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Research Paper

Resolvin D1 via prevention of ROS-mediated SHP2 inactivation protects endothelial adherens junction integrity and barrier function

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ABSTRACT

Resolvins are a novel class of lipid mediators that play an important role in the resolution of inflammation, although the underlying mechanisms are not very clear. To explore the anti-inflammatory mechanisms of resolvins, we have studied the effects of resolvin D1 (RvD1) on lipopolysaccharide (LPS)-induced endothelial barrier disruption as it is linked to propagation of inflammation. We found that LPS induces endothelial cell (EC) barrier disruption via xanthine oxidase (XO)-mediated reactive oxygen species (ROS) production, protein tyrosine phosphatase SHP2 inactivation and Fyn-related kinase (Frk) activation leading to tyrosine phosphorylation of α-catenin and VE-cadherin and their dissociation from each other affecting adherens junction (AJ) integrity and thereby increasing endothelial barrier permeability. RvD1 attenuated LPS-induced AJ disassembly and endothelial barrier permeability by arresting tyrosine phosphorylation of α-catenin and VE-cadherin and their dislocation from AJ via blockade of XO-mediated ROS production and thereby suppression of SHP2 inhibition and Frk activation. We have also found that the protective effects of RvD1 on EC barrier function involve ALX/FPR2 and GPR32 as inhibition or neutralization of these receptors negates its protective effects. LPS also increased XO activity, SHP2 cysteine oxidation and its inactivation, Frk activation, α-catenin and VE-cadherin tyrosine phosphorylation and their dissociation from each other leading to AJ disruption with increased vascular permeability in mice arteries and RvD1 blocked all these effects. Thus, RvD1 protects endothelial AJ and its barrier function from disruption by inflammatory mediators such as LPS via a mechanism involving the suppression of XO-mediated ROS production and blocking SHP2 inactivation.

1. Introduction

Many inflammatory diseases arise due to uncontrolled inflammatory response or in other words failure of resolution process [1,2]. Resolution of inflammation is now considered as an actively regulated phenomenon and understanding the signaling events that regulate the termination of inflammation is crucial in the circumvention of inflammatory diseases [1,3]. In recent years, endogenously derived lipid mediators like resolvins, protectins, maresins and lipoxins received special attention due to their anti-inflammatory properties [4–6]. These lipid mediators appear to limit excessive inflammation without any adverse effects on normal immune responses [6,7]. Resolvin D1 (RvD1) is synthesized from docosahexanoic acid (DHA) by sequential oxygenation by enzymes 15-lipoxygenase (15-LOX) and 5-LOX [5,8] and it exhibits potent anti-inflammatory effects both in vitro and in vivo [9]. It was demonstrated that RvD1 reduces ROS production, inflammatory cytokines and adhesion molecules expression and attenuates neutrophil trafficking [5,9,10]. In addition, it has been reported that RvD1 protects endothelial barrier function, although the underlying mechanisms were not well understood [11,12]. The endothelium, which acts as anti-platelet adhesion and anti-thrombotic surface for the circulating blood and with its selective barrier permeability, plays an important role in the maintenance of vascular integrity [13,14]. Disruption of endothelial barrier function facilitates passage of inflammatory cells into the tissues where these cells via expression of various proinflammatory molecules amplify the local and systemic inflammation [15,16].

Adherens junctions (AJ), gap junctions (GJ) and tight junctions (TJ) are important endothelial cell-to-cell adhesions and play an essential role in its barrier function [17,18]. Disruption of these

Abbreviations: AJ, adherens junctions; CD, chow diet; CSN, cysteine sulfonate; DHA, docosahexanoic acid; EV, Evans blue; Frk, Fyn-related kinase; GJ, gap junctions; HUVEC, human umbilical vein endothelial cells; 15-LOX, 15-lipoxygenase; LSGS, low-serum growth supplements; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RvD1, Resolvin D1; ROS, reactive oxygen species; TER, transendothelial electrical resistance; TJ, tight junctions; VE-cadherin, vascular endothelial cadherin; WB, Western blotting; IP, immunoprecipitation; WT, wild type; XO, xanthine oxidase

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junctions leads to development of gaps in the endothelial layer, which results in its increased permeability and altered function, a hallmark of various vascular diseases [19–22]. Among the cell-to-cell junctions, AJ are comprised of vascular endothelial (VE) cadherin and its binding partners catenins (α, β, γ and p120) [13]. Impaired expression of AJ proteins affects vascular morphogenesis during embryonic development and vascular permeability in the adulthood [23,24]. In response to inflammatory mediators VE-cadherin gets phosphorylated and either internalizes or degrades leading to loss of endothelial AJ integrity and barrier function [25–27]. Interestingly, both protein kinases (PKs) and protein phosphatases (PPs) have been found to be localized at the AJ, suggesting a role for phosphorylation and dephosphorylation of AJ proteins in the regulation of the maintenance of AJ integrity [19,25,28,29]. In fact, LPS via oxidant-mediated inhibition of PPs has been shown to activate PKs in the modulation of inflammation [30,31]. LPS has also been reported to disrupt endothelial barrier function in the propagation of inflammation [11,12,31,32]. Previously we have reported that arachidonic acid metabolite, 15(S)-HETE, by XO-mediated ROS production leads to activation of Src and Pyk2 in the tyrosine phosphorylation of TJ proteins affecting endothelial TJ integrity and its barrier function [33]. Based on these findings, we asked the question whether RvD1 via inhibiting XO-mediated ROS production and preventing protein tyrosine phosphatases (PTPs) inactivation and protein tyrosine kinases (PTKs) activation protects endothelial barrier function from disruption by LPS. We found that RvD1 by inhibition of LPS-induced XO-mediated ROS production, and preventing SHP2 inactivation and Frk activation, thereby reducing α-catenin and VE-cadherin tyrosine phosphorylation and their dissociation from each other protects endothelial AJ integrity and its barrier function. RvD1 exerts its protective effects on the maintenance of endothelial AJ integrity and its barrier function via activation of its receptors ALX/FPR2 and GPR32. Consistent with these observations, RvD1 also protected endothelial AJ integrity and its barrier function from LPS-induced disruption in mice arteries in vivo as well.

2. Materials and methods

2.1. Reagents

Resolvin D1 (10012554) was purchased from Cayman Chemical Company (Ann Arbor, MI). Fluorescein isothiocyanate-dextran (FD70S), LPS (L4391), PHPS1 (P0039) and xanthine oxidase kit (MAKO78-1KT) were bought from Sigma Aldrich Company (St. Louis, MO). Anti-α-catenin (SC-7894 and SC-9988), anti-β-catenin (SC-7963), anti VE-cadherin (SC-28644 and SC-9989), anti-p120 catenin (SC-23872), anti-α-tubulin (SC-23948), anti-cMyc (SC-789), anti-pLck (SC-101728), anti-Lck (SC-433), anti-pYes (SC-130182), anti-Yes (SC-8403), anti-Fyn (SC-365913), anti-Lyn (SC-15), anti-Frk (SC-166478) and anti-PTPμ (SC-56957) antibodies were procured from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Anti-pSrc (2101 S) antibody was obtained from Cell Signaling Technology (Beverly, MA). Anti-pFyn (ab192172) and anti-VE-cadherin (ab33168) antibodies were bought from Abcam (Cambridge, MA). Anti-PY20 (05–777), anti-Src (05–184), anti-pLyn (04–375), anti-PTP-PEST (05–1417) and anti-FPR2 (ABF118) antibodies and PTP assay kit (17–125) were obtained from Millipore (Temecula, CA). Anit-SHP2 (610622) and anti-SHP1 (610126) antibodies were purchased from BD Biosciences (San Jose, CA). Anti-cysteine sulfonate antibody (ADI-OSA-820-F) was bought from Enzo Life Sciences (Farmingdale, NY). Wild type SHP2 (12283) and mutant SHP2 (C459S) (12284) plasmids were received from Addgene (Cambridge, MA) [34]. Anti-GPR32 neutralizing antibody (GTX71225) was obtained from Genetex (Irvine, CA). BOC2 (07201) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Hoechst 33342 (10 mg/ml) solution (H3570), goat anti-rabbit, and goat anti-mouse secondary antibodies conjugated with Alexa Fluor 568 (A11011) or Alexa Fluor 488 (A11029) fluorochrome, ProLong Gold antifade reagent (P36930), Medium 200 (M200500), low serum growth supplements (S003K), Lipofectamine 3000 (L3000015), non-targeting siRNA (D-001810-10), Frk siRNA (S5363), and gentamycin/amphotericin solution (R01510) were bought from ThermoFisher Scientific (Carlsbad, CA). The enhanced chemiluminescence (ECL) Western blotting detection reagents (RPN2106) were obtained from GE Healthcare (Pittsburg, PA).

2.2. Animals

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained at UTHSC vivarium according to the Institutional Animal Care and Use Committee's guidelines. 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 effect of RvD1 on the protection of endothelial barrier function, mice were kept on chow diet (CD) and administered intraperitoneally with RvD1 at a dose of 10 μg/kg body weight [35] every third day for a total of one week before the administration of (5 mg/kg body weight) LPS [36]. Twenty-four hrs after the administration of LPS, the mice were sacrificed, aortas were isolated and used as required.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Invitrogen (C0035C) and cultured in Medium 200 containing low serum growth supplements (LSGS), 10 μg/ml gentamycin and 0.25 μg/ml amphotericin B. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. HUVECs between 6 and 10 passages were growth-arrested by incubating in Medium 200 without LSGS for 12 h and used to perform the experiments unless otherwise indicated.

2.4. Transfections

HUVECs were transfected with the indicated siRNA at a final concentration of 100 nM using Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. After transfections, cells were quiesced in Medium 200 without LSGS for 12 h and used as required.

2.5. Western blot analysis

Cell or tissue extracts containing an equal amount of protein from control and the indicated treatments were resolved by SDS-PAGE. The proteins were transferred electrophoretically to a nitrocellulose membrane. After blocking in either 5% (W/V) nonfat dry milk or 5% (W/V) BSA, the membrane was probed with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using enhanced chemiluminescence detection reagents as described previously [33].

2.6. Immunoprecipitation

Immunoprecipitation was performed as described by us previously [33]. Cell or tissue extracts containing an equal amount of protein from control and each treatment was incubated with the indicated primary antibody at 1:100 dilution overnight at 4 °C. Protein A/G-conjugated Sepharose CL-4B beads were added and incubation continued for an additional 1 h at room temperature and the beads were collected by centrifugation at 1000 rpm for 1 min at 4 °C. The beads were washed three times with lysis buffer and once with PBS, boiled in SDS sample buffer and analyzed by immunoblotting.