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Original Contribution

ROS-dependent Syk and Pyk2-mediated STAT1 activation is required for 15(*S*)-hydroxyeicosatetraenoic acid-induced CD36 expression and foam cell formation

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ABSTRACT

15(*S*)-Hydroxyeicosatetraenoic acid (15(*S*)-HETE), the major 15-lipoxygenase 1/2 (15-LO1/2) metabolite of arachidonic acid (AA), induces CD36 expression through xanthine oxidase and NADPH oxidase-dependent ROS production and Syk and Pyk2-dependent STAT1 activation. In line with these observations, 15(*S*)-HETE also induced foam cell formation involving ROS, Syk, Pyk2, and STAT1-mediated CD36 expression. In addition, peritoneal macrophages from Western diet-fed ApoE$^{-/-}$ mice exhibited elevated levels of xanthine oxidase and NADPH oxidase activities, ROS production, Syk, Pyk2, and STAT1 phosphorylation, and CD36 expression compared to those from ApoE$^{-/-}$:12/15-LO$^{-/-}$ mice and these events correlated with increased lipid deposits, macrophage content, and lesion progression in the aortic roots. Human atherosclerotic arteries also showed increased 15-LO1 expression, STAT1 phosphorylation, and CD36 levels as compared to normal arteries. Together, these findings suggest that 12/15-LO metabolites of AA, particularly 12/15(*S*)-HETE, might play a crucial role in atherogenesis by enhancing foam cell formation.

Introduction

Atherosclerosis is a chronic inflammatory disease of blood vessels and is one of the major causes of death and disability in the world [1,2]. It is characterized by lipid-laden foam cell accumulation in the subendothelial space as well as calcification [2,3]. Since its discovery that epitopes of oxidized low-density lipoprotein (OxLDL) colocalized with 15-lipoxygenase mRNA and protein and that the atherosclerotic arteries exhibited increased 15-lipoxygenase activity [4–7], a number of studies using genetic and pharmacological approaches have demonstrated that lipoxygenases (LOs), mainly 5- and 15-LOs, play a role in atherogenesis [8–11]. In addition, the most appreciated mechanism of the role of LOs in atherogenesis was their apparent involvement in the oxidation of LDL [12,13]. LOs catalyze stereospecific insertion of molecular oxygen into *cis*-polyunsaturated fatty acids including arachidonic acid (AA) and linoleic acid (LA), resulting in the formation of hydroperoxyeicosatetraenoic acids (HpETEs) and hydroperoxyoctadecadienoic acids (HpODEs) [14–16]. HpETEs and HpODEs are nonenzymatically converted to hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs), respectively. 15-LO1 and 15-LO2 metabolize AA mainly to 15(*S*)-HpETE [14–17], while the murine ortholog of 15-LO1, 12/15-LO, converts AA to 12/15(*S*)-HpETE [17] and atherosclerotic arteries upon incubation with AA produced more of 15-HETE compared to normal arteries [18,19]. Furthermore, many cardiovascular disease risk factors such as hypercholesterolemia, diabetes, obesity, and smoking all have been shown to be associated with increased expression and/or activity of 12/15-LO [20–23]. In addition, HETEs are prooxidants [24] and a convincing body of evidence links oxidant stress to the pathogenesis of a variety of diseases, including cardiovascular diseases, cancer, and rheumatoid arthritis [25–28]. Thus, while a mounting amount of data point out a proatherogenic role for 12/15-LO [8–10], one study showed that this lipoxygenase exerts antiatherogenic effects [29]. Despite its controversial role in atherogenesis, no mechanisms supporting its pro- or antiatherogenic effects were explored.

Since many studies have shown that cardiovascular risk factors such as hypercholesterolemia, diabetes, obesity, and smoking increase 15-LO activity [20–23] and atherosclerotic arteries produce 15-HETE [18,19], a major 15-LO1/2 metabolite of AA, we asked the question whether this eicosanoid has any influence in the pathogenesis of atherogenesis. Toward this end, we have previously reported that 15(*S*)-HETE by inducing IL-17A expression triggers inflammation [30]. In this study, we report that 15(*S*)-HETE also enhances CD36 expression and foam cell formation.

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In addition, we show that 15(*S*)-HETE-induced CD36 expression and foam cell formation require xanthine oxidase and NADPH oxidase-dependent ROS production and Syk and Pyk2-mediated STAT1 activation. Furthermore, peritoneal macrophages from Western diet (WD)-fed ApoE$^{-/-}$ mice exhibited substantially higher levels of xanthine oxidase and NADPH oxidase activities, ROS production, Syk, Pyk2, and STAT1 phosphorylation as well as CD36 expression as compared to those from ApoE$^{-/-}$:12/15-LO$^{-/-}$ mice. These observations correlated with increased lipid deposits and plaque progression in aortic roots of ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$:12/15-LO$^{-/-}$ mice in response to WD feeding. Interestingly, human atherosclerotic arteries showed increased 15-LO1 levels, STAT1 phosphorylation, and CD36 expression as compared to normal arteries. Thus, these findings provide additional evidence for the role of 12/15-LO in foam cell formation and hence in the pathogenesis of atherogenesis.

Materials and methods

Reagents

5(*S*)-HETE (34230), 12(*S*)-HETE (34570), 15(R)-HETE (34710), 15(*S*)-HETE (34720), anti-CD36 (100011), total cholesterol assay kit (10007640), and triglycerides assay kit (1001030) were obtained from Cayman Chemicals (Ann Arbor, MI). Anti-pJak2 (3771), anti-pPyk2 (3291), anti-pSrc (2101), anti-pSTAT1 (7649), anti-pSTAT3 (9133), anti-pSTAT5 (9351), anti-pSTAT6 (9364), and anti-pSyk (2715) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-CD68 (SC-9139 and SC-9154), anti-CREB (SC-58), anti-Mac3 (SC-19991), anti-p47Phox (SC-14015), anti-SR-A1 (SC-20660), anti-SR-B1 (SC-67098), anti-STAT1 (SC-464), anti-STAT3 (SC-482), anti-STAT5B (SC-1656), anti-STAT6 (SC-981), anti-Syk (SC-573), anti-β-tubulin (SC-9104), and anti-xanthine oxidase (SC-20991) antibodies and normal mouse serum (SC-45051) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Pyk2 antibodies (ab32571), HDL assay kit, and LDL/VLDL cholesterol assay kit (ab65390) were purchased from Abcam (Cambridge, MA). Anti-hSTAT-5A (MAB2174) antibody was obtained from R&D Systems (Minneapolis, MN). Brewer-modified thioglycolate medium (21176) was purchased from BD Biosciences (San Jose, CA). Apocynin (A10809), allopurinol (A8003), and oil red O (234117) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Diphenyleneiodonium chloride (BML-CN240) was from Enzo Life Sciences (Farmingdale, NY). AG-490 (658401) was purchased from Calbiochem (Billerica, MA). Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (A11034), CM-H_2DCFDA [5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate] (C6827), Lipofectin transfection reagent (15596018), and TRIzol reagent were obtained from Invitrogen (Grand Island, NY). pGL3 basic vector and Luciferase assay system (E4530) were purchased from Promega (Madison, WI). Apolipoproteins (BT927), Dil-OxLDL (BT-920), and OxLDL (BT-910) were obtained from Biomedical Technologies (Stoughton, MA). QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). T4 polynucleotide kinase was obtained from New England Biolabs (Ipswich, MA). [γ^{32}P]ATP (S.A. 3000 Ci/mmol) was from MP Biomedicals (Irvine, CA). [^{3}H]Cholesterol (S.A. 53 Ci/mmol) was bought from PerkinElmer (Waltham, MA). The enhanced chemiluminescence (ECL) Western blotting detection reagents (RPN2106) were obtained from GE Healthcare. All the phosphorothioate-modified antisense oligonucleotides (ASOs) and primers were synthesized by IDT (Coralville, IA). The phosphorothioate-modified ASOs used in this study are as follows:

hControl ASO, 5′-GGGGGUTCTCTGCGTACGGTGCUAGU-3′; hCD-36 (NM_000072) ASO, 5′-CCACAGTTCCGGTCACAGCC-3′; hCREB (NM_004379) ASO, 5′-GCUGCTTCCCTGTTCUUCAU-3′; hp47Phox (NM_000265) ASO, 5′-GUUGGGCTCAGGGTCTTCCGUCUC-3′; hPyk2 (NM_173175) ASO, 5′-CCUGUGTCCATAGCCCAGAGUACC-3′; hSTAT1 (NM_007315) ASO, 5′- GGUCUCGTGTTCTCTGUUCU-3′; hSTAT5B (NM_012448) ASO, 5′- GGUGCTCTGCCTTCTUCUGC-3′; hSyk (NM_001174168) ASO, 5′-UUCCCTGTCTTGTCTUUGUC-3′; and hXO (NM_000379) ASO, 5′-GCCUCCTCCCATTCTCTTCACUCG-3′.

Cell culture

THP1 cell cultures were maintained in a humidified 95% air and 5% CO_2 atmosphere at 37 °C as described previously [30].

ROS detection

Intracellular ROS generation was measured using membrane-permeable CM-H_2DCFDA dye as described previously [30] and expressed as relative fluorescence units (RFU).

RT-PCR

Total cellular RNA was extracted from THP1 cells using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was performed with a high capacity cDNA reverse transcription kit (Applied Biosystems). Complementary DNA (cDNA) was then used as a template for amplification using the following primers: human SR-A1 (NM_002445), forward, 5′-CCTCGTGTTTGCAGTTCTCA-3′ and reverse, 5′-CCATGTTGCTCATGTGTTCC-3′; human SR-B1 (NM_001082959), forward, 5′- CTGTGGGTGAGATCATGTGG-3′ and reverse, 5′- GCCCTTCCTTTGGAGTAACC-3′; human CD36 (NM_000072), forward, 5′- ACAGATGCAGCCTCATTTCC-3′ and reverse, 5′- GCCTTGGATGGAAGAACAAA-3′; human β-actin (NM_001101), forward, 5′- AGCCATGTACGTTGCTAT-3′ and reverse, 5′-GATGTCCACGTCACACTTCA-3′. The amplification was performed using Gene AMP PCR system 2400 (Applied Biosystems). The amplified PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and the images were captured using a Kodak In Vivo Imaging System.

Western blotting

Western blotting was performed as described previously [30].

NADPH oxidase and XO activities

NADPH oxidase and XO activities were measured as described previously [30].

Transfections

Transfections using the indicated ASO were performed as described previously [30].

CD36 promoter cloning

Using HRMVEC genomic DNA as a template, CD36 promoter fragment from −724 nucleotides (nt) to +83 nt was amplified by polymerase chain reaction using a forward primer, 5′-GGTACCTTTTGGTTGAAGAAATTTAAAGAGTT-3′ incorporating a KpnI restriction enzyme site at the 5′-end and a reverse primer, 5′- AGATCTTTCAATCAAATGCTCCAACA-3′ incorporating BglII restriction site at the 5′-end. The resulting 0.807 kb PCR product was digested with KpnI and BglII and cloned into KpnI and BglII sites of the pGL3 basic vector (Promega) to yield pGL3-hCD36. The underlined regions are KpnI and BglII sites in both the forward and the reverse primers, respectively. Site-directed mutations within the STAT-binding element at −107 nt were introduced by using the QuickChange site-directed mutagenesis kit according to manufacturer's instructions and