12/15-Lipooxygenase Mediates High-fat Diet-induced Endothelial Tight Junction Disruption and Monocyte Transmigration

A NEW ROLE FOR 15(S)-HYDROXYEICOSATETRAENOIC ACID IN ENDOTHELIAL CELL DYSFUNCTION*

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Background: The purpose of this study is to test the role of 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) in endothelial barrier function.

Results: 15(S)-HETE by increasing zonula occluden (ZO)-2 tyrosine phosphorylation disrupts tight junctions and thereby increases endothelial barrier permeability.

Conclusion: 12/15-LO and its arachidonic acid metabolite, 15(S)-HETE, play a crucial role in endothelial dysfunction.

Significance: 12/15-Lipooxygenase by increasing endothelial barrier permeability could facilitate monocyte/macrophage transmigration and enhance vascular inflammatory disease.

A convincing body of evidence suggests that 12/15-lipooxygenase (12/15-LO) plays a role in atherosclerosis. However, the mechanisms of its involvement in the pathogenesis of this disease are not clear. Therefore, the purpose of this study is to understand the mechanisms by which 12/15-LO mediates endothelial dysfunction. 15(S)-Hydroxyeicosatetraenoic acid (15(S)-HETE), the major 12/15-LO metabolite of arachidonic acid (AA), induced endothelial barrier permeability via Src and Pyk2-dependent zonula occluden (ZO)-2 tyrosine phosphorylation and its dissociation from the tight junction complexes. 15(S)-HETE also stimulated macrophage adhesion to the endothelial monolayer in Src and Pyk2-dependent manner. Ex vivo studies revealed that exposure of arteries from WT mice to AA or 15(S)-HETE led to Src-Pyk2-dependent ZO-2 tyrosine phosphorylation, tight junction disruption, and macrophage adhesion, whereas the arteries from 12/15-LO knock-out mice are protected from these effects of AA. Feeding WT mice with a high-fat diet induced the expression of 12/15-LO in the arteries leading to tight junction disruption and macrophage adhesion and deletion of the 12/15-LO gene disallowed these effects. Thus, the findings of this study provide the first evidence of the role of 12/15-LO and its AA metabolite, 15(S)-HETE, in high-fat diet-induced endothelial tight junction disruption and macrophage adhesion, the crucial events underlying the pathogenesis of atherosclerosis.

Cardiovascular diseases are the leading cause of clinical deaths and disability in the United States as well as in the rest of the world (1, 2). Atherosclerosis, which accounts for the majority of the cardiovascular disease-related deaths, is a chronic vascular inflammatory disease and is characterized by excessive deposits of oxidized lipids, inflammatory cells, and calcium in the vessel wall (3). Although the list of the causative factors of atherosclerosis keeps growing (4), the widely accepted risk factors for this disease are hypercholesterolemia, hyperglycemia, dyslipidemia, diabetes, obesity, and smoking (2). These risk factors are also associated with induced expression or enhanced activity of 12/15-lipooxygenase (12/15-LO)2 (5–8). LOs are iron containing dioxygenases that catalyze stereospecific incorporation of molecular oxygen into cis-polyunsaturated fatty acids resulting in the formation of hydroperoxyeicosatetraenoic acids, which are nonenzymatically converted to hydroxyeicosatetraenoic acids (HETEs) (9–11). 15-LO1 and its murine ortholog, 12/15-LO, metabolize arachidonic acid (AA) mainly to 12(S)- and 15(S)-hydroxyeicosatetraenoic acids (11, 12). In addition to free fatty acids, 12/15-LO was shown to oxidize low-density lipoprotein (LDL) directly in a cell-free system (13). Forced expression of 15-LO1 in cultured fibroblasts and rabbit arteries also resulted in increased oxidation of LDL compared with their controls (14, 15). The role of oxidized LDL (oxLDL) in atherosclerosis is indisputable, and the appearance of oxLDL in the vessel wall is the hallmark of the development of atherosclerosis (16, 17). Based on the ability of 12/15-LO to oxidize LDL, many studies have proposed that 12/15-LO plays a major role in the development of atherosclerosis (15, 18). However, how 12/15-LO modifies LDL is not clear.

LOs are mainly located in the cytoplasm, and their secretion has not been reported. Thus, a direct interaction between LOs and LDL is highly unlikely and is not documented in vivo. Several models were proposed to explain the role of 12/15-LO in the oxidation of LDL. Zhu et al. (19) proposed that upon expo-

1 The abbreviations used are: 12/15-LO, 12/15-lipooxygenase; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; m.o.i., multiplicity of infection; ZO, zonula occluden; AJ, adherens junction; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; TJ, tight junction; BCECF-AM, 2',7'bis(carboxyethyl)-5-(and 6)carboxyfluorescein acetoxymethyl ester.

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sure to LDL, 12/15-LO in peritoneal macrophages translocates to the plasma membrane where it oxidizes the LDL particles. Thus, although the proposed mechanisms for the involvement of 12/15-LO in atherosclerosis are debatable, the role of this enzyme in the pathogenesis of this disease is evident from the observations that: (i) incubation of atherosclerotic arteries but not healthy arteries converted AA to 15-HETE (20); (ii) selective inhibition of 12/15-LO attenuated high-fat diet-induced atherosclerosis (21); (iii) induced expression of 15-LO was detected in atherosclerotic lesions (22); (iv) overexpression of human 15-LO in vascular wall of LDLR−/− mice showed enhanced atherosclerosis (23); and (v) disruption of 12/15-LO gene in ApoE-deficient mice substantially reduced atherosclerotic lesion formation (24). Despite evidence for the role of 12/15-LO in atherosclerosis, the mechanisms by which it promotes this vascular disease are unclear.

The endothelium forms a continuous inner lining of the blood vessels that provides a selective nonthrombogenic permeability barrier between the vascular wall and blood (25, 26). In inflammatory conditions, the endothelial cells undergo phenotypic changes as characterized by the loss of their barrier function and increased leukocyte adhesion (26). Endothelial dysfunction is generally regarded as the initial step in atherosclerotic plaque formation. In the initial stages of endothelial dysfunction, tight junctions (TJs) between the endothelial cells are disrupted leading to increased paracellular permeability, also known as type I endothelial cell activation. During the subsequent type II endothelial activation, expression of inflammatory and adhesion molecules is triggered, leading to the recruitment of monocytes/macrophages to the endothelium and their subsequent transendothelialization (26). In this study, we demonstrate for the first time a new mechanism for the role of 12/15-LO in high-fat diet-induced endothelial cell (EC) barrier dysfunction. Our findings show that 15(S)-HETE, the 12/15-LO metabolite of AA, induces an EC barrier disruption by Src- and Pyk2-dependent tyrosine phosphorylation of zonula occluden (ZO)-2 and the dissociation of ZO-2 from the endothelial tight junctions (TJs), enhancing transendothelialization of monocytes/macrophages. In addition, feeding mice with a high-fat diet induces the expression of 12/15-LO in the aorta, disassembles ZO-2 from the endothelial TJs, and increases the adhesion of circulating monocytes to the TJ-disrupted endothelium. These effects were all attenuated in 12/15-LO−/− mice.

**MATERIALS AND METHODS**

**Reagents**—5(S)-HETE (34230), 12(S)-HETE (34570), and 15(S)-HETE (34720) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Recombinant human VEGF-165 (293-VE-010) was from R & D Systems (Minneapolis, MN). FITC-conjugated dextran (FD705) was obtained from Sigma. Anti-claudin-1 (51–9000 and 35–2500), anti-claudin-5 (34–1600 and 35–2500), anti-ZO-2 (71–1400 and 37–4700), BCECF-AM (B1170), Hoechst 33342 (10 mg/ml) solution (H3570), goat anti-rabbit conjugated with Alexa Fluor 568 (A11011), goat anti-mouse conjugated with Alexa Fluor 488 (A11029), and goat anti-rat conjugated with Alexa Fluor 350 (A21093) secondary antibodies, ProLong Gold Antifade mounting solution (P36930), pAd/CMV/V5-GW/lacZ vector, Medium 200 (M200500), low serum growth supplements (S003K), gentialycin/amphotericin solution (R01510), and TRZol reagent (15596–026) were from Invitrogen. High capacity cDNA reverse transcription kit (4374966) was from Applied Biosystems (Foster City, CA). Anti-Pyk2 antibodies (SC-74539) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-p-Pyk2-Tyr402 (3291S) and anti-p-Src-Tyr416 (2101S) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-PY20 (05–777) and anti-Src (05–184) antibodies and cell culture inserts (8.0 µm) for cell migration assay (P18P01250) were from Millipore (Temecula, CA). Poly carbonate membrane transwell inserts with 0.4-µm pores (3401) were purchased from Corning Inc. (Tewksbury, MA). Matrigel (354230) and rat anti-mouse Mac-3 antibodies (553322) were obtained from BD Biosciences. Pyk2 inhibitor, PF-431396, was purchased from Tocris Bioscience (Bristol, UK). Src inhibitor PP1 (PHZ1213) was from BioSource (Camarillo, CA). The CD11b MicroBeads based mouse monocye isolation kit (130–049-601) was from Miltenyl Biotech (Auburn, CA). Protein A-Sepharose (17–0780-01), Protein G-Sepharose (17–0618-01), and Ficoll-Paque (17–5446-02) were obtained from GE Healthcare. All the primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were obtained from Invitrogen and cultured in Medium 200 containing low serum growth supplements, 10 µg/ml of gentialycin, and 0.25 µg/ml of amphotericin B. THP-1 cells were purchased from ATCC and grown in RPMI 1640 medium containing 50 µM 2-mercaptoethanol, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml of streptomycin. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO2 atmosphere. HUVECs were grown to confluence in Medium 200 for 6 h and used to perform the experiments unless otherwise indicated.

**Animals**—C57BL/6 mice and 12/15-LO−/− mice (B6.129S2-Alox15tm1Fun/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained according to the institutional animal facility guidelines. All the experiments involving the use of animals were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, TN. To study diet-induced atherosclerosis, 8-week-old mice were placed on control chow diet or Western diet containing 21.2% fat, 0.2% cholesterol, 48.5% carbohydrate, and 17.3% protein (TD88137, Harlan Teklad, Madison, WI) for 3 months. All 12/15-LO−/− mice used in this study were genotyped using DNA isolated from tail biopsy and the following primers, common forward, 5′-GGC TGC CTA TGG AAT AGG TAC AG-3′; wild type reverse, 5′-CCA TAG AGC AGA CCA GCA CA-3′; and mutant reverse, 5′-GGG AGG ATT GGG AAC ACA AT-3′.

**Adenoviral Vectors**—Construction of Ad-GFP, Ad-Src, and Ad-Pyk2 were described previously (27, 28). To generate AdlacZ, pAd/CMV/V5-GW/lacZ was digested with Pael, gel purified, and transfected into HEK293A cells. The adenovirus was purified by cesium chloride gradient ultracentrifugation (29). Wherever adenoviral vectors were used, cells were transduced with adenovirus harboring GFP or the target molecule at
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40 m.o.i. overnight in complete medium. After transductions, cells were grown in serum-free medium 200 without low serum growth supplement for 48 h and used as needed.

Western Blot Analysis—HUVECs with and without an appropriate treatment were lysed, and cell extracts were prepared using cell lysis buffer (20 mm HEPES, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 mM sodium fluoride, and 1 mM sodium orthovanadate). Equal amounts of protein from control and each treatment were separated by SDS-PAGE and immunoblotted for the indicated molecules using their specific antibodies. The antigen-antibody complexes were detected by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare). The band intensities were quantified using NIH Image J.

RT-PCR—Mouse aortas were dissected out, washed in 1× PBS, and cleaned from connective and fatty tissues under a stereoscopic microscope (SMZ645, Nikon, Tokyo, Japan). Cleaned aortas were minced and homogenized with TRizol using a Dounce homogenizer. RNA from the homogenate was isolated following the manufacturer’s instructions (Invitrogen). The cDNA was generated from the isolated RNA using High Capacity cDNA reverse transcription kit following the supplier’s protocol (Applied Biosystems) and the cDNA was then diluted to 15–100 ng/μl. The cDNA was then amplified using NIS Elements AR 4.0 imaging software (Nikon, Tokyo, Japan).

Endothelial Cell Barrier Permeability—HUVECs were seeded on the apical side of the polycarbonate membrane transwell inserts with 0.4-μm pores, allowed to grow to full confluence for the formation of a monolayer, and quiesced for 6 h. FITC-conjugated dextran (Mw ~70,000) was added (100 μg/ml) to the basal chamber. 15(S)-HETE (0.1 μM) was added to both the apical and basal chambers, and, after 2 h, 100 μl of the medium from each chamber was collected, and the fluorescence intensity was measured using SpectraMax microplate fluorometer (Molecular Devices). The flux was expressed as % dextran diffused/h/cm².

Immunofluorescence—HUVECs were grown on cover slides and coverslips to confluence, quiesced, and treated with vehicle or 15(S)-HETE (0.1 μM) for various time periods. After treatment, cells were washed with PBS, fixed with 3% paraformaldehyde for 10 min at 37 °C, permeabilized in TBS containing 3% BSA and 0.1% Triton X-100 for 10 min, and blocked in 3% BSA for 1 h at room temperature. Cells were then incubated with rabbit anti-ZO-2 antibodies in combination with mouse anti-claudin-1 or anti-claudin-5 antibodies followed by goat anti-rabbit and goat anti-mouse secondary antibodies conjugated with Alexa Fluor 568 and Alexa Fluor 488, respectively, counterstained with Hoechst 33342 (1:3000 dilution in PBS) for 1 min at room temperature, and mounted onto glass slides with Prolong Gold antifade mounting medium. To study the integrity of TJ's of mouse aorta, the aortas were dissected out from mice, washed, cleaned from connective and fatty tissues under a stereoscopic microscope, opened longitudinally, subjected to appropriate treatments, washed with PBS, fixed with 3% paraformaldehyde solution containing 0.2% picric acid for 10 min at 37 °C, permeabilized in TBS containing 3% BSA and 0.1% Triton X-100 for 10 min, and blocked in 3% BSA for 1 h at room temperature. The aortas were then incubated with rabbit anti-ZO-2 antibodies followed by goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 568 and mounted onto glass slides with Prolong Gold antifade mounting medium. To make cross-sections, after appropriate treatments, the aortas were washed with PBS, fixed with 3% paraformaldehyde for 10 min at 37 °C, and frozen in OCT compound. Cryosections (5 μm) were made using a Leica cryostat (model CM3050S; Wetzlar, Germany) and stained for ZO-2 as described above. Fluorescence images of cells and artery cross-sections were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a ×40 NA 0.6 objective and AxioCam MRm camera without any enhancements. In the case of longitudinally opened arteries, a stack of fluorescence images were acquired over a 1.5 to 2.0-μm Z-focus range and the composite images were generated using Zeiss Extended Focus module. Localization of ZO-2 and claudins in the TJ's were calculated using NIS Elements AR 4.0 imaging software (Nikon, Tokyo, Japan).

Monocyte Transmigration—To study the transmigration of THP-1 cells through HUVEC monolayer, 8.0-μm cell culture transwells (Millipore) were coated with Matrigel (50%) on the dorsal side, and HUVECs were seeded on the apical side, allowed to grow to a confluent monolayer, and quiesced. Whenever adenoviral vectors were used, HUVECs were transduced with control and the indicated adenovirus prior to seeding onto the transwell. BCECF-AM-labeled quiescent THP-1 cells (1 × 10⁵ cells/well) were seeded onto the HUVEC monolayer, and treated with vehicle or 15(S)-HETE (0.1 μM) for 6 h. At the end of the treatment period, cells from the apical side were removed by a cotton swap, the membrane was cut, fixed, and the THP-1 cells transmigrated through the HUVEC monolayer to the dorsal side of the membrane were observed under a Zeiss inverted microscope (Zeiss AxioObserver Z1, type, plan-Apochromat; magnification, ×10/0.45 NA) and fluorescence images were captured with a Zeiss AxioCam MRm camera using the microscope operating and image analysis software AxioVision Version 4.7.2 (Carl Zeiss Imaging Solutions GmbH).

Monocyte Adhesion—Circulating monocytes were isolated by magnetic cell sorting method using CD11b microbeads (Miltenyi Biotech, Auburn, CA) from mouse blood. C57BL/6 (WT) mice were anesthetized by isoflurane inhalation, and peripheral blood was drawn via cardiac puncture. Blood (0.8 to 1 ml) was collected from each mouse in 150 μl of anti-coagulant (100 mM sodium citrate and 130 mM glucose, pH 6.5). The blood was diluted by 4-fold with Hanks’ balanced salt solution, layered on Ficoll-Paque (density 1.084 g/ml, GE Healthcare),...
and centrifuged for 20 min at 1000 × g at room temperature. The leukocyte-rich interface was collected and washed twice with PBS, and the CD11b-positive cells were isolated following the manufacturer’s protocol. Aortas from WT and 12/15-LO−/− mice were dissected out and cleaned free of connective and fatty tissues under a stereoscopic microscope. After appropriate treatments, each aorta was injected with 6000 to 8000 circulating monocytes isolated from WT mice, and the open ends of the aorta were closed by 6–0 nylon suture knots and incubated in DMEM containing 10% FBS for 30 min at 37 °C. The monocytes were prelabeled with BCECF-AM following the supplier’s instructions (Invitrogen). At the end of the incubation period, knots were released, and the nonadherent monocytes were flushed out by PBS. The aortas were longitudinally opened, fixed with 3% paraformaldehyde for 30 min and 3% paraformaldehyde containing 0.2% picric acid for 1 h at 37 °C, permeabilized in TBS containing 3% BSA and 0.1% Triton X-100 for 10 min, and blocked in 3% BSA for 1 h at room temperature. The aortas were then incubated with rabbit anti-ZO-2 antibodies followed by goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 568 and mounted onto glass slides with Prolong Gold antifade mounting medium. To study high-fat diet-induced recruitment of monocytes onto the endothelium, after feeding with chow diet or high-fat diet for 3 months, mice were anesthetized, perfusion fixed by injecting PBS containing 3% paraformaldehyde, and aortas were isolated and cleaned as described above. After permeabilizing, they were incubated with rabbit anti-ZO-2 and rat anti-Mac-3 antibodies followed by goat anti-rabbit and goat anti-rat secondary antibodies conjugated with Alexa Fluor 568 and Alexa Fluor 350, respectively. Fluorescence images of the luminal side of the aorta were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a × 40 NA 0.6 objective and AxioCam MRm camera without any enhancements.

**Statistics—**All the experiments were repeated three times and data are presented as mean ± S.D. Flux and transmigration assays include triplicates in each experiment and in the case of microscopic studies, six fields were examined for each group. The treatment effects were analyzed by Student’s t test, and the p values <0.05 were considered statistically significant. In the case of RT-PCR, Western blotting, and fluorescence imaging, one representative set of data is shown.

**RESULTS**

15(S)-HETE Increases EC Permeability—Loss of barrier function is a characteristic feature of endothelial cell dysfunction, which is the foremost initiation point of atherosclerosis (26). Because the majority of atherogenic risk factors are associated with increased 12/15-LO expression (5–8), and disruption of 12/15-LO gene-protected ApoE−/− mice from developing high-fat diet-induced atherosclerosis (24), we studied the role of 12/15-LO in EC barrier dysfunction. A quiescent monolayer of HUVECs, when treated with 5-, 12-, and 15(S)-HETE, the AA cleavage products of 5-, 12-, and 15-LO, the paracellular permeability of the monolayer, as measured by passive diffusion of FITC-conjugated dextran, significantly increased (Fig. 1A). Both 12(S)-HETE and 15(S)-HETE increased EC permeability almost to the same extent, and their effects were found to be equivalent to those of VEGF-A, a well characterized vascular permeability factor (Fig. 1A). Because 15(S)-HETE is the major AA metabolite of human 15-LO, we focused further studies on this lipid molecule. Claudins, which are tetraspanins, form multimeric protein complexes with intracellular adaptor molecules, ZOs, and constitute the major components of EC TJs (30–32). Hence, to test if increased EC permeability by 15(S)-HETE was due to the disruption of TJs, we tested its effects on the levels of claudin-1, claudin-5, and ZO-2. We found that 15(S)-HETE had no significant effect on the levels of these TJ proteins (Fig. 1B). Several kinases and phosphatases were reported to regulate the assembly and/or disassembly of TJs via modulation of the phosphorylation state of TJ proteins in epithelial cells as well as ECs (33–37). Therefore, we next studied the phosphorylation of TJ proteins. 15(S)-HETE, without much effect on tyrosine phosphorylation of claudin-1 and claudin-5, increased the tyrosine phosphorylation of ZO-2 by 8–10-fold (Fig. 1C). Because the tyrosine phosphorylation of TJ proteins is known to destabilize TJs (35, 36), we next studied the effect of 15(S)-HETE-induced tyrosine phosphorylation of ZO-2 on TJ stability. Claudin-1 and claudin-5 existed as a complex with ZO-2 in the resting HUVEC monolayer and, upon exposure to 15(S)-HETE, ZO-2 was found to dissociate from claudin-1 and claudin-5 in a time-dependent manner (Fig. 1D). To evaluate the implication of 15(S)-HETE-induced dissociation of claudins from ZO-2 on TJs, the quiescent HUVEC monolayer was treated with and without 15(S)-HETE for different time periods and immunostained for claudins and ZO-2. Resting HUVEC monolayer displayed intact and continuous TJs, and claudin-1, claudin-5, and ZO-2 were located mostly in the TJs (Fig. 1, E and F). Treatment with 15(S)-HETE caused a time-dependent dissociation of claudin-1, claudin-5, and ZO-2 from the TJs with a maximum effect at 30 min (Fig. 1, E and F). The 15(S)-HETE-induced dislocation of claudin-1, claudin-5, and ZO-2 from TJs appears to be transient as their reappearance in the TJs, albeit slightly, was observed at 120 min (Fig. 1, E and F).

Src and Pyk2 Mediate 15(S)-HETE-induced TJ Disruption—To understand the mechanisms of tyrosine phosphorylation of ZO-2 by 15(S)-HETE, we studied the role of nonreceptor tyrosine kinases, Src and Pyk2. 15(S)-HETE activated both Src and Pyk2 in a time-dependent manner with maximum effect at 30 min (Fig. 2A). Interfering with the activation of Src or Pyk2 by their dominant-negative mutants abolished 15(S)-HETE-induced ZO-2 tyrosine phosphorylation (Fig. 2B). Interestingly, we found that whereas blockade of Src activation inhibited Pyk2 tyrosine phosphorylation, interference with Pyk2 stimulation had no effect on Src tyrosine phosphorylation (Fig. 2B). These results suggest that Pyk2 is the downstream effector of Src in the mediation of 15(S)-HETE-induced ZO-2 tyrosine phosphorylation. Next, we tested the effect of dnSrc and dnPyk2 on 15(S)-HETE-induced dissociation of claudin-1 and claudin-5 from ZO-2. Treatment with 15(S)-HETE for 30 min led to dissociation of claudin-1 and claudin-5 from ZO-2 (Fig. 2C), and blockade of either Src or Pyk2 activation by their dominant-negative mutants attenuated this effect (Fig. 2C).

15(S)-HETE-induced EC TJ Disruption Promotes Monocyte Transmigration—If 15(S)-HETE-induced phosphorylation of ZO-2 leads to the dissociation of claudins from ZO-2 and...
thereby disrupts TJs, interfering with ZO-2 phosphorylation by blockade of Src or Pyk2 activation should prevent 15(S)-HETE-induced TJ disruption. To test this hypothesis, a quiescent HUVEC monolayer transduced with Ad-GFP, Ad-dnSrc, or Ad-dnPyk2 was treated with and without 15(S)-HETE for 30 min and immunostained for ZO-2, claudin-1, and claudin-5. As shown in Fig. 3, A and B, 15(S)-HETE induced the dislocation of both claudin-1 and claudin-5 from ZO-2 in the TJs to a signif-
**15(S)-HETE Disassembles Endothelial Tight Junctions**

**FIGURE 1.** 15(S)-HETE disrupts endothelial TJs. A, quiescent HUVECs were treated with vehicle, 0.1 μM of the indicated HETE, or 40 ng/ml of VEGF-A and dextran flux was measured as described under “Materials and Methods.” B, quiescent HUVECs were treated with vehicle or 15(S)-HETE (0.1 μM) for the indicated time periods and cell extracts were prepared and analyzed for claudin-1, claudin-5, and ZO-2 using their specific antibodies. The cell extracts were also analyzed for tyrosine phosphorylation of Src and Pyk2 as described in panel A. The blots were reprobed with anti-Src and anti-Pyk2 antibodies for normalization or to show the overexpression of dnSrc and dnPyk2. The bar graphs represent mean ± S.D. of three independent experiments. *, p < 0.01 versus vehicle control or Ad-GFP; †, p < 0.01 versus 15(S)-HETE or Ad-GFP + 15(S)-HETE. IB, immunoblot.

**FIGURE 2.** Src and Pyk2 via phosphorylation of ZO-2 disrupt its association with claudin-1 and claudin-5. A, quiescent HUVECs were treated with and without 15(S)-HETE (0.1 μM) for the indicated time periods and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for tyrosine phosphorylation of Src and Pyk2 using their specific antibodies. The blots were reprobed with anti-Src and anti-Pyk2 antibodies for normalization, and the immunocomplexes were analyzed by Western blotting for claudin-1, claudin-5, and ZO-2 using specific antibodies. The cell extracts were also analyzed for tyrosine phosphorylation of Src and Pyk2 as described in panel A. The blots were reprobed with anti-Src and anti-Pyk2 antibodies for normalization or to show the overexpression of dnSrc and dnPyk2. The bar graphs represent mean ± S.D. values of three independent experiments. *, p < 0.01 versus vehicle control or Ad-GFP; †, p < 0.01 versus 15(S)-HETE or Ad-GFP + 15(S)-HETE. IB, immunoblot.

**FIGURE 3.** 15(S)-HETE disrupts endothelial TJs. A, quiescent HUVECs, and the expression of dnSrc or dnPyk2 blocked this effect. To understand the functional significance of 15(S)-HETE-induced disruption of TJs, we measured the macromolecular transflux through the HUVEC monolayer in response to 15(S)-HETE. A quiescent HUVEC monolayer grown in transwells was treated with and without 15(S)-HETE and FITC-dextran (70,000 Da) was added to the basolateral side of the membrane. Passive diffusion of the dextran through the monolayer to the apical side of the membrane was quantitated by measuring the fluorescence intensity. 15(S)-HETE caused a 2-fold increase in the permeability of the HUVEC monolayer to dextran and this effect was blocked by Ad-dnSrc or Ad-dnPyk2 (Fig. 3C). Although many models have been proposed to explain how dysfunctional endothelium promotes leukocyte trafficking, only recently it has been shown that disruption of endothelial adherens junctions (AJs) enhances leukocyte transendothelial migration (38). To find out whether 15(S)-HETE-induced EC TJ disruption enhances transendothelial migration of monocytes, and if so, to study the role of Src and Pyk2, we plated BCECF-AM-labeled quiescent THP-1 cells on a HUVEC monolayer grown on a porous membrane (8.0 μm) support and treated with or without 15(S)-HETE. As measured by the appearance of THP-1 cells in the basal side of the membrane, 15(S)-HETE increased THP-1 cell transmigration 4-fold and this effect was completely blocked by dnSrc or dnPyk2 (Fig. 3D).
12/15-LO Gene Knock-out Protects Mice from High-fat Diet-induced Endothelial TJ Disruption—To study the role of 12/15-LO-15(S)-HETE in EC TJ disruption in vivo, we used a knock-out mouse model. It has been shown that both ECs and vascular smooth muscle cells express 12/15-LO (39, 40). To understand the role of 12/15-LO in EC TJ disruption, aortas from WT and 12/15-LO−/− mice were incubated with AA and 15(S)-HETE and analyzed for TJ integrity by immunostaining for ZO-2. As
observed in Fig. 4A, in WT aortas, TJs were disrupted both by the 12/15-LO substrate, AA, and its product, 15(S)-HETE. In the case of 12/15-LO \(^{-/-}\) aortas, AA even at 5-fold excess concentration had no effect on TJ disruption (Fig. 4B). However, as expected, addition of 15(S)-HETE did disrupt the TJs. These observations using arteries from WT and 12/15-LO \(^{-/-}\) mice in ex vivo show that 12/15-LO plays an important role in EC TJ disruption. To find whether 15(S)-HETE-induced TJ disruption in the arteries was due to Src and Pyk2-mediated ZO-2 tyrosine phosphorylation, we used a pharmacological approach. Incubation of arteries from WT mice with 15(S)-HETE increased ZO-2 tyrosine phosphorylation by 4-fold (Fig. 4C). PP1 and PF431396, potent inhibitors of Src and Pyk2, respectively (41, 42), completely blocked 15(S)-HETE-induced ZO-2 tyrosine phosphorylation. 15(S)-HETE-induced tyrosine phosphorylation of ZO-2 also caused its dissociation from Claudin-1 and Caludin-5 in a Src and Pyk2-dependent manner (Fig. 4C). Consistent with these observations, incubation of arteries from WT mice with 15(S)-HETE led to TJ disruption and these effects were also blocked by both PP1 and PF431396 (Fig. 4D). To understand the role of 12/15-LO in high-fat diet-induced TJ disruption, we used its knock-out mice. Deletion of 12/15-LO negated EC TJ disruption and monocyte adhesion were observed in aortas from WT mice that received a high-fat diet. Deletion of 12/15-LO negated EC TJ disruption and monocyte adhesion caused by the high-fat diet (Fig. 4B).

**DISCUSSION**

Four decades ago, "a specific form of endothelial injury" triggering smooth muscle cell proliferation was proposed as the key-escalating factor of atherosclerosis caused by hyperlipidemia (45, 46). The specific injury was later identified as endothelial barrier disruption, a characteristic feature of endothelial dysfunction (26). Endothelial barrier is a function of endothelial intercellular junctions that includes AJs and TJs (30). AJs participate in the initiation and maintenance of cell-to-cell and cell-to-substratum contacts, whereas TJs form stand-like structures between the neighboring ECs and regulate the paracellular permeability of the endothelium to ions and macromolecules (47). However, recent studies demonstrated that disruption of AJs results in endothelial hyperpermeability (38, 48). Although these studies linked the reported EC hyperpermeability to AJs disruption, it is quite possible that this could be due to perturbation in TJs as dynamic interactions between AJs and TJs exist (31, 49). It was also proposed that AJs are linked to the development of TJs (49), although some studies demonstrated that interfering with formation of AJs has no influence on TJ organization (50).

Endothelial TJs are comprised of transmembrane (occludin, claudins and junction adhesion molecules) and intracellular (Zona occludens 1–3, protein incorporated later into tight junctions and junction enriched and associated protein) proteins (30–32). Disruption in the interactions between these proteins perturbs the TJs, influencing the permeability of the endothelium (47). Many studies have reported that phosphorylation is one of the major mechanisms regulating TJ integrity (33–35). However, most of these studies were reported on epithelial TJs. The role of phosphorylation of TJ proteins in the regulation of endothelial TJ integrity has not been studied in detail. In this aspect, some studies showed that tyrosine phosphorylation of endothelial TJ proteins leads to loss of its barrier function (36). In the present study, we, for the first time, report that Src-Pyk2-mediated phosphorylation of ZO-2 results in TJ disassembly and enhances EC permeability in response to 15(S)-HETE. We also found that in 12/15-LO-15(S)-HETE-induced signaling leading to ZO-2 tyrosine phosphorylation, Src was an upstream regulator of Pyk2 activation. Previously, an association between Pyk2 and Src was reported in epithelial cells in response to receptor tyrosine kinase agonist EGF-induced wound healing (51). Dikic et al. (52) demonstrated that Pyk2 directly binds to Src and mediates its activation in PC12 cells in response to a G protein-coupled receptor agonist lysophosphatidic acid. In both studies, Pyk2 was observed to be the upstream modulator.
FIGURE 4. 12/15-LO plays an essential role in disassembly of ZO-2 from aortic endothelial TJs. A and B, aortas from WT (A) or 12-LO−/− (B) mice were dissected out, incubated with vehicle, AA (2.5 μM), or 15(S)-HETE (0.5 μM) for 30 min, fixed, and cryosections were made and stained for ZO-2 using its specific antibodies followed by Alexa Fluor 568-conjugated goat anti-rabbit antibodies. C, aortas from WT mice were incubated with vehicle or 15(S)-HETE (0.5 μM) in the presence and absence of PP1 (10 μM) or PF431396 (10 μM) for 30 min, tissue extracts were made and equal amounts of protein from control and each treatment were immunoprecipitated with anti-PY20 or anti-ZO-2 antibodies and the immunocomplexes were analyzed by Western blotting for ZO-2 and claudin-1, respectively, using their specific antibodies. The blot in the bottom panel was sequentially reprobed with anti-claudin-5 and anti-ZO-2 antibodies. D, arteries from WT mice were isolated, incubated with vehicle or 15(S)-HETE (0.5 μM) in the presence and absence of PP1 (10 μM) or PF431396 (10 μM) for 30 min, opened longitudinally, fixed, permeabilized, and incubated first with rabbit anti-ZO-2 antibodies followed by goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 568 and mounted onto glass slides with Prolong Gold antifade mounting medium. Fluorescence images of luminal side of the aortas were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a ×40 NA 0.6 objective and AxioCam MRm camera under Extended Focus module. The levels of ZO-2 in the TJs were calculated by measuring the percentage fluorescence localized in the TJs using Nikon’s NIS Elements AR 4.0 imaging software. The bar graphs represent mean ± S.D. of three independent experiments. *, p < 0.01 versus vehicle control; **, p < 0.01 versus 15(S)-HETE. Yellow and white arrowheads point out intact and disrupted TJs, respectively.
of Src activation. However, in the present study, we found that Src mediates Pyk2 activation in 15(S)-HETE-induced phosphorylation of ZO-2 leading to its dissociation from claudins-1/5 and thereby disrupting TJs. Based on these observations, it is conceivable that the mode of interactions between Src and Pyk2 differs in response to various agents in mediating specific cellular responses.

The hypothesis that endothelial TJs, by providing a static barrier to separate and protect thrombogenic matrix surface from flowing blood, understates the active functions of intact TJs (53). In addition to its permeability barrier function, endothelial TJs take part in cell signaling, maintenance of cell polarity, regulation of gene expression, and importantly provide “fencing” to the endothelium in intact vessels (53–55). During inflammation, endothelial TJs are disrupted, leading to activation of endothelial cells and enhancement of transendothelial migration of leukocytes (26), which marks the initiation of atherosclerosis (43, 44). In the present study, when the aortas were exposed to 12/15-LO substrate, AA, or its product, 15(S)-HETE, EC TJs were disrupted, resulting in enhanced monocyte adhesion. Studies involving aortas from 12/15-LO/H11002/H11002/H11002 revealed that oxidation of AA by 12/15-LO is essential for the observed disruption of EC TJs and the subsequent monocyte recruitment to the vessel wall. We also observed that the high-fat diet induces the expression of 12/15-LO in aorta by 3-fold. Furthermore, feeding mice a high-fat diet disrupted EC TJs and induced monocyte adhesion to the endothelium. Interestingly, deletion of the 12/15-LO gene in mice negated all these effects. Thus, these results demonstrate that 12/15-LO plays a crucial role in endothelial TJ disruption and monocyte recruitment, which is considered an important early event in the onset of atherosclerosis.

How disruption of endothelial TJs in intact arteries enhance monocyte recruitment is not known. However, one proposed mechanism is that dysfunctional endothelium expresses high levels of cell adhesion molecules such as intercellular adhesion molecule and vascular cellular adhesion molecule, which may aid in the adhesion of circulating leukocytes to the endothel-

FIGURE 5. 12/15-LO gene knock-out prevents high-fat diet-induced aortic endothelial TJ disassembly. A, WT and 12-LO−/− mice were fed with chow diet (CD) or high-fat diet (HFD) for three months. Upper panel, DNA was isolated from tail biopsy and analyzed by PCR using genotyping primers. Lower panel, aortas were dissected out and RNA was isolated and analyzed for 12/15-LO and β-actin mRNA levels by RT-PCR. B, all conditions were the same as in panel A except that aortas were dissected out, fixed, cryosections were made and immunostained for ZO-2 using its specific antibodies followed by Alexa Fluor 568-conjugated goat anti-rabbit secondary antibodies. Fluorescence images were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a ×40 NA 0.6 objective and AxioCam MRm camera without any enhancements. The bar graphs represent mean ± S.D. Six mice were used in each group. *, p < 0.01 versus CD.
lium, and the presence of inflammatory molecules at the site of inflammation may drive their migration through the endothelium (56). In line with this mechanism, studies by Bolick et al. (57) showed that 12/15-LO, via stimulating protein kinase Cε-dependent activation of NFκB, enhances intercellular adhesion molecule expression in endothelial cells and mediates endothelium-monocyte interaction. In addition, it was shown that loss of EC polarity triggered by TJ disruption influences NFκB activation leading to expression of inflammatory molecules (58). Furthermore, TJs have been reported to play a role in leukocyte extravasation (59, 60). Based on these observations as well as our findings that 12/15-LO-mediated disruption of TJs leads to monocyte adhesion, we believe that high-fat diet-induced expression of 12/15-LO enhances tyrosine phosphorylation of ZO-2, disassembles TJs, and damages the fencing function of the TJs, which may lead to the expression of cell adhesion and/or inflammatory molecules, thereby enhancing the recruitment of monocytes to the vessel wall, the events that exacerbate the progression of atherosclerosis. However, the present findings do not exclude the possibility, at least to some extent, that monocytes adhere to the endothelium, disrupt the TJs, and transmigrate through the disrupted TJs into the subendothelial space, and both of these events may be mediated via 12/15-LO expression/activation in response to high-fat diet feed.

FIGURE 6. 12/15-LO is essential for high-fat diet-induced endothelial TJ disruption and monocyte adhesion. A, aortas from WT and 12-LO−/− mice were dissected out, cleaned, and treated with vehicle, AA (2.5 μM), or 15(S)-HETE (0.5 μM) for 30 min. After treatment, 6000 to 8000 BCECF-AM prelabeled circulating monocytes (CD11b-positive cells) isolated from WT mice were injected, the open ends of the aorta were then closed and incubated for 30 min at 37 °C. At the end of the incubation period, the nonadherent monocytes were removed by washing with PBS. The aortas were opened longitudinally, fixed, permeabilized, blocked, and incubated with rabbit anti-ZO-2 antibodies followed by goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 568 and mounted onto glass slides with Prolong Gold antifade mounting medium. B, WT and 12-LO−/− mice were fed with CD or HFD for 3 months, anesthetized, perfusion fixed, aortas were isolated, cleaned, permeabilized, and incubated with rabbit anti-ZO-2 and rat anti-Mac-3 antibodies followed by goat anti-rabbit and goat anti-rat secondary antibodies conjugated with Alexa Fluor 568 and Alexa Fluor 350, respectively. Fluorescence images of the luminal side of the aortas were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a ×40 NA 0.6 objective and AxioCam MRm camera without any enhancements. Yellow and white arrowheads point out intact and disrupted TJs, respectively. The bar graphs represent mean ± S.D. Six mice were used in each group. *, p < 0.01 versus vehicle control or CD; **, p < 0.01 versus AA (WT) or HFD (WT).
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