RESEARCH COMMUNICATION

Histamine reduces ZO-1 tight-junction protein expression in cultured retinal microvascular endothelial cells

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We examined ZO-1 protein content in cultured retinal vascular endothelial cells to test the hypothesis that histamine alters tight-junction-protein expression. Histamine (10^{-9} – 10^{-4} M) causes a reversible concentration-dependent reduction of ZO-1 protein content, mediated by both H_1 and H_2 receptors. Histamine

reduces ZO-1 expression within the time associated with increased paracellular permeability. Tight-junction-protein alterations may be a novel explanation for the mechanism by which vasoactive agents increase microvascular permeability.

INTRODUCTION

Numerous studies have been performed to explain the means by which histamine increases microvascular endothelial paracellular permeability [1–10]. Majno and Palade [1] first proposed that histamine opened inter-endothelial-cell tight junctions. They and others found histamine-induced endothelial-cell retraction, both *in vivo* and in cultured endothelial cells [2,3]. Biochemical changes, including increased F-actin content, increased free cytosolic Ca^{2+} and breakdown of inositol phospholipids with release of $InsP_3$, have also been proposed to account for increased permeability [4–10]. These studies describe acute events (within seconds to minutes) involving the cytoskeleton and cytoplasmic mediators, but do not address potential alterations of tight junctions.

At least seven protein constituents of the tight junction have been identified (reviewed in [11]). Of these, ZO-1, a 220 kDa phosphoprotein, has been the best characterized [12]. It is located on the cytoplasmic face of the plasma membrane, closely associated with another protein, ZO-2. The α^- isoform of ZO-1, created by alternative splicing, is expressed by vascular endothelium [13]. The functional importance of ZO-1 protein in endothelial-cell tight junctions is suggested by: (1) its presence in the blood/brain barrier [14]; (2) its increased expression in parallel with *trans*-epithelial electrical resistance in cultured rat brain capillary endothelial cells [15]; and (3) its up-regulation by astrocyte-conditioned medium in cultured retinal capillary endothelial cells [16]; ZO-1 protein levels, therefore, respond to physiological influences and participate in the regulation of paracellular permeability through tight junctions.

To understand better the role of ZO-1 in conditions associated with microvascular permeability, we examined the effects of histamine on ZO-1 protein content in cultures of retinal capillary endothelial cells to test the hypothesis that histamine alters tight-junction expression.

MATERIALS AND METHODS

Chemicals

The monoclonal rat anti-ZO-1 antibody was obtained from Chemicon (Temecula, CA, U.S.A.), the rabbit anti-rat IgG from

Accurate (Westbury, NY, U.S.A.) and the ^{125}I -labelled anti-rat IgG from NEN Research (Boston, MA, U.S.A.). The rabbit anti-(rat GLUT1 glucose transporter) antibody was from Charles River (Southbridge, MA, U.S.A.) and the fluorescein isothiocyanate-conjugated anti-rat IgG secondary antibody was from Accurate (Westbury, NY, U.S.A.). The rabbit anti-(human von Willebrand Factor) antibody was obtained from Dako (Glostrup, Denmark).

Bovine retinal capillary endothelial-cell cultures

Bovine retinal endothelial cells were isolated and cultured as previously described [16]. The standard growth medium consisted of modified Eagle's medium with D-valine, 20% fetal-calf serum, 50 μ g/ml endothelial-cell growth supplement (Collaborative Research, Bedford, MA, U.S.A.), 16 units/ml heparin (Sigma), 5% antibiotic/antimycotic solution (Sigma) and 1% glutamine (Sigma). The cells were grown in a humidified incubator at 37 °C under air/CO₂ (19:1). By selective trypsin treatment, endothelial-cell cultures were made essentially free of pericytes as observed by phase-contrast microscopy. Verification of endothelial-cell characteristics was based on typical endothelial-cell 'cobblestone' morphology and positive immunostaining for Factor VIII-related antigen with a rabbit anti-(human Factor VIII) antibody (1:200) [17]. Endothelial cells were used for experiments between six and ten passes, after the primary culture, and only cells which had undergone ten or fewer doublings were employed in these studies.

Retinal endothelial-cell membrane preparation and immunoblotting

Crude bovine endothelial-cell membranes were prepared by rinsing confluent 75 cm² flasks twice with ice-cold PBS, scraping the flask with a 'rubber policeman', and pelleting the collected cells at 300 g for 5 min at 4 °C. The pellet was then resuspended in ice-cold buffer composed of 10 mM Tris, pH 7.4, 1 mM EGTA, 4 mM EDTA, 250 mM sucrose and the following protease inhibitors: leupeptin (2.5 μ g/ml), pepstatin (0.5 μ g/ml), aprotinin (0.5 μ g/ml), soybean trypsin inhibitor (30 μ g/ml),

Abbreviations used: GLUT1, glucose transporter GLUT1; PKC, protein kinase C.

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dithiothreitol (0.2 mM) and PMSF (0.2 mM). The cells were homogenized in a Wheaton 15 ml mortar with pestle A, and the homogenate was spun at 100 000 *g* at 4 °C for 20 min in a Beckman ultracentrifuge. The pellet was resuspended in buffer without sucrose and re-spun at 100 000 *g* for 20 min. Then the crude membrane fraction was resuspended, and the protein concentration was determined with the Bio-Rad Protein Reagent on a Hitachi spectrophotometer, with BSA as standard.

The retinal endothelial-cell membrane samples were adjusted to equal protein concentrations and resolved on SDS/6% polyacrylamide gels. The proteins were transferred to Nytran membranes overnight at 4 °C at 20 V using a Hoefer transfer chamber. The Nytran membranes were stained with Ponceau S to confirm equivalent protein loading. Non-specific protein binding was blocked with low-detergent Blotto (50 mM Tris/2 mM CaCl_2 /80 mM NaCl/3% non-fat dry milk/0.2% Tween 20, pH 8.0). The blots were incubated overnight with a monoclonal rat anti-ZO-1 antibody (1:1000), then washed with low-detergent Blotto with 0.4% Tween. The blots were incubated with rabbit anti-rat IgG (1:200) for 1 h, washed, then incubated with 2.0 μCi of ^{125}I -labelled anti-rat IgG. ZO-1 protein content was determined by excising areas on the filter corresponding to bands on the autoradiogram and counting ^{125}I radioactivity in a liquid-scintillation counter. A standard curve of ZO-1 immunoreactivity in increasing amounts of endothelial-cell membranes, from 10 to 160 μg , gave $r^2 = 0.995$. For an internal control, membranes prepared under parallel conditions were also subjected to SDS/10%-PAGE and blotted with rabbit anti-rat GLUT1 antibodies (1:500).

Immunocytochemistry of ZO-1 protein

Bovine retinal capillary endothelial cells grown to confluence on fibronectin-coated glass coverslips were washed three times with ice-cold PBS, soaked in -20 °C methanol for 4 min, rinsed with -20 °C acetone and dried at room temperature for 10 min. Non-specific binding was blocked with 3% BSA in PBS for 30 min at room temperature. The fixed cells were then incubated with rat anti-ZO-1 primary antibody (1:150) for 1 h at room temperature in a moist covered Petri dish. The cells were then rinsed three times with PBS and incubated with anti-rat IgG labelled with fluorescein isothiocyanate (1:300) for 60 min at room temperature. The cells were then washed three times with PBS, dried, and covered with Vecta-Shield and a coverslip. Coverslips which received only the secondary antibody served as negative controls.

Effects of histamine on ZO-1 protein content

The concentration-response of endothelial-cell ZO-1 protein content was tested in triplicate by adding 10^{-9} – 10^{-4} M histamine for 4 h to confluent cells. Cells were harvested, and crude membranes were prepared and blotted for ZO-1 protein. Preliminary studies determined the bioavailability of a single dose of 10^{-5} M histamine in endothelial-cell-culture medium decreased by 50% after approx. 6 h. Histamine in the culture medium was quantified by radioenzymic assay [18]. The time course of histamine-induced ZO-1 protein changes was examined by exposing confluent retinal endothelial cells to 10^{-5} M histamine in quadruplicate samples. Cells were harvested at 0.5, 1, 2, 3, 6, 24 and 48 h after a single dose of histamine and prepared as for the concentration time course. In separate studies, cells were pre-treated with histamine-receptor antagonists to determine whether histamine receptors mediate the changes in ZO-1 expression. Mepyramine (H_1 receptor blocker, $K_d = 0.8$ nM) or ranitidine (H_2 receptor blocker, $K_d = 200$ nM) at 4×10^{-9} M and 4×10^{-7} M

respectively, or both, were applied 15 min before adding 10^{-5} M histamine, to allow for receptor binding [19].

Statistical analyses

Protein-content data were compared by one-factor analysis of variance and *post hoc* Duncan's multiple range test ($\alpha = 0.05$). Results are expressed as percentages of the control value ($\pm$ S.E.M.).

RESULTS AND DISCUSSION

Histamine induces a concentration-dependent decrease in ZO-1 protein content

The effect of histamine on plasma-membrane ZO-1 content in bovine retinal microvascular endothelial cells was examined after

Figure 1 Concentration–response relationship for histamine and ZO-1 protein content in cultured retinal microvascular endothelial cells

Confluent endothelial cells were treated with histamine (10^{-4} – 10^{-9} M) for 4 h, and crude membranes were prepared and subjected to SDS/6%-PAGE (50 μg /lane). The membranes were blocked, then incubated with anti-(rat ZO-1 protein) monoclonal antibody, rabbit anti-rat IgG, and 2.0 μCi ^{125}I -anti-rat IgG. Regions of the membrane corresponding to ZO-1 were excised, counted for radioactivity in a scintillation counter, and the results were expressed as a percentage of the control ($\pm$ S.E.M.) (A) Plot of ZO-1 protein content against log[histamine]. The half-maximal effect is at $(3.3 \pm 1.6) \times 10^{-9}$ M histamine. (B) Representative autoradiogram of ZO-1 content after exposure to 10^{-9} – 10^{-4} M histamine. (C) Autoradiogram of GLUT1 from cells exposed to 10^{-9} – 10^{-5} M histamine. Results are representative of three separate experiments.

Figure 2 Histamine reduces ZO-1 protein expression in retinal vascular endothelial cells

Indirect immunofluorescence staining of ZO-1 protein in confluent retinal vascular endothelial cells with anti-ZO-1 monoclonal antibody and fluorescein isothiocyanate-labelled anti-rat IgG after methanol/acetone fixation. Images were exposed and printed under identical conditions. Compared with control ('Cnt') cells in (a), cells treated with 10^{-5} M histamine for 4 h (b) have less intense and less regular ZO-1 immunoreactivity at regions of cell-cell contact. (c) Negative control with omission of primary antibody. The bar represents 20 μ m. Results are representative of four separate experiments.

4 h of exposure to 10^{-9} – 10^{-4} M histamine. These concentrations represent the range of histamine known to stimulate endothelial-cell histamine receptors and increase permeability [3–10]. Crude membrane fractions were prepared, subjected to SDS/6%–PAGE, immunoblotted with anti-ZO-1 antibody, and quantified by scintillation counting. The concentration–response relationship between the histamine concentration in the culture medium and ZO-1 content is shown in Figure 1. The IC_{50} was $(3.3 \pm 1.6) \times 10^{-9}$ M histamine, as determined from curve-fitting, and the maximal inhibition was $52.2 \pm 8\%$ with 10^{-5} M histamine. The decrease in ZO-1 protein content was nearly linear over the range of 10^{-9} – 10^{-5} M histamine, with a maximal effect at 10^{-5} M, and a half-maximal response of approx. 3.3×10^{-9} M. ZO-1 content fell by $\sim 20\%$ with 10^{-9} M histamine, suggesting that retinal vascular endothelial cells are very sensitive to this agent. These results suggest a direct concentration-dependent reduction in ZO-1 protein expression and are similar to previously described concentration–response effects of histamine on free cytosolic Ca^{2+} and $InsP_3$ [9, 10]. In fact, 2×10^{-8} M is the threshold histamine concentration for cytosolic Ca^{2+} elevations in umbilical-vein endothelial cells [20]. GLUT1 protein expression from cells treated in an identical fashion did not change. To the best of our knowledge, this is the first time any vasoactive agent has been reported to alter the expression of a tight-junction protein. This finding may provide a new explanation for the increased vascular permeability associated with histamine.

To examine whether the reduced ZO-1 protein content might reflect a change in immunoaffinity of the protein or an intracellular redistribution, we performed two additional experiments: (a) immunocytochemistry under non-reducing conditions with methanol/acetone fixation; and (b) immunoblotting of whole-cell homogenates exposed to the same experimental pro-

tol. Immunocytochemistry showed reduced plasma-membrane-associated ZO-1 immunoreactivity, without a detectable increase in cytosolic or nuclear immunoreactivity (Figure 2). Immunoblotting of cell homogenates revealed a 35% reduction in ZO-1 protein content (results not shown), similar to the effect noted with crude membrane fractions. Taken together, these data strongly suggest that histamine transiently decreases both total cellular and plasma-membrane-associated ZO-1 protein content, rather than redistributing or altering its immunoaffinity.

Time course of histamine effect on ZO-1 protein content

The time course of ZO-1 protein content changes in confluent endothelial cells was studied from 0 to 48 h. Histamine was used at 10^{-5} M because this represents the upper range of histamine concentration which increases free cytosolic Ca^{2+} , $InsP_3$ release and endothelial-cell permeability and decreases ZO-1 expression [6–10]. The histamine content in the culture medium decreases linearly, with $\sim 50\%$ decay by 6 h, and falls to 20% of control by 10 h. At 1 h after exposure to 10^{-5} M histamine, ZO-1 protein content declines approx. 20%. ZO-1 content is significantly decreased compared with untreated controls, by $27 \pm 7.8\%$ at 2 h, $53 \pm 13\%$ at 3 h, and $28 \pm 4\%$ at 6 h respectively ($P < 0.05$ at each time point). By 24 h after histamine application, the ZO-1 content returns to within 17% of baseline values, and a small overshoot (10%) is observed at 48 h. These data suggest a rapid, reversible reduction of ZO-1 protein content. This recovery probably reflects the reduction of available histamine in the culture medium after several hours.

Small changes in ZO-1 content, such as the 20% reduction after 1 h may have physiological importance. For example, only 2% of tight junctions need to be opened to decrease *trans-*

epithelial electrical resistance by 98% [21]. Hydraulic conductivity, an index of water flux, increases across bovine retinal capillary endothelial-cell monolayers within the same time period as ZO-1 protein content falls (1–3 h) in response to 10^{-4} M histamine (J. Tarbell, personal communication). It seems likely, then, that changes in ZO-1 protein content closely parallel physiological changes in microvascular permeability, although the rate of ZO-1 decline may depend on the concentration of histamine.

Prior endothelial-cell-culture studies have shown a sequence of events following application of histamine. From the point of histamine addition to cultured endothelial cells, free cytosolic Ca^{2+} increases after 5–10 s [4,9], and InsP_3 accumulation is maximal at 20 s [10]; actin content decreases within 5 min [3,9]; and peripheral actin bands decrease and longitudinal bands increase within 10 min [7]. Permeability to water, sucrose and albumin increases over 30–60 min and continues to be elevated above control values for 2–4 h [3]. The data reported herein suggest that decreased ZO-1 protein content follows these more rapid cytosolic events. At this point it is difficult to know whether the reduction in ZO-1 protein content results from changes in the actin cytoskeleton or represents a direct effect on ZO-1 turnover, which then permits cytoskeletal alterations. However, phalloidin pretreatment of endothelial cells to stabilize actin microfilaments prevents permeability increases by histamine, which suggests that histamine may require cytoskeletal mobility to open tight junctions [20].

Previous work has shown a correlation between ZO-1 protein and permeability. In cultured rat brain capillary endothelial cells, ZO-1 protein content corresponds inversely with the permeability of the monolayer [15]. We also found an inverse relationship between histamine dose and ZO-1 content. ZO-1 protein expression increases directly with the degree of confluence in cultured bovine aortic endothelial cells [22]. Although ZO-1 protein content is similar in two strains of Madin–Darby canine kidney cells with different levels of *trans*-endothelial electrical resistance [23], ZO-1 protein content does correlate with electrical resistance in mammary epithelial cells [24]. Qualitative alterations of ZO-1 could also modify tight-junction function. Epidermal growth factor has recently been shown to increase mammary epithelial-cell permeability and tyrosine phosphorylation [25]. We did not, however, observe a change in phosphotyrosine content of ZO-1 by immunoblotting in response to histamine (T. W. Gardner, unpublished work).

Histamine-receptor effects

We next studied whether histamine receptors mediated the effect of histamine on endothelial-cell ZO-1 content. Confluent cells were exposed to the histamine-receptor antagonists, mepyramine, ranitidine, or both. After 4 h the cells were harvested, and crude membranes were prepared and immunoblotted for ZO-1. The overall effect was significant ($P < 0.05$). Histamine significantly reduces ZO-1 protein content by $45 \pm 8\%$ compared with controls ($P < 0.01$). ZO-1 immunoreactivity is reduced by $27 \pm 5.4\%$ in cells pretreated with mepyramine, and by $25.5 \pm 5.5\%$ in cells pretreated with ranitidine. These values are significantly higher than in histamine-treated cells ($P < 0.05$), and significantly less than control values ($P < 0.05$). When both mepyramine and ranitidine are given together prior to histamine, ZO-1 immunoreactivity is $98.1 \pm 6.4\%$ of untreated control values. Neither the H_1 -receptor antagonist, mepyramine, nor the H_2 -receptor antagonist, ranitidine, applied by themselves at twice their respective K_d , significantly affected ZO-1 protein content (results not shown). These results are representative of three separate experi-

ments. The results suggest stimulation of both major histamine-receptor subtypes induces a signal-transduction cascade in cultured retinal microvascular endothelial cells which modifies microvascular endothelial-cell ZO-1 protein expression.

Both H_1 and H_2 histamine-receptor stimulations increase microvascular permeability, depending on the circulatory bed. H_1 receptors increase permeability in cultured endothelial umbilical-vein cells, and H_2 receptors increase brain capillary permeability [6,26]. Both receptors mediate retinal vascular permeability in experimental diabetes [27]. In the present study we found that specific H_1 and H_2 histamine-receptor antagonists each partially prevent reduction in endothelial-cell ZO-1 expression. When mepyramine and ranitidine were applied together, the reduction of ZO-1 was completely blocked. We interpret these data to suggest that both H_1 and H_2 receptors mediate part of the observed changes in ZO-1 expression. This conclusion is supported by the observation that both receptors stimulate G-proteins, activate phospholipase C, release InsP_3 and diacylglycerol, and mobilize intracellular Ca^{2+} stores (reviewed in [28]). The receptors differ in that the H_1 receptor activates protein kinase C (PKC), whereas the H_2 receptor activates protein kinase A. PKC activation has been implicated both in the assembly of tight junctions and with increased permeability [29,30]. Schneeberger and Lynch have suggested that the effect of PKC in tight-junction assembly differs from its role in regulation of tight junctions in a steady state [31]. Our results suggest that the G-proteins and PKC activity associated with histamine H_1 and H_2 receptors may act to decrease tight-junction integrity. It is likely that these effects are cell-type- and PKC isoform-specific [31].

In summary, these data strongly suggest that histamine exerts specific effects on one tight-junction protein, ZO-1, and raise the possibility that other tight-junction components may also be altered. This finding may provide a novel explanation for increased microvascular permeability and suggests that other inflammatory mediators may act in a similar fashion. Further work is currently in progress to dissect the signal transduction of this effect and to determine whether it represents a change in ZO-1 gene expression, translation and/or protein stability.

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