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Resolvin D1 blocks H_2O_2-mediated inhibitory crosstalk between SHP2 and PP2A and suppresses endothelial-monocyte interactions

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ABSTRACT

In recent years, various studies have demonstrated a role for endogenously derived specialized proresolving mediators such as resolvins in the resolution of inflammation. In exploring the signaling mechanisms, in the present study we show that Resolvin D1 (RvD1) reduces LPS-induced endothelial cell (EC)-monocyte interactions via blocking H_2O_2-mediated PP2A inactivation, NFκB activation and ICAM1 and VCAM1 expression. In addition, we found that H_2O_2-mediated SHP2 inhibition leads to tyrosine phosphorylation and inactivation of PP2A by LPS, which in turn, accounts for increased NFκB activation and ICAM1 and VCAM1 expression facilitating EC-monocyte interactions and all these LPS-mediated responses were reduced by RvD1. Furthermore, the suppression of NFκB activation, ICAM1 and VCAM1 expression and EC and monocyte interactions by RvD1 involved its receptors ALX/FPR2 and GPR32 as inhibition or neutralization of these receptors negated its effects. Besides, pertussis toxin completely prevented the effects of RvD1 on inhibition of LPS-induced H_2O_2 production, SHP2 and PP2A inactivation, NFκB activation, ICAM1 and VCAM1 expression and EC and monocyte interactions. Together, these observations suggest that RvD1 via activation of Gi-coupled ALX/FPR2 and GPR32 receptors blocks LPS-induced H_2O_2-mediated SHP2 and PP2A inactivation, NFκB activation, ICAM1 and VCAM1 expression and EC-monocyte interactions, which could be one of the several possible mechanisms underlying the anti-inflammatory actions of this specialized proresolving mediator.

1. Introduction

In normal homeostatic conditions the endothelium exerts a negative regulatory effects on the expression of proinflammatory molecules [1,2]. However, cardiovascular risk factors including, hypercholesterolemia, hyperglycemia, hypertension, smoking and aging all alter the endothelial function, with the development of a chronic inflammation [3–5]. Dysfunctional endothelium expresses adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) [6,7]. The expression of adhesion molecules promotes the adherence of lymphocytes, neutrophils and monocytes to the endothelium and their subsequent transmigration into the vessel wall [8,9]. The infiltration of lymphocytes, neutrophils and monocytes into the vessel wall is an early event in the development of vascular inflammation [10,11]. If vascular inflammation progresses unresolved, it can lead to the development of various vascular diseases such as atherosclerosis [12,13]. In recent years, the discovery of specialized proresolving mediators that were found to possess anti-inflammatory properties [14–21], have also been shown to exert atheroprotective effects [22–24]. These lipid mediators tend to reduce ROS production, inflammatory cytokines and adhesion molecules expression, leukocyte trafficking and leukocyte-endothelial interactions [25,26]. ROS generation is the most prevailing mechanism of altered endothelial cell function [4,5]. ROS, particularly H_2O_2 inhibits protein tyrosine phosphatases, which in turn, by promoting the activation of protein tyrosine kinases can lead to activation of cellular signaling events of cell proliferation, migration, apoptosis, and inflammation [27–30]. Recently, we have demonstrated a role for RvD1 in the protection of EC barrier function from LPS-induced disruption by preventing AJ disruption [31]. Interestingly, the prevention of LPS-induced AJ disruption by RvD1 was dependent on the suppression of XO-mediated ROS production, SHP2 inactivation, Frk activation and AJ protein tyrosine phosphorylation [31].

To understand the anti-inflammatory mechanisms of RvD1 in the present study we have tested the role of phosphatases. Having demonstrated that RvD1 prevents ROS-mediated SHP2 inactivation in the protection of endothelial AJs and its barrier function, in the present study we tested the role of SHP2 in LPS-induced endothelial cell (EC)-

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monocyte interactions and the efficacy of RvD1 in suppressing these effects. Our findings show that RvD1 abrogates LPS-induced EC-monocyte interactions via inhibiting NFκB activation and ICAM1 and VCAM1 expression and these effects were dependent on the protection of SHP2 and its downstream effector PP2A from H_2O_2-mediated inactivation. Similarly, the protective effects of RvD1 against the EC-monocyte interactions were dependent on activation of its Gi-coupled ALX/FPR2 and/or GPR32 receptors, as inhibition or neutralization of these receptors or Giα suppressed the efficacy of RvD1 on blockade of LPS-induced H_2O_2-mediated SHP2 and PP2A inactivation, NFκB activation, ICAM1 and VCAM1 expression and EC-monocyte interactions.

2. Materials and methods

2.1. Reagents

Pyrrolidinedithiocarbamic acid (PDTC) (20713), Pertussis toxin (19546), QNZ (10006734) and Resolvin D1 (10012554) were purchased from Cayman Chemical Company (Ann Arbor, MI). Growth factor-reduced Matrigel (354520) and anti-SHP2 antibodies (610622) were obtained from BD Biosciences (Bedford, MA). Allopurinol (PHR1377), LPS (L4391), PEG-catalase (C4963) and PHPS1 (P0039) were bought from Sigma Aldrich Company (St. Louis, MO). Anti-IKKα/β (SC-7607), anti-IκBα (SC-341), anti-NFκB (SC-372 and SC-8008), anti-pPP2A-C(α/β) (SC-12615R), anti-PP2A-C(α/β) (SC-56950), anti-ICAM1 (SC-1511R), anti-VCAM1 (SC-13160) and anti-α-Tubulin (SC-23928) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pIKKα/β (2697), anti-pIκBα (2859), anti-pNFκB (3033) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Okadaic acid (459620), PP2A inhibitor (539620), anti-FPR2 (ABF118) antibodies and Ser/Thr phosphatase assay kit (17127) were bought from Millipore (Temecula, CA). Anti-GPR32 neutralizing antibody (GTX71225) was obtained from Genetex (Irvine, CA). Anti-cysteine sulfonate antibodies were bought from Enzo Lifesciences (Farmingdale, NY). Neutralizing ICAM1 (ab171123) and VCAM1 (ab47159) antibodies were obtained from Abcam (Cambridge, MA). BOC2 (07201) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Amplex Red Hydrogen Peroxide Assay kit (A22188), Medium 200 (M200500), low serum growth supplements (S003K), BCECF (B1170) and gentamycin/amphotericin solution (R01510) were bought from ThermoFisher Scientific (Waltham, MA). The enhanced chemiluminescence (ECL) Western blotting detection reagents (RPN2106) were obtained from GE Healthcare (Pittsburg, PA).

2.2. 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. Human THP1 cells (TIB-202) were purchased from American Type Culture Collection (Manassas, VA) grown 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 the adhesion and transmigration assays. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. HUVECs between 6 and 10 passages were used to perform the experiments unless otherwise indicated.

2.3. H_2O_2 production assay

HUVECs with and without the indicated treatments were collected by scraping and 50 μl of the cell suspension was incubated with 50 μl of 100 μM Amplex Red along with 0.2 U/ml of HRP for 30 min at 37 °C in the dark. Following the incubation, the fluorescence intensities were measured in SpectraMax Gemini XPS Spectrofluorometer (Molecular Devices) with excitation at 530 nm and emission at 590 nm. The H_2O_2 production was expressed as RFU.

2.4. Immunoprecipitation

Immunoprecipitation was performed as described by us previously [31]. Cell extracts containing equal amounts of protein from control and the indicated treatments were incubated with the indicated primary antibodies 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.

2.5. Western blot analysis

After appropriate treatments, cell extracts were prepared and 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.

2.6. Ser/Thr phosphatase assay

Ser/Thr phosphatase activity was measured by dephosphorylation of Ser/Thr phosphatase-specific phosphopeptide and the inorganic phosphate released was detected by malachite green reagent kit. To measure PP2A/B activity, the cell extracts containing equal amounts of protein were immunoprecipitated with anti-PP2A/B antibodies and the immunocomplexes were assayed for phosphatase activity as described by Narayanan et al. [32].

2.7. Monocyte adhesion

The adhesion of THP1 cells to HUVEC monolayer was measured with a fluorometric method [33]. HUVEC monolayer was grown to confluency, quiesced, treated with and without LPS (500 ng/ml) in the presence and absence of RvD1 (200 ng/ml). Wherever pharmacological inhibitors or neutralizing antibodies were used, they were added to cells 30 min prior to the stimulant. THP1 cells were labeled with 10 μM BCECF in serum-free medium for 30 min and the labeled cells were placed on the HUVEC monolayer at 8×10^4 cells/well, and incubation continued for another 1 h. After incubation, the nonadherent cells were washed off with PBS and the adherent cells were lysed in 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100. The fluorescence intensity was measured in a Spectra Max Gemini XPS Spectroflurometer (Molecular Devices) with excitation at 485 nm and emission at 535 nm. Cell adhesion was expressed as relative fluorescence units.

2.8. Monocyte transmigration

THP1 cell transmigration was measured as described previously [34]. Wherever pharmacological inhibitors or neutralizing antibodies were used, they were added to cells 30 min prior to the stimulant. HUVEC monolayer was treated with and without LPS in the presence and absence of RvD1 (200 ng/ml) for 1 h at which time the BCECF-labeled quiescent THP1 cells (1×10^5 cells/well) were added and incubation continued overnight at 37 °C. The transmigration of THP1 cells through the HUVEC monolayer was measured by capturing the images by an inverted Zeiss fluorescence microscope (AxioObserver. Z1) via a 10 × /NA 0.6 objective and AxioCam MRm camera without

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