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Dynamics and regulation of lipid droplet formation in lipopolysaccharide (LPS)-stimulated microglia

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ABSTRACT

Lipid droplets (LDs) are neutral lipid-rich organelles involved in many cellular processes. A well-known example is their accumulation in leukocytes upon activation by pro-inflammatory stimuli such as lipopolysaccharides (LPS) derived from gram-negative bacteria. A role of LDs and LD-associated proteins during inflammation in the brain is unknown, however. We have now studied their dynamics and regulation in microglia, the resident immune cells in the brain. We find that LPS treatment of microglia leads to the accumulation in them of LDs, and enhancement of the size of LDs. This induction of LDs was abolished by triacsin C, an inhibitor of triglyceride biosynthesis. LPS strongly activated c-Jun N-terminal kinase (JNK) and p38 MAPK stress signaling pathways and increased the expression of LD-associated protein perilipin-2 (ADRP) in a time-dependent manner. Immunostaining showed that perilipin-2 in LPS-treated microglia predominantly colocalized with LDs. Inhibitors of p38 α/β (SB203580) and PI3K/Akt pathway (LY294002), but not that of JNK (SP600125), reduced LPS-induced LD accumulation and eliminated the activating effect of LPS on perilipin-2. In addition, cytosolic phospholipase A_2 (cPLA$_2$-α), a key enzyme for arachidonic acid release, colocalized with LPS-induced LDs. These observations suggest that LDs may play an important role in eicosanoid synthesis in activated microglia; they provide a novel insight into the regulation of LDs in inflammatory cells of the brain and point to a potential role of p38 α/β in LPS-induced LD accumulation. Collectively, our findings imply that LD formation and perilipin-2 induction could be microglial biomarkers of inflammation in the central nervous system.

1. Introduction

Lipid droplets (LDs), also called lipid bodies, are dynamic intracellular organelles that contain a core rich in neutral lipids, such as triglycerides (TG) and cholesteryl esters [1]. Their surface is composed of a phospholipid monolayer with a unique phospholipid and fatty acid composition [2]. Initially considered as inert neutral lipid-storage compartments, LDs have become intensively studied organelles, implicated, for example, in the pathology of atherosclerosis, obesity, insulin resistance, type 2 diabetes [3], hepatic steatosis [4], cardiovascular disease, and inflammatory disorders [5,6]. The formation of LDs is a precisely regulated and inducible process which can be triggered by a variety of extracellular cues such as growth factors, long-chain fatty acids [7], oxidized low density lipoproteins [8] or by various environmental insults such as oxidative stress [9,10] and inflammatory stimuli [11]. LDs are rapidly induced under inflammatory conditions in leukocytes. In experimental models of sepsis, the interaction of bacterial lipopolysaccharide (LPS) with Toll-like receptor 4 (TLR4) leads to a considerable accumulation of LDs in macrophages [12]. LDs were shown to contain esterified arachidonic acid in their phospholipid and neutral lipid pools and proposed to be intracellular sites involved in the metabolism of arachidonic acid into eicosanoids [13,14]. Cytosolic phospholipase A_2-α (cPLA$_2$-α), also called group IVA PLA$_2$, is a central enzyme for the release of arachidonic acid from phospholipids and has been found to co-localize with LDs [15]. cPLA$_2$-α activation and release of proinflammatory lipid mediators are regulated by TLR4 signaling in LPS-activated macrophages [16].

The surface of LDs is coated with various proteins, many of which regulate their formation, growth or turnover [17]. Members of the PAT family proteins, namely perilipin, adipocyte differentiation-related protein (ADRP) and tail-interacting protein 47 kDa (TIP47),

Abbreviations: LD, lipid droplet; TG, triglyceride; TLR4, Toll-like receptor 4; LPS, lipopolysaccharides; ADRP, adipocyte differentiation-related protein; CNS, central nervous system; NO, nitric oxide; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; OA, oleic acid; BODIPY 493/503, 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene; PI3K, phosphatidylinositol 3-kinase; MKK, MAPK kinase; AP-1, activator protein-1; FFA, free fatty acid; cPLA$_2$-α, cytosolic phospholipase A $_2$ alpha

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share extensive amino acid sequence in their amino termini (PAT domain) and bind to LDs [17]. A recently implemented new nomenclature for the PAT proteins has changed the names of perilipin, ADRP and Tip 47 to perilipin 1, 2 and 3, respectively [18]. Perilipin-2 (ADRP) and perilipin-3 (Tip 47) are ubiquitously expressed in different cell types, including macrophages [19,20]. Perilipin-2 is a prominent LD-associated protein, whose expression level rises concomitantly with an increase in both LD abundance and TG level [21]. Perilipin-2 is a key regulator of LD formation and has been investigated in the context of metabolic syndrome and inflammatory diseases [5].

The present study sought to advance our understanding of the physiological roles of perilipin-2 and LDs in microglia, the resident macrophage population of the central nervous system (CNS) [22]. Microglia are of myeloid origin and share certain phenotypic characteristics with macrophages in their activated form. Upon activation microglia express cell surface markers that are also present on macrophages [23]. They respond rapidly to pathological challenges in the CNS by acquiring a reactive state and secreting proinflammatory cytokines, nitric oxide (NO) and reactive oxygen species [22,24]. Importantly, TLR4-initiated signaling underlies microglia-induced inflammation in the CNS [25]. LPS-induced activation of TLR4 triggers an activating downstream signal to mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and p38 [25]. Phosphorylation of p38 and JNK by upstream MAPK kinases leads to activation of transcription complex activator protein −1 (AP-1), which then induces the expression of proinflammatory cytokines [26]. In LPS-treated macrophages, stimulation of the binding of AP-1 to the Ets/AP-1 binding site results in increased perilipin-2 expression [27]. Although the effect of LPS on perilipin-2 protein expression in macrophages has been previously examined [27,28], this has not been investigated in microglia.

In the current study, we investigated the effects of LPS on LD status and perilipin-2 expression in murine N9 microglia cells. These cells express TLR4 and many other pathogen recognition receptors and rapidly respond to bacterial endotoxins [29]. The effect of LPS was compared with that of oleic acid (OA), a major plasma fatty acid [30]. In cells, OA is esterified and stored as triglyceride inside LDs. OA and other long chain fatty acids induce LD formation and perilipin-2 expression [7,31]. In the brain, OA is synthesized by astrocytes and acts as a neurotrophic factor for neurons [32]. Although induction of LD formation and perilipin-2 expression in response to OA has been assessed in different cell types, it has not been studied so far in microglia.

We hypothesized that stimulation of microglia with LPS may alter LD status and perilipin-2 expression via JNK and/or p38 MAPK. We show that LPS stimulation greatly increases microglial LD and TG content and induces perilipin-2 expression. We also demonstrate that pharmacological inhibition of p38 downregulates perilipin-2 expression and LD accumulation. OA-driven LD accumulation was not accompanied by p38 or JNK activation. In order to better understand the role of microglial LDs in the production of proinflammatory lipid mediators, we examined colocalization of $cPLA_2$-α with LDs. Our findings imply that LPS-driven changes in LD biogenesis and perilipin-2 expression could contribute to microglia-mediated inflammation in the CNS.

2. Materials and methods

2.1. Lipidomics analysis

Lipid analysis was performed as previously reported [33]. Prior to lipid extraction, internal standards were added corresponding to each lipid class, lipids were then extracted from whole cells by a modified Bligh and Dyer method, samples were dried under nitrogen and resuspended in chloroform. Immediately prior to injection the extracted lipids were combined with 2:1 methanol:chloroform with 0.1% (v/v) ammonium hydroxide. This was injected directly into a Q-TOF 2 mass spectrometer (Waters, Milford, MA) using a nano-esi spray source at 1 μl/min. Spectra were obtained in positive-ion mode ($PC+H^+$, $TAG+NH_4^+$) and negative-ion mode ($FFA\text{-}H^+$,$PA\text{-}H^+$, $PE\text{-}H^+$, $PG\text{-}H^+$, $PI\text{-}H^+$,$PS\text{-}H^+$,$CL\text{-}2H^+$). Acquired spectra were centroided using the Masslynx software then deconvoluted and deisotoped with excel macros.

2.2. Cell culture and treatments

Murine N9 microglia were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (+ L-glutamine, + 25 mM HEPES) (Gibco) supplemented with 5% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). Subconfluent cells were treated with lipopolysaccharides (LPS) (*Escherichia coli*, strain 055:B5) (Sigma, #62326) in medium containing 1% FBS as described in the figure legends. In experiments with oleic acid, control group cells were treated with bovine serum albumin (BSA) alone (0.1% w/v). Pharmacological inhibitors SP600125 (Calbiochem, #420119), SB203580 (Sigma, S8307) and LY294002 (Cell Signaling, #9901) were added 30 min prior to the addition of LPS (10 μg/ml). For every 10 μM of SP600125, 0.1% of DMSO was added to the cultures, as recommended by the supplier.

2.3. OA/BSA complex preparation

OA/BSA complex was formed by mixing 30 mg of sodium oleate (Nu-Check Prep, S-1120) with fatty acid-free BSA solution (5% w/v) (Sigma, A6003) for 5 h at 37 °C. After the incubation, the pH of the solution was adjusted to pH 7.4 and the solution was filtered through a 0.22 μm filter. Non-esterified OA concentration in this solution was determined with the NEFA C method kit (Wako). The final molar ratio of OA to BSA was approximately 4.4:1. Aliquots of the stock solution were stored at −20 °C.

2.4. Staining of lipid droplets

LDs in cells fixed with 4% paraformaldehyde were stained with the neutral lipid-staining fluorophores BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Invitrogen, D-3922) or HCS LipidTOX (Invitrogen, H34477). Stock solution of BODIPY 493/503 was made by dissolving the powder in DMSO. Protected from light cells were incubated with BODIPY 493/503 (20 μM, diluted in PBS) for 10 min or with HCS LipidTOX (1:200 in PBS) for 30 min at room temperature. Cells were washed at least twice with PBS.

2.5. Confocal microscopy

Images were acquired with a Zeiss LSM 510 NLO inverted confocal microscope using a Plan Achromat 63X/1.4 Oil DIC objective. Microglial cells were seeded on confocal chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY, USA) at a density of 1.5×10^4 cells/well (area of well = 0.8 cm^2) or on coverslips (area = 1.1 cm^2) at a density of 2×10^4 cells/coverlsip. Surfaces were coated with rat tail collagen (Sigma, C7661) prior to seeding. Coverslips were mounted on glass microscope slides (Fisher Scientific, 12-550-14) using glycerol-free mounting media Vectashield H-1000 (Vector) and were sealed around the perimeter with clear nail polish. Images of BODIPY 493/503-labeled LDs were acquired using an Argon 488 nm excitation laser and a 500–550 band pass (BP) filter. HCS LipidTOX-labeled LDs were detected using the HeNe 633 nm laser and a long pass (LP) 650 filter. Alexa Fluor 594 (red) was detected using HeNe 543 excitation laser and BP 565–615 IR filter. Each Z-stack image consisted of 10 to 20 optical slices taken at 0.3 μm intervals and had dimensions of $73.1\times73.1\times5.7$ μm (x, y, z) when zoomed by a factor of 2, and $97.5\times97.5\times5.7$ μm (x, y, z) when