Histamine type 1-receptor activation by low dose of histamine undermines human glomerular slit diaphragm integrity

DOI:10.1016/j.phrs.2016.10.011

Histamine Type 1-Receptor activation by low dose of histamine undermines human Glomerular Slit Diaphragm Integrity

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Abstract

Histamine has been reported to decrease the ultrafiltration coefficient, which inversely correlates with glomerular permselectivity, however the mechanism(s) underling this effect have never been investigated. This study aimed to assess whether histamine could exert a direct detrimental effect on podocyte permeability and the possible involvement of two key proteins for the glomerular slit diaphragm (SD) integrity, zonula occludens-1 (ZO-1) and P-cadherin.

The effect of histamine (100 pM-1000 nM) on coloured podocytes junctional integrity was evaluated functionally by a transwell assay of monolayer permeability and morphologically by electron microscopy. Histamine receptor ($H_{1-4}R$) presence was evaluated at both mRNA (RT-PCR) and protein (immunofluorescence) levels. The K_d and B_{max} values for [^{3}H]mepyramine were detemined by saturation binding analysis; IP_1 and cAMP production evoked by histamine were measured by TR-FRET. ZO-1, P-cadherin and vimentin expression was assessed by qRT-PCR and quantitative immunoblotting.

Histamine elicited a time- and sigmoidal dose-dependent (maximum effect at 8 h, 10 nM) increase in podocyte paracellular permeability widening the paracellular spaces. Only H_1R was predominantly localised to the podocyte membrane. Consistently, histamine elicited a sigmoidal dose-dependent increase in IP_1, but not in cAMP. Histamine exposure evoked a concentration-dependent reduction in both ZO-1 and P-cadherin and a parallel induction of vimentin mRNA expression with a maximum effect after 6 h, and protein expression with a maximum effect after 8h. These effects were prevented by the selective H_1R antagonist chlorpheniramine.

In conclusion, our data demonstrate that histamine, via the H_1R, modifies SD morphological and functional integrity, in part, by decreasing the expression of ZO-1 and P-cadherin.

Keywords: histamine, podocytes, paracellular permeability, histamine receptors, junction integrity.

Abbreviations cAMP, cyclic adenosine monophosphate; *CDH3*, cadherin 3, type 1, P-cadherin gene; ER, endoplasmic reticulum; FITC, fluorescein; *GADPH*, glyceraldehyde 3-phosphate dehydrogenase gene; GBM, glomerular basement membrane; $H_{1\text{-}4}R$, histamine receptor $_{1\text{-}4}$ subtypes; K_f, ultrafiltration coefficient; IP_1, inositol monophosphate; IP_3, inositol 1,4,5-trisphosphate; PAN, puromycin aminonucleoside; qRT-PCR, quantitative real-time PCR; SD, slit diaphragm; TBP, TATA-binding protein; *TJP1,* tight junction protein 1 gene; TR-FRET, Time-Resolved Fluorescence Resonance Energy Transfer; *VIM* vimentin gene; ZO, Zonula Occludens

1 Introduction

Histamine is a pleiotropic vasoactive amine, whose pathogenic role in microvascular endothelial paracellular permeability has been extensively studied [1-7]. Most of these studies describe acute events (within seconds to minutes), resulting in a rapid transient increase in permeability, due to a rapid formation of endothelial gaps [2-7]. Moreover, histamine has been suggested to be involved in prolonged vascular leakage by reducing Zonula Occludens (ZO)-1 protein expression in cultured retinal microvascular endothelial cells within hours [8]. The work by Takeuchi K and colleagues (2001) suggested that histamine-induced paracellular permeability might be also extended to other epithelial cells. In particular, histamine was shown to significantly downregulate ZO-1 mRNA expression in cultured human nasal epithelial cells [9]. ZO-1 contributes to the functional integrity of different permeability barriers among which the glomerular filter is one [10]. Interestingly, histamine has been previously reported to decrease the ultrafiltration coefficient (K_f) [11]. Thus, our hypothesis was that histamine could modify K_f by regulating ZO-1 expression in the podocyte.

Podocytes are parenchymal cells known to be highly dynamic and terminally differentiated. They interact with the glomerular basement membrane (GBM) and communicate through various signalling pathways at the slit diaphragm (SD). The glomerular SD represents the junction structure that links the interdigitating foot processes from neighbouring podocytes and consists of transmembrane-bridging proteins networking with a juxtaposed cytoplasmic platform of protein complexes, which in turn is linked to the actin cytoskeleton [12]. Within this cytoarchitecture, ZO-1 protein is located at the cytoplasmic face of the SD [13] and has been accepted to be one of its functional molecules; a disrupted interaction and distribution of ZO-1 in podocytes results in loss of SD structure and function [14-16]. Besides the transmembrane protein, P-cadherin, a podocyte specific adhesion protein [17] localised on adherens-type junctions, mediates calcium-dependent cell-cell bonds and its loss is recognised as a cause of barrier filtration integrity impairment [18]. Therefore, it is likely that glomerular injury affecting ZO-1 and/or P-cadherin results in loss of SD structure, podocyte detachment, K_f reduction and in a subsequent impairment of the filtration barrier integrity with proteinuria, progressive renal damage and eventual loss of renal function [12, 19, 20].

Among the histamine receptor subtypes, $H_{1-4}R$, H_1R and H_2R were first described in mammalian glomeruli [21-23], but these studies were focused on the entire glomerulus or only on stromal cells such as mesangial cells; little is known about parenchymal cells. Indeed, the data on histamine receptors expression on renal parenchymal cells arise only from our recent observations of H_1R, H_2R, H_3R and H_4R on tubular epithelial cells [24-26]. However, no such studies have to date focused on podocytes.

Thus, the present study was designed to investigate whether histamine could exert a direct detrimental effect on podocyte permeability compromising SD functional integrity, the underlying histamine receptor pharmacology, and the possible involvement of two key SD-associated proteins ZO-1 and P-cadherin.

2 Materials and Methods

2.1 Materials

All reagents and chemicals used were from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. Cell media and reagents were from Lonza group Ltd. (Allendale, NJ, USA). Hans Balanced Salt Solution was from GIBCO (Grand Island, NY). HTS Transwell inserts were from Corning Life

Sciences (Lowell, MA). RevertAid™ First Strand cDNA Synthesis Kit, GeneRuler™ 50 bp DNA Ladder, DNA Gel Loading Dye (6X), CellMask™ Orange plasma membrane stain, MagicMark™ XP Western Protein Standard and Alexa-Conjugated secondary antibodies donkey anti-Mouse IgG (A-31570), chicken anti-Goat IgG (A-21469) and goat anti-Rabbit IgG (A-11034) were from Thermo Fisher Scientific Inc. (Rockford, IL, USA). EuroTaq DNA polymerase as well as EuroGOLD Trifast™ were from Euro-clone (Milan, Italy). High Capacity cDNA Reverse Transcription Kit and Power SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Sequence-specific oligonucleotide primers were purchase from Sigma-Genosys (Milan Italy). The antibodies for histamine H_1R (H300, sc-20633), H_2R (A20, sc-33973), calnexin (AF18, sc-23954), ZO-1 (C-19, sc-8146), P-cadherin (H-105, sc-7893), vimentin (C20, sc-7557) and UltraCruz™ Autoradiography Film were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), while anti-rabbit and anti-mouse IgG HRP-linked antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA) and the swine anti-Goat IgG antibody from Cedarlane Labs (Ontario, Canada). The LANCE® Ultra cAMP Detection Kit, the IP-One HTRF® assay kit, the [^{3}H]mepyramine [PubChemCID 656400; kindly provided by Prof. Rob Leurs (20 Ci/mmol) VU University Amsterdam, Amsterdam] and the Whatman™GF/C Glass Fiber Filter Paper were from PerkinElmer Inc. (Waltham, MA, USA) and Cisbio Bioassays (France), respectively. Precision Plus Protein™ Dual Color Standards and BCA protein assay were from Pierce Bio-technology Inc. (Rockford, IL, USA) and PVDF membrane from Millipore (Bradford, MA, USA). Visiglo™ HRP chemiluminescent substrate kit was purchased from Amresco llc. (Solon, OH, USA).

Histamine dihydrochloride (PubChem CID 5818), (+/-) chlorpheniramine maleate (PubChem CID 5281068), [^{3}H]mepyramine and difenhydramine (Pubmed CID 3100) were dissolved in dimethyl sulfoxide, and the final drug concentrations were obtained by dilution of stock solutions in the experimental buffers. The final concentration of the organic solvent was less than 0.1%, which had no effect on cell viability.

2.2 Cell cultures

Immortalised human podocytes were obtained from the respective primary cells, derived from the normal portion of cortex surgically removed kidneys (n=5) for as described previously [27], by infection with a hybrid Adeno5/SV40 virus as previously described [27-29]. The line was generated in 1997, after the authorization of the local Ethical Committee (Hospital San Giovanni Battista "Molinette", Turin, Italy). Podocytes were isolated from the healthy tissue derived from kidney samples of patients who underwent unilateral nephrectomy due to local renal carcinomas as first-line treatment. To our knowledge, no other relevant pathology was diagnosed in the medical history of each patient enrolled and the derived podocytes can be reasonable assumed as healthy podocytes. Podocytes were characterised for the positive expression of nephrin, podocin, and synaptopodin and for negative expression of von Willebrand factor, CD31, and smooth muscle cell actin. Cells were cultured in DMEM containing 4.5 mg/l glucose supplemented with 10% Fetal Calf Serum, penicillin/streptomycin (100 IU/ml), and l-glutamine and the cultures were maintained at 37 °C in a 95% air/5% CO_2 humidified incubator.

2.3 Permeability assay

Podocyte monolayer permeability was determined as previously described [30, 31]. Human immortalised podocytes (40,000 cells well, 500 µl) were seeded on the top of HTS Transwell inserts (3 µm pore, 24-well plate) and cultured till confluence was achieved. Cells were washed twice with PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and then pre-treated with vehicle alone or chlorpheniramine maleate 10 µM for 10 min before exposure to histamine 0.01-1000 nM for 0-8 h. Fluorescein (FITC)-labeled bovine serum albumin was added to the bottom chambers of the transwells and medium fluorescence activity in the top chambers was measured at selected time-points with a fluorescence plate reader (excitation: 495 nm, emission: 520 nm; multiple plate reader Victor X4; PerkinElmer Inc.). Fold-change in expression with respect to the control mean was calculated for all samples.

2.4 Electron microscopy

Podocytes pre-treated with vehicle alone or chlorpheniramine maleate 10 μM for 10 min before exposure to histamine 10 nM or 0.1 nM for 0-8 h were fixed in 4% glutaraldehyde and post-fixed, after pelletting, in 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 20 kV and 50 kV. Morphometrical analysis were performed on 50KV digitized images using ImageJ 1.41 software (http://rsbweb.nih.gov/ij; NIH, USA) in 20 regions of interest (ROI) for each sample.

2.5 RT-PCR

Two μg/μl of total RNA extracted from podocytes by using RevertAid™ First Strand cDNA Synthesis Kit according to the manufacturer's instruction, were subjected to RT-PCR as previously described [26, 32]. The subsequent specific oligonucleotide sequences were used: *hH1R* forward 5' CATTCTGGGGGCCTGGTTTCTCT-3' and reverse 5'-CTTGGGGGTTTGGGATGGTGACT-3'; *hH2R* forward 5'-CCCGGCTCCGCAACCTGA-3' and reverse 5'-CTGATCCCGGGCGACCTTGA-3'; *hH3R* forward 5'-CTTCCTGCCCTAGCAGTT-3' and reverse 5'-GCAGAGAACAGCTTCGAGGTT-3' *hH4R* forward 5'-TGGAAGCGTGATCATCTCAG-3' and reverse 5'-ATATGGAGCCCAGCAAACAG-3'. PCR amplicons were resolved in an ethidium bromide-stained agarose gel (2.5%) by electrophoresis. GAPDH gene expression was used as an internal control.

2.6 Immunocytofluorescence and confocal analysis

Podocytes plated on collagen-coated cover glasses were fixed with 4% paraformaldehyde for 10 minutes at room temperature. H_4R was detected using The anti-hH_4R (374–390) antibody produced and validated for detecting both human and rodent H_4R in the School of Biological and Biomedical Sciences, Durham University [33-39]. Sections were incubated overnight with anti-H_1R (1.3 μg/ml), anti-H_2R (1.3 μg/ml) or anti-H_4R (2 μg/ml) receptor subunit at 4°C. Subsequently, for endoplasmic reticulum (ER) cells were incubated with anti-calnexin (2 μg/ml) followed by incubation with the respective Alexa-Conjugated secondary antibodies. The negative control to exclude non-specific staining by the secondary antibody (where cells were incubated with secondary antibodies alone) is reported in Supplementary Material Fig. 1. The plasma membrane was stained using CellMask™ Orange plasma membrane stain according to the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde and processed for histamine receptors staining. Nuclei were stained with Hoescht.All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica). A variable number of optical section images in the z-dimension (z-spacing, 0.42μm) were collected ensuring that images throughout the 3D cellular structure, spanning multiple confocal planes, were fully captured. Maximum projection of the confocal images were analyzed quantitatively for the extent of colocalization of $H_{1\text{-}4}$Rs with each of the sub-compartment marker proteins using the ImageJ software package.

2.7 Radioligand binding studies

The saturation binding isotherms were determined by incubating washed podocyte homogenates for 45 min at 25°C with 0–16 nM [^{3}H] mepyramine and assay buffer (50 mM phosphate buffer pH 7.4) in a total assay volume of 200 μl.The incubation was stopped by rapid dilution with ice-cold assay buffer. Non-specific binding was determine using unlabelled 10 μM difenhydramine. The bound radioactivity was separated by filtration through GF/B Glass Fiber Filter Paper that had been treated with 0.3% polyethyleneimine (PEI). Filters were washed thrice with ice-cold assay buffer and the radioactivity retained on the filters was measured by liquid scintillation counting. Protein concentrations were determined according to Bradford methods, using bovine serum albumin as a standard.

2.8 Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

The TR-FRET assay was used to evaluate both cAMP and IP_1 production by using the LANCE® Ultra cAMP Detection Kit and the IP-One HTRF® assay kit, respectively, as previously described [26] and according to their manufacturer's instruction. The energy transfer was measured by the multiple plate reader Victor X4 (excitation: 620 nm, emission: 665 nm).

2.9 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on RNA isolated by EuroGOLD Trifast™ according to the manufacturers' instructions from podocytes (80% confluence) pretreated with vehicle alone or with chlorpheniramine maleate at 10 μM and challenged with histamine in the range 3 pM-1000 nM for 0-8 and 24 h. Briefly, first-strand cDNA was produced from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit. Real-time PCR experiments were performed in 20-μl reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (*TJP1* forward: CATCAGATCATTCTGGTCGATCA and reverse: TCCGGAGACTGCCATTGC; *CDH3* forward: CCAGCTTGGGCAACATAGGGTand reverse: TCAGCTCCCGCTGAGACTACA; *VIM* forward: GGAACAGCATGTCCAAATCGATand reverse: CAGCAAACTTGGATTTGTACCATT), and the Power SYBR Green PCR Master Mix. TATA-binding protein (TBP) mRNA were used to normalise RNA inputs. The relative expression of mRNA was calculated according to the $2^{(-\Delta Ct)}$ method. Fold-change expression with respect to control was calculated for all samples.

2.10 Immunoblotting

Sixty μg of proteins, extracted by EuroGOLD Trifast™ according to the manufacturers' instructions from podocytes (80% confluence) exposed to vehicle alone or pretreated with vehicle alone or with chlorpheniramine maleate and challenged with histamine in the range 3 pM-1000 nM for 0-8 and 24 h, were subjected to SDS-PAGE using a 8 % gel. The PVDF membrane was blotted over-night with goat polyclonal anti-ZO-1, rabbit polyclonal anti-P-cadherin or goat polyclonal anti-vimentin antibodies (1 μg/ml in PBS) and then re-probed with mouse monoclonal anti-β-actin antibody (1:5000) to confirm the homogeneity of the proteins loaded. The membranes were overlaid with Visiglo™ HRP chemiluminescent substrate kit and then exposed to Hyperfilm ECL film. Densitometric analysis was performed by ImageJ software package. Fold-change expression with respect to control was calculated for all samples.

2.11 Data analysis and statistical procedures

Gene and protein expression data are expressed as fold-changes relative to the control (vehicle alone), which is represented as one-fold. For the permeability assay the mean of the control values have been calculated and all the individual control values and all the individual test values are expressed as fold-change relative to the control mean. Results are shown as mean ± SEM from 5 different experiments, and were analysed by Prism 4 software from Graphpad (CA, USA). The test for normality using the Kolmogorov-Smirnov followed by the one-way ANOVA and the *post-hoc* Dunnett's multiple comparison were performed when there was a variance homogeneity (permeability assay). In all the other cases, the non-parametric Kruskal-Wallis test was used. For concentration-response curves a four-parameter logistic equation was applied and the best-fit was obtained. Radioligand binding data were evaluated by a nonlinear, least-squares curve-fitting procedure using GraphPad Prism 5. To determine significant differences between means, the threshold for statistical significance was set to P-values < 0.05.

3 Results

3.1 Histamine compromises SD integrity in human immortalised podocytes

We tested whether the amine influences the transepithelial flow of FITC-albumin in a transwell assay of monolayer permeability. We found that histamine treatment (0.01-10 nM; 0-8 h) time- and concentration-dependently and significantly increased fluorescence intenstity in the top chamber beginning from 6 h (basal level of Fluorescence intensity = 15651 ± 950 FU; $P< 0.05$) up to 160% at 8 h ($P<0.01$; Fig. 1a) with an EC_{50} of about 0.1 nM (Fig. 1b). Notably, the 10 min pre-treatment of podocytes with chlorpheniramine maleate was effective in preventing the transepithelial flow of FITC-albumin, as demonstrated by its ability to partially prevent the effect exerted by histamine 0.1 nM (Fig. 1c).

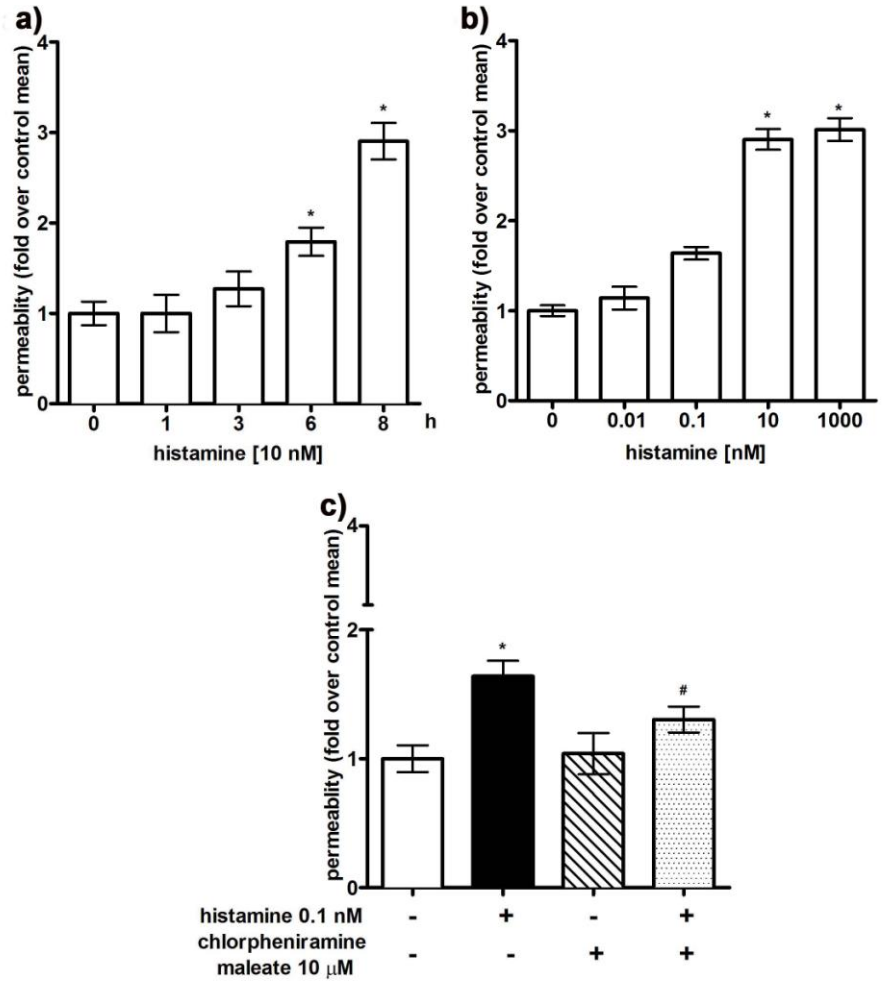

Fig. 1. Junction functional integrity of human immortalized podocyte. Transepithelial albumin permeability of podocyte monolayer was measured using FITC-BSA. (**a**) Cells were exposed to histamine 10 nM from 0 to 8 h. Data, expressed as fold-change over control mean, are means ± SEM of 5 independent experiments. Statistical analysis was performed by one-way ANOVA and Dunnett test. *$P < 0.05$ *versus* time 0. (**b**) Cells were exposed to histamine 0 - 1000 nM for 8 h. Data, expressed as fold-change over control mean, are means ± SEM of four independent experiments. Statistical analysis was performed by one-way ANOVA and Dunnett test. *$P < 0.05$ *versus* concentration 0. (**c**) Podocytes pretreated for 10 min with vehicle alone or the selective H_1R antagonist, chlorpheniramine maleate 10 μM, were exposed to histamine (0.1 nM) for 8 h. Data, expressed as fold over control, are means ± S.E.M. of 5 independent experiments. *$P < 0.05$ *versus* vehicle alone and $^{\#}P < 0.05$ *versus* histamine 0.1 nM.

The detrimental effect of H_1R activation on SD was strongly validated when the junction integrity was quantitatively evaluated by electron microscopy. The width of the intercellular space was narrow and constant throughout the contact area in the control cells (Fig. 2a and b) whereas it was significantly compromised after histamine exposure for 8 h at 0.1 nM (Fig. 2b), appearing to be discontinuous and uneven with widening of the paracellular spaces after histamine exposure for 8 h at 0.1 nM. The pre-treatment with chlorpheniramine maleate was able to ameliorate the histamine evoked-effects as intercellular spaces appeared narrower, although this was incomplete (Fig. 2a and b). Chlorphenamine treatment alone did not affect the intercellular contact area.

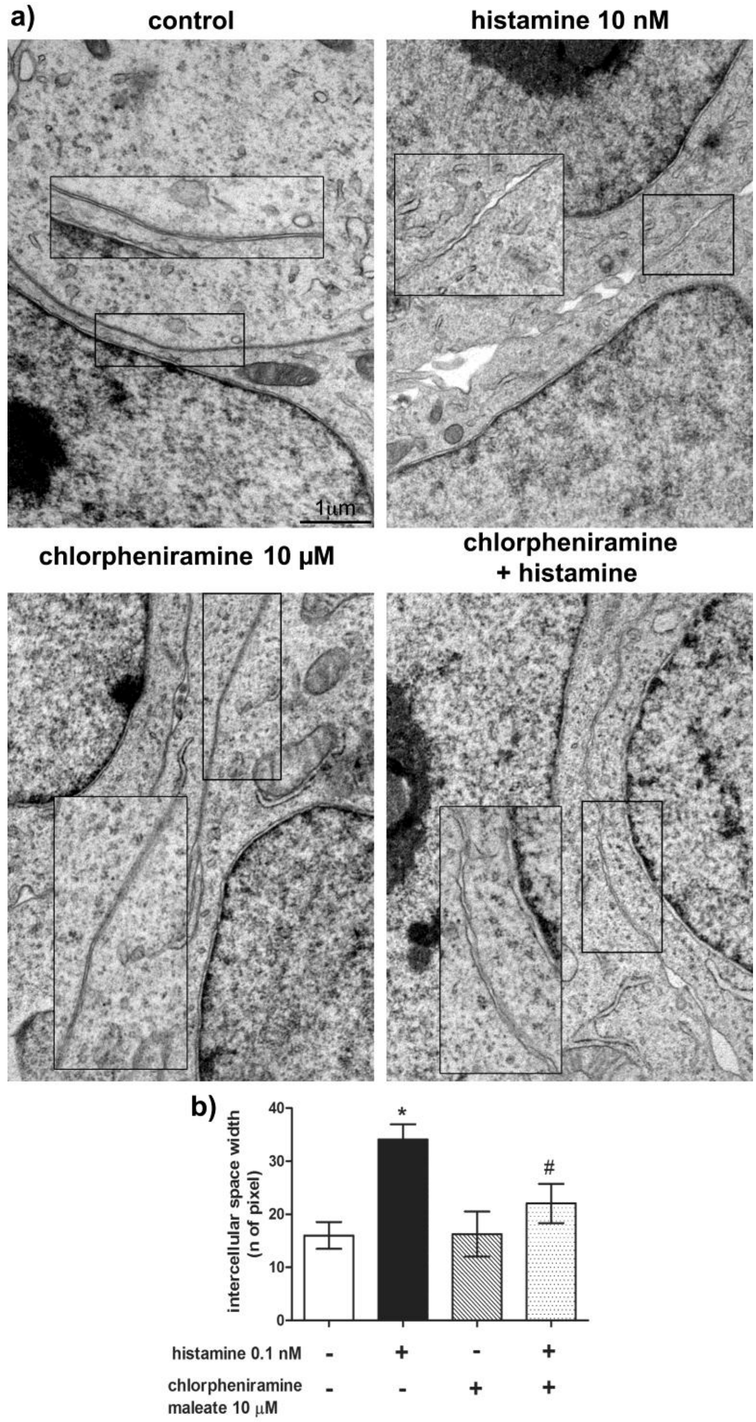

Fig. 2 Chlorpheniramine effect on junction integrity. (**a**) Representative images of ultrathin sections from human immortalized podocytes pretreated for 10 min with vehicle alone or the selective H_1R antagonist, chlorpheniramine maleate 10 μM, were exposed to histamine (0.1 nM) for 8 h. The morphological assessment of the junction integrity was carried out in three different experiments. Sections were examined under a JEM 1010 electron microscope at 20 kV. The insets show the integrity junctional level detail evaluated at 50 kV. (**b**) Quantitative analysis of the

intercellular junction morphology performed on 50KV digitized images. Data, expressed as fold-change over control, are means ± S.E.M. for n = 20 regions of interest (ROI) for each sample. *$P <$ 0.05 *versus* vehicle alone and #$P <$ 0.05 *versus* histamine 0.1 nM.

3.2 Histamine receptor expression in human immortalised podocytes

In order to further explore the underlying histamine pharmacology, the expression of histamine receptors in human immortalised podocytes was evaluated at both the gene and protein levels. Moreover, their functional expression was confirmed by TR-FRET assay evaluating the second messenger production induced by histamine. As shown in Fig. 3a, RT-PCR analysis revealed single transcripts corresponding to the predicted size for *H1R* (403 bp) and *H4R* (353 bp); *H2R* (497 bp) was at the limit of detection. No transcript for *H3R* (221 bp) was detected. Consistent results were obtained when protein expression was evaluated by immunocytofluorescence and confocal analysis (Fig. 3b and c). Indeed, H_1R showed a robust staining with a prominent membrane localisation as demonstrated by its colocalisation with CellMask™ plasma membrane stain (Pearson's coefficient, r = 0.62; Mander's coefficients threshold for channel 1, red staining, tM1 = 0.38 and Mander's coefficients threshold for channel 2, green staining, tM2 =0.93); moreover, H_1R coimmunolabelling with calnexin (r = 0.59; tM1 = 0.76 and tM2 = 0.59), suggestive of the presence of a proportion of H_1R on the ER, was also revealed. In comparison, H_4R immunolabeling showed a lower immunopositivity level, predominantly within the cytoplasm where a partial positivity was found in the ER (r = 0.21, tM1 = 0.30 and tM1 = 0.22 for the colocalization within the membrane and r = 0.29, tM1 = 0.28 and tM2 = 0.33 for the colocalization within the ER; Fig.3b and c). Very modest, if any, membrane colocalisation was revealed. The staining for H_2R was at the limit of the detection and was not possible to localise it within the membrane.

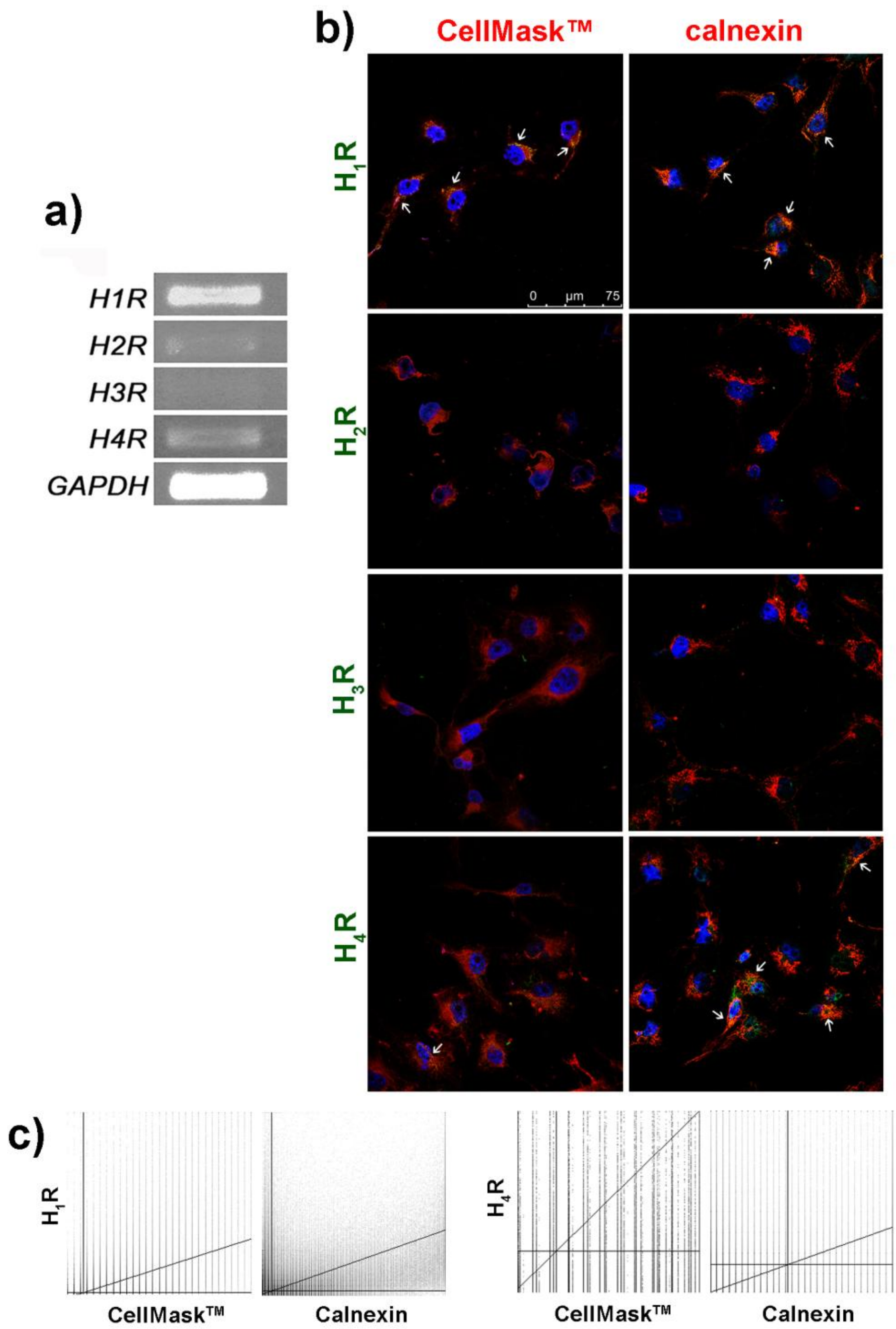

Fig. 3 Histamine receptor expression in human immortalized podocytes. (**a**) Agarose gels representative of 5 independent RT-PCR assays for cDNA from human immortalized podocyte. Single transcripts corresponding to the size predicted for *H1R* (403 bp), *H2R* (497 bp), *H3R* (221 bp), and *H4R* (353 bp) were detected. The housekeeping gene *GAPDH* was used as control. (**b**) Representative merged immunofluorescence images from 5 independent experiments where cells were labelled with specific anti-H_1 anti-H_2, anti-H_3, or anti-H_4 receptor antibodies (green),

respectively, calnexin or CellMask™ Plasma Membrane Stains (red). Nuclei were stained with Hoescht (blue). Detection of yellow colouration, shown by arrows, indicates colocalization.All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica). (**c**) Scatterplot of red and green pixel intensities of the maximum projection of the image volume in (**b**).

These data suggested the presence of H_1R on the surface of human podocytes, therefore a saturation binding study with the H_1R antagonist mepyramine were performed to quantify the receptor levels. The saturation isotherms revealed a K_d for [^{3}H] mepyramine of 7.02 ± 1.76 nM, indicating the presence of a single class of high affinity binding sites in podocyte membranes. The binding was saturable with a B_{max} estimate of 376 ± 39 fmol/mg protein (Fig. 4a).

To further confirm our findings, we tested the activation of the histamine receptors evaluating the levels of cAMP and IP_3 second messengers evoked by histamine. As shown in Fig. 4b, podocytes challenged with histamine (3 pM-10 nM) did not show any changes in cAMP levels, the second messenger predominantly coupled with G_s (such as H_2R) and with G_i (such as H_3R and H_4R) receptors. Notably, cells exposed for 1 h to histamine 3 pM-10 nM showed a concentration-dependent decrease in TR-FRET signal, indicating a sigmoidal increase of IP_1 (EC_{50} 0.15 ± 0.03 nM). The pre-treatment for 10 min with the selective H_1R antagonist chlorpheniramine maleate at either 1 μM or 10 μM shifted rightwards in a parallel fashion the curve evoked by histamine in a dose-dependent manner (chlorepheniramine maleate alone did not affecteither cAMP or IP_1 production; data not shown). Taken together, these data confirm the functional expression of H_1R on podocyte membrane.

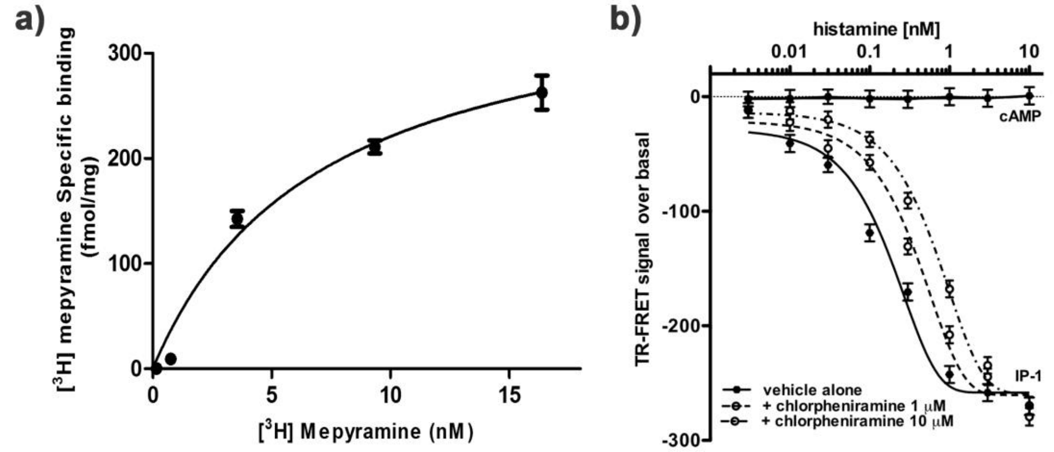

Fig. 4 Histamine receptor pharmacological profile in human immortalized podocytes. Saturation binding curve for [^{3}H]mepyramine binding to podocyte cell membrane homogenates (**a**). Data are the mean ± SD of 3 separate cell preparations determined in triplicate. (**b**) The levels of IP_1, downstream metabolite of IP_3, and of cAMP were measured, according to the manufacturer's instruction, by IP-One HTRF® assay kit (Cisbio) or by LANCE Ultra cAMP assay (PerkinElmer), respectively. Human immortalized podocytes were pretreated for 10 min with vehicle alone (black hexagon, solid line for IP_1 and white square solid line for cAMP) or the selective H_1R antagonist, chlorpheniramine maleate 1 μM (white circle, dash line) or 10 μM (white circle, dash-dot line), were exposed to histamine (3 pM – 10 nM). Results, expressed as TR-FRET signal over the basal and represented as best-fit concentration-response curve, are the mean ± SEM of 5 independent experiments run in duplicate.

3.3 Histamine affects SD protein expression in human immortalized podocyte

The effect evoked by histamine on SD associated proteins ZO-1 and P-cadherin was evaluated at both gene and protein levels. As shown in Fig. 5a and Fig. 6a, cells challenged with histamine (1 pM–10 nM up to 8 h) showed a time- and concentration-dependent reduction of both TJP1, the gene encoding for ZO-1, and CDH3, the encoding gene for P-cadherin, with a maximum of approx. 50% after 6 h. Consistently, similar results with a maximum effect at 8 h were obtained when protein expression was evaluated (Fig. 5b and c, and Fig. 6b and c).Moreover, histamine evoked a parallel induction of the intermediate filament vimentin expression (Figure 5a, b and c and Figure 6a, b and c), a mesenchymal marker associated with podocyte effacement and detachment [40].

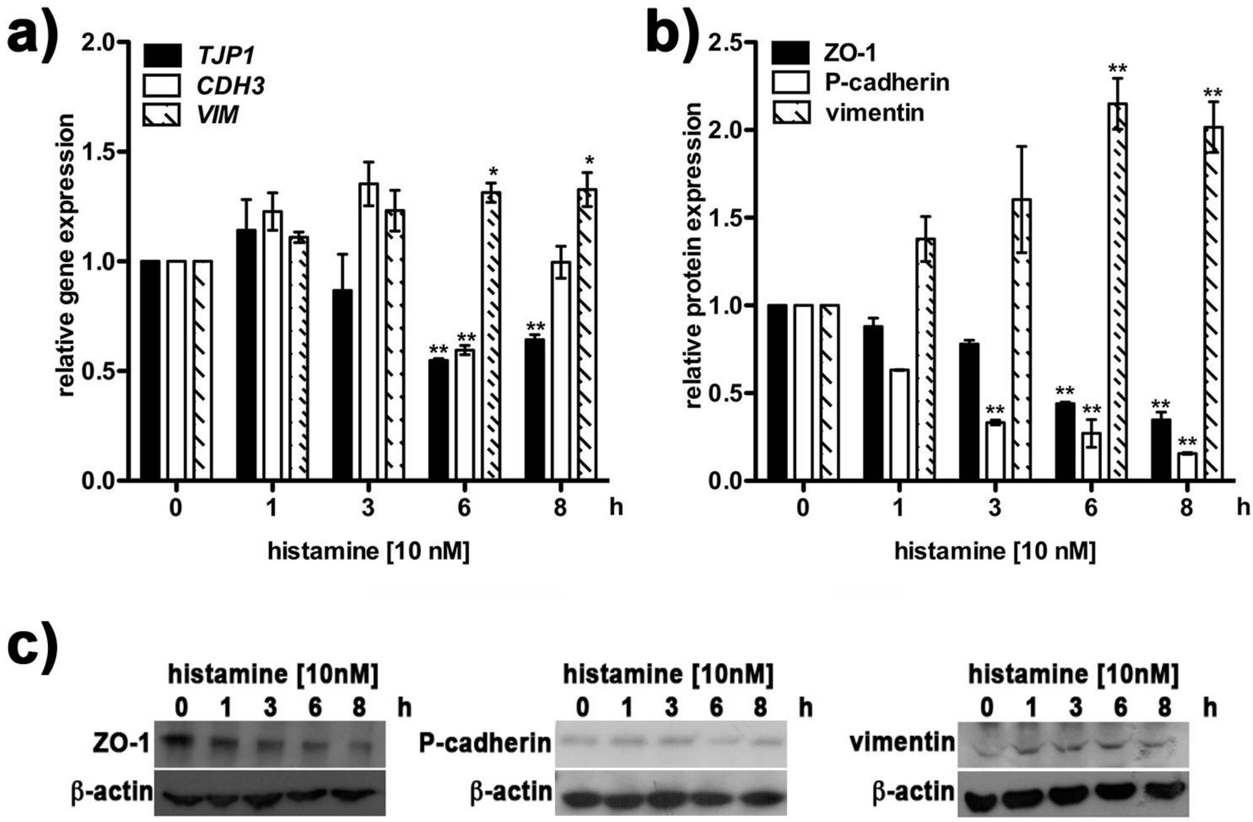

Fig. 5 Effect evoked by histamine on ZO-1, P-cadherin and vimentin expression: time-course. Effect evoked by histamine on ZO-1, P-cadherin and vimentin expression: time-course. Human immortalized podocytes were treated with histamine (10 nM) from 0 to 8 h. At selected time *TJP1*, *CDH3* and *VIM* expression was evaluated by qRT-PCR (**a**) and ZO-1, P-cadherin and vimentin protein expression was determined by Western blot analysis (**b**); results are expressed as mean ± SEM of five independent experiments; *$P< 0.05$ *versus* time 0. Pictures shown are representative of five independent immunoblotting experiments (**c**).

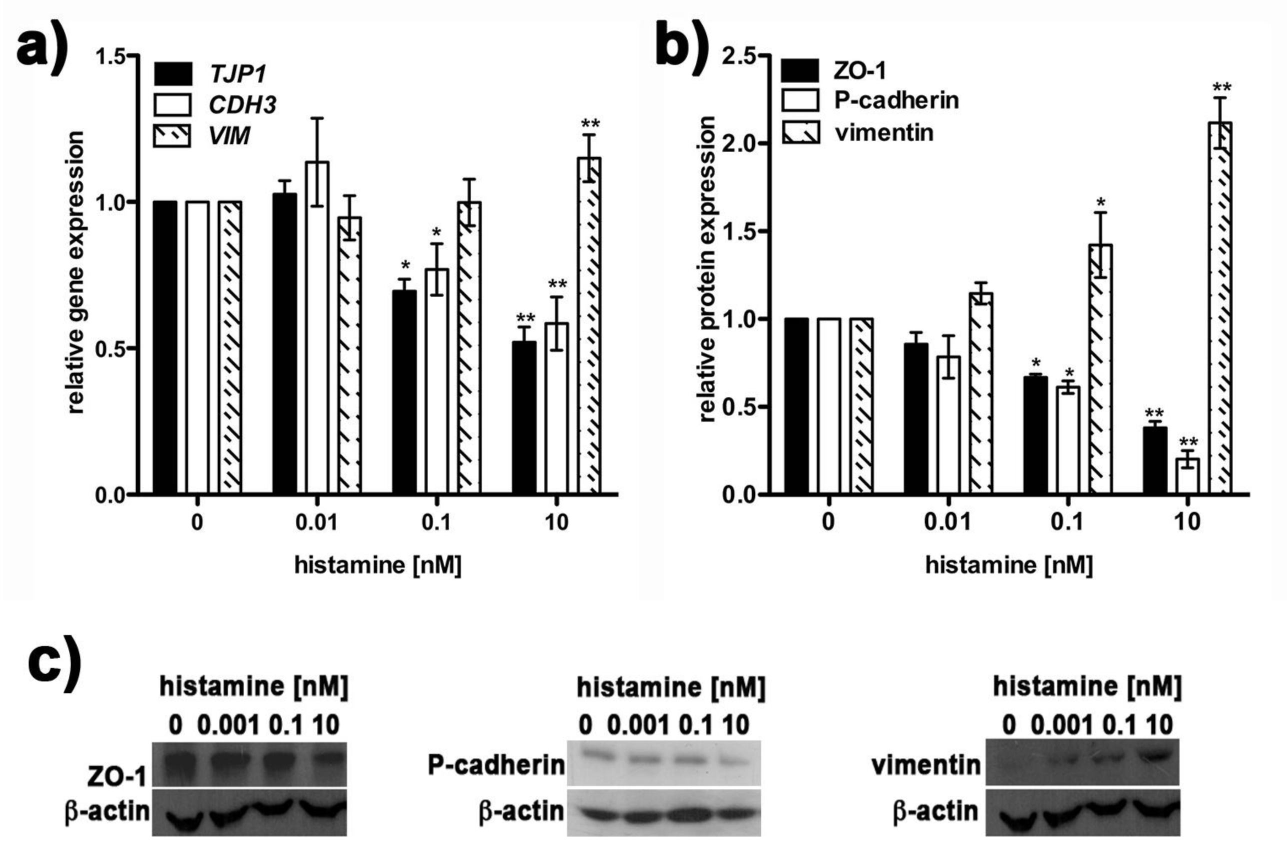

Fig. 6 Effect evoked by histamine on ZO-1, P-cadherin and vimentin expression: concentration-response. Human immortalized podocytes were treated with histamine (0-10 nM) for 6 or 8 h and processed for TJP1, CDH3 and VIM expression by qPCR (**a**) and ZO-1, P-cadherin and vimentin protein expression by Western blot analysis (**b**), respectively; results are expressed as mean ± SEM of five independent experiments, $^*P < 0.05$ *versus* 0 (vehicle alone). Pictures shown are representative of five immunoblotting independent experiments (**c**).

Notably, in our experimental condition, synaptopodin and podocin expression, both markers of podocyte differentiation, was not affected (Supplementary Material Fig. 2)

When cells were pre-treated with the selective antagonists chlorpheniramine maleate 10 μM, a parallel rightward shift of the curves evoked by histamine was observed with an increase in EC_{50} from 0.09 ± 0.03 nM to 7.62 ± 1.44 nM for ZO-1 (Fig. 7a), from 0.13 ± 0.25 nM to 8.57 ± 2.25 nM for P-cadherin (Fig. 7b) and from 2.40 ± 1.23 nM to 9.76 ± 3.23 nM for vimentin (Fig. 7c), respectively.

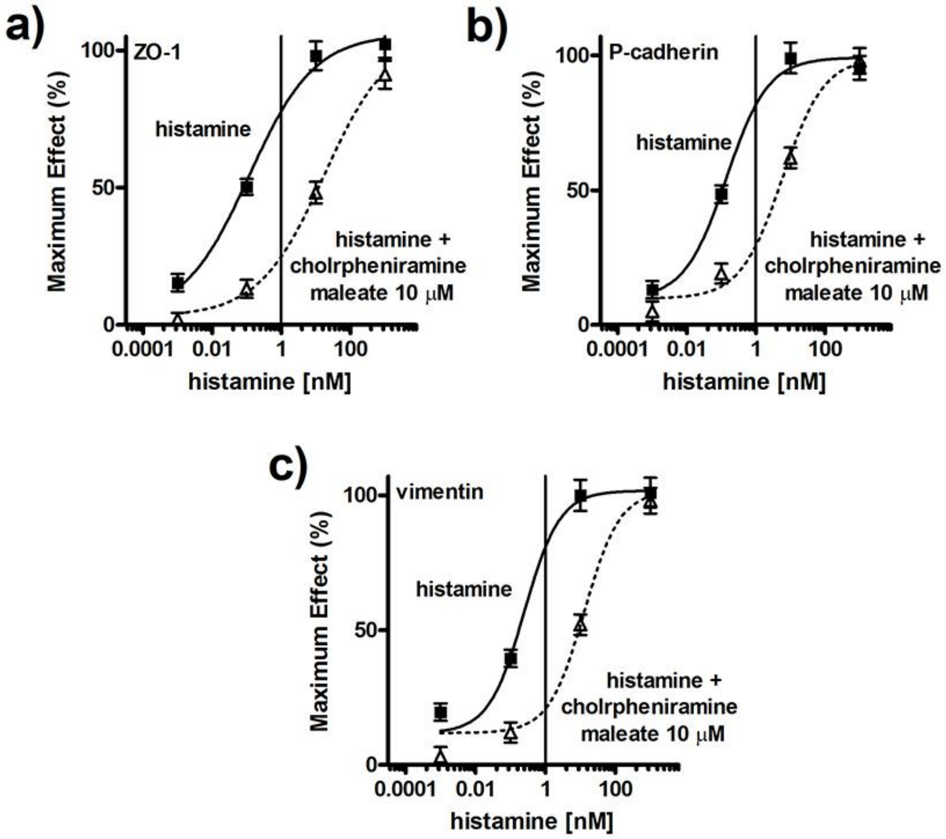

Fig. 7 Chlorpheniramine antagonism on ZO-1, P-cadherin and vimentin expression evoked by histamine. Human immortalized podocytes pretreated for 10 min with vehicle alone or the selective H_1R antagonist, chlorpheniramine maleate 10 μM were treated with histamine for 8 h and processed for ZO-1 (**a**), P-cadherin (**b**) and vimentin (**c**) protein expression. Results, represented as best-fit dose-response curve, are expressed as mean ± SEM of 5 different experiments.

4 Discussion and Conclusions

This study demonstrates for the first time that histamine, via the H_1R, exerts a detrimental direct effect on SD integrity, which results in an increase in podocyte paracellular permeability. This effect is underpinned, in part, by the down-regulation of two key proteins vital for the maintenance of SD integrity, ZO-1 and P-cadherin, with a parallel increase in vimentin expression, the latter probably promoting an epithelial-mesenchymal transition. Interestingly, our study offers, not only a new insight into the effect of histamine on podocyte integrity, but also a clear demonstration of the involvement of histamine in prolonged permeability leakage. Indeed, the extended kinetics of the herein observed events (number of hours) are in keeping and strongly support the delayed mechanism by which histamine increases paracellular permeability involving transcriptional and transductional events [9]. Similar to what was reported for the endothelial junction [41] and in keeping with the ability of histamine to alter adhesion at sites between adjacent cells [42], the results herein suggest that the production of IP_3 by histamine interaction with H_1R triggers the activation of a signalling pathway ultimately resulting in an increase in paracellular permeability.

This detrimental effect in the human podocytes is supported by the reduction of the expression of two vital proteins for the SD integrity, ZO-1 and P-cadherin. Notably, although many *in vitro* podocytes cultures showed a cellular dedifferentiation, reflected by loss of processes, and accompanied by a down-regulation of synaptopodyn, our cells displayed a robust mature phenotype as indicated by not only synaptopodin and podocin expression (Supplementary Material Fig. 2a and 2c), but also nephrin [43, 44]. Therefore, our *in vitro* system can be considered a suitable new model to study SD-associated proteins. Most notably, our data provide evidence to point to human immortalised podocytes as an interesting new *in vitro* model to study the histaminergic system in naïve human cells. Our *in vitro* cell culture of human immortalised podocytes showed unique expression pattern for both H_1R and HDC enzyme (Supplementary Material Fig. 2a and c), this suggesting that both the autocrine and paracrine effects of this amine could be studied in this system.

The unexpected low EC_{50} (ca. 0.1 nM) herein reported for both ZO-1 and P-cadherin, closely comparable to the one evoked by histamine when IP_3 was measured, could be due, in part, to the ability of cells to endogenously produce histamine; indeed, our data show that podocytes clearly express the HDC enzyme (Supplementary Material, Fig. 3 a and b) and that the enzyme is functionally acting, although with a low rate of histamine production (Supplementary Material, Fig. 3c). The atypical apparent high potency of histamine, may also reflect changes in local histamine concentrations over time, H_1R membrane expression changes and trafficking during the extended exposure period (1-8h), or a large portion of spare receptors. Moreover, splice variations [45] and polymorphisms [46] in human H_1R have been observed, and their eventually occurrence in our cells cannot be ruled out. Although these alternative forms of H_1R have not been pharmacologically characterized thus far, they could be more sensitive to histamine than the wild-type form. The similar shifts in dose-response curves with the racemic (+/-) chlorpheniramine are consistent with the range of published pA_2 values for native H_1R across species and tissues [47-49].

Notably, the similar EC_{50}s for ZO-1, P-cadherin and IP_3 led also to speculate that H_1R activation in human podocytes could initiate different and parallel signaling pathways, which converge in the SD dysregulation. For instance, it has been reported that the elevation in IP_3 triggers the protein kinase C activation and evokes calcium efflux from the ER thus promoting signals to the cytoskeleton, ultimately resulting in the increase in permeability [8, 50-52]. However, histamine could affect the cytoskeleton rearrangement through also a phospholipase C-independent mechanism, evoking the phosphorylated myosin light chains accumulation through the RhoA/Rho-associated coiled kinase pathway and activating the mitogen-activated protein kinase p38 [41, 53]. These warrant further investigation.

Radioligand binding studies demonstrate high affinity [^{3}H]mepyamine binding sites in the podocyte cells with a mean K_d value comparable to previously published studies for the human H_1R [54]. Consistent with functional data indicating that histamine activates only the canonical H_1R-mediated response (second messenger IP_3, evaluated by IP_1), and not H_2R-, H_3R- or H_4R-mediated response (second messenger cAMP), we found that H_1R is predominantly localised on the plasma membrane. Its partial intracellular presence, suggested by co-localization with calnexin, could reflect the receptor trafficking in the presence of nM histamine concentrations.

Actually, in human podocytes, we clearly detected the H_4R receptor at both the gene and protein level, although no functional evidence of H_4R were observed. These data raises the discussion of the selectivity of H_4R antibody [55, 56], however we obtained the same results using different commercial H_4R antibodies (Supplementary Material, Fig. 4), at least one of which was validated by $H_4R^{-/-}$ mice. The low level of localisation of the H_4R at the cell membrane could be a possible explanation for the lack of the functional evidence. Indeed, we found a predominantly intracellular localisation accounting for a possible receptor trafficking or for the presence of splice variants of the H_4R. Indeed, the predominant intracellular localisation of H_4R splice variants was already reported by van Rijn and colleagues (2008). However, this speculation needs to be further investigated and also we could not completely rule out the hypothesis that in this cell type, H_4R might activate alternative second messengers, such as the recruitment of ß-arrestin [57].

Consistent with our results pointing to H_1R as the only histamine receptor functionally expressed in the podocyte membrane, only the pre-treatment with the competitive H_1R antagonist, chlorpheniramine maleate, shifted the curve to the right, while JNJ7777120, a well-known H_4R antagonist prototype, as well as ranitidine, a well-known anti-H_2 antihistamine drug, were both ineffective (Supplementary Material, Fig. 5). All these molecular events, the activation of H_1R leading to the increase in IP_3 production and the co-incident reduction in ZO-1 and P-cadherin, may represent the possible mechanism underlining the detrimental effect of histamine on junction morphological and functional integrity as suggested by the comparable time- and concentration-response profiles. Notably, although P-cadherin protein expression was already affected after 3 h of histamine challenge, the permeability leakage was evident only after 6 h, when also ZO-1 expression was reduced. This evidence is in keeping with previous studies showing that modulating P-cadherin alone is not sufficient for the SD dysregulation [58].

In addition to ZO-1 and P-cadherin down-regulation a parallel increase of vimentin expression was also observed. Again the concentration-response profile of vimentin induction by histamine was closely comparable to the one evoked by histamine when IP_3 was measured and, again, only the pre-treatment with chlorpheniramine maleate shifted the curve to the right, thus indicating a pure H_1R-mediated event. The increase of mesenchymal markers, such as vimentin, has been associated with podocyte effacement, detachment and loss in diabetic nephropathy [40]. Indeed, podocyte dedifferentiation and mesenchymal transition could be a potential pathway leading to their dysfunction, thereby playing a role in the genesis of proteinuria [59].

Histamine was previously demonstrated to alter the link between adherent junction and vimentin in primary human umbilical vein endothelial cells [50]. Moreover, vimentin was also already reported to be upregulated by histamine in primary mouse brain organotypic (MBO) cultures [60]. Vimentin has been also found to be upregulated in podocytes in puromycin aminonucleoside nephrosis [61], a model characterized by the presence of high levels of histamine in the renal cortex, whose abnormal metabolism was postulated to be related both as a cause or a consequence of the pathogenenesis [21].

Interestingly, until now histamine was assumed only to affect the K_f through the H_1R and H_2R present on mesangial cells, in keeping with the theory that contraction of these cells leads to a reduction in the glomerular capillary surface area. We provide for the first time, molecular pharmacological evidence for a direct effect of histamine on human podocytes, suggestive of a possible use of antihistamines as add-on therapy to counteract the onset and progression of both albuminuria and glomeruosclerosis in different renal aetiologies. Previously, this hypothesis was dismissed by the report that histamine and diphenhydramine had no effect on the alterations in glomerular ultrafiltration that occurs shortly after administration of anti-glomerular basement membrane antibody [62] in a model of acute glomerular immune injury in the rat. However, the authors claimed that it was unlikely that later events would differ greatly regarding a role for histamine because of the effects of locally injected histamine are immediate and should be evident early. In our study, we clearly demonstrate that exposure to low concentrations of histamine promotes important later events on the SD and that these could be prevented by chlorpheniramine.

Taken together these data are suggestive of a combined pathophysiological mechanism of histamine on K_f, it evokes not only the mesangial cell contraction but also may promote delayed podocyte detachment, the loss of SD integrity, and subsequent proteinuria and eventual renal damage.

Acknowledgements

This work was funded by the Ateneo/CSP2012 (H4 Histamine Receptor As A New Pharmacological Target For The Treatment Of Diabetic Nephropathy - HISDIAN), University of Turin (ex60% 2013), University of Florence (ex60% 2014), and Wolfson Research Institute, Durham University; a preliminary report of this study was presented at the *Queen Elizabeth II Conference Centre London Pharmacology 2014, London, UK December 16-18, 2014* and at the *44th Annual Meeting of the European Histamine Research Society (EHRS), Malaga, Spain, May 6-9, 2014*, whose abstract have been published on *Inflammation Research 2015 July; 64 (Suppl1)*. The authors wish to thank Professor Rob Leurs for his kind gift of [^{3}H] mepyramine. PL Chazot would like to dedicate this paper to the memory of his mother.

Authorship contributions is listed as follow: AC Rosa, E Veglia, A Pini and PL Chazot participated in research design; AC Rosa, E Veglia, A Pini, A Moggio, PL Chazot, K Tiligada, C Grange conducted the experiments; F Premoselli has contributed to data acquisition; AC Rosa, E Veglia and A Pini analysed the data; AC Rosa, A Pini, R Fantozzi, G Miglio and PL Chazot wrote or contributed to the writing of the manuscript. All co-authors contributed and have approved the submitted version of the paper.

Conflicts of Interest

None

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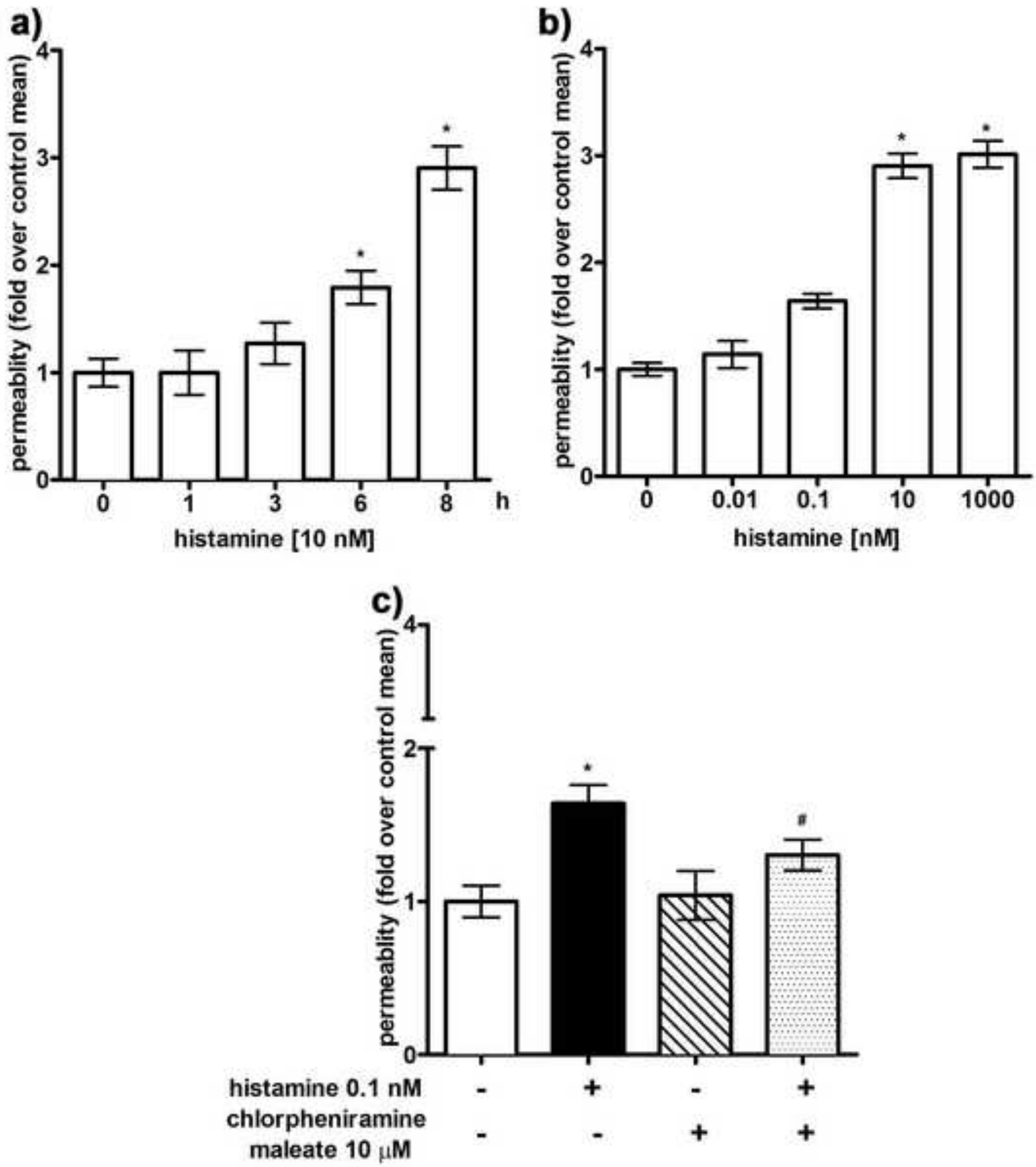

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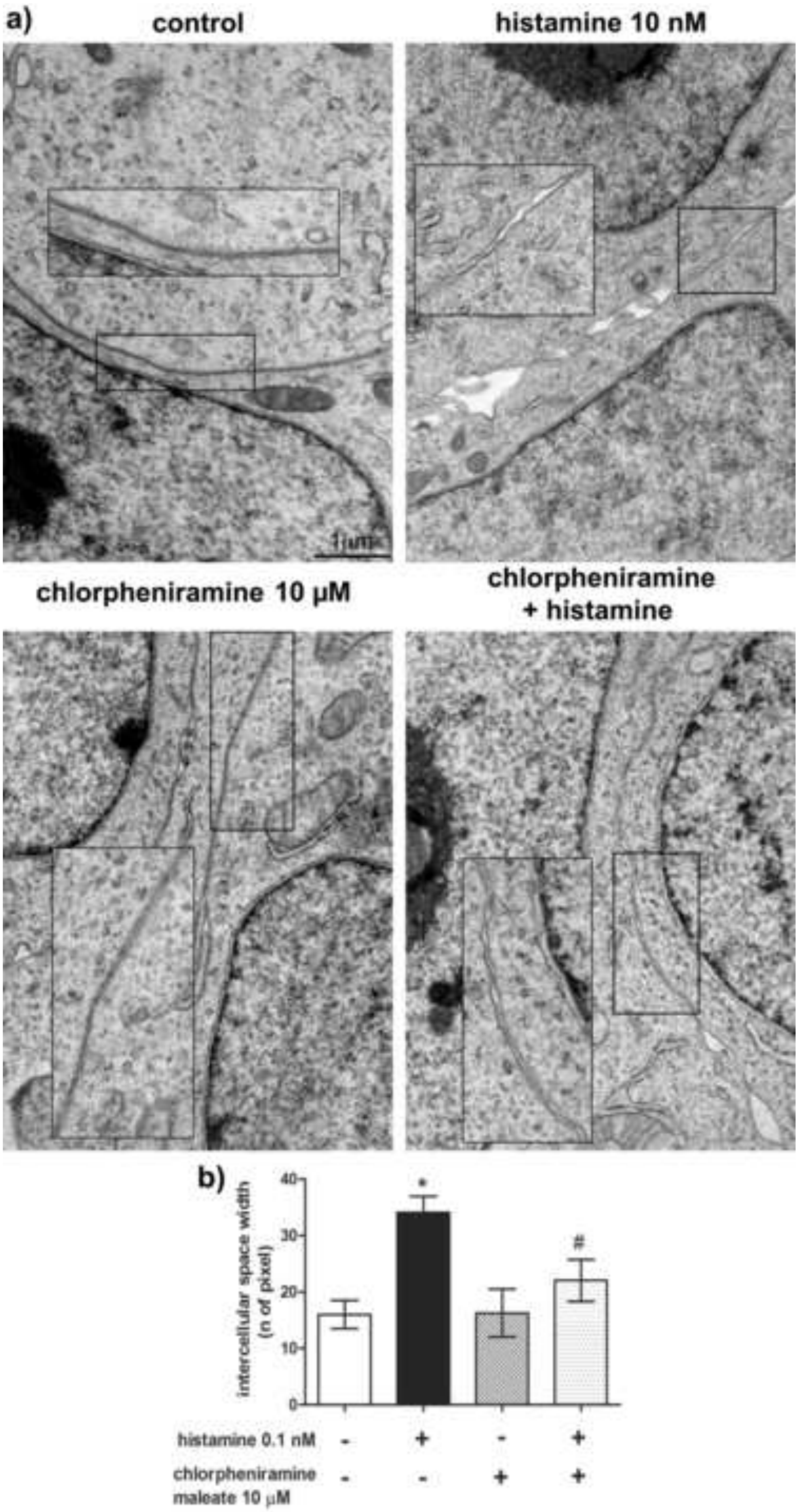

Figure(s) 3
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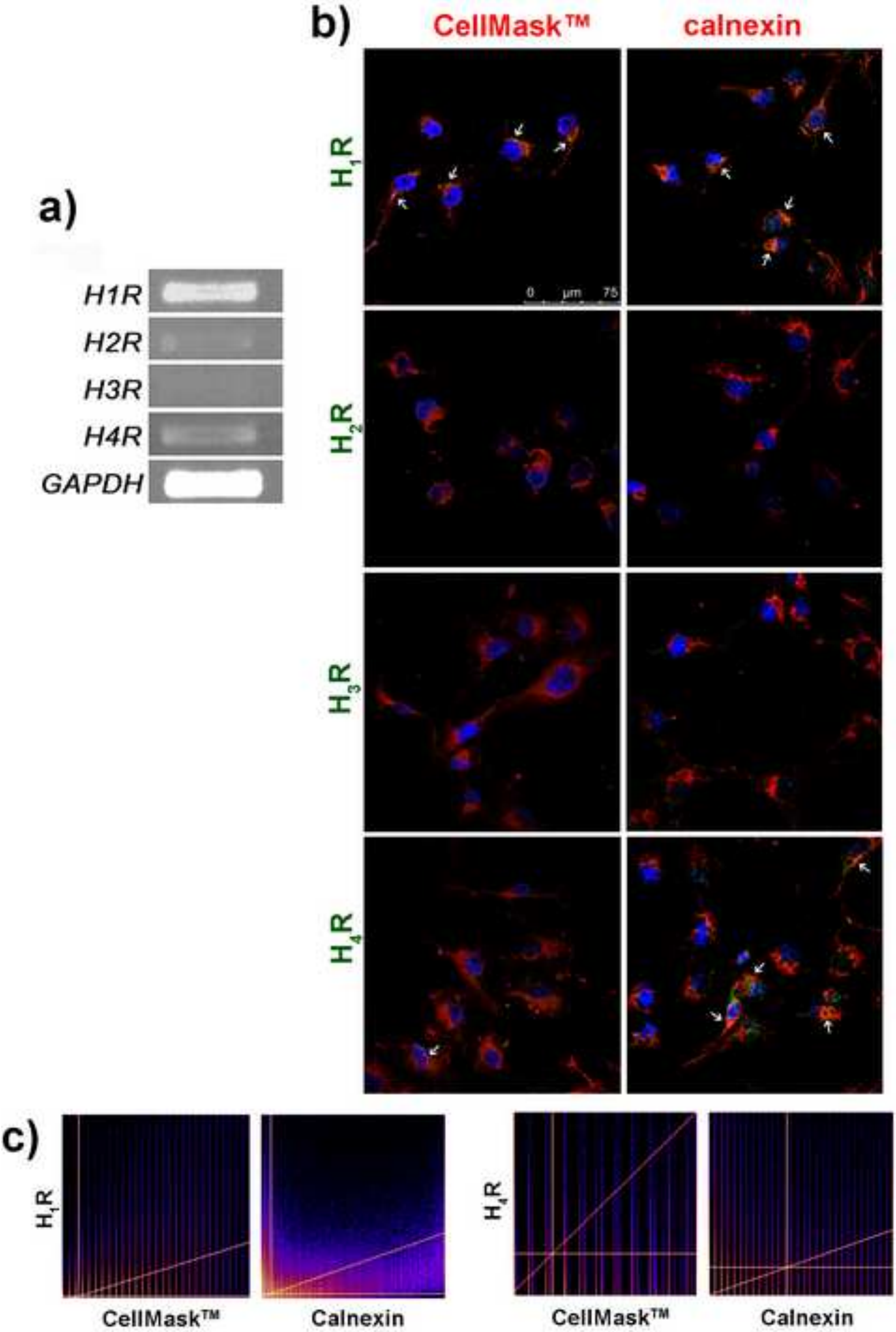
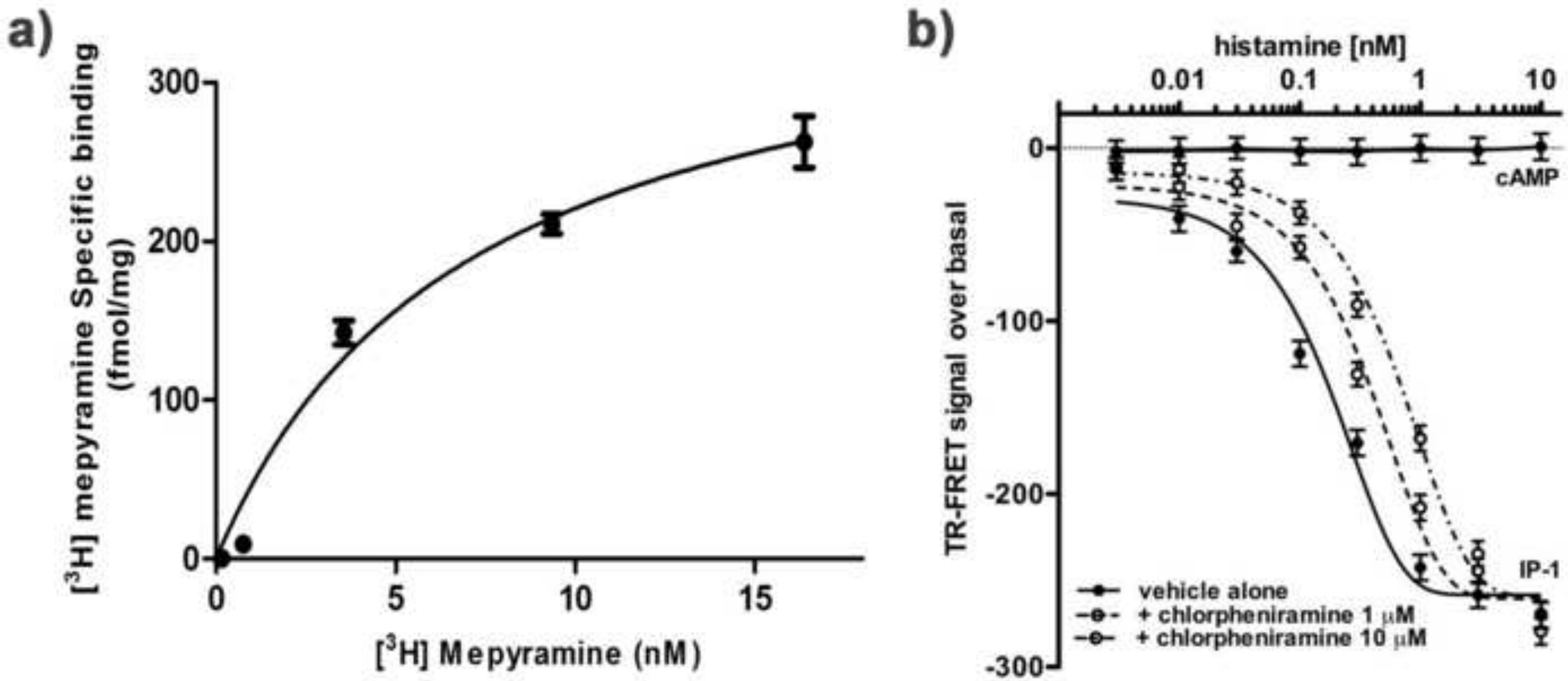
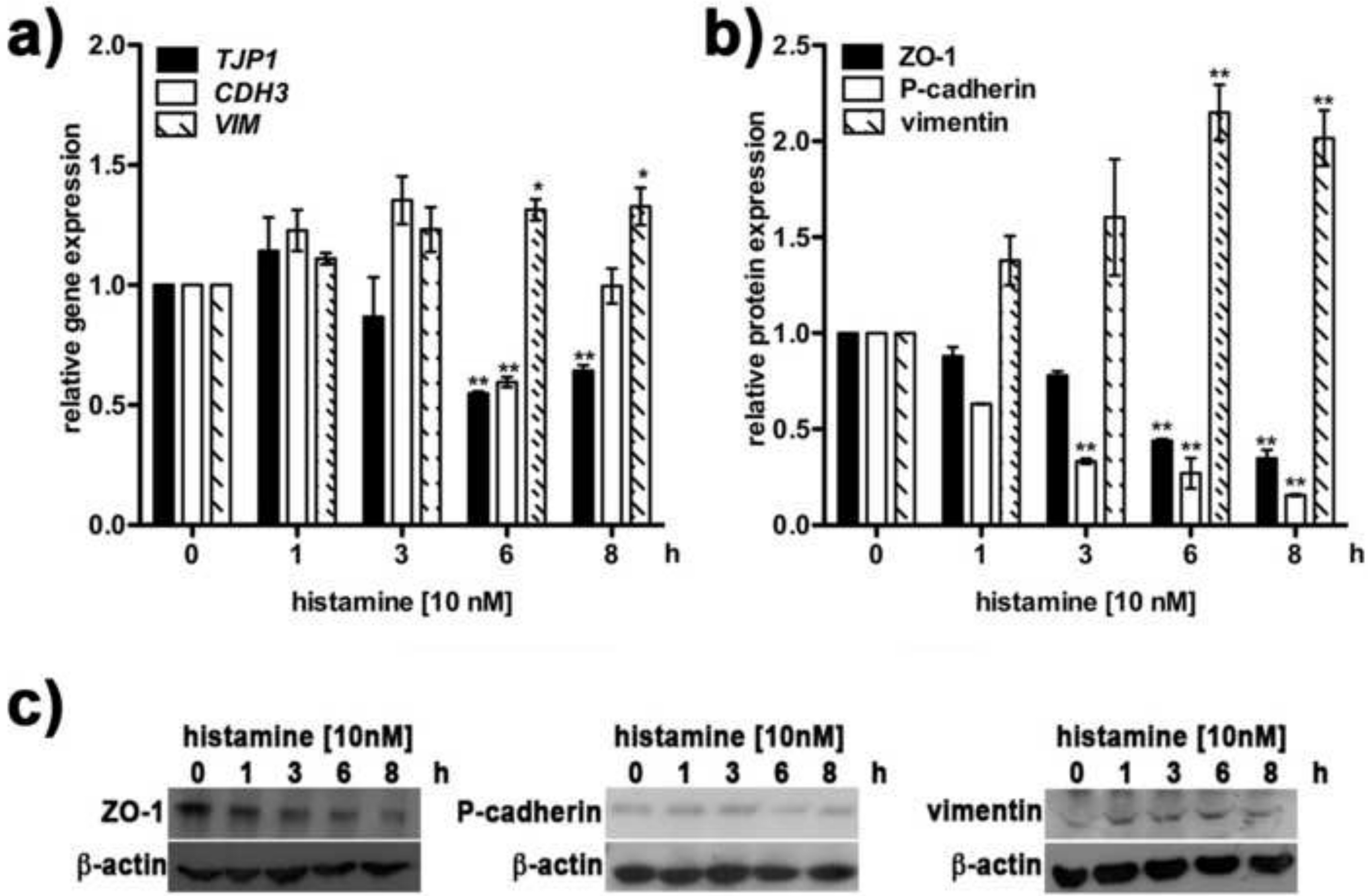
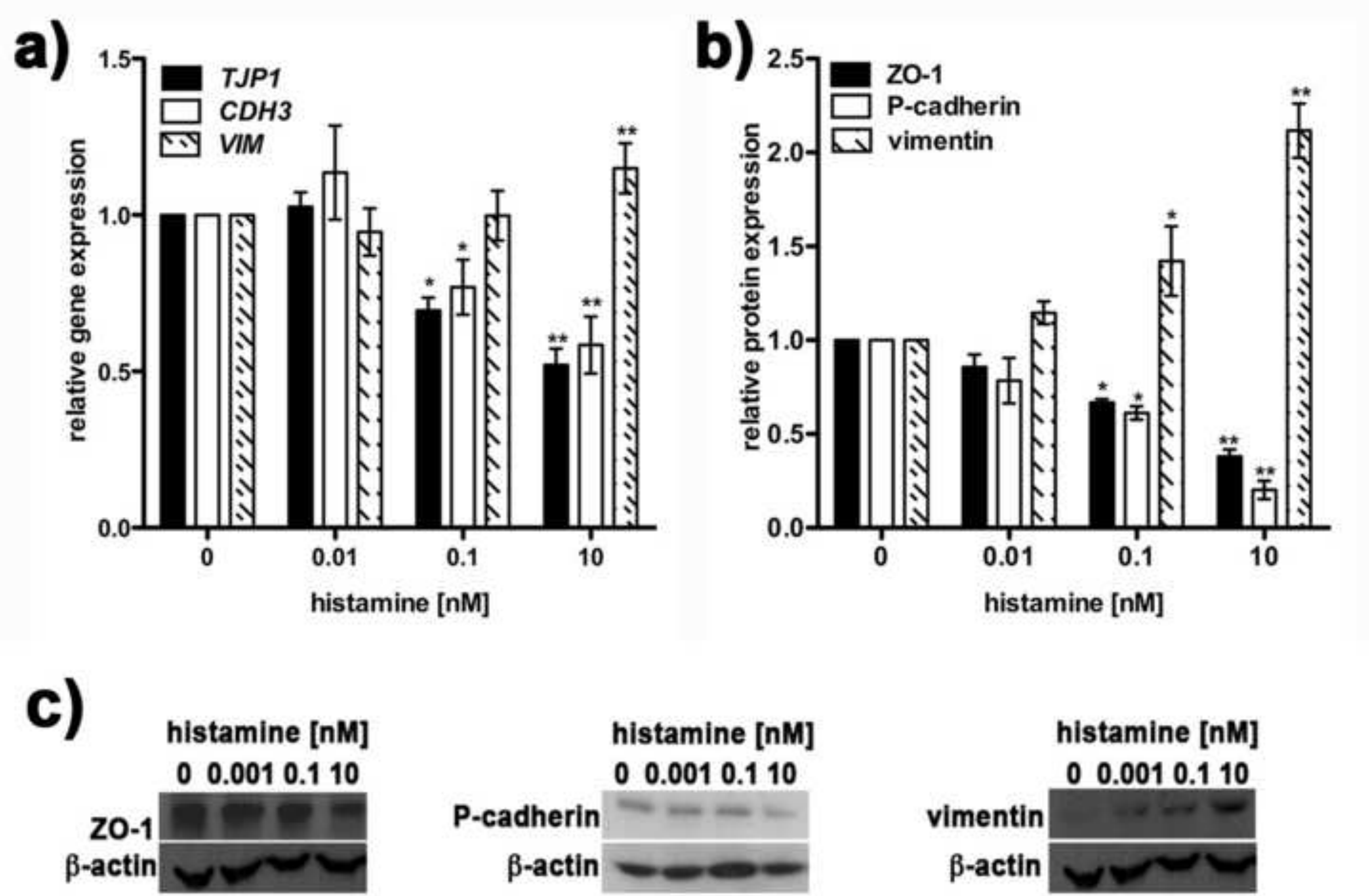


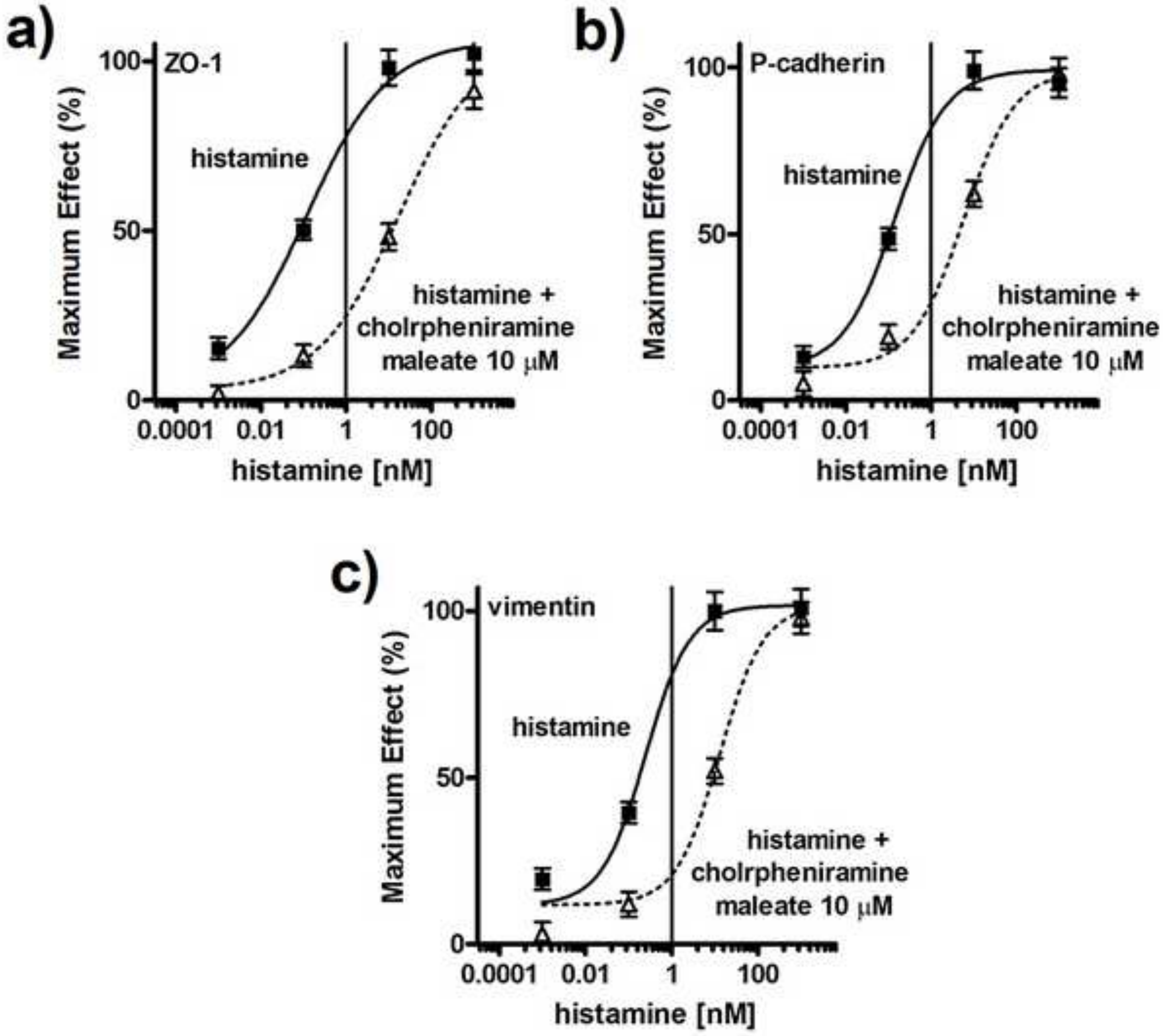
Figure(s) 4


a)
[3H] mepyramine Specific binding (fmol/mg)
300
200
100
0
0
5
10
15
[3H] Mepyramine (nM)
b)
histamine [nM]
0.01
0.1
1
10
TR-FRET signal over basal
0
-100
-200
-300
cAMP
IP-1
vehicle alone
+ chlorpheniramine 1 μM
+ chlorpheniramine 10 μM

Figure(s) 5


a)
2.0
1.5
1.0
0.5
0.0
relative gene expression
TJP1
CDH3
VIM
0 1 3 6 8 h
histamine [10 nM]
**
**
*
**
*
b)
2.5
2.0
1.5
1.0
0.5
0.0
relative protein expression
ZO-1
P-cadherin
vimentin
0 1 3 6 8 h
histamine [10 nM]
c)
histamine [10nM]
0 1 3 6 8 h
ZO-1
β-actin
P-cadherin
β-actin
vimentin
β-actin


a)
relative gene expression
1.5
1.0
0.5
0.0
TJP1
CDH3
VIM
0
0.01
0.1
10
histamine [nM]
b)
relative protein expression
2.5
2.0
1.5
1.0
0.5
0.0
ZO-1
P-cadherin
vimentin
histamine [nM]
c)
histamine [nM]
0 0.001 0.1 10
ZO-1
β-actin
P-cadherin
vimentin


a)
ZO-1
Maximum Effect (%)
100
50
0
histamine
histamine +
cholrpheniramine
maleate 10 μM
0.0001
0.01
1
100
histamine [nM]
b)
P-cadherin
Maximum Effect (%)
100
50
0
histamine
histamine +
cholrpheniramine
maleate 10 μM
0.0001
0.01
1
100
histamine [nM]
c)
vimentin
Maximum Effect (%)
100
50
0
histamine
histamine +
cholrpheniramine
maleate 10 μM
0.0001
0.01
1
100
histamine [nM]

List of chemical compounds studied in the article

1. [^{3}H]mepyramine PubChem CID 656400
2. Chlorpheniramine maleate PubChem CID 5281068
3. Difenhydramine Pubmed CID 3100
4. Histamine dihydrochloride PubChem CID 5818

Supplementary Material - Materials and Methods

Immunocytofluorescence and confocal analysis

Podocytes plated on collagen-coated cover glasses treated with vehicle alone or histamine 10 nM for 0-8 h, were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Sections were incubated overnight with goat polyclonal anti-synaptopodin 1.3 µg/ml (N14, sc-21536), rabbit anti-podocin 2 µg/ml (H130, sc-21009), goat anti-rabbit H_4R 2 µg/ml (Y19 sc-33967; Santa Cruz Biotechnology Inc., Dallas, TX, USA), or rabbit polyclonal anti-HDC 0.4 µg/ml (HPA038891; Sigma–Aldrich St. Louis, MO) at 4°C. After incubation with the respective Alexa-Conjugated secondary antibodies the nuclei were stained with Hoescht. All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica). A variable number of optical section images in the z-dimension (z-spacing, 0.42 µm) were collected ensuring that images throughout the 3D cellular structure, spanning multiple confocal planes, were fully captured.

Fluorometric quantification of histamine

Podocytes seeded at a density of 800.000 cell/ml were pelleted after one hour from medium re-challenge and the medium was collected. Both pellet and medium were processed accordingly to the standard fluorimetric protocol reported in the Histamine Methods & Tools Database (https://www.i-med.ac.at/hmtd/) for the quantification of histamine. Histmaine extracted in *n*-butanol was reconstituted in aqueous phase using 0.1 N sulphuric acid and *n*-heptane and assayed fluorometrically at 360 nm excitation (Tiligada E, et al. Pharmacol Res 2000).

Supplementary Material 1

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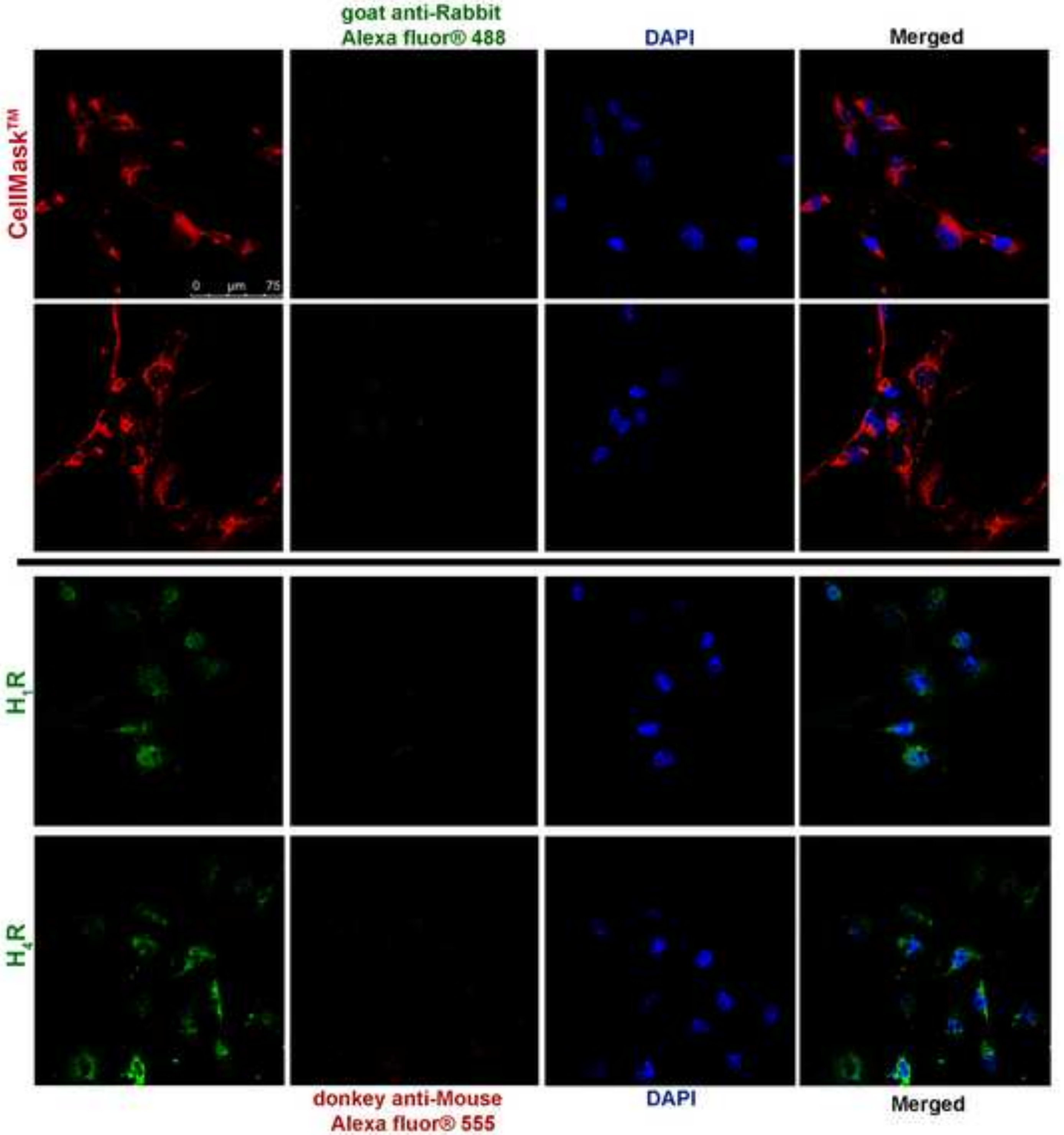

Supplementary Material Fig. 1. Nonspecific binding of the secondary antibodies in colocalization experiments. Representative maximum projection of podocyte Z sections showing the interactions between the different antibodies used. All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica).

*Supplementary Figure 2
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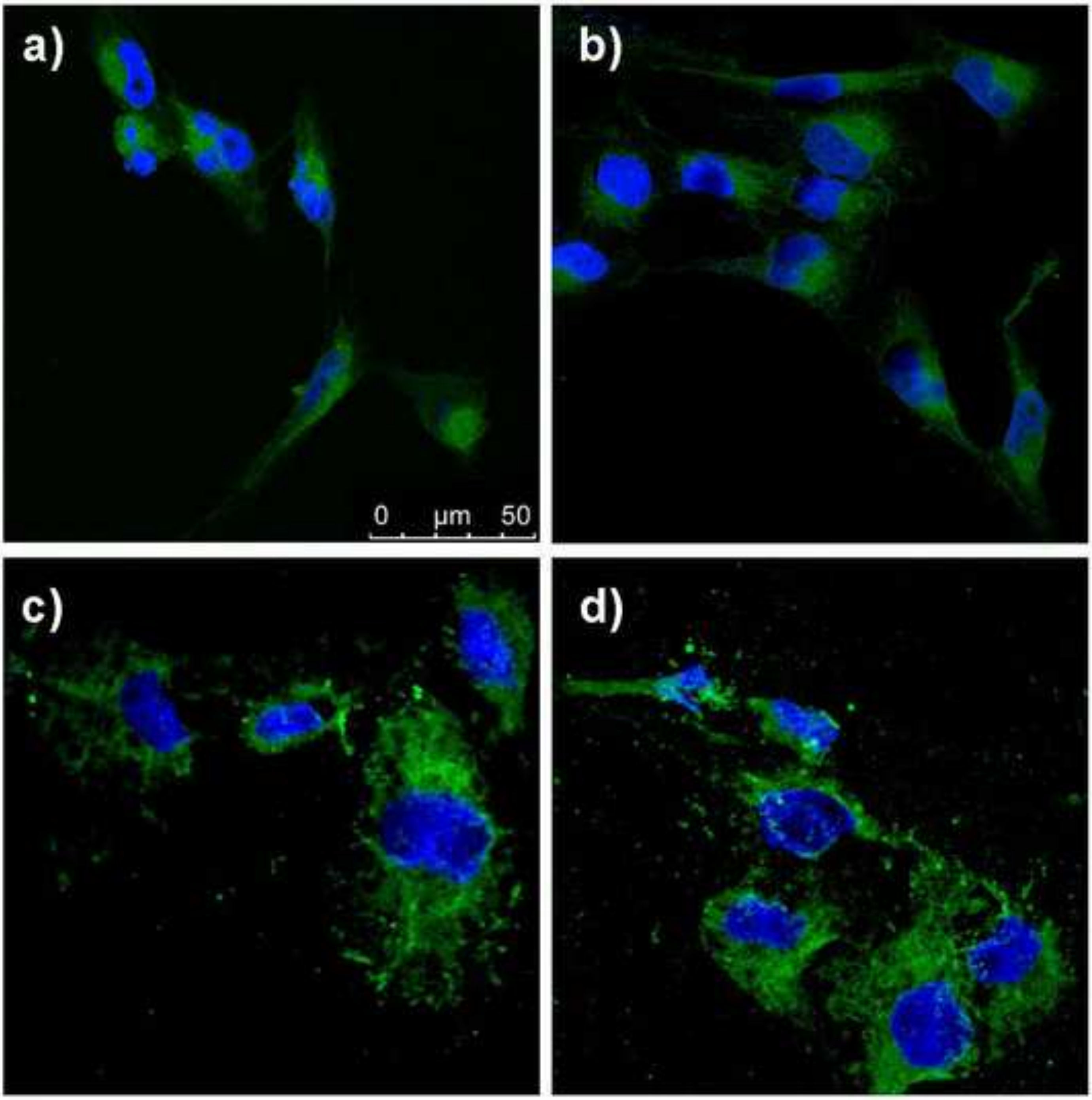

Supplementary Material Fig. 2. Synaptopodin and podocin expression in human immortalized podocytes. Representative maximum projection of the Z sections from 3 independent experiments where cells treated with histamine 10 nM for 0 (**a**) or 8 (**b**) h were labelled with specific anti-synaptopodin (**a**) and (**b**) or (**c**) and (**d**) anti-podocin antibody (green). Nuclei were stained with Hoescht (blue). All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica).

*Supplementary Figure 3
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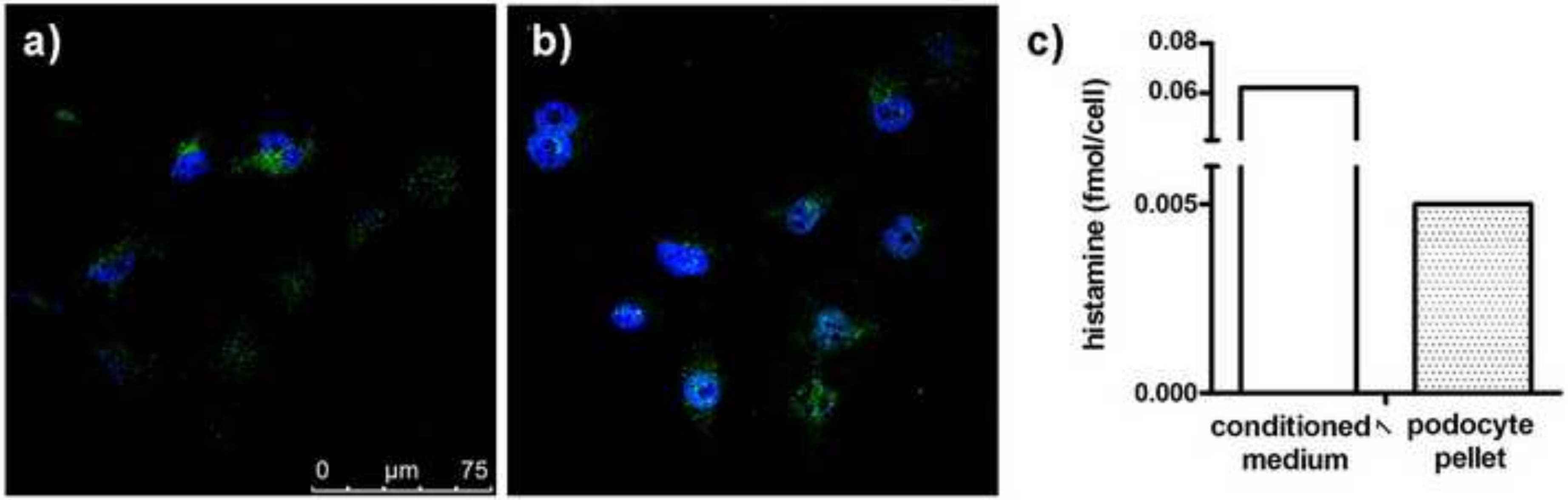

Supplementary Material Fig. 3. HDC expression and histamine production in human immortalized podocytes. Representative maximum projection of the Z sections from 3 independent experiments where cells treated with histamine 10 nM for 0 (**a**) or 8 (**b**) h were labelled with the specific anti-HDC antibody (green). Nuclei were stained with Hoescht (blue). All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica). Histamine basal production was measured in two indipendent experiments after 1h of medium rechellenge in both the conditioned medium and the podocyte pellet accordingly to the fluorometric quantification method (**c**).

*Supplementary Figure 4
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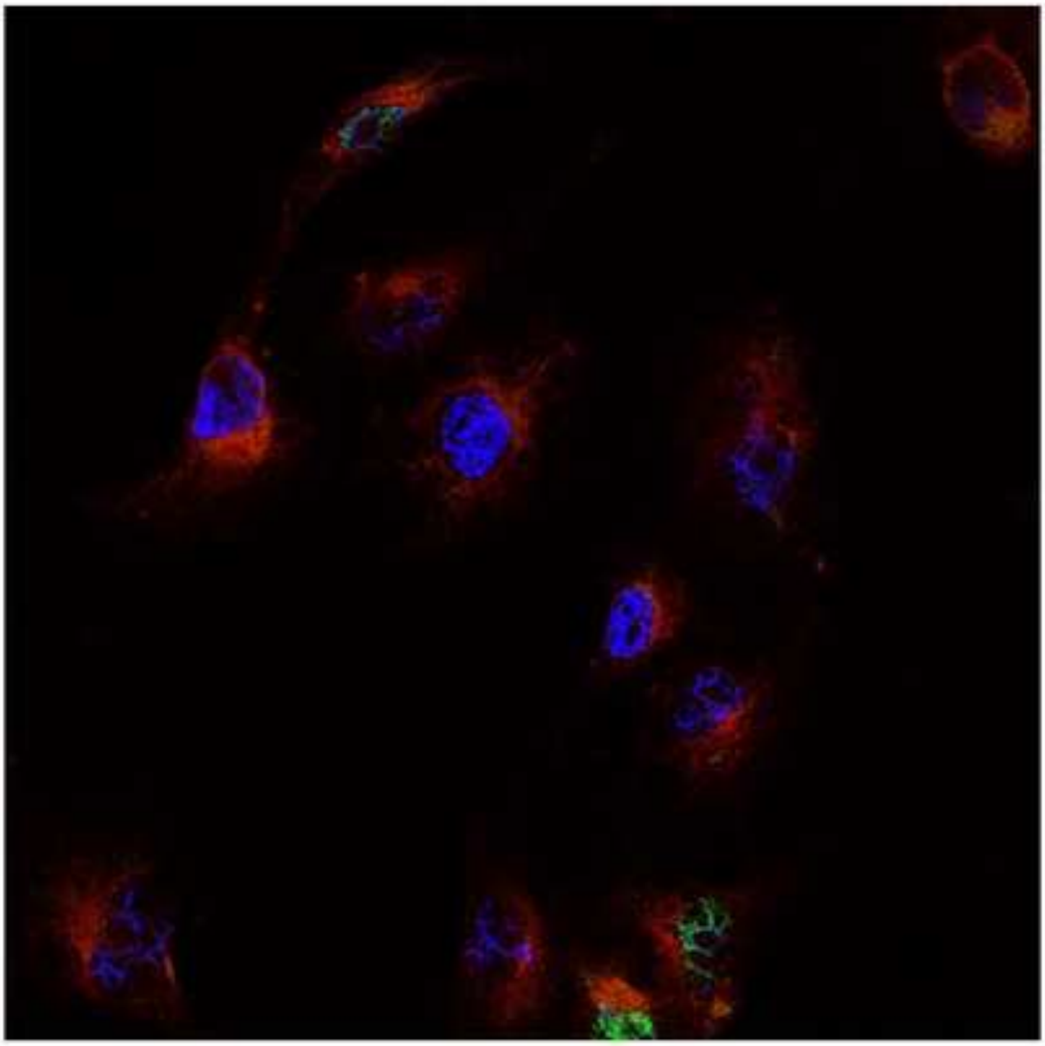

Supplementary Material Fig. 4. H_4R expression on human immortalized podocyte plasma membrane. Representative maximum projection of the Z sections from 3 independent experiments where cells were labelled with the Santa Cruz anti-H_4R antibody (Y19, sc-33967; green). and CellMask™ Plasma Membrane Stains (red). Nuclei were stained with Hoescht (blue). All the slides were examined at ×63 magnification using the SP5 Confocal Laser Scanning Microscope SMD (Leica).

*Supplementary Figure 5
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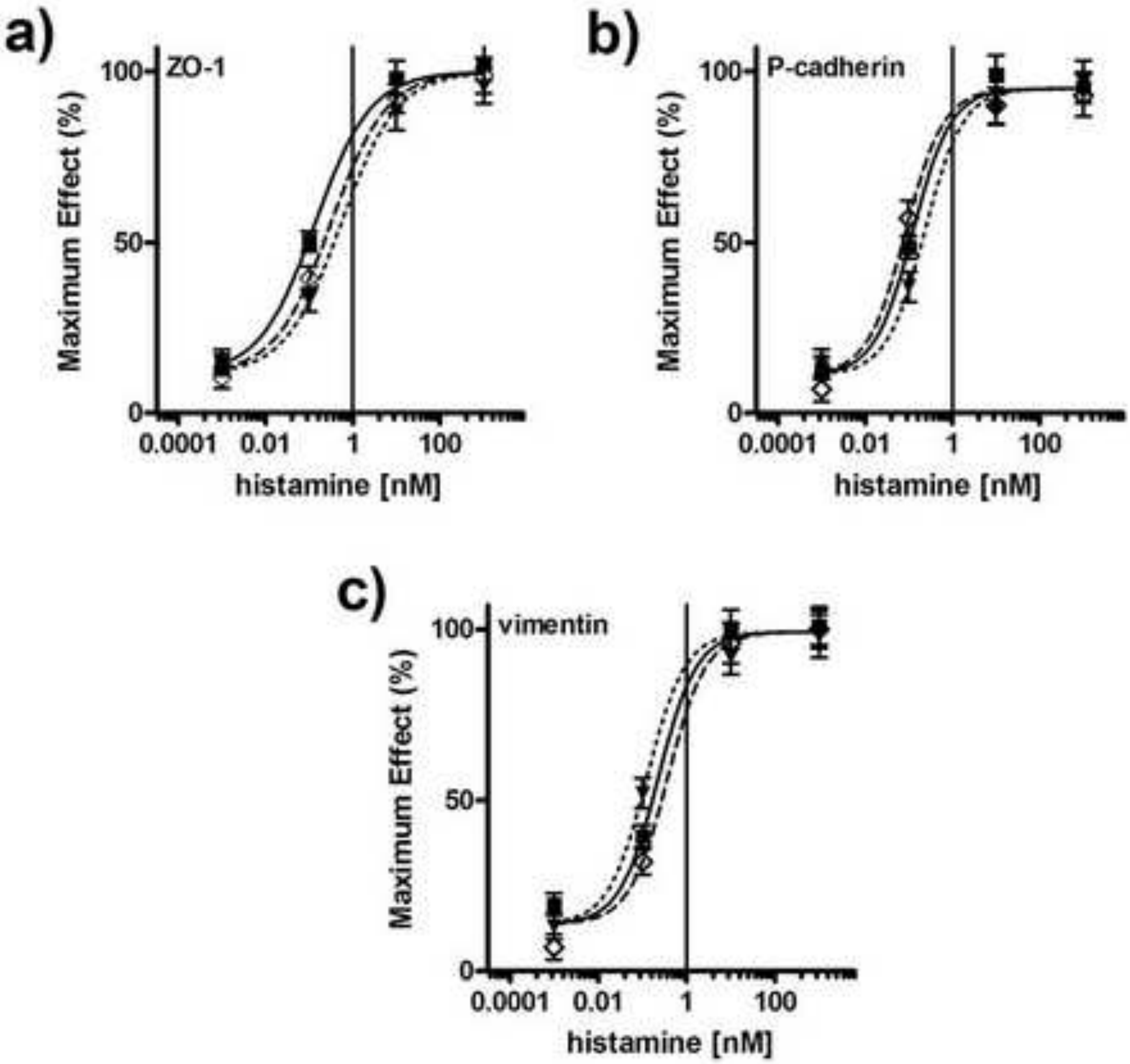

Supplementary Material Fig. 5 Ranitidine and JNJ7777120 antagonism on ZO-1, P-cadherin and vimentin expression evoked by histamine. Human immortalized podocytes pretreated for 10 min with vehicle alone (black square, straight line) or ranitidine (selective H_2R antagonist; black triangle, dotted line) or JNJ7777120 (selective H_4R antagonist; white rhombus, dashed line) 10 µM were treated with histamine for 8 h and processed for ZO-1 (**a**), P-cadherin (**b**) and vimentin (**c**) protein expression. Results, represented as best-fit dose-response curve, are expressed as mean ± SEM of 5 different experiments.

*Graphical Abstract

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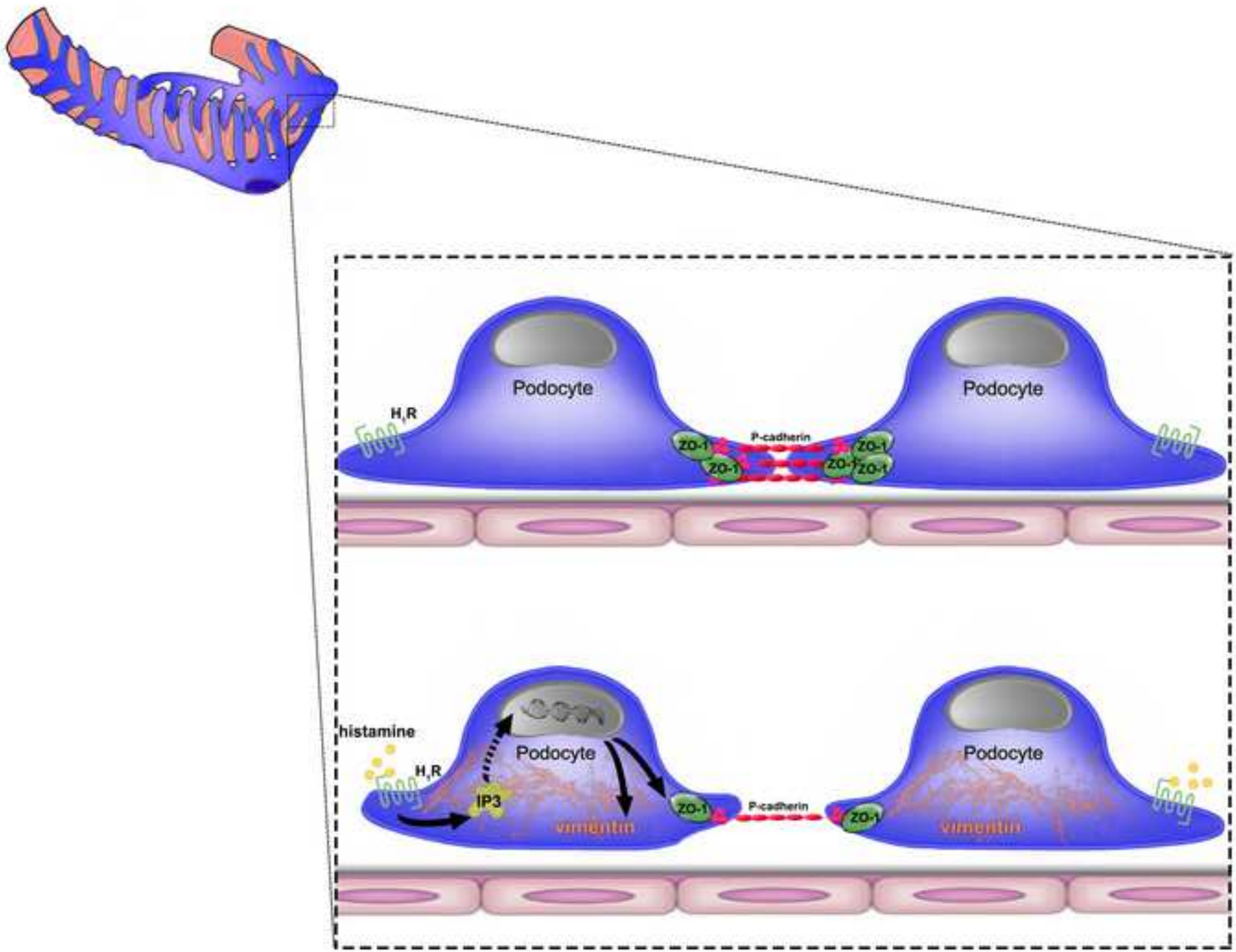

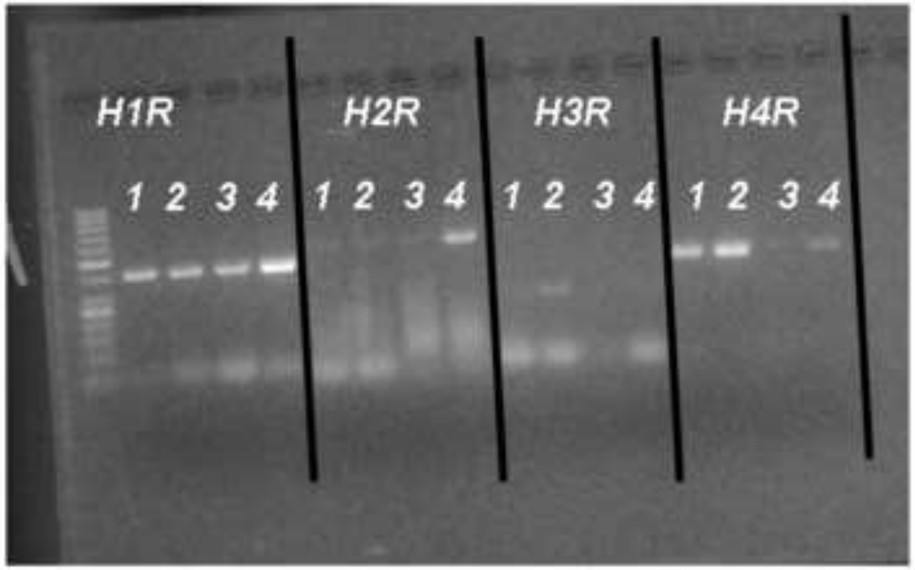

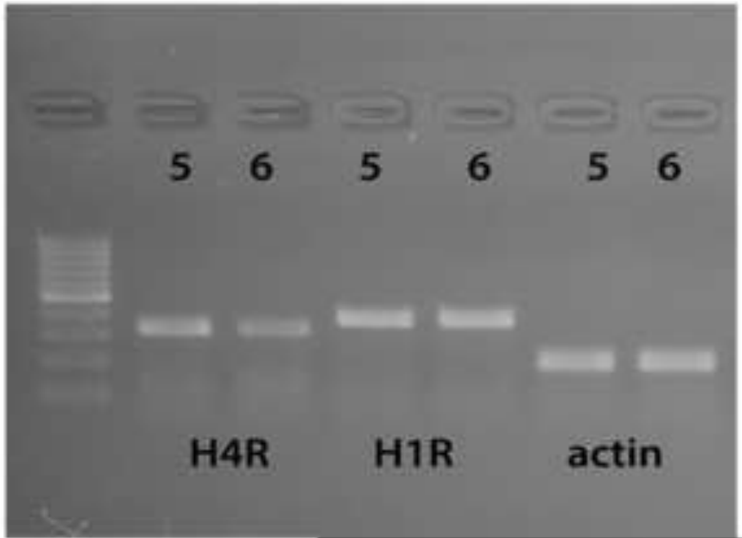

1, 3, 5 and 6 = podocytes
2 and 4 = Tubular epithelial cells
(used as positive control on the basis of our previous data, Veglia et al., 2015)

*Supplementary Figure
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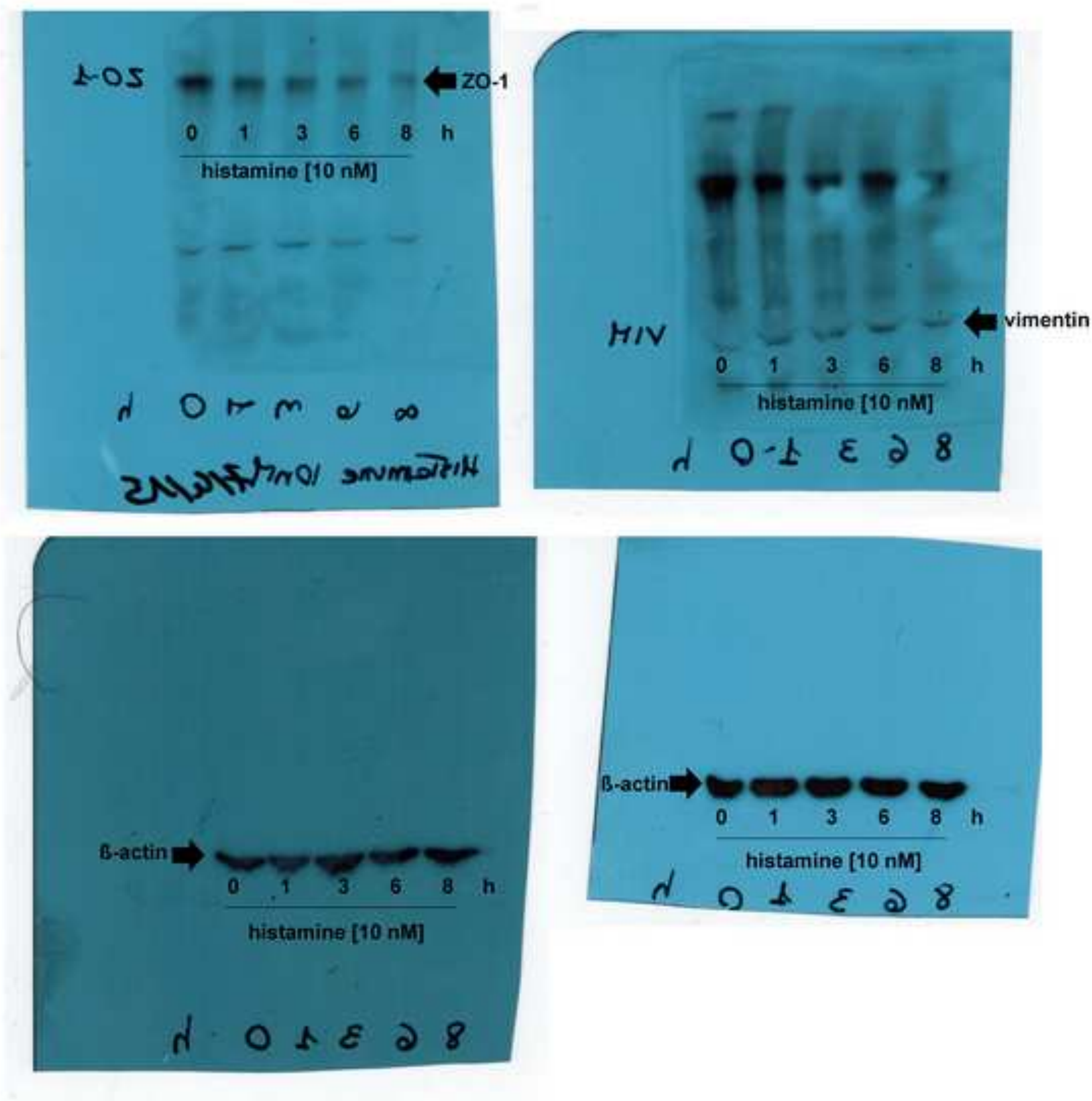

*Supplementary Figure

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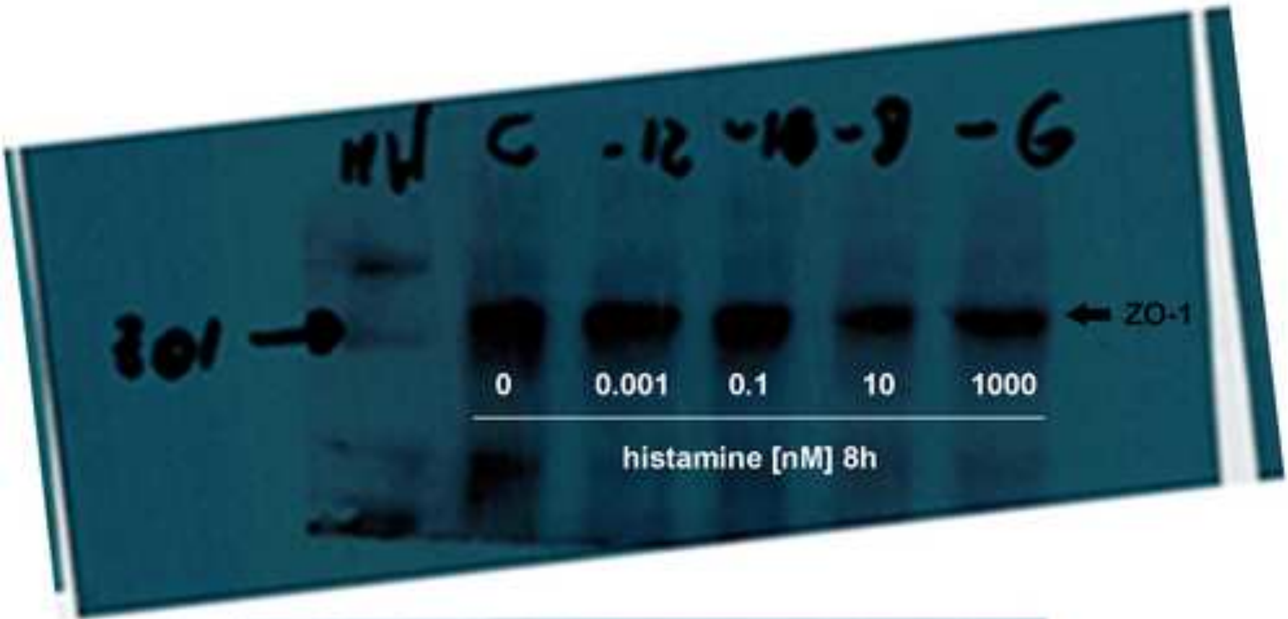

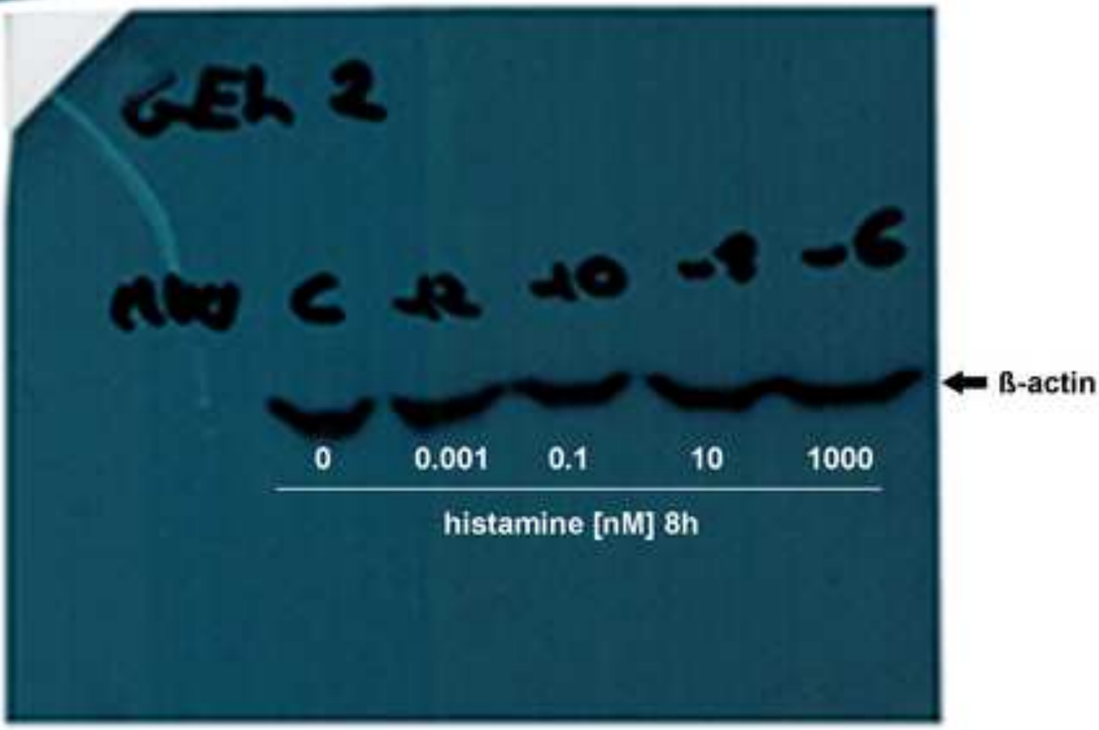

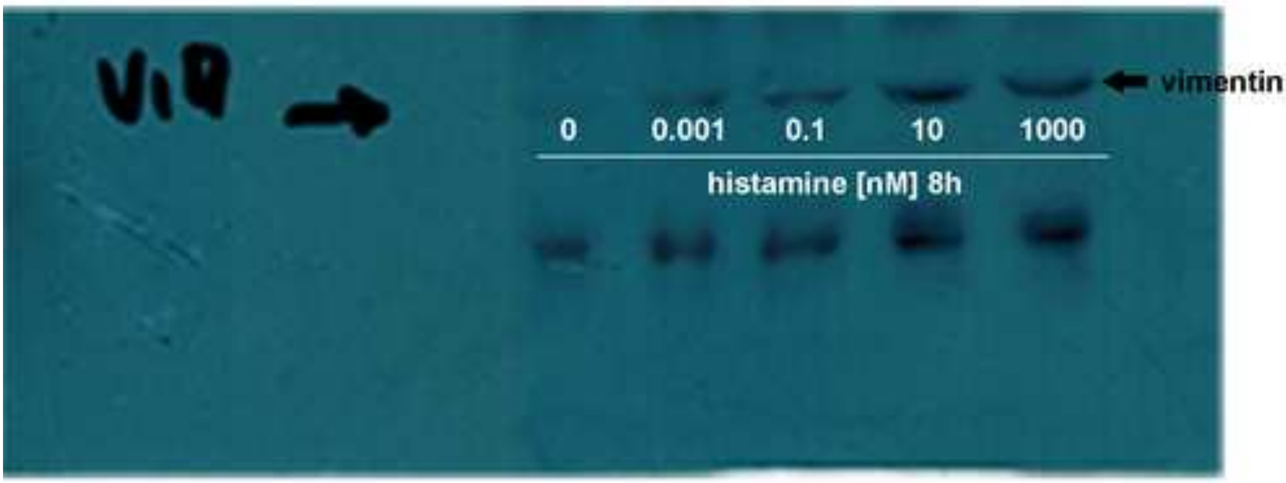

*Supplementary Figure
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***Conflict Of Interest**

Conflicts of Interest Statement

Manuscript title:

Uncontrolled Histamine Type 1-Receptor activation of podocytes affects human Glomerular Slit Diaphragm Integrity

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