5,6-DiHETE attenuates vascular hyperpermeability by inhibiting Ca\(^{2+}\) elevation in endothelial cells

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Abstract Although more than 100 lipid metabolites have been identified, their bioactivities remain unknown. In a previous study, we discovered that the production of several lipid metabolites in the intestines dramatically changed in colitis. Of these metabolites, 5,6-dihydroxyeicosatetraenoic acid (DiHETE) possesses novel anti-inflammatory activity in the vasculature. In this study, we used mouse and human umbilical vein endothelial cell (HUVEC) models to elucidate the mechanisms underlying the vascular activity of lipid metabolites, particularly those related to the release of histamine, a major proinflammatory mediator that stimulates endothelial cells to produce NO, a mediator of vascular relaxation and hyperpermeability, by activating intracellular Ca\(^{2+}\) concentration-dependent signaling. In a mouse ear, the administration of 5,6-DiHETE did not induce inflammatory reactions, and pretreatment with 5,6-DiHETE inhibited histamine-induced inflammation, specifically vascular dilation and hyperpermeability. In an isolated mouse aorta, 5,6-DiHETE treatment did not influence vascular contraction but attenuated acetylcholine-induced vascular relaxation. In HUVECs, treatment with 5,6-DiHETE inhibited histamine-induced endothelial barrier disruption and inhibited the production of NO. Most notably, 5,6-DiHETE inhibited histamine-induced increases in intracellular Ca\(^{2+}\) concentrations in HUVECs. Our findings suggest that 5,6-DiHETE attenuates vascular hyperpermeability during inflammation by inhibiting endothelial Ca\(^{2+}\) elevation, which might lead to a novel pharmacological strategy against inflammatory diseases. —Hamabata, T., T. Nakamura, Y. Tachibana, D. Horikami, and T. Murata. 5,6-DiHETE attenuates vascular hyperpermeability by inhibiting Ca\(^{2+}\) elevation in endothelial cells. J. Lipid Res. 2018. 59: 1864–1870.

Supplementary key words calcium • inflammation • lipid mediators • nitric oxide • vascular biology

Lipids and their metabolites are substances produced from membrane phospholipids or fatty acids. More than 100 lipids and their metabolites have been identified (1), but their bioactivities remain to be elucidated. In a previous study in mice, we found that the concentrations of several lipid metabolites significantly increased or decreased in colon tissue depending on the progression and healing of dextran sulfate-induced colitis (2). Severely inflamed colon tissue was found to contain higher concentrations of docosahexaenoic acid-derived 7-hydroxydocosahexaenoic acid (HDoHE). The concentrations of α-linolenic acid-derived 13-hydroxyoctadecatrienoic acid (HOTrE) and eicosapentaenoic acid-derived 5,6-dihydroxyeicosatetraenoic acid (DiHETE) were found to be elevated in the healing colon tissue. Thus, the above-mentioned lipid mediators potentially exert bioactivities that participate in the progression and/or resolution of inflammation.

During inflammation, various bioactive substances, including lipid metabolites, are secreted from the vascular tissues, as well as by infiltrating immune cells. Some of these substances stimulate hyperpermeability of the vasculature, which in turn causes plasma leakage and infiltration of immune cells into the interstice of tissue (3, 4). This phenomenon is necessary to eliminate foreign substances and to promote tissue repair. However, excessive and/or sustained vascular hyperpermeability can trigger a variety of inflammatory diseases (5, 6). Thus, the appropriate modulation of vascular permeability is crucial for managing health and disease.

The vasculature is primarily composed of endothelial cells and smooth muscle cells. Endothelial cells form a monolayer that covers the inner surface of the blood vessels, while smooth muscle cells encapsulate the outer surface.
of the endothelial layer. These two types of cells undergo coordinated action to modulate vascular reactions. Upon inflammation, some bioactive substances increase vascular permeability by modulating two major factors: blood flow and endothelial barrier function. Histamine is a major pro-inflammatory mediator that is involved in the acute immune response and strongly increases vascular permeability. It stimulates endothelial cells to produce a vascular relaxant factor NO by activating intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) -dependent signaling (7). The vascular dilations increase the downstream blood flow and intraluminal hydrostatic pressure, which in turn causes vascular hyperpermeability (8). In contrast, the endothelial barrier is formed mainly by intercellular adherens junctions consisting of vascular endothelial cadherin and the cytoskeleton (4). Histamine also disrupts the endothelial adherence junction by increasing [Ca\(^{2+}\)]\(_c\) (9).

Certain lipid metabolites, especially cyclooxygenase-derived prostanooids, are known to modulate vascular function (10). Prostaglandins E\(_2\) and I\(_2\) cause vascular dilatation and hyperpermeability (11, 12). Likewise, thromboxane A\(_2\) causes vascular hyperpermeability by disrupting endothelial barrier function (13). In addition, we recently demonstrated that prostaglandin D\(_2\) causes vascular hyperpermeability by enhancing endothelial barrier formation (14). However, there are numerous lipid metabolites whose bioactivities remain unknown.

In this study, we attempted to determine the bioactivities of 7-HDoHE, 13-HOTrE, and 5,6-DiHETE by evaluating their effects on vascular function both in vivo and in vitro.

**MATERIALS AND METHODS**

**Reagents**

The following reagents were obtained from the indicated suppliers: Evans blue, norepinephrine, and acetylcholine (Sigma-Aldrich); EGM-2 and EBM-2 (Lonza, Switzerland); formamid, histamine dihydrochloride, diphenhydramine, and Dextran, Fluorescein, 70,000 MW, Anionic, Lysine Fixable (Molecular Probes); mouse anti-eNOS (pS1177) antibody and mouse anti-eNOS antibody (BD Transduction Laboratories); goat anti-mouse IgG IRDye 800CW (LI-COR Biosciences); LaCl\(_3\) (Nacalai Tesque, Japan); and 7-HDoHE, 13-HOTrE, (±)5,6-DiHETE, (±)8,9-DiHETE, 5(\(S\)),6(\(S\))-DiHETE, and 5(\(S\)),6(\(R\))-DiHETE (Cayman). (±)5,6-DiHETE, (±)8,9-DiHETE, 5(\(S\)),6(\(S\))-DiHETE, and 5(\(S\)),6(\(R\))-DiHETE possess very close molecular structures. Before the experiments, we confirmed that there was no contamination in these compounds using LCMS8030 with the analysis program LabSolutions LCMS Ver. 5.65 (Shimadzu, Japan; supplemental Fig. S1A).

**Animals**

Male C57BL/6 mice aged 8–12 weeks were used. All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee at the University of Tokyo.

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. HUVECs were cultured in EGM-2. These cells (passages 4–9) were used for experiments after 4 h of starvation in EBM-2 supplemented with 2% FBS.

**Modified Miles assay**

Vehicle (80% acetone) or histamine (400 μg) was applied transcutaneously to the ventral surface of the mouse ears. Diphenhydramine (2.5 μg), LaCl\(_3\) (250 μg), 7-HDoHE (0.1 μg), 13-HOTrE (0.1 μg), or 5,6-DiHETE (0.1 μg) was injected intracutaneously 15 min before histamine treatment. To assess vascular permeability, Evans blue (50 mg/kg) was injected intravenously 5 min after the treatment. Mice were euthanized by cervical dislocation 30 min after the injection. The ears were dissected, dried at 55°C, and weighed. Extravasated Evans blue present in the ears was extracted in formamide and quantified spectrophotometrically (PerkinElmer Japan) at a wavelength of 610 nm.

**In vivo microscopy**

To visualize the ear vessels of the mice using a confocal microscope (ECLIPSE Ti with C1 confocal system; Nikon, Tokyo, Japan), 70 kDa FITC-dextran (10 mg/kg; Sigma-Aldrich) was injected intravenously. Mice were then positioned on the microscope stage, and their body temperatures were maintained at 37°C. Dextran leakage and vascular diameter were monitored every minute and quantified as described previously (11) using EZ-C1 Free Viewer (Nikon).

**Measurement of vascular contraction**

The aortic arteries of the mice were excised and placed in a physiological saline solution containing 136.9 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 23.8 mM NaHCO\(_3\), 1.5 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), and 0.01 mM EDTA. After removing fat and connective tissue, the aortic arteries were cut into rings. The contractile force of the vascular rings was then isometrically recorded with a force-displacement transducer (Oriente, Tokyo, Japan) connected to a strain amplifier (Yokogawa, Japan) under a resting tension of 3 mN. Precontraction was induced by norepinephrine (0.3 or 1 μM). Acetylcholine (0.03–1 μM) was added cumulatively.

**Measurement of TER**

Endothelial barrier function was evaluated by measuring transendothelial electrical resistance (TER) with the use of the xCelligence Real-Time Cell Analyzer DP system (Roche, Basel, Switzerland). This system monitors changes in TER over time across an interdigitated microelectrode at the bottom of tissue culture E-plates (Roche). Cells (8,000) were plated on E-plates and incubated until confluent. TER was measured every 60 s. For normalization, cell index values at each time point were shown as a ratio to the initial value. We quantified the maximum normalized cell index after stimulation and represented it as maximal response.

**Measurement of NO production**

HUVECs were washed three times with HEPES and incubated in HEPES supplemented with 1 mM L-arginine and 10 μM tetrahydrobiopterin. After equilibrating for 30 min, the media were exchanged, and the cells were incubated for another 30 min. Cells were subsequently stimulated with the indicated agents for 15 min and stimulated with 10 μM histamine for 5 min. Conditioned media before and after stimulation (100 μl each) were collected and centrifuged at 300 g for 3 min. The supernatants were collected and used for the measurement of nitrite and nitrate, stable metabolites of NO, by an ENO-20 NOx analyzer (Eicom, Japan). The degrees of increase in nitrite and nitrate levels after stimulation were normalized with cell protein contents.
Western blotting
HUVECs were stimulated with each reagent and then lysed with the following modified lysis buffer: 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40 substitute, 0.1% SDS, 0.1% deoxycholic acid, 50 mM NaF, 1 mM Na3VO4, and 10 mM β-glycerophosphate. Pefabloc SC (1.0 mg/ml) and cOmplete Protease Inhibitor Cocktail tablets (1 tablet/50 ml; Sigma-Aldrich) were added fresh into the lysis buffers. Protein (20 μg) was electrophoresed and blotted onto a PVDF membrane. The membrane was probed using mouse anti-human phosphorylated eNOS (S1177) antibody or mouse anti-human eNOS antibody overnight at 4°C. As secondary antibodies, goat anti-mouse IgG IRDye (S1177) antibody or mouse anti-human eNOS antibody overnight was applied for 30 min at room temperature. Bands were detected and quantified using the Odyssey system (LI-COR Biosciences).

Measurement of Ca2+ concentration
HUVECs were incubated with 3 μg Fura 2-AM containing 0.11% Cremophor EL for 30 min and washed three times with HEPES-buffered solution. Coverslips were placed into a specialized airtight chamber mounted onto the stage of microscope maintained at 37°C. HUVECs were excited at 340 and 380 nm, and the emitted fluorescence signal was collected every 3 s at 510 nm. The fluorescence ratio was determined using a fluorescence imaging system (AQUACOSMOS; Hamamatsu Photonics, Japan). After the experiments, 1 μM ionomycin was added to measure the fluorescence alteration in the presence of 0 or 1.5 mM Ca2+. Ca2+ concentration was shown as the change in the fluorescence ratio. The area under the curve (AUC) was calculated to assess the change in the fluorescence ratio 1 min after each stimulation.

Statistical analysis
The results of the experiments were expressed as means ± SEMs. Data evaluations were conducted using one-way ANOVA, followed by Bonferroni’s test for comparison between more than two groups. P < 0.05 was regarded as significant.

RESULTS
5,6-DiHETE inhibits histamine-induced vascular hyperpermeability
First, we performed the modified Miles assay to assess the effects of the lipid metabolites on vascular permeability in vivo. Figure 1A shows representative images of mouse ears, and Fig. 1B shows the results for the quantification of dye extravasation. The administration of 400 μg histamine induced blue dye extravasation (0.14 ± 0.02 μg/mg); this amount was twice the amount used in the vehicle treatment (0.06 ± 0.02 μg/mg). Histamine stimulates a histamine H1 receptor to increase [Ca2+]i, in endothelial cells. This signaling activation promotes endothelial NO production, which in turn causes vascular dilation and hyperpermeability. Pretreatment with the histamine H1 receptor blocker diphenhydramine (2.5 μg per ear; 15 min) or a Ca2+ channel blocker LaCl3 (250 μg per ear; 15 min) consistently and significantly inhibited the dye extravasation.

Pretreatment with 0.1 μg 7-HDoHE or 13-HOTrE did not influence histamine-induced dye extravasation. By contrast, pretreatment with 0.1 μg 5,6-DiHETE significantly inhibited dye extravasation (0.06 ± 0.02 μg/mg). These results suggest that 5,6-DiHETE inhibited vascular hyperpermeability.

In subsequent experiments, we focused the investigation on the inhibitory effects of 5,6-DiHETE on histamine-induced vascular activation.

5,6-DiHETE inhibits histamine-induced arterial dilation
We next investigated the effect of 5,6-DiHETE on histamine-activated vasculature in the mouse ears. Results of intravital imaging showed that 400 μg histamine caused extravasation of FITC-dextran mainly from the bifurcation area of vasculature and peripheral vascular vessels (Fig. 2A, B). The mean fluorescence intensity was significantly higher at 30 min following histamine administration (Fig. 2B). Pretreatment with 0.1 μg 5,6-DiHETE (15 min) narrowed the area of FITC-dextran extravasation (Fig. 2A) and significantly decreased the mean intensity relative to that of histamine-treated samples (Fig. 2B).

Smooth muscle contraction reduces downstream blood flow and limits vascular leakage, whereas smooth muscle relaxation increases blood flow, thereby leading to vascular leakage. We next assessed the changes in the vascular diameters of the mouse ears. Figure 2C shows the typical blood vessels of the mouse ear, and Fig. 2D shows the results of quantification of arterial diameters. The administration of 400 μg histamine increased the arterial diameter to 178% ± 13% relative to that of nonstimulated arteries, which suggested that histamine induced vascular dilation. Interestingly, pretreatment with 0.1 μg 5,6-DiHETE significantly inhibited the histamine-induced increase in arterial diameter by 132% ± 15% relative to that of nonstimulated arteries. On the other hand, Fig. 2E shows the results of the quantification of vein diameters. Histamine administration tended to increase vein diameter, but pretreatment with 5,6-DiHETE did not inhibit the histamine-induced increase in vein diameter.
5,6-DiHETE inhibits acetylcholine-induced relaxation of the aorta

We also examined the effects of 5,6-DiHETE on the contraction and relaxation of isolated mouse aortae. As shown in Fig. 3A, norepinephrine administration (0.001–1 μM) constricted the aortae in a concentration-dependent manner. By contrast, treatment with 0.001–1 μM 5,6-DiHETE did not cause contraction. Acetylcholine (0.03–1 μM) dilated mouse aortae preconstricted by 0.3 μM norepinephrine in a dose-dependent manner (Fig. 3B), whereas 0.001–1 μM 5,6-DiHETE did not influence the preconstriction. Consistent with previous results, treatment with 0.03–1 μM acetylcholine caused relaxation of mouse aortae preconstricted with 0.3 μM norepinephrine. Interestingly, pretreatment with 1 μM 5,6-DiHETE (15 min) significantly attenuated acetylcholine-induced aorta relaxation. These results suggest that 5,6-DiHETE attenuated acetylcholine-induced arterial relaxation without affecting smooth muscle contractility.

5,6-DiHETE inhibits histamine-induced endothelial barrier dysfunction

We evaluated endothelial barrier function in vitro by measuring the TER of HUVECs. Barrier disruption resulted in lower TER, whereas barrier enhancement increased TER. As shown in Fig. 4A and B, treatment with 1 U/ml thrombin significantly decreased TER, whereas treatment with an adenylate cyclase activator forskolin (1 μM) increased TER. Treatment with 5,6-DiHETE alone (0.1 μM and 0.3 μM) did not change TER.
We next examined the effects of 5,6-DiHETE under histamine stimulation. As shown in Fig. 4C and D, treatment with 10 μM histamine significantly decreased TER (minimum of 0.82 ± 0.05-fold), which was abrogated by pretreatment with 0.3 μM diphenhydramine, a histamine H1 receptor antagonist. In addition, pretreatment with 0.3 μM 5,6-DiHETE (15 min) significantly inhibited the histamine-induced barrier disruption (minimum of 0.915 ± 0.012-fold). These results suggest that 5,6-DiHETE attenuated endothelial barrier dysfunction under histamine stimulation.

We also assessed the effects of (±)8,9-DiHETE, 5(S),6(S)-DiHETE, and 5(S),6(R)-DiHETE on endothelial barrier function, but none of these isomers inhibited histamine-induced endothelial barrier dysfunction (supplemental Fig. S1B).

5,6-DiHETE inhibits histamine-induced eNOS phosphorylation and NO production in endothelial cells

Both histamine and acetylcholine lead to eNOS phosphorylation and NO production (15, 16). Thus, we examined whether 5,6-DiHETE inhibited eNOS phosphorylation and NO production in HUVECs. Treatment with 10 μM histamine (5 min) resulted in eNOS phosphorylation at serine1177, which is essential for NO synthesis in HUVECs. Figure 5A shows the representative results of Western blot analysis of total eNOS and phosphorylated eNOS levels, while Fig. 5B shows the corresponding phosphorylation levels. Pretreatment with 0.1 μM 5,6-DiHETE (15 min) significantly reduced histamine-induced phosphorylation of eNOS. As shown in Fig. 5C, 10 μM histamine stimulated NO production (0.31 ± 0.06 pmol/μg protein), which was abrogated by pretreatment with the histamine H1 receptor blocker diphenhydramine (10 μM; 15 min). Consistent with the results for eNOS phosphorylation, pretreatment with 0.1 μM 5,6-DiHETE (15 min) significantly inhibited histamine-induced NO production (0.11 ± 0.06 pmol/μg protein).

These findings show that 5,6-DiHETE inhibited eNOS phosphorylation and NO production in endothelial cells.

5,6-DiHETE inhibits the increase in Ca2+ concentrations in endothelial cells

The increase in [Ca2+]i in endothelial cells promotes NO production by phosphorylating eNOS and dilating smooth muscle cells. At the same time, the [Ca2+]i increase disrupts the endothelial barrier. There is a possibility that 5,6-DiHETE can inhibit the increase in [Ca2+]i. Figure 6A
shows the time-dependent changes in \([\text{Ca}^{2+}]\) levels in HUVECs, and Fig. 6B shows the AUC. Histamine treatment (10 \(\mu\)M) rapidly increased \([\text{Ca}^{2+}]\), in HUVECs (AUC: 12.2 ± 2.1), which was suppressed by pretreatment with the histamine H1 receptor blocker diphenhydramine (10 \(\mu\)M; AUC: 1.5 ± 0.4). Pretreatment with 0.03 \(\mu\)M 5,6-DiHETE did not inhibit the histamine-induced increase in \([\text{Ca}^{2+}]\), (AUC: 7.8 ± 2.3), while 0.1 and 0.3 \(\mu\)M 5,6-DiHETE significantly inhibited the histamine-induced increase in \([\text{Ca}^{2+}]\), in a dose-dependent manner (AUC: 5.9 ± 1.5 and 1.8 ± 1.1).

**DISCUSSION**

Our findings revealed that 5,6-DiHETE attenuates inflammation in the vasculature by inhibiting vascular hyperpermeability. Our study is the first to demonstrate the bioactivity of 5,6-DiHETE, a cytochrome P450-catalyzed metabolite produced from eicosapentaenoic acid following epoxidation of the e-5 double bond. Isomers of 5,6-DiHETE have been reported to promote aryl hydrocarbon receptor-mediated transcriptional activity, which potentially stimulates inflammatory signaling (17). However, no studies have investigated the bioactivity of 5,6-DiHETE. We originally showed that 5,6-DiHETE production is increased during the healing stage of murine colitis. 5,6-DiHETE may have a crucial role in the promotion of healing by inhibiting excessive or sustained inflammation.

Although 5,6-DiHETE concentrations produced in inflamed tissue have not been documented, Yamada et al. (1) reported that 5,6-DiHETE concentrations in normal, non-inflamed mouse heart, liver, and kidney tissues were \(< 30–70 \text{ pg/mg. In this study, we utilized a considerably higher concentration of 5,6-DiHETE (~10 ng/mg; 0.1 \(\mu\)g per ear) to evaluate its bioactivity. 5,6-DiHETE is a lipid metabolite that can be produced in and affect the local vasculature. Further investigations are required to identify the pathophysiological role of endogenous 5,6-DiHETE in inflammation.

As described above, vascular permeability is primarily determined by two factors: blood flow and endothelial barrier function. Vascular dilation increases blood flow in peripheral tissue. In turn, the increased blood flow exerts intraluminal hydrostatic pressure, which leads to plasma leakage from the blood vessels (18). In particular, nonactivated eNOS binds to caveolin found on the endothelial cell membrane and is subsequently translocated into the cytoplasm in response to an increase in \([\text{Ca}^{2+}]\). Afterward, a serine/threonine kinase Akt phosphorylates/activates eNOS to induce NO production (19, 20). Endothelial cell-derived NO causes the dilation of smooth muscle cells through cyclic guanosine monophosphate-protein kinase G signaling. The increase in \([\text{Ca}^{2+}]\), also disrupts the endothelial barrier by forming the actin stress fiber and destabilizing adherence junctions (19, 21). In this study, we found that 5,6-DiHETE inhibited the increase in \([\text{Ca}^{2+}]\), in turn attenuates vascular dilation and hyperpermeability. Interestingly, 5,6-DiHETE decreased endothelial cell activation but did not influence smooth muscle contractility. 5,6-DiHETE appeared to modulate endothelial cell-specific \([\text{Ca}^{2+}]\) channels, which attenuates both the dilation of smooth muscle cells and endothelial barrier dysfunction, resulting in the inhibition of vascular hyperpermeability.

Appropriate modulation of vascular barrier is indispensable for maintaining tissue homeostasis. Vascular dysfunction is a critical step in the pathogenesis of inflammatory disease. Given that endothelial barrier enhancement is likely to inhibit an excessive inflammatory response, identifying a novel barrier enhancer and elucidating its mechanism of action will potentially provide new therapeutic strategies for the treatment of various diseases.

This study is the first to verify the anti-inflammatory effects of 5,6-DiHETE and the mechanisms underlying its activity. However, further studies are required to identify the source cells, receptor involved, endogenous physiological activity, and precise mechanisms underlying the function of 5,6-DiHETE. In addition, we were unable to identify the bioactivities of 0.1 \(\mu\)g 7-HDoHE or 13-HOTrE by assessing vascular function. However, we cannot exclude the possibility that these lipid metabolites modulate the progression and/or healing of inflammation through mechanisms other than modulating vascular function. Further investigations are needed to clarify the abovementioned points.

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