weeks. One week after injection, the mice were exposed to either room air (normoxia) or chronic hypoxia (10 % O₂) for 4 weeks. The right ventricular systolic pressure (RVSP) was significantly higher in hypoxia + vehicle group than in normoxia + vehicle group. ET-1 mRNA expression in the lungs was comparable among all the groups. ET_B mRNA and protein expression in the lungs was significantly lower in hypoxia + vehicle group than in normoxia + vehicle group. The above changes were normalized by liraglutide treatment. The expression of phospho-eNOS and phospho-AMPK proteins in the lungs was significantly higher in hypoxia + liraglutide group than in normoxia + vehicle group. We demonstrated for the first time that liraglutide effectively improved RVSP and RV hypertrophy in hypoxia-induced PH mice by activating eNOS through normalization of impaired ET_B pathway and augmentation of AMPK pathway. Therefore, GLP-1R agonists can be promising therapeutic agents for PH.

Key words

GLP-1R • Pulmonary hypertension • Liraglutide • ET_B receptor • eNOS

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Introduction

Pulmonary hypertension (PH) is a disease affecting small pulmonary arteries and is characterized by vascular proliferation and remodeling. It results in a progressive increase in pulmonary vascular resistance and mean pulmonary arterial pressure more than 25 mm Hg (Barst *et al.* 2004), and ultimately results in right ventricular (RV) failure and death. Various endogenous components are considered to be involved in the worsening of PH. Endothelin-1 (ET-1), an endothelium-derived potent vasoconstrictor peptide with vasoproliferative activity (Yanagisawa *et al.* 1988, Miyauchi and Goto 2013, Gohar *et al.* 2016, Barton and Sorokin 2016), is considered to play an important role in the progression of PH (Miyauchi *et al.* 1993, Miyauchi and Goto 2013, Clozel 2016, Davenport *et al.* 2016). The effects of ET-1 are mediated *via* the G-protein-coupled receptors, endothelin receptor type A (ET_A) and endothelin receptor type B (ET_B) (Miyauchi and Goto 2013, Gohar *et al.* 2016, Barton and Sorokin 2016, Davenport *et al.* 2016). ET_A stimulation has been considered to induce harmful responses such as vasoconstriction and vasoproliferation. In various situations, the stimulation of ET_B was considered to induce beneficial responses, such as nitric oxide (NO) release from the endothelium (Barton and Yanagisawa 2008, Thorin *et al.* 2010, Rodriguez-Pascual *et al.* 2011, Miyauchi and Goto 2013, Davenport *et al.* 2016). Endothelin receptor antagonists (ERA) are currently in clinical use for the treatment of patients with PH (Miyauchi and Goto 2013, Clozel 2016, Davenport *et al.*

2016). In addition, prostacyclin derivatives and phosphodiesterase type 5 inhibitors (PDE5 inhibitors) are currently in clinical use (Clozel 2016). Nevertheless, PH is a devastating disease with high mortality. Therefore, development of other drugs is expected to improve the survival of patients with PH.

The glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide is an incretin hormone mimetic that is used in the treatment of diabetes mellitus. GLP-1 is secreted in a nutrient-dependent manner. It stimulates insulin secretion and inhibits both glucagon secretion and gastric emptying, thereby reducing postprandial hyperglycemia (Drucker *et al.* 2006, Irwin *et al.* 2001). GLP-1 has been shown to exhibit favorable cardiovascular effects in preclinical and clinical studies. It has been reported that GLP-1 exhibits cardioprotective and vasodilatory effects in mice (Ban *et al.* 2008). Recently, GLP-1R agonists were reported to inhibit endothelial dysfunction (Gaspari *et al.* 2013), inflammation (Liu *et al.* 2009, Arakawa *et al.* 2010), and cell proliferation (Goto *et al.* 2011). On the assumption that the aforementioned favorable effects of GLP-1R agonists occur in the pulmonary arteries, it seems likely that GLP-1R agonists would be effective in ameliorating PH. However, the effect of GLP-1R agonists on PH has not been reported to date. Therefore, in this study, we aimed to examine the effects of GLP-1R agonist on PH in a mouse model of hypoxia-induced PH. In addition, we investigated the effects of GLP-1R agonists on the lung ET systems (ET-1, ET_A, and ET_B).

Methods

PH mouse model and experimental design

Wild-type male C57BL/6J mice (8-week-old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were injected liraglutide (200 µg/kg/day) or saline intraperitoneally. This concentration of liraglutide was selected to be effective in mice experiments as previously reported (Gaspari *et al.* 2013, Shirakawa *et al.* 2012, Noyan-Ashraf *et al.* 2013). One week after injection, the mice were exposed to room air (normoxia) or chronic hypoxia (10 % O₂) for 4 weeks. All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tsukuba and conform to the NIH guidelines for the care and use of laboratory animals. We divided the mice into the following four groups: normoxia + vehicle (saline administration) group (n=6); normoxia + liraglutide

group (n=6); hypoxia + vehicle group (n=6); and hypoxia + liraglutide group (n=6).

Hemodynamic measurement

Blood pressure and heart rate were assessed non-invasively using a Volume Pressure Recording system (Kent Scientific Corporation, Torrington, USA). The mice were accustomed to the tail cuff device for 20 min before measurements.

To assess RV systolic pressure (RVSP), the mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The RV apex was exposed *via* a subdiaphragmatic incision. An apical stab was made with a 27-gauge needle containing a fiber pressure sensor (FPI-LS-PT9; FISO Technologies, Inc., Quebec, Canada). RVSP measurements were obtained at a steady state. All signals were analyzed with a signal conditioner (FPI-LS-10; FISO Technologies, Inc.) and data acquisition system (LabTrax 4, World Precision Instruments, Sarasota, FL, USA), and then analyzed using a software (LabScribe2, iWorx Systems, Inc., Dover, NH, USA).

RNA extraction and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

All lung tissues were frozen and stored at -80 °C. For preparation of total RNA, the lung tissue was homogenized using a bead kit (MagNA Lyser Green Beads; Roche Life Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Total RNA samples from the lung tissue and cultured cells were prepared using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, Netherlands), and 1 µg of total RNA was reverse transcribed to complementary DNA by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mRNA expression levels of the target genes were analyzed by the LightCycler 96 system (Roche Applied Science, Penzberg, Germany) with a Universal Probe Library (Roche Applied Science). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA was used as an internal control (#05 046 211 011; Roche Diagnostics GmbH, Mannheim, Germany). PCR amplification was performed in duplicate as follows: 1 cycle at 95 °C for 10 min and 40 cycles at 94 °C for 15 s and 60 °C for 1 min. The following primers for mouse were used in the study: ET-1: 5'-TCTCTGCTGTTTGTGGCTTG-3' (sense), 5'-GAGCTCAGCGCCTAAGACTG-3' (antisense), ET_A receptor: 5'-TGTGAGCAAGAAATTCAAAAATTG-3'

(sense), 5'-ATGAGGCTTTTGGACTGGTG-3' (antisense), ET_B receptor: 5'-TCAGAAAACAGCCTTCATGC-3' (sense), 5'-GCGGCAAGCAGAAGTAGAA-3' (antisense), eNOS (endothelial nitric oxide synthase): 5'-ATCCAGTGCCCTGCTTCA-3' (sense), 5'-GCAGGGCAAGTTAGGATCAG-3' (antisense).

Histological analysis of lung tissues

First for the morphological analysis of the lungs, the hearts and lungs were flushed with saline and then dissected (Zhang *et al.* 2017). After that, the lungs were fixed with 4 % paraformaldehyde, embedded in paraffin, sectioned into 3- μ m thick slices, and stained with Elastica van Gieson. Images were obtained using a digital microscope (Biozero BZ-X700; Keyence, Osaka, Japan). Pulmonary vascular remodeling was quantified by assessing the medial wall thickness. To determine the degree of medial wall thickness, 10 muscular arteries categorized $<50 \mu\text{m}$ and $\geq 50 \mu\text{m}$ in diameter from each lung (n=6) were randomly outlined by an observer blinded to the mouse genotype or pharmacological treatment. The degree of medial wall thickness expressed as the ratio of medial area to cross sectional area (Medial/CSA) was analyzed using the ImageJ software (Ver. 1.48, NIH, USA).

Western blotting

The lung tissues were homogenized with RIPA buffer on ice using MagNA Lyser Green Beads. The homogenate was centrifuged at 13,000 rpm for 30 min at 4 °C. The concentrations of proteins extracted from lung homogenates were determined using the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10-20 % polyacrylamide gradient gels and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 3 % skim milk in tris-buffered saline (TBS) containing 0.1 % Tween 20 (TBST), incubated with primary antibodies (1:1,000) in a solution (W00H, DRC Co., Tokyo, Japan) overnight at 4 °C, and then reacted with a horseradish peroxidase-conjugated goat anti-rabbit antibody (#7074, Cell Signaling Technology, Boston, MA, USA) or rabbit anti-mouse antibody (#7076, Cell Signaling Technology) at 1:10,000 in a solution (W00B, DRC) at 25 °C for 60 min. The following primary antibodies were used: eNOS (49G3) (#9586, Cell Signaling Technology), Phospho-eNOS (Ser 1177) (#9571, Cell Signaling Technology), AMPK (23A3)

(#2603, Cell Signaling Technology), Phospho-AMPK (Thr 172) (#2535, Cell Signaling Technology), ET_A (#AER-001, Alomone Labs, Jerusalem, Israel), ET_B (#AER-002, Alomone Labs), and GAPDH (MAB374, Merck Millipore, Billerica, MA, USA). The blots were visualized with a chemiluminescent reagent (ImmunoStar, Wako, Osaka, Japan) and CCD camera system (Light-Capture II, Atto Co., Tokyo, Japan) and analyzed with a software (CS Analyzer ver. 3.0, Atto Co.) as described previously (Sakai *et al.* 2016).

Statistics

All data are expressed as mean \pm SEM. The experimental groups were compared by one-way analysis of variance followed by Tukey's test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. The analysis was performed using IBM SPSS version 22.0 software (IBM Co., Armonk, NY, USA).

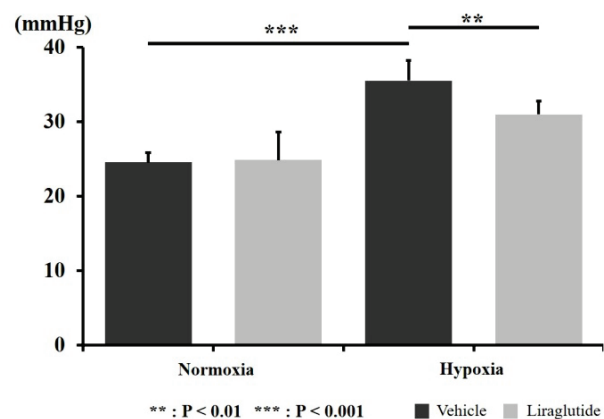


Fig. 1. Right ventricular systolic pressure (n=6 for each group). The bar graphs show the mean \pm SEM of the groups. ** $p < 0.01$, *** $p < 0.001$.

Results

Effects of liraglutide on body weight, systemic blood pressure, heart rate, right ventricle, left ventricle, RVSP, and glucose level

We investigated whether liraglutide administration attenuated hypoxia-induced PH in mice. After 4 weeks of exposure to chronic hypoxia (10 % O₂), a significant increase in both RVSP and the ratio of weight of the RV wall to body weight (RV/BW) was observed in hypoxia + vehicle group compared with the normoxia + vehicle group (Fig. 1, Table 1). Notably, hypoxia + liraglutide group showed

a significant reduction in RVSP (34.2 ± 2.2 mm Hg vs. 30.0 ± 1.5 mm Hg, $p < 0.01$, $n = 6$) and RV/BW compared with the hypoxia + vehicle group. In contrast, there were no significant differences in systolic blood pressure and ratio of weight of the left ventricular plus interventricular septum to BW among all groups. The reduction in lung weight was less than 5%, the reduction in right

ventricular systolic pressure was 13% and the reduction in right ventricular weight was 16% by liraglutide administration under hypoxia. Lung weight, heart rate, and blood sugar level were significantly reduced in the hypoxia groups compared with those in the normoxia groups (Table 1).

Table 1. Heart rate, lung weight, body weight, hemodynamic states, and blood sugar level in each group.

	Normoxia		Hypoxia	
	Vehicle	Liraglutide	Vehicle	Liraglutide
BW (g)	26.5 ± 0.3	25.7 ± 0.2	26.7 ± 0.7	25.3 ± 0.6
SBP (mm Hg)	107.5 ± 3.0	107.7 ± 4.1	108.4 ± 4.4	109.6 ± 3.2
HR (bpm)	678 ± 16	648 ± 17	$565 \pm 40^*$	595 ± 28
RV (mg)/BW (g)	0.68 ± 0.03	0.77 ± 0.10	$0.98 \pm 0.06^*$	$0.82 \pm 0.12^*\$$
LV + IVS (mg)/BW (g)	3.12 ± 0.05	$3.23 \pm 0.03^*$	3.23 ± 0.10	2.93 ± 0.12
Lung (mg)/BW (g)	4.55 ± 0.12	$4.87 \pm 0.14^*$	$6.58 \pm 0.15^*$	$6.28 \pm 0.11^*$
BS (mg/dl)	277 ± 11.5	$257 \pm 13.6^*$	$143 \pm 15.8^{**}$	$133 \pm 9.5^{**}$

Results are presented as mean \pm SEM ($n = 6$ for each group). BW, body weight; SBP, systolic blood pressure; HR, heart rate; RV, right ventricle; LV, left ventricle; IVS, interventricular septum; BS, blood sugar. * $p < 0.05$ vs. normoxia + vehicle; † $p < 0.05$ vs. normoxia + liraglutide; § $p < 0.05$ vs. hypoxia + vehicle.

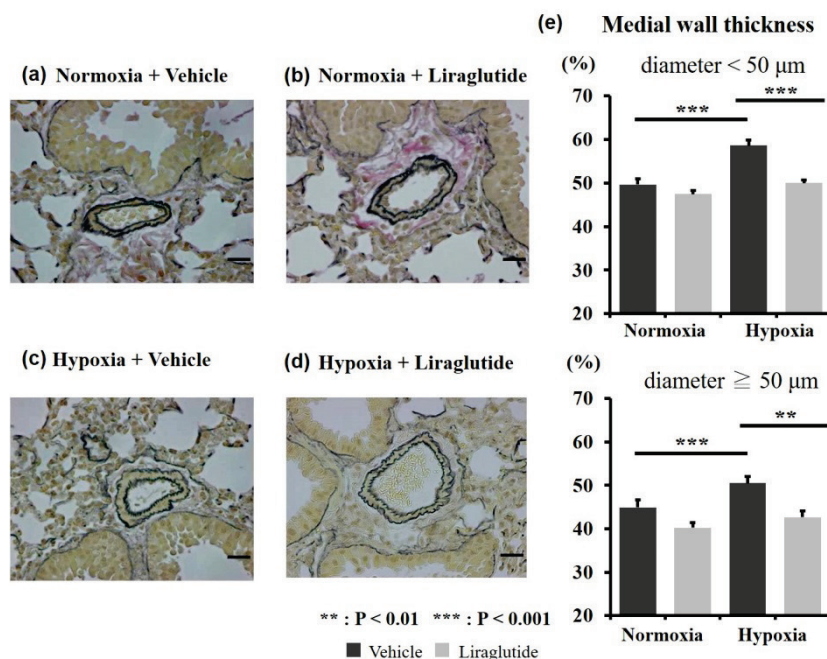


Fig. 2. Representative images of pulmonary artery in (a) normoxia + vehicle, (b) normoxia + liraglutide, (c) hypoxia + vehicle, and (d) hypoxia + liraglutide groups. The pulmonary arteries were stained with Elastica van Gieson (scale bar: $10 \mu\text{m}$). (e) These graphs show the ratio of medial area to cross sectional area of pulmonary arteries smaller than $50 \mu\text{m}$ (upper panel) and larger than $50 \mu\text{m}$ ($\geq 50 \mu\text{m}$) in diameter. Ten muscular arteries categorized $< 50 \mu\text{m}$ and $\geq 50 \mu\text{m}$ in diameter from each lung ($n = 6$) were randomly outlined by an observer blinded to the mouse genotype or pharmacological treatment. The error bars represent the mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$.

Effects of liraglutide on pulmonary medial wall thickening

To determine the degree of medial wall thickening in pulmonary arteries, Elastica van Gieson staining was performed. The representative images of lung tissues are shown in Figures 2a-2d. The medial wall

thickness of arteries categorized $< 50 \mu\text{m}$ and $\geq 50 \mu\text{m}$ in diameter increased significantly in the hypoxia + vehicle group compared with that in the normoxia + vehicle group. However, medial wall thickening in the hypoxia + liraglutide group decreased significantly compared with that in the hypoxia + vehicle group (Fig. 2e). These

results suggested that medial wall thickening was reduced by liraglutide treatment.

Effect of liraglutide on mRNA expression of eNOS and ET systems

To investigate the effect of liraglutide on the expression of eNOS and ET systems, we determined mRNA expression levels in the lung tissue by using

real-time PCR. The mRNA expression level of eNOS in hypoxia + liraglutide group was significantly higher than that in hypoxia + vehicle group (Fig. 3a). Next, we investigated the mRNA expression of ET systems. No significant difference in the mRNA expression level of ET-1 was observed among the groups (Fig. 3b). However, liraglutide normalized the reduced expression of ET_A and ET_B induced by chronic hypoxia (Figs 3c and 3d).

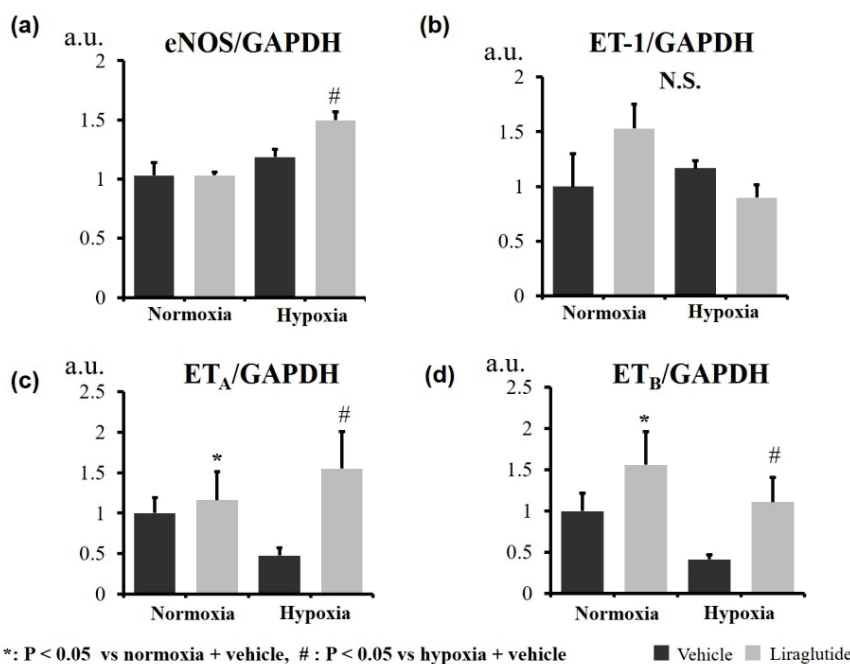


Fig. 3. Gene expression levels in the lung tissues analyzed by real-time PCR (n=6 for each group). **(a)** The mRNA expression level of endothelial nitric oxide synthase (eNOS) and **(b)** endothelin-1 (ET-1) in all groups. **(c)** Endothelin receptor type A (ET_A) and **(d)** Endothelin receptor type B (ET_B). The error bars represent the mean ± SEM. *p<0.05, vs. normoxia + vehicle, #p<0.05 vs. hypoxia + vehicle.

Effects of liraglutide on the protein expression of eNOS, phospho-eNOS, AMPK, phospho-AMPK, ET_A, and ET_B in the lungs

We determined the expression of different proteins by Western blot analysis in the lung tissues. In Figure 4a, representative expressions of eNOS, phospho-eNOS, AMPK, phospho-AMPK are shown. The expression of eNOS and AMPK was comparable among all the groups. However, the expression of phospho-eNOS and phospho-AMPK in hypoxia + liraglutide group increased compared with that in hypoxia + vehicle group (Fig. 4a).

We further examined the Western blot analysis for ET_A and ET_B. The representative expressions and the statistical analysis of these data are shown in Figures 4b, 4c and 4d. Hypoxia significantly increased the expression of ET_A and liraglutide administration under hypoxia further increased the expression of ET_A (Figs 4b and 4c). Nevertheless, hypoxia did not change the expression of ET_B whereas liraglutide administration under hypoxia significantly increased the expression of ET_B (Figs 4b and 4d).

Discussion

In the present study, we demonstrated that the GLP-1R agonist liraglutide significantly reduced RVSP in hypoxia-induced PH mice model. This study also demonstrated that chronic administration of liraglutide inhibited the medial thickening of pulmonary arteries and development of RV hypertrophy in hypoxia-induced PH mice. For the first time, we demonstrated that a GLP-1R agonist effectively normalized the reduction in ET_B expression in the lungs of mice. In addition, we demonstrated for the first time that the GLP-1R agonist liraglutide augmented the expression of phospho-eNOS protein in lung tissues of PH mice. Thus, we suggest that GLP-1R agonists are effective in ameliorating PH, and this beneficial effect is partly attributed to the normalization of reduction in ET_B-mediated eNOS activation in the pulmonary artery. The findings of this study can offer insights into a novel strategy for the treatment of PH using a GLP-1R agonist.

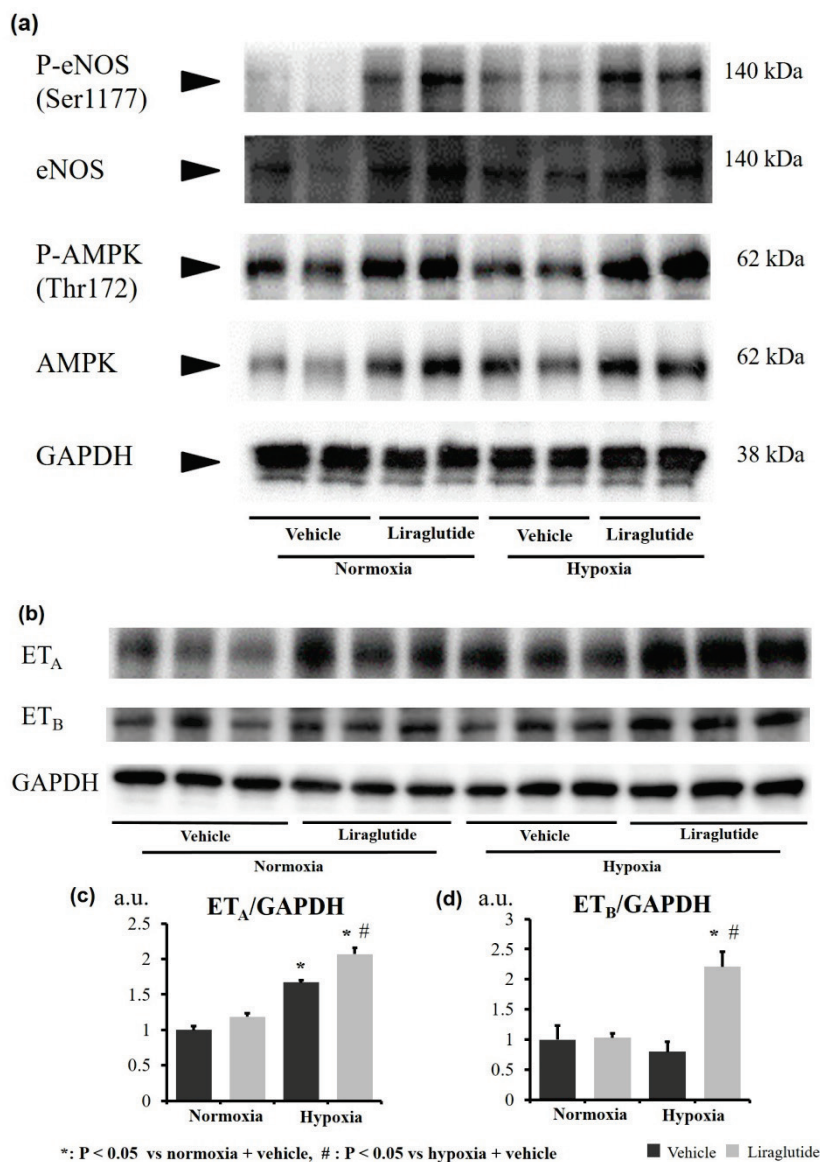


Fig. 4. Effects of liraglutide on the protein expression of eNOS, P-eNOS, AMPK, P-AMPK, ET_A , and ET_B receptors. **(a)** Representative Western blots of eNOS, P-eNOS, AMPK, P-AMPK and GAPDH in the lungs ($n=2$). **(b)** Representative Western blots of ET_A receptor, ET_B receptor, and GAPDH in the lungs ($n=3$). **(c, d)** Bar graphs show relative expressions of ET_A **(c)** and ET_B **(d)** to GAPDH (an internal control) analyzed by densitometry software. Values are expressed as the mean \pm SEM ($n=3$). * $p < 0.05$ vs. normoxia + vehicle, # $p < 0.05$ vs. hypoxia + vehicle. AMPK, adenosine monophosphate-activated protein kinase; eNOS, endothelial nitric oxide synthase; ET_A , endothelin receptor type A; ET_B , endothelin receptor type B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; P-AMPK, phospho-AMPK; P-eNOS, phospho-eNOS.

eNOS is an enzyme that catalyzes the production of NO, which plays an important role in vasorelaxation and maintaining vascular homeostasis. Notably, eNOS and NO expression levels were found to be reduced in PH patients (Giaid *et al.* 1995, Seimetz *et al.* 2011). In addition, a previous study has shown that liraglutide augmented the expression of eNOS in cultured vascular endothelial cells (Dai *et al.* 2013). Therefore, the inhibition of PH development would be partly obtained by the eNOS enhancement by liraglutide administration in the lungs of PH mice. We considered that the eNOS activation was mediated by two pathways: normalization of impaired ET_B -mediated signaling pathway and augmentation of AMPK signaling pathway (Fig. 5).

In the ET signaling pathway, ET-1 is a vasoconstrictor and proinflammatory peptide produced by vascular endothelial cells (Yanagisawa *et al.* 1988,

Miyauchi and Goto 2013, Gohar *et al.* 2016, Barton and Sorokin 2016). A positive correlation between serum ET-1 levels and mean pulmonary artery pressure has been reported (Kedzierski *et al.* 2001). Therefore, ET-1 was considered one of the major factors involved in the development of PH, and ERA is the current therapy for PH in clinical practice (Miyauchi and Goto 2013, Clozel 2016, Davenport *et al.* 2016). The various effects of ET-1 are mediated *via* ET_A and ET_B (Miyauchi and Goto 2013, Gohar *et al.* 2016, Barton and Sorokin 2016, Davenport *et al.* 2016). The stimulation of ET_A is considered to be involved in the pathogenesis and development of various diseases, such as systemic hypertension and pulmonary hypertension, atherosclerosis, and cardiac remodeling (Barton and Yanagisawa 2008, Rodriguez-Pascual *et al.* 2011, Thorin *et al.* 2010, Miyauchi and Goto 2013, Davenport *et al.* 2016). In contrast, the stimulation of

ET_B was considered to induce beneficial responses, such as increase in the production of NO and prostacyclin (PGI₂) by endothelial cells to cause vasodilatation. ET_B functions as a “clearance receptor” to prevent excessive ET_A stimulation by endogenous ET-1 (Thorin *et al.* 2010, Miyauchi and Goto 2013, Davenport *et al.* 2016). ET_A is highly expressed in vascular smooth muscle cells, but not in endothelial cells (Hosoda *et al.* 1991, Miyauchi and Goto 2013, Davenport *et al.* 2016). Stimulation of ET_A by ET-1 induces contraction and proliferation of vascular smooth muscle cells, which contributes to the development of PH (Frumkin *et al.* 2012, Rubin *et al.* 2012, Miyauchi and Goto 2013, Davenport *et al.* 2016). ET_B is highly expressed in endothelial cells (Ogawa *et al.* 1991, Miyauchi and Goto 2013, Davenport *et al.* 2016), where ET_B activation by ET-1 accelerates the production of vasodilators, such as NO and PGI₂, and the clearance of ET-1 from the circulation (Warner *et al.* 1989, de Nucci *et al.* 1988, Fukuroda *et al.* 1994, Dupuis *et al.* 1996, Miyauchi and Goto 2013). The endothelial NO generation mediated *via* the ET_B inhibits proliferation of vascular smooth muscle cells (Garg *et al.* 1989, Miyauchi and Goto 2013, Davenport *et al.* 2016). In some patients with PH, vascular ET_A, ET_B, and eNOS expression was reduced (Hall *et al.* 2011). However, in PH patients treated with bosentan and/or epoprostenol, ET_A and ET_B expression

was normalized, whereas eNOS expression was increased (Hall *et al.* 2011). Other researchers have shown that the expression of ET_A increased but ET_B decreased under hypoxia (Wang *et al.* 2013, Wang *et al.* 2017, Gao *et al.* 2013). Therefore, the expressions of ET_A and ET_B under hypoxic conditions are controversial and depend on the experimental methods. In the present study, we revealed that liraglutide increased the expression of ET_B and ET_A in the lungs in a mouse model of PH. The increase in ET_A expression may affect vascular remodeling in PH. Nevertheless, it is unclear whether the increase in ET_A expression by liraglutide is beneficial or harmful for the setting of PH. In addition, it has been reported that ET_A was not present in endothelial cells (Hosoda *et al.* 1991). Therefore, we believe that the beneficial effect of liraglutide in the mouse model of hypoxia-induced PH was at least partly due to eNOS activation mediated by the normalization of impaired ET_B signaling (Fig. 5). The number of patients with PH treated with a drug combination (e.g. ambrisentan/tadalafil) is increasing. Ambrisentan is a selective ET_A blocker. Liraglutide could stimulate ET_A-mediated vasoconstriction, whereas simultaneously liraglutide could enhance ET_B-mediated vasodilatation by increasing cGMP. It is of great interest that liraglutide could be tested in combination with an ET_A blocker because the increase in ET_A by liraglutide could be antagonized in this setting.

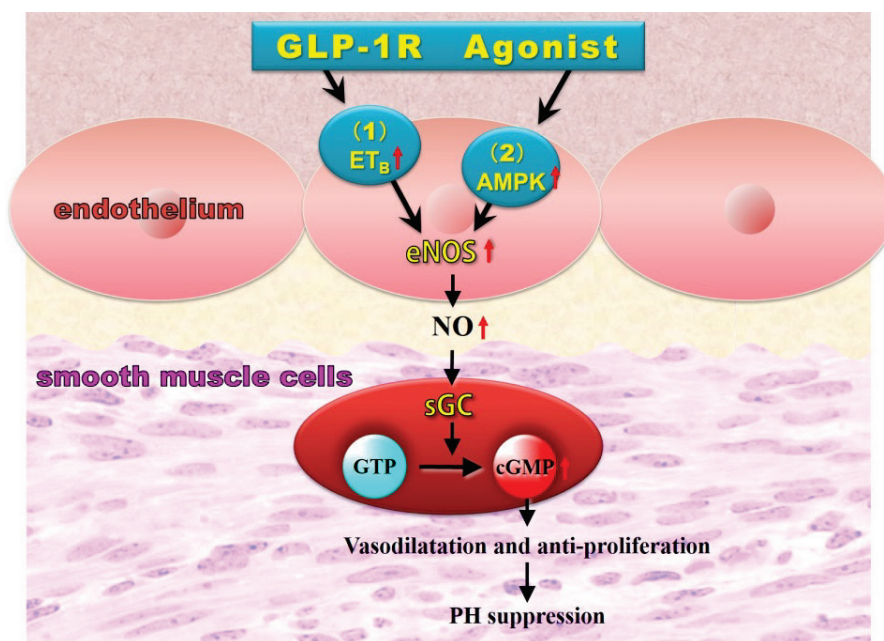


Fig. 5. The scheme of the GLP-1R agonist liraglutide activates eNOS pathway. The inhibition of PH development by liraglutide may be caused by eNOS activation in the endothelial cells of pulmonary artery. eNOS activation by liraglutide was mediated by two pathways in the mouse model of hypoxia-induced PH, namely, ET_B and AMPK signaling pathways. AMPK, adenosine monophosphate-activated protein kinase; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; ET_B, endothelin receptor type B; GLP-1R, glucagon like peptide-1 receptor; GTP, guanosine triphosphate; NO, nitric oxide; PH, pulmonary hypertension; sGC, soluble guanylate cyclase.

It has been reported that adenosine monophosphate-activated protein kinase (AMPK) causes activation of eNOS in endothelial cells (Thors *et al.* 2011, Omura *et al.* 2016). Therefore, we studied the effects of liraglutide on AMPK pathway in a mouse model of PH. The present study also demonstrated for the first time that the GLP-1R agonist liraglutide augmented the expression of phospho-AMPK protein in the lungs of a mouse model of hypoxia-induced PH. AMPK has been reported to have protective effects on endothelial cells. It was also reported that AMPK was activated by fibrates (Murakami *et al.* 2006), statins (Sun *et al.* 2006), and metformin (Hawley *et al.* 2002), which are associated with anti-arteriosclerotic actions. The present findings revealed that the activation of eNOS by liraglutide was partly induced *via* AMPK pathway in the endothelial cells of pulmonary artery. Therefore, liraglutide exerts its beneficial effects on the development of PH *via* augmentation of AMPK pathway as well as ET_B pathway (Fig. 5).

In this present study, blood sugar levels were reduced in mice under chronic hypoxia. Previous reports showed that chronic hypoxia (10 % O₂) decreased plasma glucose levels in mice because hypoxia-induced

translocation of intracellular GLUT4 to the sarcolemma increased glucose uptake in soleus muscle (Gamboa *et al.* 2011, Mackenzie *et al.* 2016). Therefore, we might suggest that lower blood sugar level in hypoxia mice is mediated *via* increased glucose uptake in skeletal muscles.

In conclusion, this is the first study to demonstrate that the GLP-1R agonist liraglutide effectively improves RVSP and RV hypertrophy in a mouse model of hypoxia-induced PH by activating eNOS *via* normalization of impaired ET_B pathway as well as augmentation of AMPK pathway in the lungs. Taken together, GLP-1R agonists are expected to be promising therapeutic agents for the treatment of PH.

Conflict of Interest

There is no conflict of interest.

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