

Endothelial, but not smooth muscle, peroxisome proliferator-activated receptor β/δ regulates vascular permeability and anaphylaxis

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Background: Remodeling of quiescent vessels with increases in permeability, vasodilatation, and edema are hallmarks of inflammatory disorders. Factors involved in this type of remodeling represent potential therapeutic targets.

Objectives: We investigated whether the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) β/δ , a regulator of metabolism, fibrosis, and skin homeostasis, is involved in regulation of this type of remodeling.

Methods: Wild-type and various *Pparb/d* mutant mice were used to monitor dermal acute vascular hyperpermeability (AVH) and passive systemic anaphylaxis-induced hypothermia and edema. PPAR β/δ -dependent kinase activation and remodeling of endothelial cell-cell junctions were addressed by using human endothelial cells.

Results: AVH and dilatation of dermal microvessels stimulated by vascular endothelial growth factor A, histamine, and thrombin are severely compromised in PPAR β/δ -deficient mice. Selective deletion of the *Pparb/d*-encoding gene in endothelial cells *in vivo* similarly limits dermal AVH and vasodilatation, providing evidence that endothelial PPAR β/δ is the major player in

regulating acute dermal microvessel remodeling. Furthermore, endothelial PPAR β/δ regulatory functions are not restricted to the skin vasculature because its deletion in the endothelium, but not in smooth muscle cells, also leads to reduced systemic anaphylaxis, the most severe form of allergic reaction, in which an acute vascular response plays a key role. PPAR β/δ -dependent AVH activation likely involves the activation of mitogen-activated protein kinase and Akt pathways and leads to downstream destabilization of endothelial cell-cell junctions.

Conclusion: These results unveil not only a novel function of PPAR β/δ as a direct regulator of acute vessel permeability and dilatation but also provide evidence that antagonizing PPAR β/δ represents an important strategy to consider for moderating diseases with altered endothelial integrity, such as acute inflammatory and allergic disorders. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Peroxisome proliferator-activated receptor β/δ , anaphylaxis, vascular permeability, endothelium, smooth muscle cells

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The structure of the vessel wall includes a layer of contractile cells (vascular smooth muscle cells [VSMCs] in large vessels and pericytes in arterioles and venules) and the internal endothelium. The endothelium performs critical functions in the control of vessel wall permeability by controlling exchanges through the transcellular and paracellular routes.¹⁻⁴ The paracellular route (based on destabilization of cell-cell endothelial junctions) is believed to be of primary importance for vessel hyperpermeability in pathophysiologic situations.^{2,5} The endothelium also regulates contractile cell functions by releasing vasorelaxing and vasoconstricting signals,⁶ thereby regulating vessel tone and diameter, as well as blood pressure.^{7,8}

Vasodilatation, vascular hyperpermeability, and plasma extravasation are hallmarks of inflammatory disorders, like allergic reactions ("type I immediate hypersensitivity" in the older nomenclature). In sensitized allergic subjects the prevalent mechanism through which allergens trigger an inflammatory reaction is activation of mast cells and basophils on binding of multivalent antigen to the IgE-loaded high-affinity IgE receptor FcεRI.⁹⁻¹² Activated mast cells release preformed mediators, such as histamine (also serotonin in rodents), proteases, and neosynthesized arachidonic acid-derived prostaglandins and leukotrienes, as well as cytokines, growth factors (eg, vascular endothelial growth factor A [VEGF-A]), and chemokines.¹³⁻¹⁶ Mediators such as histamine and VEGF-A decrease vessel tone and destabilize the endothelial barrier,^{5,17} causing systemic

Abbreviations used

AJ:	Adherens junctions
AVH:	Acute vascular hyperpermeability
BMMC:	Bone marrow–derived mast cell
DAPI:	4'-6-Diamidino-2-phenylindole dihydrochloride
EBD:	Evans blue dye
ERK:	Extracellular signal-regulated kinase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HUVEC:	Human umbilical vein endothelial cell
NO:	Nitric oxide
PPAR:	Peroxisome proliferator-activated receptor
siRNA:	Small interfering RNA
SMA:	Smooth muscle actin
VEGF-A:	Vascular endothelial growth factor A
VEGFR:	Vascular endothelial growth factor receptor
VSMC:	Vascular smooth muscle cell

plasma extravasation and vasodilatation, which results in edema, decreased blood volume, and hypotension.^{17,18} In its most severe form, systemic anaphylaxis, general vascular failure results in life-threatening altered tissue perfusion, which remains a major cause of lethality in patients with systemic shock.^{19,20} Surprisingly, although the importance of the vasculature in inflammation manifestations and allergies is widely acknowledged, therapeutic strategies targeting the vasculature to improve existing treatments have received little attention.

The 3 peroxisome proliferator-activated receptor (PPAR) isotypes PPAR α , PPAR β/δ , and PPAR γ are ligand-inducible transcription factors that form a subfamily of the nuclear hormone receptors.²¹ The canonical pathway by which PPARs regulate gene transcription involves activation by an agonist, heterodimerization with the retinoid X receptor, binding to a PPAR response element in the promoter region of their target genes, and recruitment of coactivators, which result in the activation of the target genes. PPARs are activated by natural fatty acids and fatty acid derivatives.^{21–23} Importantly, PPAR α and PPAR γ are the therapeutic targets of fibrates (lipid-lowering drugs) and thiazolidinediones (insulin-sensitizing compounds), respectively, although the use of thiazolidinediones has recently been restricted because of major side effects.^{24,25} PPAR β/δ is involved in the control of energy homeostasis, lipid metabolism, inflammation, and skeletal and heart muscle functions,^{26,27} as well as the development of the white adipose tissue, skin, gut, and placenta.^{28–30} We have identified PPAR β/δ as a key factor in skin repair^{31,32} and cancer³³ and in regulation of keratinocyte functions.^{32,34,35} Recent results show that PPAR β/δ also regulates vasculature functions. In endothelial cells, VSMCs, or both, PPAR β/δ performs proangiogenic, anti-inflammatory, antiatherosclerotic, and antihypertensive functions.^{36–39}

In this article we have shown that although PPAR β/δ is dispensable for the development of mature microvessels, its activity is a key factor for acute vascular hyperpermeability (AVH) and vasodilatation.

METHODS**Animals**

Animal procedures were performed on approval by the cantonal veterinary service of the Canton of Vaud or by the Ethics Committee of the Nord-Pas-de-Calais Region. *Pparb/d* germline-deficient animals were described

previously.³⁰ *Pparb/d*^{−/−} and control *Pparb/d*^{+/+} animals were littermates obtained from heterozygous mating. An endothelial cell-specific *Pparb/d*-deficient mouse line was obtained by crossing *Tie1-Cre* recombinase hemizygous animals (Professor R. Fässler, Max Planck Institute, Martinsried, Germany⁴⁰) and *Pparb/d*^{fl/fl} mice (Professors W. Wahli and B. Desvergne, University of Lausanne, Lausanne, Switzerland^{41,42}). Mice genotypes are labeled as follows: *Tie1-Pparb/d*^{fl/fl} mice are Cre recombinase-negative/PPAR β/δ -proficient mice, with *Pparb/d* exon 4 (encoding the N-terminal zinc finger of the DNA binding domain) flanked by LOX-P sites, and *Tie1-Pparb/d*^{−/−} mice are Cre recombinase-positive mice, with *Pparb/d* exon 4 deleted, and thereby *Pparb/d* is invalidated. A *SM22-Pparb/d*^{fl/fl} smooth muscle cell (SMC)-specific PPAR β/δ -deficient mouse line (and littermate proficient control animals) was obtained by crossing *SM22-Cre* recombinase animals (Jackson Laboratory, Bar Harbor, Me; Strain STOCK Tg[Tagln-cre]1Her/J) and *Pparb/d*^{fl/fl} mice. Mice genotypes are labeled as follows: *SM22-Pparb/d*^{fl/fl} mice are Cre recombinase-negative/PPAR β/δ -proficient mice, with the *Pparb/d* exon 4 flanked by LOX-P sites, and *SM22-Pparb/d*^{−/−} mice are Cre recombinase-positive mice, with the *Pparb/d* exon 4 deleted, and thereby *Pparb/d* is invalidated.

All mouse strains are maintained on a mixed C57BL6/SV29 background. Genotyping (presence of the Cre recombinase transgene [472-bp DNA fragment] of the *Pparb/d* invalidated allele [490-bp DNA fragment] and of the *Pparb/d* floxed allele [400-bp DNA fragment]) was performed by using PCR of genomic DNA extracted from the ear (PCR-positive control: *Glut2* gene, 230-bp DNA fragment). All animals were kept in a standard colony with a temperature- and light-controlled environment and fed with a standard laboratory diet *ad libitum*.

Miles assay

Acute vascular permeability was assessed by using the Miles assay. Evans blue dye (EBD; 100 μ L, 5% solution in 0.9% NaCl) was injected in the tail veins of 6-month-old female mice. After 10 minutes, 50 μ L of VEGF-A (100 ng/mL, PEPR100-20D-10UG; PeproTech, Rocky Hill, NJ), thrombin (10 U/mL, AG T6884-100UN; Sigma-Aldrich, St Louis, Mo), histamine (100 μ M/L, AG H7125-1G; Sigma-Aldrich), or the control (PBS) were injected intradermally into the flank skin (PBS in the left-side flank skin and vasoactive agents in the right-side flank skin). After 30 minutes, the animals were killed, and full-thickness flank skin samples were collected (standardized 1 $\times$ 1-cm surfaces, which included the entire injection site), minced, and incubated in a fixed volume of 1 mL of formamide for 48 hours at 60°C, and extravasated EBD was quantified (OD 620 nm).

Anaphylaxis

Female mice (8–12 weeks old) were injected with 100 μ g/mL anti-DNP IgE (D 8406, Sigma) or PBS (200 μ L per animal) intravenously (tail vein). Twenty-four hours later, the anaphylactic reaction was initiated by means of injection (intravenously into the tail vein) of 5 mg/mL DNP-KLH (324121-100MG; Calbiochem, Nottingham, United Kingdom)/2% EBD (200 μ L). Hypothermia was monitored with an electronic thermometer with a rectal probe. At the end of the experiment (based on temperature recovery: 50 minutes for the *Pparb/d* germline mouse strain and 100 minutes for the *Tie1-Pparb/d* and *SM22-Pparb/d* mouse strains), animals were killed, and full-thickness ear tissue samples (standardized surfaces with an 8-mm punch biopsy device) were collected, minced, and incubated in a fixed volume of 1 mL of formamide for 48 hours at 60°C. Extravasated EBD was quantified (OD 620 nm).^{43,44}

Cell cultures and transfection

Human umbilical vein endothelial cells (HUVECs; C2519A; PromoCell, Heidelberg, Germany; passages 4–7) were cultured in complete Endothelial Cell Growth Medium (211 0101, PromoCell). Cells were seeded in TPP plastic 6-well plates (0.1% gelatin coating), except for immunofluorescent stainings, for which cells were seeded on glass-bottom culture dishes (10 μ g/mL fibronectin coating). For small interfering RNA (siRNA) transfection,

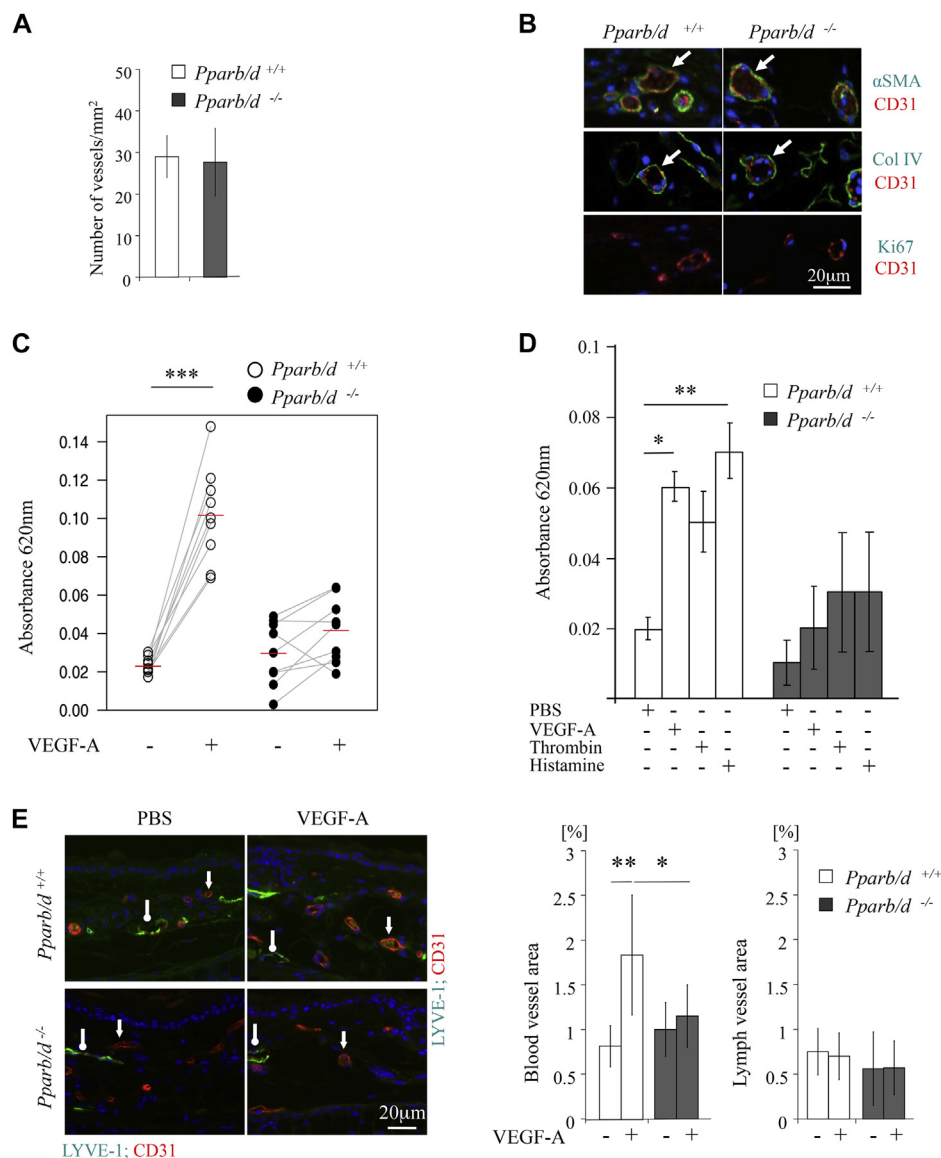


FIG 1. *Pparb/d*^{-/-} animals exhibit compromised vascular responses. **A**, Dermal vessel density. **B**, Dermal vessels. Green, αSMA, collagen IV, or Ki67; red, CD31. Arrows, αSMA- or collagen IV-positive vessels. **C** and **D**, Miles assay for EBD extravasation. Fig 1, **C**, Dots, individual animals; black lines, individual responses to treatment; red lines, mean per group. **E**, Left, Dermal vessels after intradermal injection. Green, LYVE-1 (white points); red, CD31 (white arrows). Middle and right, Blood and lymph vessel size. Data are expressed as means ± SDs (Fig 1, **A**, **B**, and **E**, n = 6; Fig 1, **C**, n = 9; Fig 1, **D**, n = 3 per group). **P* < .05 and ***P* < .01.

30% to 50% confluent HUVECs were treated with RNAimax (13778150; Life Technology, Carlsbad, Calif) and 25 nmol/L control (scrambled sequence; Thermo Scientific, Waltham, Mass) or PPARβ/δ siRNA (PPARβ/δ loss of function; GACCUGCCCCAUUCAUUG; Thermo Scientific).

Adherens junctions immunolabeling

VEGF-A (30 ng/mL) or PBS was added to confluent HUVECs 48 hours after siRNA transfection. Cells were collected 1 hour later and labeled with goat anti-VE-cadherin (C-19; Santa Cruz Biotechnology, Dallas, Tex; 1:50). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:5000). Pictures were taken with a Zeiss Axiovert 200 M microscope. Images were analyzed with ImageJ software (National Institutes of Health, Bethesda, Md).

Protein extraction and Western blotting

Forty-eight hours after siRNA transfection, VEGF-A (30 ng/mL), histamine (1 μmol/L), or PBS was added to 80% to 90% confluent HUVECs. Cells were collected 10 minutes later and lysed in TNE buffer. Phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (1:10,000, 4370; Cell Signaling Technology), total ERK1/2 (1:10,000, 4695; Cell Signaling Technology), phospho-Akt (1:2,000, 4060; Cell Signaling Technology), total Akt (1:2,000, 4685; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; loading control, 1/10,000, 2118; Cell Signaling Technology) were detected as follows: 5 μg of proteins were separated on SDS-PAGE and immunolabeled by using Western blotting. Primary antibodies were incubated for 2 hours at room temperature. Secondary antibody (anti-rabbit IgG-horseradish peroxidase 1:30,000, W4011; Promega, Madison, Wis) was incubated for 1 hour at room temperature. All antibodies were diluted in Tris buffer/1% Tween-20/1% BSA. Positive signals

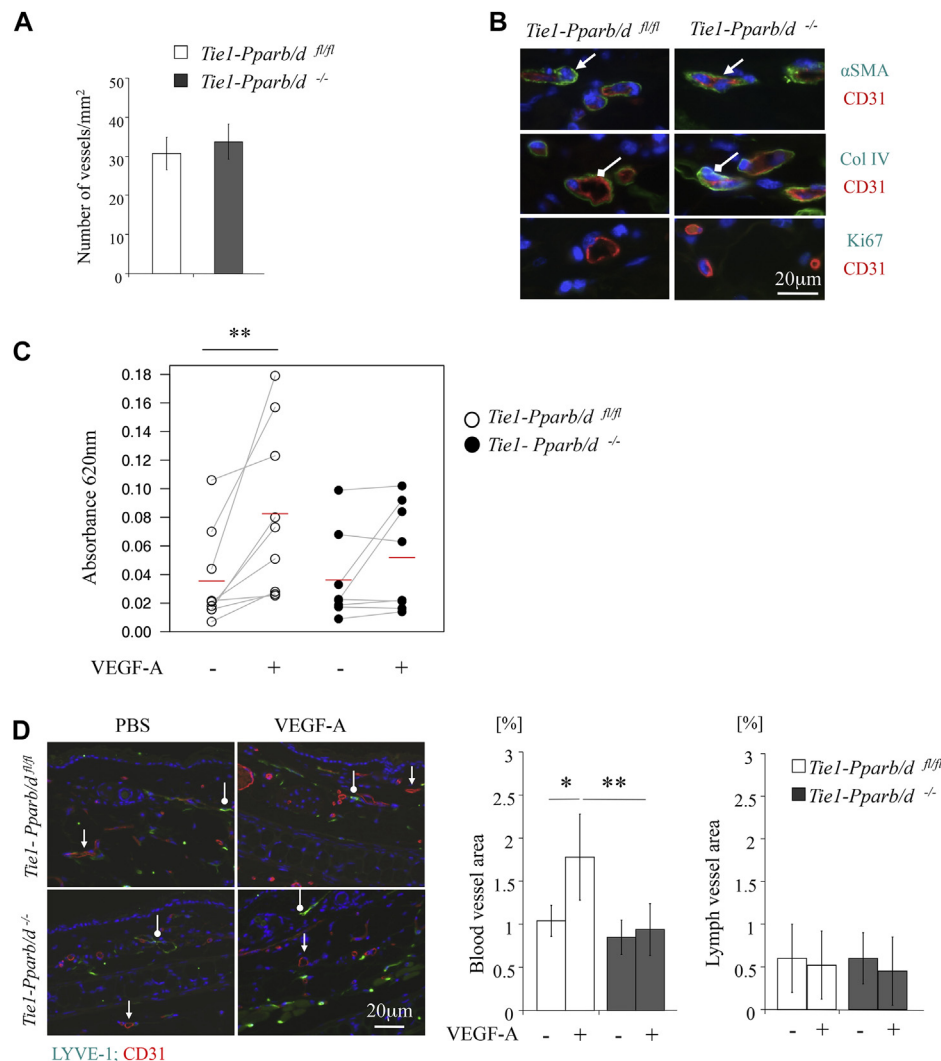


FIG 2. *Tie1-Pparb/d*^{-/-} animals exhibit compromised vascular responses. **A** and **B**, Dermal vessels as in Fig 1, **A** and **B**. **C**, Miles assay for EBD extravasation. Dots, Individual animals; black lines, individual responses to treatment; red lines, mean per group. **D**, Left, Dermal vessels after intradermal injection. Green, LYVE-1 (white points); red, CD31 (white arrows). Middle and right, blood and lymph vessel size. Data are expressed as means $\pm$ SDs (Fig 2, **A**, $n = 4$; Fig 2, **C**, $n = 9$; Fig 2, **D**, $n = 4$ -6 per group). * $P < .05$ and ** $P < .01$.

were detected with WesternBright Quantum-HRP (Advansta K-12042-D20) and Fusion FX (Vilber Lourmat, Eberhardzell, Germany) and quantified with the Bio1D software (Vilber Lourmat).

Histology and tissue immunostaining

Skin tissues were fixed (Zinc Fixative, 550523; BD PharMingen, San Jose, Calif) for 24 hours and paraffin embedded, and 4-μm-thick sections were used for standard hematoxylin and eosin or double immunostainings. Primary antibodies were as follows: CD31 (panendothelial marker, 1:50, 557355; BD PharMingen); Ki67 (proliferation marker, ab15580; Abcam, Cambridge, United Kingdom); collagen IV (basement membrane marker, 1:1000, AB756P; Chemicon, Temecula, Calif); α-smooth muscle actin (SMA) cell marker (1:400, A2547; Sigma); and LYVE-1 (lymph endothelial cell marker, 103-PA50AG; Reliatech, Wolfenbüttel, Germany) overnight at 4°C. Secondary antibodies (all from Molecular Probes, Eugene, Ore) were goat anti-rat Alexa Fluor 568 (1:100), goat anti-mouse Alexa Fluor 488 (1:400), and goat anti-rabbit Alexa Fluor 488 (1:400) for 40 minutes at room temperature. Nuclei were counterstained with DAPI (1:5000).

Characterization of dermal microvessel density and enlargement

Dermal microvessel density and enlargement after vasoactive treatments were quantified as follows. Ear sections were stained for CD31 (panendothelial marker, 1:50, 557355; BD PharMingen) and LYVE-1 (lymph endothelial cell marker, 103-PA50AG; Reliatech). Numbers of ear dermal blood and lymph vessels (vessel density) were counted on 3 standardized, nonadjacent microscopic fields of 0.7 mm². For quantification of vessel dilatation, the surface occupied by the blood or lymph vessels (which reflects the vessel size) was quantified by using computer-assisted morphometry (ImageJ software) standardized to the number of vessels and presented as a percentage of the total dermis surface subject to analysis. Three nonadjacent sections per animal (6 animals per group) were analyzed.

Statistical analysis

Results were presented as means $\pm$ SDs or SEMs, as indicated. Unless mentioned otherwise, the statistical comparison between groups was performed by using 2-way ANOVA, followed by the Bonferroni multiple comparison test. Probability was considered significant at a P value of less than .05. Two-way

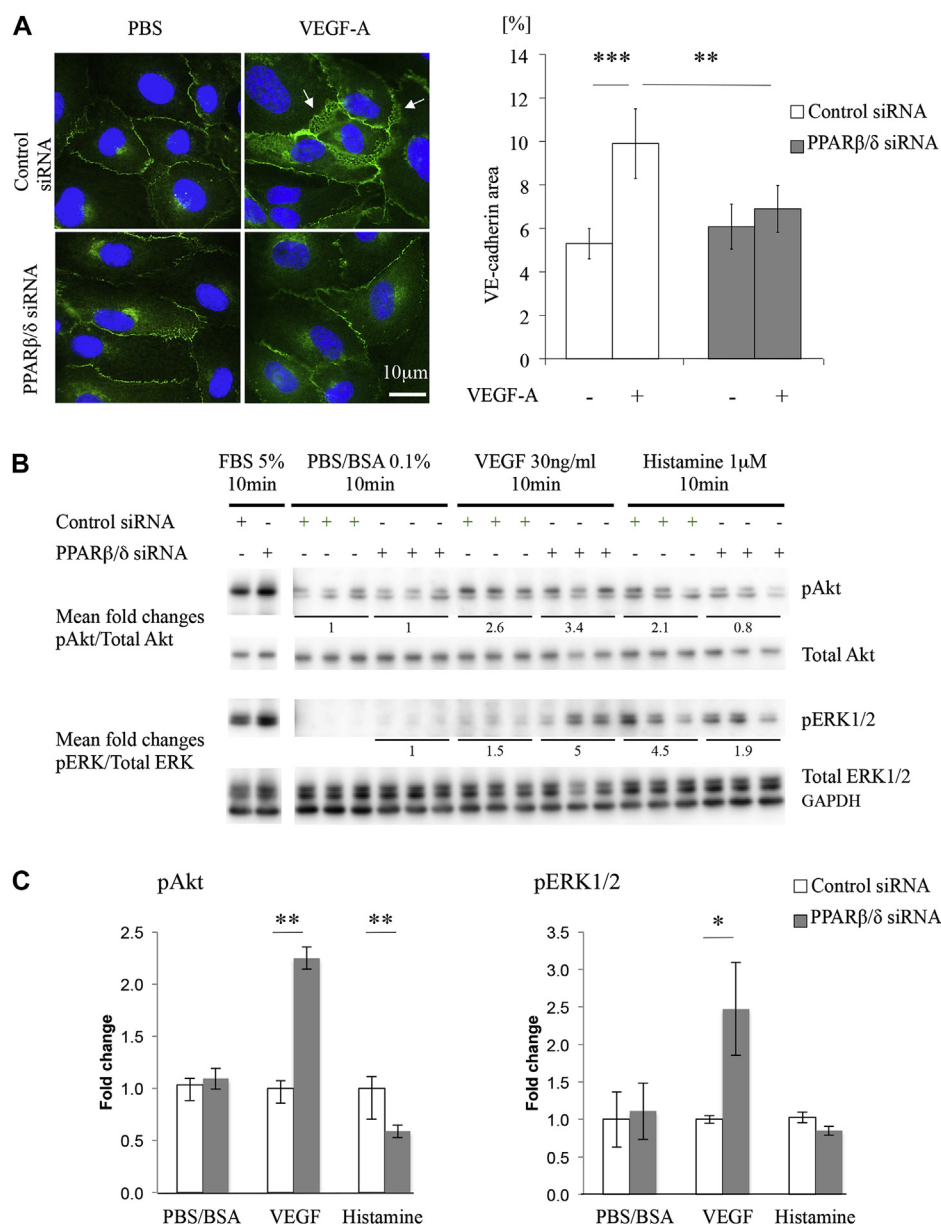


FIG 3. PPARβ/δ regulates cell-cell junction dismantling and kinase pathway activation. **A, Left**, VE-cadherin and nuclei (DAPI) stainings of HUVECs. **Right**, Area occupied by the VE-cadherin AJs. Data are expressed as means ± SDs (n = 7-9). **B**, Western blot of total ERK1/2, phosphorylated ERK1/2, total Akt, and phosphorylated Akt (upper band) from HUVECs treated as indicated. GAPDH was the loading control. **C**, Quantification of 3 independent experiments as shown in Fig 3, B. Data are expressed as means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001.

ANOVA was used to assess the effect of genotype over time on the amplitude of the anaphylactic shock. Time points were sampled at regular intervals to reduce the autocorrelation effect. Both models with and without interactions were tested and yielded similar results (with no interaction effect). Pools of independent biological replicates are shown, as indicated in the legends.

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.⁴⁵⁻⁴⁷

RESULTS

PPARβ/δ loss of function severely compromises AVH and microvessel dilatation in *Pparb/d*^{-/-} mice

To evaluate the effect of PPARβ/δ on the development and maturation of the skin microvasculature, we compared dermal

microvessels in *Pparb/d*^{-/-} and *Pparb/d*^{+/+} control animals. *Pparb/d*^{-/-} and *Pparb/d*^{+/+} animals had a similar dermal microvessel density (Fig 1, A). Next, we examined the dermal microvessel wall architecture, which is expected to comprise a quiescent endothelium lined with a continuous basement membrane covered by quiescent mural cells. Labeling with the Ki67 proliferation marker revealed no proliferating cells in the vessel walls of *Pparb/d*^{-/-} or *Pparb/d*^{+/+} mouse skin, confirming the absence of activated microvessels in the unchallenged tissues (Fig 1, B, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). Labeling with the collagen IV basement membrane marker showed that this layer tightly surrounded the endothelium in a continuous manner in

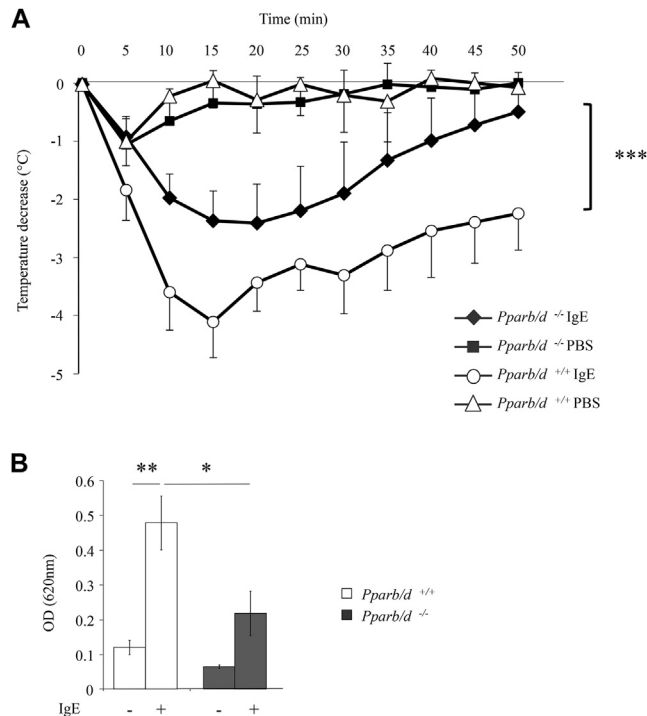


FIG 4. Passive systemic anaphylaxis-induced hypothermia and edema are less severe in *Pparb/d*^{-/-} mice than in *Pparb/d*^{+/+} mice. **A**, Hypothermia in *Pparb/d*^{+/+} or *Pparb/d*^{-/-} mice (pool of 3 experiments). **B**, Edema in the ears of *Pparb/d*^{+/+} and *Pparb/d*^{-/-} mice. Data are expressed as means $\pm$ SEMs (n = 7-10 [Fig 4, A] and n = 3-4 [Fig 4, B] for PBS-injected animals; n = 10-15 [Fig 4, A] and n = 4-6 [Fig 4, B] for IgE-injected animals). **P* < .05, ***P* < .01, and ****P* < .001.

both *Pparb/d*^{-/-} and *Pparb/d*^{+/+} dermis, whereas detection of α SMA, a marker for vascular mural cells, revealed similar microvessel wrapping in *Pparb/d*^{-/-} and *Pparb/d*^{+/+} unchallenged dermis (Fig 1, B). These observations were confirmed in whole-mount staining of dermal vessels harvested from *Pparb/d*^{-/-} and *Pparb/d*^{+/+} mouse skin (data not shown).

To explore the role of PPAR β/δ in the acute increase of permeability and vasodilatation of the microvasculature, we induced an acute response by injecting VEGF-A into *Pparb/d*^{-/-} and *Pparb/d*^{+/+} mouse dermis. We then quantified AVH, as well as basal vessel permeability, with the Miles assay. In the unchallenged dermal microvessels we observed no significant difference at baseline between the *Pparb/d*^{-/-} and *Pparb/d*^{+/+} mice (Fig 1, C). In response to intradermal injection of VEGF-A, however, *Pparb/d*^{+/+} animals exhibited a 2.5- and 5-fold increase in AVH in the ear (data not shown) and the flank dermis (Fig 1, C), respectively, compared with that seen in PBS-injected control animals. In contrast, VEGF-A injections did not significantly stimulate microvessel AVH in the dermis of *Pparb/d*^{-/-} mice. This suggests that VEGF-A-induced dermal AVH is PPAR β/δ dependent (Fig 1, C).

We further determined whether the lack of response of *Pparb/d*^{-/-} dermal vessels was specifically caused by impaired VEGF-A signaling in the *Pparb/d*^{-/-} vessel wall by comparing the AVH response of *Pparb/d*^{-/-} and *Pparb/d*^{+/+} microvessels to the intradermal injection of 2 other well-characterized activators of vascular permeability, thrombin, and histamine. Although thrombin is not involved in anaphylactic shock, it was used as an independent vasoactive inflammatory mediator known to induce hyperpermeability.⁵ Whereas *Pparb/d*^{+/+} dermal

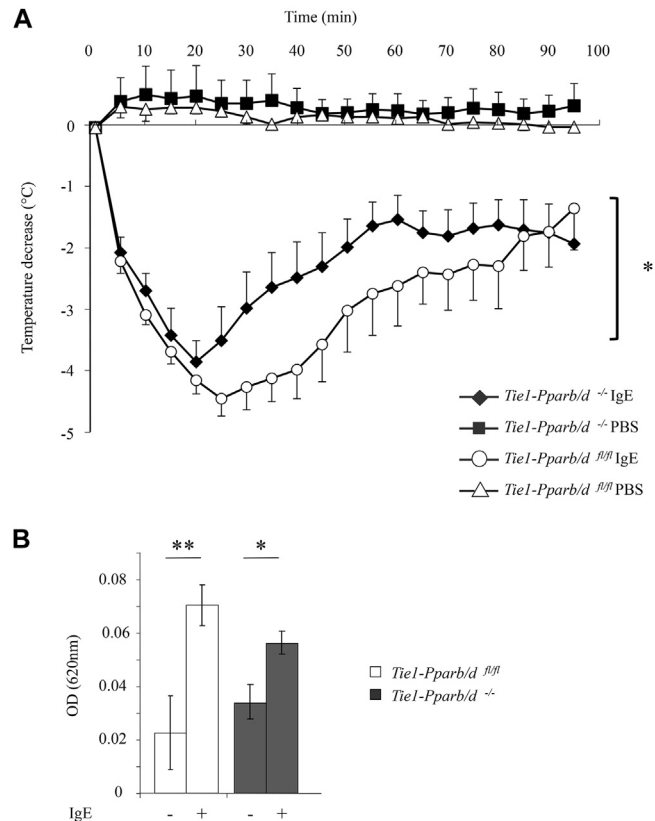


FIG 5. Passive systemic anaphylaxis-induced hypothermia and edema are less severe in *Tie1-Pparb/d*^{-/-} mice than in *Tie1-Pparb/d*^{fl/fl} mice. **A**, Hypothermia in *Tie1-Pparb/d*^{fl/fl} or *Tie1-Pparb/d*^{-/-} mice (pool of 2 experiments). **B**, Edema in the ears of *Tie1-Pparb/d*^{fl/fl} and *Tie1-Pparb/d*^{-/-} mice. Data are expressed as means $\pm$ SEMs (n = 7-9 [Fig 5, A] and n = 3 [Fig 5, B] for PBS-injected animals; n = 10-13 [Fig 5, A] and n = 4 [Fig 5, B] for IgE-injected animals).

microvessels showed a significant increased in AVH in response to the dermal injection of VEGF-A (3-fold), thrombin (2.5-fold), and histamine (3.5-fold), *Pparb/d*^{-/-} dermal microvessels showed no significant AVH in response to any of the 3 vasoactive mediators (Fig 1, D). Although lower expression levels of vascular endothelial growth factor receptor (VEGFR) 1 and 2 in the skin of *Pparb/d*^{-/-} mice might account for a lack of VEGF-A-induced AVH (see Fig E1, C), the absence of histamine- and thrombin-induced AVH shows that the compromised response of the *Pparb/d*^{-/-} microvessels to vasoactive mediators is not limited to VEGF-A and involves more general mechanisms.

VEGF-A is also known to stimulate vasodilatation. Therefore we investigated dermal microvessel enlargement in *Pparb/d*^{-/-} mouse skin. Microvessel vasodilatation was quantified by using computer-assisted morphometry on ear sections of *Pparb/d*^{-/-} and *Pparb/d*^{+/+} mice after intradermal injection of VEGF-A or vehicle (PBS) and labeling of the microvessels with endothelial (CD31) and lymph endothelial (LYVE-1) markers (Fig 1, E). In the PBS-injected dermis no significant difference was observed in the number and size of the *Pparb/d*^{-/-} and *Pparb/d*^{+/+} blood vessels (Fig 1, E, left and middle panels) or lymph vessels (Fig 1, E, left and right panels). On stimulation with VEGF-A, *Pparb/d*^{+/+} mice displayed a significant 2.2-fold increase in blood dermal microvessel size, whereas *Pparb/d*^{-/-} blood dermal microvessels did not enlarge (Fig 1, E, left and middle panels). As

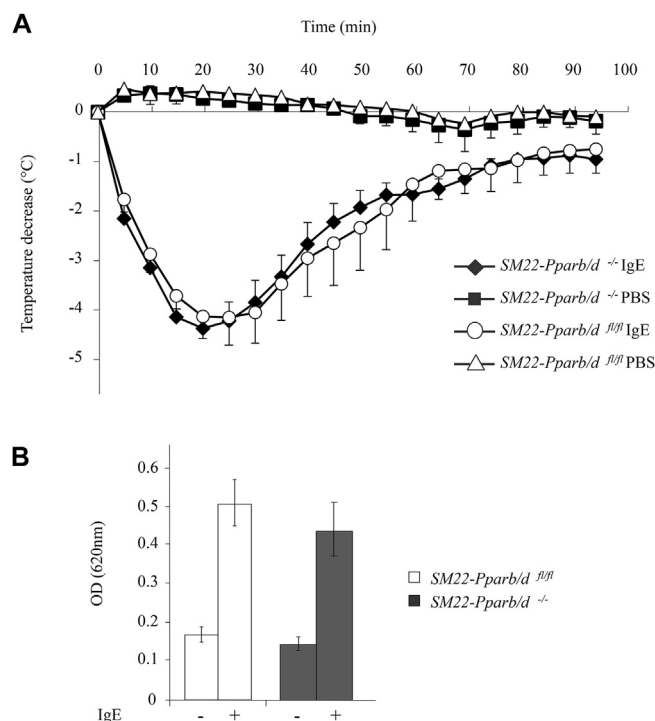


FIG 6. Smooth muscle cell-specific PPAR β/δ deficiency does not affect passive systemic anaphylaxis-induced hypothermia and edema. **A**, Hypothermia in SM22-Pparb/d^{fl/fl} or SM22-Pparb/d^{-/-} mice (pool of 3 experiments). **B**, Ear edema of SM22-Pparb/d^{fl/fl} and SM22-Pparb/d^{-/-} mice. Data are expressed as means $\pm$ SEMs (n = 7 [Fig 6, A] and n = 3 [Fig 6, B] for PBS-injected animals; n = 10 [Fig 6, A] and n = 3 [Fig 6, B] for IgE-injected animals).

expected in response to VEGF-A, lymph vessels, the diameters of which are generally much more variable compared with those of blood vessels, remained unaffected in both Pparb/d^{-/-} and Pparb/d^{+/+} animals (Fig 1, E, left and right panels).

Overall, these data suggest that PPAR β/δ is dispensable for normal dermal vascularization but is required for the acute response of dermal microvessels to vasoactive signals, such as VEGF-A, histamine, and thrombin.

Selective deletion of Pparb/d in endothelial cells is sufficient to compromise AVH and microvessel dilatation in Tiel-Pparb/d^{-/-} mice

Next, we investigated the involvement of endothelial PPAR β/δ in the mediation of AVH and vasodilatation responses to VEGF-A. Targeted deletion of PPAR β/δ in the endothelium was achieved by breeding Tiel-Cre recombinase transgenic mice⁴⁰ with a Pparb/d^{fl/fl} mouse line. Characterization of the Tiel-Cre recombinase transgenic mice showed that Tiel-driven expression of Cre recombinase results in efficient and selective gene excision in endothelial cells.⁴⁰

The appropriate invalidation of the Pparb/d gene was evidenced in the dermis of Tiel-Pparb/d^{-/-} mice but neither in the epidermis of these mice nor in the dermis or epidermis of Tiel-Pparb/d^{fl/fl}, as expected for an endothelium-targeted inactivation (see Fig E2, B, in this article's Online Repository at www.jacionline.org). Furthermore, PPAR β/δ expression was decreased as expected in Tiel-Pparb/d^{-/-} thoracic aortas and total skin, with the remaining PPAR β/δ expression levels being

attributed to the nonendothelial cells of these tissues (see Fig E2, C). The density of dermal microvessels in the unchallenged skin of Tiel-Pparb/d^{-/-} animals was similar to that of the Tiel-Pparb/d^{fl/fl} control animals (Fig 2, A). In addition, the vessel wall was similar in animals of both genotypes, exhibiting quiescent endothelial and mural cell layers (Ki67), continuous lining of the endothelium with basement membrane (collagen IV), and similar wrapping with mural cells (α SMA; Fig 2, B).

Then we compared the acute response of skin dermal microvessels after VEGF-A dermal injection. In vehicle (PBS)-injected flank skin, average basal vascular permeability was similar in Tiel-Pparb/d^{fl/fl} and Tiel-Pparb/d^{-/-} animals (Fig 2, C). In response to an intradermal injection of VEGF-A, the dermal microvessels of Tiel-Pparb/d^{fl/fl} flank skin showed a significant 2-fold increase in AVH, which in contrast was not observed in the vast majority of Tiel-Pparb/d^{-/-} flank skin (Fig 2, C). Furthermore, we observed a 1.8-fold increase in the VEGF-A-stimulated enlargement of dermal blood microvessels in the Tiel-Pparb/d^{fl/fl} dermis but no response in skin from Tiel-Pparb/d^{-/-} mice (Fig 2, D, left and middle panels). Lymph vessels did not show any significant dilatation in either animal (Fig 2, D, left and right panels).

These data show that absence of PPAR β/δ in the vascular endothelium is sufficient to compromise the acute response of microvessels to VEGF-A exposure in Tiel-Pparb/d^{-/-} animals.

PPAR β/δ loss of function compromises the dynamics of the endothelial cell-cell junction and the activity of kinase signaling pathways

Because our data show that endothelial PPAR β/δ expression is a key factor for stimulation of AVH and because VEGF-A, histamine, and thrombin are known to destabilize endothelial cell-cell junctions, thereby stimulating the paracellular permeability route, we examined whether PPAR β/δ might activate AVH by regulating endothelial paracellular permeability. We performed an siRNA-mediated PPAR β/δ knockdown in confluent HUVECs. PPAR β/δ siRNA reduced the PPAR β/δ mRNA level by 75% compared with scrambled control siRNA (see Fig E3, A, in this article's Online Repository at www.jacionline.org). The fact that no increase was observed in the expression of ANGTL4 (angiopoietin-like 4, a well-known PPAR β/δ target gene) in PPAR β/δ siRNA-transfected HUVECs after treatment with a PPAR β/δ agonist demonstrated that the transcriptional activity of PPAR β/δ was inhibited concomitantly with its decreased expression level (see Fig E3, A). Neither the PPAR β/δ agonist treatment nor the siRNA-mediated PPAR β/δ knockdown affected expression levels of adherens junction (AJ) proteins (ie, VE-cadherin and associated α -catenin, β -catenin, and p120-catenin; see Fig E3, B). However, expression of one of the VEGF-A receptors was slightly decreased (see Fig E3, C). Confluent HUVEC monolayers were then stimulated with VEGF-A, followed by VE-cadherin labeling of AJs. In vehicle-treated cells VE-cadherin was localized along the intercellular contacts in control and PPAR β/δ siRNA-treated monolayers, showing that the downregulation of PPAR β/δ expression did not interfere with AJ maturation (Fig 3, A, left panel). In VEGF-A-treated cells computer-assisted analysis of AJs revealed a reorganization of VE-cadherin from a continuous localization along the cell membranes to a diffuse distribution in control siRNA-treated cells (Fig 3, A, left panel, white arrows; quantified in Fig 3, A, right panel) but not in cells transfected with the PPAR β/δ siRNA (Fig 3, A). Importantly, siRNA-mediated inactivation of PPAR β/δ in HUVECs

affected VEGF-A- and histamine-induced phosphorylation of ERK1/2 and Akt (Fig 3, B and C). Taken together, these data demonstrate that PPAR β/δ loss of function compromises VEGF-A-induced dismantling of AJs in confluent HUVEC monolayers through a mechanism that might involve changes in signaling through ERK1/2 and Akt, also observed after histamine stimulation.

Germline and endothelial-specific invalidation of *Pparb/d* attenuates IgE-mediated systemic anaphylaxis in vivo

Next, we investigated whether PPAR β/δ was also able to regulate systemic AVH associated with IgE-dependent passive systemic anaphylaxis, in which several mediators released by mast cells play (eg, histamine, serotonin in rodents, and PAF in human subjects) or might play (VEGF) a role.

An allergic reaction was induced in PPAR β/δ -proficient and deficient animals, and EBD extravasation and hypothermia were evaluated. In sensitized and challenged *Pparb/d*^{+/+}-proficient animals, hypothermia was more pronounced, with a maximal temperature decrease of about 4°C, which was achieved after 15 minutes, whereas *Pparb/d*^{-/-} animals exhibited an average decrease of 2.2°C at that time point and recovered faster (Fig 4, A). Similarly, dye extravasation at the time of death was also 50% lower in the absence of PPAR β/δ (Fig 4, B). Thus these results suggest that PPAR β/δ expression regulates the magnitude of a prototypic allergic reaction.

Because PPAR β/δ is ubiquitously expressed and because the IgE-mediated anaphylactic reaction is primarily dependent on mast cell degranulation, we assessed the effect of PPAR β/δ deficiency *in vitro* by using bone marrow-derived mast cells (BMMCs). BMMCs differentiated normally from PPAR β/δ -deficient hematopoietic progenitors and expressed comparable levels of Fc ϵ RI (data not shown). Furthermore, IgE-induced degranulation was comparable in *Pparb/d*^{+/+} and *Pparb/d*^{-/-} BMMCs (see Fig E1, D, left panel), and the specific PPAR β/δ agonist (GW0742) or antagonist (GSK0660) had no effect on IgE-mediated BMMC degranulation (see Fig E1, D, right panel). This suggests that PPAR β/δ regulates the magnitude of systemic anaphylaxis through the control of vascular permeability.

We further confirmed the key role of endothelial PPAR β/δ expression in this process by inducing IgE-mediated anaphylaxis in *Tie1-Pparb/d*^{fl/fl} and *Tie1-Pparb/d*^{-/-} animals. The specific absence of PPAR β/δ in endothelial cells slightly affected maximal hypothermia (about 3.5°C in PPAR β/δ -deficient mice and 4°C in PPAR β/δ -proficient mice) and led to a faster recovery (Fig 5, A) and a significant 50% lower peripheral plasma extravasation (1.7-fold increase in PPAR β/δ -deficient and 3-fold increase in PPAR β/δ -proficient mice; Fig 5, B). In line with this, the *Tie1-Pparb/d*^{-/-} animals also recovered faster from histamine-induced hypothermia (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, smooth muscle cell (SMC) PPAR β/δ did not appear to play a regulatory role in this pathophysiologic context because smooth muscle cell-specific PPAR β/δ deletion in *SM22-Pparb/d*^{-/-} animals led to a similar IgE-mediated anaphylaxis as wild-type mice measured by the induced hypothermia and plasma extravasation reactions (Fig 6, A and B, and see Fig E2, D-F). This suggests that PPAR β/δ regulated IgE-mediated anaphylaxis through its expression on endothelial cells at least in part through control of their sensitivity/response to histamine.

DISCUSSION

PPAR β/δ is an important regulator of endothelial and smooth muscle cell activities. PPAR β/δ supports angiogenesis in tumor growth and ischemia recovery,⁴⁸⁻⁵¹ it displays anti-inflammatory and antiatherogenic properties in endothelial cells and VSMCs,^{36,48,52-57} and PPAR β/δ agonists were reported to be vasorelaxing compounds^{58,59} able to reduce blood pressure and heart rate in spontaneously hypertensive rats.⁶⁰

Despite this body of evidence in several pathophysiologic conditions, the involvement of PPAR β/δ in vascular responses to the acute release of vasoactive mediators occurring in patients with inflammation and allergies has not attracted any attention to date. Our work provides evidence that endothelial PPAR β/δ expression promotes local acute vasodilatation and AVH in response to allergic and inflammatory mediators. When exposed to such vasoactive mediators, the endothelium responds by increasing microvessel permeability to fluid, which provokes edema, and by releasing vasorelaxing agents, which relax VSMCs and pericytes.^{7,8} In this article we have shown that endothelium-specific PPAR β/δ loss of function plays a protective role by reducing acute local vasodilatation and edema in response to VEGF-A and histamine. This was not due to lower expression of VEGF or histamine receptors, which were expressed at similar or even higher levels in the vena cavae of PPAR β/δ -deficient animals (see Fig E1, B). Although the exact mechanisms remain to be unveiled, the molecular basis underlying PPAR β/δ -dependent regulation of AVH likely involves the ERK1/2 and Akt signaling pathways and the destabilization of endothelial cell-cell AJs. The ERK1/2 and Akt signaling pathways are 2 of the multiple pathways involved in regulation of endothelial permeability and of VE-cadherin-dependent cell-cell adhesion.^{3,61-65} The remodeling of VE-cadherin is affected by most, if not all, permeabilizing factors^{1,3,62} and correlates with reduced endothelial barrier integrity.

Interestingly, we show that PPAR β/δ siRNA-mediated loss of function compromised the VEGF-A-induced destabilization of AJs. Moreover, PPAR β/δ deficiency exacerbated VEGF-A-dependent and prevented histamine-dependent activation of ERK1/2 and Akt in human endothelial cell monolayers. Although an increase in ERK1/2 and Akt phosphorylation usually correlates with an increase in vessel permeability and VE-cadherin dynamics, exacerbated activation of these pathways after PPAR β/δ loss of function is associated with compromised AVH and VE-cadherin reorganization. It is likely that after stimulation by VEGF-A, factors downstream of ERK1/2 and Akt are affected by PPAR β/δ deficiency, thereby preventing these signaling pathways from activating VE-cadherin dynamics and, in turn, AVH. One likely candidate is the kinase cSrc, a direct PPAR β/δ target gene³³ known to be a major actor involved in VE-cadherin phosphorylation and AVH.⁶⁶⁻⁶⁹ Alternatively, actin cytoskeletal dynamics, which are involved in VEGF-induced permeability,⁶² are known to be compromised in the absence of PPAR β/δ .^{34,42} Therefore compromised cSrc expression, actin cytoskeleton dynamics, or both in PPAR β/δ loss-of-function conditions might prevent VEGF-A-induced AVH, despite activation of ERK and Akt signaling cascades.

The exact molecular mechanism of endothelial PPAR β/δ -dependent vasodilatation remains to be explored. The best-characterized vasorelaxing signals produced by endothelial cells are nitric oxide (NO) and prostacyclin, a ligand for

PPAR β/δ .^{6,70} It is to be noted that PPAR β/δ agonists were shown to indirectly restore NO bioavailability in vessels affected by chronic endothelial dysfunctions.⁷¹⁻⁷⁴ Therefore a possibility is that endothelial PPAR β/δ regulates acute vasodilatation through regulation of NO and/or prostacyclin release by endothelial cells. Importantly, the prevention of acute edema by endothelial PPAR β/δ loss of function is extended to the systemic level because anaphylaxis-associated peripheral edema is reduced in *Pparb/d*^{-/-} and endothelial *Pparb/d*^{-/-} mice. The absence of PPAR β/δ from VSMCs had no consequence on anaphylaxis and associated edema, which supports a major role of endothelial PPAR β/δ in the vascular response to this acute systemic allergic reaction.

Thus our results suggest that silencing of endothelial PPAR β/δ might have a therapeutic benefit by reducing the magnitude of edema and vasodilatation. Surprisingly, although AVH and vasodilatation are key events in allergic and other inflammatory reactions, developing therapeutic strategies targeting the vasculature remains uncommon. Yet recent advances suggest that interfering with vascular remodeling, permeability, and/or enlargement represents a promising strategy. Targeting the vasculature reduced the symptoms of experimental models of contact dermatitis,⁷⁵⁻⁷⁷ inflammatory bowel disease,⁷⁸ and Dengue and LPS-induced shock syndromes.⁷⁹ Moreover, stabilizing endothelial junctions reduced TNF- α -induced AVH,⁸⁰ and reducing microvascular hyperpermeability was shown to attenuate lung injury.⁵ Although pharmacologic activation of PPAR β/δ was reported to be of interest in the context of hypertension, diabetes, and stroke-induced vascular and neuronal damage, our data now suggest that despite potential side effects, which are to be monitored carefully, antagonizing PPAR β/δ might provide a new therapeutic strategy to moderate acute diseases with altered endothelial barrier integrity, such as acute and/or severe allergic reactions and acute inflammatory disorders, in which normal organ perfusion must be restored.^{2,81}

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Key messages

- The absence of PPAR β/δ in endothelial cells in mice severely compromises AVH and the dilatation of dermal microvessels in response to vasoactive agents.
- Endothelial, but not smooth muscle, cell PPAR β/δ is a key regulator of passive systemic anaphylaxis-induced hypothermia and edema.
- Modulation of PPAR β/δ activity is a novel and important strategy to consider for moderating diseases with altered endothelial integrity, such as acute inflammatory and allergic disorders.

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METHODS

Histamine-induced hypothermia

Tie1-PPAR β / δ ^{fl/fl} and *Tie1-PPAR β / δ ^{-/-}* female mice (8–12 weeks old) were injected with 25 mg/mL histamine (D 8406, Sigma) or PBS (200 μ L per animal) intravenously (tail vein). Hypothermia was monitored by using an electronic thermometer with a rectal probe.

Epidermal and dermal cell isolation

Full-thickness dorsal skin was removed and washed in 70% ethanol and sterile PBS. Narrow strips (2 mm) were cut and incubated in 1 mL of ammonium thiocyanate (3.8% in PBS) at room temperature for 20 minutes. The dermis was scraped with a scalpel blade. Epidermal and dermal cells were further used for genomic DNA or total RNA extraction.

Mast cell degranulation

BMMCs were prepared from PPAR β / δ -proficient and deficient mice by using 10 ng/mL stem cell factor (250-03, PeproTech) and 10 ng/mL IL-3 (213-13, PeproTech). Purity exceeded 95% after 6 weeks of culture. IgE-mediated BMMC degranulation was assessed by measuring β -hexosaminidase release, as described previously, on activation with 1 μ g/mL anti-TNP IgE (557079, BD PharMingen), followed by 0.01 to 1000 ng/mL ranging concentrations of TNP-OVA (T-5051-100; Biosearch Technologies, Novato, Calif), as indicated in Figs E1 and E2.^{E1,E2} The PPAR β / δ agonist GW0742 (100 nmol/L, 10006798; Cayman Europe) and/or antagonist GSK0660 (1 μ mol/L, AG G5797; Sigma-Aldrich) were added to the culture medium 24 hours before the degranulation was initiated.

Reverse transcription and real-time PCR

One microgram of total TRIZOL-extracted RNA was reverse transcribed with random hexamer primers by using SuperScript II Reverse Transcriptase (18064071, Life Technologies). Real-time PCR was performed with SYBR Green PCR Master Mix (04913914001; Roche Diagnostics, Mannheim, Germany) by using an Agilent Technologies Stratagene MX3000P PCR machine (40 cycles). Primers (detailed below) were designed to generate PCR amplification products of 100 to 200 bp. Expression was related to the following housekeeping genes: ribosomal protein L-27 (*RPL27*), eucaryotic elongation factor 1 α (*EEF1A*), *GAPDH*, and peptidylprolyl isomerase cyclophilin, the expression of which did not vary under the experimental conditions

being studied. qBase and JaRT (P. Meylan, 2010; proprietary software based on Hellemans et al^{E3}) software were used to analyze the data.

Human primers

Human primers were as follows: *GAPDH*, forward CATCCATGA CAACCTTTGGTATCGT, reverse CCATCACGCCACAGTTTCC; *RPL27*, forward TGTCTCTGGCTGGACGCTACT, reverse CTGAGGTGCCATCATC AATGTT; *PPAR β / δ* , forward CGGCAGCCTCAACATGG, reverse AGATCCGATCGCACTTCTCATAC. VE-cadherin, α -catenin, β -Catenin, p120-Catenin, and ANGPTL4 were all purchased from Qiagen (Hilden, Germany; sequence not provided).

Mouse primers

Mouse primers were as follows: *RPL27*, forward TCATGCCCAAGG-TACTCTGT, reverse CTGGCCTTGCCTTCAA; *EEF1A*, forward CCTGGCAAGCCCATGTGT, reverse TCATGTCACGAACAGCAAAGC; cyclophilin, forward GGCCAACGATAAGAAGAAGG, reverse ACAAAT-TATCCACTGTTTTTGGGAACA; *PPAR β / δ* , forward CGGCAGCCTCA ACATGG, reverse AGATCCGATCGCACTTCTCATAC. *Pparb/d^{fl/fl}* floxed allele and excised allele genotyping was as follows: forward GCAGCTGCT-CAGCTGCCTGC. *Pparb/d^{-/-}* invalidated allele genotyping was as follows: reverse ATGCCGAGTGCCAGGCACTTCTGGAAG. *Pparb/d^{fl/fl}* floxed allele genotyping was as follows: reverse GGACCCCGTAGTGGAAGCCCGAGGCC. *Cre* recombinase was as follows: forward AGGTGTAGAGAAGGCACTTAGC, reverse CTAATCGCCATCTTC CAGCAGG. *Glut2* was as follows: forward CCAATCCCTTGGTTCATGGTTGC, reverse CGTAAGGCCCAAGGAAGTC CTGC. Qiagen QuantiTect primers were as follows: VEGFR1 (Flt1 QT00096292); VEGFR2 (Qiagen QuantiTect KDR QT00097020); NRP-1 (Qiagen QuantiTect QT0015); H2 (Qiagen QuantiTect QT01039157). Unless mentioned otherwise, primers were purchased from Eurofins.

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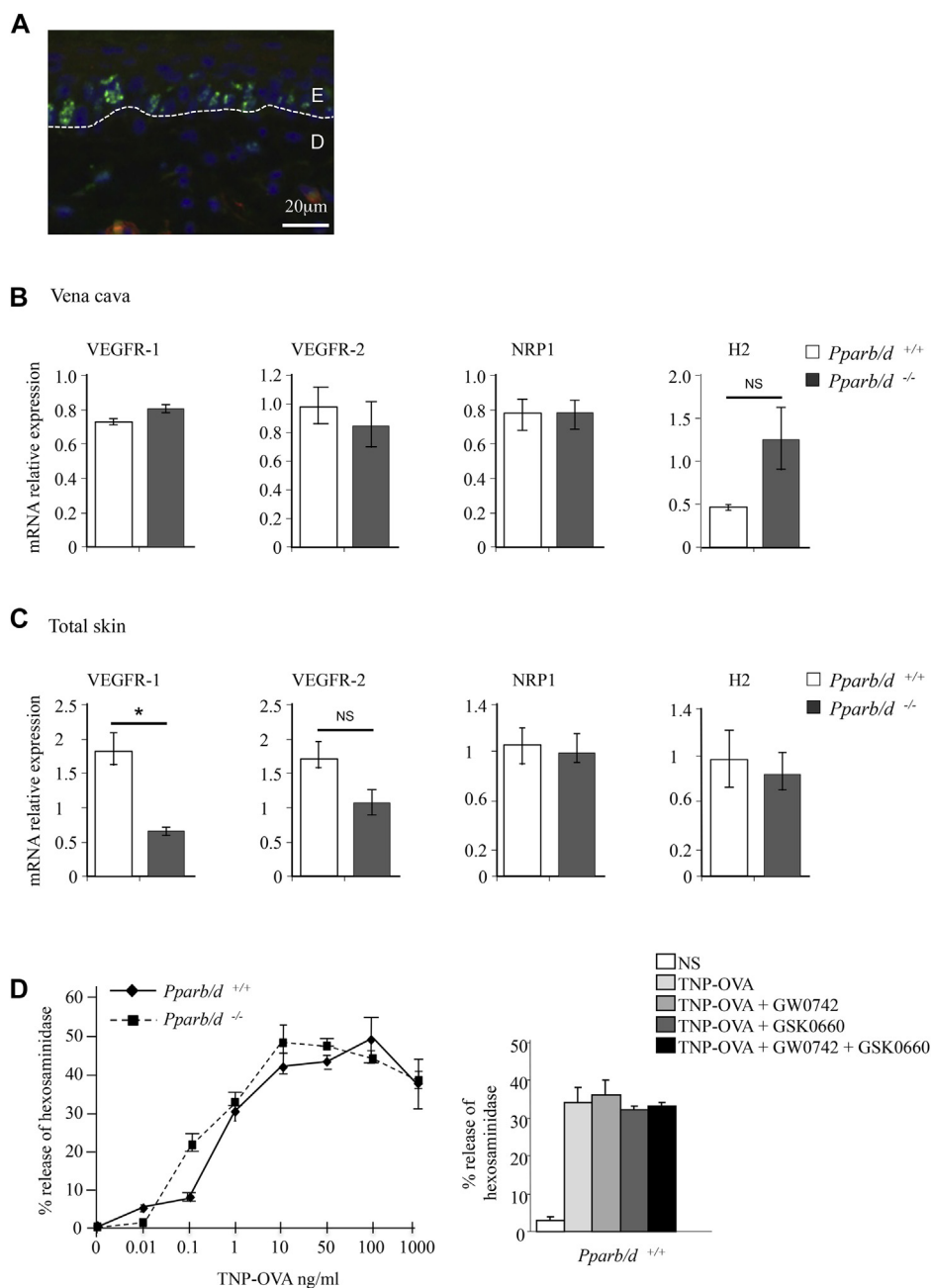


FIG E1. A, Proliferation marker positive control. *D*, Dermis; *E*, epidermis. Green, Ki67. **B and C,** Expression levels of VEGF (VEGFR-1, VEGFR-2, and neuropilin [*Nrp-1*]) and histamine (*H2*) receptors in vena cava (Fig E1, *B*) and total skin (Fig E1, *C*). Data are expressed as means $\pm$ SDs (Fig E1, *B*, $n = 3$; Fig E1, *C*, $n = 6$). **D,** BMMC IgE-induced degranulation. *Right*, TNP-OVA (20 ng/mL). Values are expressed as a percentage of total release $\pm$ SD. * $P < .05$. NS, Not significant.

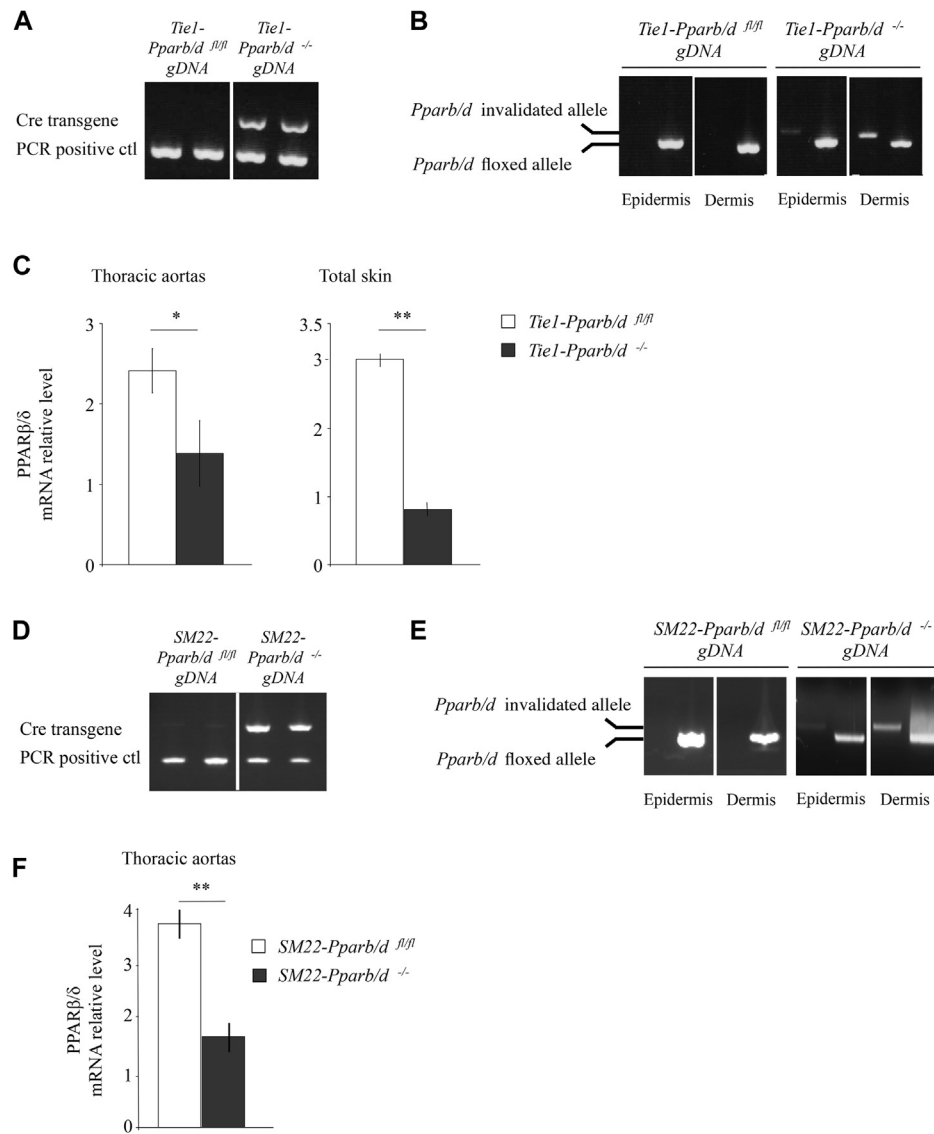


FIG E2. Characterization of *Tie1-Pparb/d* and *SM22-Pparb/d* mice. **A** and **D**, PCR detection of the *Cre* recombinase transgene in genomic DNA (*gDNA*). The PCR-positive control was the *Glut2* gene. **B** and **E**, PCR detection of *Pparb/d* invalidated and floxed/proficient alleles in genomic DNA extracted from the epidermis or dermis. **C** and **F**, *PPAR β/δ* mRNA expression levels in thoracic aortas and total skin ($n = 3$). * $P < .05$ and ** $P < .01$.

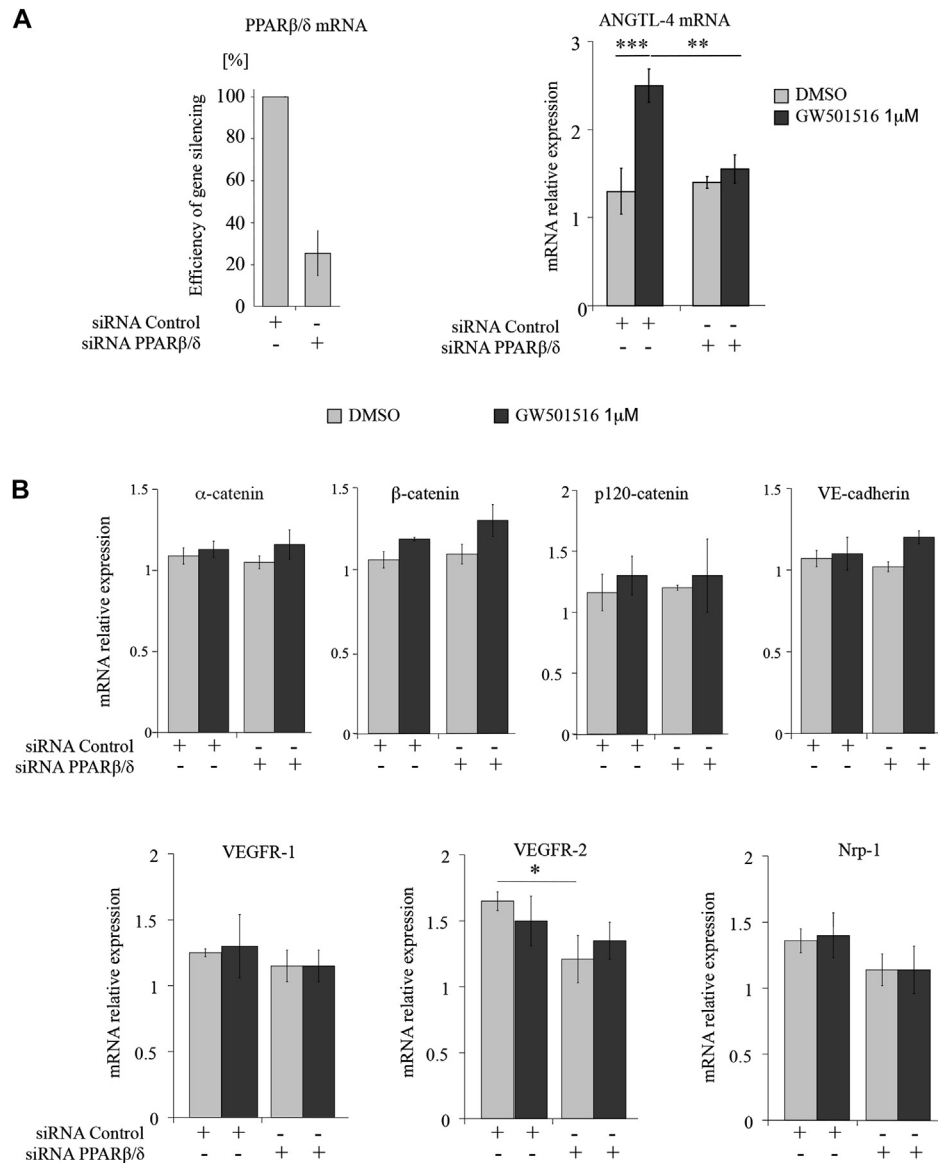


FIG E3. A, siRNA-mediated PPAR β/δ silencing in HUVECs. Expression levels of PPAR β/δ (left) and its target gene, *ANGTL4* (right), in HUVECs. *GW501516*, PPAR β/δ agonist. **B,** Expression levels of AJ proteins (α -catenin, β -catenin, p120-catenin, and VE-cadherin) and VEGF receptors (VEGFR-1, VEGFR-2, and the coreceptor neuropilin [*Nrp-1*]) in HUVECs. Data are expressed as means $\pm$ SDs (n = 3). *DMSO*, Dimethyl sulfoxide. **P* < .05, ***P* < .01, and ****P* < .001.

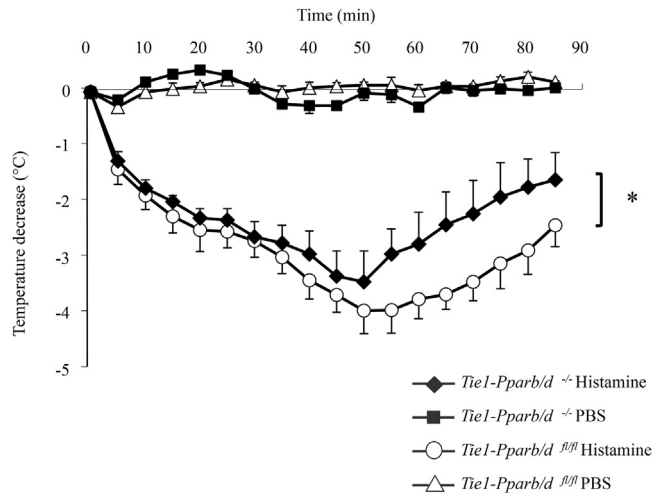


FIG E4. Histamine-induced hypothermia in *Tie1-Pparb/d*^{-/-} compared with *Tie1-Pparb/d*^{fl/fl} mice. Data are expressed as means $\pm$ SEMs ($n = 4$ for PBS-injected animals and $n = 5-7$ for histamine-injected animals). * $P < .05$.