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Levocetirizine inhibits rhinovirus-induced ICAM-1 and cytokine expression and viral replication in airway epithelial cells

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ABSTRACT

Levocetirizine inhibits the production of intercellular adhesion molecule (ICAM)-1 and secretion of interleukin (IL)-6 and IL-8, which may have beneficial effects on the pathophysiologic changes related to human rhinovirus (HRV) infection. We investigated the effects of levocetirizine on rhinovirus infection in primary human nasal epithelial cells (HNEC) and A549 cells. Cells were treated with different concentrations of levocetirizine, ranging from 0.5, 5 or 50 nM, either starting at the time of infection and continuing thereafter, or beginning 24 h before infection and continuing thereafter. Levocetirizine treatment inhibited the HRV-induced increase in ICAM-1 mRNA and protein levels, as well as the HRV-induced expression of IL-6 and IL-8 mRNA and protein levels. Viral titer, as measured by culture in MRC-5 cells, was reduced by levocetirizine. Levocetirizine treatment also reduced the increased nuclear factor-kappa B (NF-κB) expression seen with HRV infection. Levocetirizine inhibited the expression of Toll-like receptor (TLR)3 mRNA and protein levels. These findings indicate that, in HNEC and A549 cells, levocetirizine inhibits HRV replication and HRV-induced upregulation of ICAM-1, IL-6, and IL-8, TLR3 expression and NF-κB activation. The results of this study suggest that levocetirizine may have a possible clinical application in the treatment of airway inflammation caused by HRV infection.

1. Introduction

Human rhinovirus (HRV) infections are the most common acute infectious illnesses in humans, accounting for about 5–20% of acute illnesses (Gwaltney et al., 1966), including 50–60% of common colds. HRV infections also increase the risks of other common health problems, including asthma attacks, rhinosinusitis, and otitis media (Greenberg, 2003). The recognition of HRV infections of both upper and lower respiratory tracts as important causes of morbidity has led to diligent searches for antiviral agents to treat or prevent infections with HRV (Gern and Busse, 1999; van Kempen et al., 1999).

Toll-like receptor (TLR)3 is one of an evolutionary conserved receptor family that recognizes double-stranded RNA, an important intermediate of many viral life cycles including that of HRV, and responds by activating signal transduction pathways, leading to nuclear factor-kappa B (NF-κB) activation and cytokine gene expression (Gern et al., 2003; Hewson et al., 2005). It has been reported that HRV replication increases the expression of TLR3 mRNA and protein on the cell surface (Hewson et al., 2005). NF-κB is an important pro-inflammatory transcription factor, which has been reported to be required for HRV-induced upregulation of intercellular adhesion molecule (ICAM)-1 and the upregulation of several inflammatory mediators and cytokines in epithelial cells (Kim et al., 2000; Laza-Stanca et al., 2006; Papi and Johnston, 1999). Therefore, given the physiologic significance of NF-κB in HRV-related inflammation, a therapeutic approach targeted to the inhibition of NF-κB might have potential for treatment of HRV-induced airway inflammation (Edwards et al., 2007; Laza-Stanca et al., 2006). Furthermore, modulation of TLR3, which is known to have an important role in signaling pathways of NF-κB activation during viral replication, might also be useful for treatment of HRV-induced inflammation (Gern et al., 2003; Hewson et al., 2005; Sajjan et al., 2006).

Histamine H1 receptor antagonists have a long history of clinical efficacy in variety of allergic-inflammatory disorders. Recent studies have demonstrated that, in addition to their classic antihistaminic effects, several H1-antihistamines have anti-inflammatory properties that are unlikely to be related to classic histamine receptor antagonism, but are instead mediated by inhibitory effects on NF-κB (Simons, 2004). For example, levocetirizine (LCT), due to their inhibitory effects on NF-κB, inhibits the production of ICAM-1, a major receptor for HRV (Caproni et al., 2006). LCT also inhibits the production of interleukin (IL)-8, TNF-α, RANTES, and GM-CSF by epithelial cells (Ciprandi et al., 2004). These anti-inflammatory properties of LCT might modulate the pathophysiologic changes related to HRV infection. We therefore tested the effects of LCT on HRV infections in primary human nasal epithelial cells (HNEC) and

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A549 cells. The effects of this drug on the viral titer and expression levels of ICAM-1, IL-6, IL-8, NF-kB, and TLR3 were studied.

2. Materials and methods

2.1. Cell culture

Primary human nasal epithelial cells were obtained by digesting the inferior turbinate of 6 subjects removed during turbinoplasty with 0.1% dispase (Godo Shusei Co., Ltd., Tokyo, JAPAN) for 24 h at 37 °C in airway epithelial cell growth medium (AEGM, Promocell, Houston, TX, USA) containing 0.4% BPE, 10 ng/mL epithelial growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.5 μg/mL epinephrine, 6.7 ng/mL triiodothyronine, 10 μg/mL transferrin and 0.1 ng/mL retinoic. None of the subjects had any history of allergic rhinitis and asthma. This study was approved by the institutional review board of Asan Medical Center, and all study subjects gave written informed consent. The cells were separated by straining into a conical tube to which fetal bovine serum (FBS, GIBCO) was added to a final concentration of 10%, inactivating the dispase. The cells were centrifuged twice at 1200 rpm for 5 min, after which the supernatant was aspirated and discarded. Subsequently cells were washed with phosphate-buffered saline (PBS) and resuspended in AEGM and seeded at a density of 1.5×10^4 cells/mL onto Petri dishes. Culture medium was replaced every other day. Cells were grown in fully humidified air containing 5% CO_2 at 37 °C.

A549 alveolar epithelial type-II-like cells and MRC-5 human fetal lung fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were grown in F-12K Nutrient Mixture (GIBCO, Grand Island, NY), supplemented with 10% FBS, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. MRC-5 cells were grown in minimum essential medium (MEM, GIBCO) supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 100 units/mL penicillin, 100 μL/mL streptomycin and 0.25 μL/mL fungizone.

2.2. Human rhinovirus-16 infection and treatment of LCT

HRV-16 was obtained from the ATCC. Viral stocks were prepared by infection of sensitive cell monolayers (Ohio HeLa), as described elsewhere (Papi and Johnston, 1999). For RV infection, HNEC and A549 cells were plated at a density of 2×10^5 cells per well in 12-well plastic tissue culture plates (Costar, Cambridge, MA, USA) in F-12K Nutrient Mixture (GIBCO, Grand Island, NY), supplemented with 10% FBS.

LPS-free LCT provided by UCB Korea (Seoul, Korea) was dissolved in dimethyl sulfoxide (DMSO). In the control group, cells were cultured for 2 days in medium containing solvent alone (DMSO, final concentration of 0.1%, v/v), the vehicle for LCT. In the HRV infection group (HRV), the cells were infected with HRV-16 stock at a multiplicity of infection (MOI) of 1. After adsorption for 4 h at 33 °C, the viral solution was removed and the cells were rinsed with PBS and incubated at 33 °C. In the HRV plus LCT group (HRV + LCT) group, the cells were treated with 5 or 50 nM LCT at the time of RV infection and cultured in medium containing LCT for 2 days. In the LCT-only group (LCT), cells were treated with 5 or 50 nM LCT for 2 days without HRV infection. Cell viability after LCT treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with no significant cell death observed at 50 nM LCT for 2 days.

To determine if there was a difference among LCT pre-treatment before HRV infection, treatment at the time of viral infection, and post-treatment after infection, other sets of experiments were conducted. In these experiments, the cells and supernatants of LCT-only group were collected after incubation with LCT for 3 days. In the HRV + LCT group, cells were treated with 0.5, 5, or 50 nM LCT for 24 h before viral infection. After adsorption of virus for 4 h, the cells were rinsed with PBS and incubated at 33 °C for 2 more days in the media containing LCT. In other experiment, cells were infected with HRV first for 4 h and rinsed with PBS, and the cells were incubated at 33 °C for 2 more days in the media containing 50 nM LCT after 4 h.

Using real-time PCR, we evaluated the inhibitory effect of LCT on HRV replication according to the amount of HRV infection load. Cells were pre-treated with 50 nM LCT for 24 h and the cells were infected with HRV-16 stock at a MOI of 0.01, 1, or 5 for 4 h. After rinse with PBS, the cells were incubated at 33 °C for 2 more days in the media containing 50 nM LCT.

2.3. Detection of ICAM-1 and TLR3

Surface expression levels of ICAM-1 and TLR3 were measured by flow cytometry, as previously described (Jang et al., 2006). Fluorescein-isothiocyanate-conjugated mouse antihuman ICAM-1 antibody (Serotec, Oxford, UK), or isotype-specific control antibody (mouse immunoglobulin G1-fluorescein isothiocyanate; Serotec), or TLR3 antibody (Santa Cruz Biotechnology, California, USA) was used. After extensive washing in PBS, each cell preparation was fixed in 200 μL 1% paraformaldehyde/PBS, and fluorescence was measured using single-color flow cytometry (FACSCalibur Becton Dickinson, Franklin Lakes, NJ, USA).

2.4. Quantification of IL-6 and -8

Immunoreactive IL-6 and IL-8 were quantitated using dual-antibody ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer's protocol. The sensitivity limit of each kit was 10 pg/mL. Data were expressed in pg/mL and were obtained by extrapolation from a standard curve generated in parallel with each experiment.

2.5. Real-time PCR analysis for IL-6, -8, ICAM-1, TLR3, and HRV replication

After treatment of 50 nM LCT, cells were lysed and total RNA was extracted using Trizol agent (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. Two micrograms of total RNA were converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For each reaction, 4 μL 5× first-strand buffer (50 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μL of 0.1 M dTT, 5 U RNAsin, 500 μM dNTP mix, 200 pmol Oligo-dT, 25 U Superscript II reverse transcriptase and sterile water were added to the RNA to a volume of 20 μL. This reaction mixture was then incubated for 1 h at 37 °C. The cDNA products were stored in aliquots at −80 °C until needed. Real-time quantitative PCR was performed in triplicate in 96-well plates; each 20 μL reaction mixture consisted of 10 μL 2× SYBR I Mix (Applied Biosystems), 2.5 μL 900 nM forward and reverse primers and 1 μL cDNA. Oligonucleotide PCR primer pairs were designed as follows: ICAM-1, 5′-CTGCAGACAGTGACCATC-3′ (forward) and 5′-GTCCAGTTTCCCGGACAA-3′ (reverse); IL-6, 5′-TCTCCACAAGCGCC-TTCG-3′ (forward) and 5′-CTCAGGGCTGAGATGC CG-3′ (reverse); IL-8, 5′-TTTTGCCAAGGAGTGCTAAAGA-3′ (forward) and 5′-AACCCT-CTGCACCCAGTTTTC-3′ (reverse); TLR3, 5′-TTGCCTTGTATCTACTTT-TGGG G-3′ (forward) and 5′-GCGGCTGGTAATCTTCTGAGTT-3′ (reverse); HRV-16, 5′-GCACTTCTGTTTCCCC-3′ (forward) and 5′-CGGACACCCAAAGTAG-3′ (reverse).

After reverse transcription, the PCR reaction was performed in an ABI7900HT real-time sequence detection system programmed for 40 cycles of denaturation for 30 s at 95 °C, annealing for

30 s at 60 °C, and extension for 30 s at 72 °C. Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also was assayed using reagents obtained from Applied Biosystems.

2.6. Assessment of activated NF-κB

2.6.1. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared by centrifugation and suspension in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM.

EGTA, 1 mM DTT, and the protease inhibitors LIST). Cells were kept on ice for 15 min and then 0.5% NP-40 was added, after which the cells were vortexed for 10 s and centrifuged at 16,000 × *g* for 1 min at 4 °C. The pellets were resuspended in buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 25% glycerol and protease inhibitors), rocked for 15 min and centrifuged at 16,000 × *g* for 5 min at 4 °C. The supernatant obtained was the nuclear extract used in EMSA reactions. The nuclear protein was assayed for protein determination using the Bio-Rad protein assay kit (Richmond, CA). The remainder of the nuclear protein extract was frozen at −80 °C until used.

These reactions were performed using consensus NF-κB oligonucleotide (Santa Cruz Biotechnology, California, USA) radiolabeled as described previously (Basu et al., 1998) using 50 ng NF-κB oligonucleotide (AGTTGAGGGGACT-TTCCCAGGC), 70 μCi ^{32}P-ATP, 1 μL T4 polynucleotide kinase, 1.5 mL of 10× T4 polynucleotide buffer made up to 15 mL with double-distilled water. The EMSA reactions were carried out at room temperature for 20 min with nuclear proteins (10 μg), [r^{32}P-ATP]-labeled NF-κB consensus oligonucleotides (0.5 ng) (NEN, Boston, MA, USA), BSA (20 mg), pdI-dC (2 mg), Buffer D+ (2 mL), Buffer F (4 mL) and DTT (1 mM) in a total of 20 mL. Buffer D+:20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, and 0.25% NP40; Buffer F: 20% Ficoll 400, 100 mM HEPES pH 7.9, and 300 mM KCl. At the end of the reactions, the reactants were loaded on non-denaturing 6% polyacrylamide gels using a running buffer containing 50 mM Tris, pH 7.5, 0.38 M glycine and 2 mM EDTA. After the run was completed, the gels were dried and exposed to X-ray film for autoradiography at −80 °C. All reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. The gels were scanned using a PhosphorImager system (STORM, Molecular Dynamics).

2.6.2. Western blot analysis

Antibodies to NF-kB and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary horseradish peroxidase-conjugated donkey anti-rabbit antibodies were purchased from Amersham (Arlington Heights, IL). Protein concentrations were measured with BCA protein assay reagents (Pierce). Equivalent amounts of proteins (20 μg) were mixed with an equal volume of 2× Laemmli sample buffer, boiled and resolved by electrophoresis in 4–20% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad). Membranes were incubated in blocking solution containing 5% non-fat dry milk overnight to inhibit non-specific binding. The membranes were then incubated with specific antibody (1–3 μg/mL) for 2 h. After washing in Tris/0.1% Tween 20 for 30 min the membranes were incubated for another 60 min with horseradish peroxidase-conjugated secondary antibody. The membranes were then washed and developed with enhanced chemiluminescence (ECL Western Blotting Kit, Amersham). Differences in protein expression were quantified by integrated density analysis (Eagle Sight software; Stratagene) and normalized to β-actin expression on the same membrane.

2.7. Measurement of viral titer

MRC-5 cells were plated at a density of 3×10^5 cells per well in 96-well microplates (Falcon Labware, Oxnard, CA, USA) in 100 μL of MEM supplemented with 10% FBS. On the day of the experiment, the supernatant was removed and 125 μL of 5% medium was added to each well. 25 μL of serial 10-fold dilutions of each specimen was added and the plates were incubated at 33 °C for 7 days. Viral cytopathic effects were assessed using an inverted microscope. The amount of specimen required to infect 50% of MRC-5 cells (50% tissue culture infection dose, $TCID_{50}$) was determined, and the viral titer was expressed as $TCID_{50}$/mL.

2.8. Statistical analysis

Differences among groups were analyzed using the Kruskal-Wallis test, and between group differences were analyzed using the Mann–Whitney *U* test. All data are reported as the mean ± SD of independent experiments. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of ICAM-1

Using real-time PCR, it was found that HRV infection increased the level of ICAM-1 mRNA expression in HNEC, but this increase was reduced by LCT treatment. However, LCT alone had no effect on ICAM-1 mRNA levels in the absence of HRV infection (Fig. 1A). Post-treatment after HRV infection also reduced the mean level of ICAM-1 mRNA expression (Fig. 1B).

It was observed, using flow cytometry, that the mean fluorescence intensity of control HNEC and A549 cells was not changed significantly in the LCT groups (Fig. 1C and D). HRV infection increased the surface expression of ICAM-1, the magnitude of which was reduced by treatment with LCT at concentrations greater than 5 nM. In HNEC and A549 cells, HRV infection increased surface expression of ICAM-1 from 3.16 and 6.30 to 5.36 and 9.74, respectively, but these were reduced to 3.57 and 7.49 with 50 nM LCT in the HRV + LCT group, respectively. The differences in expression level between the control and HRV groups and between the HRV and the HRV + LCT groups were significant. LCT treatment significantly reduced the HRV-induced increases in ICAM-1 mean fluorescence intensity, regardless of whether the cells were treated with LCT before or at the time of HRV infection in HNEC and A549 cells.

3.2. Viral titers

When the supernatants were cultured in MRC-5 cells, it was found that the mean viral titer in the supernatant of the HRV group was $10^{4.88}$ and $10^{5.25}$ $TCID_{50}$/mL in nasal epithelial cells and A549 cells, respectively (Fig. 2). The mean viral titer was significantly reduced with LCT treatment in HRV-infected nasal epithelial cells and A549 cells. In the HRV + LCT group (50 nM), the mean titer was reduced to $10^{2.44}$ and $10^{3.13}$ $TCID_{50}$/mL, respectively. The magnitude of reduction was also similar between the cells pretreated with LCT for 24 h before HRV infection and the cells treated at the time of the infection with LCT in HNEC and A549 cells. Using real-time PCR, we also investigated the inhibitory effect of LCT on HRV replication according to the infection load. LCT treatment significantly decreased the viral replication at a MOI of 1 and 5 (Fig. 2C).

3.3. Quantity of IL-6 and IL-8

Using real-time PCR, we found that HRV infection increased the levels of IL-6 and IL-8 mRNA expression in HNEC, but these increases

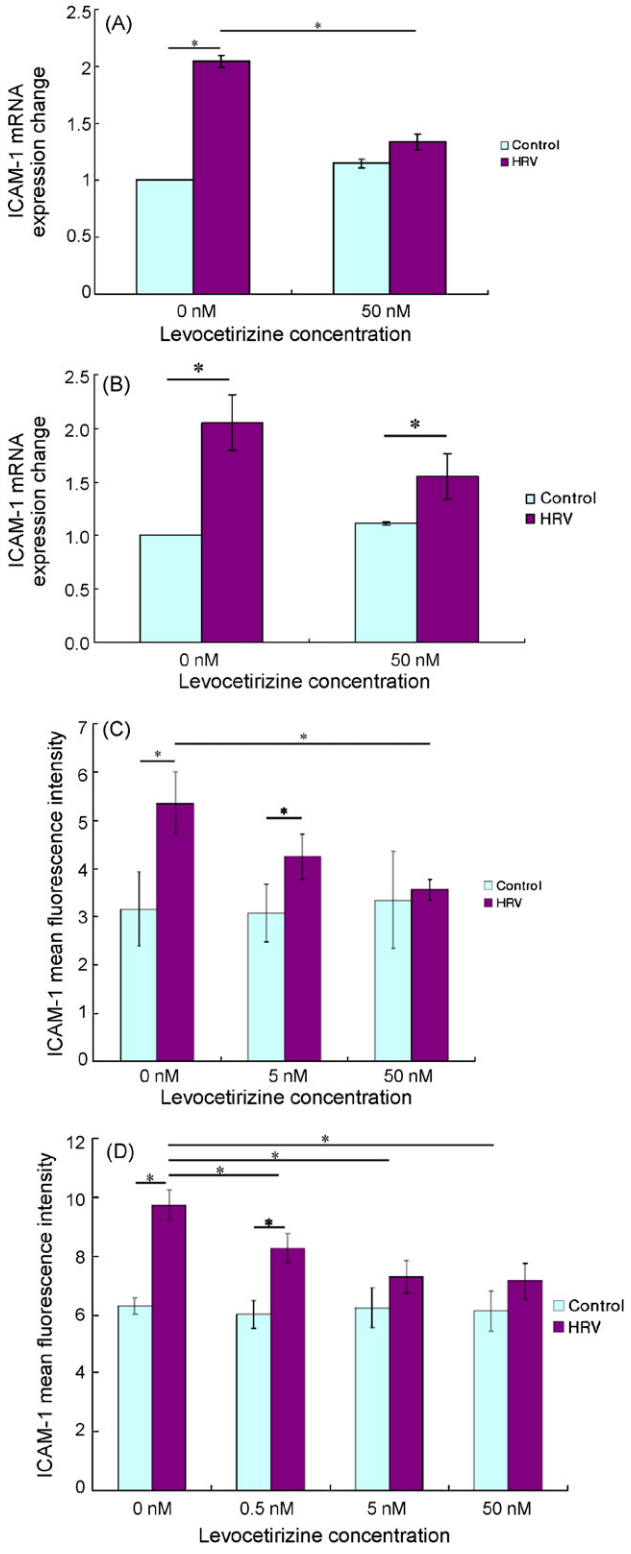

Fig. 1. Real-time PCR of intracellular adhesion molecule-1 (ICAM-1) mRNA in human rhinovirus (HRV)-infected primary nasal epithelial cells treated with levocetirizine (LCT). Primary nasal epithelial cells were treated with LCT (50 nM) (A) at the time of HRV infection without pretreatment ($n = 6$) and (B) 4 h after HRV infection ($n = 3$). HRV infection increased the level of ICAM-1 mRNA expression, but this was reduced by LCT treatment. The induction of ICAM-1 mRNA was calculated using control samples as 1. Effects of LCT on ICAM-1 mean fluorescence intensity in (C) primary nasal epithelial cells ($n = 6$) and (D) A549 cells ($n = 6$). HRV infection increased the surface expression of ICAM-1, which was reduced by LCT at doses greater than 5 nM. Each bar represents the mean ± SD of independent experiments. Significant differences are indicated by $^{*}p < 0.05$.

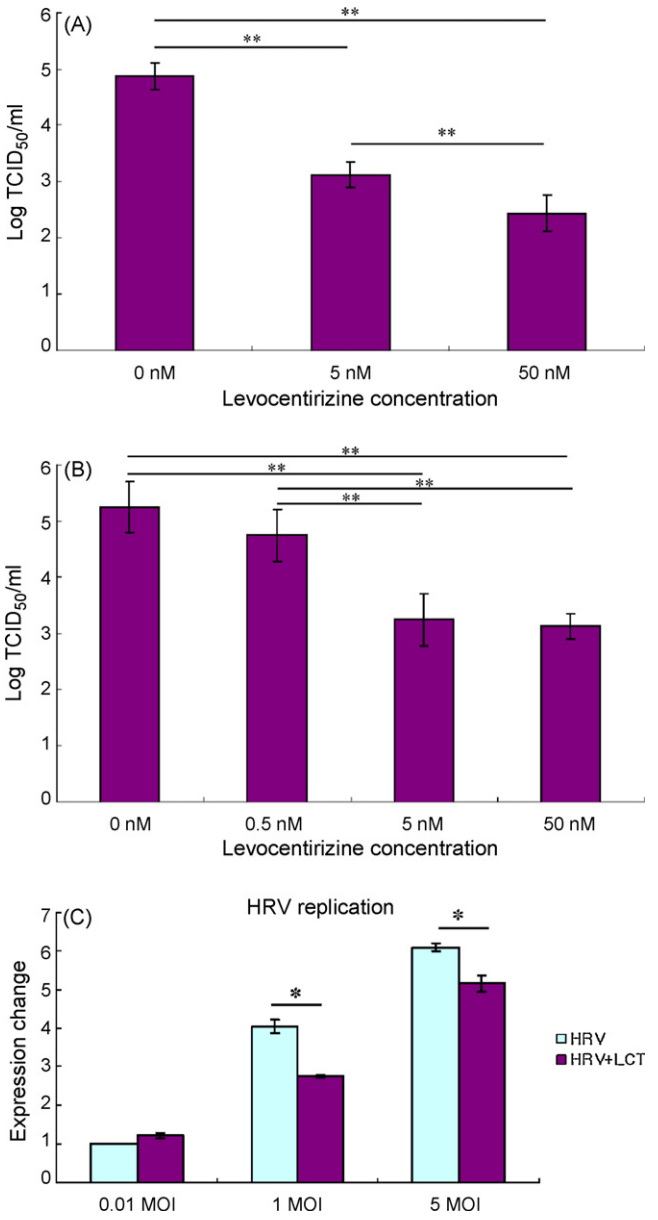

Fig. 2. Viral titers in supernatants of human rhinovirus (HRV)-infected (A) primary nasal epithelial cells ($n = 6$) and (B) A549 cells after levocetirizine (LCT) treatment for 2 days ($n = 6$). (C) Inhibitory effect of LCT on HRV replication according to a multiplicity of infection (MOI) ($n = 3$). LCT treatment inhibited HRV-induced increase in viral titer. Each bar represents the mean ± SD of independent experiments. Significant differences are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$.

were reduced by LCT (50 nM) treatment. However, LCT alone had no effect on IL-6 mRNA levels but had some effect on the increase of IL-8 mRNA in the absence of HRV infection (Fig. 3A and B).

When the levels of HRV-induced cytokine secretion were measured using ELISA in HNEC and A549 cells, it was found that the mean concentrations of IL-6 and IL-8 in the control groups were increased with HRV infection, but these increases were significantly reduced with LCT treatment.

In HNEC and A549 cells, the mean concentration of IL-6 in the control group was 288.5 and 222.2 pg mL^{-1}, and was 566 and 446.7 pg mL^{-1} in the HRV group, respectively. In HNEC treated with 5 nM, and 50 nM LCT at the time of HRV infection, the IL-6 levels were significantly reduced to 413 and 386.5 pg mL^{-1}, respectively (Fig. 3C). Post-treatment after HRV infection also significantly reduced the mean concentration of IL-6 (Fig. 3D). In A549 cells

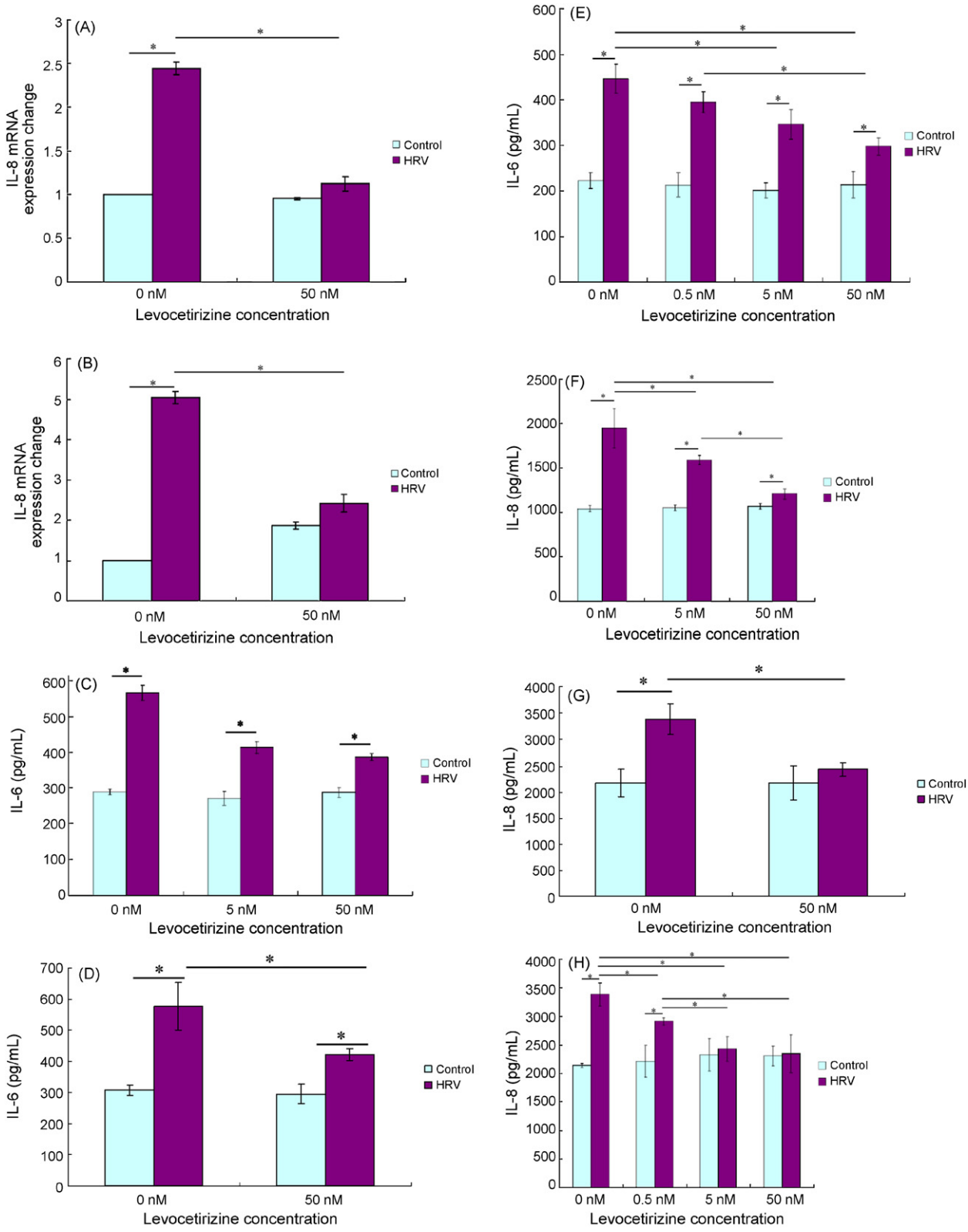

Fig. 3. Real-time PCR of (A) interleukin (IL)-6 and (B) IL-8 mRNA in human rhinovirus (HRV)-infected primary nasal epithelial cells treated with levocetirizine (LCT) ($n = 6$). Primary nasal epithelial cells were treated with LCT (50 nM) at the time of HRV infection without pretreatment. HRV infection increased the level of IL-6 and IL-8 mRNA expression, but this was reduced by LCT treatment. The induction of IL-6 and IL-8 mRNA was calculated using control samples as 1. Effects of LCT on IL-6 secretion in primary nasal epithelial cells (C) at the time of HRV infection ($n = 6$) and (D) after HRV infection ($n = 3$), and (E) A549 cells ($n = 6$). Secretion of IL-6 was increased in the control group after HRV infection, but this was reduced with 5 or 50 nM LCT treatment. LCT alone had no effect on IL-6 secretion in the control group. Effects of LCT on IL-8 secretion in primary nasal epithelial cells (F) at the time of HRV infection ($n = 6$) and (G) after HRV infection ($n = 3$), and (H) A549 cells ($n = 6$). Secretion of IL-8 was increased in the control group after HRV infection, but this was reduced with 5 or 50 nM LCT treatment. LCT alone had no effect on IL-8 secretion in the control group. Each bar represents the mean ± SD of independent experiments. Significant differences are indicated by $^{*}p < 0.05$.

treated with 0.5 nM, 5 nM, and 50 nM LCT at the time of HRV infection, the IL-6 levels were also significantly reduced to 394.7, 345.5 and 297.5 pg mL^{-1}, respectively (Fig. 3E).

Similar results were observed for IL-8: in HNEC and A549 cells, the mean concentration of IL-8 in the control group was 1041.2 and 2141.5 pg mL^{-1}, and was 1950.2 and 3381 pg mL^{-1} in the HRV group, respectively. In HNEC treated with 5 nM, and 50 nM LCT at the time of HRV infection, the IL-8 levels were significantly reduced to 1588.7 and 1206.5 pg mL^{-1}, respectively (Fig. 3F). Post-treatment after HRV infection also significantly reduced the mean concentration of IL-8 (Fig. 3G). In A549 cells treated with 0.5 nM, 5 nM, and 50 nM LCT at the time of HRV infection, the IL-6 levels were also significantly reduced to 2913.3, 2431 and 2350.8 pg mL^{-1}, respectively (Fig. 3H).

The inhibitory effect of LCT on RV-induced IL-6 and IL-8 secretion was similar between the cells pretreated for 24 h before HRV infection and the cells treated at the time of the infection in HNEC and A549 cells.

3.4. NF-κB activation

NF-κB activation after stimulation with HRV in HNEC was examined by EMSA. HNEC were treated with LCT (50 nM) at the time of HRV infection. LCT alone had no effect on NF-κB activation in the control cell groups. EMSA results showed increased binding of the NF-κB probe after HRV infection, but the magnitude of this increase was reduced by LCT (Fig. 4A).

Western blot analysis demonstrated that HRV infection significantly increased NF-κB protein expression by up to 3.46 ± 0.15-fold compared with control groups but post-treatment after viral infection significantly reduced HRV-induced NF-κB protein expression by 1.62-fold (Fig. 4B).

3.5. Expression of TLR3

Using real-time PCR, HRV infection was shown to increase TLR3 mRNA expression, but this was significantly reduced with LCT (50 nM) treatment in HNEC. However, LCT alone had some effect on the increase of TLR3 mRNA in the absence of HRV infection (Fig. 5A). Post-treatment after HRV infection also significantly reduced the level of TLR3 mRNA expression (Fig. 5B).

Flow cytometry results showed that the mean fluorescence intensity of control HNEC was not significantly different from that in the LCT group. HRV infection increased the surface expression of TLR3 from 7.89 to 10.62, which was reduced to 7.47 with LCT 50 nM (HRV + LCT group). The differences in surface expression between the control and HRV groups and between the HRV and the HRV + LCT groups were significant (Fig. 5C).

4. Discussion

LCT is the R-enantiomer of racemate cetirizine, which is a carboxylated metabolite of hydroxyzine (Curran et al., 2004; Walsh, 2006). In the management of allergic airway diseases, LCT has been shown to have several anti-inflammatory effects in addition to their classic antihistaminic effects (Walsh, 2006). To evaluate its potential role in modulating HRV-related inflammation, we assayed the effects of LCT at doses of 5 and 50 nM on HRV-induced changes in viral titer, ICAM-1 expression, and cytokine production in HNEC and A549 cells. Skin cetirizine levels increased to peak values of 1.6–2.4 ng/mL after a single oral dose of 10 or 20 mg of cetirizine (Petersen et al., 1999). In this study, 5 nM of LCT corresponded to about 1.94 ng/mL.

We found that ICAM-1 mRNA and protein were constitutively expressed by HNEC and A549 cells and that their levels were significantly increased with HRV-16 infection, which is in agreement with previous observations (Jang et al., 2006; Papi et al., 2002). Although,

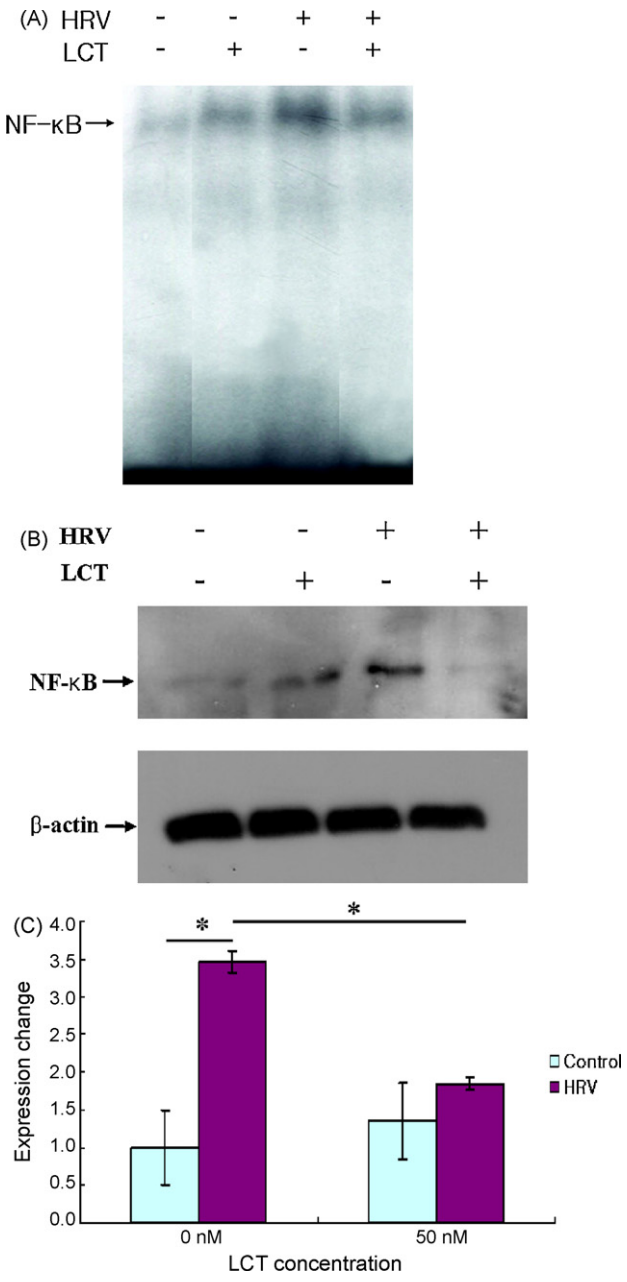

Fig. 4. (A) Electrophoretic mobility shift assay for nuclear factor-kB (NF-κB) activation after stimulation with human rhinovirus (HRV) in primary nasal epithelial cells. Lane 1: control group; lane 2: group treated with levocetirizine (LCT) only; lane 3: group infected with HRV only; lane 4: group infected with HRV and treated with LCT. Control indicates the NF-κB binding activity level without any stimulation. The arrow indicates specific NF-κB DNA complexes. Primary nasal epithelial cells were treated LCT (50 nM) at the time of HRV infection. HRV infection increased the level of NF-κB activation, which was reduced by LCT treatment. (B) Western blot analysis shows that post-treatment of LCT significantly reduced the level of HRV-induced NF-κB. Data are representative of three independent experiments. Each bar represents the mean ± SD of independent experiments. Significant differences are indicated by $^{*}p < 0.05$.

in the absence of infection, treatment with LCT did not affect the levels of baseline mRNA expression or ICAM-1 specific fluorescence intensity, LCT reduced the HRV-induced increases in ICAM-1 mRNA and specific fluorescence intensity, regardless of whether the LCT were added before, after, or at the time of HRV infection. The effects of LCT on ICAM-1 expression were in agreement with the results of previous investigations that showed inhibitory effects of LCT on ICAM-1 upregulation (Arnold et al., 1999).

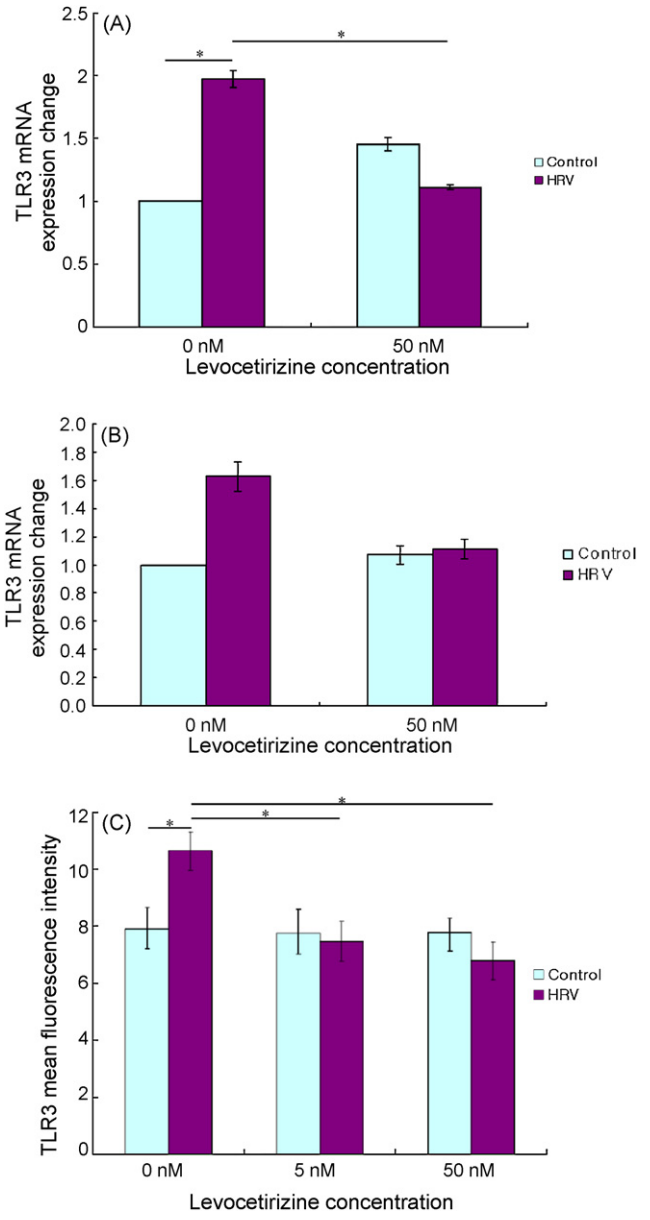

Fig. 5. Real-time PCR of Toll-like receptor (TLR)3 mRNA in human rhinovirus (HRV)-infected primary nasal epithelial cells treated with levocetirizine (LCT). Primary nasal epithelial cells were treated with LCT (50 nM) (A) at the time of HRV infection without pretreatment ($n = 6$) and (B) 4 h after HRV infection ($n = 3$). HRV infection increased the level of TLR3 mRNA expression, but this was reduced by LCT treatment. The induction of TLR3 mRNA was calculated using control samples as 1. (C) Effects of LCT on TLR3 mean fluorescence intensity in primary nasal epithelial cells ($n = 6$). HRV infection increased the surface expression of TLR3, which was reduced by LCT at doses greater than 5 nM. Each bar represents the mean ± SD of independent experiments. Significant differences are indicated by $^{*}p < 0.05$.

It was found that LCT inhibited the HRV-induced secretion of IL-6 and IL-8 in HNEC and A549 cells, but had no effect on the constitutive production of these cytokines. IL-6 and IL-8 are the inflammatory mediators that are associated with the development of viral URI symptoms. IL-6 plays an important role in the development of symptoms of the common cold by inducing pyrexia (van Kempen et al., 1999). IL-8 is a strong chemoattractant for neutrophils, and can also activate the recruited neutrophils, resulting in the release of their cytotoxic granular content (van Kempen et al., 1999). HRVs are potent stimulators of IL-6 and IL-8 production, and there is substantial evidence for a direct correlation between the IL-6 and IL-8 levels and symptom severity in experimental HRV infection (Gern and Busse, 1999). Therefore, our results suggest that LCT may relieve the symptoms of HRV infection by inhibiting the HRV-induced secretion of IL-6 and IL-8.

During the replication cycle of most viruses, double-stranded RNA (dsRNA), which is recognized by TLR3, is produced (Johnsen et al., 2006). Subsequent to the recognition of dsRNA, TLR3 activates a signaling cascade that elicits a NF-κB-mediated immune response that has critical importance in HRV-related inflammation (Hewson et al., 2005). Thus, it can be speculated that the inhibition of TLR3 production might have potential modulatory effects on the pathophysiologic changes induced by HRV infection. In the present study, we found that LCT inhibited HRV-induced TLR3 expression and NF-κB activation in HNEC, which has not been reported previously. Thus, it is conceivable that the inhibition of NF-κB activation may be caused by the decrease of TLR3 production with LCT treatment, thereby resulting in reduced expression of ICAM-1 and cytokines.

We found that LCT reduced viral titers in the supernatants of HRV-infected HNEC and A549 cells. LCT treatment also significantly decreased viral replication even at a high MOI. In this study, LCT alone had no significant effect on the expression of ICAM-1, IL-6, IL-8, TLR3, and NF-κB activation in the absence of HRV infection but it reduced HRV-induced expression of inflammatory markers even after viral infection, regardless of treatment point. Previous studies have reported that HRV infection upregulates ICAM-1 expression in airway epithelial cells, thereby facilitating further viral attachment and entry (Jang et al., 2006; Papi and Johnston, 1999; Papi et al., 2002). Therefore, the decrease of HRV-induced ICAM-1 expression may reduce the reattachment of replicated viruses released from epithelial cells to the cells and it may influence the viral titers. However, our results suggest that LCT may inhibit viral replication not only by the inhibition of HRV-induced ICAM-1 upregulation but presumably by the suppression of cytoplasmic viral growth including genome replication, protein synthesis, assembly, or release of virus. Further studies are required to gain greater insight into the mechanisms of action by which LCT inhibits viral replication in respiratory epithelium.

As in our study, other second-generation antihistamines loratadine and desloratadine have also been reported to inhibit HRV-induced ICAM-1 expression and NF-κB activation (Papi et al., 2001). However, in contrast to the inhibitory effect of LCT on viral replication seen in our study, loratadine and desloratadine did not reduce the viral titer. Furthermore, the effects of loratadine and desloratadine on cytokine secretion have not been studied. These slightly different results compared with our study therefore indicate the specific beneficial roles of LCT in HRV infection, which may not be a general characteristic of all antihistamines. A recent study showed that a log-1 reduction in viral shedding results in a significant clinical benefit in HRV-infected patients (Gern et al., 2007). We found that LCT treatment significantly reduced viral titer by about log-2, suggesting that LCT may have a potentially beneficial role in treatment of HRV infections.

Several different drugs have been assessed for efficacy in treatment of HRV infections. Antiviral agents are undergoing clinical trials, but as yet show little clinical benefit and are associated with the added complication of antimicrobial resistance. So far, symptomatic treatments targeted for HRV-induced symptoms are regarded as a mainstay of treatment (Turner, 2001). HRV is a major cause of asthma exacerbation (Mallia and Johnston, 2006). Thus, prevention of HRV infection in these asthma patients by use of a drug that is primarily beneficial for their asthma or allergic rhinitis but also effective for prevention and treatment of HRV infection would be an attractive and practical management strategy for this serious healthcare issue. Therefore, in this context, the effects of LCT on HRV infection, if proved beneficial by confirmation of our *in vitro* data in further *in vivo* clinical trials, could contribute to reducing HRV-related morbidity and also prevent asthma exacerbation.

In conclusion, we have shown that LCT inhibits HRV replication and HRV-induced upregulation of ICAM-1, IL-6, and IL-8, TLR3 expression and NF-κB activation. The results of this study suggest that LCT may have a possible clinical application in the treatment of airway inflammation caused by HRV infection.

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