Inhibitory activity of loratadine and descarboxyethoxyloratadine on histamine-induced activation of endothelial cells

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Summary

Background The allergic inflammatory reaction is characterized by leucocyte adherence and infiltration processes which are controlled by the expression of adhesion molecules on the surface of vascular endothelium. One of the main mediators implicated in allergic reactions is represented by histamine. Histamine is a potent activator of endothelial cells (EC); it induces the expression of P-selectin on the surface of endothelium and the secretion of IL-6 and IL-8.

Objectives Loratadine (L), a histamine H₁-antagonist, and one of its active metabolites, descarboxyethoxyloratadine (DCL), were studied at different concentrations for their ability to reduce the histamine-induced activation of human umbilical vein EC (HUVEC).

Methods HUVEC were stimulated in the presence of histamine at 10⁻⁴ M, 10⁻⁵ M and 10⁻⁶ M. We assessed by ELISA the expression of P-selectin on EC surface, as well as cytokine production in EC supernatants of 24 h culture.

Results Our results showed that for a 10⁻⁴ M-histamine stimulation, L and DCL have a similar inhibitory effect on P-selectin expression (IC₅₀ = 13 × 10⁻⁷ M and 23 × 10⁻⁷ M, respectively). L and DCL inhibited significantly IL-6 and IL-8 secretion induced by histamine with a more powerful efficiency of the active metabolite. For the dose of 10⁻⁴ M histamine, a 50% inhibition of IL-6 secretion was obtained for a dose of DCL equal to 2.6 × 10⁻⁷ M whereas the same magnitude of effects were only reached for a higher concentration of L (0.3 × 10⁻⁷ M). Similar results were obtained for IL-8 (IC₅₀ = 0.2 × 10⁻⁷ M for L and 10⁻⁸ M for DCL). Analysis of IL-8 mRNA expression by RT-PCR was in accordance with these data.

Conclusion These results demonstrate that both L and DCL are active to reduce the histamine-induced activation of EC. Interestingly, DCL seems to be effective at lesser concentrations especially to inhibit cytokine secretion.

Keywords: allergy, histamine, H₁-receptor antagonist, human endothelial cells, P-selectin, interleukin (IL)-6, IL-8


Introduction

Loratadine is a non-sedating selective peripheral H₁-receptor antagonist which is active following oral administration and currently used in the treatment of allergic disorders. In vitro studies previously showed that Loratadine has anti-allergic properties, resulting in mast cell stabilization, by inhibiting leukotrienes and histamine release from human lung fragments [1] and from MC9 cells, a murine mast cell line [2]. In addition to H₁ receptor blocking activity, Loratadine has the capacity to inhibit histamine release from human basophils [3]; to attenuate PAF-induced eosinophil chemotaxis and O₂ generation [4]. It was also
Materials and methods

Materials

Loratadine and DCL were provided by Schering-Plough (Levallois-Perret, France). Loratadine was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was 0.005% in the highest concentration of Loratadine used. Solutions of DMSO corresponding to the different final concentrations used to dissolve Loratadine were added to HUVEC: it was demonstrated that they did not affect cell activation and cell viability (data not shown). DCL was dissolved in RPMI 1640 medium (Gibco).

Endothelial cell culture

Isolation and culture

Human umbilical vein endothelial cells (HUVEC) were obtained as previously described [16]. Briefly, HUVEC were collected after a 15 min 0.2% collagenase (type IV, Sigma) treatment of umbilical vein and centrifugated (700 g, 10 min). After washings, cells were resuspended in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum (v:v; Gibco, France)-2 mm L-glutamine-25 µg/mL endothelial cell growth supplement (Sigma Chemical, St Louis, MO, USA)-100 µg/mL heparin (Choay, France)-100 U/mL penicillin G–10 µg/mL streptomycin. Then HUVEC were cultured in 35 mm diameter tissue culture wells at 37 °C, 5% CO2. At confluence, cells were trypsinized and subcultured at 10^4 cells/well in 96-well plates coated with 0.5% gelatin in PBS. All the experiments were performed with cells at confluence.

Stimulation

At confluence, HUVEC were washed with RPMI medium and incubated with L or DCL at different concentrations. Then, endothelial cells were stimulated in the presence or in the absence of various concentrations of histamine (10^{-6} M, 10^{-5} M or 10^{-4} M) for 10 min (P-selectin expression) or 24 h (cytokine production). Supernatants were recovered and kept at −20 °C until IL-6 and IL-8 quantifications. After washing twice with PBS, endothelial cells were fixed for 10 min at 4 °C with 0.5% glutaraldehyde in PBS, washed twice with 0.1 M PBS pH 7.4 containing 5 mM EDTA–0.1% (w:v) bovine serum albumin (BSA), and incubated (for one hour at room temperature or overnight at 4 °C) with the same buffer.

Enzyme Linked ImmunoSorbent Assay (ELISA)

The expression of P-selectin (GMP140) was measured by ELISA. This was performed on glutaraldehyde-fixed endothelial cells using the method described by Pober et al. [17] with some modifications. Briefly, fixed endothelial cells were washed with the PBS-BSA-EDTA buffer before being incubated for one hour with 100 µL/well of anti-GMP140 monoclonal antibody (MoAb) (Becton Dickinson, France) at the defined dose of 1.4 µg/mL. Cells were washed twice again and incubated for 1 h with 100 µL/well of 1:5000 (v:v) diluted peroxidase labelled anti-mouse IgG(H+L) goat antiserum (Diagnostic Pasteur, France). After four additional washings, 100 µL/well of 0.1 M citrate buffer pH 5.0 containing 0.03% H2O2 and 0.4 mg/mL o-phenylenediamine was added for 30 min, after which the reaction was stopped with 100 µL/well of 4N HCl. Optical density (OD) was read in a multiwell scanning spectrophotometer at 492 nm. All analyses were performed in triplicate.
Inhibitory activity of loratadine

Fig. 1. Time course of inhibition of histamine-induced P-selectin expression by Loratadine. HUVEC were pretreated with increasing concentrations of Loratadine (10^{-8} - 10^{-5} M) for the same time as the histamine stimulation (0) or one hour (1 h) and three hours (3 h) before the addition of histamine (10^{-5} M, 10 min). The effect of Loratadine on P-selectin expression was measured by ELISA. The results are expressed as the inhibition percentage of P-selectin expression (one experiment representative of two). The positive control was the P-selectin level obtained with 10^{-5} M histamine at 10 min. □, L (0); ◦, L (1 h); ○, L (3 h).

IL-6 and IL-8 production in EC supernatants was quantified using commercial kits (CLB, Amsterdam, the Netherlands). Results were expressed in nanograms per millilitre for IL-8 and in picograms per millilitre for IL-6 quantification.

Cytokine mRNA expression Endothelial cells were cultured with or without H_{1}-antagonists for 3 h and then stimulated with a 10^{-4} M histamine solution for 4 h at 37 °C and in the presence of 5% CO_{2}. Total cellular RNA was isolated by a guanidium isothiocyanate method and purified by cesium chloride modification. The RNA was reverse-transcribed to first strand cDNA using Moloney Mouse Leukaemia Virus (MMLV)-reverse transcriptase (Life Technologies, Paisley, UK) and oligo(dT) primers (Boehringer Mannheim, Germany). The first strand cDNA was amplified with primer sets for GAPDH (Glyceraldehyde 3 phospho-dehydrogenase), (Eurogentec) or human IL-8 (Eurogentec) in a 25 μL reaction using Taq polymerase (Perkin Elmer, New Jersey, USA), MgCl_{2}, dNTP and digoxigenin-11-dUTP (Boehringer Mannheim, Germany). Nucleotide sequences for oligonucleotide 5'- and 3'-primers, respectively, were as follows: GAPDH GTCTTCCACCATGGAGA and CCAAGTTGTACATGGATGACC; IL-8 TGGGACGCCTTCTGATGTT and AACTTCTCCAC-ACCCCTCTG. A 19-cycle PCR was performed concomitantly for GAPDH and IL-8 in a DNA thermal cycler (cyclone integra biosciences) using a denaturation step at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The number of cycles was previously determined by a kinetic experiment and corresponded to optimal conditions. When all the cycles are achieved, a primer extension period of 7 min at 72 °C was added. After PCR, 10 μL of the products was electrophoresed on a 1.5% agarose-ethidium bromide gel. The cDNA transfer to nylon membrane was accomplished by capillary blotting for 18 h. After transfer, membranes were dried and fixed under UV light. The cDNA were revealed by means of an antidigoxigenin antibody labelled with alkaline-phosphatase.

Fig. 2. Inhibition of histamine-induced P-selectin expression by Loratadine and DCL. HUVEC were pretreated with increasing concentrations of Loratadine or DCL (10^{-8} - 10^{-5} M) 1 h before the addition of histamine (10^{-5} M) for 10 min. (a) The effect of Loratadine on P-selectin expression was measured by ELISA. The results are expressed in OD × 10^{3} as the mean ± SEM of five experiments. *P<0.05 and **P<0.01 in comparison with cells stimulated by histamine alone. (b) Comparison of Loratadine (D) and DCL (◦). The results are expressed as the inhibition percentage of P-selectin expression. The positive control was the P-selectin level obtained with 10^{-5} M histamine; mean of five experiments. *P<0.05 and **P<0.01 in comparison with cells stimulated by histamine alone.
in a chemiluminescent system according to the manufacturer protocol (Boehringer Mannheim, Germany). The optical density of each band was read using a computer equipped with the Bio-Profil sofware (Vilber Lourmat). Results for IL-8 were expressed as percentage of GAPDH optical density.

**Statistical analysis**

Results are expressed as mean ± SEM for P-selectin expression and cytokine production. The percentage of inhibition was calculated as follows:

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\% \text{ inhibition} = \frac{[\text{histamine with H}_1\text{-receptor antagonist - medium alone} - \text{histamine alone - medium alone}]}{\text{histamine alone - medium alone}} \times 100
\]

The student's t-test for paired data was used to analyse the effect of H1-receptor antagonist (data obtained in the absence and in the presence of the drug were compared). P-values lower than 0.05 were regarded as significant.

**Results**

**P-selectin expression on the EC surface**

Preliminary experiments showed that the inhibitory effect of Loratadine on histamine-stimulated endothelial cells was influenced by the time of pretreatment by Loratadine. As shown in Fig. 1, the optimal effect was obtained when the cells were preincubated for 1 h with Loratadine before the histamine stimulation. This time was chosen for all subsequent experiments. The expression of P-selectin was enhanced after a stimulation of HUVEC for 10 min with \(10^{-5}\) M and \(10^{-3}\) M histamine and was maximal for \(10^{-4}\) M. When HUVEC were preincubated for 1 h with increasing amounts of \(L\) (from \(10\) to \(8\) M to \(10^{-3}\) M), Loratadine inhibited the histamine-induced P-selectin expression (Fig. 2a: \(P<0.01\) for \(10^{-5}\) M to \(10^{-3}\) M, \(P<0.05\) for \(10^{-4}\) M). The activity of Loratadine was dose-dependent (Fig. 2a).

Results obtained with DCL were similar even if the active metabolite seemed to be less efficient in comparison with \(L\) (Fig. 2b: \(P<0.05\) only for \(10^{-3}\) M and \(10^{-6}\) M). Fifty percent inhibition of \(10^{-3}\) M histamine-stimulated EC was obtained with \(L\) at the concentration of \(13\times10^{-9}\) M or with DCL at the concentration of \(23\times10^{-9}\) M.

**IL-6 production by EC**

Contrary to the inhibition of P-selectin expression, the
preincubation of endothelial cells with Loratadine for 1 h did not represent optimal conditions (data not shown). A longer time (corresponding to 3 h) was required to inhibit significantly the cytokine release by histamine-stimulated endothelial cells. As shown in a previous report [14], histamine is able to enhance IL-6 secretion by HUVEC in a dose-dependent manner. This observation was confirmed in this study where endothelial cells were stimulated with different doses of histamine (10⁻⁶ M, 10⁻⁵ M and 10⁻⁴ M). IL-6 production was evaluated in endothelial cells supernatants collected after 24 h of culture. The dose-dependent inhibitory effect of L and DCL was clearly demonstrated for each concentration of histamine (Fig. 3a and 3b, respectively). The highest concentration of L (10⁻⁵ M) was the most effective (P<0.01 with His 10⁻⁶ M and 10⁻⁵ M; P<0.05 with His 10⁻⁴ M) but a significant inhibition was detectable at lower Loratadine levels and also with a less potent histamine activation (Fig. 3a: P<0.05 in all situations except for the lowest concentration of L associated with a 10⁻⁴ M- or 10⁻⁶ M-histamine stimulation). DCL was tested at lower concentrations (Fig. 3b) as it seemed to be more powerful than L (Fig. 3c) and demonstrated to inhibit histamine-induced IL-6 secretion at the amount of 10⁻¹¹ M (P<0.05 in all cases except for DCL 10⁻⁶ M, 10⁻⁸ M and 10⁻¹² M associated with a 10⁻⁶ M-histamine stimulation). The concentrations of L and DCL inducing a 50% inhibition of IL-6 production were, respectively, 0.3×10⁻⁸ M and 2.6×10⁻¹² M for a 10⁻⁴ M-histamine stimulation.

**IL-8 production by EC**

As shown recently [15], we confirmed that histamine dose-dependently increased IL-8 production by endothelial cells. L and DCL have been used at the same concentrations as mentioned above. In the same way, the pretreatment with L or DCL inhibited the histamine-induced IL-8 production dose dependently (Fig. 4a: whatever the dose of His, P<0.01 for L 10⁻⁵ M or 10⁻⁶ M and P<0.05 for L 10⁻⁷ M or 10⁻⁸ M; Fig. 4b: P<0.05 in all cases except for DCL 10⁻¹² M associated with a 10⁻⁶ M- or 10⁻⁵ M-histamine stimulation). DCL seemed also to be more powerful than L to inhibit the IL-8 secretion by EC (Fig. 4c). For a 10⁻⁴ M histamine stimulation, the concentrations of histamine receptor antagonist inducing 50% of inhibition was estimated to 0.2×10⁻⁶ M and 10⁻⁹ M for L and DCL, respectively.

**Fig. 4.** Inhibition of histamine-induced IL-8 secretion by Loratadine and DCL. HUVEC were pretreated with increasing concentrations of Loratadine (10⁻⁸–10⁻⁶ M) or DCL (10⁻¹²–10⁻⁶ M) 3 h before addition of histamine (10⁻⁶–10⁻⁴ M). IL-8 was measured in supernatants 24 h after stimulation. (a) The effect of Loratadine. The results are expressed in pg/mL as the mean of six experiments. (b) The effect of DCL. The results are expressed in pg/mL as the mean of six experiments. (c) Comparison of Loratadine and DCL. The results are expressed as the inhibition percentage of IL-8 secretion. The positive control was the IL-8 level obtained with histamine at 10⁻⁴ M; mean of six experiments.
IL-8 mRNA expression by EC

When examined by RT-PCR (Fig. 5), mRNA for IL-8 was detectable in EC at basal level (lane 1) and was strongly expressed after a $10^{-4}$ M histamine stimulation (lane 2). The pretreatment with Loratadine inhibited this histamine-induced IL-8 mRNA expression dose-dependently (lanes 3, 4, 5: L at $10^{-5}$ M, $10^{-6}$ M and $10^{-7}$ M, respectively). Similar results were obtained with DCL (data not shown).

Discussion

The present findings are in accordance with previous studies demonstrating that histamine induces the transient expression of P-selectin on the surface of EC [13] and that histamine is able to augment dose-dependently the secretion of IL-6 [14] and IL-8 [15], as the specific mRNA expression of these two cytokines by EC. Because of the rapid degradation of histamine, it is difficult to evaluate precisely the histamine concentration into tissues. Except Foreman et al. who used high doses of histamine ($10^{-3}$ M to $5 \times 10^{-3}$ M) to induce P-selectin expression on EC [18], numerous studies used histamine at the same concentration range as we have done ($10^{-6}$ M to $10^{-4}$ M). This scale of histamine concentrations is reported to be compatible with the levels measured in tissues after mast cell degranulation [19-21]. In this report, we showed that Loratadine (in the concentration range of $10^{-3}$ M to $10^{-2}$ M) and DCL (in the concentration range of $10^{-12}$ M to $10^{-8}$ M) were able to inhibit histamine-induced EC activation at doses which are consistent with physiological conditions. Although it is clear that a concentration of $10^{-5}$ M of Loratadine is never reached in vivo, we believe that these results may have clinical relevance since the maximal serum concentration of Loratadine measured in humans, 1 h after a single oral administration of 10 mg Loratadine, was approximatively $5 \times 10^{-8}$ M, that is to say in the range of the concentrations tested in our experimental procedure. When Loratadine and DCL were compared, it appeared that Loratadine exhibited a dose-dependent and more marked activity on P-selectin expression than on cytokine release. On the contrary, the active metabolite DCL, that expresses also a dose-dependent inhibitory effect, seems to be more powerful than Loratadine to reduce the cytokine release.

The role of P-selectin in allergic inflammation is largely discussed: P-selectin is produced constitutively, stored intracellularly in Weibel-Palade bodies of HUVEC [22] and capable of being rapidly translocated to the cell surface after endothelium exposure to different mediators such as thrombin, histamine, C5a, IL-3 [23]. P-selectin is implicated in the adhesion of neutrophils [24] and eosinophils [25] to EC. Since Loratadine is a good inhibitor of P-selectin expression on histamine-stimulated HUVEC, it is tempting to speculate that it could modulate the inflammatory reaction by reducing the infiltrate of leucocytes at the first step of adhesion.

Concerning IL-6, this pleiotropic proinflammatory cytokine is produced in different allergic diseases following an allergen challenge [26,27] and can be considered as a major mediator of the inflammatory response [28]. Interestingly, IL-6 deficient mice are unable to develop an efficient inflammatory response [29]. As for IL-8, its production has numerous consequences on tissular environnement. IL-8 is chemoattractant in vitro for neutrophils, lymphocytes, basophils [30,31] and primed eosinophils [32]. The histamine release by basophils can also be modulated by IL-8: at low concentration ($10^{-8}$ M), IL-8 inhibited the histamine release induced by IL-3 or Histamine Releasing Factors [33] but at high concentration ($10^{-5}$ M), IL-8...
induced the histamine release by basophils [34]. These data could suggest that, by inhibiting the IL-6 and IL-8 production by histamine-stimulated EC, DCL and to a lesser extent Loratadine, could participate in the downregulation of the late phase reaction and especially the leucocyte recruitment.

In summary, the effect of Loratadine and DCL on the activation of EC by a stimulus compatible with pathophysiological conditions observed in allergic diseases may be of importance in the understanding of the mechanisms of action of H1-blockers. The significance of these results may be of relevance for the treatment of allergic diseases since cell adhesion molecules expression and cytokine production by EC could be inhibited by H1 receptor antagonists. By reducing these functions, Loratadine and DCL could limit the leucocyte recruitment in inflammatory reactions. Consequently Loratadine and DCL appear at least in our experimental conditions to present some interesting anti-inflammatory properties.

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References


