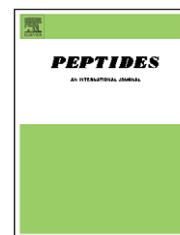


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Ghrelin and obestatin: Different role in fetal lung development?

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ABSTRACT

Ghrelin and obestatin are two proteins that originate from post-translational processing of the preproghrelin peptide. Various authors claim an opposed role of ghrelin and obestatin in several systems. Preproghrelin mRNA is significantly expressed in airway epithelium throughout lung development, predominantly during the earliest stages. The aim of this study was to evaluate the role of ghrelin and obestatin in fetal lung development *in vitro*. Immunohistochemistry studies were performed at different gestational ages in order to clarify the expression pattern of ghrelin, GHS-R1a, obestatin and GPR39 during fetal lung development. Fetal rat lung explants were harvested at 13.5 days post-conception (dpc) and cultured during 4 days with increasing doses of total ghrelin, acylated ghrelin, desacyl-ghrelin, ghrelin antagonist (D-Lys(3)-GHRP-6) or obestatin. Immunohistochemistry studies demonstrated that ghrelin, GHS-R1a, obestatin and GPR39 proteins were expressed in primitive rat lung epithelium throughout all studied gestational ages. Total and acylated ghrelin supplementation significantly increased the total number of peripheral airway buds, whereas desacyl-ghrelin induced no effect. Moreover, GHS-R1a antagonist significantly decreased lung branching. Finally, obestatin supplementation induced no significant effect in the measured parameters. The present study showed that ghrelin has a positive effect in fetal lung development through its GHS-R1a receptor, whereas obestatin has no effect on lung branching.

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1. Introduction

Ghrelin is a 28-aminoacid acylated peptide mainly synthesized in the endocrine A-like cells of the oxyntic mucosa of the stomach, that was originally described as a potent growth hormone (GH) secretagogue interacting with the growth hormone secretagogue receptor subtype 1a (GHS-R1a) [23,26,30,34]. Moreover, ghrelin is a pleiotropic peptide with

multiple roles described in various organs and systems as recently reviewed by Leite-Moreira and Soares [25].

Throughout lung development, preproghrelin mRNA is significantly expressed in airway epithelium, mainly during the pseudoglandular stage, suggesting that ghrelin might act as regulator of fetal lung development by autocrine/paracrine mechanisms [37]. In this sequence, Santos et al demonstrated that ghrelin expression was increased in hypoplastic lungs

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from fetuses and newborns with congenital diaphragmatic hernia (CDH), whereas maternal treatment with ghrelin was able to partially attenuate pulmonary hypoplasia in a CDH experimental model [31]. On the other hand, in other adult pulmonary diseases, ghrelin also seems to have beneficial effects suggesting a role for ghrelin system in lung pathophysiology [5,18,32,38].

Ghrelin has the unique characteristic of having an acylated group on one of its serine residues (Ser 3). This acylation by *n*-octanoic acid is unstable but it is necessary for the binding of ghrelin to the GHS-R1a. Therefore, acylated ghrelin is often referred to as “active” ghrelin, although desacyl-ghrelin represents >90% of total circulating ghrelin in the adult rat. Even though the acylated form of ghrelin has been recognized as the major active orexigenic molecule regulating energy balance [7,19], recent data provides evidence that differential influences of the acylated and

non-acylated forms of the peptide must be considered [13]. Though the two ghrelin forms have a good chemical correlation, they may have different actions [1,4,13,36]. Obestatin, is a newly discovered peptide encoded by the preproghrelin and originated from post-translational processing of the preproghrelin peptide [41]. The effects of obestatin are mediated through the orphaned receptor GPR39, which belongs to the family of class A rhodopsin receptors that also include the ghrelin receptor (GHS-R1a) and motilin receptor [26,41]. Although ghrelin and obestatin have the same origin, several authors claim that their roles might be opposite. In fact, obestatin appears to act as an anorexic hormone, decreasing food intake, gastric emptying, jejunal motility and body weight gain, antagonizing the actions of ghrelin when both peptides are co-administered [41]. However, in other systems obestatin is devoid of action, for instance it has no effect on GH secretion [41]. Thus, the aim of this work was

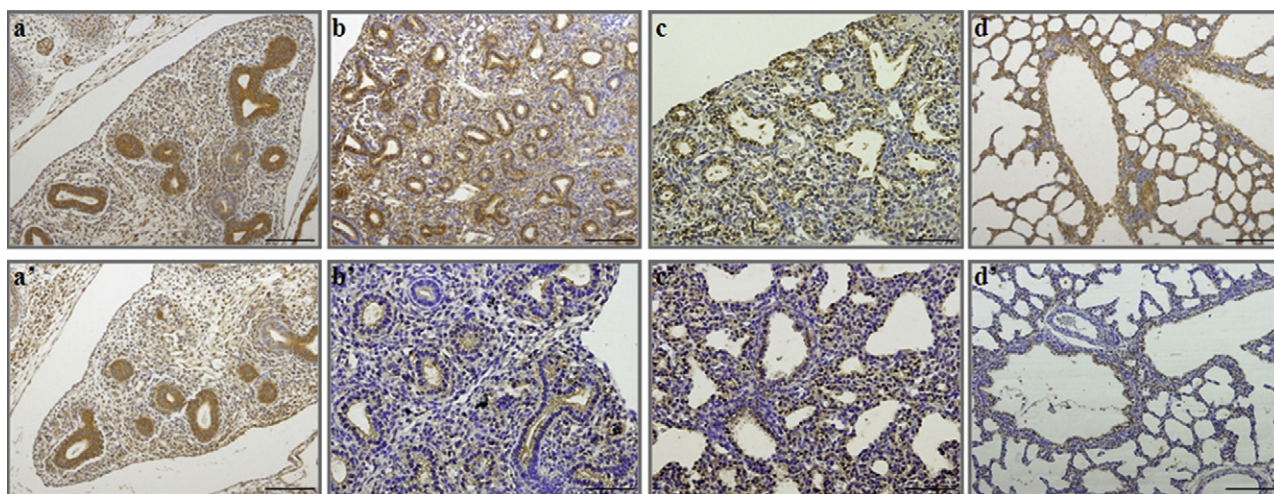


Fig. 1 – Ghrelin (a–d) and GHS-R1a (a'–d') expression pattern during normal rat lung development. IHC studies showed that in normal fetal lung, ghrelin and GHS-R1a expression was localized to the primitive airway epithelium (a and a': 15.5 dpc; b and b': 17.5 dpc; c and c': 19.5 dpc; d and d': 21.5 dpc). Scale bar, 15.5, 17.5 and 21.5 dpc = 100 μ m; 19.5 dpc = 50 μ m.

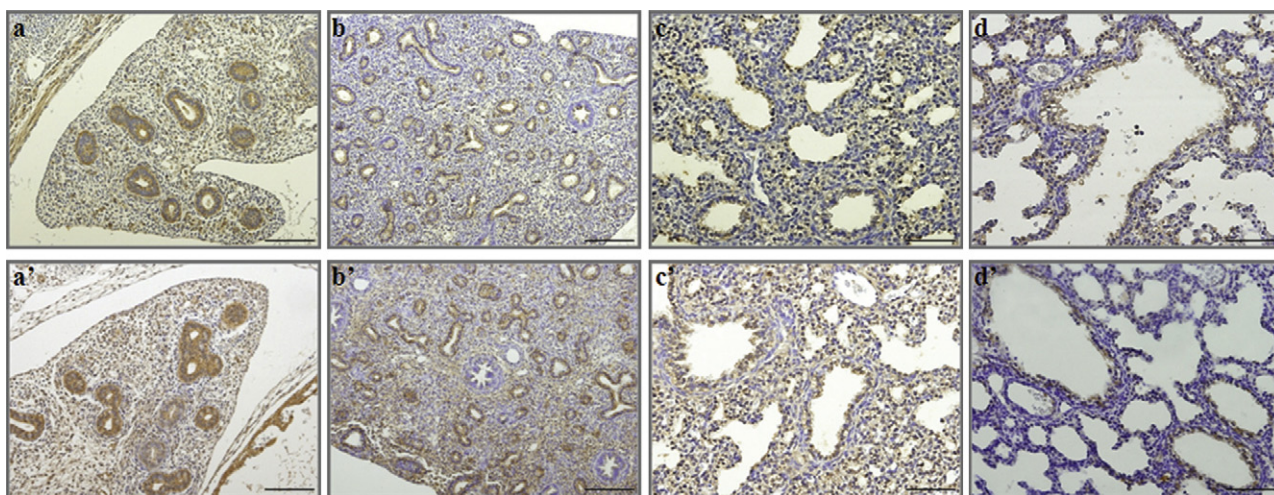


Fig. 2 – Obestatin (a–d) and GPR39 (a'–d') expression pattern during normal rat lung development. IHC studies showed that in normal fetal lung, obestatin and GPR39 expression was localized to the primitive airway epithelium (a and a': 15.5 dpc; b and b': 17.5 dpc; c and c': 19.5 dpc; d and d': 21.5 dpc). Scale bar, 15.5 and 17.5 dpc = 100 μ m; 19.5 and 21.5 dpc = 50 μ m.

to clarify whether ghrelin and obestatin have different or concurrent effects during fetal lung development.

2. Materials and methods

2.1. Animal model and experimental design

Animal experiments were performed according to the Portuguese law for animal welfare. Animals were housed in an accredited mouse house and treated as specified in the 'Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health's (National Institutes of Health Publication No.85-23, revised 1996). Sprague–Dawley female rats (225 g, Charles-River, Barcelona, Spain) were maintained in appropriate cages under controlled conditions, fed with commercial solid food and after mating they were checked for introital plugging. All pregnant rats were sacrificed by decapitation. For immunohistochemistry studies, fetuses were harvested by caesarean section at 15.5, 17.5, 19.5 and 21.5 dpc. For lung explant cultures, fetuses were harvested at 13.5 dpc.

2.2. Immunohistochemistry studies (IHC)

Immunostainings were performed on formalin-fixed and paraffin-embedded lung explants. Sections (5 μ m) were placed

on SuperFrost® Plus slides (Menzel-Glaser, Braunschweig, Germany). The ghrelin (1:50 dilution), obestatin (1:100 dilution) and GPR39 antibodies (1:100 dilution) were obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). The GHSR antibody (1:100) was obtained from Acris Antibodies GmbH (Hiddenhausen, Germany).

After dewaxing in xylene and rehydration in ethanol, antigen retrieval was achieved by boiling in 10 mM citrate buffer followed by cool down at room temperature. The samples were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase and blocked with 5% BSA (Roche, Penzberg, Germany). Incubation of the primary antibody was performed at 4 °C overnight. Negative control reactions included omission of the primary antibody. Incubation with the UltraVision detection system anti-polyvalent horseradish peroxidase (Lab Vision Corporation, Fremont, CA, USA) was carried according to manufacturer's instructions. To visualize the peroxidase activity in sections, diaminobenzidine tetrahydrochloride was used. The slides were counterstained with hematoxyline and photographed with Olympus BX61 microscope (Olympus, Tokyo, Japan).

2.3. Fetal lung explant cultures

Fetuses were removed by caesarean section at 13.5 dpc. Harvesting and dissection of the lungs was made in DPBS

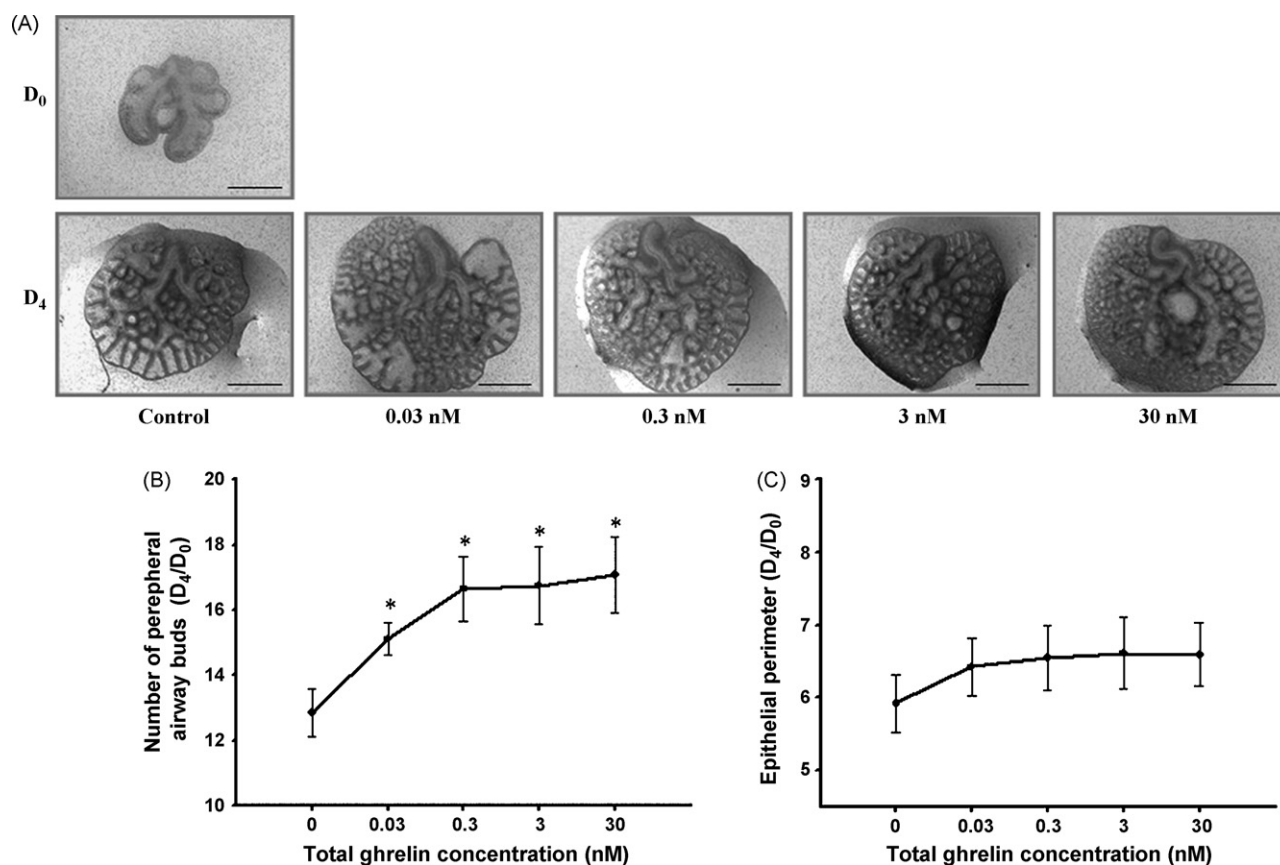


Fig. 3 – Branching morphogenesis in rat lung explant system treated with different total ghrelin doses. A: upper panel is representative of lung explants at D₀. Bottom panel represents lung explants treated with different total ghrelin doses, at D₄; B: total number of peripheral airway buds; C: epithelial perimeter. Scale bar, 6349 μ m (all images at same magnification). Numeric results are expressed as D₄/D₀ ratio. $p < 0.05$; *vs. control (0 nM).

(Lonza, Basel, Switzerland) under a dissection microscope (Leica MZFLIII, Wetzlar, Germany). The lungs were transferred to Nucleopore membranes with an 8 μ m pore size (Whatman, Clifton, NJ, USA) and incubated in a 24-well culture plates from Costar (Corning, NY, USA). Membranes were presoaked in DMEM (Lonza) for 1 h before the explants were placed on them. Floating cultures of the explants were incubated in 200 μ L of 50% DMEM, 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 100 μ g/mL streptomycin, 100 units/mL penicillin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal calf serum (Invitrogen). Fetal lung explants were incubated in a 5% CO₂ incubator at 37 °C for 96 h, and the medium was replaced every 48 h. Increasing doses of total ghrelin (0 nM, *n* = 10; 0.03 nM, *n* = 10; 0.3 nM, *n* = 10; 3 nM, *n* = 10; 30 nM, *n* = 12), acylated ghrelin (0 nM, *n* = 10; 0.03 nM, *n* = 12; 0.3 nM, *n* = 10; 3 nM, *n* = 10; 30 nM, *n* = 10), desacyl-ghrelin (0 nM, *n* = 10; 0.03 nM, *n* = 10; 0.3 nM, *n* = 10; 3 nM, *n* = 10; 30 nM, *n* = 10), ghrelin antagonist—D-Lys(3)-GHRP-6 (0 nM, *n* = 10; 10 nM, *n* = 10; 100 nM, *n* = 10; 1000 nM, *n* = 10) or obestatin (0 nM, *n* = 8; 0.5 nM, *n* = 8; 5 nM, *n* = 8; 50 nM, *n* = 8; 100 nM, *n* = 8; 500 nM, *n* = 9) were added daily. The total (rat, 1–28), acylated (rat, 1–5), and desacyl-ghrelin as well as ghrelin antagonist were obtained from the Peptides International (Louisville, KY, USA). Obestatin was obtained from Anaspec, Inc. (San Jose, CA, USA).

2.4. Morphometric analysis

The branching morphogenesis was monitored daily by photographing the explants. At day 0 (D₀: 0 h) and 4 (D₄: 96 h) of culture, the total number of peripheral airway buds (branching) in all lung explants was determined, whereas the epithelial perimeter and total explant area were measured using AxionVision Rel. 4.3 (Carl Zeiss, Gottingen, Germany). For all experimental conditions, the results of branching and epithelial perimeter were expressed as D₄/D₀ ratio.

2.5. Statistical analysis

All quantitative data are presented as mean \pm SEM. For statistical analysis one-way ANOVA was performed, using SigmaStat 3.5 (Systat, San Jose, CA, USA). The Student–Newman–Keuls test was used for post-test analysis. Statistical significance was set at *p* < 0.05.

3. Results

3.1. IHC studies

IHC studies revealed that ghrelin and GHS-R1a were predominantly expressed in primitive lung epithelium during all

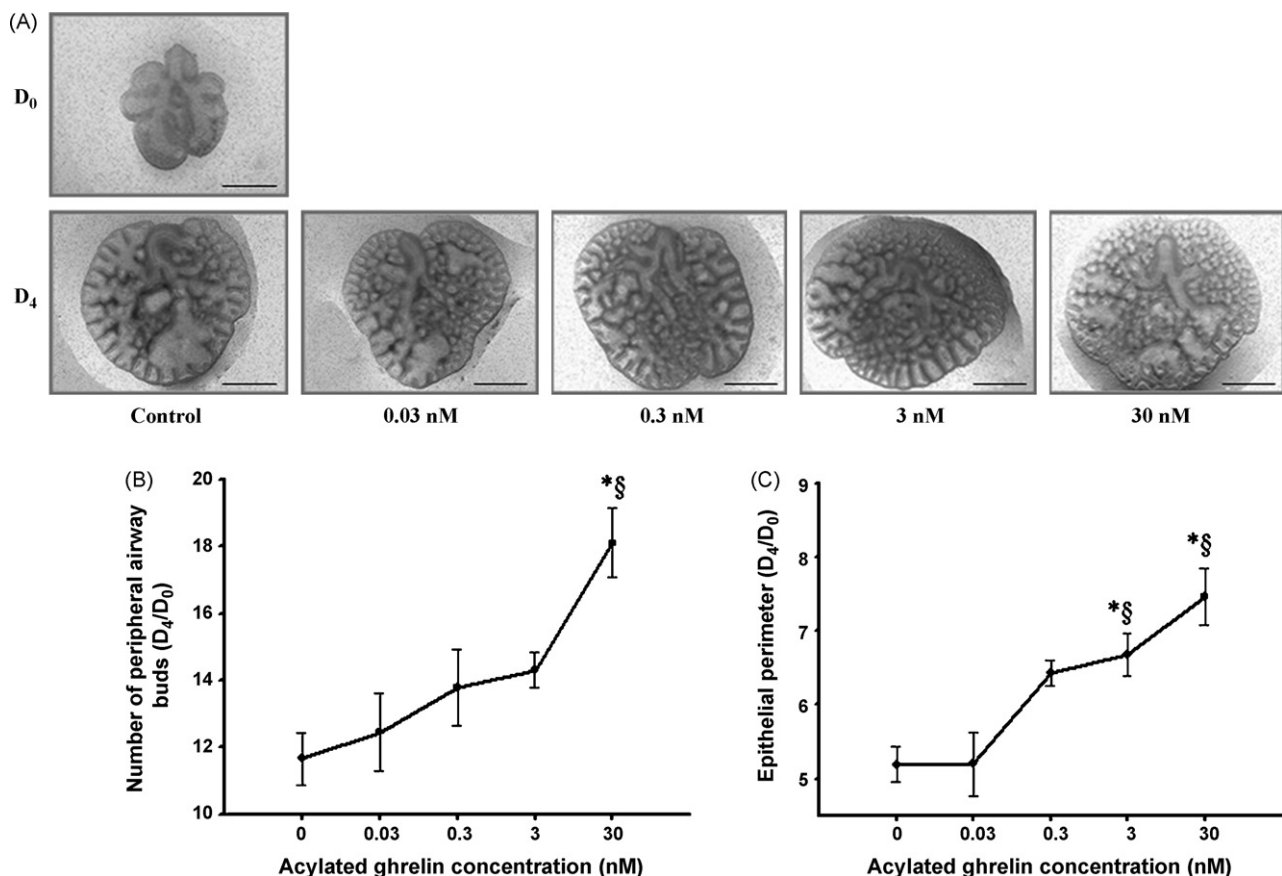


Fig. 4 – Branching morphogenesis in rat lung explant system treated with different acylated ghrelin doses. A: upper panel is representative of lung explants at D₀. Bottom panel represents lung explants treated with different acylated ghrelin doses, at D₄; B: total number of peripheral airway buds; C: epithelial perimeter. Scale bar, 6349 μ m (all images at same magnification). Numeric results are expressed as D₄/D₀ ratio. *p* < 0.05; * vs. control (0 nM), § vs. 0.03 nM.

studied gestational ages (Fig. 1). These studies also showed that obestatin and GPR39 were expressed in primitive lung epithelium throughout all studied gestational ages (Fig. 2).

3.2. Total, acylated and desacyl-ghrelin supplementation studies

For the current protocol 243 lung explants were cultured. In order to evaluate ghrelin role during lung morphogenesis, fetal lung explants were treated with different doses of recombinant total, acylated and desacyl ghrelin.

In Fig. 3A, representative examples of fetal lung explants treated with increasing doses of total ghrelin are illustrated. Total ghrelin appears to have an enhancing effect on lung explants growth in all the studied doses. The results of morphometric analysis on fetal lung explants are summarized in Fig. 3B and C. Increasing doses of total ghrelin induced a progressive and positive effect on total number of peripheral airway buds (Fig. 3B), although this effect was not evident on epithelial perimeter (Fig. 3C).

In Fig. 4A, representative examples of fetal lung explants treated with increasing acylated ghrelin doses are illustrated. Acylated ghrelin appears to have an enhancing effect on lung explants growth in a dose dependent way. The results of morphometric analysis of fetal lung explants are summarized in Fig. 4B and C. Increasing acylated ghrelin doses induced a

positive effect on total number of peripheral airway buds as well as on epithelial perimeter. Treatment with 30 nM of acylated ghrelin induced a maximal and statistically significant growth regarding the number of peripheral airway buds (Fig. 4B). Treatment with 3 and 30 nM of acylated ghrelin induced a maximal and statistically significant growth regarding the epithelial perimeter (Fig. 4C).

In Fig. 5A, representative examples of fetal lung explants treated with increasing desacyl-ghrelin doses are illustrated. Desacyl-ghrelin appears to have no effects on lung explants growth at all the studied doses. The results of morphometric analysis on fetal lung explants are summarized in Fig. 5B and C. No significant differences were observed in lungs, neither on the total number of peripheral airway nor on epithelial perimeter.

3.3. GHS-R1a antagonist supplementation studies

In order to clarify the involvement of GHR-S in the response to ghrelin, lung explants were treated with D-Lys(3)-GHRP-6 on the lung development was examined. The D-Lys(3)-GHRP-6 is a specific receptor antagonist of GHS-R1a that is able to interact with the GHS-R1a inhibiting the interaction of ghrelin with this receptor. In Fig. 6A, representative examples of fetal lung explants treated with increasing GHS-R1a antagonist doses are illustrated. GHS-R1a antagonist seems to have an

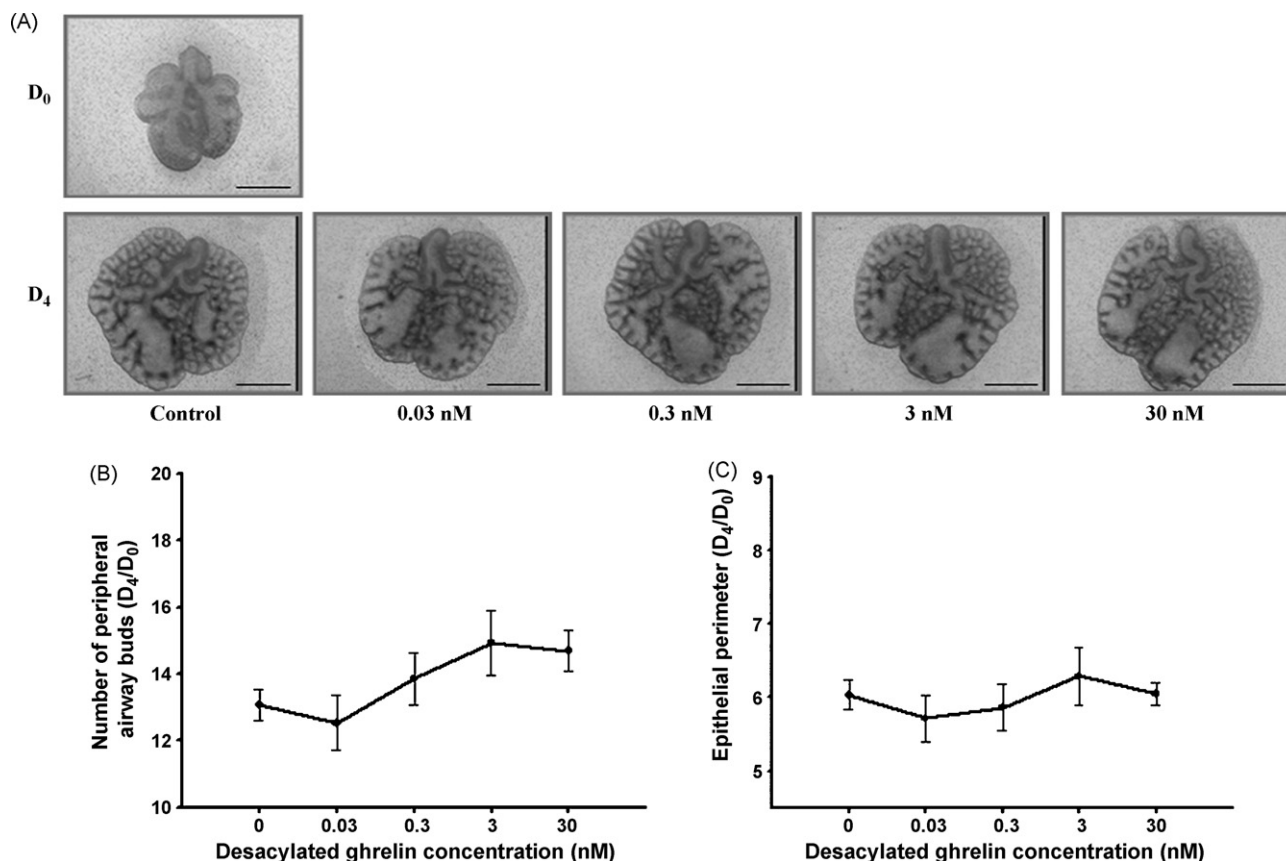


Fig. 5 – Branching morphogenesis in rat lung explant system treated with different desacyl-ghrelin doses. A: upper panel is representative of lung explants at D₀. Bottom panel represents lung explants treated with different desacyl-ghrelin doses, at D₄; B: total number of peripheral airway buds; C: epithelial perimeter. Scale bar, 6349 μ m (all images at same magnification). Numeric results are expressed as D₄/D₀ ratio.

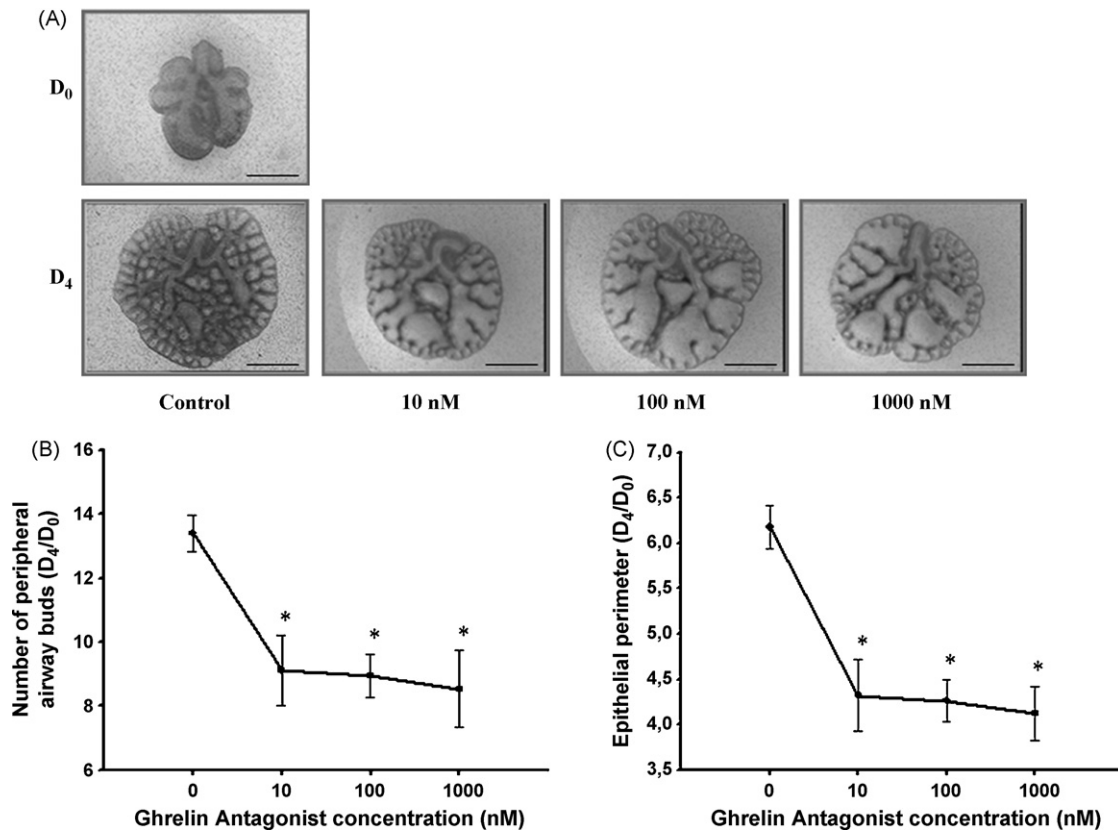


Fig. 6 – Branching morphogenesis in rat lung explant system treated with different GHS-R1a antagonist doses. A: upper panel is representative of lung explants at D₀. Bottom panel represents lung explants treated with different GHS-R1a antagonist doses, at D₄; B: total number of peripheral airway buds; C: epithelial perimeter. Scale bar, 6349 μ m (all images at same magnification). Numeric results are expressed as D₄/D₀ ratio. $p < 0.05$; *vs. control (0 nM).

inhibitory effect on lung explants growth with all the studied doses. The results of morphometric analysis on fetal lung explants are summarized in Fig. 6B and C and confirm this inhibitory effect on total number of peripheral airway buds and epithelial perimeter.

3.4. Obestatin supplementation studies

In order to evaluate obestatin role during lung morphogenesis, fetal lung explants were treated with different doses of recombinant rat obestatin. In Fig. 7A, representative examples of fetal lung explants treated with increasing obestatin doses are illustrated. Obestatin appears to have no effects on lung explants growth at all the studied doses. The results of morphometric analysis on fetal lung explants are summarized in Fig. 7B and C and corroborate the lack of effects.

4. Discussion

This study confirms that ghrelin has a developmental effect during fetal lung growth likely through GHS-R1a receptor. In contrast, although obestatin is expressed in the fetal lung, it does not seem to have a physiological relevant role.

These results demonstrate that ghrelin, obestatin and their respective receptors (GHS-R1a and GPR39) are expressed in

lung epithelium throughout all studied gestational ages, suggesting their involvement in lung development mechanisms. Ghrelin was first identified in the neuroendocrine cell compartment of the oxyntic mucosa of the stomach [7,23]. Subsequently, it was also demonstrated that ghrelin is expressed in the pituitary gland, arcuate nucleus of the hypothalamus, duodenum, jejunum, ileum, colon, testis and also in branching organs such as kidney, pancreas, mammary gland and placenta [7,12,16,23,27]. Volante et al. [37] had already demonstrated that ghrelin is expressed in neuroendocrine cells of the bronchial wall during lung development. Moreover, Santos et al. [31] documented by *in situ* hybridization and immunohistochemistry that ghrelin is expressed in fetal lung epithelium. So, these studies corroborate the present results. Relatively to GHS-R1a expression pattern, it has already been described that this ghrelin receptor is widely expressed in different systems such as the hypothalamic-pituitary, cardiovascular, immune, gastrointestinal, and reproductive, reflecting the pleiotropic activity of its ligand [40]. In the present study, it is documented that GHS-R1a is expressed in fetal lung. Previously, Santos et al. [31] performed PCR studies in human and rat lungs, but GHS-R1a could not be amplified. This apparent contradiction might be explained by the use of different experimental techniques (immunohistochemistry to study protein expression and PCR to study mRNA expression).

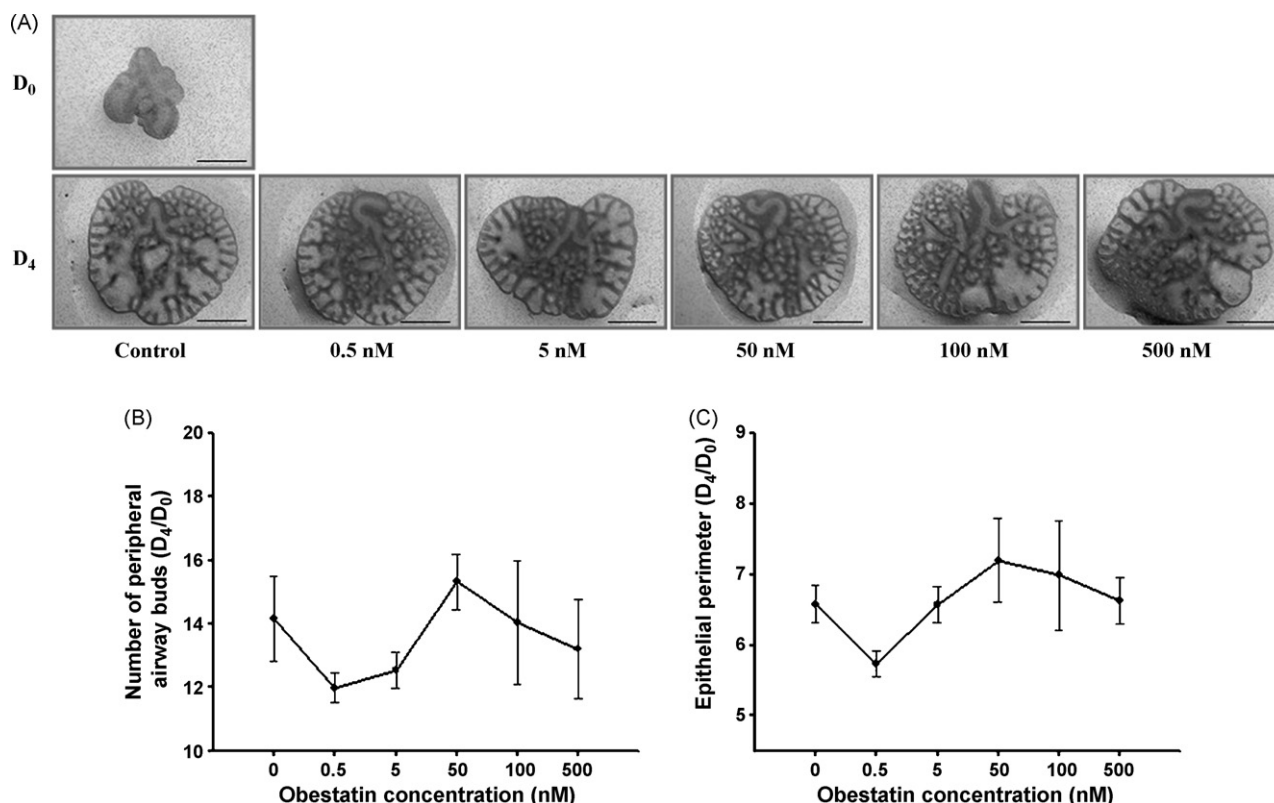


Fig. 7 – Branching morphogenesis in rat lung explant system treated with different obestatin doses. A: upper panel is representative of lung explants at D_0 . Bottom panel represents lung explants treated with different obestatin doses, at D_4 ; **B:** total number of peripheral airway buds; **C:** epithelial perimeter. Scale bar, 6349 μm (all images at same magnification). Numeric results are expressed as D_4/D_0 ratio.

Regarding obestatin expression, it was previously described that obestatin is expressed in gastric mucosa, myenteric ganglion cells, Leydig cells of the testis and pancreatic islets [9,42]. The present work demonstrates that obestatin is also expressed in lung epithelium. Moreover, it is provided the first immunohistochemical evidence of the GPR39 expression in fetal epithelial lung cells. GPR39 expression was only previously demonstrated by RT-PCR analysis, in the gastrointestinal tract, liver, pancreas, kidney, adipose tissue and central nervous system.

In order to clarify whether ghrelin and obestatin play some role on lung morphogenesis, supplementation studies were performed, using a fetal lung explants culture system. Both total and acylated ghrelin induced a positive effect on fetal lung branching, confirming *in vitro* the results described *in vivo* by Santos et al. [31]. On the other hand, supplementation with desacyl-ghrelin showed no effect and ghrelin antagonist supplementation induced an inhibitory effect on lung growth. These results clearly suggest that fetal lung effects of ghrelin are mainly mediated through a GHS-R1a pathway. These findings are in agreement with previous studies in other organs. In this work, D-Lys(3)-GHRP-6 was the ghrelin antagonist used. This ghrelin antagonist has been being used in many other studies in order to characterize the subtype of receptor involved in the various actions of ghrelin [3,6,8,10,11,17,21,22,28,29,33,43]. After obestatin description, several authors suggested that it might have an opposite effect to ghrelin. For instance, obestatin has an

anorexigenic effect and has a sleep-promoting effect [34,35,43]. In this work it was demonstrated the expression of obestatin and GPR39 in fetal lung, however these peptides did not have any effect in lung growth. This observation is in agreement with the literature that describes that obestatin do not have a physiological role and that ghrelin and obestatin do not necessarily present opposed effects. The initial claim of obestatin role on inhibition of food intake, body weight gain, satiety signalling, gastric emptying and intestinal motility in rodents, through the activation of the GPR39 receptor, has not been easily replicated [2,14,15]. Moreover, Yamamoto et al. showed that the intracerebroventricular injection of obestatin did not have a stimulating effect of the pituitary hormones secretions (plasma GH, PRL, ACTH and TSH levels) despite the presence of GPR39 in the pituitary [39]. Furthermore, it was demonstrated that ghrelin is synthesized by cardiomyocytes and has a direct effect on its viability, however Iglesias et al. did not find any relevant metabolic or viability effects of obestatin [20]. In the same trend, Lago et al. claim that, unlike ghrelin, obestatin does not exert any relevant activity in chondrocytes [24].

5. Conclusion

The present study showed that ghrelin has a positive effect in fetal lung development through its GHS-R1a receptor, whereas obestatin has no effect on lung branching.

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