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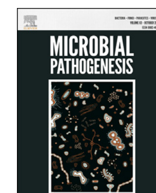

Evaluation of the expression level of 12/15 lipoxygenase and the related inflammatory factors (CCL5, CCL3) in respiratory syncytial virus infection in mice model

Vahid Salimi [a], Ali Ramezani [a], Habibollah Mirzaei [a], Alireza Tahamtan [a], Ebrahim Faghihloo [b], Farhad Rezaei [a], Maryam Naseri [a], Louis Bont [c], Talat Mokhtari-Azad [a], Masoumeh Tavakoli-Yaraki [d, *]

[a] Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Paediatrics, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands
[d] Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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A B S T R A C T

Human respiratory syncytial virus (RSV) is a leading cause of acute respiratory infection during early childhood and imposes a great burden on patients, parents, and society. Disease is thought to be caused, at least partially, by an excessive immune response. Pulmonary leukocyte infiltration is the result of a coordinated expression of diverse chemokines with distinct cellular specificities. Lipoxygenases (LOXs), as a key enzyme catalyzing deoxygenation of poly unsaturated fatty acids, regulate inflammation and have been suggested to play an important role in the immune response in viral infection. To expand our understanding on the possible role of LOX in respiratory viral infection, we studied the 12/15- lipoxygenase expression in RSV-related airway inflammation, and the related inflammatory chemokines, Chemokine (C-C motif) ligand 5 (CCL5) and Chemokine (C-C motif) ligand 3(CC L3) in both lung tissue and Bronchoalveolar lavage (BAL) fluid during experimental RSV infection. RSV infection induced mRNA expression of CCL5 and CCL3 in both BAL and lung tissue cells. In addition RSV infection enhanced expression of 12/15-LOX in both BAL and lung cells. In conclusion, we confirm that RSV infection leads to the increased expression of 12/15 LOX and the related chemokines CCL5 and CCL3 in BAL fluid and lung tissue cells suggesting that the 12/15 LOX pathway could serve as a candidate target for prevention and treatment of RSV infection.

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1. Introduction

Respiratory syncytial virus (RSV) is the primary cause of bronchiolitis and pneumonia in infants and young children [1]. It's so common that nearly all children have been infected with the virus during the first year of life [2]. In addition, adults become reinfected despite the presence of antibodies [3]. During RSV infection, lung epithelium and alveolar macrophages are the first cells that are infected [4]. It was shown that early inflammatory and immune responses of the host may be crucial in response to RSV infection [5].

RSV bronchiolitis is associated with development of a wide range of pro-inflammatory cytokines and chemokines and an extensive inflammatory infiltration in the lower airways, comprising neutrophils monocytes, T cells and eosinophils [6]. RSV infection has been shown to induce Chemokine (C-C motif) ligand 5 (CCL5) and Chemokine (C-C motif) ligand 3(CC L3) activity *in-vivo* and *in-vitro* which are present in inflammatory infiltrates or in respiratory secretions of RSV-infected children [7]. However, the exact mechanisms of RSV-induced airway disease, controlling the

List of abbreviations: BAL, Bronchoalveolar lavage; CCL5, Chemokine (C-C motif) ligand 5; CCL3, Chemokine (C-C motif) ligand 3; cDNA, complementary DNA; DEPC, Diethylpyrocarbonate; FBS, fetal bovine serum; HEp-2, human laryngeal carcinoma; H&E, hematoxylin and eosin; IMDM, Iscove's Modified Dulbecco's Media; LOXs, Lipoxygenases; PFU, plaque-forming units; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RSV, Human respiratory syncytial virus.

* Corresponding author.
E-mail addresses: tavakoli.m@iums.ac.ir, masoumeh.tavakoli@gmail.com (M. Tavakoli-Yaraki).

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influx of specific inflammatory cells and its long-term consequences such as recurrent wheezing in later life are not well understood [5,6].

Eukaryotic cells respond to a large number of distinct extracellular signals and environmental stresses, and the responses usually involve signal transduction pathways that lead to the activation of specific sets of genes. Thus, to define the innate host response to the infection, considerable effort has been focused on the transcriptional activation of cellular genes by viruses [8]. Besides cytokines and chemokines, lipid mediators are critically involved in the development of pulmonary inflammation and play important roles in the pathophysiology of allergen-induced inflammation and lung remodeling in asthma [9].

Lipoxygenases (LOX) are a family of enzymes capable of incorporating oxygen into unsaturated fatty acids [9]. The 15(S)-lipoxygenase (15-LOX) pathway has several roles in the pathogenesis of inflammatory lung disorders and may thus constitutes a potential drug target [10]. 15-LOX plays a janus role in inflammation with pro-inflammatory and anti-inflammatory effects on cell cultures and primary cells and even opposite effects on atherosclerosis in two different animal species [11].

A high homology exists between 12/15-LOX in mice and 15-LOX in humans. Thus, the murine 12/15-LOX is considered as the mouse ortholog of human 15-LOX [9]. Murine 12/15-LOX and human 15-LOX have 74% identity in primary structure, and both are dual-specificity lipoxygenases [12]. 12/15-LOX is expressed in a variety of tissues, with the highest expression levels in monocytes and macrophages [9]. A variety of vascular cells are able to express 12/15-LOX, including endothelial cells, smooth muscle cells, and immune cells [12].

Although several studies have investigated the nature of interaction between RSV, human bronchial cell and immune cell infiltration, however information on the effect of RSV replication on pro-inflammatory mediators such as LOX is limited. To ascertain the importance of 12/15-lipoxygenase in the RSV infection, we infected mice by RSV and analyzed the expression level of 12/15-LOX and related inflammatory chemokine (CCL5 and CCL3) in both lung and BAL cells.

2. Material and methods

2.1. Cell culture

HEp-2 (human laryngeal carcinoma), cell line was cultured in Iscove's Modified Dulbecco's Media (IMDM; Sigma-Aldrich, Biochrom AG, Germany) supplemented with 10% fetal bovine serum (FBS), and penicillin/streptomycin (Sigma-Aldrich, Biochrom AG, Germany) at 37 °C in 5% CO2.

2.2. RSV preparation

The A2 strain of RSV was grown in HEp-2 cells supplemented with 1% heat-inactivated FBS and concentrated by polyethylene glycol as described previously [13]. Aliquots of the RSV viral suspension were flash frozen and stored at −80 C. The virus titer of the purified RSV pools was 5.8*10e9 plaque-forming units (PFU) per milliliter as determined by an Agarose plaque assay. No mycoplasma contamination was detected in these preparations.

2.3. RSV infection in vivo

Female BALB/c mice (6–7 weeks old), were purchased from Institute Pastor of Iran and housed in an animal care facility. Mice were fed mouse maintenance diet and water ad libitum. The animals were allowed one week to acclimatize before initiating experiments and housed in groups according to experimental setup. All experiments with animals were approved by the Animal Ethics Committee at the Tehran University of Medical Sciences. Mice were anesthetized mildly by intraperitoneal administration of chloral hydrate (0.229 mg/g body weight) and challenged intranasally with 1 × 10e7 PFU of RSV A2 (volume 50 μl) in sterile phosphate-buffered saline (PBS). In a mock-infected group, mice were given PBS intranasally. Mice were weighed daily and monitored for signs of illness.

2.4. Bronchoalveolar lavage (BAL) fluid and lung collection

On 5th, day after RSV infection, mice were sacrificed using a fatal dose (100–150 μl) of pentobarbital injected i. p. BAL cells were collected by inserting a syringe with a cannulated needle into the trachea and gentle aspiration, 2 times with 1 ml of PBS. BAL was centrifuged and the cell pellet was suspended in 100 μl of PBS and counted using a hemocytometer. The cell suspensions were then transferred onto a microscope slide (Thermo Scientific, United Kingdom) using a Cytospin centrifuge and slides were stained with hematoxylin and eosin (H&E) (Reagena, Gamidor, United Kingdom). At least 200 cells per sample were counted by direct microscopic observation [14]. After collecting the BALF, the lungs were removed. The BAL cells and lung tissues were quick-frozen and stored at −80 °C.

2.5. Real-time RT-PCR assay

The total RNA from the BAL cells the lung tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) dissolved in 30 mL of Diethylpyrocarbonate (DEPC) water. RNA samples were quantified using a Nanodrop spectrophotometer (Nanodrop Technologies). RNA extracts were treated with RNase-free DNase I (Roche), then complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a high capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) and incubated at 37 °C 95 °C for 5 min to stop the reaction. Real-time RT-PCR was performed on cDNA samples using the SYBRs Premix ExTapTM II (Takara, Japan). The reaction conditions were as follows: 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5s and extension at 60 °C for 34s. The PCR data were analyzed using the detection system (Step One Plus Real-time PCR system, Applied Biosystems Co.,USA). Duplicate CT values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems). The amount of target (2−ΔΔCT) was obtained by normalizing to the level of the housekeeping gene endogenous β-actin. The following primers were used for the analysis: β-actin, forward, 5′-GCTCTGGCTCCTAGCACCAT-3′, and reverse, 5′-GCCACCGATCCACACAGAGT-3′; 12/15 LOX, forward, 5′-ATC GGT ACG TGG TGG GAA TG -3′, and reverse, 5′-TGA CAC CAG CTC TGC AGT TC-3; CCL3, forward, 5′-CAAGTCTTCTCAGCGCCA-TATG-3 and reverse, 5′-CGTGGAATCTTCCGGCTGTA-3; CCL5, forward, 5′- AAGTTCAGCTGCCCCATCTG-3′, and reverse, 5′-AGCATGCCTGGGTGGAAGT-3.

2.6. Statistics

The Statistical software package GraphPad Prism (GraphPad, San Diego, CA) software was used for analysis. Data were analyzed for statistical significance using the two-tailed Student's *t*-test. Data are expressed as the mean ± SEM. Correlations and differences were considered significant for $p < 0.05$.

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