Histamine Induces Tyrosine Phosphorylation of Endothelial Cell-to-Cell Adherens Junctions

Paraskevi Andriopoulou, Pilar Navarro, Adriana Zanetti, Maria Grazia Lampugnani, Elisabetta Dejana

Abstract—Endothelial adherens junctions (AJ) promote intercellular adhesion and may contribute to the control of vascular permeability. These structures are formed by a transmembrane and cell-specific adhesive protein, vascular endothelial (VE)-cadherin, which is linked by its cytoplasmic tail to intracellular proteins called catenins (α-catenin, β-catenin, and plakoglobin) and to the actin cytoskeleton. Little is known about the functional regulation of AJ in endothelial cells. In this study, we analyzed the effect of histamine on AJ organization in cultured endothelial cells. We first observed that histamine induced detectable intercellular gaps only in loosely-confluent cells, whereas this effect was strongly reduced or absent in long-confluent cultures. Despite this difference, in vitro permeability was augmented by histamine in both conditions. In resting conditions, tyrosine phosphorylation of AJ components and permeability values were higher in recently-confluent cells than in long-confluent cells. Histamine did not affect the phosphorylation state of AJ in long-confluent cells but strongly increased this parameter in long-confluent cultures. In addition, in long-confluent cells, histamine caused dissociation of VE-cadherin from the actin cytoskeleton measured by a decrease of the amount of the molecule in the detergent-insoluble fraction of the cell extracts. Dibutyryl cAMP was able to prevent the effect of histamine on both tyrosine phosphorylation of AJ components and on endothelial permeability. The effect of histamine was specific for VE-cadherin because the phosphorylation state of neural (N)-cadherin, the other major endothelial cadherin, was unchanged by this agent. Hence AJ components are a target of histamine activation cascade; we suggest that induction of tyrosine phosphorylation of VE-cadherin and catenins contributes to the histamine effect on permeability, even in absence of frank intercellular gaps and cell retraction. (Arterioscler Thromb Vasc Biol. 1999;19:2286-2297.)

Key Words: endothelial cells ■ inflammatory mediators ■ adhesion molecules ■ cell-to-cell interactions

Endothelial cells lining blood vessels regulate the passage of plasma proteins and circulating cells between blood and the underlying tissues. This function is finely regulated by different systems. In general, plasmatic components traverse the endothelial barrier through both transcellular and paracellular pathways.1,2 The transcellular pathway defines the passage of plasma solutes through the endothelial cytoplasm by the action of highly regulated vesicular systems such as caveolae3,4 and vesiculo-vacuolar organelles5 and by the formation of fenestrae.6 The paracellular pathway is essentially regulated by the opening or weakening of intercellular junctions.1,2

The structure and the molecular components of endothelial cell-to-cell junctions have been partially characterized. Endothelial cells present tight junctions and adherens junctions (AJ), which have a general organization similar to that described in epithelial cells (for review see References 7 and 8). In addition, other adhesive proteins such as platelet endothelial cell adhesion molecule-1, S-endo 1/muc 18, endoglin, or CD347,8 have been found to be concentrated at intercellular contacts in the endothelium. AJ are of particular interest because they are ubiquitous along the vascular tree.7 AJ are formed by transmembrane adhesive proteins belonging to the cadherin superfamily that are bound through their cytoplasmic tail to a complex network of cytoskeletal proteins called catenins, which in turn promote the anchorage of the complex to the actin cytoskeleton. Cadherins are responsible for homotypic calcium dependent adhesion between the cells.9–11 In the endothelium, the major cadherin consistently found at intercellular contacts is VE-cadherin, which is cell specific.7,8 AJ are dynamic structures that may vary in composition and adhesive strength according to the cellular requirements. We have previously found that at early stages of confluency, VE-cadherin and catenins are phosphorylated on tyrosine.12 When the junctions mature, such as at late stages of confluency, tyrosine phosphorylation strongly decreases. These observations are consistent with previous papers that reported that phosphorylation in tyrosine of AJ, such as in src transformed cells13,14 or in cells treated with phosphatase inhibitors,15,16 is associated with weakening of their adhesive properties.

Received September 23, 1998; revision accepted March 3, 1999.
From the Laboratory of Vascular Biology, Mario Negri Institute for Pharmacological Research, Milan (P.A., P.N., A.Z., M.G.L., E.D.); and Universita’ degli Studi dell’Insubria, Dipartimento di Scienze Cliniche e Biologiche, Facolta’ di Medicina e Chirurgia, 21100 Varese (E.D.), Italy. Present address of P.A., Department of Pharmacology, University of Patras, Medical School, Patras, Greece. Correspondence to Dr E. Dejana, Mario Negri Institute, Via Eritrea 62, 20157 Milano, Italy. E-mail dejana@irfmn.mnegri.it
© 1999 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

2286
The increase in microvascular permeability by inflammatory mediators such as histamine has been correlated with activation of the cellular contractile cytoskeleton and the formation of small gaps between adjacent endothelial cells in vitro and in vivo.17-25 These observations suggest that the cohesive properties of intercellular junctions should also be affected. However, the direct evidence that the molecules at interendothelial junctions may be a target of the histamine signaling pathway is still missing.

In this study, we found that histamine activation of endothelial cells causes increase of monolayer permeability in vitro even without the opening of apparent intercellular gaps. This effect is accompanied by tyrosine phosphorylation of VE-cadherin from the cytoskeleton. These observations indicate that histamine can directly affect AJ functional properties and suggest that this may be one of the mechanisms through which this agent reduces intercellular cohesion and increases vascular permeability.

**Methods**

All reagents were purchased from Sigma Chemical Co unless indicated otherwise.

**Antibodies**

Mouse mAbs against the extracellular domain of human VE-cadherin were clone TEA 1.31 and clone BV9.26 Antihuman N-cadherin polyclonal antibody against the whole molecule (serum 144727 was a kind gift from Dr B. Geiger [Weizmann Institute, Rehovot, Israel]). Mouse mAbs against α-catenin, β-catenin, and plakoglobin were purchased from Transduction Laboratories.

For immunofluorescence microscopy, fluorescein (FITC)- and rhodamine (TRITC)-conjugated secondary antibodies (reactive with either mouse or rabbit IgG) were purchased from Dakopatts. For immunofluorescent detection, goat antimouse IgG peroxidase-conjugated and protein A peroxidase-conjugated were from Pierce. rhodamine (TRITC)-conjugated secondary antibodies (reactive with mouse or rabbit IgG) were purchased from Dakopatts.

**Cell Culture**

Medium 199, fetal calf serum, and other culture reagents were Medium 199, fetal calf serum, 2 mmol/L glutamine, 100 u/mL penicillin, 100 mg/mL streptomycin, 2.5 µg/mL fungizone, 10 ng/mL epidermal growth factor, and 1 µg/mL hydrocortisone. Coating of either tissue culture plastic or glass coverslips was as for HUVEC.

Details on the production and characterization of HMEC-1 cell line were previously reported.29

**Culture Density and Treatments**

Cells were seeded at different initial densities to reach various stages of confluency after the same time in culture (72 to 96 hours). For HUVEC, initial and final cell density and definition of the recently- and long-confluent stage, respectively, were previously described in detail.26 For HMEC, seeding density was 25 000 and 120 000 cells/cm² to obtain recently- and long-confluent monolayers, respectively, after 72 to 96 hours culture. Briefly, recently-confluent cultures reached confluency no longer than 18 hours before the experiment. These cultures presented cells maximally spread along their entire periphery and formed a low-density monolayer without apparent discontinuities. Long-confluent cultures reached confluency 48 to 72 hours before the experiment and were 2.5- to 4-fold denser than recently-confluent monolayers at the time of the experiment (cell densities were 40 000 to 50 000 cells/cm² and 140 000 to 160 000 cells/cm², respectively). Last change of medium was for all cultures no less than 24 hours before the experiment.

Histamine was 10−4 M, unless otherwise specified. Dibutyryl cAMP (dBcAMP) (10−3 M) was added to the cells 3 hours before histamine and maintained on the cells during treatment with histamine.

For filipin treatment, cell layers were washed once with serum free culture medium containing 0.1% BSA; filipin (3 µg/mL) in 0.1% BSA containing medium (or 0.1% BSA containing medium alone for controls) was then added for 10 minutes, as previously described.30 Cells were then washed once with 0.1% BSA containing medium before further incubation in the presence or absence of histamine as indicated. For permeability assay on filipin treatment (see below), both fluorescein isothiocyanate-dextran (FITC-dextran) and fluorescein isothiocyanate-albumin (FITC-albumin, bovine) were used. For immunofluorescence microscopy after filipin, cells were incubated for 30 minutes with FITC-albumin (10 µg/mL), washed three times with ice cold 0.1% BSA containing medium, and fixed as described below.

**Immunoprecipitation and Western Blot Analysis**

In these experiments in which tyrosine phosphorylation was studied, to maintain phosphorylation, cells had to be treated with a combination of vanadate (100 µmol/L) and hydrogen peroxide (200 µmol/L) to give rise to pervanadate, a potent inhibitor of P-tyr phosphatases.13 This was done 7 minutes before extraction. After this period, cells were put on ice and washed 4 times with ice cold CaCl2 and MgCl2 PBS, which still contained vanadate (300 µmol/L) and hydrogen peroxide (600 µmol/L). Extraction buffer 4°C for these samples also contained vanadate (300 µmol/L) and hydrogen peroxide (600 µmol/L).

For immunoprecipitation, cell extracts were preclarified by incubation with uncoupled protein G or protein A-Sepharose CL-4B (Pharmacia LKB Biotechnologie.) for 1 hour at 4°C. Supernatant was collected and immunoprecipitated with protein G or protein A-sepharose, respectively, coupled to mAb TEA 1.31 or polyclonal antibody 1447 against N-cadherin for 1.5 hours at 4°C under continuous mixing. This was followed by 5 washings with ice-cold 10 mmol/L Tris-HCl, 150 mmol/L NaCl (TBS) containing vanadate (300 µmol/L) and hydrogen peroxide (600 µmol/L) for samples treated in vivo with pervanadate. Protein separation by sodium dodecyl sulfate (SDS)-electrophoresis was under reducing conditions.

Western blot analysis of the various cell extracts was carried out essentially as described.29 Nitrocellulose on which proteins had been electrotransferred was blocked with 10% low fat milk in CaCl2 and MgCl2 PBS. PBS, without CaCl2 and MgCl2, was used for P-tyr detection, as reactivity of anti-P-tyr RC20 is inhibited by divalent cations. Incubation of nitrocellulose sheets with the appropriate monoclonal or polyclonal antibodies at the optimal dilution was either for 1 hour at room temperature or overnight at 4°C. This was sequentially followed by incubation with goat antimouse IgG peroxidase-conjugated (1 µg/mL) for monoclonal antibodies or protein A peroxidase-conjugated (1 µg/mL) for polyclonal antibodies. Anti-phosphotyrosine RC20 was horseradish peroxidase-conjugated and therefore did not require any further reaction before detection as described below. The immunoreactive bands were revealed using an ECL Western blotting detection kit (Amersham) and the signal was recorded on autoradiography films (Reflection). The nature of the bands recognized by anti-phosphotyrosine antibody was proved as described previously in details.31 Detergent solubilization for the analysis of Triton X-100 soluble and insoluble fractions was carried out essentially as reported in detail.26 The antigens were visualized by ECL method (Amersham) and the density of each band quantitatively determined measuring the
optical density per area in arbitrary units using the Kontron Bildanalyse Software.

**Immunofluorescence Microscopy**

The procedure has been described previously in detail. Briefly, cells on glass coverslips were fixed with 3% formaldehyde freshly prepared from paraformaldehyde (PAF) and permeabilized with 0.5% Triton X-100 for labeling with VE-cadherin antibodies. For labeling with either N-cadherin, p120, α-catenin, β-catenin, or plakoglobin antibodies, cells were fixed and permeabilized at the same time using 3% PAF with 0.5% Triton X-100 for 3 minutes followed by 3% PAF alone for 15 minutes. Incubation with the first antibody was followed by either rabbit antimouse or swine antirabbit TRITC-conjugated secondary antibodies, depending on the first antibody used, in the presence of fluorescein (FITC)-labeled phaloidin (2 μg/mL). Coverslips were mounted in Mowiol 4 to 88 (Calbiochem Int) and examined under a Zeiss Axiohot microscope. Images were recorded on Kodak T MAX P3200 films with constant exposure of 40 seconds.

**Cell Layer Barrier Properties**

Transwell units (6.5-mm diameter, 0.4-μm pore size polycarbonate filters, Corning Costar Corporation) coated with human fibronectin (7 μg/mL) were used. Culture medium in the upper and lower compartments was 100 and 600 μL, respectively, as suggested by the manufacturer. Cells were cultured for 72 hours without medium change. To test for barrier property fluorescein (FITC)-conjugated dextran (38 900 daltons, final concentration 1 mg/mL) was added, immediately followed by histamine when indicated. At the indicated time points, a 50-μL sample was removed from the lower compartment and replaced with 50 μL culture medium and incubation continued as specified. After diluting the sample to 1 mL with PBS-Ca2+ and Mg2+ free, fluorescein content was measured in a fluorimeter (Kontron Instruments) at 492 nmol/L absorbance and 520 nmol/L emission wavelengths, respectively. In some experiments, as indicated, fluorescein (FITC)-albumin (from bovine serum) was used at a final concentration of 1 mg/mL.

To evaluate transcellular transport of FITC-dextran, a method was adopted that has been described previously. Cells were incubated with FITC-dextran for 30 minutes. Plates were put on ice and cells layers washed 4 times with ice cold culture medium containing 0.5% BSA (at each washing step the Transwell unit was also transferred to fresh ice-cold culture medium containing 0.5% BSA to wash also the lower face of the filter). Cells were then incubated for 25 minutes in culture medium containing 0.5% BSA either in the presence or in the absence of histamine, as indicated. Fluorescence content was measured in both the lower and upper compartments to determine the transport to either the basal or apical direction.

**Results**

**Effect of Histamine on Adherens Junction Organization**

We first examined whether histamine activation of cultured endothelial cells caused any change in cadherin and catenin localization at intercellular contacts.

Cells were stained with specific antibodies and the distribution of AJ components was analyzed by immunofluorescence microscopy.

Figure 1 shows that when recently confluent (no more than 18 hours confluency), HUVEC were activated with histamine gaps that appeared along the intercellular contacts. This effect was already apparent at 1 to 5 minutes but reaches its maximum at 25 minutes (Figures 1a to 1d). Intercellular gaps disappeared within one hour after histamine activation (not shown). VE-cadherin was only partially absent at the borders of the gaps but remained unchanged in the areas where intercellular contacts were still intact.

Histamine also induced a marked change in actin organization. In activated cells, actin microfilaments formed a thick network of parallel stress fibers running along the all cell body. This effect followed the same time course as gap formation but presented a slower recovery because at 1 hour after cell activation, stress fibers were still somehow better organized than in control cells (not shown). When HUVEC were kept confluent for a longer time (48-hours postconfluence), histamine activation did not induce detectable intercellular gaps (Figures 1e to 1h). Actin, however, showed reorganization in thick stress fibers (Figure 1h).

To extend the study to microvascular cells, we used an endothelial cell line (HMEC) derived from human dermal foreskin microvasculature that, as previously characterized, expresses endothelial markers and presents an endothelial cobblestone morphology.

In a way comparable with HUVEC, histamine induced gaps between the cells (Figures 2a to 2d) in recently-confluent HMEC. This was accompanied by VE-cadherin disappearance in the areas of cell detachment. As in HUVEC, histamine induced profound changes in actin organization with the appearance of a well organized stress fiber network. The time course of gap formation and actin rearrangement was similar to that observed in recently-confluent HUVEC reaching its maximum at 25 minutes (Figure 2). In long-confluent HMEC, histamine was essentially inactive in inducing detectable gaps but was still capable of modifying actin organization increasing the number and organization of actin stress fibers (Figures 2e to 2h). VE-cadherin was still present at intercellular contacts (Figure 2g).

As discussed above, VE-cadherin is linked inside the cell to catenins that mediate its anchorage to the actin cytoskeleton. It was therefore of interest to examine whether histamine could change catenin (α-catenin, β-catenin, and plakoglobin) distribution at junctions. In general, the results show that catenin behavior always paralleled that of VE-cadherin in both recently- and long-confluent cells, using both HUVEC and HMEC. As an example, in recently-confluent HMEC, catenins were lost from the borders of the gaps in a way comparable with VE-cadherin, but they remained at junctions in the areas where cell-to-cell contacts were still present (Figure 3). In long-confluent cells, intercellular gaps were undetectable, and catenin distribution did not change after histamine and essentially overlapped VE-cadherin staining along the cell borders (not shown).

Data presented in Figures 1, 2, and 3 have been obtained using 10^{-4} M histamine. Decreasing histamine concentration to 10^{-5} M, comparable morphological changes were observed (not shown).

In all types of experiments (see below), HMEC presented a reproducible response to histamine in contrast to the variability observed using HUVEC from different cultures. For this reason, most of our work and the data presented below were done using HMEC.

**Histamine Increases Permeability of Early- and Long-Confluent Cells**

We asked whether despite the lack of detectable intercellular gaps histamine could still increase permeability in long-confluent endothelial cells as compared with recently-confluent cells.
**Figure 1.** Effect of histamine on VE-cadherin distribution in recently- and long-confluent HUVEC. HUVEC monolayers with recently (a to d) or long (e to h) established cell-cell contacts were exposed to histamine ($10^{-4}$ M; c, d, g, and h) for 25 minutes and then processed for immunofluorescence microscopy. Cells were double stained for VE-cadherin and F-actin to analyze the organization of cell-cell contacts and the general condition of the cell layer. Controls, a, b, e, and f. Cell retractions were evident in recently-confluent HUVEC on histamine treatment (c and d, arrowheads). In long-confluent HUVEC the distribution of VE-cadherin did not appear to be significantly modified by histamine. In response to histamine, F-actin stress fibers increased in both recently- and long-confluent cells. Bar, 20 μm. The figure reports a typical experiment out of 6 performed.
Figure 2. Effect of histamine on VE-cadherin distribution in HMEC at different stages of confluency. Cells were analyzed by immunofluorescence microscopy and double stained for VE-cadherin and F-actin. HMEC recently-(a to d) and long-confluent (e to h) showed distinct responses to histamine (10^{-4} M for 25 minutes). In recently-confluent HMEC, gaps were formed between cells, in correspondence with such retractions VE-cadherin disappeared from cell margin. In long-confluent HMEC, no frank retractions were evident, whereas VE-cadherin distribution appeared subtly modified assuming in some contacts a more fragmented (less compact) pattern. A strong increase in F-actin stress fibers was induced by histamine in both conditions of cell density. Bar, 20 μm. The figure reports a typical experiment out of 10 performed.
Figure 3. Effect of histamine on catenin distribution in HMEC. Recently-confluent HMEC were treated with histamine (10^{-4} M for 25 minutes) and processed for immunofluorescence microscopy. Cells were double stained for one catenin (a and c, β-catenin; e, α-catenin; or g, plakoglobin) and actin stress fibers (b, d, f, and h). Histamine induced retractions of cell-cell contacts as also presented in Figure 2. In these regions, cytoplasmic catenins were no longer detectable at cell margin (arrowheads), as reported in Figure 2 (c and d) for the transmembrane VE-cadherin. The distribution of plakoglobin and α-catenin in control cells (not shown) is comparable with that of β-catenin shown in panel a. The effect of histamine in increasing F-actin stress fibers is also evident (d, f, and h). Bar, 20 μm. The figure reports a typical experiment out of 10 performed.
Permeability was measured in vitro by seeding the cells on Transwell filters and measuring the amount of FITC-dextran (38,900 daltons mw), which could pass through the monolayers. In the absence of histamine, long-confluent monolayers presented a significantly lower permeability than early-confluent cells (Figures 4A and 4B, white symbols).

When recently-confluent cells were exposed to histamine, permeability increased and peaked within 25 minutes (Figure 4A). After this time, histamine and control curves paralleled indicating no further effect of histamine on permeability. In long-confluent cells (Figure 4B), the increase in permeability induced by histamine was more marked in comparison with recently-confluent cultures (120% and 46%, respectively). Also in long-confluent cells, the maximal effect of histamine was observed at 25 minutes, after which no further increase was observed and the curves of the control and histamine treated cells became parallel.

In a typical experiment using recently-confluent cells, the concentration of FITC-dextran in the lower compartment after 25 minutes was 27.6 and 41.6 μg/ml for control and histamine treated cells, respectively. In long-confluent cells it was 13.6 and 30.4 μg/ml for control and histamine treated cells, respectively.

When FITC albumin was used (see below, Figure 5), in a typical experiment its concentration in the lower compartment after 25 minutes was 41.6 and 104.4 μg/ml for control and histamine long-confluent treated cells, respectively.

Histamine and Transcellular Permeability In Vitro

In addition to paracellular permeability, histamine might augment transcellular permeability by increasing the dynamic transport of the solutes through the intracellular vesicular systems. We studied whether this pathway could contribute to the increase in permeability observed in long-confluent cells where intercellular gaps were undetectable.

We measured the endothelial transcellular passage of FITC-dextran as previously described.31 Endothelial cells were preincubated with FITC-dextran for 30 minutes. The label was then washed away from the culture and the amount of dextran released in the lower and upper wells measured as described.31 In a typical experiment, the fluorescent units in the upper wells were 19.1 ± 2 and 25.9 ± 0.5 in the absence or presence of histamine, respectively (at 25 minutes using 10⁻⁴ M histamine), and in the lower wells, 3.7 ± 0.9 and 7.9 ± 1 in absence or presence of histamine, respectively. In parallel wells, the total passage of FITC-dextran to the lower compartment resulted in 75 ± 10 and 174 ± 4 fluorescence units without and with histamine, respectively. These data indicate that the contribution of the transcellular pathway to the total permeability measured (FITC-dextran passage to the basal compartment) is very low (approximately 4% to 5% of the total).

As an additional approach, we considered the contribution of the vesicular transport system to histamine induced permeability. As previously reported, the treatment of endothelial cells with filipin, a sterol binding agent, induces the disappearance of the caveolar transport system.30 As shown in Figure 5, exposure of endothelial cells to filipin did not significantly change the increase in permeability induced by histamine. The concentration of filipin used was able to abolish albumin containing vesicles as reported previously30 (Figure 5A).

These data strongly suggest that, in our experimental conditions, the vesicular transport pathways do not significantly contribute to the observed histamine effect.
Histamine Induces Tyrosine Phosphorylation of Adherens Junction Components

VE-cadherin and associated catenins can undergo tyrosine phosphorylation and, as discussed above, tyrosine phosphorylation of adherens junction components correlates with inactivation of their adhesive properties. It was therefore of interest to investigate whether histamine could induce tyrosine phosphorylation of VE-cadherin and catenins.

As reported in Figure 6, according to previously published data, recently-confluent cells present a higher degree of phosphorylation in tyrosine of VE-cadherin, β-catenin, and plakoglobin as compared with long-confluent cultures. In long-confluent cultures, VE-cadherin and catenins were usually poorly phosphorylated on tyrosine, and in many cases the signal was undetectable.

Histamine did not change tyrosine phosphorylation of adherens junctions in recently-confluent cells, but it did so in long-confluent cultures (Figure 6A). This effect was mostly evident for β-catenin and plakoglobin (Figure 6A), whereas, as previously reported, α-catenin did not seem to be a good target for phosphorylation.

Tyrosine phosphorylation was not accompanied by any significant change in the amount of β-catenin or plakoglobin associated with VE-cadherin (Figure 6B), and the complex remained essentially unchanged in both recently- and long-

confluent cells after histamine. Only a slight but consistent decrease in β-catenin and increase in plakoglobin content was observed comparing recently- with long-confluent cells according to previously published observations (see Figure 6B).

Figure 7 reports the time course of histamine induced adherens junction phosphorylation. Tyrosine phosphorylation of VE-cadherin and catenins was already detectable at 1 minute after histamine addition and remained stable up to 25 minutes.

The adhesion strength of cadherins is deeply influenced by their binding to the actin cytoskeleton. We therefore examined the association of VE-cadherin to the cytoskeleton before and after histamine treatment. This was done indirectly by measuring the amount of VE-cadherin present in the detergent soluble and insoluble fractions of cell extracts.

As shown in Figure 8, a larger amount of VE-cadherin was found in the insoluble fraction of long-confluent cells as compared with recently-confluent cells. Histamine induced a shift of VE-cadherin from the insoluble to the soluble fraction in long-confluent cells without significantly affecting this parameter in recently-confluent cells.

We then studied whether the inhibition of the histamine effect on endothelial permeability could be related to inhibi-
tion of tyrosine phosphorylation of AJ components. To this purpose we treated the cells with dcAMP. According to previous work, the treatment of long-confluent cells with dcAMP inhibited histamine induced increase in permeability (Figure 9B). In parallel, tyrosine phosphorylation of both VE-cadherin and catenins was inhibited (Figure 9A).

Herbimycin A treatment of endothelial cells, at a concentration of $1.2 \times 10^{-6}$ M for 18 hours, reduced by 42% to 35% the increase in permeability induced by histamine ($10^{-4}$ M at 25 minutes) (not shown). As described, N-cadherin was very poorly phosphorylated on tyrosine in both recently- and long-confluent cultures, and histamine did not change this pattern (Figure 10C). As a control in the same cells, histamine was able to increase significantly the extent of tyrosine phosphorylation of VE-cadherin and catenins (Figure 10C).

**Discussion**

In this study, we address the question of whether intercellular junctions and in particular AJ could be a target of histamine activation in endothelial cells.
We first observed that, as described, histamine causes rearrangement of actin cytoskeleton and intercellular gap formation in endothelial cells. In our conditions, however, gaps were apparent only at early and not at late stages of confluency indicating that the effect of histamine may change as a function of junction maturation.

However, when we measured permeability, by an in vitro assay, histamine was effective in increasing endothelial permeability in both recently- and long-confluent cells, strongly suggesting that the presence of frank discontinuities in the endothelial monolayers was not the absolute requirement for the histamine effect.

We therefore investigated whether other types of functional modifications could occur in long-confluent cultures on exposure to histamine.

In previous work, we found that AJ components (in particular, VE-cadherin, β-catenin, and plakoglobin) may be differentially phosphorylated on tyrosine in function of the state of maturation of intercellular junctions. In particular, AJ components presented a higher phosphorylation state when the cells were loosely confluent as compared with cells with well established intercellular contacts.

In this study, we confirm this early observation, and in addition, we report that histamine is able to upregulate the tyrosine phosphorylation levels of AJ components in long-confluent cells. In loosely-confluent cells, the state of phosphorylation of VE-cadherin and catenins is already high, and histamine cannot further increase it. In contrast, in long-confluent cells, AJ phosphorylation is low and frequently undetectable, and histamine is able to increase this parameter to levels comparable with loosely-confluent cells.

Tyrosine phosphorylation is an important regulatory mechanism for AJ. In general, it has been associated with disruption of the adhesive properties of these structures and to a more marked cell motility and invasive behavior. It is therefore tempting to speculate that the induction of tyrosine phosphorylation of AJ components induced by histamine could be related to the increase in permeability induced by this substance. Consistent with this possibility is the observation that recently-confluent cells present high levels of AJ phosphorylation and high permeability values. Histamine does not further increase AJ phosphorylation in these cells but induces intercellular gaps that are likely responsible for the further increment in cell permeability. In long-confluent cells, histamine induces levels of phosphorylation in tyrosine comparable with those of recently-confluent cells, and this is accompanied by a marked increase in permeability even in the absence of apparent opening of intercellular gaps.

Many studies are available on the mechanism of action of histamine in inducing vascular permeability. Some evidences indicate that histamine induces endothelial cell retraction and this effect is regulated by the phosphorylation of myosin light chain. Cell contraction may cause small gaps at cell junction, which may be responsible for increased permeability. A recent report, however, shows that histamine did not significantly change isometric cell tension but rather decreased the centrifugal forces that tether the cells to each other. This observation is in agreement with the possibility that part of the histamine effect is mediated by the reduction of adhesion strength at intercellular junctions.

dcAMP is known to improve endothelial barrier function. This action involves cAMP-dependent protein kinase and inhibition of phosphorylation of the myosin light chain, which results in a decrease in endothelial cell centripetal forces. In this study, we found that dcAMP also blocks the effect of histamine on AJ phosphorylation. This effect paralleled the inhibition of the increase in permeability induced by histamine. In addition, treatment of the cells with a tyrosine kinase inhibitor such as herbimycin A reduced histamine induced permeability.

Overall these observations suggest that phosphorylation in tyrosine of AJ induced by histamine may be related to a decrease in intercellular adhesion strength.

However, a recent study shows that the decrease in transendothelial electrical resistance induced by histamine is not modified by genistein, suggesting that tyrosine kinase inhibition is not relevant for this effect. A possible explanation for this discrepancy, in comparison with our data on herbimycin (see above), may be due to the fact that TEER and cell permeability do not always overlap (see for instance, Reference 31).

In previous work, we found that VE-cadherin association to the cell cytoskeleton is required for a full control of paracellular permeability. As reported here and in previous work, phosphorylation in tyrosine of VE-cadherin by histamine is accompanied by a decrease in the amount of this protein linked to the cell cytoskeleton. A possibility is that histamine, changing the phosphorylation state of VE-cadherin and catenins, causes their dissociation from actin, which is then responsible for a decrease in intercellular tethering.

In this study, we did not investigate the mechanism through which histamine induces tyrosine phosphorylation of AJ. The observation that, in contrast to VE-cadherin, N-cadherin is not affected by histamine suggests that the effect of histamine is specifically directed to junctional associated mediators. Activation of src is a possibility because this kinase was found to be present at junctions and its activation induces phosphorylation of AJ components. However, histamine might also inhibit specific junctional phosphatase(s). Different phosphatases have been described to be associated with AJ and influence their phosphorylation state. Endothelial cells express several phosphatases, but it is still unknown which one(s) is responsible for modulation of AJ phosphorylation.

In conclusion, adherens junction components are a target of histamine activation of endothelial cells, and it is conceivable that this activity contributes to histamine induced increase in endothelial permeability. A previous report showed that histamine reduced ZO-1 synthesis. ZO-1 is a junctional protein associated with AJ and TJ. Even if this activity is different to that reported here, overall these data are consistent with an effect of histamine on junction organization.

In this study, we were unable to evidence an important contribution of the transcellular pathway in histamine induced permeability. This may be due to the fact that cultured cells may have lost specialized vesicle shuttling systems and may be better models for measurements of changes in paracellular as compared with transcellular permeability.

Some recent reports show that permeability increasing agents, such as vascular endothelial growth factor/vascular permeability factor, histamine, or serotonin, increase the
function of the vesicular (vesiculo-vacuolar organelles) transport system.49 These authors do not observe interendothelial gap formation or passage of tracers through interendothelial junctions. For this reason, they suggest that previously published work on endothelial gap formation in vivo is related, at least in part, to the properties of the tracer used and not to the actual effect of permeability inducing agents. The data reported here may in part reconcile these conflicting observations because even in the absence of detectable cell retraction and gap formation, the permeability control by AJ may be affected by histamine.

Acknowledgments
This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, Italian National Research Council (Progetto Finalizzato Biotecnologie CNR 97.01299.PF49), European Community (projects: Biomed 2: BMH4 CT 96-0669, BMH4 CT95-0875 and BMH4 CT98-3380 and Biotech 2: BIO4 CT96-0036 and BIO4 CT98-0337) and Istituto Superiore di Sanita’ (Progetto Sostituzioni funzionali, organi artificiali e trapianti di organo). The authors thank the Cell Culture Development Section of the Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga, for the generous gift of the human microvascular endothelial cell line (HMEC).

References


Histamine Induces Tyrosine Phosphorylation of Endothelial Cell-to-Cell Adherens Junctions
Paraskevi Andriopoulou, Pilar Navarro, Adriana Zanetti, Maria Grazia Lampugnani and Elisabetta Dejana

doi: 10.1161/01.ATV.19.10.2286
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/19/10/2286

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/