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Effect of prenatal steroidal inhibition of sPLA2 in a rat model of preterm lung

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ABSTRACT

Introduction: Secretory phospholipase A2 regulates surfactant catabolism and inflammatory cascade. This enzyme is correlated with compliance, oxygenation and major outcomes in various forms of acute respiratory failure. Steroids inhibit secretory phospholipase A2 in cell culture and are widely used to boost surfactant production before preterm delivery. No data are available about the effect of antenatal steroids on secretory phospholipase A2 in the offspring: we aimed to study this effect in a rat model of preterm lung.

Material and methods: Fifteen pregnant Wistar rats were randomized to receive betamethasone, dexamethasone or placebo at 20 and 21 days gestation. Newborn rats were supported for 8 h and then sacrificed: lung tissue was analysed for secretory phospholipase A2 expression and activity, inflammatory mediators and protein content. Lipidomics was analysed using liquid chromatography-mass spectrometry.

Results: Secretory phospholipase A2 expression was significantly reduced by antenatal steroids ($p < 0.001$). Secretory phospholipase A2 activity, TNFα and lysophosphatidylethanolamine, a product of phospholipase reaction, were lowest in betamethasone-treated rats ($p < 0.001$). There was a strong correlation between secretory phospholipase A2 activity and lysophosphatidylethanolamine ($r = 0.75$; $p = 0.001$) and this remained significant after adjustment for total proteins or phospholipids.

Conclusions: Antenatal steroids decrease secretory phospholipase A2 in rat model of preterm lung.

1. Background

Secretory phospholipases A2 (sPLA2) represent a widely distributed group of enzymes that are secreted into the extracellular space and catalyse the hydrolysis of ester bonds, at the sn-2 position of phospholipids [1]. sPLA2 subtype −IIA is mainly secreted by alveolar macrophages, shows a preference for certain anionic phospholipids, such as phosphatidylethanolamine (PEA) and represents the foremost pulmonary form of the enzyme [1,2]. sPLA2-IIA may, through its enzymatic activity, hydrolyse surfactant phospholipids and regulate the first step of the inflammatory

Abbreviation list: ARDS, acute respiratory distress syndrome; lyso-PEA, lyso-phosphatidylethanolamine; PEA, phosphatidylethanolamine; lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; PBS, phosphate buffered saline; sPLA2, secretory phospholipase A2; TNFα, tumor necrosis factor-α.

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cascade in the alveoli [1,2]. Thus, sPLA2-IIA plays a dual role for surfactant catabolism and lung tissue inflammation. Because of this dual role, sPLA2-IIA is known to be crucial during clinical conditions characterized by surfactant dysfunction and lung inflammation, such as acute respiratory distress syndrome (ARDS) and meconium aspiration syndrome [3,4]. In these cases, sPLA2 is correlated to mortality, length of mechanical ventilation, oxygenation impairment and other major outcomes [5–8]. Preterm babies are commonly affected by surfactant deficiency, but they can also have a high degree of lung inflammation: this may further impact on surfactant composition and function, through sPLA2 activity. This latter has shown significant correlations with respiratory system compliance and oxygenation deficit also in preterm neonates [9].

While specific sPLA2 inhibitors are still at early investigational stages, steroids are well known to block sPLA2-IIA expression in cell culture experiments [10]. Antenatal steroids are used as a cornerstone prenatal therapy to boost surfactant production and they are administered to women at risk to give birth before 34 weeks' gestation [11,12]. Despite antenatal steroid administration being a part of the routine care, no data are available about their effect on the sPLA2-IIA pathway in the preterm lung.

Our aim is to study the effect of antenatal administration of the two commonly used synthetic steroids in clinical practice (betamethasone and dexamethasone) on the sPLA2 pathway in a well-known animal model mimicking the human preterm lung.

2. Materials and methods

2.1. Animal model

We employed a Wistar rat model often used for neonatal lung research. These rats show structural features similar to saccular and alveolar phases of human lung development: rat lung at birth is histologically equivalent to the human preterm lung at 28 weeks gestation (saccular phase) [13,14]. In detail, pregnant Wistar rats were supported at room temperature with a normal circadian rhythm and free access to food and water; at the 20th day of gestation, they were randomised to receive intravenous dexamethasone (Kern PharmaSL, Barcelona, Spain; 0.4 mg/kg/day), betamethasone (Merck Corp. Whitehouse Station, NJ, USA; 0.4 mg/kg/day) or an equivalent volume of NaCl 0.9% saline solution as placebo on the 20th and 21st days of gestation (five animals per group) [15,16]. Natural delivery occurred at the 22nd day of gestation and five newborn rats per study group ("dexa", "beta" or "control") were studied. Newborn rats were kept with their mothers for 8 h and then killed by intra-peritoneal administration of pentobarbital [15,16]. The Animal Health Care Committee of the University of Salamanca approved the study protocol and all experiments were performed following EU guidelines on animal research.

2.2. Tissue preparation

To study lipidomics, sPLA2 mRNA and activity, right lungs were perfused with phosphate buffered saline (PBS). Lung tissue was then divided in portions of 20 mg, immediately frozen in liquid nitrogen, stored at −80 °C and thawed only once for the study. Before freezing, one portion was homogenized (Omni kit, Omni International inc, Waterbury, CT, USA) in 600 μl PBS and centrifuged (3000 rpm; 10 min) in order to assay TNFalfa, sPLA2 activity and to perform lipidomic analyses, as follows.

To study immunohistochemistry, left lungs were fixed using an intra-tracheal infusion of 4% buffered paraformaldehyde solution; a constant airway pressure of 20 cmH_2O was applied during the infusions. The trachea was then ligated, the lungs immersed in the fixative and embedded in paraffin within 48 h for immunohistochemistry [13].

2.3. Molecular biology

Total RNA was extracted from lung tissue samples using a specific kit (RNeasy minikit, Qiagen, Valencia, CA, USA) and stored at −80 °C. RNA concentration was measured by spectrophotometry and quality analysis was performed in a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Then, 1 μg of total RNA was reverse-transcribed to cDNA using a high capacity kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR, 4 ng of cDNA (1:10 dilution), 4 μl of Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and 12 μl of distilled water were loaded into 96 well plates. TaqMan protocol and probes (Applied Biosystems) were used as follows: TNFα Rn01525859_g1, context sequence CCAACAAGGAGGAGAAGTTCCCAAA, amplicon size 92; sPLA2-IIA Rn00580999_m1, context sequence CAAATCTCCTGCTCTACAAACCAGG, amplicon size 94; GAPDH (as reference) Rn01462661_g1, context sequence TCTTGTGCAGTGCCAGCCTCGTCTC, amplicon size 74. mRNA transcript levels were obtained by SDS Software and analysed with RQ Manager (Applied Biosystems, Foster City, CA, USA). Analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method.

TNFα was measured using a high sensitivity rat-specific ELISA kit (detection limit 11 pg/mL; CV 5%; BioVendor Laboratornı, Modrice, Czech Republic). Secretory and cytosolic phospholipases (weighting approximately 14 and 80 kDa, respectively) were separated, as previously published [7]. Then, sPLA2 total activity was measured with a non-radioactive method, using hexadecanoylthio-1-ethyl phosphorylcholine as substrate (detection limit 3.85 IU/mL; CV 10%), as previously described [7,17]. Results were corrected for total protein content, which was measured using the Bradford method [18]. Total phosholipid content was also measured, as previously published [19]. All measurements were performed in triplicate by investigators blinded to the study group.

2.4. Immunohistochemistry

One tissue microarray was built and each lung was assayed in triplicate, as previously published [14]. Immunostaining was performed automatically using a Bond polymer kit (Vision Biosystems, Newcastle, UK), consisting of a polymeric horseradish peroxidase-linker antibody, according to manufacturer's instructions. A polyclonal rabbit anti-sPLA2-IIA antibody (Abcam, Cambridge, MA, USA) was used in 1/100 dilution. Immunostaining was performed simultaneously to minimize inter-assay variability. The intensity of immunostaining was evaluated by two independent skilled pathologists blinded for the study group using a 0–3 score, multiplied for the number of cells (%) being stained [20].

2.5. Lipidomic analysis

PEA and lyso-PEA were assayed since they respectively are a main substrate and product of reaction of sPLA2-IIA [1]. Phosphatidylcholine (PC) and lyso-PC were also assayed, as control. Lipidomic assays were performed as previously described [21]. In detail, lung tissue lipid extracts were solubilized in 150 μL of methanol. The liquid chromatography-mass spectrometer consisted of a Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters, Millford, MA), operated in positive/negative electrospray ionization mode. Full scan spectra (50–1500 Da) were obtained. Mass accuracy and reproducibility were maintained by using an