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The apelinergic system in the developing lung: Expression and signaling☆

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ABSTRACT

Apelin and its receptor APJ constitute a signaling pathway best recognized as an important regulator of cardiovascular homeostasis. This multifunctional peptidergic system is currently being described to be involved in embryonic events which extend into vascular, ocular and heart development. Additionally, it is highly expressed in pulmonary tissue. Therefore, the aim of this study was to investigate the role of apelinergic system during fetal lung development. Immunohistochemistry and Western blot analysis were used to characterize apelin and APJ expression levels and cellular localization in normal fetal rat lungs, at five different gestational ages as well as in the adult. Fetal rat lung explants were cultured *in vitro* with increasing doses of apelin. Treated lung explants were morphometrically analyzed and assessed for MAPK signaling modifications. Both components of the apelinergic system are constitutively expressed in the developing lung, with APJ exhibiting monomeric, dimeric and oligomeric forms in the pulmonary tissue. Pulmonary epithelium also displayed constitutive nuclear localization of the receptor. Fetal apelin expression is higher than adult expression. Apelin supplementation inhibitory effect on branching morphogenesis was associated with a dose dependent decrease in p38 and JNK phosphorylation. The results presented provide the first evidence of the presence of an apelinergic system operating in the developing lung. Our findings also suggest that apelin inhibits fetal lung growth by suppressing p38 and JNK signaling pathways.

1. Introduction

The existence of the apelinergic system began to unfold in 1998 when APJ (angiotensin II receptor-like 1), a formerly orphan G-protein coupled receptor (GPCR), and its ligand were finally paired by Tatemoto et al. [45]. Apelin was identified as an endogenous ligand for APJ receptor. This peptide is translated as a 77-amino acid precursor, which undergoes proteolytic maturation generating shorter active apelin peptides. Apelin-36 was the first of these shorter C-terminal sequences being described to bind and activate APJ [45]. Also Apelin-17, Apelin-13 and its pyroglutamyl isoform which is resistant to degradation, (Pyr1)-Apelin-13, were proven to act as functional ligands of APJ and in some cases exhibited much higher biological activity than Apelin-36 [45,17,18,25,46].

Apelin receptor was originally identified as a receptor related to angiotensin II receptor 1, due to high homology between the two receptor proteins [38]. This receptor is 380 amino acids long, consists of seven transmembrane domains and also includes a signal sequence that allows agonist-independent nuclear localization, a feature that may be cell-specific [27]. GPCRs are the estimated targets of nearly half of all currently available clinically used drugs [11] and are key components of the signal transduction machinery [35]. Binding of apelin to APJ activates second messenger signaling cascades after coupling to G proteins, which results in activation of central signaling molecules such as mitogen-activated protein kinases (MAPKs) and the PI3K/AKT pathway that are responsible to instigate multiple biological responses [1,28–31].

The apelinergic system has a widespread pattern of distribution in human and animal tissues and its established physiological actions are extensive. Overall, apelin and APJ mRNA transcripts and respective peptides, are abundantly present in central nervous system and also in peripheral tissues such as vascular endothelium, heart, lung, kidney and mammary gland [18,25,40,32,10,21], suggesting a functional role of apelin/APJ in these tissues. Remarkably the bulk of the studies report cardiovascular actions of apelin/APJ. Moreover, this peptidergic system has been proposed to have a role in body fluid homeostasis, immunologic modulation, diabetes and

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obesity. [39,12,26,22,4]. Recently the apelinergic system has been described to promote embryonic and tumor angiogenesis [19,9,43]. Growing evidences of apelin/APJ involvement in embryonic events currently extend beyond vascular development, into ocular [20] and heart development [42,49,14].

Interesting findings regarding the apelinergic system clearly reveal that the lung is one of the organs with strongest expression of both ligand and receptor. Furthermore, emerging evidences of this system's implications in embryonic development prompt the speculation that there might be an underlying role in embryonic lung development. However, the expression profile of this pair of proteins and their functional role during normal lung development is hitherto unknown. So far, the effects of apelin on lung development have been described only in one study that reports attenuation of lung injury in neonatal rats exposed to prolonged hyperoxia [47]. Therefore we proposed to investigate the apelin–APJ system during fetal lung development. In this report a thorough characterization of both components of the apelinergic system by immunohistochemistry and Western blot in several stages of fetal lung development is provided. We further investigated the role of this system in branching morphogenesis of the lung and the intracellular effectors implicated.

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, Spain) were maintained in appropriate cages under controlled conditions, fed with commercial solid food and after mating they were checked for vaginal plug. The average pregnancy length in normal adult rat is of 21.5 days and the day of plugging was defined as gestational day 0.5 for time dating purposes. Pregnancies were confirmed by regular weightings. At different time-points [13.5, 15.5, 17.5, 19.5, and 21.5 days postconception (dpc)], pregnant female rats were sacrificed by rapid decapitation and fetuses were harvested by cesarean section. Fetuses were also sacrificed by decapitation, fetal and adult lungs were excised, processed and collected for immunohistochemistry (IHC) or Western blot analysis. Regarding lung explant cultures, fetuses were harvested at 13.5 dpc and their lungs were dissected, cultured *in vitro* for four days and then collected for Western blot analysis.

2.2. Western blot analysis

Protein lysates of 13.5 and 15.5 dpc excised lungs and protein lysates of pooled lung explants were obtained by homogenization of the fetal tissue with a pellet pestle motor (Kontes, USA) on ice; as for lungs of later gestational ages and adult samples a mini bead beater (Biospek Products Inc., USA) was used for homogenization. Different pooled lung samples for each gestational age and also the adult were used and three independent experiments were performed. Proteins were obtained according to Kling et al. [24]. Whole protein concentration was quantified by the Bradford method [3]. Either twenty-five or ten micrograms of protein were loaded onto 12.5% or 10% acrylamide minigels under denaturing and reducing conditions, electrophoresed for approximately 2 h at 100 V at room temperature and then transferred onto nitrocellulose membranes (Hybond™-C Extra GE Healthcare Life Sciences, UK) in a wet transfer system for 1 h. Blots were probed with antibodies to apelin [1:500; Apelin (FL-77), Santa Cruz Biotechnology Inc., USA], apelin receptor [1:500; APLNR (H-300), Santa Cruz Biotechnology Inc.] and non-phosphorylated and phosphorylated forms of p38, p44/42 (ERK1/2) and JNK (1:1000; Cell Signaling Technology Inc., USA) according to the manufacturer's instructions. For loading control, blots were reprobed with β-tubulin antibody (1:150 000 Abcam, UK), which were previously incubated with EzWay™ Antibody Erasing Buffer (Komabiotech Inc., Korea) for primary and secondary antibody removal. Afterwards blots were incubated with a secondary horseradish peroxidase conjugate and developed with Super Signal West Femto Substrate (Pierce Biotechnology Inc., USA). The chemiluminescent signal was captured using a Chemidoc XRS (BioRad, USA) apparatus and subsequent densitometric analysis of nonsaturating bands was performed using Quantity One software (BioRad).

2.3. Immunohistochemistry

Immunostaining was performed on paraformaldehyde-fixed and paraffin-embedded excised lungs and embryos. Five micrometers sections were placed onto glass microscope slides. Primary antibodies for apelin [1:50; Apelin (M-77), Santa Cruz Biotechology Inc.] and apelin receptor (APJ, Abcam Inc.) were used. Tissue sections were deparaffinized in xylene and rehydrated in ethanol, boiled in 10 mM citrate buffer for antigen retrieval and cooled down at room temperature. Incubation with the primary antibody occurred at 4 °C overnight. Negative control reactions included omission of the primary antibody and immunoreactive apelin and APJ staining were not observed in these cases. Sections were incubated with a labeled streptavidin–biotin immunoenzymatic antigen detection system (UltraVision Large Volume Detection System Anti-Polyvalent, Horseradish Peroxidase, Lab Vision Corporation, USA) according to manufacturer's instructions. For visualization of the immune reaction, a diaminobenzidine tetrahydrochloride solution (Dako, Denmark) was used. Sections were finally counterstained with hematoxylin. The slides were observed and photographed with Olympus BX61 microscope (Olympus, Japan). The pictures presented are representative of six animals ($N = 6$), twelve samples were examined for each gestational age as well as the adult and three independent experiments were performed.

2.4. Fetal lung explant cultures

Lungs were removed from 13.5 dpc embryos, harvested and dissected under a stereomicroscope (SZX16, Olympus). These were transferred to porous membranes (Isopore™ membrane filters, Millipore, USA) previously presoaked in DMEM (Invitrogen, UK) and incubated in twenty-four-well culture plates (Nunc, Denmark). Three explants per membrane were positioned in a well-separated arrangement and these floating cultures were incubated at an air-medium interface in a chemically defined medium containing 50% DMEM, 50% F-12 nutrient mixture (Invitrogen) and supplemented with 100 mg/mL penicillin, 100 units/mL streptomycin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma–Aldrich, USA) and 10% FCS (Invitrogen). Fetal lung explants were incubated in a 5% CO_2 incubator at 37 °C for 96 h, and the medium was replaced every 48 h. Cultures were supplemented daily with several doses of (Pyr1)-Apelin-13 (Bachem, Switzerland) ranging from 10^{-11} to 10^{-5} M.