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Modulation of transcriptional responses by poly(I:C) and human rhinovirus: Effect of long-acting β_2-adrenoceptor agonists

Christopher F. Rider [a], Anna Miller-Larsson [b], David Proud [a], Mark A. Giembycz [a], Robert Newton [a,*]

[a] Airways Inflammation Research Group, Snyder Institute for Chronic Diseases, Faculty of Medicine, University of Calgary, Calgary, 3330 Hospital Drive NW, AB, Canada T2N 4N1
[b] AstraZeneca R&D Mölndal, Pepparedsleden 1, Mölndal SE-43183, Sweden

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ABSTRACT

Exacerbations of asthma, a chronic inflammatory respiratory disease, are associated with viral upper respiratory tract infections involving human rhinovirus. Although glucocorticoids (corticosteroids) effectively control airways inflammation in many asthmatics, human rhinovirus-associated exacerbations show reduced glucocorticoid responsiveness. Using human bronchial epithelial BEAS-2B cells, we show that human rhinovirus reduced glucocorticoid-inducible activation of glucocorticoid response element (GRE) reporter systems in a time- and concentration-dependent manner. The synthetic double-stranded viral RNA mimetic, polyinosinic:polycytidylic acid (poly(I:C)), also reduced activation of GRE reporter systems in BEAS-2B and pulmonary A549 cells. In addition, poly(I:C) decreased transcription from cAMP response element (CRE)-, TATA-, simian virus 40- and nuclear factor-kappa B (NF-κB)-dependent reporter systems. The effects of poly(I:C) on GRE-reporter activation were countered by the long-acting β_2-adrenoceptor agonists, formoterol and salmeterol. Likewise, increased expression of the gene cyclin-dependent kinase inhibitor 1C (CDKN1C; p57^{KIP2}) by dexamethasone was reduced by poly(I:C), but was substantially enhanced by the addition of formoterol. Poly(I:C) induced the expression of interleukin-8 (IL8; CXCL8) and this was significantly decreased by dexamethasone, formoterol or their combination. This confirms that not all transcriptional responses were attenuated by poly(I:C) and that decreased glucocorticoid-dependent transcription can be counteracted by the addition of long-acting β_2-adrenoceptor agonists. These data show how human rhinovirus may attenuate glucocorticoid-induced transcription to reduce anti-inflammatory activity. However, addition of long-acting β_2-adrenoceptor agonist to the glucocorticoid functionally restored this response and shows how glucocorticoid plus long-acting β_2-adrenoceptor agonist combinations may prove beneficial during virus-induced exacerbations of asthma.

1. Introduction

Asthma is a heterogeneous chronic respiratory disease characterized by airways inflammation, hyperresponsiveness and reversible bronchoconstriction (Bousquet et al., 2000). In many asthmatics, symptoms and lung function vary over time, with occasional exacerbations in which lung function deteriorates acutely and can become life threatening or even fatal (Peters, 2003; Reddel et al., 2009). While many factors contribute, asthma exacerbations are frequently associated with viral upper respiratory tract infections, leading to a temporal relationship between upper respiratory tract infection outbreaks and hospitalization for asthma (Johnston et al., 2006; Proud, 2011). Indeed, 80% of the wheezing episodes in children and more than half of those in adults were upper respiratory tract infection-associated, with human rhinovirus detected in ~65% of cases (Grünberg and Sterk, 1999; Jackson and Johnston, 2010; Proud, 2011).

Human rhinovirus has a single-stranded, positive sense, RNA genome encased in a capsid of four viral proteins. During replication, a double-stranded RNA intermediate is generated with the complementary negative strand serving as template for further copies of the genome (Proud and Leigh, 2011; Proud, 2011). Activation of inflammatory pathways following human rhinovirus infection results in the production of cytokines and chemokines, including interleukin-8 (IL8), chemokine (C–C motif) ligand 5 (CCL5; RANTES) and interleukin-1β (IL1B) and leads to lung inflammation (Grünberg and Sterk, 1999; Proud, 2011).

Inflammation in asthma is typically reduced by glucocorticoids (otherwise known as corticosteroids) and these, in an inhaled form, are recommended for all but the mildest asthmatic patients (Barnes,

* Correspondence to: Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1. Tel.: +1 403 210 3938; fax: +1 403 210 7944.
E-mail addresses: cfrider@ucalgary.ca (C.F. Rider), anna.miller-larsson@astrazeneca.com (A. Miller-Larsson), dproud@ucalgary.ca (D. Proud), giembycz@ucalgary.ca (M.A. Giembycz), rnewton@ucalgary.ca (R. Newton).

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2006). Glucocorticoids bind the glucocorticoid receptor (NR3C1), inducing translocation from the cytoplasm to the nucleus, where it may inhibit the function of inflammatory transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1. Alternatively, the glucocorticoid receptor can interact with glucocorticoid response elements (GREs) to enhance gene transcription (Barnes, 2006; Newton and Holden, 2007). Many such glucocorticoid-induced genes, including cyclin-dependent kinase inhibitor 1C (CDKN1C; $p57^{KIP2}$), TSC22 domain family member 3 (TSC22D3; GILZ), and dual specificity phosphatase 1 (DUSP1; MKP-1), show functional properties consistent with anti-inflammatory activity (Chang et al., 2003; Eddleston et al., 2007; Newton and Holden, 2007).

As glucocorticoids do not directly control bronchoconstriction, asthmatics are prescribed short-acting β_2-adrenoceptor agonists, which act on β_2-adrenoceptors to activate the adenylyl cyclase/cAMP/protein kinase A pathway and elicit bronchodilatation (Giembycz and Newton, 2006). However, large multicentre clinical trials convincingly show that inhalers combining a long-acting β_2-adrenoceptor agonist with a glucocorticoid provide superior symptom control in adult asthmatics than is achieved by increasing, even quadrupling, the dose of glucocorticoid monotherapy (Ducharme et al., 2010; Giembycz et al., 2008; Pauwels et al., 1997). Conversely, in many asthmatics, inflammation induced during human rhinovirus-associated exacerbations shows reduced glucocorticoid responsiveness and may be termed glucocorticoid resistant. Having previously shown that glucocorticoid-dependent transcription can be reduced by tumor necrosis factor α (TNF) and other inflammatory stimuli (Rider et al., 2011), we sought to determine the effect of human rhinovirus or poly(I:C), a double stranded RNA viral mimetic, on glucocorticoid-inducible gene expression in human pulmonary epithelial cells.

2. Materials and methods

2.1. Cell culture and drugs

Human bronchial (BEAS-2B) and type II alveolar (A549) epithelial cells were cultured in Dubecco's modified Eagle's medium/F12 plus 14 mM $NaHCO_3$ or Dubecco's modified Eagle's medium (Invitrogen; Carlsbad, CA), respectively, supplemented with 10% fetal calf serum (Canadian sourced; Invitrogen) and 2 mM L-glutamine. Prior to experiments, plates were incubated in serum-free medium overnight before fresh serum free medium containing drugs or cytokines was added. Formoterol furoate (formoterol) and salmeterol xinafoate (salmeterol) were dissolved in dimethyl sulfoxide (Sigma-Aldrich; St. Louis, MO) with a final dimethyl sulfoxide concentration of $<0.1\%$ on the cells. Dexamethasone and poly(I:C) were dissolved in hank's balanced salt solution and phosphate buffered saline respectively (Sigma-Aldrich, St. Louis, MO). TNF (R&D systems; Minneapolis, MN) was dissolved in phosphate buffered saline containing 0.1% bovine serum albumin.

2.2. Human rhinovirus and poly(I:C) treatments

Human rhinovirus type 16 stocks were propagated in WI-38 fibroblasts (American Type Culture Collection; Manassas, VA) and purified by sucrose density centrifugation. Viral titers were determined on monolayers of WI-38 cells and expressed as log 50% tissue culture infective doses per ml ($TCID_{50}$/ml), as previously described (Hudy et al., 2010; Sanders et al., 2001). BEAS-2B cells were infected with human rhinovirus at various $TCID_{50}$/ml in serum free media and cultured at 34 °C (rather than 37 °C) in 5% CO_2. Poly(I:C) was mixed with 10 μl/ml of the transfection reagent lipofectamine 2000 (Invitrogen) in serum free media and incubated for 30 min, prior to addition to cells (1 in 10 dilution) for between 6 and 78 h.

2.3. Reporter constructs and luciferase assays

BEAS-2B and/or A549 cells were stably transfected with the plasmids: pGL3-Control.neo, a plasmid containing simian virus 40 promoter and enhancer sequences driving a luciferase gene (Catley et al., 2004); pGL3.neo.TATA to create BEAS-2B TATA reporter cells; pGL3.neo.TATA.2GRE, a 2 × GRE containing luciferase reporter plasmid (Chivers et al., 2006; Kaur et al., 2008); 6κB.tkluc.neo, a plasmid containing 6 consensus NF-κB binding sites upstream of a luciferase gene, to create 6κbtk cells (Bergmann et al., 2000); pGL3.neo.TATA.3κBu, which contains the upstream NF-κB site of the prostaglandin-endoperoxide synthase 2 (PTGS2; COX-2) gene promoter (Holden et al., 2007), or a cAMP-responsive reporter that contains 6 × cAMP response elements (CREs) driving a luciferase gene (Catley et al., 2004). A549 and BEAS-2B reporter cell lines were cultured in medium containing 0.75 and 0.25 mg/ml G-418 (Geneticin; Promega, Madison, WI) respectively and plated in G-418 free media for experiments. Luciferase assays were performed according to the manufacturer's guidelines (Firefly Luciferase Assay Kit; Biotium, Hayward, CA).

2.4. Cell viability assay

Cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. After aspiration of 24-well plates, 200 μl/well of MTT solution (1 mg/ml in hanks balanced salt solution) was added and plates incubated for 30 min at 37 °C. Following aspiration, 200 μl/well of dimethyl sulfoxide was added to lyse cells, before measurement of absorbance at 584 nm.

2.5. RNA isolation and real-time-PCR

RNeasy Mini Kits (QIAGEN, Valencia, CA) were used to extract total RNA from cells cultured in 12 well plates. The qScript cDNA synthesis kit (Quanta, Gaithersburg, MD) was used to prepare cDNA from 0.5 μg aliquots of RNA, prior to 1:4 dilution in DNase-free water. Real-time-PCR was performed with a 7900HT instrument (Applied Biosystems Inc., Foster City, CA) on 2.5 μl aliquots of cDNA using SYBR GreenER chemistry (Invitrogen) in a reaction volume of 10 μl. Amplification conditions of 50 °C, 2 min; 95 °C, 10 min; then 40 cycles of 95 °C, 15 s; 60 °C, 1 min, were used before melt curve analysis to confirm primer specificity (King et al., 2009). PCR primers were: CDKN1C forward: 5′-CGG CGA TCA AGA AGC TGT C-3′, reverse: 5′-GGC TCT AAA TTG GCT CAC CG-3′, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5′-TTC ACC ACC ATG GAG AAG GC-3′, reverse: 5′-AGG AGG CAT TGC TGA TGA TCT-3′, IL8 forward: 5′-GCA GCT CTG TGT GAA GGT GC-3′, reverse: 5′-AAA GGT TTG GAG TAT GTC TTT ATG CA-3′.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from cells following experiments and frozen prior to quantification of IL8 release by sandwich ELISA, according to the manufacturer's instructions (DuoSet ELISA kit; R&D systems).

2.7. Statistical analysis

All data are presented as means ± S.E.M. Version 5.01 of GraphPad Prism (GraphPad Software, San Diego, CA) was utilized to perform statistical analysis using repeated measures one- or two-way analysis of variance (ANOVA) with Bonferroni's correction for paired tests or Dunnett's tests, as appropriate.