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Antiviral and anti-inflammatory activity of budesonide against human rhinovirus infection mediated via autophagy activation

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ABSTRACT

Human rhinovirus (HRV) infection causes more than 80% of all common colds and is associated with severe complications in patients with asthma and chronic obstructive pulmonary disease. To identify antiviral drug against HRV infection, we screened 800 FDA-approved drugs and found budesonide as one of the possible drug candidates. Budesonide is a corticosteroid, which is commonly used to prevent exacerbation of asthma and symptoms of common cold. Budesonide specifically protects host cells from cytotoxicity following HRV infection, which depend on the expression of glucocorticoid receptor. Intranasal administration of budesonide lowered the pulmonary HRV load and the levels of IL-1β cytokine leading to decreased lung inflammation. Budesonide regulates IL-1β production following HRV infection independent of inflammasome activation. Instead, budesonide induces mitochondrial reactive oxygen species followed by activation of autophagy. Further, the inhibition of autophagy following chloroquine or bafilomycin A1 treatment reduced the anti-viral effect of budesonide against HRV, suggesting that the antiviral activity of budesonide was mediated via autophagy. The results suggest that budesonide represents a promising antiviral and anti-inflammatory drug candidate for the treatment of human rhinovirus infection.

1. Introduction

Human rhinovirus (HRV) is a non-enveloped virus belonging to the *Picornaviridae* family. HRV consist of a viral capsid and a single strand of positive sense RNA (Puhakka et al., 1998). HRV is one of major causative agents of viral pulmonary infection, which triggers acute exacerbation of lower respiratory tract diseases including asthma and chronic obstructive pulmonary disease (COPD) (Johnston et al., 1995; Nicholson et al., 1993; Seemungal et al., 2000, 2001). Despite tremendous efforts to develop effective antiviral drugs for HRV infection, no treatment is currently available. Several medications have been used for the prevention of asthma exacerbation (O'Byrne et al., 2001; O'Byrne, 2011; Busse et al., 2001; Hanania et al., 2011). Budesonide is a corticosteroid, which can be used in combination with a long-acting beta agonist such as formoterol to prevent exacerbation of asthma and symptoms of common cold (Davies et al., 2011). Budesonide is a commonly used to treat children with asthma (Silverman and Otley, 2011) and is generally used as a nasal spray to treat allergic rhinitis (Wang and Zhang, 2015).

IL-1β is an important pro-inflammatory cytokine in host immunity.

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However, excessive production of IL-1β also triggers acute lung injury and also exacerbates acute lung inflammation (Kolb et al., 2001). Transcription of pro-IL-1β is mediated via activation of NF-κB signaling (Bonizzi and Karin, 2004). The maturation and secretion of IL-1β is regulated by the inflammasome, in which activation of caspase 1 by several nucleotide-binding oligomerization domain-like receptors (NLRs) is critical for the IL-1β activation (Martinon et al., 2002). The levels of IL-1β were enhanced in the primary bronchial epithelial cells by the calcium flux-dependent activation of NLRP3 and NLRC5 inflammasome (Triantafilou et al., 2013).

In the current study, we showed that budesonide has antiviral activity against rhinovirus *in vitro* and *in vivo*, which correlates with the controlled IL-1β production in the lungs of HRV1B-infected mice after budesonide treatment. We found that budesonide enhanced the levels of mitochondrial reactive oxygen species (ROS), which in turn activated autophagy. Chemical inhibitors of autophagy attenuated the antiviral activity of budesonide *in vitro* and *in vivo*, suggesting that activation of autophagy pathway might be responsible for the antiviral activity of budesonide.

2. Materials and methods

2.1. Cell culture, viruses, and reagents

Human rhinovirus 1B (HRV1B), coxsackievirus B3 (CVB3), and enterovirus 71 (EV71) were obtained from ATCC (Manassas, VA, USA) and HRV1B was propagated by infection at 33 °C in HeLa cells, and CVB3 and EV71 were propagated by infection at 37 °C in Vero cells. HeLa cells and Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). Budesonide, rupintrivir, chloroquine diphosphate salt and bafilomycin A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxycycline was purchased from Clontech (Mountain View, CA, USA). For Tet-on system, the GCR shRNA-transfected HeLa cells were treated with doxycycline (2 μg/mL) in culture media every 2 days for 7 days.

2.2. Mice and virus infection

Four-week-old, female BALB/c mice and C57BL/6 mice were purchased from SPL animal company (Orient Bio Inc, Sungnam, Korea), and IL-1R$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in accordance with the guidelines, and stabilized for 7 days at Kangwon National University. Mice were infected intranasally with HRV1B (1.8×10^7 pfu/mouse) three times with 10 μL/PBS at intervals of 10 min. Budesonide was intranasally administered with 0.1 mg/kg after HRV1B infection.

2.3. SRB assay

The SRB assay was used to measure the antiviral activity as previously reported (Song et al., 2014). The day before the experiment, HeLa and Vero cells (3×10^4 cells/well) were seeded in a 96-well culture plate. HeLa cells were used for HRV1B infection, and Vero cells were used for CVB3 and EV71. After 24 h, the HRV1B infection medium was replaced with 30 mM $MgCl_2$, 1% FBS in MEM media. The CVB3 and EV71 were replaced with a medium containing 1% FBS in MEM. The HRV1B-infected HeLa cells were incubated at 33 °C and 5% CO_2, and CVB3-, EV71-infected Vero cells were incubated at 37 °C and 5% CO_2, resulting in CPE. After 48 h, the 96-well culture plate was washed with PBS, and fixed in 70% acetone (100 μL/well) at 30 min, followed by staining with 0.4% SRB (Sigma-Aldrich, St, Louis, MO, USA) in 1% acetic acid. The precipitated SRB crystals were solubilized with 10 mM unbuffered tris-based solution 100 μL/well. The absorbance was read on a SpectraMax i3 microplate reader (Molecular Devices, Palo Alto, CA, USA) at 562 nm.

2.4. Real-time PCR

Total RNA was extracted from HeLa cells and mice lung lysate with a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with RNase inhibitor, oligo (dT) 15 primers, dNTP mixture, and Moloney murine leukemia virus reverse transcriptase with 5× buffer, according to the established protocol (Promega, Madison, WI, USA). Quantitative real-time PCR (qPCR) analysis was performed to amplify complementary deoxyribonucleic acid (cDNA), using the THUNDERBIRD® SYBR® qPCR mix (Toyobo, Osaka, Japan), and CFX96 optics module real-time PCR system (Bio-Rad, Hercules, CA, USA). We used the following primers: HRV 5′-NCR sense, 5′-TCC TCC GGC CCC TGA ATG-3′ and HRV 5′-NCR-antisense, 5′-GAA ACA CGG ACA CCC AAA G-3′; and human β-actin-sense, 5′-CCA TCA TGA AGT GTG ACG TGG-3′ and human β-actin-antisense, 5′-GTC CGC CTA GAA GCA TTT GCG-3′. The PCR conditions were as follows: 95 °C for 3 min for 1 cycle and 95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; for 35 cycles.

2.5. Cytokine and chemokine assays

The cytokine levels were evaluated in the supernatants of HRV1B-infected mouse lung lysate using the IL-1β and IL-6 ELISA kits (eBioscience, San Diego, CA, USA). The lung tissue was homogenized in CK28 (Bertin Technology, Orsay, France) using 2.8-mm ceramic beads with 600 μL PBS, and using a Minilys homogenizer (Bertin Technology) at 6000 rpm, for 15 s, twice at −20 °C. The clear supernatants were collected following centrifugation at 10,000*g* for 10 min, at 4 °C (Song et al., 2017). The absorbance was read at 450 nm using a SpectraMax 340 (Molecular Devices).

2.6. Histological analysis

The HRV1B-infected lungs of mice were washed in PBS, and fixed with 4% (w/v) formaldehyde overnight. The HRV1B-infected lungs were dehydrated in serial gradients of ethanol and xylene, and embedded in paraffin. The tissues were sliced into 5-μm-thick sections and stained with hematoxylin and eosin. After HRV1B infection, the lungs with severe inflammation (score: 0–4) were graded for inflammation, edema, and cellular infiltration, according to the guidelines. A pathologist evaluated the degree of inflammation microscopically (200×) and was blinded to the scores. The means of lung inflammatory score were graded for severity (absent, minimal, mild, moderate, marked).

2.7. Western blot

SDS-PAGE was carried out as described previously (Hong et al., 2017). Protein levels were evaluated in the lysate of HRV1B-infected HeLa cells with primary antibodies including: LC3B:2775S, AMPK and ACC Antibody Sampler Kit: 9957 and α-tubulin: 2144 (Cell Signaling Technologies, Denver, MA, USA), SQSTM1 monoclonal antibody (p62): H00008878-M01 and anti-Glucocorticoid Receptor antibody: GTX101120 (GeneTex, Inc, Irvine, CA, USA), anti-rabbit Cytoskeletal Actin Antibody: A300-491A (Bethyl Laboratories, Montgomery, TX, USA), anti-caspase-1 p20 antibody: 06-503 (EMD Millipore, Burlington, MA, USA), anti-caspase-1 antibody: sc-622 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-NLRP3 antibodies: #AG-20B-0014-C100 (AdipoGen, San diego, CA, USA), anti-mouse IL-1β antibody: AF-401-NA (R&D Systems, Minneapolis, MN, USA), and secondary antibodies including: anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technologies), and goat anti-mouse IgG F(ab′)2, polyclonal antibody (HRP conjugate) (Enzo Life Sciences., Farmingdale, NY, USA). The elevated chemi-luminescence substrate used was femtoLUCENT™ PLUS HRP Kit (G-biosciences, St. Luise, MO, USA). Images were obtained with ImageQuant™ LAS 4000 mini system (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and analyzed using