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Purpose. Although endothelium-dependent nitric oxide (NO)–mediated dilation of retinal arterioles has been well described, the role of endothelium-derived hyperpolarizing factor (EDHF) in the retinal arteriolar response remains unclear. In the current study, we examined the contribution of EDHF to the retinal arteriolar dilation to the inflammatory agent histamine and investigated the signaling mechanisms underlying this vasomotor activity.

Methods. Porcine retinal arterioles were isolated, cannulated, and pressurized without flow for functional study by using video microscopic techniques. The immunohistochemical staining was performed to determine histamine receptor subtypes.

Results. Histamine (0.1–30 μM) produced concentration-dependent dilation of retinal arterioles in a manner sensitive to H1- and H2-receptor antagonists chlorpheniramine and famotidine, respectively. Histamine-induced vasodilation was almost abolished after endothelial removal. In the intact vessels, vasodilation to histamine was partially inhibited by the inhibitors of cyclooxygenase (indomethacin), NO synthase (N^G-nitro-L-arginine methyl ester, L-NAME), or Ca^{2+} -activated K^+ (K_{Ca}) channels (apamin plus charybdotoxin). Combination of the above inhibitors abolished histamine-induced vasodilation. Residual vasodilation in the presence of indomethacin and L-NAME was further reduced by the cytochrome P450 enzyme inhibitor sulfaphenazole but not by the gap junction inhibitor carbenoxolone or the hydrogen peroxide scavenger catalase. Immunohistochemical signals for H1- and H2-receptor expression were found only in the endothelium.

Conclusions. The endothelium plays an essential role in the dilation of porcine retinal arterioles to histamine via H1- and H2-receptor activation. The EDHF derived from cytochrome P450 contributed in part to this vasodilation via K_{Ca} channel activation, in addition to the endothelial release of NO and prostanoids.

Keywords: histamine, EDHF, retinal blood flow, endothelium, isolated porcine retinal arterioles

The vascular endothelium consists of a monolayer of cells lining the inner wall of the vasculature. It plays a critical role in many homeostatic processes, including the trafficking of nutrients and immune cells between blood and underlying tissue, maintenance of blood fluidity, regulation of permeability, formation of new vasculature, and the control of vascular tone for blood flow regulation.[1] The retinal blood flow is regulated by the activity of smooth muscle cells (i.e., contraction or relaxation) in small resistance arterioles. This vasomotor activity can be governed by the release of vasoactive factors from the endothelium.[2] The release of endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO) and prostacyclin (PGI_2) in response to various neurohumoral and shear stress stimulations[3] has been well described in different organs and tissues,[4] including the retina.[5] However, some of the endothelium-dependent vasodilations are resistant to the blockade of NO synthase and cyclooxygenase but sensitive to agents that prevent membrane hyperpolarization.[6,7] Therefore, the existence of an additional pathway that involves the release of endothelium-derived hyperpolarizing factor (EDHF) has been suggested.[8,9]

The endothelial hyperpolarization initiated by the activation of Ca^{2+}-activated K^+ (K_{Ca}) channels[9] or gap junctions[10] has been suggested to play a critical role in mediating EDHF-induced vasodilatory responses. It has been shown that an EDHF-induced vasodilation could be prevented by a combined administration of apamin and charybdotoxin (ChTX), which selectively inhibit small- and intermediate/large-conductance K_{Ca} channels, respectively, in a number of vascular beds.[11] Although the chemical nature of EDHF and its signaling pathways are not fully understood, hydrogen peroxide (H_2O_2) and epoxyeicostrienoic acids (EETs), which are synthesized by superoxide dismutase and cytochrome P450 enzyme CYP2C9, respectively, have been suggested to be involved in the

signaling of EDHF in some vascular beds.[9] While previous studies have reported that EDHF is involved in agonist-induced relaxation of rat mesenteric arterioles,[12] the direct role of EDHF in regulation of retinal arteriolar tone remains unknown.[13,14]

Histamine is known to be an important mediator for inflammatory reactions[15] exerted by four different subtypes of histamine receptors, namely, H1, H2, H3, and H4.[16] Histamine is present in the retina,[17] and the enzymes responsible for its formation and inactivation also have been found in the retinal tissue.[18] Histamine causes dilation of bovine large retinal arteries[19] and increases retinal blood flow in rats[20] and humans.[21–24] Plasma histamine level has been found to increase by 5-fold in the patients with diabetes.[25] It is likely that the increased histamine may contribute to the regulation of blood flow, activation of immune and inflammation systems, and pathogenesis of diabetic complication and retinopathy. However, the histamine-induced vasomotor activity in retinal arterioles that control blood flow and its distribution has not been characterized. Although EDHF has been suggested to play a considerable role in mesenteric vasodilation to histamine,[26] the importance of this dilation in retinal arterioles remains undetermined. Herein, we examined the contribution of EDHF to retinal arteriolar dilation to histamine and investigated the signaling mechanisms involved in this vasomotor activity.

Materials and Methods

Animal Preparation

The Animal Care Committee of Asahikawa Medical University approved all animal procedures, which were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. After the pigs (age, 16–24 weeks either sex; weight, 25–35 kg) were killed in a local slaughterhouse, one of the eyes was enucleated immediately and transported to the laboratory in a moist chamber on ice. In each eyeball, only one retinal arteriole was isolated and a total of 75 pigs (i.e., vessels) were used for this study.

Isolation and Cannulation of Microvessels

The techniques for visualization, identification, isolation, cannulation, and pressurization of the retinal arterioles have been described previously.[27–30] Briefly, the isolated retinal arterioles (90–110 μm in situ) were cannulated with a pair of glass micropipettes and pressurized to 55 cm H_2O intraluminal pressure without flow by using two independent pressure reservoir systems.[31] The internal diameter of the isolated vessels was recorded continuously by using videomicroscopic techniques throughout the experiments.[27]

Control Experiment

The cannulated and pressurized arterioles were bathed in the physiologic saline solution (PSS; 36°C–37°C) containing albumin (0.1%). After the development of stable basal tone (~30–40 minutes), the concentration-dependent response of retinal arterioles to histamine (0.1–30 μM) was examined. The vessels were exposed to each concentration of histamine until a stable diameter was established (~3 minutes). After completing the concentration-dependent response, the vessels were washed with PSS and allowed to reestablish the basal tone (~30–40 minutes). The reproducibility of the vascular response to histamine was then examined in the same vessels ($n = 6$).

Role of Histamine Receptors and the Endothelium in Histamine-Induced Dilation

To determine the involvement of histamine receptor subtypes, the vessels were pretreated with respective H1, H2, H3, or H4 histamine receptor antagonist chlorpheniramine (H1; 1 μM),[32] famotidine (H2; 10 μM),[33] and thioperamide (H3/H4; 1 μM),[34] for 30 minutes, and the concentration-dependent responses to histamine were reexamined. In another set of experiments, the endothelium of the isolated arterioles was disrupted by intraluminal perfusion of the nonionic detergent CHAPS (0.4%) for 1 to 2 minutes[35] and the vascular response to histamine was reevaluated. To ensure that the vascular smooth muscle function was uncompromised by CHAPS treatment during denudation, the concentration-dependent dilation of the vessel in response to the endothelium-independent vasodilator sodium nitroprusside (SNP; 0.1–100 μM) before and after denudation was examined. Only vessels that exhibited normal basal tone, showed no vasodilation in response to endothelium-dependent vasodilator bradykinin (10 nM),[35] and maintained unaltered vasodilation in response to SNP after removal of the endothelium were accepted for further study.

Role of EDRF and K_{Ca} in Retinal Arteriolar Dilation

We assessed the involvement of PGI_2, NO, and K_{Ca} in mediating the vascular response to histamine by using the known effective concentration of the specific inhibitors of cyclooxygenase (indomethacin, 10 μM),[28,36] NO synthase (L-NAME, 10 μM),[27,28] and K_{Ca} channels (combination of apamin 0.1 μM and ChTX 0.1 μM),[37–39] respectively.

Chemical Nature of EDHF and the Role of Gap Junctions

Since H_2O_2 and the CYP enzyme metabolites EETs have been suggested to be involved in the EDHF-induced vasodilation in some vascular beds,[9] we tested this possibility by pretreating the vessels with the effective concentration of H_2O_2 scavenger catalase (1000 U/mL)[40,41] and the CYP2C9 enzyme inhibitor sulfaphenazole (10 μM),[42] respectively. The role of gap junctions in histamine-induced responses was tested by the gap junction inhibitor carbenoxolone (100 μM).[43] In these studies, the cyclooxygenase inhibitor (indomethacin, 10 μM) and NO synthase inhibitor (L-NAME, 10 μM) were present throughout the experiment to eliminate the confounding effects from prostaglandins and NO.[44]

Response to Sodium Nitroprusside

The smooth muscle relaxing agent SNP (0.1–100 μM) was used to probe endothelium-independent vasodilation. The vascular response to SNP was examined in the presence of various pharmacologic antagonists, as mentioned previously.

All drugs were administered extraluminally unless otherwise stated. The vessels were incubated with each pharmacologic inhibitor for a minimum of 30 minutes.

Immunohistochemistry

Immunohistochemical detection of vascular histamine receptors was performed after preparation of cryomicrotome sections of the retinal arterioles as described previously.[30] The following specific primary antibodies were used: anti-H1 receptor antibody (1:100; Aviva Systems Biology, San Diego, CA, USA), anti-H2 receptor antibody (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-endothelial NO

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synthase (eNOS) antibody (1:100; Santa Cruz Biotechnology), and the anti-α-smooth muscle actin antibody (1:100; Sigma-Aldrich Corp., St. Louis, MO, USA). The slides then were incubated with fluorescein isothiocyanate (FITC)-conjugated antibody (1:100; Santa Cruz Biotechnology), Alexa Fluor 647-conjugated antibody (1:200; Abcam Ltd., Cambridge, UK), and Cy3-conjugated antibody (1:100; GE Healthcare Life Sciences, Piscataway, NJ, USA), observed for green (FITC) and red (Alexa Fluor 647 and Cy3) staining, and analyzed with a confocal microscope (Fluoview FV 1000, Olympus, Tokyo, Japan). Merged images were created by using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Chemicals

All drugs were purchased from Sigma-Aldrich Corp. Famotidine was dissolved in dimethyl sulfoxide (DMSO). Indomethacin and sulfaphenazole were dissolved in ethanol; other drugs were dissolved in PSS. All subsequent dilutions of these drugs were prepared in PSS. The final concentrations of DMSO and ethanol in the vessel bath were less than 0.1%. Vehicle-controlled studies indicated that these final solvent concentrations did not affect the arteriolar diameter.[28]

Data Analysis

At the end of each experiment, the vessels were relaxed in ethylenediaminetetraacetic acid (1 mM) calcium-free PSS to obtain its maximal diameter at 55 cm H_2O intraluminal pressure.[27] The diameter changes in response to histamine and SNP were normalized to this maximal vasodilation and expressed as percentage maximal dilation.[27] Data are reported as the mean ± standard error of the mean (SEM); n represents the number of vessels studied. Statistical comparisons of the changes in resting tone caused by antagonists were performed by using the paired t-test. Statistical comparisons of vasomotor responses to the same agonist under various treatments were performed by using 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni multiple-range test when appropriate. One-way ANOVA followed by Dunnett's post hoc comparison was used to determine the significance of changes in the baseline diameter in response to different concentrations of agonists.

Results

Dilation of Retinal Arterioles to Histamine

In this study, all vessels ($n = 75$) developed a similar level of basal tone (i.e., constricted to 61% ± 1% of maximal diameter). The average resting and maximal vessel diameters were 62 ± 1 and 102 ± 1 μm, respectively. Histamine-induced concentration-dependent dilation of the retinal arterioles and the highest concentration (30 μM) elicited approximately 85% of maximal dilation (Fig. 1). The histamine-induced dilation was reproducible and did not deteriorate at ~30 to 40 minutes after the first application (Fig. 1).

Role of Endothelium

In this series of studies, 10 vessels were subjected to the denudation protocol. After perfusion with CHAPS, 3 of the 10 vessels lost basal tone and two showed partial inhibition by the endothelium-dependent vasodilator bradykinin. These apparently damaged or partially denuded vessels were excluded from further study. The remaining five vessels maintained basal tone (control 64% ± 2% versus denudation 64% ± 2%; $P = 0.18$) and the vasodilation induced by bradykinin (10 nM) was abolished (control 83% ± 2% versus denudation: 1% ± 1%). In addition, these vessels exhibited normal vasodilation in response to SNP (Table). In these five denuded vessels, the dilation in response to the lower concentrations of histamine (0.1–10 μM) was almost abolished, and the response to the highest histamine concentration (30 μM) was reduced from 87% to 6% ($P < 0.001$, Fig. 2).

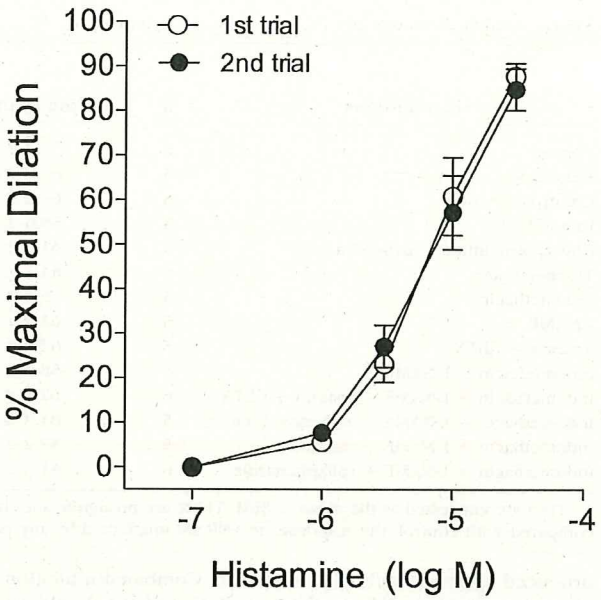

FIGURE 1. Response of isolated retinal arterioles to histamine. There was no significant difference between the two trials ($n = 6$).

Role of Histamine Receptors

Blockage of the H1 receptor by chlorpheniramine significantly reduced histamine-induced vasodilation (Fig. 3). The H2-receptor antagonist famotidine exhibited a stronger inhibitory effect than chlorpheniramine on the histamine-induced vasodilation. Combined administration of chlorpheniramine and famotidine abolished histamine-induced vasodilation (Fig. 2). On the other hand, H3- and H4-receptor antagonist thioperamide did not have any effect on the vasodilator response to histamine. These histamine receptor antagonists did not alter the basal tone of retinal arterioles.

Localization of the Histamine Receptor in the Retinal Arterioles

Immunohistologic stainings showed that the endothelium of retinal arterioles expresses eNOS and both histamine H1 and H2 receptors (Figs. 4A, 4B). The H1 and H2 receptors were not detectable in the vascular smooth muscle cells (Figs. 4C, 4D). No staining signals were detected in the control experiments in which the specific histamine receptor antibodies were omitted (data not shown).

Role of EDRFs and K_{Ca} Channels

Indomethacin, L-NAME, and K_{Ca} channel inhibitors (apamin plus ChTX) partially inhibited the maximal histamine-induced vasodilation from 89% to 42%, 38%, and 66%, respectively (Fig. 5). Indomethacin and L-NAME exhibited equal inhibition (~50%) on vasodilation to histamine and ChTX plus apamin

TABLE. Resting Diameters and Dilation of Retinal Arterioles to SNP

Interventions	n	Resting Diameter, μm	% Maximum Dilation			
			0.1 μM	1 μM	10 μM	100 μM
Control	6	62.7 ± 2.9	6.3 ± 0.9	29.4 ± 3.3	60.9 ± 3.6	83.0 ± 3.0
Denudation	5	65.0 ± 1.7	5.7 ± 0.4	23.8 ± 1.1	57.6 ± 2.0	85.9 ± 1.2
Chlorpheniramine	6	69.2 ± 4.5	4.8 ± 0.5	27.6 ± 2.5	53.2 ± 1.9	84.2 ± 1.5
Famotidine	5	58.6 ± 4.0	6.6 ± 1.0	31.5 ± 2.4	55.7 ± 3.0	86.3 ± 2.3
Chlorpheniramine + famotidine	5	61.0 ± 4.5	7.0 ± 1.1	32.3 ± 2.9	55.6 ± 3.9	86.7 ± 2.9
Thioperamide	5	64.4 ± 3.3	6.3 ± 0.6	29.1 ± 6.7	59.6 ± 5.7	83.4 ± 1.9
Indomethacin	5	66.0 ± 2.4	6.6 ± 0.7	30.0 ± 6.2	60.4 ± 4.9	85.7 ± 3.9
L-NAME	5	61.8 ± 3.2	6.3 ± 1.7	32.2 ± 1.1	68.5 ± 3.2	88.2 ± 1.5
Apamin + ChTX	5	62.0 ± 3.0	5.9 ± 0.8	26.8 ± 3.5	65.1 ± 5.1	88.2 ± 2.8
Indomethacin + L-NAME	6	58.8 ± 3.8	5.8 ± 0.9	27.1 ± 3.3	61.6 ± 3.6	85.2 ± 3.6
Indomethacin + L-NAME + apamin + ChTX	6	62.5 ± 3.1	6.1 ± 0.6	26.0 ± 3.1	65.7 ± 3.9	88.9 ± 2.5
Indomethacin + L-NAME + carbenoxolone	5	61.8 ± 1.8	5.2 ± 0.5	24.3 ± 4.2	63.3 ± 2.9	85.7 ± 2.4
Indomethacin + L-NAME + catalase	5	57.2 ± 3.0	4.2 ± 0.6	22.7 ± 2.4	59.5 ± 2.3	83.7 ± 3.2
Indomethacin + L-NAME + sulfaphenazole	6	61.3 ± 2.1	5.3 ± 0.6	22.3 ± 1.8	59.2 ± 2.5	85.0 ± 2.4

Data are expressed as the mean ± SEM. There are no significant changes in the resting diameters. Based on 2-way repeated measures ANOVA, compared with control, the responses to SNP are unaffected by any perturbations.

produced approximately 30% inhibition. Combined inhibition of cyclooxygenase, NO synthase, and K_{Ca} channels almost completely blocked the histamine-induced relaxation (Fig. 5). These pharmacologic inhibitors did not significantly alter resting vascular tone.

Role of EDHF and Gap Junction

The residual vasodilation to histamine in the presence of indomethacin and L-NAME was further reduced after treating the vessels with CYP450 enzyme inhibitor sulfaphenazole but not with the H_2O_2 scavenger catalase or the gap junction inhibitor carbenoxolone (Fig. 6).

Response to SNP

The resting diameters and dilations of retinal arterioles to SNP in the absence of endothelial cells (i.e., denudation) and in the presence of various pharmacologic inhibitors were not significantly altered (Table).

DISCUSSION

The present study is the first to show that the endothelium plays an essential role in the dilation of porcine retinal arterioles to histamine via H1- and H2-receptor activation. In addition, the endothelium-derived vasodilators prostanoid and NO partially contributed to the retinal vasodilatation induced by histamine. The CYP450 enzyme metabolites, which act as EDHF, also contributed to histamine-induced vasodilation via K_{Ca} channel activation.

In isolated bovine retinal arteries, histamine at a concentration of 1 mM elicits 60% vasodilation.[19] On the other hand, intravitreal administration of 1 mM histamine induces approximately 10% vasodilation in rat retinal arteries, but the actual concentration of histamine acting on the retinal arteries is

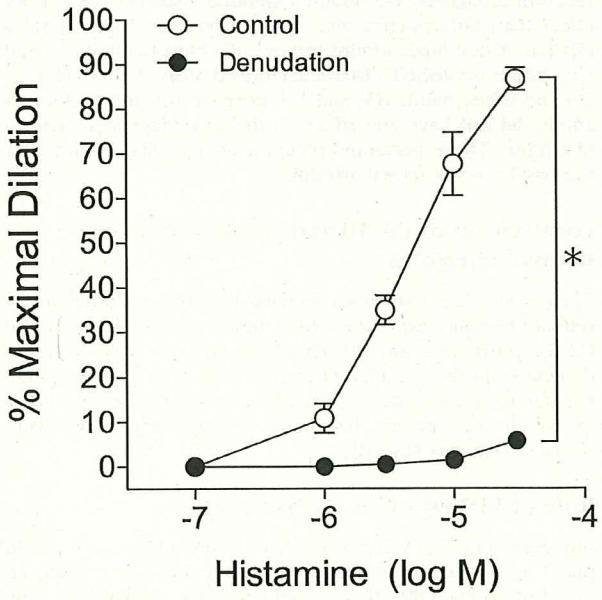

FIGURE 2. Effect of the removal of the endothelium by perfusion with 0.4% CHAPS. *$P < 0.05$ versus control.

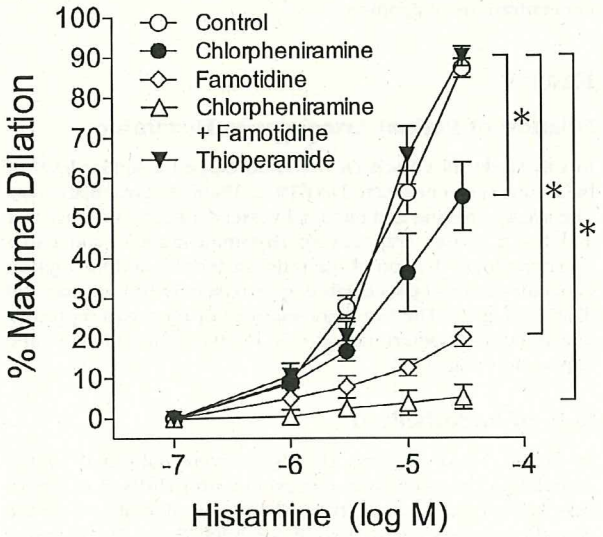

FIGURE 3. Effect of incubation with H1 antagonist chlorpheniramine (1 μM), H2 antagonist famotidine (10 μM), or H3 and H4 antagonist thioperamide (1 μM). *$P < 0.05$ versus control.

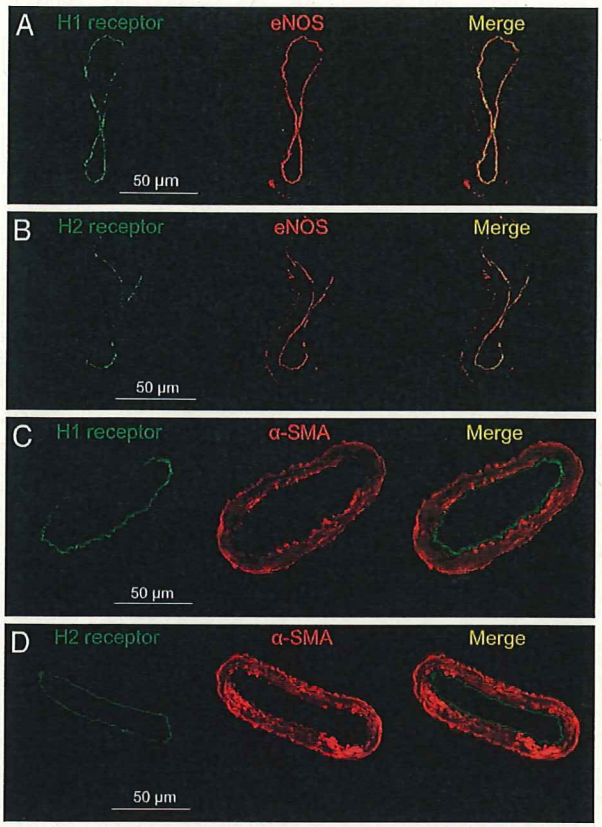

FIGURE 4. Immunohistochemical analysis of H1 and H2 receptors in retinal arterioles. (**A**) Staining with anti-H1 receptor (*green*) and anti-eNOS (*red*) antibodies shows expression of the H1 receptor and eNOS. The merged image shows overlapping staining (*yellow*) of the H1 receptor with eNOS. (**B**) Staining with anti-H2 receptor (*green*) and anti-eNOS (*red*) antibodies shows expression of the H2 receptor and eNOS. The merged image shows overlapping staining (*yellow*) of the H2 receptor and eNOS. (**C**) Staining with anti-H1 receptor (*green*) and anti-α-SMA (*red*) antibodies shows expression of the H1 receptor and SMA. The merged image shows no overlapping staining of the H1 receptor and SMA. (**D**) Staining with anti-H2 receptor (*green*) and anti-α-SMA (*red*) antibodies shows expression of the H2 receptor and SMA. The merged image shows no overlapping staining of the H2 receptor and SMA. The images are representative of three separate experiments.

unclear.[20] In the current study, 30 μM histamine elicited approximately 85% dilation of pressurized porcine retinal arterioles (Fig. 1). Although the experimental settings are different, the pig retinal arterioles appear to be more sensitive to histamine as compared with other species. In humans, the basal plasma concentration of histamine is approximately 0.3 to 1.0 ng/mL (2.7–9.0 nM),[45] and a 5-fold increase is reported in patients with diabetes.[25] Although these plasma levels of histamine are not sufficient to cause retinal arteriolar dilation, the elevation of histamine concentration in the local retinal tissue under disease states may have an impact on the retinal circulation.[17,18] Since histamine can be synthesized in the neuroretina, its release is likely to influence nearby vasculature to exert vasodilation, as retinal arterioles are sensitive to extraluminal administration of histamine as demonstrated in the present study.

Histamine tachyphylaxis has been reported in human airway smooth muscle.[46] Benedito et al.[19] also have reported histamine tachyphylaxis in bovine retinal arteries that are

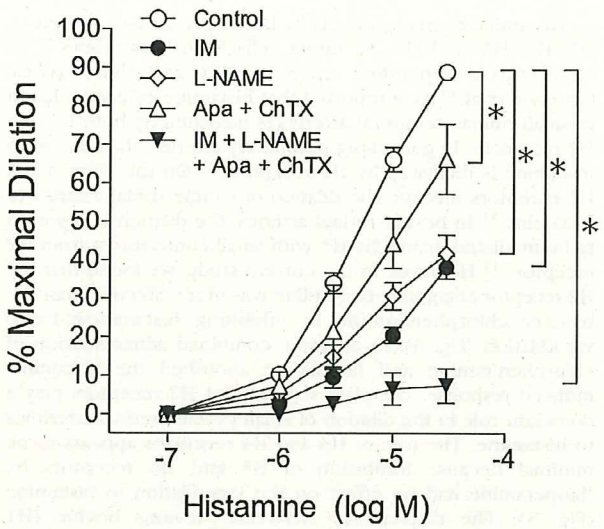

FIGURE 5. Effect of incubation with cyclooxygenase inhibitor indomethacin (IM, 10 μM), NO synthase inhibitor L-NAME (10 μM), or SKCa inhibitor apamin (Apa, 0.1 μM) plus IKCa inhibitor ChTX (0.1 μM), or incubation with all four inhibitors. *$P < 0.05$ versus control.

preconstricted with 10 μM prostaglandin $PGF_{2\alpha}$. In the current study, no apparent tachyphylaxis was observed in porcine retinal arterioles (Fig. 1). Although the reasons for these contradictory findings are unclear, the differences in species (bovine versus porcine), experimental setup (vascular ring wired myograph versus pressurized isolated vessel), and vessel size (~240 vs. ~100 μm internal diameter) may contribute to the observed discrepancy. The use of preconstrictor $PGF_{2\alpha}$ might also have altered the vascular tone and triggered the histamine desensitization mechanism in the previous bovine vessel study[19] as seen in other vascular preparations.[47,48]

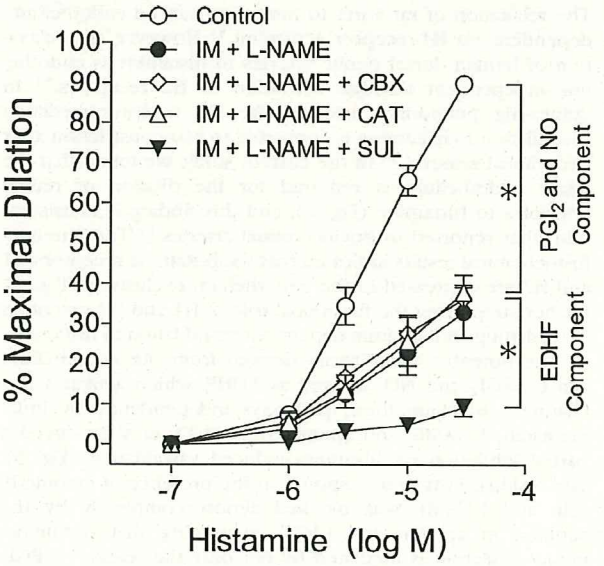

FIGURE 6. Effect of incubation with gap junction inhibitor carbenoxolone (CBX, 100 μM), H_2O_2 scavenger catalase (CAT, 1000 U/mL), and CYP450 enzyme inhibitor sulfaphenazole (SUL, 10 μM). To isolate the EDHF component of the histamine-induced vasodilator response, IM (10 μM) and L-NAME (10 μM) were added throughout the experiment. *$P < 0.05$ between groups.

Histamine exerts its effects by binding to its four receptors, H1, H2, H3, and H4, on target cells in various tissues.[16] In vasculatures, depending upon species and tissue types, Ottosson et al.[49] have reported that histamine-induced dilation in small human temporal arteries is mediated by both H1 and H2 receptors. In guinea-pig pulmonary arteries, the dilation to histamine is mediated by H1 receptors.[50] On the other hand, H2 receptors mediate the dilation of canine spinal arteries to histamine.[51] In bovine retinal arteries, the dilation is reported to be mediated mainly by H1 with small contribution from H2 receptors.[19] However, in the current study, we found that the H2-receptor antagonist famotidine was more effective than H1-blocker chlorpheniramine in inhibiting histamine-induced vasodilation (Fig. 3). In addition, combined administration of chlorpheniramine and famotidine abolished the histamine-induced response. Our data suggest that H2 receptors play a dominant role in the dilation of small porcine retinal arterioles to histamine. The role of H3 and H4 receptors appears to be minimal because inhibition of H3 and H4 receptors by thioperamide had no effect on the vasodilation to histamine (Fig. 3). The discrepancy between previous bovine (H1 dominant)[19] and current porcine (H2 dominant) study on retinal vascular dilation to histamine is unclear. The different approaches (the pressurized vascular segment versus stretched vascular ring), the development of vascular tone (spontaneous basal tone versus external preconstrictor), vessel size (~100 vs. 250 μm), and species difference (porcine versus bovine) may contribute to the observed inconsistent results. Interestingly, in healthy humans, histamine dilates retinal arteries and veins in a manner sensitive to H1-receptor antagonist diphenhydramine[24] but not to H2-blocker cimetidine.[23] However, the role of H2 receptors cannot be completely excluded because the efficacy of cimetidine in blocking H2 receptors in the above human study has not been tested.[23] Our finding on the prominent role of H2 receptors in mediating retinal arteriolar dilation is consistent with the finding in human ophthalmic arteries.[52]

The role of endothelium in mediating the histamine response is inconsistent across different tissues and species. The relaxation of rat aorta to histamine can be endothelium-dependent via H1-receptor activation.[53] However, the relaxation of human dorsal penile arteries to histamine is endothelium-independent through activation of H2 receptors.[54] In guinea-pig pulmonary arteries, the H1 receptor–mediated vasodilation to histamine is converted to vasoconstriction after endothelial removal.[50] In the current study, we found that the intact endothelium is required for the dilation of retinal arterioles to histamine (Fig. 2), and this finding is consistent with that reported in bovine retinal arteries.[19] The immunohistochemical results indicated that the histamine receptors H1 and H2 are expressed in the endothelium exclusively (Fig. 4), further supporting the functional role of H1 and H2 receptors in mediating endothelium-dependent vasodilation to histamine.

The potential vasodilators derived from the endothelium can be PGI_2 and NO, as well as EDHF, which activates K_{Ca} channels. Blocking these pathways independently by indomethacin, L-NAME, and apamin plus ChTX only produced a partial inhibition on histamine-induced vasodilation (Fig. 5). The residual histamine response in the presence of indomethacin and L-NAME was blocked almost completely by the addition of apamin and ChTX, suggesting that histamine-induced dilation is mediated by not only the release of PGI_2 and NO but also the K_{Ca} channel activation.

Since L-NAME can block both constitutive endothelial (eNOS) and inducible (iNOS) NOS isoforms, the contribution of iNOS to histamine-induced vasodilation cannot be completely excluded in the present study. Although the gene that encodes iNOS is not thought to be expressed in normal vessels,[55] a recent study[56] has reported that hypoxia stimulation for 5 to 10 minutes can relax preconstricted porcine retinal arterial rings with preserved perivascular retinal tissue in a manner sensitive to an iNOS inhibitor. The source of NO appears to be from the iNOS expressed in the perivascular retinal tissues during hypoxia. It is worth noting that our studies were performed in the arterioles devoid of retinal tissue and that the vasodilation to histamine was observed within a few minutes in a manner dependent upon the intact endothelium. It has been shown that histamine can stimulate rapid eNOS phosphorylation/activity[57] and NO release[58] from cultured endothelial cells in a matter of minutes. Based on the signaling characteristics in response to histamine, it is likely that eNOS contributes to such a rapid response in our studies. We found that blockade of prostanoids by indomethacin partly inhibited the histamine-induced vasodilation. Since both vasoconstrictor and vasodilator prostanoids[59] can be blocked by indomethacin, the reduced histamine-induced vasodilation by indomethacin suggests the blockage of vasodilator prostanoids in the present study. It has been reported that the vessel relaxation to histamine can be attenuated by cyclooxygenase blockade in bovine retinal arteries[19] and that histamine can induce the release of vasodilator prostanoids,[53] possibly PGI_2,[60] from the arterial wall. Since indomethacin did not alter resting vascular tone, it is speculated that histamine activates cyclooxygenase for PGI_2 release and subsequently leads to dilation of retinal arterioles in our study.

This is the first report on the involvement of K_{Ca} channels in mediating histamine-induced vasodilation in small retinal arterioles. The vasodilations through the activation of K_{Ca} channels have been shown to be the main mechanism of EDHF-induced vasodilation in various tissues,[11] especially the vasodilators related to CYP450 metabolites such as EETs.[61] Similar to the finding of K_{Ca} channel inhibition, administration of CYP450 enzyme inhibitor sulfaphenazole in the presence of indomethacin and L-NAME abolished vasodilation to histamine (Fig. 6), suggesting the involvement of CYP450 enzymes in this vasodilation. Although some evidence has suggested that activation of myoendothelial gap junctions and release of H_2O_2 can contribute to the EDHF-induced vasodilation in certain vascular beds,[9] these proposed mechanisms appear not to apply to the retinal arterioles, since H_2O_2 scavenger catalase or the gap junction inhibitor carbenoxolone had no effect on histamine-induced vasodilation (Fig. 6). Under the inhibition of cyclooxygenase and NOS pathways, sulfaphenazole and K_{Ca} channel inhibitors (apamin plus ChTX) produced comparable inhibition of histamine-induced vasodilation (Figs. 5, 6), suggesting that EETs may contribute to the dilation of the retinal arterioles by activating K_{Ca} channels. Interestingly, an earlier study[19] has reported that the dilation of large bovine retinal arterioles (~240 μm) to histamine was mediated solely by prostanoids and NO. This discrepancy may be related to the use of smaller sizes of arterioles in our study because the importance of EDHF was found to increase with decreasing vessel size.[12,62]

Histamine might be a useful agent to facilitate studies on the physiologic and pathophysiologic regulation of vasomotor function by endothelium-derived vasodilators such as NO, prostanoids, and EDHF in the retinal microcirculation because it activates three major endothelium-dependent vasodilators from the retinal arteriolar wall. It has been shown that the EETs/EDHF-mediated vasodilation is sensitive to high glucose exposure by inhibiting CYP450 activity under oxidative stress.[63] We previously have found that retinal arterial blood flow is reduced in patients with type 2 diabetes mellitus with minimal retinopathy,[64] suggesting that the early blood flow dysregulation leads to the subsequent diabetic retinopathy. Since EDHF can play compensatory roles in vasoregulation,

especially under conditions with compromised function of NO and prostanoids,[65] the selective EDHF deficiency might have a significant impact on retinal blood flow regulation during disease development. Further study is needed to examine the effect of diabetes on retinal arteriolar dilation to histamine in relation to the pathogenesis of diabetic retinopathy in humans.

In conclusion, we showed that histamine elicits potent dilation of the small retinal arterioles through activation of the H1 and H2 receptors in the retinal endothelial cells. The endothelium-dependent dilation is mediated by the activation of cyclooxygenase, NO synthase, and CYP450 enzymes. The EETs, derived from CYP450, might exert EDHF properties by activating K_{Ca} channels for vasodilation.

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