Oxatomide inhibits Interleukin-8 release from respiratory epithelial cells*

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Abstract

Background: Oxatomide, a H1-receptor antagonist, exerts besides its well-known anti-allergic potential an array of anti-inflammatory activities. We wondered whether oxatomide might influence the release of IL-8 from human epithelial cells activated with agonists of TLR2, which mainly expresses on airway epithelial cells.

Methodology: We used the human lung epithelial cell line A549 and primary Human nasal epithelial cell line for our in vitro studies. IL-8 releases from these cell lines were determined by IL-8 enzyme immunoassay. NF-κB was analysed by Luciferase reporter assay. Confluent epithelial cell monolayer were pre-incubated with oxatomide for 30 min and afterwards activated with lipoprotein as a TLR2 agonist for 24 h.

Results: Epithelial cells stimulated with lipoprotein showed a significantly increased release of IL-8. Pre-incubation with oxatomide diminished the IL-8 release from cells activated with lipoprotein in a significant manner. Furthermore, activity of the NF-κB was determined by luciferase reporter assay. Besides, oxatomide inhibits expression of MIP2, a homologue of human IL-8, and neutrophilic infiltration in nasal membrane of mice intranasally exposed with lipoprotein. These results suggest that oxatomide reduced the release of IL-8 from respiratory epithelial cells stimulated with lipoprotein.

Conclusion: Therefore, oxatomide might exert anti-inflammatory effects beyond its H1-receptor antagonistic activity in the course of inflammatory respiratory tract disorders such as acute bacterial rhinitis.

Key words: H1-receptor antagonists, IL-8, nasal epithelial cell, NF-κB

Introduction

Oxatomide, a carboxylated metabolite of hydroxyzine, belongs to the new generation of H1-receptor antagonists (1). A positive therapeutic effect of oxatomide has been shown in allergic rhinoconjunctivitis, chronic urticaria, and pollen-associated asthma (2,3). There has also been an accumulation of evidence that oxatomide shows some anti-inflammatory activities unrelated to its histamine antagonism, e.g. the chemotactic activity of granulocytes, T cells (4) and the adhesion of eosinophils to endothelial cells was inhibited (5). It has been shown that the production of newly generated pro-inflammatory mediators such as reactive toxic oxygen radicals and lipid mediators from eosinophils (6), basophils and mast cells (7) was counter-regulated by oxatomide. Besides, LTD4 in middle ear of chinchillas with otitis media with effusion was reported to be suppressed by oxatomide (8). It has also been shown that epithelial cells play a crucial role during...
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acute inflammatory and infectious processes by secreting interleukin-8 (IL-8) \(^{9-12}\). The multifactorial pro-inflammatory cytokine IL-8, a member of the chemokine super-gene family \(^{13}\), has chemotactic activity toward neutrophils as well as T lymphocytes \(^{14,15}\). In addition, neutrophils are activated by IL-8, enhanced expression of Clusters of differentiation (CD) 11b/CD18, transendothelial migration, generation of reactive oxygen products and exocytosis \(^{16}\). As a direct consequence of this potenent neutrophil-activating potential, IL-8 plays a major role in the pathogenesis of many acute inflammatory reactions, including acute rhinitis \(^{16,17}\). Epithelial cells recognize microbial pathogens by a set of germ line-encoded receptors that are referred to as pattern-recognition receptors. Toll-like receptors (TLRs) function as the major epithelial pattern-recognition receptor in recognition of a pathogen-associated molecular patterns \(^{18}\). Activation of TLRs, in turn, leads to induction of direct antimicrobial effector pathways that can result in elimination of the invading pathogens \(^{19}\). Activation of TLRs also induces the expression of co-stimulatory molecules and the release of IL-8 that instruct the acquired immune response \(^{20}\). To date, 10 members of the human TLR family have been cloned. Among these, TLR2 has been shown to mediate responses to a variety of Gram-positive products, including peptidoglycan, lipoprotein, lipoteichoic acid and lipoarabinomannan. It is clear that TLR2 plays a crucial role in host defense against both Gram-positive and -negative bacteria \(^{21,22}\). Interestingly, in the upper respiratory tract, it has been reported that TLR2 is expressed dominantly in epithelial cells \(^{23}\). We wondered whether IL-8 production by human epithelial cells stimulated with lipoprotein via TLR2 might also be influenced by the H1-receptor antagonist oxatomide. We used the human pulmonary type II epithelial cell line A549 and primary Human nasal epithelial cells (HNECs) for our in vitro study. We also assessed the in vivo role of oxatomide in a murine acute rhinitis model.

**Methods**

**Reagents**

Synthetic Lipid A was provided by Ono Pharmaceutics. Synthetic lipoprotein (palmitoyl-Cys(RS)-2,3-di(palmitoyloxy)-propyl-Ala-Gly-OH)) was provided by Bachem (Bubendorf, Switzerland). Anti-phospho-Inhibitor κ Bα (iκBα) mAb and anti-iκBα polyclonal Ab were obtained from New England Biolabs (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Anti-murine MIP-2 monoclonal antibody was purchased from Genzyme-Thecne (Technhe, AN'ALYZA immuno-assay system; Genzyme).

**Cells**

Cells of the human A549, pulmonary epithelial cell line (ATCC CCL 185, Rockville, MD, USA) show features of type II alveolar epithelial cells and produce surfactants \(^{24}\). The cells were grown in FK12 medium containing 10% (v/v) fetal calf serum (FCS), 100 µg/ml streptomycin, 100 IU/ml penicillin and 20 mmol/l sodium hydrogen carbonate. Normal human nasal tissues (500 mg) were obtained from two patients with chronic sinusitis after receiving informed consent from the patients. HNECs were transfected with human papillomavirus 16 (HPV-16) E6 and E7 open reading frames. These cells were designated HNEC-1 and HNEC-2, respectively, and used between passages 50 and 60. HEK293 and U937 as a human monocytes were obtained from ATCC.

**Cell culture conditions**

A549 cells, HNEC-1 and HNEC-2 were cultured at 37°C in a water-saturated atmosphere containing 5% CO\(_2\). Only cell prepartions with a viability greater than 95%, determined by a trypan blue exclusion test, were used. The buffers and cell media were prepared using pyrogen-free water. Confluent A549, HNEC-1 and HNEC-2 monolayers (10° cells) were cultured in 1 mL RPMI-1640 (10% FCS (v/v)) with antibiotics in 24-well plastic tissue culture plates (Costar, Cambridge, MA, USA). Confluent monolayers were pre-incubated with oxatomide (0.01-10 µg/ml) for 30 min. After pretreatment, the cells were washing with PBS twice. The range of doses used, 0.01-10 µg/ml, corresponds to the physiologi- plasmatic concentration range of oxatomide. After 24 h of culture, the cell supernatants were collected and stored at -70°C until IL-8 determination.

**Mice**

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The mice were bred in our institute under specific pathogen-free conditions. Eight- to 10-week-old female mice were used for the experiments.

**Reverse transcription-PCR**

Total cellular RNA from A549, HNEC-1, HNEC-2 and U937 was extracted with RNAzol B (Tel-Test, Friendswood, TX, USA) using a single-step isolation method \(^{25,26}\) according to the manufacturer’s recommendation. RNase-free DNase (Takara Biochemi- cals, Shiga, Japan) was used to remove genomic DNA based on methods described previously \(^{27}\). Two micrograms of extracted RNA was reverse-transcribed into first-strand cDNA at 42°C for 40 min using 100 U/ml of reverse transcriptase (RT) (Takara Biomedicals, Shiga, Japan) and 0.1 µM oligo(dT) adapter primer (Takara Biomedicals, Shiga, Japan) in a 50 µl reaction mixture. PCR amplification of cDNA was performed using oligonucleo- tide primers specific for TLR1 (Sense: 5'-AGTTGTCAGCGATGACTCAGGATG TGTTCCG-3' Antisense: 5'-GATCAAGTACCTTGACCGGG-3'), TLR2 (Sense: 5'-GGCTTCTCTGTCTTGACC-3' Antisense: 5'-GGCTTCTCTGTCTTGACC-3'), TLR4 (Sense: 5'-TTGTAATCCGTTGACC-3' Antisense: 5'-GGCTTCTCTGTCTTGACC-3'), TLR5 (Sense: 5'-AGCACCAGGCTTCACTGGG-3' Antisense: 5'-AGTCAAGTACCTTGATCCTCTGG-3'), TLR9 (Sense: 5'-AGTCAAGTACCTTGATCCTCTGG-3' Antisense: 5'-AGTCAAGTACCTTGATCCTCTGG-3').

**CATCTG ACTGCATTAAGG-3' Antisense: 5'-GACTTCCTCTTCATCAGG-3' Antisense: 5'-CAGTCAAATTCGTGCAGAAGGC-3'), TLR3 (Sense: 5'-AGCCACCTGAAGTTGGAACCAGGAAGAC G-3'), TLR2 (Sense: 5'-TTGTATTCAAG GTCT GGCTGG-3' Antisense: 5'-GATCAAGTACCTTGATCCTGGG-3' , TLR2 (Sense: 5'-AGTTGTCAGCGATGACTCAGGATG TGTTCCG-3' Antisense: 5'-GATCAAGTACCTTGATCCTGGG-3', TLR4 (Sense: 5'-TTGTAATCCGTTGACC-3' Antisense: 5'-GGCTTCTCTGTCTTGACC-3').
transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 1% BSA in TBST for 1 h, and Western blot analysis was performed as described previously (33), followed by detection using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

Luciferase assays
A549 cells were transiently transfected with 2 μg of pGL3-Nuclear Factor-κB (NF-κB)/Luc (a luciferase reporter construct containing a consensus NF-κB binding sequence) and 0.2 μg of an internal control by Lipofectamine (Life Technologies) according to the manufacturer's instructions. Twenty-four hours after the transfection, some cells were pretreated with oxatomide or curcumin for 30 min followed by the addition of 1 μg/ml lipoprotein. After 8-h incubation with lipoprotein, cells were lysed, and the luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Toyo Ink, Tokyo, Japan).
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according to the manufacturer’s instructions. Background luciferase activity was subtracted, and the data are presented as means ± SD of triplicate samples (34).

Acute rhinitis model
C57Bl/6 mice (females, 6 weeks old) were intranasally injected with 10 µg of lipoprotein 2 times per day for 2 days. 2 mg of Oxatomide in 200 µl of saline was daily inoculated with an intragastric intubation into mice. Twenty four hours after the last injection of lipoprotein, mice were killed and then nasal membrane tissues were stained by the HE-staining method. The counts of neutrophils and macrophages were performed under the light microscope. Then, we collected the lysates from nasal membrane using ice-cold lysis buffer and analyzed the expression of MIP-2 by western blot assay.

Statistical analysis
The statistical significance of the data was determined by Student’s t-test. A value of p<0.05 was taken as significant.

Results
Expression of TLR on respiratory epithelial cells
We examined the expression of TLRs mRNA on A549 cells, HNEC-1 and HNEC-2 by RT-PCR. As shown in Figure 1, both A549 cells and HNECs showed TLR2 and TLR6 mRNA expression, however, HNECs expressed no TLR4 mRNA. A549 cells and HNEC1 expressed TLR6 mRNA. But A549 cells and HNECs expressed no TLR9 mRNA. TLR1, TLR5, TLR7, TLR8 and TLR10 mRNAs were also not expressed in A549 cells and HNECs. U937, as a control, markedly expressed high level of TLR2, TLR3, TLR4, TLR6 and TLR9 mRNA. RT-PCR analysis of β-actin expression confirmed the quality of all RNA preparations used for RT-PCR. No band was detected in the non-RT sample by PCR.

Induction of IL-8 production
IL-8 producing activities in A549 cells and HNECs after stimulation with lipoprotein (as a TLR2 and TLR6 ligand) and lipid A (as a TLR4 ligand) were examined (Figure 2). Lipoprotein significantly induced IL-8 production in both A549 cells and HNECs, whereas stimulation of these cells with lipid A resulted in no induction of IL-8 production.

Effects of oxatomide on IL-8 production by A549 cells and HNECs
As shown in Figure 3, pre-incubation of A549 cells and HNECs with oxatomide in the dose range of 0.01-10 µg/ml significantly diminished the lipoprotein-induced IL-8 release. The IL-8 release from A549 cells stimulated with lipoprotein for 24 h was significantly inhibited by oxatomide even at 0.1 µg/ml. Furthermore, the releases of IL-8 from both HNEC-1 and HNEC-2 was also inhibited by pre-treatment with oxatomide at 1 µg/ml.

Lipoprotein-induced NF-κB activation may be regulated by oxatomide
To determine whether the inhibitory effect of oxatomide on IL-8 production correlated with the inhibition of NF-κB activity, we performed a luciferase reporter assay. A549 cells were transfected with a luciferase reporter construct containing an NF-κB recognition sequence and then treated with oxatomide and lipoprotein. As shown in Figure 4A, concentration-dependent inhibition of NF-κB activity by oxatomide correlated well with the inhibition of IL-8 production, suggesting that NF-κB inhibition may be essential for the regulation of lipoprotein-mediated IL-8...
Acute nasal inflammatory diseases are infectious, have a bacterial etiology, and cause inflammatory responses elicited by nasally pathogenic exposure. In these responses, nasal epithelial cells are thought to play important roles as the initial point of contact with pathogens. It has been shown that the extravasation step of neutrophil granulocytes, e.g., directed diapedesis from the vascular space into the interstitium and the alveolar spaces, during inflammatory tissue responses is highly potentiated by IL-8 (35-37). Therefore, diminished IL-8 cytokine levels induced by treatment with oxatomide might be of value with respect to reduced neutrophilic infiltration rates (38) and decreased levels of activation of neutrophils (39). In this study, we analyzed the oxatomide-dependent inhibition of IL-8 release from human respiratory cells (A549 cells) and from primary human nasal epithelial cells HNECs. Results of RT-PCR indicated that both A549 production by oxatomide. We then analyzed the effects of oxatomide on iκBα phosphorylation of iκBα by Western blotting. As shown in Figure 4B, iκBα was significantly phosphorylated by lipoprotein 15 min after stimulation with lipoprotein. However, in the case of pretreatment with oxatomide, phosphorylation of iκBα was markedly inhibited even at 0.01 µg/ml (Figure 4C). These data correlate with results of the luciferase assay.

In vivo role of oxatomide on in a murine acute rhinitis model
To determine the effects of oxatomide on acute rhinitis, we established a murine acute rhinitis model. C57Bl/6 mice were intranasally (i.n.) injected with lipoprotein for 2 days. After intranasal injection, the skin around the nose was severely inflamed (Figure 5A). Neutrophil infiltration was clearly observed in the nasal membrane (Figure 5B). MIP-2, a homologue of human IL-8, expressed in the nasal membrane compared with naive mice (Figure 5C). In contrast, intragastric (i.g.) inoculation of oxatomide inhibited nasal skin inflammation and neutrophil infiltration (Figure 5A and B). Interestingly, MIP-2 expression was also inhibited by i.g. inoculation of oxatomide.

Discussion
Acute nasal inflammatory diseases are infectious, have a bacterial etiology, and cause inflammatory responses elicited by nasally pathogenic exposure. In these responses, nasal epithelial cells are thought to play important roles as the initial point of contact with pathogens. It has been shown that the extravasation step of neutrophil granulocytes, e.g., directed diapedesis from the vascular space into the interstitium and the alveolar spaces, during inflammatory tissue responses is highly potentiated by IL-8 (35-37). Therefore, diminished IL-8 cytokine levels induced by treatment with oxatomide might be of value with respect to reduced neutrophilic infiltration rates (38) and decreased levels of activation of neutrophils (39). In this study, we analyzed the oxatomide-dependent inhibition of IL-8 release from human respiratory cells (A549 cells) and from primary human nasal epithelial cells HNECs. Results of RT-PCR indicated that both A549
cells and HNECs express TLR2 and TLR6. A549 cells expressed TLR4, but HNECs did not. Recently, bacterial components have been reported to stimulate cells via TLR2, TLR4 and TLR9. Neither HNECs nor A549 cells expressed TLR9. Lipoprotein, as a TLR2 and TLR6 agonist, promoted IL-8 production by epithelial cells, though lipid A, as a TLR4 agonist, did not enhance IL-8 production by epithelial cells, including A549 cells, which expressed TLR4 mRNA. These results indicate that lipoprotein stimulated nasal epithelial cells mainly via TLR2. TLR2 may act as a essential role in the production of IL-8 by epithelial cells, including A549 cells, which expressed TLR4 mRNA. Pretreatment of the cells with oxatomide significantly diminished lipoprotein-induced IL-8 release from A549 cells and HNECs. It was also found in the present study by luciferase assays and western blot analysis that lipoprotein induces IL-8 production and NF-κB activation in A549 cells and HNECs. The 5′-flanking regions of genes coding for IL-8 contain putative binding sites for the transcription factor NF-κB (40,41). NF-κB complexes are sequestered in the cytoplasm by inhibitory molecules, named IκBα (42). Upon cell stimulation with cytokines or viruses, IκBα become degraded by phosphorylation on two serine residues at positions 32 and 36, allowing migration of activated NF-κB into the nucleus and binding to the DNA-NF-κB binding site (43). Pretreatment with oxatomide results in inhibition of the phosphorylation of IκBα as shown by Western blot analysis from the luciferase assay, NF-κB activation was also inhibited by addition of oxatomide in a dose- dependent manner. These data indicate that oxatomide inhibited the IL-8 production from nasal epithelial cells stimulated with lipoprotein mainly via TLR2, by down-regulating the NFκB activity. Finally, we analyzed the in vivo role of oxatomide using a murine acute rhinitis model. Interestingly, i.g. inoculation of oxatomide resulted in inhibition of nasal skin inflammation and neutrophil infiltration in the nasal membrane. Furthermore, expression of the murine chemokine MIP-2, a functional homologue of human IL-8, was also inhibited in the nasal membranes of mice i.g. inoculation with oxatomide. These in vivo data confirm the actual inhibitory effect of oxatomide on inflammatory reaction in nasal mucosal linings.

Conclusion
In conclusion, our results suggest that a TLR2 ligand mainly acts on respiratory epithelial cells and stimulates IL-8 production via NF-κB activation. Oxatomide clearly inhibited NF-κB activation and IL-8 production. Using a murine acute rhinitis model, we confirmed the in vivo inhibitory role of oxatomide in MIP-2 expression, a functional homologue of human IL-8, and neutrophil infiltration of the nasal membrane. There has been accumulating data on the immunomodulating in vitro effect of antihistamines in previous reports (44-46). This anti-inflammatory potential of oxatomide, so called second generation of antihistamine, might be of great value for the treatment of inflammatory reaction in the upper respiratory tract, including acute rhinitis.

Authorship contribution
HK made this project and supervised all experimental processes with his immunological background and as otolaryngologist. NA, IM, TF, YS, KS, YH, and QI have done all experiments together and produced data under the supervision of HK. TY mainly took care of animal experiments, based on his background as veterinarian. EP supervised all experiments from clinical point of view and gave us supervision of writing this article in English.

Conflict of interest
All authors declare that they have no conflict of interest or funding.

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