



Antiallergic drugs, azelastine hydrochloride and epinastine hydrochloride, inhibit ongoing IgE secretion of rat IgE-producing hybridoma FE-3 cells

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Abstract

We asked whether or not antiallergic drugs, azelastine hydrochloride and epinastine hydrochloride, inhibit IgE secretion from IgE-producing hybridoma FE-3 cells. FE-3 cells were cultured in the presence of azelastine or epinastine for 24 h, washed in phosphate-buffered saline, and then recultured in the medium in the absence of the antiallergic drugs. IgE levels in the cultured medium as well as those in the cytoplasm of FE-3 cells were measured by enzyme-linked immunosorbent assay. mRNA levels of Cε, activation-induced cytidine deaminase (AID), XBP-1, and Bip were estimated by northern blot or reverse transcriptase polymerase chain reaction analysis. The activities of nuclear factor-kappa B (NF-κB) were analyzed by electrophoretic mobility shift assay (EMSA). Phosphorylation of I kappa B alpha (IκBα) was analyzed by immunoprecipitation followed by western blot analysis. IgE levels in the cultured medium and in the microsome fraction were lower on the treatment with 10⁻⁵ M azelastine or epinastine than those on the treatment with vehicle. Cε and AID mRNA levels were lower on the treatment with 10⁻⁵ M azelastine than those on the treatment with vehicle, but were not decreased on the treatment with 10⁻⁵ M epinastine. XBP-1 and Bip mRNA levels were not altered following treatment of the antiallergic drugs. Azelastine at 10⁻⁵ M, but not epinastine, reduced DNA binding activity of NF-κB and also diminished IκBα phosphorylation, leading to sustaining IκBα protein levels. These findings suggest that azelastine exerts its inhibitory effect on the IgE secretion from FE-3 cells through the inhibition of Cε mRNA expression, and that the inhibitory effect of epinastine is, at least in part, due to suppression of IgE synthesis at the post-transcriptional level.

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1. Introduction

Mast cells, basophils and eosinophils, which have a capacity to produce a large number of inflammatory mediators, are effector cells involved in acute and subacute allergic responses. The degranulation of the effector cells is induced by interaction with IgE, resulting in initiation of type I allergic reaction. In clinical field, several histamine H₁ receptor antagonists are used for the treatment of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis by relying upon their inhibitory effects on the release of mediators from the effector cells.

Histamine H₁ receptor antagonists such as azelastine hydrochloride (azelastine) and epinastine hydrochloride (epinastine) are known to have antiallergic and antiinflammatory effects apart from antihistaminergic actions *in vivo* and *in vitro*. Azelastine reportedly inhibits the IL-2 production of splenic T cells (Furie et al., 1994), the release of leukotrienes from rat basophilic leukemia (RBL)-1 cells (Hamasaki et al., 1996), and TNF-alpha, IL-6 and IL-8 secretion from mast cells (Hide et al., 1997; Kempuraj et al., 2003; Matsuo and Takayama, 1998; Nakata and Hide, 1998). Administration of azelastine inhibits the number of cells infiltrating into the bronchial mucosa such as eosinophils, T lymphocytes, and cells expressing mRNA of IL-4 or IL-5 (Shimojo, 1998). Preincubation with azelastine causes a dose-dependent inhibition of intra and extracellular

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platelet activating factor (PAF)-like activity from asthmatic and non-asthmatic eosinophils as well as neutrophils or macrophages (Shindo and Fukumura, 1996; Shindo et al., 1997a,b). On the other hand, epinastine inhibits the scratching behavior in mice associated with passive cutaneous anaphylaxis mainly through an antagonistic action on histamine H₁ receptors (Inagaki et al., 2000). Epinastine has a suppressive effect on eosinophilic chemotaxis through inhibiting eosinophil CD11b expression (Matsukura et al., 2003) and IL-8 release in allergic patients (Kohyama et al., 1997). Epinastine also provides antileukotriene and antibradykinin activities (Tasaka, 2000). However, not much is available on the effect of antiallergic or antihistaminergic drugs on the production of IgE, which is another key factor involved in allergic reaction. In previous reports, azelastine was shown to have an inhibitory effect on IgE levels and IgE distribution in rats apart from its well-known antiinflammatory effects (Hanashiro et al., 1994, 2002). These findings led us to believe the possibility that certain antiallergic drugs inhibit IgE production *in vivo*. However, the issue of whether the inhibitory effect of azelastine on IgE production is a direct effect on B cells (or plasma cells) remains to be elucidated.

We have established IgE-producing hybridoma FE-3 cells by hybridizing mouse myeloma cells (SP2/0-Ag14/SF) with rat spleen cells that were isolated from Brown–Norway rats immunized with DNP-*Ascaris suum* (Hanashiro et al., 1996). The cDNA sequence of the Cε3 and Cε4 domains of IgE FE-3 was identical to that of rat IgE IR162 (Tokeshi et al., 2001), indicating that IgE produced from the hybridoma cells is encoded in rat immunoglobulin locus. FE-3 cells are expected to be available not only for supply source of monoclonal IgE antibodies but also for various allergic experiments as a model of plasma cells that constitutively produce IgE antibodies. The aim of the present study was to determine whether antiallergic drugs inhibit ongoing IgE secretion from IgE-producing cells. We show herein that azelastine hydrochloride and epinastine hydrochloride inhibit in a distinct manner IgE secretion of IgE-producing hybridoma FE-3 cells.

2. Materials and methods

2.1. Materials

Azelastine hydrochloride (azelastine) and epinastine hydrochloride (epinastine) were provided by Eisai Pharmaceutical Co. (Tokyo, Japan) and by Boehringer Ingelheim KG (Ingelheim, Germany), respectively. Since azelastine has low solubility to water, it was dissolved in methanol (final concentration of methanol was 0.1%), and then diluted with growth medium to the final concentrations, 1.0×10^{-7} M to 1.0×10^{-5} M. Epinastine was dissolved in phosphate-buffered saline (PBS) and diluted with growth medium to the final concentrations, 1.0×10^{-7} M to 1.0×10^{-5} M.

2.2. Cell culture

FE-3 cells were grown in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.3 mg/ml of L-

glutamine, 0.1 mg/ml of pyruvic acid, 100 μg/ml of penicillin, 100 μg/ml of streptomycin and supplemented with 10% fetal bovine serum (FBS) (Sanko Junyaku, Tokyo, Japan), as described previously (Hanashiro et al., 1996). The cells were cultured at 37 °C in a humidified water-jacketed incubator (Astec Inc., Fukuoka, Japan) under 5% CO₂/95% air atmosphere. The cells (2×10^5 cells/ml) were incubated with or without azelastine or epinastine at different concentrations (10^{-7} , 10^{-6} or 10^{-5} M) for 24 h, washed in PBS, and then subjected to subsequent assays. In the case of determination of IgE levels in the cultured medium, the cells were adjusted to a cell density equivalent between dishes (2×10^5 cells/ml) and then recultured in the growth medium for 0.5, 1, 2 or 3 h. Supernatants were harvested by centrifugation and stored at –70 °C until assay and cell pellets were used for further analysis. The cell viability was evaluated by the trypan blue dye exclusion test.

2.3. Determination of IgE by IgE capture-enzyme-linked immunosorbent assay (ELISA)

IgE-capture ELISA was performed as described previously (Hanashiro et al., 1996; Nakasone et al., 2001). In brief, the ELISA plate was coated with 5 μg/ml of monoclonal anti-IgE antibody (BB-9) overnight at 4 °C. After blocking with 1% gelatin-PBS, the wells were incubated with the conditioned medium or cell lysate of FE-3 cells for 1 h at room temperature. Following washing, the wells were incubated with 1 μg/ml of biotinylated DNP-*Ascaris suum* in 1% gelatin-phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 30 min at room temperature. After further washing, the wells were incubated with peroxidase-conjugated streptavidin (ZYMED Laboratories, CA, USA) in 1% gelatin-PBST for 30 min at room temperature, followed by final washing and addition of substrate solution (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, MO, USA). Spectrophotometric readings were then made using $\lambda_1=415$ nm and $\lambda_2=492$ nm wavelength filters of a dual wavelength microplate reader MTP-300 (CORONA Electric, Ibaraki, Japan).

2.4. Microsome fractionation

The microsome fractionation was performed according to the cytoskeleton stabilizing buffer (CSB) method (Abe et al., 1992). In short, FE-3 cells (1×10^7 cells), treated with azelastine or epinastine for 24 h, were collected by centrifugation at $250 \times g$ at 4 °C, and suspended in 200 μl of the CSB containing 0.2 M sucrose, 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 5 mM Hepes–KOH, pH 7.5, followed by being homogenized with 20 strokes of a Dounce homogenizer. After centrifugation at $450 \times g$, the supernatant was transferred to another tube, and the pellet was dissolved as fraction A in 100 μl of the lysis buffer containing 1% NP-40, 150 mM NaCl, 0.2 M sucrose, 2 mM EGTA, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 5 mM Hepes–KOH, pH 7.5. After further centrifugation of the supernatant at $18,800 \times g$, the supernatant was collected as fraction C, and the pellet was dissolved in the lysis buffer as fraction B that

presumably contains the bulk of microsome fraction associated with the fragment of cytomembrane and cytoskeleton (Abe et al., 1992). The Bradford system (Bio-Rad, CA, USA) was used to determine the protein concentration.

2.5. RNA isolation and northern blot analysis

Total RNA (10 µg), prepared from FE-3 cells (2×10^6 cells) using Purescript™ RNA isolation kit (Gentra Systems, MN, USA) according to the manufacturer's instructions, was resolved by gel electrophoresis under denaturing conditions and transferred to a nylon membrane. Equal RNA loading and transfer to nylon membranes were confirmed by staining ribosomal RNA on the membranes with methylene blue (Herrin and Schmidt, 1988). Probes for detecting Cε, XBP-1, Bip, and β-actin mRNA were respectively prepared by reverse transcriptase polymerase chain reaction (RT-PCR) and labeled with [³²P]. The cDNAs used as probes were amplified using the following primers: Cε (F), 5'-CTTACCTGTCTGGT TTTGGACCTGG-3'; Cε (R), 5'-C GGA GGAAGTGTACCAGGC-3'; XBP-1 (F), 5'-CTCAGAGGC AGAGTCCAAGG -3'; XBP-1 (R), 5'-GGTCCCCACTGAC AGAGAAA-3'; Bip (F), 5'-CGTATGTGGCCTTCACTCT-3'; Bip (R), 5'-CTGCATGGGTGACCTTCTTT-3'; β-actin (F), 5'-G AGCTATGAGCTGCCTGACGG-3'; β-actin (R), 5'-TTGC GGTGCACGATGGA-3'. (Pre)hybridization was performed with the ExpressHyb™ hybridization solution (BD Clontech, CA, USA). Radioisotope signals were detected and analyzed by Bio-imaging Analyzer System-1500 (BAS-1500) (Fuji Photo Film, Tokyo, Japan).

2.6. Reverse transcription (RT) and polymerase chain reaction (PCR) for determination of activation-induced cytidine deaminase (AID) mRNA expression

Reverse transcriptions (RTs) were carried out according to the standard procedures. In brief, 1 µg of total RNA in 18.8 µl of reaction mixture (4 µl of 5×RT buffer, 4 µl of 2.5 mM dNTP mixture, 2 µl of 25 mM MgCl₂, 2 µl of 0.1 M DL-dithiothreitol (DTT), and RNase free water as the rest of mixture) and 0.2 µl of 100 µM synthetic oligo(dT) primer were mixed. After incubated at 70 °C for 10 min, the reaction mixture was cooled down on ice. The reaction was initiated by adding 1 µl of M-MLV RTase and then the mixture was incubated at 42 °C for 50 min. The cDNAs encoding AID and β-actin were amplified by the PCR method with Program Temp Control System PC-800 (Astec Inc., Fukuoka, Japan). The reaction was performed in a final volume of 20 µl of reaction mixture, which consisted of 20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM each primer, 1 µl of Taq DNA polymerase (Promega, WI, USA) (final concentrations), and 1 µl of sample. Primer sequences are as follows: AID (F), 5'-GCCACCTTCG CAACAAGT-3'; AID (R), 5'-CGCGGTGAAAATCCTCAG-3'; β-actin (F), 5'-3'; β-actin (R), 5'-TTGCGGTGCACGATG GAGGG-3'. The PCR conditions were as follows: a single 5 min at 94 °C; 36 cycles (24 cycles for β-actin) of 94 °C for 30 s, 52 °C (58 °C for β-actin) for 30 s and 72 °C for 1 min; and a single 5-min extension at 72 °C. The PCR products were separated on a

2%-agarose gel, visualized with ethidium bromide, and analyzed using the Gel Doc 1000 UV fluorescent gel documentation system (Bio-Rad Lab., CA, USA) with the accompanying Multi-Analyst software (version 1.0).

2.7. Preparation of nuclear extracts

FE-3 cells (5×10^6 cells) were homogenized in ice-cold buffer A (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.5 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.5 mM PMSF and Na₃VO₄) and incubated on ice for 15 min, followed by centrifugation at 450 ×g at 4 °C for 5 min. The isolated nuclear pellets were resuspended in ice-cold buffer B (20 mM Tris–HCl, pH 8.0, 25% glycerol, 400 mM NaCl, 0.5 mM EDTA, 5 mM spermidine, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF and 1 mM DTT), and incubated on ice for an additional 15 min. The nuclear extracts were collected by centrifugation of the nuclear homogenates at 18,800 ×g at 4 °C for 5 min.

2.8. Electrophoretic mobility shift assay (EMSA)

The oligonucleotides used for EMSA were as follows (only upper strands are given): consensus NF-κB oligonucleotide (long), 5'-GAATCAAAAGGGAAGTCC AAGGCTGCTA-3'; consensus NF-κB oligonucleotide (short), 5'-AAGGGAAGTCCAA-3'; mutated NF-κB oligonucleotide (mt), 5'-AAGGGAAGT**AGT**AA-3' (mutated nucleotides in bold italics). Double-stranded oligonucleotides were radiolabeled using T4 polynucleotide kinase and [γ-³²P]ATP. Nuclear extracts (5 µg), prepared from FE-3 cells, were first incubated at room temperature for 30 min in 20 µl of a binding mixture of 50 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 8% glycerol, 1 µg salmon sperm DNA, 6 µg bovine serum albumin and 1 mM DTT, followed by a further 15-min incubation after addition of 0.45 ng of radiolabeled oligonucleotide probe. Aliquots (5 µl) of reaction mixture were separated in running a 4.5% polyacrylamide gel containing 4% glycerol in 0.25× TBE buffer at room temperature. For a competition assay, nuclear extracts were preincubated with 50-fold excesses of nonlabeled consensus NF-κB oligonucleotide (short) or mutated oligonucleotide for 15 min before addition of the [³²P]-labeled probe. For supershift assay, nuclear extracts were preincubated with 2 µg each of anti-p50 (sc-114X; Santa Cruz Biotech., CA, USA) or anti-p65 (sc-372X; Santa Cruz Biotech., CA, USA) for 15 min before addition of the [³²P]-labeled probe. The gels were dried and exposed to Imaging Plate (IP) (Fuji Photo Film, Tokyo, Japan) at room temperature. Radioisotope signals were detected and analyzed by Bio-imaging Analyzer System-1500 (BAS-1500) (Fuji Photo Film, Tokyo, Japan).

2.9. Immunoprecipitation and western blot analysis

Cytoplasmic extracts were prepared in a buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 5 mM EDTA, 10 mM NaF, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM Na₃VO₄. The cytoplasmic extracts were then subjected to immunoprecipitation with anti-IκBα (sc-1643; Santa Cruz Biotech., CA, USA), pulled down by Protein A sepharose beads,

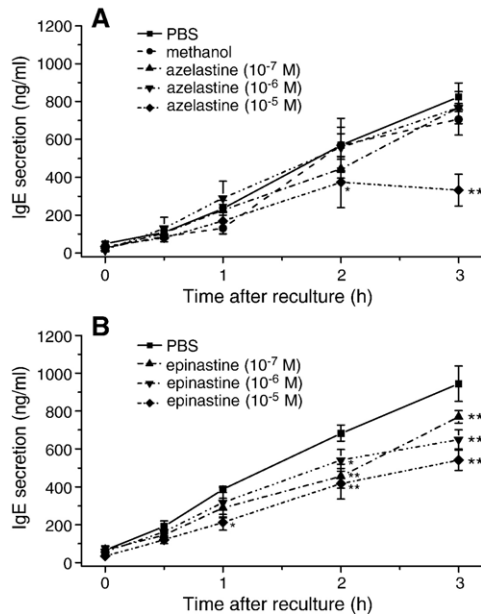


Fig. 1. Inhibitory effects of antiallergic drugs on IgE secretion from FE-3 cells. The cells (2×10^5 cells/ml) were incubated in the growth medium with azelastine (A) or epinastine (B) at different concentrations (10^{-7} , 10^{-6} or 10^{-5} M), or with vehicle (methanol or PBS) for 24 h, washed by PBS, adjusted to a cell density equivalent between dishes, and then recultured in the growth medium for 0.5, 1, 2 or 3 h. IgE levels in the cultured medium were determined as described in Materials and methods. Data are indicated as means $\pm$ S.D. ($n=5$). * $P<0.05$ or ** $P<0.01$ (vs. vehicle).

and washed twice with TNET [10 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100 and 1 mM EDTA] and once with TNE [10 mM Tris (pH 8.0), 150 mM NaCl and 1 mM EDTA]. The beads were boiled in $2\times$ SDS sample buffer and eluates were analyzed by western blotting using anti-phospho I κ B α (sc-8404; Santa Cruz Biotech., CA, USA). ECL Plus Western Blotting Detection System (Amersham Biosciences, Sweden) was employed, and membranes were exposed to Fuji X-ray film (RX-U) (Fuji Photo Film, Tokyo, Japan).

2.10. Statistical analysis

Data are expressed as means $\pm$ standard deviations (S.D.) of the mean, if not specifically otherwise mentioned. Statistical analysis was performed by unpaired Student's *t*-test for between-group comparisons. For comparison of more than two groups, analysis of variance (ANOVA) was used. If the ANOVA was significant, Fisher's protected least significant difference (Fisher's PLSD) was used as a *post hoc* test. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Effects of antiallergic drugs on IgE secretion from FE-3 cells

To test the effect of the antiallergic drugs on the IgE secretion of FE-3 cells, the cells were treated with azelastine or epinastine for 24 h, washed by PBS, adjusted to a cell density equivalent between dishes, and recultured for another 0.5, 1, 2, or 3 h. Both azelastine

and epinastine were found to suppress IgE secretion from FE-3 cells (Fig. 1A and B). The levels of IgE released in the cultured medium were significantly lower on the treatment with 10^{-5} M azelastine at 2 and 3 h after reculture than those on the treatment with vehicle ($P<0.05$, $P<0.01$, Fig. 1A). At 10^{-7} and 10^{-6} M, however, azelastine was not found to suppress the IgE levels in the medium. On the other hand, IgE levels in the medium were significantly lower on the treatment with epinastine (10^{-7} to 10^{-5} M) than those on the treatment with vehicle 2 and 3 h after reculture (2 h: $P<0.01$, $P<0.05$, $P<0.01$; 3 h: each concentration, $P<0.01$), and were also lower at the concentration of 10^{-5} M 1 h after reculture ($P<0.05$, Fig. 1B). It was observed that the concentration of epinastine required for reducing IgE secretion was lower than that of azelastine at any time after reculture. In contrast, the reduction of IgE secretion on the treatment with azelastine (52.9% vs. methanol) was larger than that on the treatment with epinastine (41.3% vs. PBS) at 10^{-5} M 3 h after reculture.

3.2. Effects of antiallergic drugs on intracellular IgE levels of FE-3 cells

The levels of IgE synthesized in the FE-3 cells were decreased by 22% when cells were treated with azelastine ($P<0.05$, Fig. 2A),

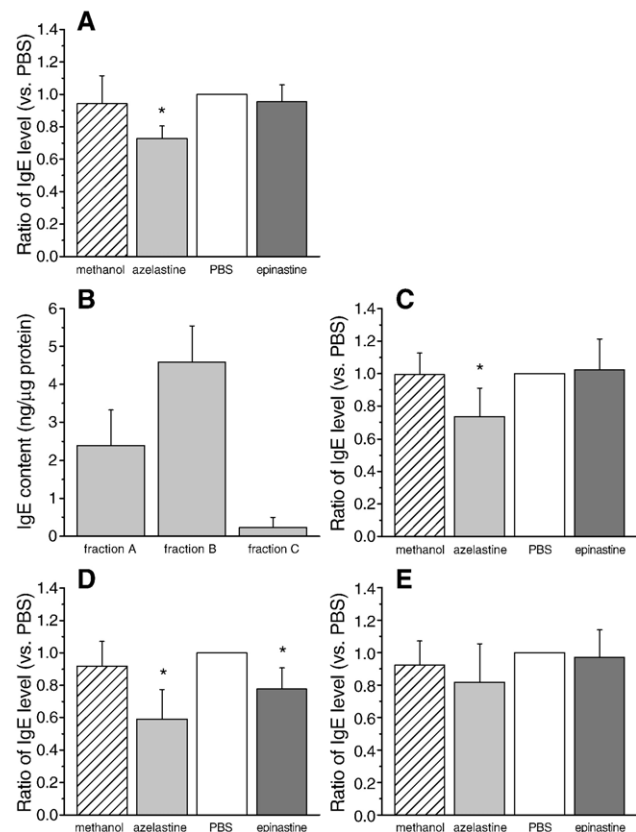


Fig. 2. Effects of antiallergic drugs on intracellular IgE levels of FE-3 cells. The cells (2×10^5 cells/ml) were incubated in the growth medium with azelastine or epinastine at 10^{-5} M, or with vehicle (methanol or PBS) for 24 h. The procedure for microsomal fractionation was described in Materials and methods. IgE levels in the microsomal fraction were determined by using IgE-capture ELISA. A: whole cell lysate; B: IgE content in each fraction; C: fraction A; D: fraction B; E: fraction C. Data are indicated as means $\pm$ S.D. ($n=5$). * $P<0.05$ (vs. vehicle).

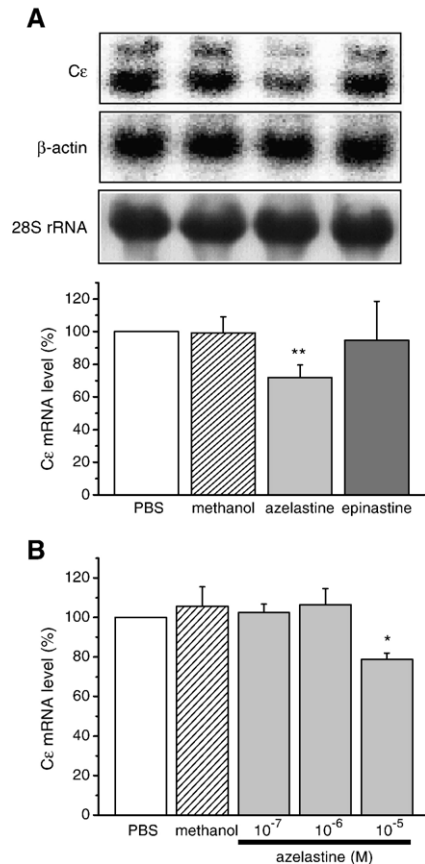


Fig. 3. Effects of antiallergic drugs on Cε mRNA levels of FE-3 cells. A: Effects of antiallergic drugs on Cε mRNA levels of FE-3 cells following 24-h drug exposure. The cells (2×10^5 cells/ml) were incubated in the growth medium with azelastine or epinastine at 10^{-5} M, or with vehicle (methanol or PBS) for 24 h. The determination of Cε mRNA levels was performed by northern blot analysis. The probe for Cε-chain as well as for β-actin was prepared by RT-PCR as described in Materials and methods. Equivalent loading of RNA samples was confirmed by probing with β-actin cDNA. The Cε-chain/β-actin ratio that was given when cells were treated with PBS was adjusted to 100%. Data are indicated as means $\pm$ S.D. ($n=5$). $**P<0.01$ (vs. methanol). B: Inhibitory effect of azelastine at different concentrations on Cε mRNA levels of FE-3 cells following 24-h drug exposure. The cells were incubated in the growth medium with azelastine at different concentrations (10^{-7} , 10^{-6} or 10^{-5} M), or with vehicle (methanol or PBS) for 24 h. The Cε-chain/β-actin ratio that was given when cells were treated with PBS was adjusted to 100%. Data are indicated as means $\pm$ S.D. ($n=5$). $*P<0.05$ (vs. methanol).

but not decreased when treated with epinastine (Fig. 2A). As IgE is a constitutive secretory protein, microsome fraction was further prepared and employed to test the level of IgE synthesized in cells under the condition without the influence of other intracellular proteins on IgE-capture ELISA. Calculating the ratio of IgE content in each fraction to that in the fraction B, we found that the ratio of IgE recovered from the fractions A, B, and C was 1.1: 1: 0.3, respectively. The purity of IgE in the fraction B was, however, approx. 2- and 20-fold higher than those in the fractions A and C, respectively (Fig. 2B). In the fractions A and C, IgE levels in the cells treated with epinastine were not significantly different from those in the cells treated with vehicle (PBS) (Fig. 2C and E). Approx. 28% reduction in the IgE levels of the cells treated with azelastine was observed in the fraction A ($P<0.05$, Fig. 2C),

but no significant change was observed in the fraction C (Fig. 2E). In the case of the fraction B, IgE levels were decreased by 36% in the cells treated with azelastine, and by 23% in the cells treated with epinastine as compared to the values on the PBS-treated cells ($P<0.05$, respectively, Fig. 2D), indicating that both of azelastine and epinastine exhibit the suppressive effects on the IgE protein synthesis.

3.3. Effects of antiallergic drugs on Cε mRNA expression of FE-3 cells

Two bands were observed in northern blot analysis for determination of Cε mRNA of FE-3 cells. Based on the report (Steen et al., 1984) concerning rat plasmacytoma IR162, which has a property of rat IgE production, we speculate that the faster migrating band observed corresponds to the mRNA for the secreted form of IgE, and that the slower band corresponds to the mRNA for the membrane-bound form of IgE (Fig. 3A). In the present study, our results indicated that azelastine, but not epinastine, suppressed the Cε mRNA levels of FE-3 cells. Treatment of cells with 10^{-5} M azelastine led to almost 30% reduction in the Cε mRNA levels after 24-h drug exposure, but did not affect β-actin mRNA levels ($P<0.01$, Fig. 3A). The inhibitory effect of azelastine on the Cε mRNA levels was not observed at the concentration of 10^{-7} and 10^{-6} M (Fig. 3B). Epinastine was not observed to decrease Cε mRNA levels (Fig. 3A).

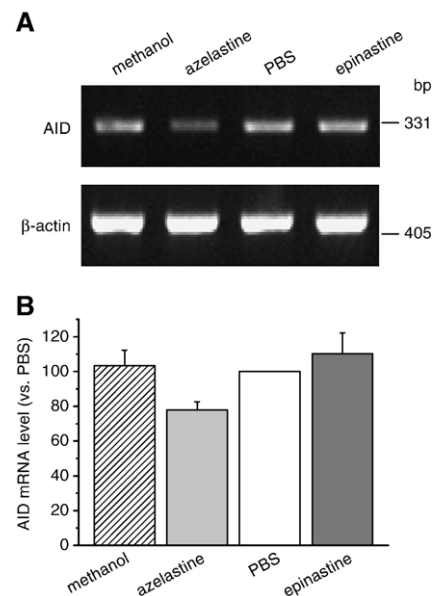


Fig. 4. Effects of antiallergic drugs on AID mRNA expression of FE-3 cells. The cells (2×10^5 cells/ml) were incubated in the growth medium with azelastine or epinastine at 10^{-5} M, or with vehicle (methanol or PBS) for 24 h, and then total RNA was prepared. AID mRNA expression was confirmed by semiquantitative RT-PCR. The numbers of PCR cycles within the linear range of PCR amplifications were determined for AID and β-actin, respectively (36 cycles for AID; 24 cycles for β-actin). A: A representative semiquantitative RT-PCR experiment from three independent experiments. B: The AID/β-actin ratio that was given when cells were treated with PBS was adjusted to 100%. Results from three experiments plus standard deviations of the mean are plotted. $*P<0.05$ (vs. methanol).

3.4. Effects of antiallergic drugs on AID mRNA expression of FE-3 cells

Although FE-3 cells have undergone class switch recombination and somatic hypermutation, AID mRNA expression in the cells can still be detected by RT-PCR. We therefore tested if azelastine has an inhibitory effect on AID mRNA expression of FE-3 cells. The numbers of PCR cycles within the linear range of PCR amplifications were determined for AID and β -actin, respectively (36 cycles for AID; 24 cycles for β -actin). Our finding was that azelastine at 10^{-5} M ($P < 0.05$, Fig. 4), but not epinastine, inhibited AID mRNA expression (Fig. 4).

3.5. Effects of antiallergic drugs on NF- κ B DNA binding activity

To investigate the mechanism of the inhibitory action of azelastine, we tested whether it reduces DNA binding activity of NF- κ B, which has an important role in IgE gene expression. The identification of putative *cis*-regulatory binding sites of NF- κ B performed by using the matinspector (www.genomatix.de/cgi-bin/matinspector/matinsjpector.pl), which utilizes the transfac library of matrices (www.gene-regulation.de/) to locate consensus matches in nucleotide sequences, indicated that rat hs1,2 element

(GenBank™ accession no. A28224) has a putative NF- κ B binding site and 4 c-Rel binding sites. Therefore, we examined the specificity of DNA protein complexes on the electrophoretic mobility shift assay (EMSA) using the oligonucleotides that contain consensus NF- κ B binding site. The patterns in the binding of nuclear proteins to the oligonucleotides are shown in Fig. 5. Of the two major complexes observed, the slower migrating complexes (indicated by an asterisk) were competed by specific oligonucleotides, but not by mutated oligonucleotides (Fig. 5A). On the other hand, the faster migrating complex (indicated by a double asterisk) was not competed even by specific oligonucleotides. The slower migrating complexes were supershifted by both anti-p50 and anti-p65, but not by control antibodies. These results indicate that the slower migrating complexes contain a NF- κ B family, p50 and p65. Next, nuclear extracts derived from cells treated with azelastine or epinastine for 24 h were subjected to this assay. The ratio of NF- κ B/the unidentified band indicated by a double asterisk that was given when cells were treated with PBS was adjusted to 100%. The signal of slower migrating complexes was reduced on the nuclear extracts derived from cells treated with azelastine, but not on the cells treated with epinastine, as compared to the signal on vehicle (Fig. 5B). The significant reduction in the signal was found by treatment with azelastine ($P < 0.05$, Fig. 5B), indicating that NF- κ B activation is suppressed by 10^{-5} M azelastine.

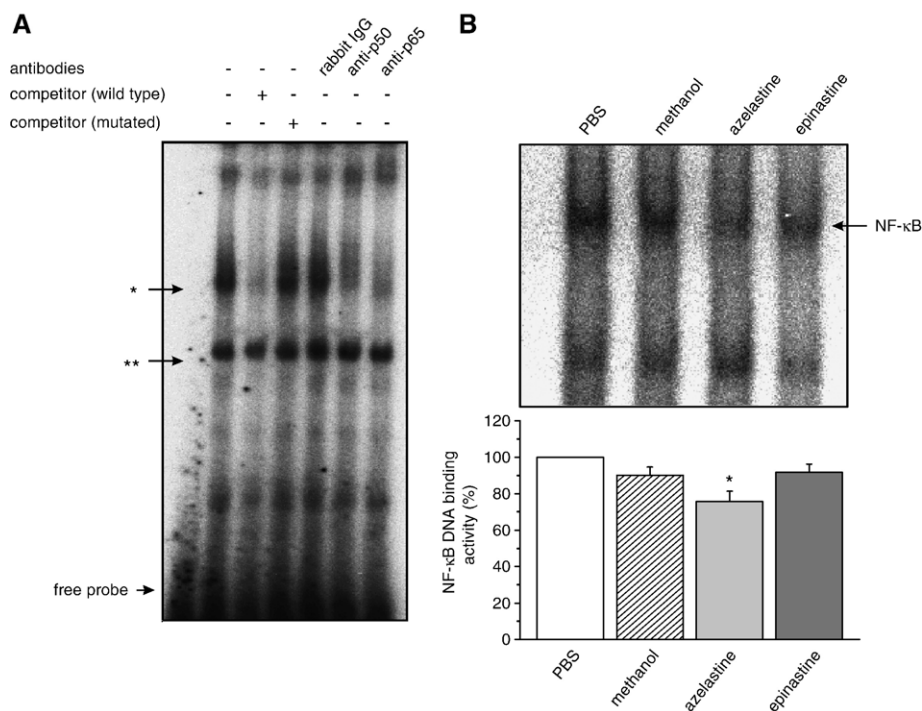


Fig. 5. Effects of antiallergic drugs on NF- κ B DNA binding activity. A: Specificity of DNA protein complexes on an electrophoretic mobility shift assay (EMSA). Nuclear extracts (5 μ g), prepared from FE-3 cells, were first incubated at room temperature for 30 min in 20 μ l of a binding mixture, followed by a further 15-min incubation after addition of radiolabeled oligonucleotide probe (consensus NF- κ B oligonucleotide; long). Aliquots (5 μ l) of reaction mixture were separated in a polyacrylamide gel run in 0.25 $\times$ TBE buffer at room temperature. For a competition assay, nuclear extracts were preincubated with 50-fold excesses of nonlabeled consensus NF- κ B oligonucleotide (short) or mutated oligonucleotide before addition of [^{32} P]-labeled probe. For a supershift assay, nuclear extracts were preincubated with 2 μ g each of anti-p50 or anti-p65 before addition of [^{32} P]-labeled probe. B: Effects of antiallergic drugs on the NF- κ B DNA binding activity following 24-h drug exposure. The cells (2×10^5 cells/ml) were incubated in the growth medium with 10^{-5} M azelastine or epinastine, or with vehicle (methanol or PBS) for 24 h. NF- κ B DNA binding activity was calculated by comparing the signal intensity ratio between NF- κ B and the unidentified band indicated by a double asterisk on each reagent to the corresponding ratio, adjusted to 100%, on the vehicle (PBS). Data are indicated as means $\pm$ S.D. of triplicate determination. * $P < 0.05$ (vs. methanol).

3.6. Effects of azelastine on the protein and phosphorylation levels of I κ B

To ask whether the reduction of the NF- κ B DNA binding activity in the FE-3 cells treated with azelastine for 24 h was due to inhibition of I κ B phosphorylation, cytoplasmic lysate was subjected to immunoprecipitation followed by western blot analysis. As expected, phospho-I κ B/I κ B protein ratio was reduced by approx. 25% and I κ B protein level was sustained following azelastine treatment as compared to vehicle treatment (Fig. 6), indicating that azelastine suppresses I κ B phosphorylation, thereby preventing ubiquitination and subsequent degradation of I κ B.

3.7. Effects of antiallergic drugs on mRNA expressions of the genes involved in the unfolded protein response

In plasmacytes, XBP-1 and other regulators lead to cessation of cell cycle, inhibit isotype switching and somatic hypermutation, and induce abundant immunoglobulin synthesis and secretion (Calame et al., 2003; Iwakoshi et al., 2003; Lin et al., 2003). XBP-1 has an important role not only for differentiation of B cells, but also for the unfolded protein response. The unfolded protein response allows cells to tolerate conditions of endoplasmic reticulum stress caused by misfolded proteins (Ma and Hendershot, 2004; Shen et al., 2004; Sriburi et al., 2004). Our hypothesis was that inhibition of IgE secretion from FE-3 cells by epinastine causes accumulation of IgE protein in the cytoplasm, thereby inducing the unfolded protein response. We examined the effect of antiallergic drugs, especially epinastine, on the expression of the genes involved in the unfolded protein response, including XBP-1 and Bip. Treatment of FE-3 cells

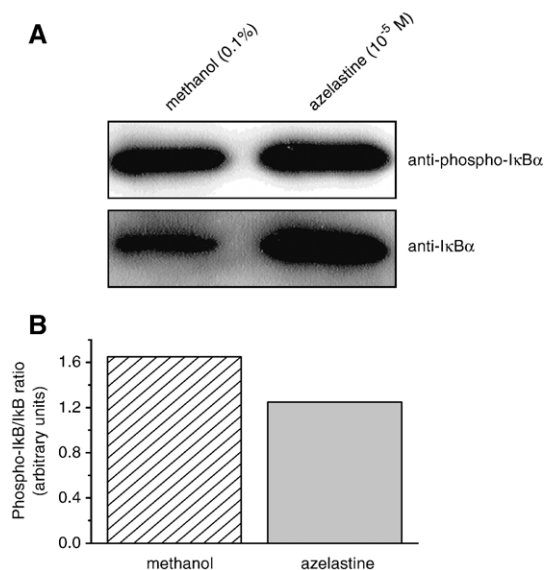


Fig. 6. Inhibitory effect of azelastine on the protein and phosphorylation levels of I κ B. Cytoplasmic extracts were immunoprecipitated by anti-I κ B α antibody coupled to Protein G Sepharose beads, followed by western blot analysis using anti-phospho-I κ B α . Results are representative of at least two independent experiments.

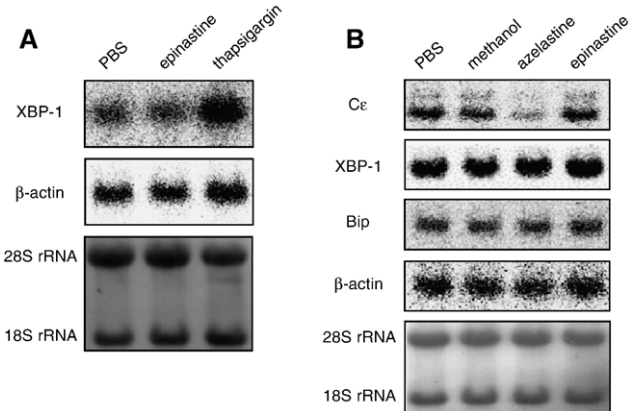


Fig. 7. Effects of antiallergic drugs on the mRNA expressions of the genes involved in the unfolded protein responses. XBP-1, Bip, and C ϵ mRNA expressions were determined by northern blot analysis as described in Materials and methods. A: Effects of antiallergic drugs on XBP-1 mRNA expression of FE-3 cells. The cells (2×10^5 cells/ml) were incubated in the growth medium with epinastine at 10^{-5} M, vehicle (PBS), or thapsigargin for 6 h, and then total RNA was prepared. Thapsigargin (1 μ M), a cell permeable inhibitor of Ca²⁺ ATPase, was used for inducing the unfolded protein response. B: Effects of antiallergic drugs on the mRNA expressions of XBP-1 and Bip in the FE-3 cells. The cells (2×10^5 cells/ml) were incubated in the growth medium with azelastine or epinastine at 10^{-5} M, or with vehicle (methanol or PBS) for 24 h, and then total RNA was prepared. Similar results were obtained in at least two independent experiments.

with thapsigargin, which induces unfolded protein response by inhibiting Ca²⁺ ATPase, augmented XBP-1 mRNA expression as shown in Fig. 7. No obvious effect of the drugs employed was however observed on the mRNA expressions of XBP-1 and Bip (Fig. 7), suggesting that epinastine treatment does not trigger the unfolded protein response.

4. Discussion

In this work, we demonstrate that both azelastine and epinastine inhibit IgE secretion from FE-3 cells. Epinastine could be regarded as a more potent inhibitor than azelastine from the viewpoint that the concentration of epinastine required for inhibiting IgE secretion is lower than that of azelastine as shown in Fig. 1. In contrast, azelastine exhibits a potent inhibition of IgE secretion compared to epinastine at highest concentration of drugs examined (10^{-5} M). These results appeared to be dependent on the difference in the mechanisms of action for inhibiting IgE secretion between azelastine and epinastine. Indeed, it has been revealed that azelastine, but not epinastine, has an inhibitory effect on the C ϵ mRNA expression, and that IgE synthesis is however suppressed by treatment with epinastine as well as with azelastine. These results suggest that azelastine and epinastine exert their effects on transcriptional and post-transcriptional level, respectively.

Our findings indicate that the inhibitory action of azelastine on IgE secretion is, at least in part, due to inhibition of C ϵ mRNA expression through reduction of NF- κ B activity involved in C ϵ gene expression. Our finding that I κ B phosphorylation was significantly decreased and I κ B protein level was sustained following azelastine treatment appears to indicate that the reduction of NF- κ B activity observed is due to inhibition of

I κ B phosphorylation by azelastine, thereby preventing ubiquitination and subsequent degradation of I κ B. FE-3 cells have a feature similar to plasma cells from the point of view that the class-switch recombination is accomplished in the C ϵ gene locus, presenting possibility that C ϵ gene expression could be predominantly regulated on the E μ and/or 3' IgH enhancer region (Michaelson et al., 1996; Ong et al., 1998; Sepulveda et al., 2004). In the human 4B6 B cell, which is an IgM⁺, IgD λ ⁺ cell line with an early germinal center phenotype and undergoes spontaneous switching to IgG, IgE and IgA, the hs1,2 element is shown to be the strongest IgH 3' enhancer using promoter assay (Kim et al., 2004). hs1,2 element also has an important role in plasmacytes such as U266, a human myeloma IgE λ ⁺ cell line (Kim et al., 2004). Transcription factors including Oct-1, Oct-2, HoxC4 and NF- κ B play a pivotal role in transcription of IgH genes (Arulampalam et al., 1997; Dariavach et al., 1991; Sepulveda et al., 2004). Of these transcription factors, we examined DNA binding activity of NF- κ B, because (1) NF- κ B is known to play a pivotal role in the expression of immunoglobulin heavy chain gene in mature B cells as well as in germline transcription in immature B cells (Geha et al., 2003; Sepulveda et al., 2004) and (2) azelastine reportedly inhibits NF- κ B DNA binding activity through inhibition of Ca²⁺ influx in mast cells thereby abrogating cytokine secretion (Kempuraj et al., 2003; Yoneda et al., 1997). Azelastine inhibits the secretion of IL-6, TNF- α and IL-8 from human mast cells through the inhibition of intracellular Ca²⁺ levels and of NF- κ B activation (Kempuraj et al., 2003). Azelastine (10⁻⁵ M) suppresses activation of NF- κ B in human monocytes, peripheral blood lymphocytes and gingival fibroblasts, leading to inhibition of cytokines and NO generation (Yoneda et al., 1997). Azelastine blocks L-type Ca²⁺ current in *in vitro* studies in smooth muscle cells from guinea pig trachea and ileum (Li et al., 2001). Ca²⁺- and PKC-dependent pathways accelerate I κ B α degradation in T cell lines (Steffan et al., 1995), suggesting that the reduction of NF- κ B activity by azelastine observed in the present study is due to abrogation of I κ B α degradation through its inhibitory effect on Ca²⁺- and/or PKC-dependent pathways. We cannot, however, rule out the possibility that azelastine acts on other transcription factors such as AP-1 and Oct-1/2, which possibly function on IgH 3' enhancer region (Kim et al., 2004). Further studies are necessary to identify the enhancer region of IgH where NF- κ B binds and functions to clarify the mechanism for inhibitory action of azelastine on C ϵ gene expression in rat.

AID, which is an enzyme expressed only in activated B cells, induces class switch recombination and somatic hypermutation, thereby causing modifications of immunoglobulin genes in B cells upon response to antigen (Dedeoglu et al., 2004; Durandy, 2003; Honjo et al., 2004). Class switch recombination is an event on the genes of immunoglobulin heavy chain constant region, leading B cells to secrete antibodies of different isotypes. Somatic hypermutation, on the other hand, is an event on the genes of immunoglobulin variable region, leading to the selection of B cells expressing a B cell receptor with high affinity for antigen. So far, there are at least two hypotheses for molecular mechanism of DNA cleavage by AID (Honjo et al., 2004): 1) editing by AID of mRNA that has not yet been identified to generate specific en-

donucleases for class switch recombination; and 2) cytosine deamination in DNA induced by AID, followed by DNA cleavage by base excision repair enzymes. The inhibition of AID mRNA expression by azelastine observed here is probably due to abrogation of NF- κ B DNA binding activity, as NF- κ B is reportedly involved in AID gene expression in the human B cell lines (Dedeoglu et al., 2004). Based on our results, we propose the possibility that azelastine is capable of acting on the class switch recombination through inhibition of AID during differentiation process of B cells.

In the present study, IgE protein levels in the microsome fraction were suppressed by treatment with epinastine as well as with azelastine. Our finding that epinastine did not inhibit C ϵ mRNA levels, but inhibited IgE protein synthesis of FE-3 cells, presents the possibility that its inhibitory effects on IgE secretion of FE-3 cells are due to its effect on IgE synthesis at post-transcriptional level. Kohyama et al. have reported that epinastine does not inhibit IL-8 mRNA levels, but inhibit IL-8 secretion from human eosinophils (Kohyama et al., 1997). They did not, however, observe the inhibition of IL-8 protein levels in the cells, probably due to employment of whole cell extracts for ELISA in their experiments. Epinastine is often called a mast cell stabilizer, because of its inhibition of the chemical mediator release from effector cells (Amon et al., 2000), but the mechanism underlying its effect still remains to be fully elucidated. Epinastine appears to differentially act on different signal transduction pathways as seen in the following reports. Epinastine inhibits histamine release from human skin mast cells induced by anti-IgE and calcium ionophore A23187. In contrast, epinastine has no apparent effect on the release of histamine induced by substance P (Amon et al., 2000) and of RANTES (regulated on activation normal T cell expressed and secreted) (Amon et al., 2000; Kamei et al., 1992) from eosinophil. Epinastine has no influence on the physiochemical property of lipid bilayers of cells (Kamei et al., 1992), indicating that inhibition of histamine release exerted by epinastine is unrelated to non-specific membrane stabilization. In general, Ca²⁺ plays an important role in both constitutive and regulated vesicular transport within the cell (Ahluwalia et al., 2001; Logan et al., 2003; Silver and Pappas, 2005). Then one possible explanation for the inhibitory action of epinastine on the effector cells is that it reduces intracellular Ca²⁺ levels, leading to modulation of the intracellular transport and secretion of proteins. In fact, epinastine inhibits Ca²⁺ incorporation into guinea pig lung mast cells, Ca²⁺ release from the intracellular Ca²⁺ store of rat mast cells, and calmodulin activity (Kamei et al., 1992). Epinastine also augments adenylate cyclase activity, but does not affect phosphodiesterase activity, leading to increase of cAMP contents, followed by inhibition of O₂ generation and Ca²⁺-calmoduline dependent process (Fukuishi et al., 1995; Kamei et al., 1992). These reports also raise the possibility that epinastine inhibits vesicular transport of IgE, leading to accumulation of IgE proteins in FE-3 cells, thereby causing the cells to make the unfolded protein response. Protein synthesis is generally downregulated in the unfolded protein response, whereas unfolded protein response induces a group of specific proteins including XBP-1 and molecular chaperones

such as Bip (Ma and Hendershot, 2004; Shen et al., 2004; Sriburi et al., 2004). Expressions of XBP-1 and Bip mRNA were not, however, augmented by treatment of FE-3 cells with epinastine. Further studies are necessary to identify the intracellular molecules that epinastine targets at the post-transcriptional level with regard to IgE production.

Taken together, these findings suggest that azelastine exerts its inhibitory effect on the secretion of IgE through the inhibition of Cε mRNA expression in the FE-3 cells, and that the inhibitory effect of epinastine is at least in part due to suppression of IgE synthesis at the post-transcriptional level. The present findings also demonstrate the possibility that azelastine and epinastine relieve the symptom of allergic diseases not only through their strong antihistamine and antimedator actions on effector cells such as mast cells, basophils and eosinophils, but also through inhibiting ongoing IgE secretion of IgE-producing cells.

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