Abstract  Endothelial dysfunction is a hallmark of multiple inflammatory diseases. Leukocyte interactions with the endothelium have significant effects on vascular wall biology and pathophysiology. Myeloperoxidase (MPO)-derived oxidant products released from leukocytes are potential mediators of inflammation and endothelial dysfunction. 2-Chlorofatty acids (2-CIFAs) are produced as a result of MPO-derived HOCl targeting plasmalogens phospholipids. Chlorinated lipids have been shown to be associated with multiple inflammatory diseases, but their impact on surrounding endothelial cells has not been examined. This study tested the biological properties of the 2-CIFA molecular species 2-chlorohexadecanoic acid (2-ClHA) on endothelial cells. A synthetic alkyne analog of 2-ClHA, 2-chlorohexadec-15-ynoic acid (2-ClHyA), was used to examine the subcellular localization of 2-CIFA in human coronary artery endothelial cells. Click chemistry experiments revealed that 2-ClHyA localizes to Weibel-Palade bodies. 2-ClHyA promote the release of P-selectin, von Willebrand factor, and angiopietin-2 from endothelial cells. Functionally, 2-ClHA and 2-ClHyA cause neutrophils to adhere to and platelets to aggregate on the endothelium, as well as increase permeability of the endothelial barrier which has not been tied to the release of angiopietin-2. These findings suggest that 2-CIFAs promote endothelial cell dysfunction, which may lead to broad implications in inflammation, thrombosis, and blood vessel stability.—Hartman, C. L., M. A. Duerr, C. J. Albert, W. L. Neumann, J. McHowat, and D. A. Ford. 2-Chlorofatty acids induce Weibel-Palade body mobilization. J. Lipid Res. 2018. 59: 113–122.

The intimate relationship between leukocytes and the endothelium is critical to an appropriate inflammatory response, producing both antiinflammatory and antioxidant mediators (1, 2). Conversely, a dysregulated inflammatory response can overwhelm the immune system, causing dysfunction of the blood-endothelium interface. Endothelial dysfunction is a hallmark of multiple inflammatory diseases, including the initiating events of atherosclerosis, myocardial ischemia/reperfusion injury, and multiorgan failure in sepsis (3–5). During inflammation, the activated endothelium leads to the release of factors, which mediate leukocyte adherence, coagulation, and changes in endothelial barrier function (6). The accelerated rate of the leukocyte-produced reactive oxygen species, as well as the release of proteolytic enzymes, can cause tissue damage and contribute to organ failure (7, 8).

Myeloperoxidase (MPO), the most abundant protein in the neutrophil, is a key antimicrobial enzyme released by neutrophils after degranulation (9, 10). Hence, levels of plasma MPO have been correlated with the systemic activation of neutrophils and inflammatory diseases (11–13). MPO converts neutrophil-produced hydrogen peroxide into HOCl, which acts as a potent antimicrobial defense by oxidizing proteins, free amino acids, carbohydrates, DNA, and lipids (14–17). The vinyl ether bond of plasmalogens, a major phospholipid molecular class in endothelial cells, monocytes, neutrophils, and many tissues in the human body, is targeted by HOCl, resulting in the production of 2-chlorofatty aldehydes (2-CIFALDs) (18–26). 2-CIFALDs are readily oxidized by intracellular fatty aldehyde dehydrogenase to the stable metabolites, 2-chlorofatty acids (2-CIFAs) (27–29). Biosynthesized 2-CIFAs can be exported...
out of neutrophils, but much remains unknown as to the role of 2-ClFAs in circulation and in microenvironments, such as the blood-endothelial interface (29, 30). During inflammation, 2-ClFAs are produced at the site of neutrophil infiltration, and, once released, 2-ClFAs could alter nearby cell function (31).

Chlorinated lipids, including 2-CIFALD and 2-CIFA, have been linked to inflammatory diseases, including endotoxemia and atherosclerosis (27, 32–34). Activated neutrophils and monocytes isolated from human blood have elevated levels of 2-CIFA, approaching 20 μM, which may be suggestive of the concentration at the site of production near the leukocyte-endothelial interface (28, 35). The concentration of 2-CIFA in the systemic circulation is lower, ranging from 1 to 100 nM, due to dilution in the volume of blood. As demonstrated in rats, lipopolysaccharide-mediated endotoxemia results in tripling the levels of plasma 2-CIFA compared with naive rats (27). Furthermore, mice exposed to a sublethal dose of chlorine gas were found to have plasma levels of 2-CIFA greater than 100 nM (32). Chlorinated lipids have been shown to elicit recruitment of macrophages to the lung, disrupt the blood-brain barrier, and induce apoptosis (32, 35, 36). The underlying mechanisms of endothelial dysfunction, seen in multiple inflammatory diseases, remain to be completely understood.

The present study was designed to elucidate a common mechanism by which 2-ClFAs alter the blood-endothelial cell interface. By using a novel click chemistry approach, results demonstrate that the 2-CIFA species 2-chlorohexadecanoic acid (2-CIHA) localizes to Weibel-Palade bodies in human coronary artery endothelial cells (HCAECs). Weibel-Palade bodies, unique to endothelial cells, are exocytic storage granules that release crucial bioactive mediators after exposure to an external stimulus (37, 38). Accordingly, further studies show that 2-CIHA induces surface expression of P-selectin and E-selectin, as well as the release of von Willebrand factor (VWF) from Weibel-Palade bodies. Consequently, 2-CIHA increases adherence of platelets and neutrophils to HCAECs. Furthermore, 2-CIHA stimulates the release of angiopoietin-2 from the Weibel-Palade bodies and the loss of HCAEC barrier function.

MATERIALS AND METHODS

Materials

Cell culture supplies were from Sigma-Aldrich (Vienna, Austria), VWR (Vienna, Austria), Lonza (Basel, Switzerland), or Cell Applications Inc. (San Diego, CA). Click-it Cell Reaction Buffer Kit was from Thermo Fisher Scientific (Waltham, MA; catalog no. C10269). Monoclonal mouse anti-calnexin (catalog no. ab31290), anti-cytochrome c oxidase subunit IV (anti-COX IV; catalog no. ab202545), anti-Golgi matrix protein 130 (anti-GM130; catalog no. ab169276), anti-VWF (catalog ab20435), and Cy2-labeled goat anti-mouse (catalog no. ab6944) antibodies were from Abcam (Cambridge, UK). Anti-P-selectin (catalog no. sc-8419) and anti-E-selectin (catalog no. sc-5262) antibodies were from Santa Cruz (Dallas, TX). HRP-labeled goat anti-mouse IgG antibody was from Bio-Rad (Hercules, CA). All other chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific.

Instrumentation

NMR spectroscopy was performed with a JEOL ECS-400 NMR spectrometer. Mass spectrometry (MS) was performed with a Thermo Fisher Scientific triple quadrupole Quantum Ultra mass spectrometer. For experiments applying LC/MS, a Thermo Fisher Surveyor LC system was coupled to the Quantum Ultra. LC/MS data analysis was completed by using XCalibur software (Thermo Fisher). GC/MS analysis was performed by using a Hewlett Packard 6890/5973. GC/MS data analysis was completed by using MSD ChemStation GC/MS Data Analysis.

Synthesis of BSA-conjugated 2-ClHyA

The alkyne analog of 2-ClHA, designated as 2-chlorohexadec-15-ynoic acid (2-ClHyA), was synthesized following synthetic schemes used for other probes (36, 39). Briefly, hexadec-7-ynol (Alfa Aesar; catalog no. B22113) was converted to hexadec-15-yn-10-oic acid by using sodium hydride (Sigma-Aldrich; catalog no. 452912) and diaminopropane (Sigma-Aldrich; catalog no. D23602) (36). The alcohol of hexadec-15-ynol was oxidized to an aldehyde in a solution of 2-iodoxybenzoic acid (Sigma-Aldrich; catalog no. 661384) in DMSO (Sigma-Aldrich; catalog no. D2650), which resulted in hexadec-15-ynal (36). This intermediate aldehyde was chlorinated by using N-chlorosuccinimide (Sigma-Aldrich; catalog no. 109681) and proline (Sigma-Aldrich; catalog no. P0380) for 1 h (39). The aldehyde was then oxidized by using pyridinium dichromate (Sigma-Aldrich; catalog no. 214698) to the carboxylic acid for the final product, 2-ClHyA. A portion of hexadec-15-ynal was not chlorinated to synthesize hexadec-15-ynoic acid.

After each step, products were purified by flash chromatography (30 g of silica gel, high-purity grade, pore size 60 Å; Sigma-Aldrich; catalog no. 227196). Products were eluted with 30 ml of ethyl acetate/hexane/toluene/acetonic acid (30/10/60/5, v/v/v/v), and 1 ml fractions were collected and analyzed by TLC (vide infra).

Column fractions were loaded onto 60 Å silica gel TLC plates (Millipore Sigma; catalog no. 105721). Mobile phase for TLC was composed of ethyl acetate/hexane/toluene/acetonic acid (30/10/60/5, v/v/v/v). TLC plates were visualized with phosphomolybdic acid (TCI America; catalog no. P1910). TLC lanes used for purification were not stained but scraped and extracted with 8 ml of chloroform/methanol (1/1, v/v) supplemented with 0.1% acetic acid. The solution was centrifuged, and the supernatant was removed. After a second extraction with chloroform/methanol (1/1, v/v), the combined extracts from the silica were dried under nitrogen and suspended in methanol.

Lipids were then conjugated to fatty acid-free BSA, which was used for all cell treatments. Briefly, 5 mg of 2-ClHyA or HyA was dried down and added to sterile-filtered PBS. A BSA blank was made with PBS alone. Each solution was heated to 70°C for 10 min, and 1 M NaOH was added dropwise until the solution was clear. After heating to 70°C for 30 min, a sterile-filtered 6% BSA (w/v; MP Biomedicals; catalog no. 152401) in PBS solution was added while shaking. The resulting solutions were aliquoted and stored at −20°C. Final solutions were quantified by pentafluorobenzyl bromide (PFB-Br) derivatization. The 2-ClHyA and 2-ClHyA were converted to their respective PFB ester derivative as previously described (28). The 2,3,4,5,6-pentafluorobenzoic acid (Sigma-Aldrich; catalog no. 101052) and N,N-diisopropylethylamine (Sigma-Aldrich; catalog no. D125806) in a solution containing acetonitrile was added to dried lipid. After reaction for 1 h at 45°C, PFB ester derivatives were suspended in ethyl acetate and subsequently subjected to GC/MS analysis using negative ion chemical ionization.

NMR analyses

To confirm the structures of 2-ClHyA and HyA, samples were dried down and suspended in CDCl3, to be analyzed with NMR.
NMR spectra were collected on a JEOL ECS-400 NMR spectrometer. 1H NMR spectra were reported in ppm from tetramethylsilane on the d scale. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet, etc.; m, multiplet; b, broadened; obs, obscured; Abq, AB quartet), coupling constants, and assignments or relative integration where appropriate. 13C NMR spectra were reported in ppm from the central deuterated solvent peak (multiplicities indicated when determined). Grouped shifts are provided where an ambiguity has not been resolved.

Cell culture

HCAECs (Lonza; catalog no. CC-2585) were grown in EGM-2MV medium containing 5% FBS (Lonza; catalog no. CC-3202). HCAECs were used through passage 8 for these experiments. HCAECs were treated with BSA-conjugated HA, HyA, 2-CHyA, or 2-CHiHyA in growth medium containing 5% FBS.

MTT assay

The metabolic activity of HCAECs was examined by using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were plated in a clear 96-well plate and grown to confluence. Cells were treated with the indicated concentrations of BSA-conjugated lipids in phenol red-free growth medium with 5% FBS for the indicated time points up to 18 h. MTT (Sigma-Aldrich; catalog no. M2128; 1.2 mM, in 100 µl of serum-free, phenol red-free medium) was added to cells for 4 h. Seventy-five microliters of medium was removed, and 50 µl of DMSO was added for 10 min to lyse the cells. Absorbance was read at 540 nm on an Enspire multimode plate reader and corrected for background absorbance. Triton at a concentration of 0.1% in PBS was used as a positive control. MTT reduction is expressed as percent MTT reduction (BSA blank is designated as 100%).

Detection of HyA and 2-CHyA in HCAEC by immunofluorescence

Cells were plated on sterile coverslips in 6-well plates and grown to confluence. Cells were treated with 10 µM HyA or 2-CHyA for 30 min as described above. After washing with PBS, cells were fixed with formalin for 10 min. Cells were permeabilized with 0.25% Triton X-100 for 10 min. Cells were subsequently washed with 3% (w/v) BSA in PBS and labeled with 5 µM azide-carboxytetramethylrhodamine (azide-TAMRA) (Sigma-Aldrich; catalog no. 760757) by using the Click-It Cell Reaction Buffer Kit (Thermo Fisher; catalog no. C10269) following manufacturer’s protocols.

To identify subcellular localization of 2-CHyA and HyA, cells were subjected to antibody treatment against known organellar markers. After the click reaction, cells were washed with 3% BSA in PBS. Cells were incubated with primary antibodies against COX IV (1:500), GM130 (1:142), calnexin (1:2,000), VWF (1:100), and P-selectin (1:500) overnight at 4°C. Cells were washed three times with PBS and incubated with Cy2-labeled goat anti-mouse IgG secondary antibody (1:500) for 2 h. The coverslips were mounted onto microscope slides with a Vectashield solution containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories; catalog no. H1200).

Confocal microscopy

A Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) with a 63 x 1.4 oil immersion objective was used to acquire images. Cy2 fluorescence was excited at 488 nm and detected between 500 and 540 nm. Azide-TAMRA fluorescence was excited at 545 nm and detected between 570 and 650 nm. DAPI fluorescence was detected between 440 and 470 nm. All fluorescence signals were acquired simultaneously. Total corrected cell fluorescence was calculated by using ImageJ. The fluorescence was measured in at least 30 cells in five fields of view per condition.

Selectin surface expression

HCAECs were plated in a 24-well plate and grown to confluence. Cells were treated with 10 µM BSA-conjugated lipids in growth medium with 5% FBS for 30 min (P-selectin) or 1 h (E-selectin). Thrombin (0.05 IU/ml; Sigma-Aldrich; catalog no. T6884) was used as a positive control. Cells were fixed with 1% paraformaldehyde overnight. Cells were washed with PBS and blocked with PBS containing BSA and fish gelatin for 1 h. Cells were incubated with primary antibodies (1:50) against P-selectin and E-selectin for 1 h at 37°C. Cells were washed with PBS and incubated with HRP-conjugated goat anti-mouse secondary antibody (1:5000) for 30 min at 37°C. Cells were washed with PBS and incubated with 3,3,5,5-tetramethylbenzidine (TMB; Sigma-Aldrich; catalog no. T0440) substrate system for 30 min in the dark. The color reaction was stopped with 8 N H2SO4 and absorbance was read at 450 nm. Values were normalized to the secondary antibody alone.

VWF and angiopoietin-2 release

HCAECs were plated in a 24-well plate and grown to confluence. Cells were pretreated with vehicle, Bisindolylmaleimide I (1 µM; Cayman Chemical; catalog 13298), or 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; 10 µM; Sigma-Aldrich; catalog no. B1205) for 30 min. Cells were treated with 10 µM BSA-conjugated lipids in growth medium with 5% FBS for 30 min. PMA (100 ng/ml; Millipore Sigma; catalog no. 524400) was used as a positive control for these studies. Supernatant was removed and centrifuged for 2,000 g for 10 min to remove cellular debris. Human VWF ELISA (Abcam; catalog no. ab108918) and human angiopoietin-2 Quantikine ELISA (R&D Systems; catalog no. DANG20) were used to quantify VWF and angiopoietin-2 in the supernatant.

Platelet and neutrophil adherence to HCAECs

Adherence of platelets to HCAECs was determined as previously described (40). Briefly, HCAECs were grown to confluence in a 24-well plate and treated with 10 µM BSA-conjugated lipids in growth medium for 30 min. PMA and thrombin were used as positive controls. Meanwhile, platelets were isolated from the whole blood of healthy volunteers as previously described (41) and as authorized by Saint Louis University Institutional Review Board Protocol 12909. Platelet-rich plasma was collected by cent rifuging whole blood for 15 min at 100 g. The plasma was gently removed and centrifuged for 20 min at 2,000 g. Platelets were washed and stained with Calcein-AM (2.5 µmol/liter; Thermo Fisher; catalog no. C3100MP) for 15 min at 37°C in the dark. Fluorescence-labeled platelets (50 × 10⁶ cells in 300 µl) were subsequently added to each well containing HCAECs and incubated for 20 min at 37°C. Following multiple washes with PBS, bound platelets were lysed in lysis buffer (1% NP40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 µg/ml soybean trypsin inhibitor) for 10 min, and fluorescence was measured with a plate reader (excitation at 492 nm, emission at 535 nm).

For neutrophil adherence assays, HCAECs were grown to confluence in a 12-well plate and treated with 10 µM BSA-conjugated lipids in growth medium for 30 min. PMA and thrombin were used as positive controls. Neutrophils were prepared from whole blood of healthy volunteers as previously described (42, 43) and as authorized by Saint Louis University Institutional Review Board Protocol 9952. Whole blood was anticoagulated with EDTA (final concentration 5.4 mM) prior to the isolation of neutrophils by
using a Ficoll-Hypaque gradient. Five hundred microliters (4 × 10⁶ cells/ml) was subsequently added to each well containing HCAECs and incubated for 20 min. Following multiple washes, bound neutrophils were lysed with 0.2% Triton X-100, and MPO activity was measured in HBSS containing BSA, 1,9-dimethyl-methylene blue, and 0.5% hydrogen peroxide. Samples were incubated for 15 min at room temperature. Sodium azide (1%) was added to stop the reaction. Absorbance was measured at 460 nm.

Endothelial cell permeability

HCAECs were grown to confluence on Transwell polycarbonate filters (Corning Inc., Corning, NY) mounted in a chamber insert. Resistance across cells was monitored daily by using an EVOM voltmeter (World Precision Instruments). Once the resistance remained consistent for 3 consecutive days, experiments were performed. BSA-conjugated lipids (10 μM) were added to each well, and then the resistance across each well was monitored at 15, 30, and 60 min, followed by 2, 4, 8, and 24 h.

Statistical analyses

Student's t-test was used for comparisons between two groups. ANOVA with the Dunnett post hoc test was used for comparisons between three or more groups to the control condition. All data are presented as mean ± SEM unless otherwise noted.

RESULTS

Characterization of 2-ClHyA

The synthetic scheme of 2-ClHyA is shown in supplemental Fig. S1A. These reactions yielded adequately pure (>98%) HyA and 2-ClHyA. To confirm 2-ClHyA structure, 1H and 13C NMR were performed (see complete NMR assignment in supplemental Table S1). The terminal alkyne proton absorption (labeled “a” in supplemental Fig. S1B; see inset) is clearly seen at 1.92 ppm as a triplet (J = 2.80 Hz). To further characterize 2-ClHyA, both 2-ClHA and 2-ClHyA were converted to their respective PFB ester derivatives and analyzed by GC/MS. The respective ion intensity ratios and proposed fragmentation patterns are shown in supplemental Fig. S1C, D. The intensity ratio of fragment

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Fig. 1. Subcellular distribution of 2-ClHyA in HCAECs. Cells were grown to confluence on a sterile coverslip and treated with the indicated concentrations of BSA-ClHyA, 10 μM BSA-ClHA, or 10 μM BSA-HA for 30 min. Cells were fixed with formalin, permeabilized with Triton X-100, and subjected to click chemistry with azide-TAMRA (red). A: Cells were mounted in DAPI-containing solution (blue) and imaged with a Leica SP5 microscope. All images were taken by using the same settings, and representative images from each concentration are shown. B: Total corrected cell fluorescence was measured by using ImageJ from HCAECs treated with selected concentrations of BSA-ClHyA following 30 min incubations. C: Total corrected cell fluorescence was measured from HCAECs treated with BSA-ClHyA (500 nM or 1 μM) in the presence or absence of either 10 μM 2-ClHA or HA following 30 min incubations. Mean ± SEM. *** P < 0.001 for comparisons with 2-ClHA treatment.
ions (supplemental Fig. S1C) of 289/291 and 285/287 for 2-ClHA and 2-ClHyA, respectively, demonstrate the presence of a 3:1 ratio of chlorine isotopes (35Cl/37Cl), indicative of monochlorination of these fatty acids. The expected fragments of \(m/z\) 255 and 181 were detected for 2-ClHA, while the expected fragments of \(m/z\) 251 and 181 were detected for 2-ClHyA. The fragmentation patterns are shown in supplemental Fig. S1D.

**Effect of 2-ClHA and 2-ClHyA on the metabolic activity of HCAECs**

To examine the effects of 2-ClFA on the endothelium, HCAECs were used for these studies. To ensure that there were no differential effects of 2-ClHyA compared with 2-ClHA on the metabolic activity in HCAECs, an MTT assay was performed (supplemental Fig. S2). There were no significant differences in metabolic activity in HCAECs after 2-ClHyA and 2-ClHA treatment at all time points and concentrations assessed. Similarly, MTT reduction was not significantly decreased in HCAECs treated with hexadecanoic acid (HA).

**Visualization of 2-ClHyA in HCAECs**

To visualize 2-ClHyA intracellularly, HCAECs were incubated with BSA-conjugated 2-ClHyA and then fixed, permeabilized, and subjected to a click chemistry reaction with azide-TAMRA. Fluorescence, as an indicator of the subcellular fate of 2-ClHyA, was detected as red punctate foci in the perinuclear region (Fig. 1A) in cells treated with concentrations ranging from 500 nM to 10 \(\mu\)M. Total cell fluorescence, corrected for background, was calculated in cells from multiple fields of view. Total corrected cell fluorescence increased with increasing concentrations of 2-ClHyA (Fig. 1B). To demonstrate the specificity of 2-ClHyA for localization shared with 2-ClHA, 2-ClHyA was outcompeted with simultaneous 2-ClHA treatment (either 10- or 20-fold molar excesses of 2-ClHA) (Fig. 1A, C). Simultaneous treatment with either 10- or 20-fold molar excesses of the nonchlorinated fatty acid, HA, did not alter 2-ClHyA subcellular localization or total cell fluorescence, highlighting the specificity of 2-ClHyA for 2-ClHA localization to the punctate foci. Alternatively, these results may indicate that 2-ClHA uptake by HCAECs is mediated by a unique mechanism compared with HA.

The 2-ClHyA accumulation in HCAECs increased over time, until reaching a plateau at 2 h. Representative images are shown in Fig. 2A. After images containing multiple fields of view were collected, the total corrected cell fluorescence and the percent of cells containing foci (number of cells containing foci/total number of cells \(\times 100\)) were calculated, as shown in Fig. 2B, C.

![Representative images](https://example.com/representative_images)

**Fig. 2.** Time course of 2-ClHyA uptake by HCAECs. Cells were grown to confluence on a sterile coverslip and treated with 10 \(\mu\)M BSA-Cl-HyA for the indicated time points. Cells were fixed with formalin, permeabilized with Triton X-100, and subjected to click chemistry with azide-TAMRA (red). Cells were mounted in DAPI-containing solution (blue) and imaged with a Leica SP5 microscope. All images were taken using the same settings. A: Representative images from selected time points are shown. Total corrected cell fluorescence (B) and percent of cells containing red foci (C) were measured. The percent of cells containing foci was calculated as follows: (no. of cells containing foci/total no. of cells) \(\times 100\). Error bars indicate SEM.
Subcellular localization of 2-ClHyA

Colocalization analyses with known organelle markers were performed to identify 2-ClHyA subcellular localization in HCAECs. Immunofluorescence studies revealed that 2-ClHyA colocalized with the Weibel-Palade bodies (as indicated by P-selectin and VWF) and the mitochondria (as indicated by COX IV) (Fig. 3). Interestingly, after 2-ClHyA treatment, the density of VWF staining of Weibel-Palade bodies was decreased, and the Weibel-Palade bodies appeared rounded when compared with control treatment (supplemental Fig. S3). In contrast to 2-ClHyA, the localization of the nonchlorinated control HyA was most apparent with the Golgi and endoplasmic reticulum (ER; supplemental Fig. S4). HyA localization was not as striking as 2-ClHyA localization to the Weibel-Palade bodies.

Effect of 2-ClHA and 2-ClHyA on selectin surface expression and neutrophil adherence

Based on colocalization with known markers of the Weibel-Palade bodies, 2-ClHA and 2-ClHyA were examined for functional properties related to the Weibel-Palade bodies. Both 2-ClHA and 2-ClHyA significantly increased P-selectin and E-selectin surface expression compared with treatments with either BSA vehicle (control) or HA (Fig. 4A, B). The increase in P-selectin surface expression elicited by 2-ClHA was comparable to that after thrombin treatment (supplemental Fig. S5A). The relative response of E-selectin surface expression was more robust for thrombin (supplemental Fig. S5B). A modest increase in selectin surface expression was also observed in HA-treated HCAECs compared with BSA vehicle-treated HCAECs.

The selectin adhesion molecules are primarily responsible for the adherence of leukocytes to the endothelium (44, 45). Because these molecules are increased after 2-CIFa treatment, the adherence of neutrophils to HCAECs after 2-CIFA treatment was examined. Both 2-ClHA and 2-ClHyA treatments of HCAECs significantly increased the amount of neutrophils adhered to HCAECs compared with treatments with either BSA vehicle (control) or HA (Fig. 4C). Again, this increase was comparable to the increase in neutrophil adherence using thrombin and PMA as positive controls (supplemental Fig. S5C). A modest increase in neutrophil adherence was also observed in HA-treated HCAECs compared with BSA vehicle-treated HCAECs.

Effect of 2-ClHA and 2-ClHyA on VWF release and platelet adherence

The major component of Weibel-Palade bodies, acting as a backbone for formation, is VWF (38). In HCAECs, both 2-ClHA and 2-ClHyA significantly increased the release of VWF compared with treatments with BSA vehicle or HA (Fig. 5A). Pretreatment with Bis I (protein kinase C inhibitor) or BAPTA-AM (intracellular calcium chelator) reduced 2-ClHA-elicited or 2-ClHyA-elicited VWF release to levels similar to control treatments. This demonstrated that VWF release induced by both 2-ClHA and 2-ClHyA is dependent on protein kinase C and calcium. As a positive control in these studies, Bis I-sensitive or BAPTA-AM-sensitive VWF release was observed in response to PMA (supplemental Fig. S5E). Because VWF mediates platelet adherence to the endothelium (44, 45), we further demonstrated that...
2-Chlorofatty acids alter endothelial function

both 2-ClHA and 2-ClHyA significantly increased the amount of platelets adhered to HCAECs compared with BSA vehicle or HA treatments (Fig. 5B). The increases in platelet adherence by using thrombin and PMA as positive controls were greater than that elicited by 2-ClHA (supplemental Fig. S5F).

Angiopoietin-2 release as a mechanism of HCAECs permeability

Another key protein stored in Weibel-Palade bodies is angiopoietin-2, which contributes to endothelial cell permeability as an antagonist to Tie2 signaling pathways (46). 2-ClFAs was examined for both release of angiopoietin-2 from HCAECs and alterations in endothelial cell electrical resistance. 2-ClHA and 2-ClHyA significantly increased angiopoietin-2 release from HCAECs compared with controls, in a concentration-dependent manner (Fig. 6A). The levels of angiopoietin-2 release by 2-ClHA and 2-ClHyA approached the levels stimulated by the positive control, PMA (supplemental Fig. S5D). Furthermore, 2-ClHA and 2-ClHyA significantly decreased resistance across HCAECs, an indicator of endothelial cell permeability (Fig. 6B). Additionally, HA treatment elicited a significant release of angiopoietin-2 from HCAECs compared with control conditions, which was accompanied by a slight decrease in resistance across HCAECs. It should, however, be appreciated that 2-ClHA-stimulated and 2-ClHyA-stimulated angiopoietin-2 release from HCAECs, as well as decreased resistance across HCAECs, were significantly greater than those HA-elicited changes.

DISCUSSION

MPO-derived HOCl targets plasmalogen phospholipids, resulting in the liberation of a novel family of chlorinated lipids including 2-CIFALDs and 2-CIFAs at the blood-endothelial interface (18–26). 2-CIFALDs are the product of HOCl oxidation of plasmalogens. Longer-lived 2-CIFAs are released from neutrophils or can be produced as a result of 2-CIFALD oxidation in endothelial cells (27–30). Identifying the role of these chlorinated lipids in endothelial function may provide new insights into communication between leukocytes and endothelium. Others have shown that 2-CIFALD alters eNOS signaling and endothelial function (36, 47). However, the role of 2-CIFa is poorly understood, and the endothelial subcellular distribution of 2-CIFAs is unknown.

To determine 2-CIFa localization in endothelial cells, we synthesized a “clickable” analog of 2-ClHA, termed 2-ClHyA, containing an alkyne bond at the omega end of the fatty acid. HCAECs were treated with this analog, and then the analog was clicked to the fluorescent probe, azide-TAMRA, to determine subcellular distribution.
reaction used in these studies was the copper-catalyzed Huisgen 1,3-dipolar cycloaddition reaction, which is ideal, as the reaction is selective, quick, and minimizes unwanted byproducts (48, 49). This approach revealed the selective, uniform distribution of 2-ClHyA-containing foci throughout HCAECs, which increased over time and then plateaued after 2 h of treatment. The plateau of 2-ClHyA uptake may reflect the complete saturation of the binding sites for 2-ClHyA in Weibel-Palade bodies or the intracellular metabolism of the lipid. These foci were associated with Weibel-Palade bodies, as determined by colocalization with VWF and P-selectin. 2-ClHyA treatment altered the shape (cigar-like to round) and abundance of Weibel-Palade bodies detected by VWF staining. Changes in the shape of VWF-detected Weibel-Palade bodies has been attributed to packaging of VWF and maturation of Weibel-Palade bodies (50), acidification of Weibel-Palade bodies (50), and the process of Weibel-Palade body exocytosis and release of body contents (51).

Interestingly, 2-ClHyA also localizes to the mitochondria, a key player in calcium homeostasis and cell respiration. Based on the well-established role of calcium in Weibel-Palade body mobilization, 2-CIFAs may alter intracellular calcium levels and further contribute to endothelial dysfunction and inflammation in a mitochondria-mediated pathway. The importance of 2-ClHyA localization to the mitochondria and the impact on mitochondrial respiration needs to be examined in future studies.

Multiple lines of evidence support that the localization of the alkyne analog, 2-ClHyA, is analogous with the localization of 2-ClHA. First, 2-ClHyA localization to the Weibel-Palade bodies was reduced in competition studies with 10- to 20-fold molar excesses of 2-ClHA. In contrast, 2-ClHyA localization was not reduced in competition studies with 10- to 20-fold molar excesses of the nonchlorinated fatty acid, HA. These studies demonstrate the shared properties of 2-ClHA and 2-ClHyA while ruling out nonspecific lipid effects. Second, 2-ClHA and 2-ClHyA were shown to elicit similar metabolic responses from HCAECs, as assessed by the MTT assay. Finally, both 2-ClHA and 2-ClHyA, compared with HA, elicited...
nearly identical changes in multiple endothelial cell responses including: 1) increased plasma membrane surface expression of P-selectin and E-selectin; 2) increased neutrophil adherence to endothelial cells; 3) release of VWF; 4) increased platelet adherence to endothelial cells; 5) release of angiopoietin-2; and 6) decreased endothelial cell permeability barrier. Collectively, both the confocal microscopy studies and the functional studies support that 2-ClHyA is a 2-ClHA analog sharing the same subcellular localization and biological properties in HCAECs.

Additional studies investigated the properties of the nonchlorinated fatty acid, HA, on endothelial function. By using the click analog of HA to assess subcellular distribution, it was clear that the intracellular distribution of HyA in HCAECs was different than that of 2-ClHyA. HyA predominantly localized to the Golgi with some localization also observed in the mitochondria, ER, and Weibel-Palade bodies. In contrast to the robust increases in P-selectin and E-selectin in response to CIFA, there were minor, yet significant, increases in membrane surface expression of P-selectin and E-selectin in response to HA. Minor changes elicited by HA were accompanied with corresponding levels of functional outcomes, including modest neutrophil adherence to and reduced resistance across HCAECs. The changes in endothelial barrier function elicited by HA have been reported by others, demonstrating that the permeability of the endothelium is impacted by HA at high concentrations (>20 µM) for long time periods (24 h) (52).

Endothelial cell biology is modulated by the status of Weibel-Palade bodies, which store multiple bioactive proteins poised to be released from the endothelial cell (38, 53, 54). The present findings suggest an intimate relationship between neutrophils and the endothelium mediated by neutrophil-derived lipid products that potentially enhance blood-endothelial interaction due to 2-CIFA targeting Weibel-Palade bodies. A key research direction that remains is revealing the underlying mechanism responsible for 2-CIFA selectively associating with Weibel-Palade bodies. We speculate that there are specific proteins associated with Weibel-Palade bodies that bind 2-CIFA. In addition to determining the mechanism responsible for 2-CIFA association with Weibel-Palade bodies, it will also be important to determine the mechanism by which 2-CIFA causes Weibel-Palade body mobilization. The changes in shape of VWF-stained Weibel-Palade bodies may indicate that 2-CIFA leads to acidification. Both actomyosin-dependent and -independent mechanisms have been attributed to Weibel-Palade body un packaging (51, 55), and our observations in morphological changes in VWF staining are consistent with both of these mechanisms.

These studies used a click analog of the novel neutrophil-derived oxidation product, 2-CIFA, to gain insights into the subcellular localization in neighboring endothelial cells. Surprisingly, these experiments revealed that 2-CIFA specifically localizes to the Weibel-Palade bodies in HCAECs. This revelation led to the discovery that 2-CIFA leads to profound changes in endothelial biology through the mobilization of the contents of Weibel-Palade bodies. Thus, 2-CIFA targeting the Weibel-Palade bodies causes endothelial cell dysfunction, which may alter multiple processes, such as leukocyte extravasation, thrombosis, and endothelial barrier function.

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