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Lipopolysaccharide impairs mucin secretion and stimulated mucosal immune stress response in respiratory tract of neonatal chicks

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ABSTRACT

The chicken immune system is immature at the time of hatching. The development of the respiratory immune system after hatching is vital to young chicks. The aim of this study was to investigate the effect of LPS on respiratory mucin and IgA production in chicks. In this study, we selected 7 days old AA broilers of similar weigh randomly; LPS atomized at 1 mg/kg body weigh dose in the confined space of 1 cubic meter. The chickens exposed for 2 h. Then collect samples after 4 h and 8 h respectively. Compared to control, LPS inhibited mucus production in BALF, caused a rising trend of the concentration of IgA in serum and BALF, and increased the protein expression of IgA in lung tissue. And LPS treat induced a decreasing trend of the mRNA expression of IL-6 and TGF-β and significantly decreased the gene expression of TGF-α and EGFR after 4 h. After 8 h the LPS suppressed the TGF-β mRNA expression significantly. In addition, LPS treatment stimulated airway epithelial cilia sparse after 4 h. Therefore, results proved: LPS can impair mucin expression and stimulated mucosal immune stress reaction of respiratory tract. This study suggested that LPS involved in respiratory tract mucosal immune response in chicks by regulating gene expression of cytokines and epithelial growth factors.

1. Introduction

There is increased prevalence of airway inflammation and diseases in intensive poultry production system. The respiratory mucosa is directly connected with the outside, which is susceptible to all kinds of inhaled harmful factors. Immunoglobulin A (IgA)-dependent mucosal immune and non-specific natural immune factors (such as mucous cilia clearance system) act as the first line of defense, and IgA plays an important role in the immune exclusion to infection pathogen in mucosal epithelium (Antunes and Cohen, 2007; Phalipon et al., 2002). Recent studies have suggested that IgA might be sensitive and specific biomarkers of the stress response in pigs (Muneta et al., 2010) and humans (Allgrove et al., 2008; Gallina et al., 2011).

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, can be inoculated from the environment via inhalation of dust or contaminated water (Castellan et al., 1987). LPS exposure elicits the release of pro-inflammatory cytokines, which is part of the host innate immune response. Administration of LPS is used as an experimental model of endotoxaemia and induces a systemic inflammatory response, allowing makes an investigation of the interaction between immune and neuroendocrine systems (Kanitz et al., 2002; Tuchscherer et al., 2004; Wright et al., 2000). Repeated LPS exposure emulates chronic inflammatory response, similar to the status during infection of gram-negative bacteria (Rakhshandeh and de Lange, 2012). In addition, injection of LPS not only decreases body weight gain but also elevates cloacal temperature by stimulating the immune inflammatory response in broiler chickens (Lai et al., 2009; Xie et al., 2000; Yang et al., 2011). In mammals, repeated challenge of LPS stimulates an immune response, resulting in an increase in IgA concentrations of salivary (Escribano et al., 2014; Iqbal et al., 2014a, 2014b).

Mucins are the major component of airway mucus, and they provide a protective barrier against pathogenic agents. Mucin provides important protection role in epithelial surfaces of various tissues such as intestine, lungs, pancreas, kidney, etc. (Hollingsworth and Swanson, 2004; Thornton et al., 2008). The misregulation of mucin is a characteristic feature of many diseases, such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, gastrointestinal inflammatory diseases, and lung, colon, and pancreatic carcinomas (Balague et al., 1995; Copin et al., 2000; Rose and Voynow, 2006; Tam et al., 2011; Van Seuningen et al., 2001). In airway, mucins are mainly produced by goblet cells and submucosal gland cells (Kim et al., 1997). Sixteen mucin genes encoding the protein backbone of mucins have been identified in the airway of humans, and mucin 5AC (MUC5AC), mucin

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5B (MUC5B), and mucin 2 (MUC2) are the principal gel-forming mucins secreted in the airway (Ali and Pearson, 2007). Overproduction of mucus and mucins (mucin hypersecretion) is associated with inflammation caused by bacterial infection. There is increasing evidence that LPS stimulated mucin secretion in Calu-3 cells (lung carcinoma) (Gosalia et al., 2013), colonic epithelium (Enss et al., 1996), middle ear epithelium (Hunter et al., 1999), and airway (Li et al., 1997; Luan et al., 2014). However, LPS can also induce impairment in mucin synthesis in stomach and salivary, which reflected in the impairment of mucus coat function, mucosal injury, and the onset of disease (Slomiany and Slomiany, 2006; Slomiany and Slomiany, 2011). Hence, we hypothesized that LPS could change the secretion of mucin and IgA in airway of chicks.

The purpose of this study was to evaluate the effects of LPS on the secretion of IgA and mucins in the airways and the expression of relevant genes in lung of neonatal chicks.

2. Materials and methods

2.1. Experimental animals

One-day-old broilers were obtained from a local hatchery and reared in an environmentally controlled room (rearing room). The brooding temperature was maintained at 35 °C (65% relative humidity, RH) for the first 2 days and was then gradually reduced to 30 °C on day 7. All of the chickens had free access to food and water during the rearing period. The experimental procedures were approved by the University and were performed in accordance with the "Guidelines for the Use of Experimental Animal" of the Ministry of Science and Technology (Beijing, P. R. China).

2.2. Treatment

At 7 days of age, 32 broilers around the mean body weight (180 g ± 2 g) were selected and divided into two groups. The two groups of chicks were respectively moved to an air-tight chamber with 1 cubic meter in a separate testing room. The environment of the testing room was kept the same as that of rearing room. All the facilities and environment of two air-tight chambers was same. One group of chicks was exposed to atomized LPS solution (*Escherichia coli*, Sigma, resolved in saline) at a dose of 1 mg/kg body weigh in the confined chamber (LPS treatment). The other group of chicks was exposed to same volume of atomized saline (control). During the two-hour exposure treatment, the behavior of chicks was observed and feed and water were withdrawal. After two hours exposure, all the chicks were moved back to normal environment. After treatment, the chicks had free access to feed and water.

2.3. Collection of blood and BALF

Eight chicks were randomly selected from each group at 4 and 8 h respectively. Blood samples were obtained from a wing vein using a heparinized syringe. Plasma was obtained by centrifugation at 400 × *g* for 10 min at 4 °C, and stored at − 20 °C for further analysis. After blood sampling, the chickens were sacrificed by cervical dislocation and the trachea and lungs were exposed. The trachea was cannulated with a catheter and lavaged three times with 1 mL of 0.9% saline. The lavage fluid was centrifuged at 3000 rpm for 5 min and stored at − 20 °C for further analysis.

2.4. Measurement

2.4.1. IgA concentration

IgA concentration was determined with a double antibody sandwich ELISA method. The specific goat-anti-chicken IgA antibody was obtained from the American Bethyl Company (A30-103A-17). The goat anti-chicken IgA detection antibody (100 μL) was added to each well and incubated at 37 °C for 1 h. After five washes, the plates were blocked with 100 μL of PBS containing 1% BSA overnight, and the plates were then washed five times. Thereafter, 100 μL of standard or sample was added and incubated at 37 °C for 1 h. After washing, 100 μL of HRP Solution A was added and incubated at 37 °C for 30 min. The plate was washed, and 100 μL of TMB substrate solution was added. After development at 37 °C under dark conditions for 30 min, the reaction was stopped by adding 100 μL of stop solution. The absorbance was measured at 450 nm using a plate reader.

2.4.2. Mucin concentrations

The mucin concentrations were measured according to the phenol sulfuric acid method described by Dubois et al. (DUBOIS et al., 1956).

2.4.3. RNA isolation and analysis

The gene expression levels in the lung and trachea were quantified using quantitative real-time PCR. The total RNA was isolated with Trizol (Invitrogen, San Diego, CA, USA). The quality and quantity of the RNA were determined by agarose gel electrophoresis and a biophotometer (Eppendorf, Germany), respectively. RT reactions (10 μL) contained 500 ng total RNA, 5 mmol/L MgCl2, 1 μL of RT buffer, 1 mmol/L dNTP, 2.5 U AMV, 0.7 nmol/L oligo d (T) and 10 U ribonuclease inhibitor (TaKaRa, Dalian, China). Real-time PCR analysis was conducted using the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster, CA, USA). Each RT reaction served as a template in a 20 μL PCR reaction that contained 0.2 μmol/L of each primer and SYBR green master mix (Takara, Dalian, China). The real-time PCR reactions consisted of pre-denaturation at 95 °C for 10 s followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 40 s. A standard curve was plotted to calculate the efficiency of the real-time PCR primers. β-Actin was used as the housekeeping gene, and the results of the relative mRNA quantification were verified with the β-actin levels (ΔCT). The comparative CT method ($2^{-\Delta\Delta CT}$) was used to quantitate the mRNA expression in accordance with Wang et al. (2013). The primer sequences refer to our previous study (listed in Table 1) (Fan et al., 2015).

2.4.4. Western blotting analysis

The samples obtained from the lung tissue were homogenized on ice using radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl at pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride) and were centrifuged at 12,000 *g* for 5 min at 4 °C. The protein concentration was determined using the BCA assay kit (Beyotime, Jiangsu, China). The samples were boiled at 100 °C for 5 min in 5 × sample buffer. Protein extracts (80 μg) were electrophoresed in 7.5% SDS polyacrylamide gels (Bio-Rad, Richmond, CA). The protein was transferred onto polyvinylidene fluoride (PVDF) microporous membrane (Millipore) at 200 mA, 4 °C for 2 h. The membranes were blocked for 1 h and immunoblotted overnight at 4 °C with goat anti chicken IgA polyclonal antibody (A30-101A, Bethyl Laboratories, Inc. USA), β-actin mouse mAb (Beyotime, China), respectively. The blots were then soaked with anti-goat or anti-mouse IgG-conjugated horseradish peroxidase (Beyotime, China) for 4 h at 4 °C. Membranes were then visualized by exposure to Hyperfilm ECL (Beyotime, China). Films were scanned, and specific bands were quantified using ImageJ 1.43 software (National Institutes of Health, USA). The band intensity was normalised to the β-actin band in the same sample.

2.4.5. SEM (scanning electron microscopy) analysis

The paraformaldehyde-fixed tracheas were further fixed with 2% glutaraldehyde. Following postfixation with 1% osmium tetroxide, the samples were dehydrated by a series of gradient alcohol. After freeze-