

RESEARCH ARTICLE

Glycolysis is integral to histamine-induced endothelial hyperpermeability

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Abstract

Histamine-induced vascular leakage is a core process of allergic pathologies, including anaphylaxis. Here, we show that glycolysis is integral to histamine-induced endothelial barrier disruption and hyperpermeability. Histamine rapidly enhanced glycolysis in endothelial cells via a pathway that involved histamine receptor 1 and phospholipase C beta signaling. Consistently, partial inhibition of glycolysis with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) prevented histamine-induced hyperpermeability in human microvascular endothelial cells, by abolishing the histamine-induced actomyosin contraction, focal adherens junction formation, and endothelial barrier disruption. Pharmacologic blockade of glycolysis with 3PO in mice reduced histamine-induced vascular hyperpermeability, prevented vascular leakage in passive cutaneous anaphylaxis and protected from systemic anaphylaxis. In conclusion, we elucidated the role of glycolysis in histamine-induced disruption of endothelial barrier integrity. Our data thereby point to endothelial glycolysis as a

Abbreviations: 2-DG, 2-deoxyglucose; 3PG, 3-phosphoglycerate; 3PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; DHAP, dihydroxyacetone phosphate; DMSO, dimethyl sulfoxide; ECAR, extracellular acidification rate; F1, 6bP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FAJs, focal adherens junctions; G6P, glucose-6-phosphate; H₁R-H₄R, histamine receptor 1-4; HDMEC, human dermal microvascular endothelial cells; HPMEC, human pulmonary microvascular endothelial cells; HRMEC, human retinal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; MLC2, myosin light chain II; OCR, oxygen consumption rate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PKA, protein kinase A; PLCβ, phospholipase C β; RhoA, Ras homolog gene family, member A; ROCK, Rho-Associated coiled-coil-containing protein kinase.

Constantinos M. Mikelis and David Sprott contributed equally to this work.

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novel therapeutic target for human pathologies related to excessive vascular leakage, such as systemic anaphylaxis.

1 | INTRODUCTION

The endothelial barrier of blood vessels controls the transport of solutes and plasma proteins between blood and interstitium. Regulated endothelial permeability contributes to tissue homeostasis, whereas increases in vascular permeability are essential for physiological processes including neovascularization and tissue repair.¹ Contrastingly, disruption of the endothelial barrier leads to endothelial hyperpermeability, which is a hallmark of pathologies associated with vascular leakage and edema formation, such as pulmonary edema or anaphylactic shock.²⁻⁴

The integrity of the endothelium is mainly regulated by endothelial cell-cell junctions, such as adherens junctions, through a complex system of transmembrane junctional proteins linked to cytoskeletal binding proteins. The endothelial cell-cell adherens junction protein VE-Cadherin plays a key role as a gatekeeper of the endothelial barrier and of vascular integrity.⁵⁻⁷

Histamine is a major inducer of vascular hyperpermeability, and hence, a central component of permeability-related human pathologies, such as allergy and anaphylaxis.⁸ Histamine is released from preformed stores in mast cells and basophils and leads to a rapid increase in vascular permeability within minutes. Histamine acts through its four G-protein-coupled receptors (GPCRs), histamine receptor 1 to 4 (H₁R to H₄R).⁹ In endothelial cells, histamine induces the disruption of the endothelial barrier through the H₁R and two downstream pathways, one involving the Ras homolog gene family, member A (RhoA) and Rho-Associated coiled-coil-containing protein kinase (ROCK), and the second involving phospholipase C β (PLC β)-dependent increases in cytoplasmic calcium levels. The two pathways converge to promote regulatory myosin light chain II (MLC2) phosphorylation, which stimulates the contractile activity of the actin cytoskeleton. The tension generated by actomyosin contraction promotes formation of focal adherens junctions (FAJs) and the consequent formation of junctional gaps thereby disrupting the continuity of the endothelial barrier.^{10,11}

Endothelial border disruption is conceivably an energy-dependent process and may, therefore, require adaptation of the cellular energy metabolism. However, potential metabolic alterations in histamine-stimulated endothelial cells have not been defined. Endothelial cells are highly glycolytic and despite having abundant access to oxygen in the bloodstream, they generate the majority of their ATP production from glycolysis.¹² On the contrary, endothelial cells have a relatively

low mitochondrial activity and do not rely on oxidative phosphorylation (OXPHOS).^{13,14} Accordingly, strategies targeting elevated endothelial glycolysis have gained attention in the context of vascular endothelial dysfunction and related diseases.¹⁵

Here, we showed that endothelial cells rapidly up-regulate glycolysis upon histamine treatment and identified this activation event as crucial for the induction of downstream processes, such as actin cytoskeleton polymerization, FAJs formation, and disruption of endothelial cell-cell contacts. Importantly, pharmacological inhibition of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3)-mediated glycolytic activation by the small molecule inhibitor 3PO^{16,17} stabilized endothelial junctions, attenuated endothelial reactions to histamine in vitro and prevented histamine-mediated vascular leakage and anaphylactic responses in vivo. These findings, therefore, suggest a potential novel therapeutic treatment for life-threatening systemic anaphylaxis.

2 | METHODS

2.1 | Mice

Animal studies were carried out according to the protocols approved by the Institutional Animal Care and Use Committees of the Texas Tech University Health Sciences Center and the University of Pennsylvania. C57BL/6 mice, purchased from the Jackson or the Charles River Laboratories, were used at the age of 8 weeks or older.

2.2 | In vivo permeability assay (Miles assay)

Histamine-induced vascular permeability changes were determined using Evans Blue dye (Fisher Scientific), as previously described.¹⁸ C57BL/6 mice were injected intraperitoneally (i.p.) with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (0.07 mg/g; R&D Systems) diluted in 100 μ L DMSO as previously described.¹⁶ Vehicle control-treated mice received equal amounts of DMSO. Two hours after 3PO injection, mice were anesthetized by i.p. injections of ketamine and xylazine or by isoflurane inhalation and were injected intravenously (iv) with sterile Evans Blue dye (100 μ L, 1% in normal saline). After 1 minute 5, 15, or 50 ng/ μ L of histamine hydrochloride

(Fisher Scientific) diluted in 20 μ L PBS were injected into the shaven back skin, whereas an equal amount of PBS was injected as control. Under these conditions, most agents causing vascular permeability promote the extravasation of Evans Blue within 3–5 minutes, reaching a maximum effect at approximately 10–15 minutes.¹⁹ Mice were euthanized after 10 minutes and ~ 1 cm² of skin containing the site of injections was excised. The skin punches were incubated in 500 μ L formamide (Sigma-Aldrich) at 55°C for 48 hours and the Evans Blue content was determined by absorption at 595 nm. Absorption values of Evans Blue extravasation in histamine-injected skin punches were normalized to the values of control-injected (PBS) skin punches in the same mouse.

2.3 | Passive cutaneous anaphylaxis

Local anaphylactic response was measured as previously described.^{10,20} Briefly, 30 ng of anti-Dinitrophenol-IgE (anti-DNP-IgE; Sigma-Aldrich) diluted in 20 μ L of sterile 0.9% NaCl were injected into the dorsal skin of the right ear of C57BL/6 mice. The left ear of mice received an equal volume of saline and served as control. Twenty-four hours later, the passively immunized mice were challenged by an iv injection of 0.5 mg of Dinitrophenol Human Serum Albumin (DNP-HSA; Sigma-Aldrich) together with 0.08 μ g/g Evans Blue (Fisher Scientific) in normal saline. Two hours before DNP-HSA challenge, mice were injected i.p. with 3PO (0.07 mg/g; R&D Systems) diluted in 100 μ L DMSO, whereas vehicle control-treated mice received an equal amount of DMSO. Mice were euthanized 30 minutes after the challenge and ear biopsies were collected. Evans Blue dye was extracted in 400 μ L of formamide (Sigma-Aldrich) at 55°C for 24 hours and quantified by measuring light absorption at 595 nm. Absorption values of Evans Blue extravasation in anti-DNP-IgE injected ear biopsies were normalized to the values of control injected (saline) ear biopsies in each mouse.

2.4 | Active systemic anaphylaxis

Active systemic anaphylaxis model was performed as previously described.^{10,20} Briefly, C57BL/6 mice were immunized through i.p. injection of 1 mg Bovine Serum Albumin (BSA; Fisher Scientific) and 300 ng Pertussis toxin (PTX; Invitrogen) in normal saline. Fourteen days later, mice were injected i.p. with 0.07 mg/g 3PO (R&D Systems) diluted in 100 μ L DMSO, whereas vehicle control-treated mice received an equal amount of DMSO. Two hours later, mice were challenged with iv injection of 2 mg BSA. Mouse survival was monitored for 120 minutes after the challenge.

2.5 | Primary cells and treatments

Primary Human Pulmonary Microvascular Endothelial Cells (HPMEC; PromoCell), Human Dermal Microvascular Endothelial Cells (HDMEC; PromoCell), and Human Retinal Microvascular Endothelial Cells (HRMEC; Cell Systems) were cultured on 0.2% gelatin-coated plates in Endothelial Cell Growth Medium MV (PromoCell) at 37°C and 5% CO₂. Human Umbilical Vein Endothelial Cells (HUVEC; Lonza) were cultured on 0.2% gelatin-coated plates in Endothelial Cell Growth Medium-2 (EGM-2; Lonza) at 37°C and 5% CO₂. All endothelial cells were used in low passages (up to the fifth passage). For in vitro stimulations of HPMEC, cells were prestarved and stimulated in Endothelial Cell Basal Medium MV (PromoCell) supplemented with 1% FCS (GIBCO), unless otherwise stated. For partial inhibition of glycolysis, particularly of PFKFB3-mediated glycolysis, cells were pretreated with 20 μ M 3PO (Tocris) (Schoors et al, 2014). Histamine (Sigma-Aldrich) was freshly dissolved and added to confluent endothelial monolayers at different concentrations.

2.6 | Extracellular acidification rate measurement

For real-time analysis of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), cells were analyzed with a Seahorse XF-96 Extracellular Flux Analyzer (Agilent). Endothelial cells (1.5 or 2.5×10^4 cells / well) were plated on gelatin-coated XF-96 cell culture plates (Agilent) and allowed to form monolayers overnight. The cells were incubated for 1h in unbuffered Seahorse XF Base medium (Agilent) supplemented with 1 g/L glucose (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), and 1 mM sodium pyruvate (Thermo Fisher Scientific), at pH 7.4 under room air at 37°C and the ECAR or OCR measurement was conducted in the same medium for the different time points, as indicated in the figure legends. For cell treatments, medium with or without histamine (1–100 μ M) was injected into the cultures or the background wells via the seahorse injection ports; the values of the respective background wells were used for background correction. Where indicated, histamine receptor 1 (H₁R) inhibitor Diphenhydramine hydrochloride (10 μ M; Tocris), phospholipase C (PLC) inhibitor U-73122 (5 μ M; Cayman Chemical), protein kinase A (PKA), and cyclic AMP (cAMP) inhibitor H-89 dihydrochloride (10 μ M; Tocris), Ras homolog gene family, member A (RhoA) Rho Inhibitor I (1 μ g/mL; Cytoskeleton), AKT inhibitor MK2206 (2.50 μ M; Cayman Chemical), and glycolysis attenuator 3PO (20 μ M, Tocris) were injected into the cell culture microplates 30 minutes before histamine injection.

Cells treated with Rho inhibitor I were additionally preincubated with the inhibitor for 4h before medium exchange. Appropriate vehicle controls (DMSO and H₂O) were used. In the experiments, in which inhibitors were used, HPMEC were treated with 100 μ M histamine. ECAR and OCR data were baselined²¹⁻²³ and set as 100% for each well at the time point prior to histamine injection, by using the Wave Desktop 2.3 software (Agilent). Area under the Curve analysis of seahorse ECAR data^{24,25} was performed with GraphPad Prism 6 (GraphPad Software Inc) using ECAR values from the first time point after histamine injection and the time points thereafter.

2.7 | Glucose uptake

Glucose uptake was measured using a cell-based glucose uptake kit (Cayman Chemical) according to manufacturer's instructions. Briefly, HPMEC were seeded in 96-well clear bottom plates, coated with 0.2% gelatin and left overnight to form monolayers. Cells were then washed with glucose-free Agilent Seahorse XF Assay Medium (Agilent) and incubated in the same medium supplemented with 200 μ g/mL 2-NBDG (a fluorescently labeled deoxyglucose analog) in the presence or absence of 100 μ M histamine for 30 minutes. 2-NBDG cellular uptake was measured in a Synergy HT multidetection microplate reader (Biotek Instruments). Glucose uptake in untreated cells was set as 100% in each experiment.

2.8 | Phosphofructokinase (PFK) activity

PFK (PFK1 and PFK2) enzymatic activity was determined in HPMEC monolayers treated with medium supplemented with or without 100 μ M histamine for 5 minutes by using a PFK Activity Colorimetric Assay Kit (BioVision) and analyzed with a Synergy HT multidetection microplate reader (Biotek Instruments) according to manufacturer's instructions. PFK activity of unstimulated cells was set as 100% in each experiment.

2.9 | ATP to ADP ratio

ATP to ADP ratio was measured in HPMEC monolayers treated with medium supplemented with or without 100 μ M histamine for 2 minutes, by using an ADP:ATP ratio assay kit (Sigma-Aldrich) and analyzed with a Synergy HT multidetection microplate reader (Biotek Instruments) according to the manufacturer's instructions. ATP:ADP ratio of untreated HPMEC was set as 100% in each experiment.

TABLE 1 MRM transitions (Q1/Q3) for each detected metabolite and corresponding retention time

Metabolite	Q1/Q3	Retention time (minutes)
Glucose-6-Phosphate	259/97	5.32
Fructose-6-Phosphate	259/97	5.50
Fructose-1,6-bisPhosphate	339/79	6.18
Dihydroxyacetone Phosphate	169/97	4.92
Glycerol-3-Phosphate	171/79	4.71
3-Phosphoglycerate	185/97	5.46
Phosphoenolpyruvate	167/79	5.66
Pyruvate	87/87	5.30
Lactate	89/89	4.00
Taurine	124/80	2.44

2.10 | ATP measurement

HPMEC were pretreated with 20 μ M 3PO for 4 hours or with 50 mM 2-DG (Sigma-Aldrich) for 30 minutes; control samples received DMSO. Cells were then stimulated with 100 μ M histamine for 2 minutes. Thereafter, they were lysed and ATP levels were determined with a luminescent ATP Detection Assay Kit (Abcam) according to the manufacturer's instructions using a Synergy HT multidetection microplate reader (Biotek Instruments). Average values of DMSO-treated control samples were set as 1 in each experiment.

2.11 | Metabolite quantitation

HPMEC monolayers were treated with medium supplemented with or without 100 μ M histamine for 30 minutes, washed with cold PBS and quenched with an ice-cold solution containing 20% methanol, 0.1% formic acid, 10 μ M D4-aurine, 3 mM NaF, 1 mM phenylalanine, and 100 μ M EDTA. Cell extracts were then collected, frozen immediately, lyophilized overnight and resuspended in water before mass spectrometry analysis. Targeted analysis of glycolytic intermediates was performed by mass spectrometry using a ABSCIEX 5500 QTRAP equipped with SelexION for differential mobility separation (DMS) and acquired using multiple reaction monitoring (MRM) in negative mode. Samples were injected onto a Hypercarb column (3 μ m particle size, 4.6 $\times$ 100 mm, Thermo Fisher Scientific) at a flow rate of 0.6 mL/min on an ACQUITY UPLC System. Chromatographic separation was achieved with a gradient of ionic strength: mobile phase A contained 3 mM ammonium formate and 20 μ L EDTA; mobile phase B consisted of 30 mM ammonium formate, 20 μ M of EDTA, and 10% acetonitrile (ROTISOLV LC-MS Grade).

The gradient had a duration of 10 minutes with the following profile: 0 minute, 100% A; 1 minute, 100% A; 8 minutes, 0% A; 9 minutes, 100% A; 10 minutes, 100% A. Samples were ionized by electrospray into the mass spectrometer with the following source parameters: CUR: 20, CAD: 7, IS: -4500, TEM: 550, GS1: 70, and GS2: 60. DMS was used as an additional separation axis (DT: low, MD: 2-propanol, MDC: high, DMO: 3, and DR: off). DMS-related Separation Voltage (SV) and Compensation Voltage (CoV) for each metabolite was optimized before each experiment. Peak identification and chromatographic separation of all metabolites was confirmed with known standards. Table 1 contains the individual MRM transitions (Q1/Q3) and respective retention times. Peaks were integrated with EIMaven v0.6.1 (Elucidata) and concentrations calculated by interpolation from a standard curve of known concentrations and shown as fold change relative to the control (histamine-untreated) group. Each sample was run twice and its values were averaged. Endogenous taurine was used for normalization, that is, as a surrogate for cell density.

2.12 | Live cell imaging

For real-time ratiometric monitoring of intracellular ATP:ADP ratios, the fluorescent biosensor PercevalHR²⁶ was used. FUGW-PercevalHR, a gift from Gary Yellen (Addgene plasmid #49083), was lentivirally produced as described.²⁷ HPMEC were transduced with the lentivirus and 3 days later treated with medium supplemented with or without 100 μ M histamine. Monolayers were imaged with an Olympus IX 83 spinning disc microscope detecting emission at 525 nm while exciting at 405 nm (corresponding to ADP) and 488 nm (corresponding to ATP). Time series were acquired with a 63x objective for 15 minutes taking a Z-stack (0.7 μ m optical slice thickness) every 25 seconds. After the initial 5 minutes of acquisition, cells received medium with 100 μ M histamine or an equal volume of unsupplemented medium as control. To compute images of ATP:ADP ratios, in each pixel intensities acquired at 488 nm excitation (ATP) were divided by values at 405 nm excitation (ADP). Z-stacks were converted to maximum intensity projections using Fiji software (Schindelin et al, 2012). Resulting ratiometric images were displayed as red-to-yellow pseudocolor gradient for enhanced visibility.

2.13 | Cytosolic calcium measurement

Cytosolic free Ca^{2+} concentration was measured as previously described.²⁸ Briefly, HUVEC were cultured to 80%-90% confluence and pretreated with 20 μ M 3PO or vehicle

(DMSO) in M199 medium without FBS for 3 hours. Cells were then washed once with Hanks' balanced salt solution (HBSS) and incubated with 1 μ M of the calcium dye Fura-2 AM in Dulbecco's modified phosphate-buffered saline (DPBS) containing 20 μ M 3PO or vehicle for 1 hour. Subsequently, cells were washed twice with DPBS and assay buffer (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM Glucose, 1.2 mM MgCl_2 , and 1.5 mM CaCl_2) was added. Readings were taken at excitation wavelengths of 340 and 380 nm with emission detection at 510 nm in both cases (using a plate reader (BioTek Synergy^{Mx}) for 8 cycles (~3 minutes), after which 100 μ M histamine in medium or only medium were added and readings of another 18 cycles (~7 minutes) were acquired. Data are displayed as ratios of 510 nm emissions at 340 nm and 380 nm excitation (340/380).

2.14 | Measurement of F-actin to G-actin ratio

Cellular F-actin to G-actin ratio was determined as previously described.²⁹ Briefly, HPMEC were grown to confluence on gelatin coated 96-well black-walled, clear-bottom microplates (Thermo Fisher Scientific), and pretreated with 20 μ M 3PO or vehicle control (DMSO) for 4 hours, followed by addition of 100 μ M histamine in medium or only medium for 5 minutes. After washing with PBS and fixation with 10% formalin in PBS solution, cells were permeabilized with 0.1% Triton X-100, blocked with 1% BSA, 5% goat serum in PBS for 1 hour at RT and stained with Alexa Fluor 488 DNase I for monomeric G-actin (Molecular Probes) and Alexa Fluor 568 Phalloidin for polymeric F-actin (Invitrogen) for 30 minutes at RT. After the cells were washed three times with PBS, quantification of G-actin and F-actin was performed with a Synergy HT multidetection microplate reader (Biotek Instruments). F-actin to G-actin ratio of control DMSO-treated HPMEC was set as 100% in each experiment.

2.15 | Immunoblot analysis

HPMEC monolayers were pretreated with 20 μ M 3PO or vehicle control (DMSO) for 4 hours followed by addition of 100 μ M histamine or medium for 6 minutes, washed with ice-cold PBS, and lysed in RIPA lysis buffer supplemented with 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitor cocktail (Santa Cruz Biotechnology). Cell lysates were centrifuged and the total protein concentration was determined with BCA Protein Assay Kit (Thermo Fisher Scientific). Protein extracts were diluted with NuPAGE LDS Sample Buffer

(Thermo Fisher Scientific) and NuPAGE Sample Reducing Agent (Thermo Fisher Scientific), separated in NuPAGE 4%-12% polyacrylamide gradient Bis-Tris Protein Gels (Invitrogen), and transferred onto nitrocellulose membranes (Amersham Biosciences). Equal loading was verified by Ponceau Red staining. Membranes were blocked with TBS-T buffer containing 5% w/v nonfat dry milk (BD Biosciences) and incubated overnight with primary antibodies followed by incubation for 1 hour with the appropriate HRP-linked secondary antibodies (R&D Systems). The primary antibodies used were against phospho-VE-Cadherin Tyr-685 (CP1981; ECM Biosciences), actin (ab3280; Abcam), phospho-Src Tyr-416 (2101; Cell Signaling), and phospho-MLC2 Thr18/Ser19 (3674; Cell signaling). Staining detection was performed using SuperSignal West Pico or Femto Chemiluminescent Substrates (Thermo Fisher Scientific) with a FUSION FX7 Multimaging System (Peqlab) as previously described.³⁰

2.16 | Immunofluorescence

Immunostaining of HPMEC monolayers was performed in 8-well glass bottom chamber slides (Ibidi). Glass surfaces were coated through subsequent treatment with poly-D-lysine solution (Thermo Fisher Scientific) for 30 minutes, 0.5% glutaraldehyde for 15 minutes at 4°C and 0.25 mg/mL rat tail collagen I solution (Thermo Fisher Scientific) for 10 minutes, followed by 70% ethanol for 30 minutes and endothelial cell culture medium (PromoCell) for another 30 minutes. Between the aforementioned treatments, surfaces were washed with PBS. HPMEC were plated at a density of 2.5×10^4 /well and allowed to form confluent monolayers over 2 days. Cells were treated with 20 μ M 3PO or vehicle control (DMSO) for 4 hours, after which they were stimulated with 20 μ M histamine or medium for 4 minutes. Monolayers were immediately fixed with 10% formalin in PBS solution for 30 minutes, stained with antibodies against Vinculin (V9131; Sigma-Aldrich) and VE-Cadherin (AF938; R&D systems), followed by staining with Alexa Fluor 488 labeled donkey anti-mouse (A21202; Thermo Fisher Scientific) and Alexa Fluor 647 labeled donkey anti-goat (A21447; Thermo Fisher Scientific) secondary antibodies. Additionally, Alexa Fluor 568 Phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Merck) were used to label F-actin and nuclei, respectively. Percentages of cells forming FAJs were determined as previously described.¹⁰ Briefly, in each field of view, cells exhibiting zipper-like structures positive for Vinculin, VE-Cadherin, and F-actin were counted and divided by the number of total visible cells. Fluorescence images were acquired in randomly chosen fields of view using either a Zeiss Observer Z.1 wide field microscope or a Leica SP 5 confocal microscope and

image analysis was performed with ZEN Imaging Software (Zeiss) and FIJI software. Images were pseudocolored; the display color of the channels was set as to optimize clarity of merged images.

2.17 | In vitro endothelial permeability assays

Real time-resolved macromolecular permeability was measured across the monolayers of HPMEC on a 0.4 μ M polycarbonate filter membrane separating luminal and abluminal compartments and trypan blue-labeled albumin flux from luminal to abluminal compartment was calculated as previously described.³¹ Briefly, 2.5 and 9.5 mL of HBSS supplemented with 1.3 mM CaCl_2 , 1.2 mM MgCl_2 , and 2% FCS were added into the luminal and abluminal compartments, respectively, and 60 μ M of trypan blue-labeled albumin was added to the luminal compartment to create a concentration gradient. HPMEC monolayers were treated with 3PO (20 μ M) or vehicle control (DMSO) for 4 hours, followed by stimulation with or without 1 mM histamine solution. Trypan blue-labeled albumin diffusion across the two compartments was recorded in real time by a spectrophotometer (Specord 10, Zeiss). Trypan blue was measured at 600 nm wavelength and subtraction of a second measurement at 720 nm was done simultaneously. For each well, absorption values at the time point of addition of histamine or control medium were set as 100%.

For fluorescence microscopy-based permeability assessment, an in vitro vascular permeability imaging assay was used (EMD Millipore). HPMEC were seeded on glass surfaces coated with biotinylated-gelatin and grown to confluence. Monolayers were then pretreated with 20 μ M 3PO or vehicle control (DMSO) for 4 hours followed by treatment with medium supplemented with FITC-streptavidin in the presence or absence of 100 μ M histamine for 5 minutes. HPMEC monolayers were fixed with 10% formalin in PBS solution, stained with DAPI and fluorescence pictures were acquired on a Zeiss Observer Z.1 wide field microscope with 40x objective. In each image, the percentage of the FITC-streptavidin-positive area (representing areas of leakage) was determined with FIJI software.

2.18 | Statistical analysis

Mann-Whitney *U* test was used for comparison of two groups and two-way ANOVA followed by Sidak's post hoc analysis or one-way ANOVA with Holm-Sidak's multiple comparisons test was used for comparison of more than two groups. Mantel-Cox test was used in active systemic anaphylaxis assay. A value of $P < .05$ was considered to be

statistically significant. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc)

3 | RESULTS

3.1 | Histamine triggers rapid activation of glycolysis in endothelial cells

Histamine rapidly induces vascular leakage through actomyosin contractility and endothelial border disruption.¹⁰ As the aforementioned processes are energy-demanding and endothelial cells are dependent on glycolysis for their bioenergetic demands,¹⁴ we hypothesized that alterations in glycolytic activity may be necessary for the endothelial cells to adapt to the histamine challenge. To this end, we examined real-time changes in glycolysis of human pulmonary microvascular endothelial cells (HPMEC) upon stimulation with histamine, by measuring their extracellular acidification rate (ECAR). Histamine induced a rapid and immediate increase in ECAR in a concentration-dependent manner (Figure 1A). Given the heterogeneity of endothelial cells from different vascular beds, we repeated the experiment with endothelial cells derived from different vascular beds. Similar to the effect observed in HPMEC, histamine rapidly increased ECAR in primary human dermal microvascular endothelial cells (HDMEC) and in umbilical vein (HUVEC) endothelial cells (Figure 1B,C), whereas hardly any ECAR enhancement was detected in human retinal microvascular endothelial cells (HRMEC) (Figure S1). In contrast to ECAR values, oxygen consumption rates (OCR) were largely unaffected by histamine in HPMEC, HDMEC, and HUVEC and only a minor increase was detected shortly after histamine treatment (Figure S2).

We further confirmed the immediate increase in glycolytic activity triggered by histamine by detecting enhanced glucose uptake (Figure 1D) and PFK activity (Figure 1E) in histamine-treated HPMEC. Additionally, we performed targeted metabolomic analysis for the glycolytic pathway intermediates in HPMEC treated with or without histamine. We observed that histamine increased the levels of most glycolytic metabolites in HPMEC. Specifically, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6bP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), pyruvate, and lactate were significantly higher in histamine-stimulated endothelial cells (Figure 1F).

We next tested whether increased glycolysis in histamine-treated endothelial cells could lead to alterations in cellular ATP:ADP ratios. Indeed, histamine stimulation acutely increased the total endothelial ATP to ADP ratio (Figure 2A). Additionally, using the genetically encoded fluorescent biosensor PercevalHR, we observed that histamine treatment

enhanced cellular ATP:ADP ratios in the nucleus and, intriguingly, instantly led to accumulation of ATP:ADP-rich hotspots at endothelial cell borders (Figure 2B). These results collectively show that endothelial cells rapidly boost glycolysis to fulfill their bioenergetic demands in response to histamine.

3.2 | Histamine induces glycolysis via the H₁R/PLC β signaling pathway

To elucidate the molecular mechanisms involved in histamine-induced glycolysis, we investigated the involvement of possible signal transduction pathways. First, we tested whether H₁R, which has a central role in histamine-induced endothelial permeability,¹⁰ was also involved in histamine-induced glycolysis upregulation. Pharmacological inhibition of H₁R abolished histamine-induced elevations in ECAR (Figure 3A and B), whereas pharmacological inhibition of H₂R, H₃R, or H₄R failed to prevent histamine-induced ECAR (data not shown). Moreover, we observed that pharmacological inhibition of PLC β , that acts downstream of H₁R, reduced the histamine-induced elevation in ECAR (Figure 3A and B). In contrast, activities of AKT, PKA, cAMP, and Rho (Figure 3A and B) were all dispensable for histamine-mediated glycolysis activation. These data suggest that histamine-dependent glycolysis induction in endothelial cells specifically requires the H₁R/PLC β pathway.

3.3 | Histamine-mediated disruption of endothelial barrier integrity is mediated by enhanced glycolysis

We next examined whether the rapid glycolysis induction by histamine contributed to disruption of the endothelial barrier. To test this hypothesis, we first inhibited endothelial glycolysis with 2-deoxyglucose (2-DG). However, 2-DG treatment disrupted barrier integrity under baseline conditions, indicating that severe blockage of glycolytic flux by 2-DG stressed endothelial cells and negatively affected their viability (data not shown). Therefore, to achieve a milder inhibition of glycolysis, which would also be therapeutically more relevant, we used 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), a small molecule inhibitor of the glycolysis activator PFKFB3.^{14,16,17} To test the extent of 3PO-mediated effects on endothelial energy metabolism, we analyzed ATP levels in histamine-stimulated HPMEC that were pretreated with 3PO, 2-DG, or vehicle (DMSO) (Figure S3). As expected, glycolysis blockage with 2-DG caused a pronounced drop in ATP, while partial glycolysis inhibition with 3PO treatment led to a weak decrease in ATP levels. Furthermore, pretreatment

of endothelial cells with 3PO moderately reduced ECAR levels, both under baseline and histamine-stimulated conditions (Figure S4). At the same time, 3PO did not affect H₁R protein levels (data not shown) and it had no influence on downstream signaling, as judged by unaltered

histamine-induced cytosolic Ca²⁺ levels of 3PO-treated endothelial cells (Figure S5).

Next, we tested whether mild glycolysis reduction through 3PO treatment affected permeability-related effects of histamine. Endothelial barrier disruption by histamine is linked to

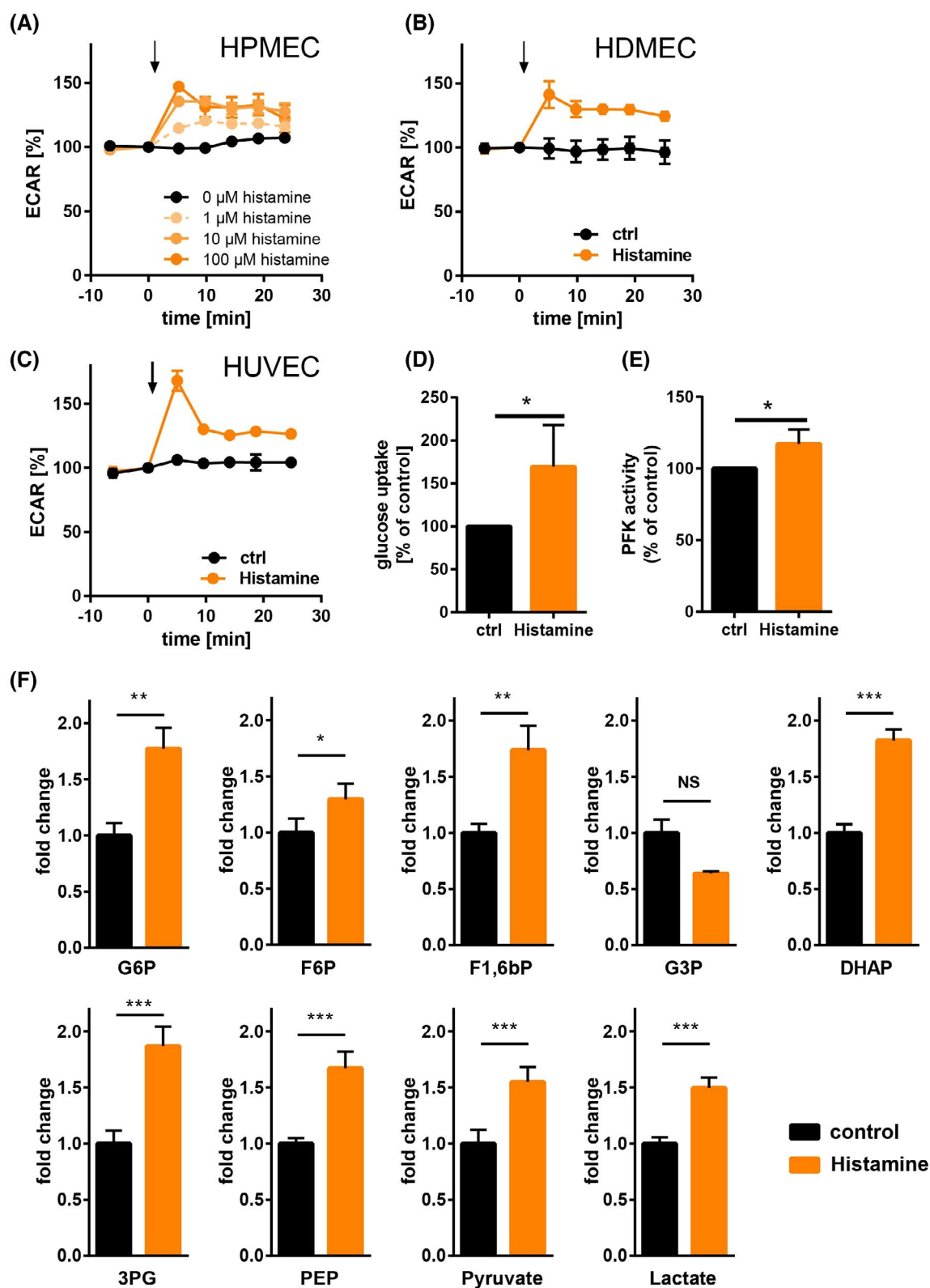


FIGURE 1 Histamine induces rapid activation of glycolysis in endothelial cells. A, Real-time changes in extracellular acidification rates (ECAR) of HPMEC treated with the indicated concentrations of histamine or left untreated as control (only medium = 0 μ M histamine). Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow) ($n = 3$ different HPMEC donors). B-C, Real-time changes in ECAR of HDMEC (B) or HUVEC (C) treated with 20 μ M histamine or left untreated as control (only medium, control). Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow) (B-C; $n = 4$ experiments). D, Glucose uptake assessed by 2-NBDG internalization in HPMEC left unstimulated (control) or treated with histamine for 30 minutes. Glucose uptake of unstimulated cells was set as 100% in each experiment ($n = 4$). E, PFK activity in HPMEC left unstimulated (control) or treated with histamine for 5 minutes. PFK activity of unstimulated cells was set as 100% in each experiment ($n = 6$). F, Targeted metabolomic analysis for glycolytic intermediates of HPMEC that were left unstimulated (control) or treated with histamine for 30 minutes. Data are shown as fold change relative to the control group ($n = 18$ HPMEC cultures; pooled from three independent runs). G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6bP, fructose-1,6-bisphosphate; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate. Data are means $\pm$ SEM. *: $P < .05$, **: $P < .01$, ***: $P < .001$ (D-F: Mann-Whitney U test)

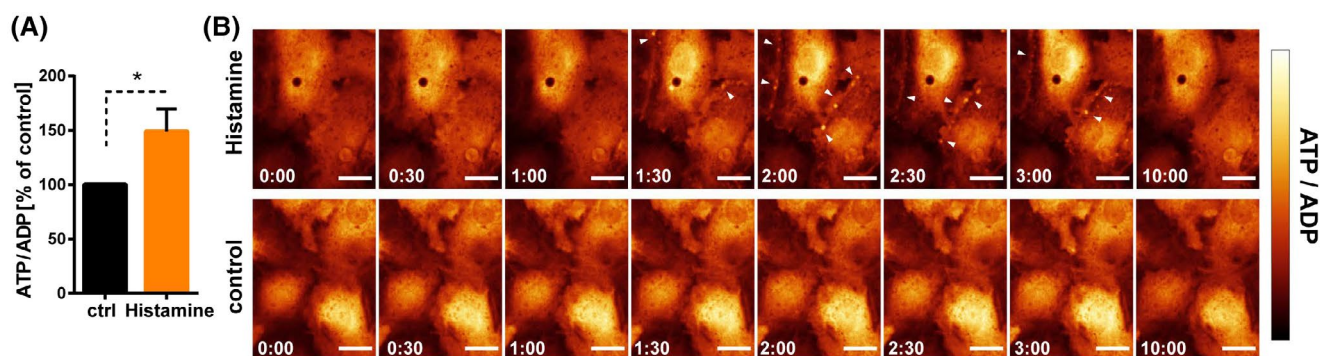


FIGURE 2 Histamine increases the ATP:ADP ratio in endothelial cells. A, ATP:ADP ratio in HPMEC left unstimulated (control) or treated with histamine for 2 minutes. ATP:ADP ratio of unstimulated cells was set as 100% in each experiment ($n = 4$ experiments). Data are means $\pm$ SEM. *: $P < .05$ (Mann-Whitney U test). B, Time-resolved imaging of cellular ATP:ADP ratios in HPMEC upon stimulation with histamine or of cells left unstimulated (control). Arrowheads indicate local enhancement of ATP to ADP ratios at cell borders. HPMEC expressing the biosensor PercevalHR were imaged via spinning disc microscopy for 10 minutes after addition of medium without (control) or with histamine. Exposure wavelengths were set to 405 and 488 nm and emission detection was set at 525. Quotients of pixel values acquired at 488 and 405 nm excitation were formed to compute pixel values corresponding to ATP:ADP ratios. Grey scales are displayed as red-to-yellow pseudocolor gradient. Displayed images are maximum intensity projections of complete Z-stacks. Scale bars = 20 μ m. A representative experiment out of three is shown

actomyosin-driven contractility and rearrangement of cortical actin into radial actin stress fibers anchored to adherens junctions.¹⁰ Attenuation of glycolytic activity with 3PO prevented histamine-induced formation of actin stress fibers and disruption of adherens junctions in endothelial cell monolayers, as assessed by VE-cadherin and F-actin staining (Figure 4A). Additionally, 3PO pretreatment of HPMEC blunted the histamine-induced increase in the F-actin to G-actin ratio (Figure 4B) and in MLC phosphorylation (Figure 4C).

The increase in endothelial permeability requires weakening of the adhesive function of VE-Cadherin. Specifically, phosphorylation of VE-cadherin at Tyr685 is involved in endothelial barrier disruption.³² Histamine-mediated phosphorylation of VE-cadherin at Tyr685 as well as phosphorylation of the upstream permeability-promoting Tyrosine-Protein Kinase SRC-1 (Src)³³ in HPMEC were both inhibited by 3PO-treatment (Figure 4C). On the contrary, histamine-induced ERK 1/2 phosphorylation was unaffected by 3PO pretreatment (data not shown), indicating that 3PO does not influence permeability-unrelated histamine receptor signaling.

These results suggest that mild inhibition of glycolysis in endothelial cells suppresses histamine-induced cytoskeleton changes and endothelial junction opening.

The tension generated by actomyosin contraction promotes the relocalization of VE-cadherin adhesion complexes to zipper-like structures (designated FAJs), which are characterized by the presence of vinculin and associated F-actin stress fibers.³⁴ Accordingly, attenuation of endothelial glycolysis with 3PO prevented the histamine-induced formation of FAJs at endothelial cell-cell contacts (Figure 4D-E). Together, inhibition of glycolysis with 3PO counteracts the effect of histamine on diminishing endothelial barrier integrity.

3.4 | Glycolysis inhibition prevents histamine-induced vascular hyperpermeability in vivo and anaphylactic shock

We next investigated the role of glycolysis in endothelial hyperpermeability induced by histamine in vitro.

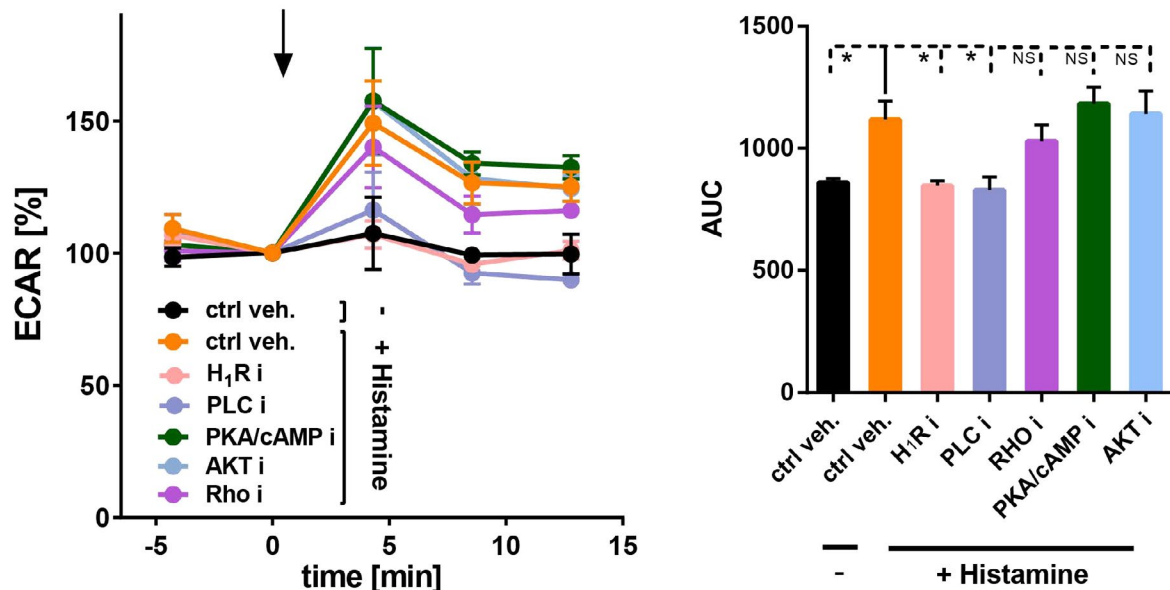


FIGURE 3 Histamine induces glycolysis via an H₁R / PLC β signaling pathway. A, Time-resolved measurement of ECAR changes in HPMEC that were left untreated (only medium with vehicle control) or treated with histamine-containing medium in the presence of vehicle control or pharmacological inhibitors against H₁R, PLC β , PKA, and cAMP, RhoA, or AKT. Inhibitors or respective control vehicles were preincubated in cultures before histamine or medium treatment, as described in the Methods. Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow). B, Area under the curve analysis of ECAR changes upon histamine stimulation (AUC analysis was performed for the area between the first and the third ECAR measurement time point after histamine injection). Data are means $\pm$ SEM (n = 3 experiments). *: $P < .05$ (1-way ANOVA with Holm-Sidak's multiple comparisons test). NS, nonsignificant

Histamine-induced hyperpermeability was abrogated almost completely in 3PO-treated HPMEC monolayers, as assessed by trypan blue-labeled albumin (Figure 5A) and FITC-labeled streptavidin leakage (Figure 5B).

To determine the physiological relevance of our in vitro findings, we next examined whether 3PO treatment could prevent histamine-induced vascular hyperpermeability in vivo. Strikingly, mice pretreated with 3PO displayed markedly attenuated histamine-induced vascular leakage in two independent models: (i) in the Miles assay, where Evans blue tissue extravasation was triggered directly by local histamine administration (Figure 6A) and (ii) in a passive cutaneous anaphylaxis model, in which Evans blue tissue extravasation was triggered by local mast cell degranulation following antigen exposure in mice pretreated with respective antihapten-specific IgEs (Figure 6B).

To further strengthen our aforementioned in vivo data, we examined the effect of 3PO treatment in a mouse model of active systemic anaphylaxis, in which mice were first sensitized to BSA, and then, systemically challenged with BSA. Consistent with our findings that glycolysis regulates histamine-induced vascular leakage (Figure 6A-B), pretreatment with 3PO protected mice from anaphylactic shock caused by injection of the allergen and from associated lethality (Figure 6C). These data suggest a profound effect of mild glycolysis inhibition in preventing vascular leakage and related pathologies in vivo.

4 | DISCUSSION

Our present findings demonstrate that glycolysis activation is an integral component of histamine-induced endothelial hyperpermeability, which is a key pathomechanistic element of anaphylactic reactions. Endothelial barrier disruption by histamine requires the immediate adaptation of energy supply and endothelial cells engage preferentially in glycolysis rather than oxidative phosphorylation.³⁵ The dependency of histamine-induced endothelial hyperpermeability on glycolysis can be likely attributed to the enhanced and immediate demand for local energy and metabolite production required for reorganization of cell-cell junctional connections and actomyosin contractility. Cellular compartmentalization of multienzymatic glycolytic complexes to specific cellular structures has been previously reported in different cells and can promote local ATP generation and other cellular functions.^{14,35,36}

The importance of enhanced endothelial glycolysis was demonstrated also in other scenarios, where endothelial cells switch from quiescence to an active state. For example, in angiogenesis the glycolytic enzymes PFKFB3 and pyruvate kinase 2 (PKM2) were found to be crucial for migration and proliferation of endothelial cells.^{12,37,38} Furthermore, partial inhibition of glycolysis with 3PO suppressed pathological angiogenesis and led to tumor vessel normalization.³⁹ Additionally, PFKFB3-mediated

endothelial glycolysis promotes endothelial inflammation and leukocyte recruitment in the models of pulmonary hypertension and acute lung injury and 3PO treatment alleviated lung edema and protected mice from LPS-induced acute lung injury.^{40,41}

We observed that, unlike other endothelial cell types tested here, retinal endothelial cells did not display enhanced glycolysis in response to histamine. It seems plausible that these cells are protected from the effects of histamine, as they contribute to formation of the blood-retina-barrier, and

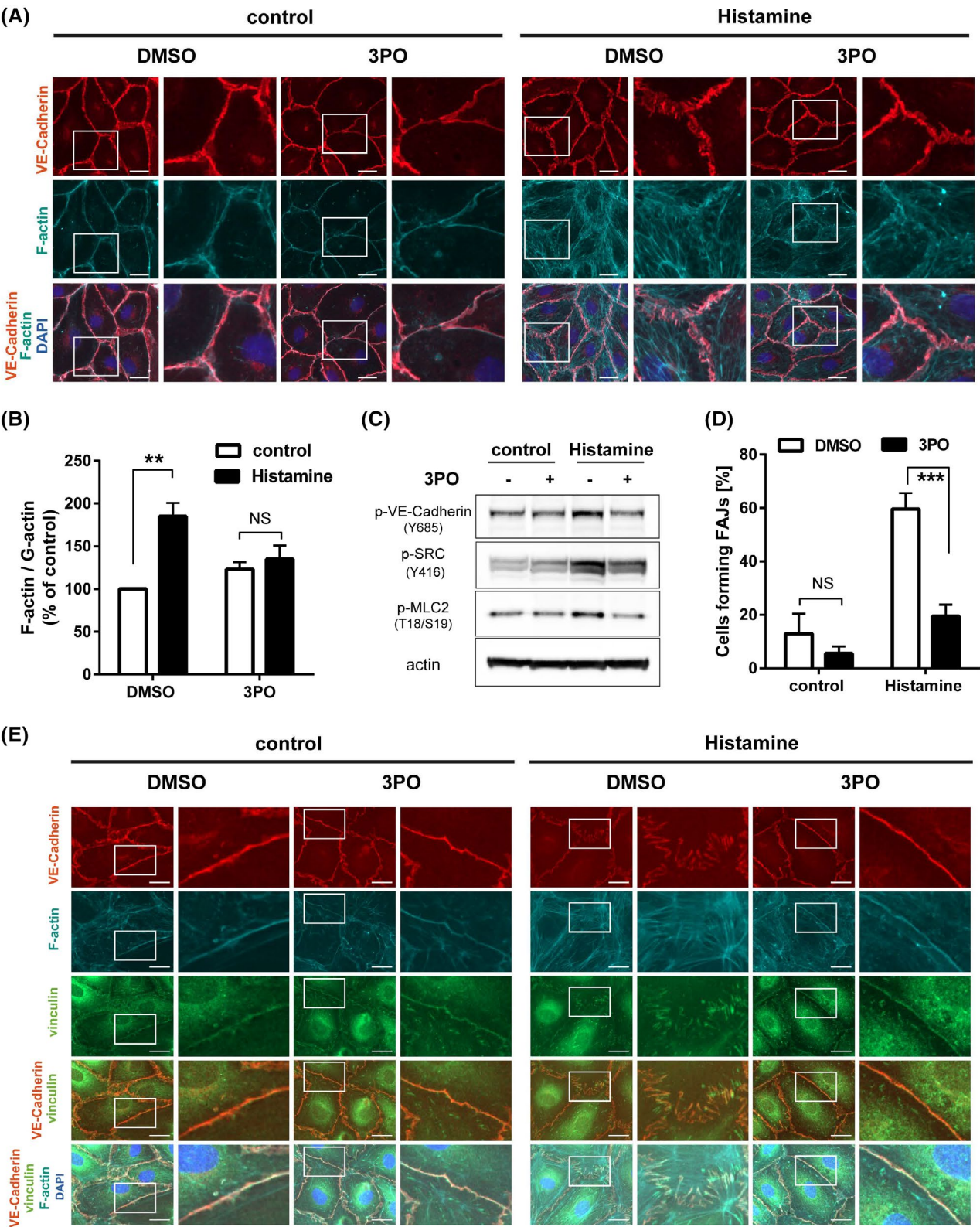


FIGURE 4 Attenuation of endothelial glycolysis blocks the formation of histamine-induced F-actin stress fibers and FAJs. A, Representative fluorescence images of HPMEC monolayers that were pretreated with 3PO or vehicle (DMSO) for 4 hours followed by addition of histamine or medium (control) for 4 minutes. Cells were stained for VE-cadherin (red), F-actin (with rhodamine-phalloidin, cyan) and nuclei with DAPI (blue). The areas indicated by the square are displayed in higher magnification on the right side of the original image. Pseudocolored images are shown. Scale bars = 20 μ m. Representative images from one experiment out of 3 are shown. B, F-actin to G-actin ratio of HPMEC pretreated with 3PO or vehicle (DMSO) for 4 hours followed by addition of histamine or medium (control) for 5 minutes. F-actin to G-actin ratio of control-treated (DMSO, no histamine) HPMEC was set as 100% in each experiment ($n = 3$). C, Representative cropped blot images of HPMEC pretreated with 3PO or vehicle (DMSO, -) for 4 hours followed by addition of histamine or medium (control) for 6 minutes. Cell lysates were immunoblotted for phospho-VE-Cadherin (Y685), phospho-SRC (Y416), phospho-MLC2 (T18/S19), and actin. One experiment representative of 6 experiments is shown. D-E, HPMEC monolayers were pretreated with 3PO or vehicle DMSO for 4 hours and subsequently treated with histamine or medium (control) for 4 minutes. Cells were fixed and stained for VE-cadherin (red), vinculin (green), F-actin was labeled by rhodamine-phalloidin (cyan) and nuclei were labeled with DAPI (blue). In (D), quantification of the percentage of cells forming focal adherens junctions (FAJs; defined as colocalization of vinculin, F-actin, and discontinuous VE-cadherin signal in interendothelial junctions) is shown ($n = 5$ experiments). In (E), representative fluorescence images are depicted. Images are shown in pseudocolor. The areas indicated by the square are shown in higher magnification on the right side of the original image. Scale bars = 20 μ m. Data are means $\pm$ SEM. **: $P < .01$, ***: $P < .001$ (B, D: 2-way ANOVA with Sidak's multiple comparisons test). NS, nonsignificant

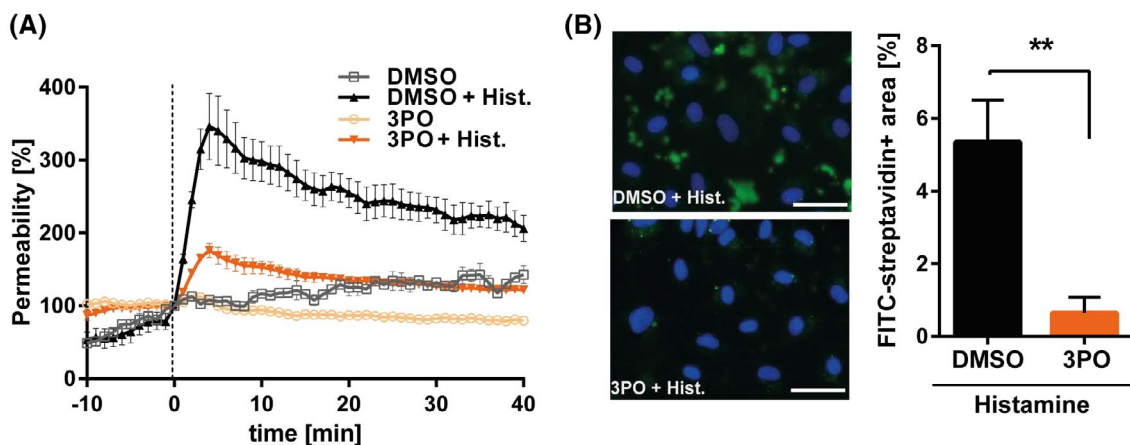


FIGURE 5 Inhibition of glycolysis reduces histamine-induced endothelial permeability. A, In vitro macromolecular permeability assay in HPMEC monolayers pre-exposed to 3PO or DMSO (control) for 4 hours followed by treatment with histamine or medium, as described in the Methods. Increase in permeability and its recovery was recorded in real-time for 40 minutes. Values at the time point of addition of histamine or medium (indicated by the vertical dotted line) were set as 100% in each well ($n = 3$ experiments). B, Staining of FITC-streptavidin leakage through histamine-treated endothelial monolayers. HPMEC were pretreated with 3PO or vehicle (DMSO) for 4 hours, after which FITC-streptavidin was added in the presence or absence of histamine for 5 minutes, as described in the Methods. (left) Representative fluorescence images of FITC-streptavidin leakage (green) through endothelial monolayers are displayed; nuclei are visualized with DAPI staining (blue). (right) Percentage of the FITC-streptavidin-leakage-positive area was quantified in each image ($n = 6$ images per condition; data are from one experiment, representative of three). Data are means $\pm$ SEM. **: $P < .01$; (B: Mann-Whitney U test)

therefore, may form tighter and less responsive monolayers than endothelial cells from other vascular beds.⁴² In fact, a previous study demonstrated that H_1R signaling was protective for vascular integrity of brain endothelial cells, to which retinal endothelial cells are related.⁴³ Moreover, considering the role of histamine as a neurotransmitter,⁴⁴ it seems reasonable that brain and retinal endothelial cells should be able to maintain their vascular integrity in the presence of histamine. Other reports have noted that HRMEC do increase permeability similarly to other endothelial cell types in response to some factors, such as VEGF, but are unresponsive to others, such as IL-6.⁴⁵⁻⁴⁷ Hence, the distinct responses to histamine

of HRMEC, compared to endothelial cells from other vascular beds, provides further evidence for the heterogeneity of vascular endothelial cells from different tissues.⁴⁸

We found that in several endothelial cell types histamine stimulation causes an acute boost in glycolysis. This was accompanied by enhanced glucose uptake, elevated levels of glycolytic metabolites and enhanced PFK1 activity. These effects could be partially explained through allosteric activation by fructose-2,6-bisphosphate (F2,6P2), the metabolic product of PFKFB3.^{39,49} We demonstrated that glycolysis activation occurs downstream of histamine-induced signaling and is required for endothelial junction disruption.

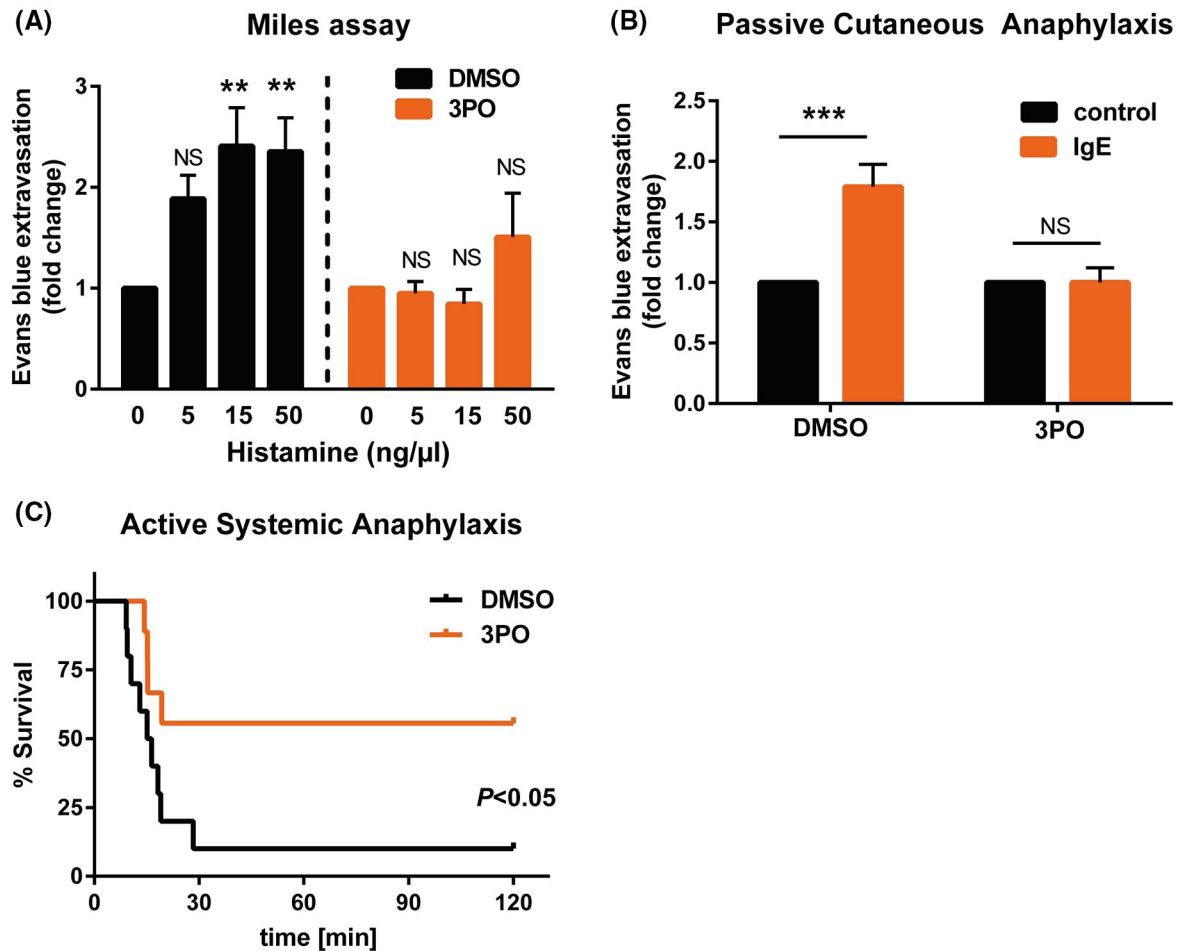


FIGURE 6 Pharmacological blockade of glycolysis impairs histamine-induced vascular permeability and anaphylactic reaction in vivo. A, Quantification of histamine-mediated vascular leakage in vivo via Miles Assay in vehicle control-treated (DMSO) and 3PO-treated mice. Evans Blue extravasation in skin biopsies was determined after subdermal injection of 0, 5, 15, and 50 ng/μL of histamine diluted in PBS. Mice were injected i.p. with either 3PO or vehicle (DMSO) 2 hours prior to histamine application. Data were expressed as the ratio of Evans Blue extravasation promoted by histamine over the Evans Blue extravasation promoted by the control injection (PBS, indicated as 0 ng/mL histamine) in the same mouse. Values of control (0 ng/μL histamine)-treated samples were set as 1 for each mouse ($n = 4$ mice per group). B, Induction of passive cutaneous anaphylaxis was conducted in mice injected into ear skin with anti-DNP-IgE or with normal saline (control). Twenty-four hours later, mice received iv injections of DNP-HSA with Evans Blue dye in normal saline to trigger dye leakage. Mice were either treated with DMSO or 3PO by i.p. injections 2 hours before injection of DNP-HSA. Data were expressed as the ratio of Evans Blue extravasation promoted by anti-DNP-IgE over Evans Blue extravasation promoted by the control injection (saline) of the same mouse. Values of saline injected (control) ear biopsies were set as 1 for each mouse ($n = 11$ mice per group). C, Assessment of mouse survival in vehicle control-treated (DMSO) versus 3PO-treated mice during active systemic anaphylaxis. Mice were sensitized with BSA and PTX and challenged 14 days later with BSA. Mouse survival was monitored for 120 minutes. 3PO or DMSO treatment took place by i.p. injections 2h before the second BSA challenge ($n = 9$ -10 mice per group). Data are means $\pm$ SEM. **: $P < .01$, ***: $P < .001$ (A-B: 2-way ANOVA with Holm-Sidak's multiple comparisons test; C: Mantel-Cox test). NS, nonsignificant

Specifically, histamine directly and rapidly activated glycolysis in endothelial cells via H_1R , the same receptor implicated in endothelial barrier disruption, and the downstream $PLC\beta$ pathway. Glycolysis triggered by histamine was in turn required for MLC phosphorylation and related actomyosin contractility, as well as for VE-cadherin Tyr685 phosphorylation and the resulting loss of endothelial barrier integrity.

We further demonstrated that endothelial glycolysis activation is essential for vascular hyperpermeability induction, as mild glycolysis attenuation with 3PO¹⁷ abolished

histamine-induced hyperpermeability. These findings are consistent with a previous report, in which 3PO promoted vascular integrity and EC interconnectivity in quiescent and VEGF-treated endothelial cell monolayers via inhibition of VE-Cadherin endocytosis.³⁹ Histamine-induced permeability, in contrast to permeability induced by other factors like VEGF, does not involve VE-cadherin endocytosis for disruption of endothelial barrier continuity but rather the rapid formation of FAJs, in which vinculin associates with VE-Cadherin to control force-dependent remodeling driven by actomyosin contractility.¹⁰ Consistently, the

histamine-dependent formation of FAJs was also mediated by increased glycolysis, as it was abrogated in 3PO-treated cells.

Importantly and of direct translational relevance, mice pretreated with 3PO were resistant to vascular leakage when challenged directly with histamine or indirectly in the model of passive cutaneous anaphylaxis, in which vascular leakage is caused by mast cell degranulation and secretion of histamine among other permeability-increasing factors. Strikingly, mild glycolysis inhibition by 3PO protected mice from systemic anaphylaxis and associated lethality. One percent of anaphylaxis-related hospitalizations have a fatal outcome,⁵⁰ while the burden of allergic diseases remains substantial.⁵¹ In conclusion, our findings not only provided new insights into the molecular mechanisms underlying histamine-induced glycolysis and the downstream disruption of endothelial barrier integrity, but are also therapeutically important. Indeed, treatment with 3PO may offer a novel approach to combat pathological conditions associated with vascular leakage elicited by the acute release of histamine.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

A. Ziogas designed and performed research, supervised research, analyzed, and interpreted data and wrote the manuscript. M. S. Sajib, J. H. Lim, A. Das, A. Witt, E. Hagag, N. Androulaki, S. Grossklaus, and T. Grinenko performed research. T. C. Alves and M. Gerlach performed experiments and interpreted data. T. Noll and P. Mirtschink provided analytical tools and helped in research design. G. Hajishengallis interpreted data and edited the paper. T. Chavakis co-designed research and edited the paper. CM Mikelis designed and supervised research and edited the paper. D. Sprott designed and performed research, supervised research, analyzed, and interpreted data and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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FIGURE S1

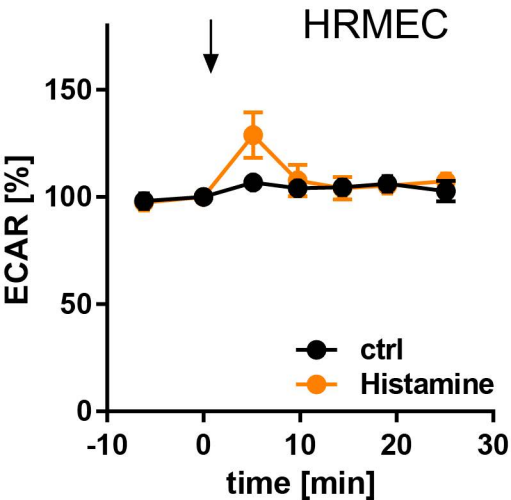


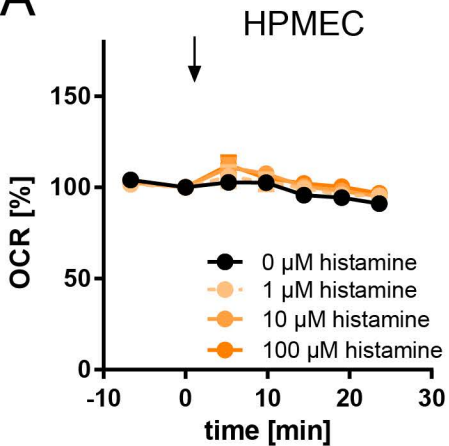
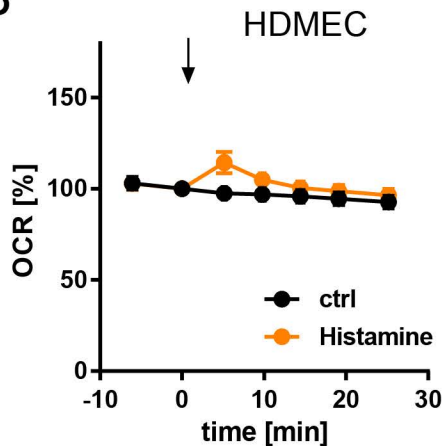
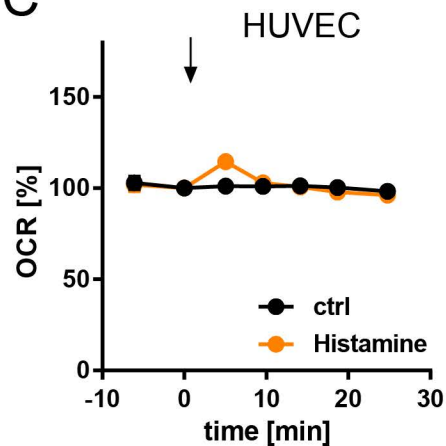
FIGURE S2**A****B****C**

FIGURE S3

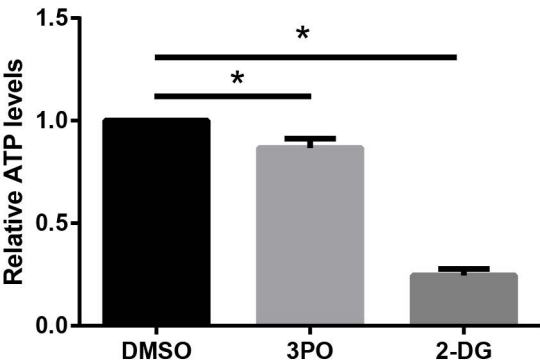


FIGURE S4

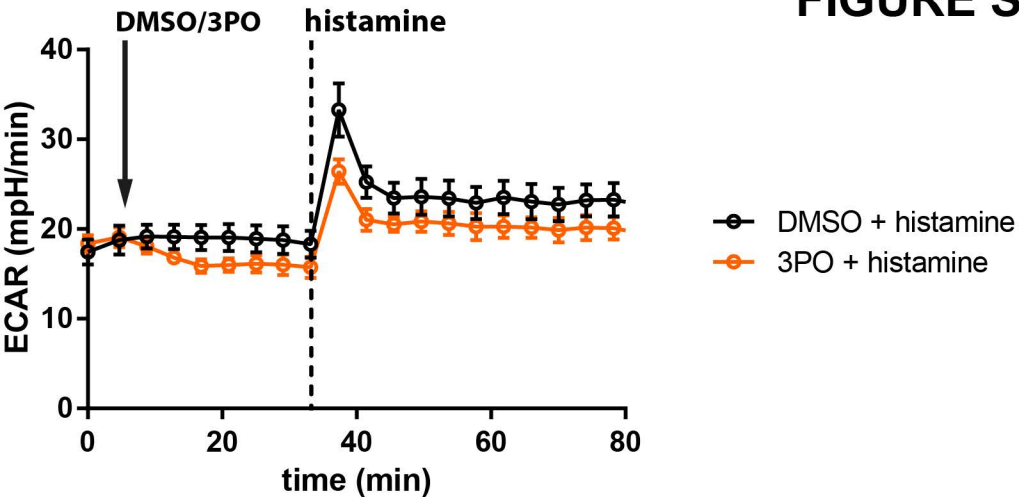
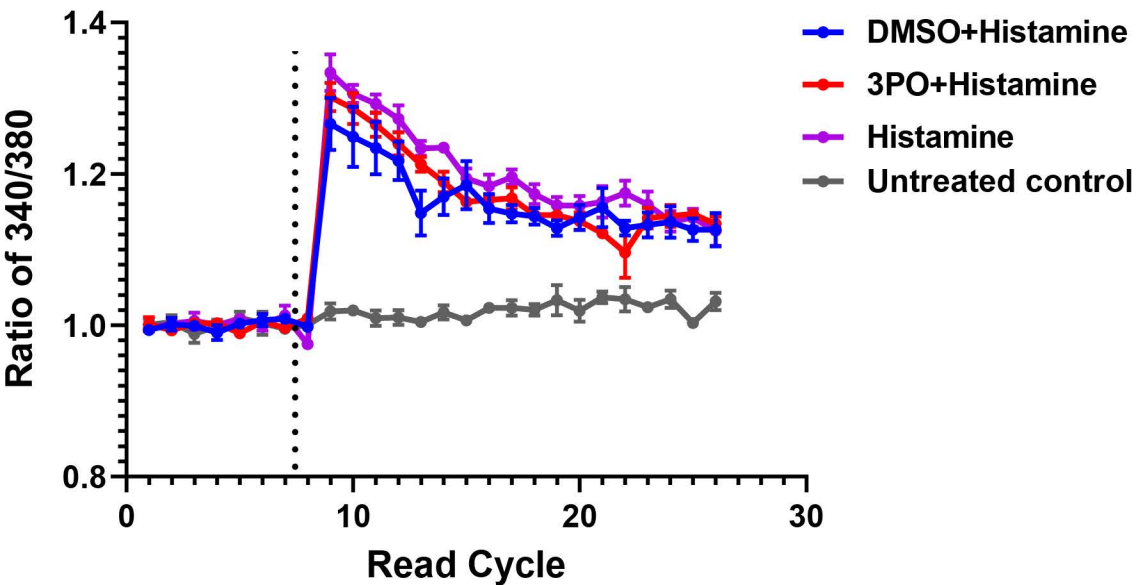


FIGURE S5

Supplemental Data Legends

Figure S1: Measurement of extracellular acidification rates (ECAR) upon histamine treatment in retinal endothelial cells

Real-time changes in ECAR of human retinal microvascular endothelial cells (HRMEC) treated with 20 μ M histamine or left untreated as control (only medium, control). Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow) (n=5 experiments).

Figure S2: Measurement of endothelial oxygen consumption rates (OCR) upon histamine treatment

(A) Real-time measurement of OCR in HPMEC that were treated with the indicated concentrations of histamine or left untreated as control (only medium = 0 μ M histamine). Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow) (n=3 different HPMEC donors). (B-C) Real-time measurement of OCR in HDMEC (B) or HUVEC (C) that were treated with 20 μ M histamine or left untreated as control (only medium, control). Values were baselined and set as 100% for each well at the time point prior to histamine injection (indicated by the arrow) (B-C; n=4 experiments).

Figure S3: Inhibition of glycolysis reduces ATP levels in histamine-stimulated HPMEC

HPMEC were pretreated with 20 μ M 3PO for 4 h (partial glycolysis inhibition) or with 50 mM 2-DG for 30 min (total glycolysis inhibition). DMSO pretreatment was used as control. Cells were then stimulated with histamine for 2 min, after which they were lysed and ATP levels were determined. Average values of DMSO-treated control samples were set as 1 in each experiment (n= 3 experiments). Data are expressed as means $\pm$ SEM. *: $p < 0.05$ (1-way ANOVA with Holm-Sidak's multiple-comparisons test).

Figure S4: 3PO treatment leads to a moderate reduction in glycolysis

Real-time changes in ECAR of HPMEC that were treated with histamine-containing medium in the presence of vehicle control (DMSO) or 3PO. DMSO/3PO treatment (arrow) and histamine treatment (dotted line) were performed at the indicated time points. Graph depicts data from a representative experiment. Data are expressed as means $\pm$ SEM. Similar results were obtained in 4 independent experiments.

Figure S5: 3PO does not directly interfere with histamine receptor signalling

Dynamic changes in Ca^{2+} efflux after histamine stimulation were detected with the ratiometric fluorescent dye fura-2 AM in human umbilical vein endothelial cells (HUVEC). Cells were pretreated with 3PO or vehicle (DMSO) for 4 h and stimulated with histamine at the indicated measurement cycle (black vertical dotted line between cycle 8 and 9) or left untreated (n=2). Values were baselined and set as 1 at cycle 1. Graph data are expressed as means $\pm$ SEM.