Biochimica et Biophysica Acta 1791 (2009) 1066–1075

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Lipid bodies in oxidized LDL-induced foam cells are leukotriene-synthesizing organelles: a MCP-1/CCL2 regulated phenomenon

Adriana R. Silva [a], Patricia Pacheco [b], Adriana Vieira-de-Abreu [b], Clarissa M. Maya-Monteiro [b], Barbara D'Alegria [a,b], Kelly G. Magalhães [b], Edson F. de Assis [b], Christianne Bandeira-Melo [c], Hugo C. Castro-Faria-Neto [b], Patricia T. Bozza [b,*]

[a] Laboratório de Inflamação, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil
[b] Laboratorio de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz; Av. Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, RJ, Brazil
[c] Laboratório de Inflamação, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 23 September 2008
Received in revised form 9 June 2009
Accepted 23 June 2009
Available online 30 June 2009

Keywords:
Atherosclerosis
Foam cell
Lipid droplet
Lipoxygenase
Leukotriene
Inflammation
MCP-1/CCL2
ERK
Macrophage

ABSTRACT

Lipid-laden foam macrophages are emerging as key players in early atherogenesis. Even though cytoplasmic lipid bodies (lipid droplets) are now recognized as organelles with cell functions beyond lipid storage, the mechanisms controlling lipid body biogenesis within macrophages and their additional functions in atherosclerosis are not completely elucidated. Here we studied oxLDL-elicited macrophage machinery involved in lipid body biogenesis as well as lipid body roles in leukotriene (LT) synthesis. Both *in vivo* and *in vitro*, oxLDL (but not native LDL) induced rapid assembly of cytoplasmic lipid bodies-bearing ADRP within mice macrophages. Such oxLDL-elicited foamy-like phenotype was a pertussis toxin-sensitive process that depended on a paracrine activity of endogenous MCP-1/CCL2 and activation of ERK. Pretreatment with neutralizing anti-MCP-1/CCL2 inhibited macrophage ADRP protein expression induced by oxLDL. By directly immuno-localizing leukotrienes at their sites of synthesis, we showed that oxLDL-induced newly formed lipid bodies function as active sites of LTB_4 and LTC_4 synthesis, since oxLDL-induced lipid bodies within foam macrophages compartmentalized the enzyme 5-lipoxygenase and five lipoxygenase-activating protein (FLAP) as well as newly formed LTB_4 and LTC_4. Consistent with MCP-1/CCL-2 role in ox-LDL-induced lipid body biogenesis, in CCR2 deficient mice both ox-LDL-induced lipid body assembly and LT release were reduced as compared to wild type mice. In conclusion, oxLDL-driven foam cells are enriched with leukotriene-synthesizing lipid bodies – specialized organelles whose biogenic process is mediated by MCP-1/CCL2-triggered CCR2 activation and ERK-dependent downstream signaling – that may amplify inflammatory mediator production in atherosclerosis.

1. Introduction

A hallmark of atherosclerosis is the fatty streak, an accumulation of lipid-laden macrophages namely foam cells beneath the endothelial layer in arteries. Since it is the earliest recognizable lesion of atherosclerosis, much attention is focused on understanding the etiology of this phenomenon and the roles of foam cells in disease. Uncontrolled oxLDL uptake by macrophages through scavenger receptors causes triglyceride and cholesterol loading, followed by cholesterol esterification and storage of cholesteryl esters (CEs) in cytoplasmic lipid bodies or lipid droplets [1–4].

Cytoplasmic lipid bodies are increasingly recognized as dynamic and functionally active organelles [5–7]. Lipid bodies in macrophages are composed of triglycerides and cholesterol esther enriched core, which is surrounded by a monolayer of phospholipids. In addition to lipids, a variable and diverse group of proteins have been found associated to lipid bodies. Perilipin, adipose differentiation related protein (ADRP) and TIP47 – members of the PAT family – are – structural proteins considered essential for lipid storage and metabolism (reviewed in [8]). ADRP is associated with cytoplasmic lipid bodies in all types of cells examined and is described as a specific protein marker for lipid bodies [9,10], including in monocyte/macrophages [11–13]. In addition, ADRP has been implicated in atherosclerosis progression as increased ADRP levels are observed in atherogenic plaques [14,15] and ADRP gene deletion leads to reduced

Abbreviations: ADRP, adipose differentiation related protein; AA, arachidonic acid; CCR2, CC chemokine receptor 2; COXs, cyclooxygenases; $cPLA_2$, cytosolic phospholipase A_2; cysLTs, cysteinyl leukotrienes; ERK, extracellular signal-regulated kinase; FLAP, five lipoxygenase-activating protein; oxLDL, oxidized low density lipoprotein; LO, lipoxygenase; LTs, leukotrienes; LPS, lipopolysaccharide; IL, interleukin; i.p., intraperitoneal; MCP-1/CCL2, monocyte chemotactic protein-1/CC Ligand 2; MAP, mitogen-activated protein; PAF, platelet-activating factor; WT, wild type

* Corresponding author.
E-mail address: pbozza@ioc.fiocruz.br (P.T. Bozza).

1388-1981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbalip.2009.06.004

numbers of lipid droplets in foam cells in atherosclerotic lesions and protects the mice against atherosclerosis in apolipoprotein E-deficient mice [16].

Lipid bodies from different cell types, including a monocytic cell line, are the location of key enzymes involved in cholesterol metabolism and for fatty acid synthesis, suggesting that both anabolic and catabolic steps of lipid metabolism take place at lipid bodies [17–22]. In addition, proteins involved in cell signaling and membrane trafficking including proteins of Rab family, small GTPases, PI3k, PKC and MAP kinase; and proteins involved in cell–cell communications and inflammation including cytokines and eicosanoid-forming enzymes have been localized in lipid bodies adding to the concept that lipid bodies are not inert fat depots but dynamic and multifunctional organelles (reviewed in [5,7]). In inflammatory cells, lipid bodies have been associated to arachidonic acid metabolism. Indeed, leukocyte lipid bodies are sites of esterified arachidonic acid storage and compartmentalize the enzymes involved in its metabolic conversion into eicosanoids including cyclooxygenase (COX) and 5-lipoxygenase (5-LO) [13,23–29]. Moreover, it has been recently demonstrated that depending on the cell type and stimulatory conditions lipid bodies are sites for 5-LO- and COX-derived eicosanoid synthesis [13,30–32].

The 5-LO pathway has been linked to atherosclerosis in both experimental mouse models and in humans [33–35]. In fact, histological analysis revealed an abundance of 5-LO in macrophages and foam cells from human atherosclerotic lesions [21,33]. Furthermore, the carotid-artery intima-media thickness, an indicator of systemic atherosclerosis, correlates with the incidence of variant 5-LO promoter genotypes in patients who lack the common human promoter allele [36] and higher 5-LO expression and increased LTB_4 production has been associated with atherosclerotic plaque instability [21,37]. However, the mechanisms involved in the enhanced leukotriene production in atherosclerotic lesions and the contributions of foam macrophages and their cytoplasmic lipid bodies to this phenomenon is not well-understood.

Foam cell is not only a cell with abnormalities in lipid handling, it is now established that it actively contributes to further development and amplification of the inflammatory response [38]. Determining the mechanisms of foam macrophage lipid metabolism, and biogenesis and functions is essential for the understanding of the pathophysiology of atherosclerosis and other inflammatory processes. Our results show that lipid bodies triggered by oxLDL exhibited LTB_4 and LTC_4 synthetic capacity. Moreover, we demonstrated that foam cell formation, ADRP expression and functions in inflammatory mediator production is highly regulated by MCP-1/CCL2 through its cognate receptor CC chemokine receptor 2 (CCR2) and mitogen-activating protein (MAP) kinase – ERK – downstream signaling.

2. Materials and methods

2.1. Purification and oxidation of human LDL

Human LDL was isolated from plasma after density adjustment to 1.3 g/mL with KBr. A gradient of plasma and saline solution (NaCl 0.9%) was centrifuged for 2 h 40 min/150,000 ×g. After centrifugation, the band of lipoproteins with density between 1,019 and 1,062 was collected and dialyzed overnight. LDL was treated with a PAF acetylhydrolase inhibitor (AESBF 200 μM; Pentapharm A.G. Laboratories (Basel, Switzerland) before oxidation with $CuSO_4$ (10 μM) for 18 h/37 °C. Control LDL was not subjected to oxidation and EDTA (40 μM) was added to this material.

2.2. Animals

C57BL/6 mice of both sexes weighing 20–25 g were obtained from Fundação Oswaldo Cruz Breeding Unit. CCR2-deficient mice (in a homogeneous C57BL/6 background) [39] were kindly provided by Dr. Kuziel and bred at the Instituto Oswaldo Cruz experimental animal facility. Animals were caged with free access to food and fresh water in a room at 22–24 °C and a 12 h light–dark cycle. Animal protocols were in agreement to the animal care guidelines of the National Institute of Health and were approved by the Fundação Oswaldo Cruz Animal Welfare Committee.

2.3. Induction of lipid body formation in vitro

Peritoneal macrophages (10^6cells/mL) were obtained by washed with PBS cavities of C57Bl/6 mice and the leukocytes were stimulated with LDL, oxLDL (200 or 400 μg lipoprotein/mL) or vehicle (PBS) at 37 °C for 6 h. In selected experiments, peritoneal macrophages were treated with anti-MCP-1/CCL2 monoclonal antibody (10 μg/mL; R&D Systems, Minneapolis, MN), IgG isotype control (10 μg/mL; Sigma), pertussis toxin (100 ng/mL, Calbiochem) or ERK phosphorylation inhibitor U 0126 (10 μM; Cayman Chemicals, Ann Arbor, Michigan) at 37 °C for 30 min before vehicle or oxLDL stimulation.

The cell viability, determined by trypan blue dye exclusion at the end of each experiment, was >90%. The cells were centrifuged in a cytocentrifuge (550 rpm/5 min) onto glass slides (10^5 cells/slide) and stained with oil red O (ORO, Sigma) or osmium tetroxide (Electron Microscopy Science (Fort Washington, PA). In brief, leukocytes on cytospin slides were fixed in 3.7% formaldehyde in Ca^{2+}-Mg^{2+}-free HBSS (pH 7.4) for 30 min. For oil red O staining, cells were rinsed in propylene glycol 85%, stained in oil red O 0.5% for 10 min, rinsed in propylene glycol 85% (5 min) and counterstained with hematoxylin for 30 s. For osmium staining, cells were rinsed in 0.1 M cacodylate buffer, 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in H_2O, and then dried and mounted. The morphology of fixed cells was observed, and osmium-stained lipid bodies were enumerated by light microscopy with a 100× objective lens in 50 consecutively scanned leukocytes.

Alternatively, lipid bodies were quantified by the measurement of ORO fluorescent area. The measurement of the area of lipid bodies was obtained with a 60 objective (at least four fields per slide). The images were transformed into black and white pictures and analyzed with Image 2D (GE Healthcare). The spots were determined by automatic spot detection, and the total area of fluorescent lipid bodies was obtained for each field and divided by the number of cells in the respective field.

2.4. Modified LDL-induced peritonitis and lipid body formation in vivo

CCR2-deficient mice or C57Bl/6 wild type received an intraperitoneal injection (i.p.) of LDL or oxLDL — 200 or 400 μg lipoprotein/cavity. After 6 h the animals were sacrificed in a CO_2 gas chamber, the peritoneal cavity was washed with 3 mL of PBS. The peritoneal wash was recovered and lipid body formation in leukocytes was evaluated on cytosmears stained as described above. The cell-free peritoneal fluid was stored at −20 °C for LT quantification as described below.

2.5. ADRP immunoblot-detection

ADRP protein expression was detected by Western blotting in peritoneal macrophages 6 h after oxLDL stimulation *in vitro*. In brief, macrophage lysates were subjected to SDS-PAGE in 5–15% acrylamide gradient SDS-polyacrylamide gels and transfered onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline-Tween (TBST; 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Membranes were incubated with anti-ADRP polyclonal antibody (Research Diagnostics Inc., Flanders, NJ), or anti-β-actin monoclonal antibody (BD Transduction Laboratories) in TBST with 1% nonfat dry milk, followed by incubation with