12/15-Lipoxygenase-dependent ROS production is required for diet-induced endothelial barrier dysfunction

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Abstract  
To understand the mechanisms of 15(S)-HETE-induced endothelial cell (EC) barrier dysfunction, we examined the role of xanthine oxidase (XO). 15(S)-HETE induced junction adhesion molecule A (JamA) phosphorylation on Y164, Y218, and Y280 involving XO-mediated reactive oxygen species production and Src and Pyk2 activation, resulting in tight junction (TJ) disruption leading to increased vascular permeability in response to HFD. Besides, it selectively permits the movement of molecules into and out of the bloodstream, and this semipermeable capacity of the endothelial monolayer depends majorly on cell-to-cell connections, namely adherens junctions (3, 4). HFD mice showed enhanced XO expression and its activity in the artery, which was correlated with increased aortic TJ disruption and these responses were inhibited by allopurinol.

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Supplementary key words  
xanthine oxidase • tight junction • 15(S)-hydroxyeicosatetraenoic acid • 15-lipoxygenase • reactive oxygen species

Endothelium, which is constituted by a monolayer of endothelial cells (ECs), is the inner most lining of the blood vessels, provides nonplatelet adherent nonthrombotic surface to the circulating blood, and releases vasoactive substances involved in the regulation of vascular tone (1, 2). Besides, it selectively permits the movement of molecules into and out of the bloodstream, and this semipermeable capacity of the endothelial monolayer depends majorly on cell-to-cell connections, namely adherens junctions (3, 4). HFD mice showed enhanced XO expression and its activity in the artery, which was correlated with increased aortic TJ disruption and these responses were inhibited by allopurinol.

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Abbreviations: AA, arachidonic acid; AJ, adherens junction; CD, chow diet; EC, endothelial cell; HFD, high-fat diet; HUVEC, human umbilical vein endothelial cell; JamA, junction adhesion molecule A; LO, lipoxygenase; PBMC, peripheral blood mononuclear cell; RFU, relative fluorescence unit; rJamA, recombinant junction adhesion molecule A; ROS, reactive oxygen species; TJs, tight junctions; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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atherosclerosis (16, 17). Furthermore, some evidence suggests that XO, via production of ROS, is involved in EC dysfunction (18, 19).

Previous work from our laboratory showed that 15(S)-HETE, the 15-lipoxygenase (LO) metabolite of arachidonic acid (AA), disrupts EC TJs causing an increase in its barrier permeability (20, 21). We also found that 15(S)-HETE increases XO activity in macrophages (22). These observations led us to hypothesize that XO might be involved in 15(S)-HETE-induced EC barrier dysfunction. To test this, we have studied the role of XO in 15(S)-HETE-induced EC TJ disruption and barrier dysfunction. Our findings suggest that XO, via ROS production and Src and Pyk2 activation, mediates 12/15-LO-15(S)-HETE-induced junction adhesion molecule A (Jama) tyrosine phosphorylation leading to endothelial TJ disruption and barrier dysfunction in response to high-fat diet (HFD) consumption.

MATERIALS AND METHODS

Reagents

5(S)-HETE (3420), 12(S)-HETE (34570), 15(R)-HETE (34710), 15(S)-HETE (34720), and XO kit (10010895) were purchased from Cayman Chemical Co. (Ann Arbor, MI). P-F3343 was obtained from Toeris Bioscience (Bristol, UK), BioSource (Camarillo, CA). Growth factor-reduced Matrigel (354520) was purchased from BD Biosciences (Bedford, MA). Harlan Teklad (Madison, WI). All primers and oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Cell culture

Human umbilical vein ECs (HUVECs) were obtained from Invitrogen and cultured in Medium 200 containing low serum growth supplements, 10 μg/ml gentamycin, and 0.25 μg/ml amphotericin B. THP1 cells which were supplied by the ATCC were grown in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Human peripheral blood mononuclear cells (PBMCs) were purchased from BioreclamationIVT (Baltimore, MD), suspended in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin and used in transmigration assays. Cultures were maintained at 37°C in a humidified 95% air and 5% CO2 atmosphere. HUVECs between 6 and 10 passages were used, cells are transduced with the indicated adenovirus at 40 multiplicity of infection (moi) overnight in complete medium. After transductions, cells were allowed to reach to 70% confluency in complete medium and growth-arrested for 24 h before being used.

Adenoviral vectors

Construction of Ad-GFP, Ad-dnSrc, Ad-dnPyk2, and Ad-LacZ are described previously (20). Wherever adenoviral vectors were used, cells were transduced with the indicated adenovirus at 40 multiplicity of infection (moi) overnight in complete medium. After transductions, cells were allowed to reach to 70% confluency in complete medium and growth-arrested for 24 h before being used.

Animals

WT (C57BL/6) and 12/15-LO−/− mice (B6129S-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 animals were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, TN. To study the diet-induced effect on 15(S)-HETE, mice were kept on chow diet (CD) or HFD for 3 months. Mice were allowed to eat ad libitum. When mice were administered with allopurinol, it was added (6.8 g/l) to drinking water ad libitum.

Western blot analysis

Mice were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, TN. To study the diet-induced effect, mice were kept on chow diet (CD) or HFD for 3 months. Mice were allowed to eat ad libitum. When mice were administered with allopurinol, it was added (6.8 g/l) to drinking water ad libitum.

Mass spectroscopy

Quiescent HUVECs were treated with 0.1 μM 15(S)-HETE for 30 min, immunoprecipitated with anti-Jama antibodies, and the software and expressed as relative fluorescence units (RFUs). Levels were quantified with Nikon’s NIS Elements AR 3.1 imaging software and expressed as relative fluorescence units (RFUs).

Immunoprecipitation

An equal amount of protein from cell or tissue extract was immunoprecipitated with anti-Jama antibodies, and the immunoprecipitate was subjected to SDS-PAGE and Western blot analysis.
immunocomplexes were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue R250 staining. The JamA band was excised and subjected to in-gel digestion with trypsin. The resulting peptides were analyzed by LC-MS/MS to identify the phosphorylated amino acid residues at the Bioanalytical and Mass Spectrometry facility at University of Alabama at Birmingham School of Medicine as described previously (23).

**Construction of JamA expression vector**

The human JamA (NM_016946) coding sequence was cloned from enriched cDNA by PCR amplification. RNA was isolated from HUVECs and cDNA synthesized by reverse transcription reaction with Superscript III First-Strand synthesis system for RT-PCR (catalog number 1808051, Invitrogen) following the manufacturer’s instructions. To enrich JamA mRNA, PVDF membrane (≈3 mm²) pieces were cultured with primers 5'-CTTCTATGCTTCCTGAGGGAG-3' and 5'-ATACCCATTCCGGCTACACAG-3' were inoculated with cDNA at ambient temperature for 30 min and eluted in 10 mM Tris-HCl (pH 8.0) for 5 min at 60°C. The eluted cDNA was used as a template to amplify JamA coding region using the forward primer 5'-ATGGGGACAAAGGCAGGAATGGGTAGAAGGTGGGGAAC-3' and the reverse primer 5'-TCACACCAGGAAATGAGGAGGTT-3' with the high fidelity TaKaRa Ex Taq DNA polymerase. Restriction sites BglII and KpnI were incorporated to the amplified JamA coding region by PCR using the forward primer 5'-TCGACCCGGCCCTAGCTCCAGGAGGAATGGGTAGAAGGTGGGGAAC-3' and the reverse primer 5'-GTCGAGGCCTAGAGGTT-3' (the restriction enzyme sites are underlined). The resulting 930 bp PCR product was then digested with BglII and KpnI and then inserted into the BglII/KpnI digested pCMV-Myc-C vector (catalog number 635689, Clonetech) to generate recombinant JamA vector. The vector was then sequenced and DNA sequencing.

**Transmigration**

Endothelial transmigration was measured as described previously (21). Whenever adenoviral vectors were used, HUVECs were transduced with the control or the indicated adenovirus prior to seeding onto the transwell. In the case of inhibitors, they were added to cells just 30 min prior to the treatment. HUVECs were treated with or without 15(S)-HETE for 30 min, at which time the BCEO-FAM-labeled quiescent THP1 cells (1 × 10⁵ cells/well) or PBMCs (1 × 10⁵ cells/well) were added and incubation continued overnight at 37°C. The transmigration of THP1 cells or PBMCs through the HUVEC monolayer was measured by capturing the images by a fluorescence microscope.

**ROS production**

Intracellular ROS production was measured using membrane permeable CM-H₂DCFDA as described previously (22). After the treatment, the cells were washed with 10 mM CM-H₂DCFDA in PBS and then suspended in 300 μl of the resuspending solution and incubated in the dark for 30 min at 37°C before the measurement. ROS production was measured using SpectraMax Gemini XS spectrofluorometer (Molecular Devices). Wherever adenoviral vectors or plasmids were used, cells were transduced or transfected with these viral or plasmid vectors, respectively, prior to seeding onto the transwell. The flux was expressed as percent dextran diffused per hour per square centimeter.

**Site-directed mutagenesis**

The tyrosine (Y) residues at 52, 83, 107, 164, 218, and 280 in JamA were mutated to phenylalanine (F) with QuickChange site-directed mutagenesis kit (catalog number 200519, Stratagene). The Y52F, Y83F, Y107F, Y164F, Y218F, and Y280F mutants were amplified using primers with 930 bp PCR product was then digested with BglII and KpnI and then inserted into the BglII/KpnI digested pCMV-Myc-C vector (catalog number 635689, Clonetech) to generate recombinant JamA vector. The vector was then sequenced and DNA sequencing.

**Flow assay**

HUVECs were grown to a monolayer on the apical side of a polycarbonate membrane of the transwell (0.4 μm pore size) and growth-arrested for 6 h. The monolayer was treated with and without 15(S)-HETE for 30 min, at which time, FITC-conjugated dextran (molecular mass ~70,000 Da) was added to the basal chamber at 100 μg/ml concentration, and after 2 h the fluorescence intensity of the medium from each chamber was measured using SpectraMax Gemini XS spectrophotometer (Molecular Devices).

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Fig. 1. 15(S)-HETE induces tyrosine phosphorylation of JamA in the disruption of TJs. A–C: Quiescent HUVEC monolayer was treated with vehicle or 15(S)-HETE (0.1 μM) for the indicated time periods and cell extracts were prepared. An equal amount of protein from control and each treatment was either analyzed by Western blotting for the steady state levels of JamA and occludin using their specific antibodies (A) or immunoprecipitated with pTyr (PY20) antibodies followed by immunoblotting with the indicated antibodies (B). C: Equal amounts of proteins from control and 30 min of the indicated HETE-treated HUVECs were analyzed for JamA tyrosine phosphorylation as described in (B). D: All the conditions were the same as in (B) except that an equal amount of protein from control and each treatment was immunoprecipitated with anti-JamA antibodies and the immunocomplexes were analyzed by Western blotting for the indicated proteins using their specific antibodies. E: Quiescent HUVEC monolayer was treated with vehicle or 15(S)-HETE (0.1 μM) for the indicated time periods, fixed, and double immunofluorescently stained for JamA and occludin using rabbit anti-JamA and mouse anti-occludin antibodies followed by developing with Alexa Fluor 568-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies, respectively. 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 TJ-localized JamA levels were quantified using NIS Elements AR 3.1 imaging software (Nikon, Japan). The bar graphs in B, D, and E represent mean ± SD values of three experiments. *P<0.05 versus vehicle control.
Fig. 2. Src and Pyk2 mediate 15(S)-HETE-induced JamA tyrosine phosphorylation. A: Cell extracts of control and the indicated time periods of 15(S)-HETE (0.1 μM)-treated HUVECs were analyzed by Western blotting for pSrc and pPyk2 levels using their phospho-specific antibodies and normalized for their total levels using their total antibodies. B, C: HUVECs that were transduced with Ad-GFP, Ad-dnSrc, or Ad-dnPyk2 (40 moi) and quiesced, were treated with vehicle or 15(S)-HETE (0.1 μM) for 30 min, and cell extracts were prepared. An equal amount of protein from control and each treatment was immunoprecipitated with anti-pTyr or anti-JamA antibodies and the immunocomplexes were analyzed by Western blotting for the indicated proteins. Equal amounts of protein from the same cell extracts were also analyzed by Western blotting for phospho and total Src and Pyk2 levels to show the effects of dnSrc and dnPyk2 on the phosphorylation of Src.
antibodies, followed by Alexa Fluor 568-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies. Fluorescence images of the luminal side of aortas were captured and quantified as described above in immunofluorescence staining of HUVECs.

Miles assay

The level of vascular permeability was determined by quantitative measurement of the Evans Blue dye diffusion into the aorta according to Miles test (24). WT and 12/15-LO\(^{-/-}\) mice that were on either CD or HFD for 3 months were anesthetized, and 0.1 ml of 1% Evans Blue dye was injected into the inferior vena cava. After 30 min, blood vessels were perfused with PBS through the left ventriculum and aortas were isolated. Evans Blue dye was extracted from the arteries by incubating in formaldehyde at 55°C for 24 h, cleared by centrifugation and the optical density was measured at 610 nm. Vascular permeability was expressed as the amount of Evans Blue extravasated per milligram of artery.

Statistics

All the experiments were performed three times and the data are presented as mean ± SD. The treatment effects were analyzed by one-way ANOVA followed by Student’s t-test and P values <0.05 were considered to be statistically significant.

RESULTS

15(S)-HETE induces tyrosine phosphorylation in the disruption of EC TJs

15(S)-HETE caused tyrosine phosphorylation in the endothelial monolayer, followed by the disruption of EC TJs. The disruption of EC TJs due to 15(S)-HETE occurred in a time-dependent manner with maximum increases between 5 min and 30 min and declining thereafter (Fig. 1B). The specificity experiment showed that 15(S)-HETE was found to be more potent than 5(S)-HETE or its enantiomer 15(R)-HETE in the stimulation of JamA tyrosine phosphorylation (Fig. 1C). However, 12(S)-HETE, that is preferentially produced by the murine ortholog of human 15-LO1, namely, 12/15-LO (25), also stimulated JamA tyrosine phosphorylation almost to the same level as that of 15(S)-HETE.

Because 15(S)-HETE induced JamA tyrosine phosphorylation more robustly than occludin, we next focused the rest of the study on its role in 15(S)-HETE-induced TJ disruption and barrier dysfunction. Coimmunoprecipitation assays showed that JamA dissociates from occludin in response to 15(S)-HETE in a time-dependent manner with a maximum effect at 30 min and starting reassociation at 120 min (Fig. 1D). JamA dissociation/reassociation with occludin was correlated with its state of tyrosine phosphorylation. Co-localization for JamA and occludin by double immunofluorescence staining revealed that both JamA and occludin were present in the TJs, and upon treatment with 15(S)-HETE, they both were found dislocated from the TJs (Fig. 1E).

Src and Pyk2 mediate 15(S)-HETE-induced JamA tyrosine phosphorylation

Previous work from our laboratory showed that Src and Pyk2 mediate 15(S)-HETE-induced ZO2 tyrosine phosphorylation leading to TJ disruption (20). Therefore, to understand the mechanism(s) involved in 15(S)-HETE-induced JamA tyrosine phosphorylation, we tested the role of Src and Pyk2. Based on our previous observations, 15(S)-HETE enhanced tyrosine phosphorylation of both Src and Pyk2 in a time-dependent manner with maximum increase at 30 min and declining thereafter (Fig. 2A). Coimmunoprecipitation assays showed that JamA disassociates from occludin in response to 15(S)-HETE-induced ZO2 tyrosine phosphorylation, and the phosphorylation of both Src and Pyk2 was attenuated at 30 min (Fig. 2B). The specificity experiment showed that 15(S)-HETE-induced JamA tyrosine phosphorylation is via Src-Pyk2 signaling and inhibition of JamA tyrosine phosphorylation by dnSrc and dnPyk2 is able to protect EC TJs and its barrier permeability from 15(S)-HETE-induced disruption.

Identification of the 15(S)-HETE-induced tyrosine phosphorylation sites in JamA

To identify the tyrosine residues phosphorylated by 15(S)-HETE, we performed mass spectrometry. We observed six tyrosine residues, namely Y52, Y83, Y107, Y164, Y218, and Y280, that are phosphorylated in 15(S)-HETE-treated ECs (Fig. 3A, B). To identify which of these tyrosine residues showed increased phosphorylation in

and Pyk2, respectively, or their over expression. D: All the conditions were the same as in (B) except that after quiescence, the HUVEC monolayer was treated with vehicle or 15(S)-HETE (0.1 μM) for 30 min and stained for JamA and occludin as described in Fig. 1E. E–G: All the conditions were as in (B) except that cells were subjected to 5(S)-HETE (0.1 μM)-induced dextran flux (E), monocyte (F), or leukocyte (G) transmigration assays. The bar graphs represent mean ± SD values of three experiments. *P < 0.05 versus vehicle control or Ad-GFP or Ad-LacZ; **P < 0.05 versus Ad-GFP + 15(S)-HETE or Ad-LacZ + 15(S)-HETE.
Fig. 3. Phosphorylation of tyrosine residues 164, 218, and 280 is required for 15(S)-HETE-induced JamA dissociation from occludin leading to EC TJ disruption and barrier dysfunction. A: The amino acid sequence of JamA is shown along with tandem MS confirmed phosphorylated amino acid residues (Y52, Y83, Y107, Y164, Y218, and Y280) as indicated in red/yellow. B: Quiescent HUVECs were treated with 15(S)-HETE (0.1 μM) for 30 min and cell extracts were prepared. Cell extracts were immunoprecipitated with anti-JamA antibodies and resolved on SDS-PAGE, stained with Coomassie Brilliant Blue, and the molecular mass band corresponding to JamA was subjected to high resolution mass spectrometric analysis (Orbitrap Velos) with fragmentation carried out in both Collision-induced dissociation (CID) and Electron transfer dissociation (ETD) modes. The resulting MS/MS scans are shown for the confirmed phosphorylated tyrosine residues indicated in (A). The phosphorylated amino acid residues for each spectra indicated were manually confirmed and included a minimal probability score of 100%, localization score of >98%, A-score of >20 (with the exception of Y107 at 13.4), charge state of ±2, delta ppm of ±3, and Sequest “XCorr” score of >4.0 (with the exception of Y280 at 3.8). Of note, all cysteine residues were carbamidomethylated with iodoacetamide resulting in an increased mass of 57.0293 Da. C, D: HUVECs were transiently transfected with empty vector (EV) or Myc-tagged rJamA expression vector with or without Y52F, Y83F, Y107F, Y164F, Y218F, or Y280F mutations, grown to confluence, quiesced, treated with vehicle or 15(S)-HETE (0.1 μM) for 30 min and an equal amount of protein from control and each treatment was immunoprecipitated with anti-pTyr or anti-Myc antibodies and the immunocomplexes were analyzed by Western blotting using its specific antibodies to show the expression of rJamA. E: All the conditions were the same as in (C) except that after quiescence and
response to 15(S)-HETE, we constructed a Myc-tagged rJamA expression vector and mutated the tyrosine (Y) residues to phenylalanine (F) by site-directed mutagenesis. Then, ECs were transfected with WT or mutant rJamA expression vectors, treated with 15(S)-HETE, and the rJamA tyrosine phosphorylation and its association/dissociation with occludin was measured. In response to 15(S)-HETE, WT and Y52F, Y83F, and Y107F mutant rJamAs showed ~5- to ~7-fold increase in their tyrosine phosphorylation as compared with control (Fig. 3C). In contrast, Y164F, Y218F, and Y280F mutant rJamAs failed to be phosphorylated by 15(S)-HETE (Fig. 3C), suggesting that 15(S)-HETE stimulates JamA phosphorylation on these tyrosine residues. Consistent with their phosphorylation states, while the WT and Y52F, Y83F, and Y107F rJamA mutants showed dissociation from occludin, the Y164F, Y218F, and Y280F rJamA mutants remained complexed with endogenous occludin and TJs (Fig. 3D, E). Similarly, while WT and Y52F, Y83F, and Y107F rJamA mutants failed to protect ECs from 15(S)-HETE-induced increase in their permeability, the Y164F, Y218F, and Y280F rJamA mutants exhibited resistance to this effect of 15(S)-HETE (Fig. 3F). These observations demonstrate that Y164, Y218, and Y280 residues of JamA are critical for its capacity to form a TJ complex with occludin and maintain barrier function.

XO-dependent ROS-induced JamA tyrosine phosphorylation and TJ disruption

To gain additional support for the role of JamA tyrosine phosphorylation in 15(S)-HETE-induced disruption of aortic endothelial TJs, we first tested the effect of catalase and its pharmacological inhibitors on 15(S)-HETE-induced change in XO activity and JamA tyrosine phosphorylation. Based on these observations, we examined the role of XO in 15(S)-HETE-induced Src, Pyk2, and JamA tyrosine phosphorylation as well as EC barrier dysfunction. Allopurinol abrogated 15(S)-HETE-induced tyrosine phosphorylation of Src, Pyk2, and JamA and prevented JamA dissociation from occludin (Fig. 4F–H). In accordance with these findings, double immunofluorescence staining for JamA and occludin pointed out that inhibition of XO activity by allopurinol prevents 15(S)-HETE-induced dissociation of JamA and occludin from TJs (Fig. 4I). 15(S)-HETE-induced EC permeability as well as monocyte transmigration through the EC monolayer were also attenuated by allopurinol (Fig. 4J, K). These results indicate that XO-dependent ROS production is required for 15(S)-HETE-induced Src-Pyk2-mediated JamA tyrosine phosphorylation, JamA dissociation from occludin, TJ disruption, and EC barrier dysfunction. These results infer that 15(S)-HETE-induced XO activity appears to be upstream to Src-Pyk2 via regulating JamA tyrosine phosphorylation.

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XO role in endothelial TJ disruption
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Fig. 4. XO-dependent ROS production is required for 15(S)-HETE-induced JamA tyrosine phosphorylation. A, B: Quiescent HUVECs were treated with vehicle or 15(S)-HETE (0.1 μM) for the indicated time periods and ROS production (A) and XO activity (B) were measured. C, D: Quiescent HUVECs were treated with vehicle or 15(S)-HETE (0.1 μM) in the presence and absence of either XO inhibitor, allopurinol (30 μM) (C) or PEG-catalase (25 U/ml) (D) for 30 min and ROS production was measured. E: All the conditions were the same as in (D) except that after the indicated treatments cell extracts were prepared and an equal amount of protein from control and each treatment was analyzed for phospho and total Src, Pyk2, and JamA levels as described in Fig. 2B. F–H: All the conditions were same as in (C) except that after the indicated treatments cell extracts were prepared and an equal amount of protein from control and each treatment was analyzed by Western blotting for pSrc and pPyk2 levels using their phospho-specific antibodies and the blots were reprobed for total Src and Pyk2 levels for normalization (F) or immunoprecipitated with anti-pTyr (G) or anti-JamA (H) antibodies and the immunocomplexes were analyzed by Western blotting for JamA and occludin using their specific antibodies. In (G), an equal amount of protein from
enhanced trapping of leukocytes onto the dysfunctional endothelium.

**Deletion of 12/15-LO gene blocks XO activity and protects aortic endothelial TJs from HFD-induced disruption**

In humans the sources of 15(S)-HETE production are 15-LO1 and 15-LO2 (25, 28, 29); however, in rodents neither of these LOs is expressed. Rather, 12/15-LO, the mouse homolog of 15-LO1 is expressed and possesses the capacity to convert AA to 12(S)-HETE and 15(S)-HETE, and both appear to exhibit at least some similar cellular responses (30, 31). Therefore, to validate the role of 15(S)-HETE in EC TJ disruption and barrier dysfunction, here we used a 12/15-LO knockout mouse model. WT, but not 12/15-LO<sup>−/−</sup>, mice fed with HFD for three months showed an increase in the expression of XO and its activity as compared with control diet (Fig. 7A–C). Consistent with these results, feeding with HFD also disrupted aortic EC TJs, as demonstrated by decreased JamA staining, and this effect was correlated with an increase in aortic endothelial permeability and leukocyte adhesion only in WT mice, but not in 12/15-LO<sup>−/−</sup> mice (Fig. 7D, E). To confirm the role of XO in HFD-induced vascular permeability, WT mice were fed with HFD, alone or in combination with administration of allopurinol for 3 months, and XO expression, along with Pyk2, and JamA tyrosine phosphorylation from occludin, endothelial TJ integrity, and vascular permeability were measured. Allopurinol treatment with 15(S)-HETE disrupted endothelial TJs and occludin, and decreased endothelial permeability as compared with untreated TJs. These results confirmed these effects on the functional protein expression and occludin.

AJs and TJs are essential structural components of the endothelial barrier. AJs form cell-to-cell and cell-to-substratum contacts, the TJs, by forming strand-like structures, close the clefts between the neighboring ECs and regulate the paracellular permeability of the endothelium to ions and macromolecules (3); although, some reports demonstrated that disruption of AJs also results in endothelial hyperpermeability (32). Because dynamic interactions between AJs and TJs are required to modulate the EC barrier function (33, 34), the EC hyperpermeability caused by AJ disruption could still be attributed to perturbation in TJs as well. In fact, it was proposed that AJs are linked to the development of TJs (34); however, some studies disputed this claim, as interfering with the formation of AJs has no adverse effects on TJ organization (35). Endothelial TJs are comprised of transmembrane (occludin, claudins, and junction adhesion molecules) and intracellular (zonula occludens 1-3, protein incorporated later into TJs, and junction enriched and associated protein) proteins (33, 36, 37). A large body of evidence shows that interference in the interactions between TJ proteins perturbs the TJs affecting the barrier permeability of the endothelium (38). It was further observed that the TJ formation or disintegration depends on the phosphorylation state of TJ proteins (39–41). However, most of these studies were focused on epithelial TJs, and therefore, the role of phosphorylation of TJ proteins in the preservation or perturbation of endothelial TJ integrity and its barrier function has not been well-studied. In this context, a few studies, including ours, showed that phosphorylation of TJ proteins leads to endothelial TJ disruption and loss of its barrier function (20, 21, 42, 43). In the present study, we have extended our previous observations on the role of tyrosine phosphorylation of TJ proteins in the disruption of TJ integrity and barrier function to JamA. We found that 15(S)-HETE induced XO conversion product of AA, induces tyrosine phosphorylation of JamA leading to its dissociation from occludin, disrupting TJs and occludin, and decreasing endothelial permeability as compared with control diet. Consistent with these effects on the phosphorylation of all TJ proteins, the phosphorylation of occludin, claudins 1/5, ZO2, and JamA tyrosine phosphorylation from occludin, endothelial TJ integrity, and vascular permeability were measured. Arteries from WT mice fed with HFD, alone or in combination with administration of allopurinol for 3 months, and XO expression, along with Pyk2, and JamA tyrosine phosphorylation from occludin, endothelial TJ integrity, and vascular permeability were measured. Allopurinol treatment with 15(S)-HETE disrupted endothelial TJs and occludin, and decreased endothelial permeability as compared with untreated TJs. These results confirmed these effects on the functional protein expression and occludin.

**DISCUSSION**

Previously, we have also demonstrated that Src- and Pyk2-dependent phosphorylation of ZO2 leads to its dissociation from occludin and TJs affecting their barrier function. These tyrosine residues appear to be crucial for the capacity of JamA in forming a TJ complex with occludin. Furthermore, because these three tyrosine residues are present in the carboxyl terminus, it may be suggested that JamA interacts with other TJ proteins in establishing functional TJs via its carboxyl region. Previously, we have also demonstrated that Src- and Pyk2-dependent phosphorylation of ZO2 leads to its dissociation from claudin 1/5 affecting endothelial TJs and barrier function. In view of these observations, it may be concluded that 15(S)-HETE disrupts endothelial TJs and their barrier function by tyrosine phosphorylation of several TJ proteins, including ZO2 and JamA, and affecting their interactions with other TJ proteins such as claudins 1/5 and occludin, respectively. These observations may also infer that 15(S)-HETE triggers simultaneous phosphorylation of several TJ proteins leading to disruption of EC TJ complexes and barrier function in a coordinate manner, as blockade of the same cell extracts was also analyzed by Western blotting for JamA levels for normalization. I: All the conditions were the same as in (C) except that quiescent the HUVEC monolayer, after treatment with vehicle or 15(S)-HETE (0.1 μM), was stained for JamA and occludin and the fluorescence images were captured and the TJ-localized JamA levels were measured as described in Fig. 1E. J, K: All the conditions were the same as in (C) except that cells were subjected to 15(S)-HETE (0.1 μM)-induced dextran flux (J) or monocyte transmigration (K) assays. The bar graphs represent mean ± SD values of three experiments. *P < 0.05 versus vehicle control; **P < 0.05 versus 15(S)-HETE.
Fig. 5. Src and Pyk2 mediate 15(S)-HETE-induced JamA tyrosine phosphorylation and TJ disruption ex vivo. A–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 and tissue extracts were prepared. An equal amount of protein from control and each treatment was immunoprecipitated with anti-pTyr (A) or anti-JamA (B) antibodies and the immunocomplexes were analyzed by Western blotting for the indicated proteins using their specific antibodies. The same tissue extracts were analyzed by Western blotting for total JamA levels shown in (A) or for pSrc and pPyk2 levels and normalized to their total levels in (C). D: Aortas from WT mice were incubated with and without 15(S)-HETE (0.5 μM) in the presence and absence of PP3 (10 μM) for 30 min, tissue extracts were prepared and analyzed for Src, Pyk2, and JamA tyrosine phosphorylation as described in (A) and (C). E, F: All the conditions were same as in (A) except that after the treatments, the aortas were opened longitudinally, either left alone (E) or further incubated with BCECF-labeled leukocytes for 60 min (F), fixed, permeabilized, blocked and stained for JamA using rabbit anti-JamA antibodies, followed by developing with Alexa Fluor 568-conjugated goat anti-rabbit secondary antibodies. The immunofluorescence images were captured and the TJ-localized JamA levels were quantified as described in Fig. 1E. The bar graphs represent mean ± SD values of three experiments or six animals. *P < 0.05 versus vehicle control; **P < 0.05 versus 15(S)-HETE.
could allow the transendothelialization of leukocytes and monocytes, the hallmarks in the initiation of inflammation. In the present study, we observed that when the EC monolayer or aortas were exposed to 15(S)-HETE, their TJs were disrupted and caused enhanced leukocyte and monocyte adhesion and transmigration. Furthermore, feeding WT, but not 12/15-LO/H11002/H11002 mice with HFD also disrupted aortic endothelial TJs and exhibited increased leukocyte adhesion to the endothelium. In addition, hypercholesterolemia and hyperlipidemia were reported to induce the expression of 12/15-LO and generate 15(S)-HETE (46–48). Furthermore, feeding WT, but not 12/15-LO/H11002/H11002 mice with HFD also disrupted aortic endothelial TJs and exhibited increased leukocyte adhesion to the endothelium. In addition, hypercholesterolemia and hyperlipidemia were reported to induce the expression of 12/15-LO and generate 15(S)-HETE (46–48). Furthermore, a number of studies have demonstrated 12/15-LO plays a role in atherogenesis (49–51). These findings, along with the observations that the effects of HFD feeding on TJ integrity and barrier function were substantially downregulated in 12/15-LO/H11002/H11002 mice, may suggest that 12/15-LO plays a crucial role in endothelial TJ disruption and leukocyte or monocyte phosphorylation of any of these proteins is sufficient in restoring TJs and barrier function. In both ZO2 and JamA tyrosine phosphorylation, Src was found to act upstream to Pyk2. An association between Pyk2 and Src was reported in epithelial cells in response to EGF-induced wound healing (44). Similarly, Pyk2 was found to bind to Src and mediate its activation in PC12 cells in response to lysophosphatidic acid (45). In both of these reports, Pyk2 was observed to act upstream to Src. Based on these observations and the present findings, it can be viewed that the spatiotemporal interactions between Src and Pyk2 differ in response to various agents in mediating their cellular effects.

In addition to their role in the regulation of barrier permeability, endothelial TJs play a role in the maintenance of cell polarity (39), signaling (38), and gene expression (4, 5). Most importantly they provide fencing to the endothelium in intact blood vessels. Therefore, the disruption of TJs may result in leaky EC barrier permeability, which
Fig. 7. 12/15-LO gene knockout protects aortic endothelium from HFD-induced TJ disruption and hyperpermeability. A, B: Aortas from WT and 12/15-LO⁻/⁻ mice that were fed with CD or HFD for 3 months were isolated and analyzed for XO levels by Western blotting using XO specific antibodies (A) or its activity using a kit (B). The blot in (A) was normalized for α-tubulin. C: All the conditions were the same as in (A) except that after isolation, the arteries were fixed in OCT compound, cross-sections were made and stained for XO and CD31 using rabbit anti-XO and rat anti-CD31 antibodies followed by Alexa Fluor 568-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-rat secondary antibodies. D: All the conditions were the same as in (A) except that after dissection the arteries were opened longitudinally and stained for JamA and leukocytes using rabbit anti-JamA and mouse anti-CD45 antibodies followed by Alexa Fluor 568-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies. E: After feeding with CD or HFD, the mice were anesthetized and 0.1 ml of 1% Evans Blue (EB) dye was injected into the inferior vena cava. After 30 min, the blood vessels were perfused with PBS through the left ventricle and the aortas were isolated and photographed. After taking the pictures, the aortas were minced, incubated in formaldehyde at 55°C for 24 h and the optical density was measured at 610 nm. The aortic endothelial permeability was expressed as nanograms of EB dye extravasated per milligram of tissue. F–J: WT mice were fed with CD or HFD, alone or in combination with allopurinol (6.8 g/l) for 3 months and either arteries were isolated and analyzed for XO activity (F), phospho and total Src, Pyk2, and JamA dissociation from occludin (H), endothelial TJ integrity (I), or vascular leakage (J) as described in (B), (A), (D), and (E), respectively. The bar graphs represent mean ± SD values of six animals. *P < 0.05 versus CD; **P < 0.05 versus WT (HFD).
recruitment to the dysfunctional endothelium, which are considered to be early events in atherogenesis (1, 2). However, how the disruption of endothelial TJs enhances the leukocyte and monocyte recruitment is not known. One proposed mechanism is that dysfunctional endothelium expresses cell adhesion molecules, such as ICAM and VCAM, which may aid in the adhesion of circulating leukocytes and monocytes to the perturbed endothelium, and the chemotactic molecules such as MCP1 produced by the dysfunctional endothelium may enhance their migration through the endothelium. Support toward this mechanism can be drawn by the findings that 12/15-LO, via stimulating protein kinase Cα-dependent activation of NFκB, enhances ICAM expression in ECs and mediates endothelium-monocyte interaction (52). In addition, it was shown that TJ disruption influences NFκB activation leading to expression of inflammatory molecules and promotes leukocyte extravasation (53, 54). Based on all these observations, we believe that HFD-induced expression of 12/15-LO enhances the tyrosine phosphorylation of TJ proteins such as JamA and ZO2, disrupts 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, it is also possible that monocytes adhere to the endothelium, disrupt TJs, and thereby mediating JamA tyrosine phosphorylation affecting endothelial TJs and barrier function. 12/15-LO has been reported to mediate LDL oxidation as well. In brief, 15-LO1-15(S)-HETE via XO-dependent ROS production leading to Src and Pyk2-mediated tyrosine phosphorylation of TJ proteins such as JamA perturbs aortic EC TJs and their barrier function and thereby promotes transendothelialization of leukocytes and monocytes in the pathogenesis of atherosclerosis in response to HFD. 

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