



Increased serum soluble vascular endothelial cadherin levels in patients with chronic spontaneous urticaria

Tao Chen, PhD^{*}; Zai-pei Guo, MD[†]; Wen-ju Wang, MD^{*}; Li-xin Fu, MD^{*}; Qiao-mei Sun, MB^{*}; Pei-mei Zhou, MD^{*}

^{*} Department of Dermato-Venereology, Chengdu Second People's Hospital, Chengdu, Sichuan, China

[†] Department of Dermato-Venereology, West China Hospital of Sichuan University, Chengdu, Sichuan, China

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ABSTRACT

Background: Chronic spontaneous urticaria (CSU) is a common skin disease characterized by recurrent itchy wheals with or without angioedema that lasts longer than 6 weeks. Vascular endothelial (VE)-cadherin is an endothelial cell-specific adhesion molecule that plays critical roles in angiogenesis and endothelial permeability.

Objective: To investigate serum levels of soluble VE (sVE)-cadherin in patients with CSU.

Methods: Serum levels of sVE-cadherin in patients with CSU, patients with atopic dermatitis, and healthy controls were determined by enzyme-linked immunosorbent assay. In addition, changes in sVE-cadherin serum levels were compared in patients with CSU before and after H₁ antihistamine treatment. Furthermore, the effects of histamine on sVE-cadherin release by HMEC-1 cells were determined by enzyme-linked immunosorbent assay. The inhibition effects of H₁ antihistamine and H₂ antihistamine on sVE-cadherin release, VE-cadherin phosphorylation, and VE-cadherin disruption were evaluated in histamine-treated HMEC-1 cells by western blot and immunofluorescence.

Results: Serum levels of sVE-cadherin in patients with CSU were significantly higher than those in patients with atopic dermatitis and healthy controls. Serum sVE-cadherin levels in patients with CSU were correlated with the severity of CSU according to Urticaria Activity Scores. Furthermore, serum sVE-cadherin levels in patients with CSU at pretreatment decreased after H₁ antihistamine treatment. In addition, histamine markedly induced sVE-cadherin release in HMEC-1 cells. Moreover, H₁ antihistamine, but not H₂ antihistamine, significantly inhibited sVE-cadherin release in histamine-treated HMEC-1 cells. Western blot data showed that histamine induced phosphorylation of VE-cadherin in HMEC-1 cells, which was blocked by H₁ antihistamine.

Conclusion: The present data showed serum levels of sVE-cadherin are increased in patients with CSU. Histamine-induced sVE-cadherin release from endothelial cells could play a role in the pathogenesis of CSU.

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Introduction

According to the guideline of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA[2]LEN), the European Dermatology Forum (EDF), and the World Allergy Organization (WAO),¹ urticaria can be divided into 2 major types: spontaneous urticaria and inducible urticaria. Chronic spontaneous urticaria (CSU) is defined as the spontaneous appearance of itchy wheals with or without angioedema that lasts longer than 6 weeks. Although the actual

pathogenesis of CSU is far from clear, abundant evidence has indicated that the release of histamine from activated mast cells and basophils and increased microvascular permeability induced by vascular active substances, including histamine, play crucial roles in the development of this disease.

The integrity of the vascular barrier is dependent on organized tight and adherens junctions in endothelial cells. Vascular endothelial (VE)-cadherin is an endothelial cell-specific adhesion molecule located at the junctions between endothelial cells and is involved in angiogenesis and vascular permeability. It has been reported that vascular active substances (such as vascular endothelial growth factor), proinflammatory cytokines (such as tumor necrosis factor- α), chemokines, and oxidative stress can increase microvascular permeability by targeting VE-cadherin.^{2–5} A previous study also indicated that histamine can induce the tyrosine phosphorylation of VE-cadherin, lead to the dissociation of this

Reprints: Zai-Pei Guo, MD, Department of Dermato-Venereology, West China Hospital of Sichuan University, No 37, Guoxue Alley, Chengdu, 610041 Sichuan, China; E-mail: guozp930@163.com.

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Table 1
Cohort Demographics^a

	CSU	AD	Controls
Patients, n	54	28	42
Age (y), mean (range)	24.5 (11–41)	21 (13–35)	22 (10–39)
Male/female, n	25/29	13/15	20/22
Disease activity, median (IQR)	3 (1–6) ^b	34.6 (18.8–82.5) ^c	NA
ASST, median (IQR)	1 (1–2)	NA	NA

Abbreviations: AD, atopic dermatitis; ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; IQR, interquartile range; NA, not applicable.

^aData in the table showed no significant difference among groups.

^bDisease activity in patients with CSU was assessed according to daily Urticaria Activity Scores.⁹

^cDisease activity in patients with AD was assessed by the Scoring of Atopic Dermatitis index.¹⁰

protein, and increase endothelial permeability in human dermal microvascular endothelial cell line (HMEC-1) cells.⁶ In addition, in some immune and inflammatory conditions, soluble VE (sVE)-cadherin can be released into the circulation because of the proteases of the extracellular domain of VE-cadherin. Increased serum sVE-cadherin levels have been reported in patients with Henoch-Schönlein purpura, urticarial vasculitis, allergic vasculitis, and Behcet disease.^{7,8} To explore whether histamine-induced sVE-cadherin release might be associated with the pathogenesis of CSU, this study investigated serum levels of sVE-cadherin in patients with CSU and compared the changes of sVE-cadherin serum levels in patients with CSU before and after H₁ antihistamine treatment. Furthermore, the effects of histamine on sVE-cadherin release by HMEC-1 cells were determined. The inhibition effects of H₁ and H₂ antihistamines on sVE-cadherin release, VE-cadherin phosphorylation, and VE-cadherin cleavage were evaluated in histamine-treated HMEC-1 cells in this study.

Methods

Ethics Statement

This human research was approved by the ethics committee of Chengdu Second People's Hospital (Chengdu, China; number 2015007). All patients were Chinese and gave written informed consent.

Patients and Control Cohorts

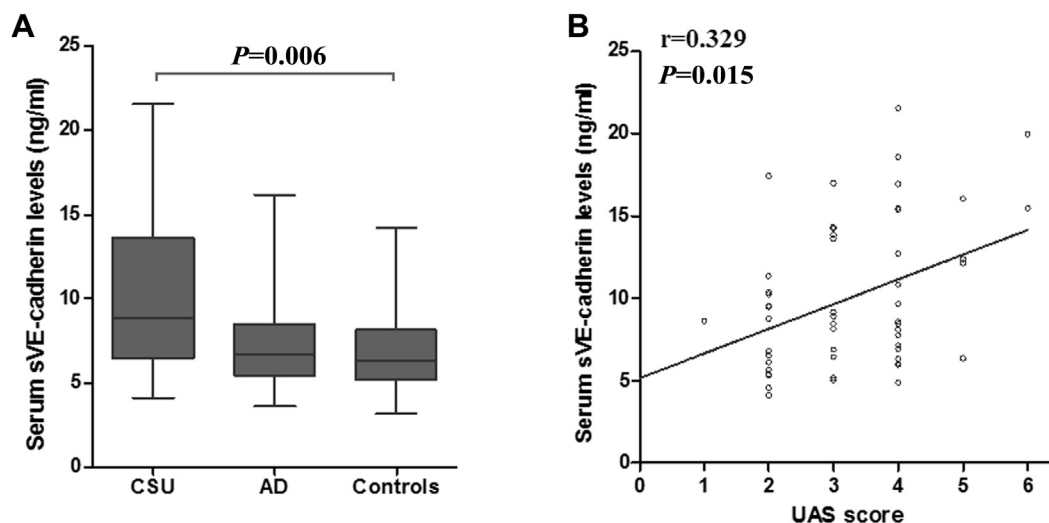
Fifty-four patients with CSU, 28 patients with atopic dermatitis (AD), and 42 age- and sex-matched healthy controls were enrolled in this study. CSU was confirmed and diagnosed by Chengdu Second People's Hospital. In addition, 42 healthy controls were recruited among healthy subjects who were free of other diseases. A detailed history and physical examination were conducted for each patient and the basic demographic information from each group is listed in Table 1. The severity of CSU was evaluated according to weekly and daily Urticaria Activity Scores (UAS7 and UAS).⁹ The autologous serum skin test (ASST) was performed in each patient with CSU.¹¹ The disease severity in patients with AD was determined by the Scoring of Atopic Dermatitis index.¹⁰ All patients and healthy subjects were not treated 2 weeks before this study. Of these, 21 patients with CSU were treated with oral H₁ antihistamine (cetirizine 10 mg once daily or fexofenadine 60 mg twice daily) at the licensed dose for 2 weeks and were not taking any other medications. Patients with CSU who received antihistamines were selected randomly. None of the healthy subjects in the study were taking any medications. Serum samples from these patients before and after treatment were obtained.

Assay for sVE-Cadherin Levels

Levels of sVE-cadherin in serum and cultured supernatants were detected with commercially available enzyme-linked immunosorbent assay kits (catalog number DCADV0; R&D Systems Inc, Minneapolis, Minnesota) according to the manufacturer's instruction.

Cell Culture

The HMEC-1 cells were obtained from the Centers for Disease Control and Prevention (Atlanta, Georgia). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, New York) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), and 100 U/mL of penicillin, 100 U/mL of streptomycin, and 2 mmol/L of L-glutamine (Gibco). In this study, HMEC-1 cells were seeded in plates at 1×10^5 cells/cm². After a 48-hour incubation, HMEC-1 cells were treated with 100 μ mol/L of histamine at different time points or histamine (100 μ mol/L for 20 minutes) plus different antihistamines.



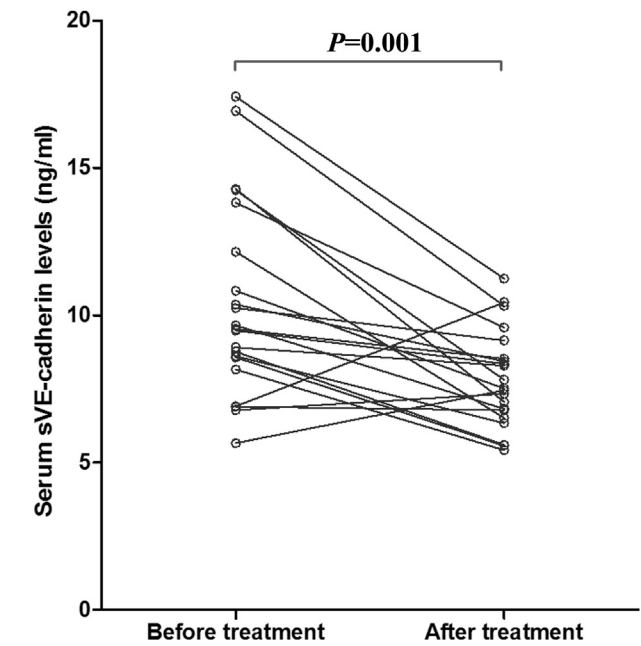


Figure 2. H₁ antihistamine decreases serum levels of sVE-cadherin in patients with chronic spontaneous urticaria. Wilcoxon sign-rank test for paired data was performed to compare serum sVE-cadherin levels in patients with chronic spontaneous urticaria before and after H₁ antihistamine treatment. sVE, soluble vascular endothelial.

Cell Viability Assay

The effects of histamine and different antihistamines on cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (1 × 10⁴ cells/well) and treated with histamine and different antihistamines (10 μmol/L, 100 μmol/L, and 1 mmol/L) for 0.5 hour. Cells were washed twice with phosphate buffered saline. Then, the MTT solution (0.5 mg/mL) was added to each well and cells were incubated at 37°C for 4 hours. After centrifugation, 150 μL of dimethyl sulfoxide was added to each well for formazan solubilization. The optical densities at 570 nm were determined using a microplate reader.

Western Blot Analysis

The expression of phosphorylated VE-cadherin in HMEC-1 cells was measured by western blot. Cells were pretreated with different antihistamines (10 μmol/L for 20 minutes; H₁ antihistamine [fexofenadine, cetirizine, or loratadine], H₂ antihistamine [cimetidine or ranitidine], or H₁ antihistamine plus H₂ antihistamine [fexofenadine plus ranitidine]) and then exposed to 100 μmol/L of histamine for 20 minutes. Histamine and antihistamines were obtained from Sigma-Aldrich (St. Louis, Missouri). After treatment, cells were collected and lysed in ice-cold RIPA buffer (EMD Millipore, Billerica, Massachusetts) containing 1% phenyl-methylsulfonyl fluoride. The samples were vortex mixed for lysis for a few seconds every 15 minutes at 4°C for 1 hour and centrifuged at 13,000 rpm for 20 minutes at 4°C. Then, the samples were heated at 98°C for 10 minutes and briefly cooled on ice. Then, total protein was extracted. Protein samples of 40 μg were

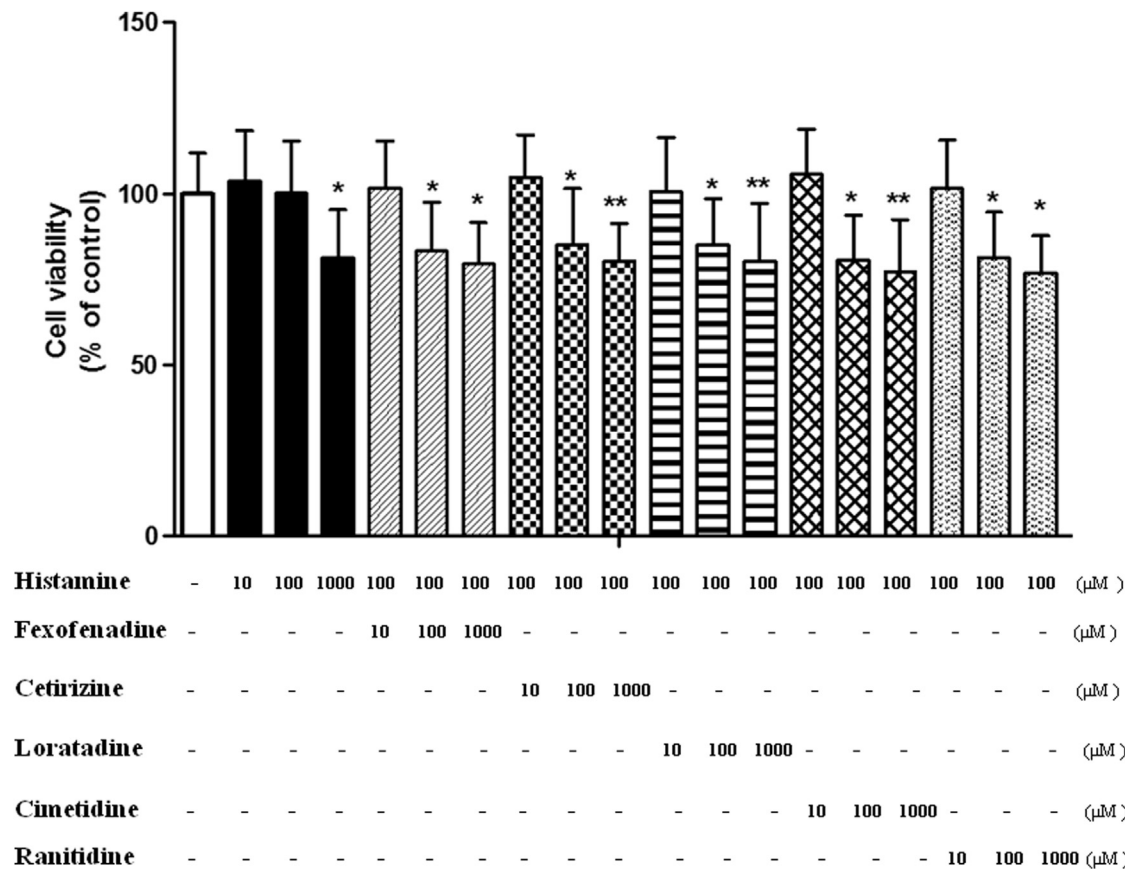


Figure 3. Effects of histamine and different antihistamines on percentage of cell viability of HMEC-1 cells. Cells were incubated with various concentrations of histamine and different antihistamines (10 μmol/L, 100 μmol/L, and 1 mmol/L) for 0.5 hour. All data are presented as mean ± SD (n = 10 per group). *P < .05, **P < .01 compared with control group.

electrophoresed on 12% Tris-glycine gels, subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Subsequently, membranes were incubated with anti-phosphorylated VE-cadherin antibody (clone C13F9; Cell Signaling Technology, Beverly, Massachusetts) or glyceraldehyde 3-phosphate dehydrogenase at 4°C overnight and with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour. The expression of phosphorylated VE-cadherin was determined with enhanced chemiluminescence reagents and exposure to a Kodak x-ray film (Eastman Kodak, Rochester, New York). The results were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase.

Immunofluorescence

The expression of VE-cadherin in HMEC-1 was detected by immunofluorescence. HMEC-1 cells, grown on glass coverslips, were pretreated with different antihistamines (10 μ mol/L for 20 minutes; H₁ antihistamine [fexofenadine, cetirizine, or loratadine], H₂ antihistamine [cimetidine or ranitidine], or H₁ antihistamine plus H₂ antihistamine [fexofenadine plus ranitidine]) and then exposed to 100 μ mol/L of histamine for 20 minutes. Then, cells were fixed using 4% paraformaldehyde (Sigma-Aldrich), washed 3 times in phosphate buffered saline, and then incubated with primary antibody at 4°C overnight (anti-VE-cadherin antibody, 1:50 dilution; clone D87F2; Cell Signaling Technology). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole for 5 minutes. Fluorescence images were captured using laser scanning confocal microscopy (Olympus, Tokyo, Japan).

Statistical Analysis

Each experiment was carried out at least 3 times. The data presented in Table 1 and Figures 1 and 2 were not normally distributed and are presented as median $\pm$ interquartile range with non-parametric statistical tests (Mann-Whitney *U* Test, Spearman test, or Wilcoxon sign-rank test). The data in the other figures were normally distributed and are presented as mean $\pm$ SD with parametric tests (1-way analysis of variance). A *P* value less than .05 was set as statistically significant.

Results

Increased Serum Levels of sVE-Cadherin in Patients With CSU

Serum sVE-cadherin levels in patients with CSU, patients with AD, and control subjects were detected by enzyme-linked immunosorbent assay. As shown in Figure 1A, serum levels of sVE-cadherin (8.84 ± 6.90 ng/mL) in patients with CSU were significantly higher than those in patients with AD (6.67 ± 2.53 ng/mL) and controls (6.35 ± 2.86 ng/mL). Correlation analysis was performed to explore the relation of serum sVE-cadherin levels to disease severity in patients with CSU. As shown in Figure 1B, a positive correlation between serum sVE-cadherin levels and UASs was observed in patients with CSU. No significant correlation was found between UAS7 and sVE-cadherin levels. The prevalence of positive ASST results in the CSU group was 44.4%. Serum sVE-cadherin levels in patients with CSU did not differ by positive or negative ASST results (*P* > .05).

H₁ Antihistamine Decreases Serum sVE-Cadherin Levels in Patients With CSU

In this study, 21 patients with CSU were treated with H₁ antihistamine (cetirizine or fexofenadine) at the licensed dose for 2 weeks. Serum levels of sVE-cadherin were compared in patients with CSU before and after H₁ antihistamine treatment. As shown in Figure 2, serum sVE-cadherin levels in patients with CSU before

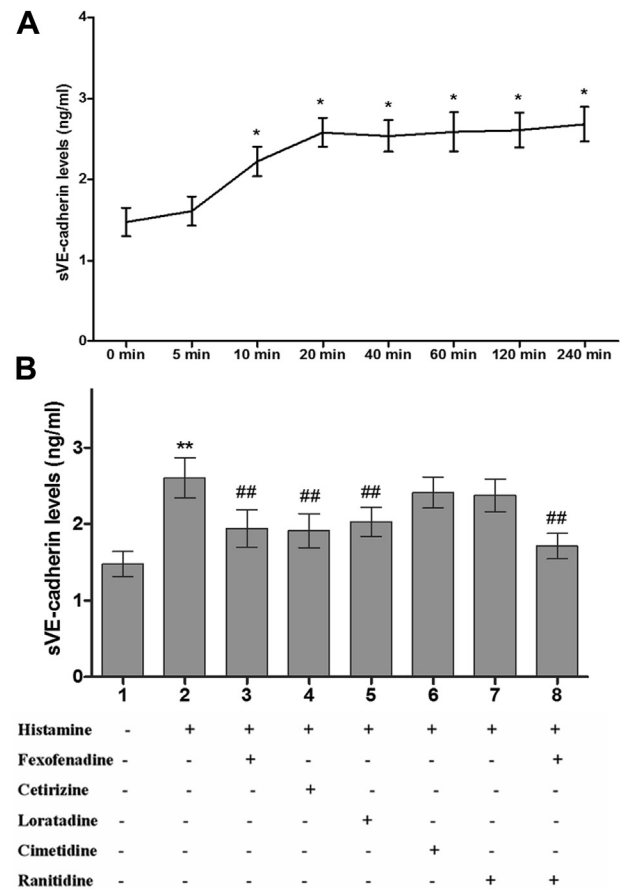


Figure 4. Histamine upregulates but H₁ antihistamine inhibits sVE-cadherin release from HMEC-1 cells. The release of sVE-cadherin in the supernatants from HMEC-1 cells was detected by enzyme-linked immunosorbent assay. (A) HMEC-1 cells were treated by histamine at different time points. (B) HMEC-1 cells were pretreated with different antihistamines (10 μ mol/L for 20 minutes; H₁ antihistamine [fexofenadine, cetirizine, or loratadine], H₂ antihistamine [cimetidine or ranitidine], or H₁ antihistamine plus H₂ antihistamine [fexofenadine plus ranitidine]) and then exposed to 100 μ mol/L of histamine for 20 minutes. Values are expressed as mean $\pm$ SD (*n* = 6). One-way analysis of variance was used for analysis. ***P* < .01 compared with control group; ##*P* < .01 compared with group treated with 100 μ mol/L of histamine. sVE, soluble vascular endothelial.

treatment (9.53 ± 3.57 ng/mL) decreased after treatment (7.52 ± 1.74 ng/mL; *P* = .001).

Effects of Histamine and Different Antihistamines on Percentage of Cell Viability of HMEC-1 Cells

Cells were treated with histamine and different antihistamines at concentrations ranging from 10 μ mol/L to 1 mmol/L for 0.5 hour. The MTT assay was performed to determine cytotoxicity. As presented in Figure 3, no significant detriment to cell viability was observed when cells were treated with less than 100 μ mol/L of histamine and less than 10 μ mol/L of different antihistamines for 0.5 hour. However, after exposure to 1 mmol/L of histamine or 100 μ mol/L of different antihistamines for 0.5 hour, cell viability was significantly decreased in the control group. Thus, 100 μ mol/L of histamine and 10 μ mol/L of different antihistamines were used as the maximum doses throughout subsequent experiments to exclude toxicity.

Histamine Induces sVE-Cadherin Release From HMEC-1 Cells

A previous study indicated that histamine can induce the dissociation of VE-cadherin in HMEC-1 cells.⁶ This study

investigated the effects of histamine on sVE-cadherin release in HMEC-1 cells at different time points. As presented in Figure 4A, as soon as 10 minutes after histamine treatment, sVE-cadherin levels were significantly increased in the supernatants from HMEC-1 cells compared with those from untreated HMEC-1 cells. Moreover, 20 minutes after histamine treatment, sVE-cadherin levels in the supernatants from HMEC-1 cells reached their peak value. This study also investigated the effects of different antihistamines on sVE-cadherin release in histamine-treated HMEC-1 cells. HMEC-1 cells were pretreated with different antihistamines (10 μ mol/L for 20 minutes) and then incubated with 100 μ mol/L of histamine for 20 minutes. As shown in Figure 4B, H₁ antihistamine (fexofenadine, cetirizine, or loratadine) or H₁ antihistamine plus H₂ antihistamine (fexofenadine plus ranitidine) markedly blocked the release of sVE-cadherin in histamine-treated HMEC-1 cells ($P < .01$ for all comparisons). However, there was no significant difference between the fexofenadine group and the fexofenadine plus ranitidine group ($P > .05$).

Histamine Induces VE-Cadherin Phosphorylation

The expression of phosphorylated VE-cadherin in HMEC-1 cells was detected by western blot. As presented in Figure 5A, histamine obviously upregulated expression levels of phosphorylated VE-cadherin in HMEC-1 cells. H₁ antihistamine (fexofenadine, cetirizine, or loratadine) and H₁ antihistamine plus H₂ antihistamine (fexofenadine plus ranitidine) prominently blocked the phosphorylation of VE-cadherin in histamine-induced HMEC-1 cells. No significant difference was observed between the fexofenadine group and the fexofenadine plus ranitidine group.

Histamine Leads to VE-Cadherin Disruption

The disruption of VE-cadherin in histamine-treated HMEC-1 cells was observed by double staining for VE-cadherin, fluorescein isothiocyanate, and 4'-6-diamidino-2-phenylindole. As presented in Figure 5C, abundant expression of VE-cadherin was observed in untreated HMEC-1 cells. Obvious VE-cadherin cleavage was found

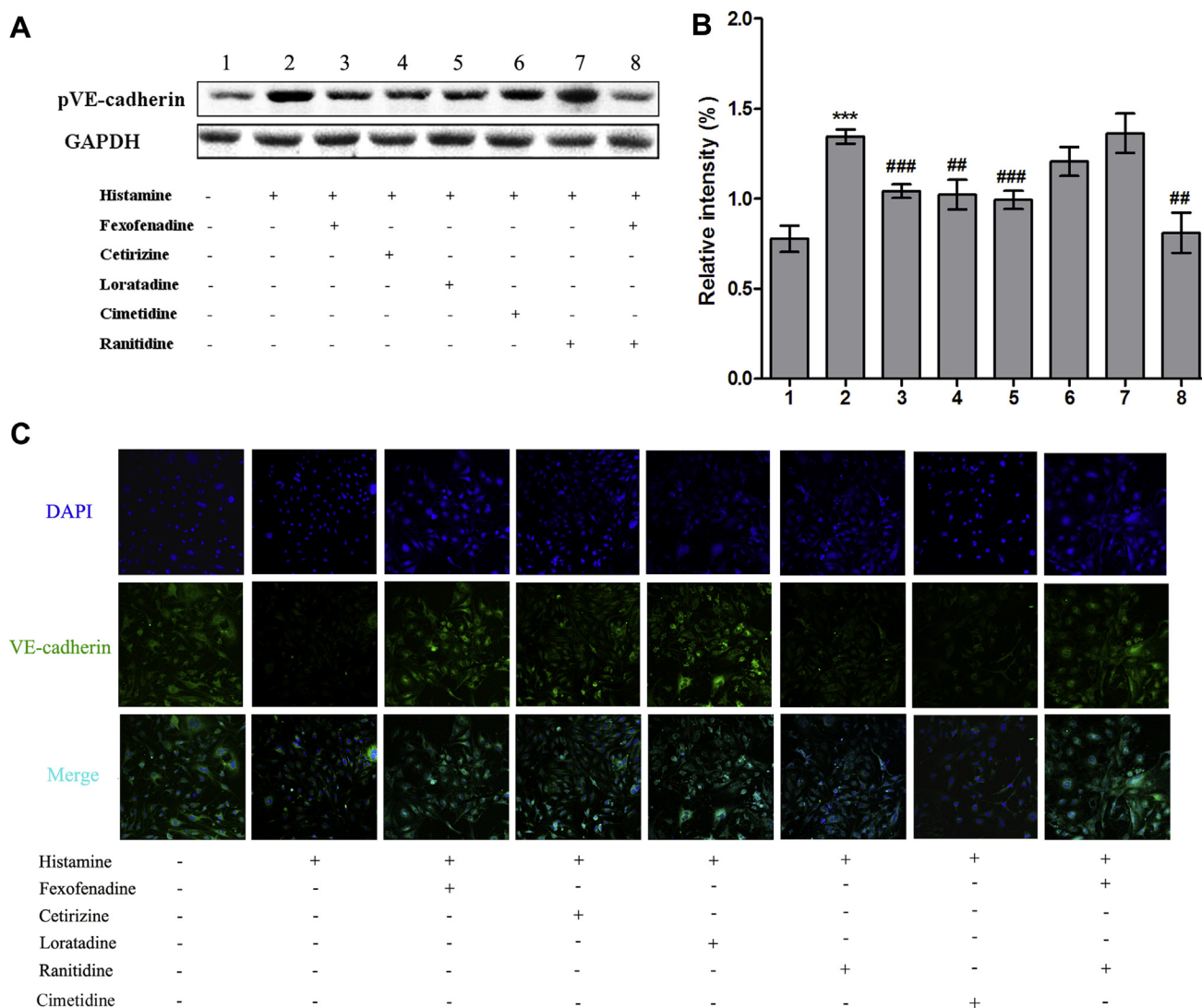


Figure 5. Histamine induces but H₁ antihistamine blocks VE-cadherin phosphorylation and VE-cadherin disruption in HMEC-1 cells. HMEC-1 cells were pretreated with different antihistamines and then exposed to 100 μ mol/L of histamine for 20 minutes. (A) Afterward, protein samples were obtained to measure expression levels of phosphorylated VE-cadherin by western blots. Representative blots from 3 experiments are presented. (B) Intensity was measured by densitometry. (C) After treatment, the expression of VE-cadherin in HMEC-1 cells was observed by immunofluorescence double staining for VE-cadherin, fluorescein isothiocyanate, and DAPI. VE-cadherin was stained green and nuclei were stained blue. Values are expressed as mean $\pm$ SD ($n = 6$). One-way analysis of variance was used for analysis. *** $P < .001$ compared with control group; ## $P < .01$, ### $P < .001$ compared with histamine-treated group. DAPI, 4'-6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VE, vascular endothelial.

in histamine-treated HMEC-1 cells. In contrast, H₁ antihistamine (fexofenadine, cetirizine, or loratadine) and H₁ antihistamine plus H₂ antihistamine (fexofenadine plus ranitidine) attenuated the change.

Discussion

Increased endothelial permeability is a vital pathophysiologic feature in urticaria. The adhesion molecule VE-cadherin plays essential roles in endothelial barrier integrity. In this study, we found that patients with CSU had significantly higher sVE-cadherin serum levels compared with patients with AD and control subjects. Moreover, serum levels of sVE-cadherin were closely associated with the severity of CSU (UAS but not UAS7). The data suggest that the release of sVE-cadherin could play a role in the pathogenesis of CSU. Serum sVE-cadherin could be a new biomarker for evaluating the current severity of CSU.

The release of sVE-cadherin represents a signal for VE-cadherin damage in endothelial cells. A previous study also established that histamine can induce VE-cadherin damage.⁶ Similarly, the present results showed that sVE-cadherin serum levels were decreased in patients with CSU after H₁ antihistamine treatment. In contrast, the results also indicated that histamine can lead to the secretion of sVE-cadherin in the supernatant from HMEC-1 cells. Moreover, 20 minutes after histamine treatment, sVE-cadherin levels in the supernatants from HMEC-1 cells reached their peak value. Hence, we suggest that histamine-induced sVE-cadherin release is a rapid and transient process in CSU. Such results also could explain the correlation between sVE-cadherin levels and UAS but not UAS7. Furthermore, we found that histamine can induce VE-cadherin phosphorylation and cleavage in HMEC-1 cells. This suggests that higher serum levels of sVE-cadherin might, at least in part, be induced by histamine from dermal microvascular endothelial cells. Histamine, a monoamine neurotransmitter, can activate 4 G-protein-coupled receptors (H₁, H₂, H₃, and H₄ receptors).^{12,13} Of these, H₁ and H₂ receptors are most commonly investigated in the pathogenesis of CSU. According to the EAACI, GA(2)LEN, EDF, and WAO guideline,¹ H₁ antihistamine, especially modern second-generation H₁ antihistamine, is strongly recommended as first-line treatment in urticaria. Furthermore, Adderley et al¹⁴ found that multiple signaling pathways contribute to histamine-induced endothelial barrier dysfunction through the H₁ receptor. Some previous studies have reported that the combination of H₁ and H₂ antihistamines have additional therapeutic effects compared with H₁ antihistamine alone.^{15,16} To determine whether the H₁ or H₂ receptor is involved in histamine-induced sVE-cadherin release and to provide experimental evidence for supporting the clinical use of H₁ or H₂ antihistamine in the treatment of urticaria, the effects of different antihistamines on sVE-cadherin release, VE-cadherin phosphorylation, and VE-cadherin damage in histamine-induced HMEC-1 cells were investigated in this study. The results demonstrated that H₁ antihistamine, but not H₂ antihistamine, markedly blocked histamine-induced sVE-cadherin release, VE-cadherin

phosphorylation, and VE-cadherin cleavage in HMEC-1 cells. Conversely, no further improvement was found in the H₁ plus H₂ antihistamine treatment group. Therefore, histamine-induced sVE-cadherin release, VE-cadherin phosphorylation, and VE-cadherin disruption in HMEC-1 cells might be associated with activation of the H₁ receptor. H₁ antihistamine, but not H₂ antihistamine, might attenuate histamine-induced VE-cadherin damage.

In conclusion, this study provides the first observation of serum levels of sVE-cadherin in patients with CSU. We showed that patients with CSU have significantly increased sVE-cadherin serum levels. The release of sVE-cadherin might be associated with the activation of the H₁ receptor in histamine-treated endothelial cells. Histamine-induced sVE-cadherin release from endothelial cells could play a role in the pathogenesis of CSU.

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