Ghrelin ameliorates hypoxia-induced pulmonary hypertension via phospho-GSK3β/β-catenin signaling in neonatal rats

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Abstract

Effective treatment and/or prevention strategies for neonatal persistent pulmonary hypertension of the newborn (PPHN) have been an important topic in neonatal medicine. However, mechanisms of impaired pulmonary vascular structure in hypoxia-induced PPHN are poorly understood and consequently limit the development of effective treatment. In this study, we aimed to explore the molecular signaling cascades in the lungs of a PPHN animal model and used primary cultured rat pulmonary microvascular endothelial cells to analyze the physiological benefits of ghrelin during the pathogenesis of PPHN. Randomly selected newborn rats were exposed to hypoxia (10–12%) or room air and received daily s.c. injections of ghrelin (150 µg/kg) or saline. After 2 weeks, pulmonary hemodynamics and morphometry were assessed in the rats. Compared with the control, hypoxia increased pulmonary arterial pressure, right ventricle (RV) hypertrophy, and arteriolar wall thickness. Ghrelin treatment reduced both the magnitude of PH and the RV/(left ventricle+Cseptum (Sep)) weight ratio. Ghrelin protected neonatal rats from hypoxia-induced PH via the upregulation of phosphorylation of glycogen synthase kinase 3β (p-GSK3β)/β-catenin signaling and associated with β-catenin translocation to the nucleus in the presence of growth hormone secretagogue receptor-1a. Our findings suggest that s.c. administration of ghrelin improved PH and attenuated pulmonary vascular remodeling after PPHN. These beneficial effects may be mediated by the regulation of p-GSK3β/β-catenin expression. We propose ghrelin as a novel potential therapeutic agent for PPHN.

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Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by abnormal pulmonary vascular tone, reactivity, and structure. A sustained elevation of pulmonary vascular resistance (PVR) at birth leads to extrapulmonary right-to-left shunting of blood and severe hypoxemia (Clark et al. 2000). PPHN patients are usually full-term or post-term infants who have had perinatal asphyxia, meconium aspiration, diaphragmatic hernia, pneumonia, or sepsis. Further research is required to fully understand the mechanism(s) behind these types of lung disorders to enable the development of effective treatments.

Lung development during the saccular period occurs between embryonic day 17-5 and postnatal day 5 and the alveolar stage occurs during the postnatal weaning period in mouse and rat (De Langhe & Reynolds 2008). During this time, alveolar septation initiates and leads to a tremendous increase in the surface area of the lung. Exposure to hypoxia during this critical period can impair both alveolar and pulmonary vascular structure and function. Postnatal exposure to 10–15% oxygen during the first 3 weeks of life impairs lung development, characterized by decreased alveolarization and reduced lung vascular development (Massaro et al. 1989, Blanco et al. 1991, Keith et al. 2000, Tang et al. 2000). Deruelle et al. (2006) found that exposure of infant rats to hypoxia for 14 days impairs alveolarization as reflected by reduced radial alveolar counts and decreased vascular volume density. The pathogenesis of PH appears to involve an initial, active vasoconstriction of pulmonary resistance vessels that may progress to fixed luminal narrowing, elevated PVR, thrombi formation, and, ultimately, right heart failure and death (Hirenallur et al. 2008). After the structural remodeling of pulmonary vessels has occurred, therapeutic interventions to restore PVR to normal levels are largely ineffective. Therefore, drug therapies targeting cellular pathways that mediate the PH and reversible rise in PVR may be advantageous.
Ghrelin is a 28-amino acid-peptide originally isolated from rat stomach as an endogenous ligand for the growth hormone (GH) secretagoge receptor (GHSR; Kojima et al. 1999). The ghrelin gene peptides include acylated ghrelin, unacylated ghrelin, and obestatin. Acylated ghrelin exerts its central and peripheral effects through the GHSR-1a. Indeed, acylated ghrelin was demonstrated to act as an autocrine/paracrine factor; regulating cell proliferation and survival, apoptosis, inflammation, cardiovascular and gastric functions, metabolism, angiogenesis, development, and reproduction (Muccioli et al. 2007, Chanoine et al. 2009, van der Lely 2009). Ghrelin is able to attenuate the development of pulmonary artery hypertension in a monocrotaline-treated adult animal model (Henriques-Coelho et al. 2006). However, this monocrotaline-induced PH model, including the response to potential therapeutic treatments, differs considerably from PH induced by chronic hypoxia (CH). A previous study has shown that ghrelin can directly stimulate the production of nitric oxide (NO) from vascular endothelial cells through PI 3-kinase-dependent signaling events that mimic the effects of insulin (Iantorno et al. 2007). PPHN in utero causes sustained alteration of fetal pulmonary artery endothelial cell (PAEC) phenotypes, as determined in vitro. Also, endothelial NO synthetase (eNOS) protein expression was decreased in PPHN PAECs and NO-enhanced growth and tube formation was observed in PPHN PAECs (Gien et al. 2007). There is increasing evidence that ghrelin has a potent vasodilator effect. We hypothesize that ghrelin would prevent PPHN by altering signal transduction pathways.

However, the cellular and molecular mechanisms underpinning these changes are still relatively unknown. Recently, several studies trying to understand the cellular and molecular changes that lead to pressure-activated hypertrophy and subsequent heart failure have revealed that the NF-κB, transforming growth factor β, Hedgehog, and Wnt pathways mediate the left ventricular (LV) hypertrophy (de Jesus Perez et al. 2009, Hou et al. 2009, Laumanns et al. 2009). Other researchers concentrated on the roles for Wnt/β-catenin in respiratory system development, including segregation of the conducting airway and alveolar compartments, specialization of the mesenchyme, and establishment of tracheal asymmetries and tracheal glands (De Lange & Reynolds 2008, Flozak et al. 2010). However, possible specific signaling events that act on the lung as a pulmonary vascular response to pressure overload in neonates remain to be determined.

In this study, using a CH-induced PPHN model in rats, we first explored the molecular signaling cascades and gene expression patterns in lung tissue. We then addressed whether ghrelin protected neonatal rats from hypoxia-induced PH, including its effects on hemodynamics and pulmonary vasculature. Finally, we also analyzed signaling transduction pathways regulated by ghrelin.

### Materials and methods

**PPHN animal model and ghrelin treatment**

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols conformed to the guidelines that were established by the Animal Laboratory Center of Zhejiang University. Pregnant Sprague–Dawley rats were maintained for at least 1 week before giving birth. Pups were delivered naturally at full term and maintained under normal atmospheric conditions for the first 48 h of life to allow for successful transition to postnatal life. Pups were treated with CH to induce PPHN as described.

Table 1 Quantitative real-time reverse transcriptase-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession no.</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birc5</td>
<td>NM_022274.1</td>
<td>5′-AGGACCCACGGATCTACACTTC-3′</td>
<td>64.8</td>
<td>107</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>NM_053357.2</td>
<td>5′-ATTCGGATAGGGTGCCGCTG-3′</td>
<td>65</td>
<td>118</td>
</tr>
<tr>
<td>Cdh1</td>
<td>NM_031334.1</td>
<td>5′-TGGGATATCCAGGCTCTC-3′</td>
<td>64.6</td>
<td>123</td>
</tr>
<tr>
<td>Lef1</td>
<td>NM_130429.1</td>
<td>5′-GGGTGCTTCTGTTTGGAC-3′</td>
<td>64.4</td>
<td>145</td>
</tr>
<tr>
<td>Pparg</td>
<td>NM_001145366.1</td>
<td>5′-GGGCTTCAGGAACACACCT-3′</td>
<td>65</td>
<td>175</td>
</tr>
<tr>
<td>Wnt1</td>
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<td>5′-GGGTCTACGAGGCTTCCT-3′</td>
<td>64.9</td>
<td>59.8</td>
</tr>
<tr>
<td>Actb</td>
<td>NM_031144.2</td>
<td>5′-GACCTACGTCGACGTCTTG-3′</td>
<td>64.9</td>
<td>150</td>
</tr>
</tbody>
</table>
previously (Deruelle et al. 2006). Pups were kept in a hypoxic chamber (10–12% O₂) for 14 days. Inspired oxygen concentrations within the hypoxic chamber were continuously monitored. Anhydrous calcium chloride and soda lime were used for CO₂ absorption. Chambers were opened briefly (<10–15 min) for cleaning and other administration. Rats were divided into four groups (n=10/each group): 1) hypoxia and vehicle treatment (s.c., 0.2 ml) daily for 14 days; 2) hypoxia and ghrelin (Phoenix Biotech Co., Ltd, Beijing, China) treatment (s.c., 150 mg/kg (Schwenke et al. 2008), 0.2 ml) daily for 14 days; 3) sham control, sham chamber was not subjected to hypoxia and was treated with the vehicle control (s.c., 0.2 ml) daily for the same period; and 4) ghrelin control (s.c., 150 μg/kg, 0.2 ml), ghrelin treatment daily for 14 days in normoxic rats. Animals were anesthetized by sodium pentobarbital (50 mg/kg, i.p.) and killed on postnatal day 16. Lung tissue was stored in liquid nitrogen for latter RNA isolation and protein extraction or fixed in formalin and embedded in paraffin for immunostaining. Serum active ghrelin (EZRGRA-90K, Millipore, Billerica, MA, USA) was determined by ELISA according to the manufacturer’s instructions.

Hemodynamic studies and morphometric analysis

Hemodynamic studies can be seen in the online supplement for detailed protocol, see section on supplementary data given at the end of this article. The atria were removed, and the right ventricle (RV) wall was separated from the LV and septum (Sep). RV and LV weights were expressed as the ratio of the RV to the LV+Sep weight (RV/LV+Sep, Fulton’s ratio; Schermuly et al. 2004, 2005, Dumitrascu et al. 2006).

Immunohistochemistry and semi-quantification

Immunohistochemical staining of alpha smooth muscle actin (α-SMA) and GHSR-1a was performed on paraffin-embedded lung tissue (n=6, see online supplement for detailed protocol, see section on supplementary data given at the end of this article). For small pulmonary arteries (lumen diameter (LD) <100 μm) with positive α-SMA staining, medial thickening was expressed as α-SMA_area/LD (Kranenburg et al. 2002).

Table 2 Cardiovascular variables and body weight of four groups of rats (n=10). Ghrelin (150 μg/kg; s.c.) was administered once a day. Data are presented as mean±S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Normoxia + ghrelin</th>
<th>Hypoxia</th>
<th>Hypoxia + ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>37.04±1.28</td>
<td>38.04±1.48</td>
<td>24.35±0.31*</td>
<td>25.56±0.25†</td>
</tr>
<tr>
<td>RV/LV+ Sep</td>
<td>0.18±0.01</td>
<td>0.16±0.02</td>
<td>0.51±0.09*</td>
<td>0.27±0.02†</td>
</tr>
<tr>
<td>mRVSP (mmHg)</td>
<td>20.02±1.02</td>
<td>19.19±2.12</td>
<td>37.29±3.15*</td>
<td>23.51±0.82†</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>305.67±24.82</td>
<td>313±21.84</td>
<td>279.33±23.08</td>
<td>333.67±20.94</td>
</tr>
<tr>
<td>Ghrelin (pg/ml)</td>
<td>29.50±9.36</td>
<td>36.75±7.57</td>
<td>27.38±5.29</td>
<td>43.75±4.94†</td>
</tr>
</tbody>
</table>

*Significant difference from normoxia values (P<0.05), †Significant difference between hypoxia + ghrelin and hypoxia (P<0.05).

Figure 1 Photomicrographs of lung tissue sections from neonatal rats (A) normoxia group, (B) hypoxia, (C) normoxia + ghrelin, and (D) hypoxia + ghrelin showing α-smooth muscle actin staining (brown) in pulmonary vascular smooth muscle cells from small (LD <100 μm) vessels (original magnification: ×200). (E) Graphic representations of ratio of α-SMA_ area/LD measurements (mean±S.E.M.) using image analysis. *P<0.05 hypoxia versus normoxia; †P<0.05 hypoxia + ghrelin versus hypoxia. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-10-0143.
PCR array analysis of signal transduction pathways

RNA was extracted from lung homogenates using an RNeasy tissue kit and treated with DNase to remove contaminating DNA (Qiagen; see online supplement for detailed protocol, see section on supplementary data given at the end of this article). Detection and quantification of gene expression in the lung were performed using an RT² Profiler rat signal transduction pathway finder PCR array (catalog no.PARN-014A, SA Biosciences, Frederick, MD, USA) according to the manufacturer’s instructions. The relative expression of each gene compared with the expression in control animals was calculated using the $2^{-\Delta \Delta Ct}$ method, using five housekeeping genes as controls ($n=3$, see online supplement for detailed protocol, see section on supplementary data given at the end of this article).

Wnt pathway verification by qRT-PCR analysis and western blot analysis

Quantitative real-time reverse transcriptase (qRT)-PCR analysis and western blot analysis can be seen in the online supplement for detailed protocol, see section on supplementary data given at the end of this article (for primers, see Table 1).

Administration of ghrelin receptor antagonist and PI3K/AKT inhibitor

Rat pulmonary microvascular endothelial cells (RPMECs) were isolated using a modification of the technique described previously by John et al. (2006). Cells were exposed to 10% fetal bovine serum, endothelial cell growth factor (80 µg/ml), and in the presence or absence of CoCl₂ (a classic hypoxia mimetic, 0, 50, 100, 200, 400, and 800 µmol/l, Sigma) for 72 h. The inhibitors D-Lys³-GHRP-6 (ghrelin receptor-selective antagonist; 100 µmol/l) and LY294002 (PI3K/AKT inhibitor; 10 µmol/l) were added 90 min prior to addition of ghrelin (100 nmol/l). For time-course experiments, cells were treated with ghrelin (100 nmol/l) for 0, 5, 10, 15, 30, or 60 min. For dose–response experiments, cells were treated with ghrelin for 15 min at concentrations of 0, 0·1, 1, 10, 100, or 1000 nmol/l. Fresh media and drugs were added to the cells every 48 h. Cells were characterized by routine fluorescence immunohistochemistry. The cells were incubated for 1 h with the primary antibodies rabbit anti-GHSR-1a (1:200 dilution) and monoclonal mouse anti-CD31 (1:200 dilution).

Apoptosis assay

Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling assays using the cell apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The sections of the RPMECs were fixed according to the manufacturer’s instructions. The number of apoptotic cells was counted in ($\times$200) fields for quantification.

Luciferase reporter assay

Cells were plated to form 60–80% confluent cultures in 96-well dishes in triplicates and transfected with TOP-FLASH plasmids (Millpore, Billerica, MA, USA) and the pRL-TK plasmid (Promega) was co-transfected to normalize for transfection efficiency using...
Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed 48 h after transfection using a dual-luciferase reporter assay system (Promega).

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analysis was performed by one-way ANOVA. $P<0.05$ was found and considered significant. All data analysis was carried out using the SPSS software for Windows (version 13.0; SPSS, Inc., Chicago, IL, USA).

Results

The effects of ghrelin on growth and survival

The body weight of the rat pups was $5.86 \pm 0.46$ g on postnatal day 1 and $10.43 \pm 0.72$ g on postnatal day 3. Normoxia pups grew faster than hypoxia-exposed pups. On postnatal day 16, the body weight of the hypoxia + ghrelin group was slightly higher than that of the hypoxia-exposed control pups (Table 2). No deaths occurred in any of the groups. Room air-exposed pups did not show any signs of illness or mortality during the first 16 days after birth. The levels of active ghrelin measured in hypoxia + ghrelin animals versus hypoxia group were $43.75 \pm 4.94$ and $27.38 \pm 5.29$ pg/ml ($P<0.05$) respectively (Table 2).

Ghrelin improved hemodynamics and RVH in chronically hypoxic rats

To confirm whether 2-week hypoxic exposure-induced PH in neonatal rats, RV systolic pressure (RVSP, a marker of systolic pulmonary arterial pressure), and the RV/(LV + Sep) ratio (a marker of RV hypertrophy (RVH)) were measured. In the hypoxia group, a significant increase in mean RVSP (mRVSP), above the control value, was observed ($37.29 \pm 3.15$ vs $20.02 \pm 1.02$ mmHg, $P<0.05$). However, hypoxia did not modify heart rate. Daily administration of ghrelin during hypoxia significantly attenuated the development of neonatal PH (Table 2). There was also a significant increase in the RV/(LV + Sep) ratio in the hypoxia group ($0.51 \pm 0.09$ vs $0.18 \pm 0.01$, $P<0.05$), indicating RVH (Table 2). Therefore, 14 days of exposure to hypoxia induced PH with RVH in neonatal rats. Ghrelin treatment reduced both the magnitude of PH and the RV/(LV + Sep) ratio (Table 2).

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Ghrelin attenuated CH-induced medial thickening of pulmonary arteriole and increased GHSR-1a protein

A significant increase was seen in the α-SMAarea/LD ratio for hypoxia rats compared with control rats (194.2 ± 8.8 vs 88.9 ± 4.8, P < 0.000). A significant decrease in the α-SMAarea/LD ratio was observed for ghrelin-treated rats (107.4 ± 6.8; P < 0.000) versus vehicle-treated hypoxia (Fig. 1). GHSR-1a was found to be expressed in pulmonary vascular endothelial cells and hypoxia decreased GHSR-1a protein expression (Fig. 2, Supplementary Figure 1, see section on supplementary data given at the end of this article).

Surprisingly, significant differences in the integrated optical density of GHSR-1a staining were observed between the groups with and without ghrelin treatment in the pulmonary arteriole. The results were also verified by western blot analysis (hypoxia: 0.7 ± 0.2 vs hypoxia + ghrelin group: 1.6 ± 0.3; P < 0.05; Fig. 2).

Ghrelin regulated gene expression profiles of signal transduction pathways associated with PPHN in lung tissue

To understand how ghrelin attenuates hypoxia-induced PH in neonatal rats and its effect on the PPHN signaling pathway, we screened the expression of 84 genes representative of 18 different signal transduction pathways using a PCR array from SA Biosciences (Supplementary Figure 2, see section on supplementary data given at the end of this article). Totally, two signal transduction pathways were significantly upregulated by a threshold of R3.0 when hypoxia rats were compared with the control group: Wnt pathway (lymphoid enhancer factor 1 (Lef1), 4.79; Wnt1, 3.87) and CREB pathway (Cyp19a1, 4.87; Egr1, 7.79; Fos, 3.86; Fig. 3). We also found that the Wnt pathway (Birc5, 5.05; Myc, 3.09; Pparg, 4.82) and the PI3K/AKT pathway (Fn1, 3.60) were upregulated in hypoxia + ghrelin rats compared with the hypoxia group (Fig. 3 and Supplementary Table 1, see section on supplementary data given at the end of this article).

Ghrelin protected neonatal rats from hypoxia-induced PH via the regulation of phosphorylation of glycogen synthase kinase 3β (p-GSK3β)/β-catenin signaling

The mRNA levels of several Wnt pathway genes in lung tissues were assessed by qRT-PCR analysis. Both Wnt1 and Cdh1 were significantly elevated, whereas β-catenin and Pparg were significantly decreased in CH rats (saline treated) compared with normal controls. However, ghrelin abrogated the CH-induced down-regulation of β-catenin mRNA expression (Fig. 4).

Western blot analysis detected p-AKT, GSK3β, p-GSK3β, and the β-catenin proteins in the lung lysates.
Notably, a significant increase in expression of p-GSK3β/GSK3β and β-catenin was detected in ghrelin-treated CH rats. This finding indicates that ghrelin is a stimulatory factor for GSK3β inactivation that leads to stabilization and nuclear accumulation of β-catenin in the lungs during neonatal PH. Upregulation of p-GSK3β correlated with an increase in the levels of β-catenin (Fig. 5). There was also a statistically significant increase in the expression of the p-AKT protein in the CH group compared with the control group (Fig. 5). 

Ghrelin activated p-GSK3β/GSK3β signaling pathways and associated with β-catenin translocation to the nucleus

Treatment of RPMECs with ghrelin rapidly activated p-AKT in a time- and dose-dependent manner (Supplementary Figure 3, see section on supplementary data given at the end of this article). The way by which AKT regulates its effect is by phosphorylating many effector proteins, including the cytoplasmic protein kinase GSK3β. GSK3β phosphorylation was increased after 30 min of ghrelin treatment and lasted for 120 min (Supplementary Figure 2, see section on supplementary data given at the end of this article). The inhibition of ghrelin receptor with [D-Lys3]-GHRP-6 reduced ghrelin-induced AKT phosphorylation and Ly294002 also reduced GSK3β phosphorylation (Fig. 6).

We investigated the effects of ghrelin on CoCl2-induced hypoxia in RPMECs. In our study, hypoxia induced RPMECs apoptosis (Supplementary Figure 4, see section on supplementary data given at the end of this article) and increased phosphorylation of AKT (Ser473), but it was not paralleled with a progressive increase in phosphorylation of GSK3β. The effect of hypoxia + ghrelin on phosphorylation of AKT was completely blocked by pretreatment of the cells with [D-Lys3]-GHRP-6 (100 μmol/l), indicating that ghrelin effect is mediated by GHSR-1a receptor. In contrast, hypoxia downregulated phosphorylation of the GSK3β expression and these effects reversed in the presence of ghrelin (Fig. 6). Also, we found that ghrelin-induced phosphorylation of GSK3β is associated with β-catenin translocation to the nucleus under hypoxia condition. The β-catenin immunoreactivity was primarily observed in the nucleus and cytoplasm under basal and hypoxia conditions respectively. However, β-catenin exhibited an increased staining pattern when cells were treated with ghrelin under hypoxia, suggesting that β-catenin has translocated to the nucleus. In contrast, ghrelin-induced nuclear translocation of β-catenin was inhibited after the cells were pretreated with inhibitor Ly294002 (Fig. 7). We also transfected TOPflash into RPMECs derived from neonatal rats and found that the relative luciferase activity was about 1.9- to 2.5-fold increased in ghrelin-treated cell concentrations from 0.1–1000 nmol/l compared with control. Collectively, these findings support that ghrelin activated the canonical Wnt pathway (Fig. 8).

Discussion

In this study, we report that continuous administration of ghrelin attenuated neonate PH, RVH, and improved pulmonary vascular remodeling. We newly found that ghrelin reverses these pathological changes via regulation of p-GSK3β/β-catenin signaling in PPHN. Since its discovery in 1999, ghrelin has been implicated as an important modulator of numerous physiological functions, including food intake, pancreas development, and cardiovascular processes (Henriques-Coelho et al. 2004, Wang et al. 2007). At the
pulmonary level, ghrelin seems to have an important role in fetal lung development (Volante et al. 2002), and the adult human lung is also a source of ghrelin mRNA expression (Gnanapavan et al. 2002). Most of the activity of the ghrelin–GHSR-1a signaling pathway is thought to be involved in the regulation of vascular tone (Nagaya et al. 2001, Wiley & Davenport 2002). GHSR-1a is expressed in many cell types and tissues, including cardiomyocytes, vascular endothelium, and monocytes, raising the possibility that ghrelin may also have important physiological actions in peripheral tissues that are GH independent (Iantorno et al. 2007). However, there is controversy about whether the GHSR-1a receptor is the sole receptor for ghrelin or just one of a group of receptors for this ligand. But, the active ghrelin form is capable of binding GHSR-1a. Our results suggest that increasing active ghrelin level in serum may be connected with higher GHSR-1a protein in the neonatal pulmonary vascular wall. These mechanisms as they pertain to active ghrelin and GHSR-1a have not been characterized; therefore, in this study, we investigated the ghrelin-mediated receptor and downstream of GHSR-1a in the activation of AKT (Iantorno et al. 2007). We studied whether GHSR-1a mediates AKT activation using a cellular model exclusively expressing the GHSR-1a and we have then characterized the signaling machinery involved. Taking all of these results into consideration, it seems reasonable to propose that ghrelin may modulate PH. With regard to decreased RV overload, primarily favoring recovery of RV function and pulmonary vascular remodeling, the results of our study showed that the medial vessel area $\alpha$-SMA$^+$ in pulmonary arterioles was significantly reduced by ghrelin, indicating that attenuation of PH is, at least partially, mediated by the ghrelin–GHSR-1a signaling pathway.

In our PCR array results, we filtered the Wnt signaling pathway from 18 different signal transduction pathways. The Wnt signaling cascades can be divided into canonical and noncanonical pathways. The best-characterized canonical Wnt pathway is the $\beta$-catenin-dependent pathway. In this study, in the absence of active Wnt ligands, $\beta$-catenin is bound to the scaffold proteins Axin and adenomatous polyposis coli (APC) and constitutively phosphorylated at four N-terminal residues via interaction with casein kinase I and GSK3$\beta$. In the presence of Wnt ligands, Wnt ligand binds to Fzd receptors, complexed with low density lipoprotein receptor-related protein (LRP), resulting in the inhibition of the GSK3$\beta$/APC/Axin complex. The activity of GSK3$\beta$ is inhibited by phosphorylation of serine 9. Subsequently, $\beta$-catenin is neither phosphorylated nor degraded and accumulates in the cytoplasm where it is translocated into the nucleus and acts as a co-transcriptional activator of Lef1/T-cell factor regulating downstream target genes such as Pparg, Tnc, and Myc (Van Scoy et al. 2008, Königshoff & Eickelberg 2010).

**Figure 7** $\beta$-Catenin protein fluorescence immunohistochemistry analysis; (A) normoxia; (B) CoCl$_2$ (100 $\mu$mol/l) treatment of 72 h induced RPMECs in hypoxic condition; (C): ghrelin (100 nmol/l) treatment of normoxia RPMECs 30 min; (D) ghrelin (100 nmol/l) treatment of hypoxic RPMECs 30 min; (E) Ly294002 (10 $\mu$mol/l) treatment of normoxia RPMECs 30 min; (F) Ly294002 (10 $\mu$mol/l) pretreatment of hypoxic RPMECs 30 min, ghrelin (100 nmol/l) 30 min. Full color version of this figure available via http://dx.doi.org/10.1530/JME-10-0143.

**Figure 8** Increased TOPflash promoter activity in ghrelin-treated RPMECs. RPMECs were cotransfected with 0.8 $\mu$g of TOPflash and 0.2 $\mu$g of pRL-TK plasmids. The cells were lysed 48 h after transfection, and the ratio of firefly luciferase to Renilla luciferase signal was quantified. Fold changes of luciferase activity relative to control are shown. Values represent fold changes expressed as the mean $\pm$ S.E.M. $^*P<0.05$ compared with control, $^*P<0.05$ compared with ghrelin 0-1 nmol/l group.
Wnt signaling has recently been suggested to regulate smooth muscle cell precursor proliferation and development in embryonic lung development and adult lung disease. Histological analysis of human PH samples also demonstrated increased levels of α-SMA and Axin (Cohen et al. 2009). PPHN is characterized by a dramatic increase in pulmonary vascular smooth muscle proliferation and eventual narrowing of these vessels. It is, therefore, likely that Wnt signaling genes play a role in the lung of PPHN. To understand how each of these genes regulates pathogenesis of PPHN and how ghrelin reverses neonatal PH, we used qRT-PCR to verify the PCR array results.

The mRNA expression of canonical Wnt pathway genes, β-catenin, and Pparg was significantly decreased in chronic hypoxic neonatal rats (saline treated) compared with control rats. This finding is consistent with the results of Takayasu et al. (2007), who reported that the Wnt signaling pathway is downregulated in nitrogen-induced hypoplastic lungs in the early stages of lung development. On the other hand, these changes were accompanied by the downregulation of Pparg, a key nuclear transcription factor, in the lung parenchyma. Some researchers proposed that upregulation of Pparg by the exogenous administration of the Pparg agonist rosiglitazone would mitigate hypoxia-mediated upregulation of Wnt signaling (Dasgupta et al. 2009). Although the exact mechanism by which Pparg downregulates in signaling pathway is unclear, both physical and functional interactions between β-catenin and Pparg involving the Lef1 binding domain of β-catenin and the β-catenin binding domain of Pparg have been reported (Liu et al. 2006). Moreover, our findings with ghrelin treatment prevented the CH-induced decrease of β-catenin, mRNA. Regarding the underlying mechanism, β-catenin, the intracellular binding partner of E-cadherin, appears to be a good candidate to participate in contact-dependent regulation of epithelial-to-mesenchymal transition. Indeed, β-catenin signaling has been implicated in branching morphogenesis of the mouse embryonic lung (De Langhe et al. 2005), although its role in promoting organ fibrosis and the formation of the pulmonary vasculature remains to be defined.

The question remained of how the ghrelin–GHSR-1α interactions play a role in the downstream signaling pathway of PPHN. As described above, the Wnt pathway (which involves GSK3β) is an important regulatory mechanism in the developmental growth of the heart (Eisenberg & Eisenberg 2007, Nagy et al. 2010). Wnt signaling in lung development regulates multiple steps in organogenesis, including cell proliferation, differentiation, and lineage specification. Wnt genes play as mediators of epithelial mesenchymal interactions in the developing lung. In our studies, we found hypoxia induced endothelial cell death and ghrelin protected RPMECs from apoptosis. In addition, a number of studies have suggested that GSK3β plays a pivotal role in cardiac hypertrophy (Kerkela et al. 2007). In essence, activated GSK3β prevents hypertrophic growth of cardiac myocytes, probably through phosphorylation and inhibition of transcriptional regulators including β-catenin. We further verified the canonical Wnt pathway key regulatory protein and selected ghrelin-related signaling protein levels for western blot analysis. In the Rossi study, the involvement of ghrelin in the proliferation of human aortic endothelial cell and the role of transduction pathways were investigated. They confirmed the involvement of PI3K/AKT pathways in ghrelin-induced endothelial cell proliferation (Rossi et al. 2008). Ghrelin and ghrelin receptor/Gq protein mediated activation of AKT in cultured endothelial cells and intact vessels. Inhibiting AKT with pharmacological inhibitors or small interference RNA markedly attenuated ghrelin-induced eNOS activation and NO production (Xu et al. 2008). In our study, we observed that GHSR-1α activation by ligand binding in the presence of ghrelin elicited a rapid increase in AKT (serine 473) phosphorylation. In our PPHN rat model and RPMECs, hypoxia induced the degradation of β-catenin in parallel with the decrease in phosphorylation of GSK3β in serine 9. An important function of p-GSK3β is to maintain the stability and function of β-catenin. However, ghrelin treatment significantly increased p-GSK3β and β-catenin expressions in PPHN rat model and RPMECs and these effects were inhibited after the cells were pretreated with the PI3K/AKT inhibitor Ly294002. After ghrelin treatment under hypoxia, β-catenin translocates into the cell nucleus, although its role in function as a transcription factor to regulate α-SMA gene expression remains to be defined. Also, further studies are needed to determine other downstream genes ghrelin–β-catenin may regulate to reverse PPHN.

In conclusion, this study demonstrated that s.c. administration of ghrelin improved PH and attenuated pulmonary vascular remodeling. These beneficial effects of ghrelin might be mediated by the activation of p-GSK3β/β-catenin activity. These data suggest that ghrelin is a potential candidate for a new therapeutic agent against PPHN.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-10-0143.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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