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Selective activation of CB2 receptor improves efferocytosis in cultured macrophages

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ABSTRACT

Aims: Recent evidence indicates that the defective ability to clear apoptotic cells by macrophages (efferocytosis) and the resultant apoptotic cells accumulation in atherosclerotic plaques play an important role during the progression of unstable plaques. The cannabinoid type 2 receptor (CB2), has recently been emerging as a new target to reduce vulnerability and promote stability of plaques, however, the underlying mechanisms have not been studied in detail. In the present study, we investigated whether selective activation of CB2 improves efferocytosis of macrophages.

Main methods: RAW264.7 macrophage line and primary-isolated peritoneal lavage macrophages from C57bl/6J mice were cultured. The efferocytosis of macrophages was analyzed by using flow cytometry or confocal microscopy; and the possible mechanisms involved in regulation of efferocytosis were also explored by using molecular biology methods.

Key findings: We found that JWH-133 and HU-308, selective agonists of CB2 receptor, concentration-dependently increased the phagocytosis of apoptotic cells in normal-cultured and oxidative low density lipoprotein (OxLDL) -loaded RAW264.7 and primary macrophages. JWH-133 and HU-308 also up-regulated expressions of tyrosine kinase family phagocytic receptors MerTK, Tyro3 and Axl, reduced levels of TNF-alpha and reactive oxygen species (ROS) induced by OxLDL, and inhibited activation of RhoA GTPase.

Significance: The selective activation of CB2 improves efferosytosis of normal-cultured and OxLDL-loaded macrophages, which might provide a novel mechanism on how CB2 activation reduces vulnerability and promotes stability of atherosclerotic plaques.

1. Introduction

Unstable plaque is the pivotal mechanism leading to acute atherothrombotic vascular disease, such as myocardial infarction, sudden cardiac death, and stroke [1]. Defective ability to clear apoptotic cells (efferocytosis) by macrophages and the resultant apoptotic cells accumulation in plaques are recently established to cause plaque instability, necrosis and rupture in advanced atherosclerosis [2]. Although our understanding on the molecular and cellular biology of efferocytosis is still limited, it may open up novel therapeutic opportunities to treat advanced atherosclerosis by enhancing efferocytosis of macrophage and decreasing accumulation of apoptotic cells [3,4].

Efferocytosis is a physiologic function of phagocyte system. In normal condition, efferocytosis is rapid and high efficient, which is responsible for the clearance of apoptotic cells or cell debris, pathogens and other antigens. When the efficiency of efferocytosis is compromised, tissue necrosis, pathological inflammation, and /or autoimmunity often ensue. For example, in advanced atherosclerosis, the defective efferocytosis of macrophages serves as one of the primary mechanisms leading to vulnerability of plaques and triggering acute atherothrombotic events [2]. Recently, oxidized lipids [5], proinflammatory tumor necrosis factor alpha (TNF-alpha) [6], smoking [7,8] and mutation of MERTK receptor [9], a member of tyrosine kinase receptor family, have been implicated to undermine efferocytosis of macrophages. In contrast, Lovastatin [10], and fish oil diet [11] were found to improve the clearance of apoptotic cells in macrophages.

In the past decade, the endocannabinoid system has attracted wide attention in a number of chronic inflammatory diseases including atherosclerosis [12,13]. Expression of CB2 is down-regulated in advanced carotid atherosclerosis [14], and activation of CB2 limits inflammatory response, inhibits the progression of atherosclerosis and promotes the stability of plaques [14,15]. Recently, the augmentation of phagocytosis to zymosan in macrophages has been documented to be linked with CB2 activation [16,17]. In the present study, we test the hypothesis

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that CB2 activation improves efferocytosis of macrophages, which may be involved in mediating the anti-atherogenic properties of CB2 agonists.

2. Materials and methods

2.1. Materials

The present manuscript doesn't contain clinical studies or patient data. The protocol involving animals was approved by the Experimental Animal Ethics Committee in Shanghai Jiaotong University School of Medicine. C57BL/6J mice were purchased from the Chinese Academy of Sciences, Shanghai Laboratory Animal Center. The cell culture reagents and materials were purchased from Gibco Life Technologies (USA) and Corning Life Science (USA), respectively. The selective CB2 agonists JWH133 and HU308 were obtained from Tocris (Cat. No 1343 and Cat. No 3088, respectively). Oxidative low density lipoprotein (OXLDL) was ordered from Kalen Biomedical, LLC. (Cat. No 770202-4). The primary anti-Mertk and anti-Tyro3 antibodies were purchased from Abcam Company (Cat. No ab137673 and Cat. No ab109231, respectively), and anti-Tubulin antibody from Bioworld Technology, Inc. (Cat. No BS1699), and the primary anti-Axl antibody from Santa Cruz (Cat. No sc-1096). The ECL Western Blotting Detection Kit was bought from Pierce (USA). G-LISA RhoA Activation Assay Biochem Kit and Total RhoA ELISA Biochem Kit were ordered from Cytoskeleton Inc. (Cat. No BK124 and Cat. No BK150, respectively), and TNF-alpha ELISA kit from R&D Systems, Inc. (Cat. No MTA00B), and Reactive Oxygen Species Assay Kit from Beyotime Company (Cat. No S0033). CFSE CellTrace™, Cell-Tracker DiI and Dead Cell Apoptosis Kit were all from Invitrogen Technologies (Cat. No C34554, Cat. No C7001 and Cat. No V13242, respectively).

2.2. Cell culture

The primary peritoneal lavage macrophages were isolated from C57BL/6J mice (20–30 g) under ketamine/xylazine (100/20 mg/kg, i.p) anesthesia and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) as previously described. The mouse RAW264.7 macrophage cell line (ATCC, Manassas, VA, USA) was cultured in the same culture medium.

2.3. Induction of apoptosis

Apoptosis in RAW264.7 macrophages was induced by using UV radiation for 15–20 min (254 nm, 20 J/cm2), followed by culturing the cells for an additional 7–8 h [5]. Apoptosis was quantitatively analyzed with flow cytometry using Annexin-V/propidium iodide (AV/PI) dual staining (shown as in *Supplement S1*). In a typical experiment, a population of macrophages over 40% apoptosis and <5% necrosis were collected and used to study efferocytosis.

2.4. Measurement of the efferocytosis of macrophages

Before measurement of efferocytosis, apoptotic cells were pre-labeled by FITC-conjugated CFSE CellTrace™, meanwhile the work macrophages were pre-labeled by phycoerythrin (PE)-conjugated Cell-Tracker DiI and cultivated for 20 h with serum-free medium. Subsequently the starved macrophages were continuously incubated for 24 h by the pretreatment with the CB2 agonist JWH-133, HU308 alone or in combination with OxLDL. The treated cells were then added a certain ratio (5:1) of apoptotic macrophages and co-incubated for 45 min. After removed off noningested apoptotic cells with PBS washing, cells were then collected and performed measurement of phagocytosis index by calculating the index of engulfing apoptotic cells by work macrophages. In brief, the phagocytosis index of RAW264.7 cells was measured by using flow cytometry analysis as described previously [18,19], and the phagocytosis index in primary peritoneal lavage macrophages was visualized and analyzed with confocal microscopy as reported by Liu B. et al. [20]. The phagocytosis index was calculated by using the following formula: phagocytosis index in flow cytometry analysis = (number of phagocytized apoptotic cells with FITC and PE double-fluorescence/number of total cells with PE fluorescence) × 100% 100% in triplicate experiment; phagocytosis index detected with confocal microscopy = (number of phagocytized apoptotic cells with green FITC fluorescence inner work cells with red PE fluorescence/number of total work cells with red PE fluorescence) × 100% in 3 different fields each slide.

2.5. Western blotting analysis

Expressions of Mertk, Tyro3 and Axl protein, the main members of tyrosine kinase family phagocytic receptors in phagocytes, were detected with Western blotting as previously described [21]. In brief, after pretreatment for 24 h with JWH133, HU308 alone or in combination with OxLDL, RAW264.7 cells were lysed and total protein was extracted. 30 μg of the protein samples were boiled with 5 × laemmli sample buffer, then resolved in 10% SDS–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. After blocking with 5% non-fat dry milk in TBS, the blots were incubated with diluted primary antibody (1:1000) overnight at 4 °C. After washing, the blots were incubated with secondary antibody and visualized by using an ECL Western Blotting Detection Kit. Finally, the optical density of immunoblot bands was analyzed with ImageJ software (NIH, USA).

2.6. Quantitative real-time polymerase chain reaction analysis

After treated for 24 h with JWH133, HU308 or vehicle, the mRNA levels of Mertk, Tyro3, Axl and GAPDH in RAW264.7 cells were determined by using the SYBR® Premix Ex Taq TM kit (Takara, Japan) and an ABI7900 analyzer (Applied Biosystems, USA). The following primers were used: Mertk sense: 5′-AGACCTCCACACCTTCCTGTTA-3′, and anti-sense: 5′-TTCCTGTTGCTCAGATACTCCA-3′; Tyro3 sense: 5′-CTCCAGAACCCGTAACCATTTA-3′, and anti-sense: 5′-GGGCTTCACAAGAAAACTCTGT-3′; Axl sense: 5′-AGATTTACGACTACCTGCGTCA-3′; and anti-sense:5′-GCCTTCAGTGTGTTCTCCAAGT-3′; GAPDH sense: 5′-GTGAAGGTCGGAGTCAACG-3′ and anti-sense: 5′-TGAGGTCAATGAAGGGGTC-3′. Total RNA (2 μg) was first reverse-transcribed to cDNA with the Revertaid™ First Stand cDNA Synthesis Kit. Real-time polymerase chain reaction (PCR) was performed as follows: preliminary denaturation at 95 °C for 120 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 15 s. Relative expression of mRNA was determined with the ΔΔCt method using GAPDH as the internal control.

2.7. Measurement of TNF-alpha level

TNF-alpha was induced with OxLDL in cultured RAW264.7 macrophages, and the level in culture supernatants was analyzed quantitatively by mouse TNF-alpha ELISA Kits according to the manufacturer's instructions with reference to our previous study [22]. Concentration of TNF-alpha was expressed as pg/ml; the data were from three separate experiments in duplicate.

2.8. Detection of ROS level

Generation of ROS in RAW264.7 macrophages was determined with the ROS assay kit by monitoring the oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) with flow cytometry as described previously [22]. The level of ROS was determined with mean fluorescence intensity (MFI) from data in three separate experiments in duplicate.