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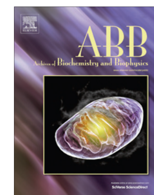

Cholesterol load of microglia: Contribution of membrane architecture changes to neurotoxic power?

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ABSTRACT

Considerable evidence provides a link between hypercholesterolemia and ageing-related neurodegenerative diseases. The present study was aimed to provide a complex view on the effects caused by cholesterol- and cholesterol 5α,6α-epoxide-load in microglia, with particular emphasize put on membrane proteins.

Prolonged application of oxysterol significantly enhanced LPS-stimulated association of cytosolic NADPH-oxidase factor p47[phox] with detergent-resistant microdomains (DRMs) in BV-2 cells. Although the treatment with both sterols does not influence the portion of CD36 receptor in DRMs, its apparent surface-cellular expression was altered. Even though sterol-treatment potentiated oxidant production by microglia, as well as their phagocytosis, these effects, however, appeared to be independent of cholesterol profusion in the membrane. In addition, oxysterol-treatment resulted in a loss of DRMs-associated activity of 26S proteasome, the protease critically regulating both protein homeostasis and immune signaling in microglia. Oxysterol relatively ameliorated cytotoxic effects of inflammed microglia on co-cultured PC12 cells.

The outcomes of this study suggest that cholesterol and cholesterol oxides can differentially modulate microglia resulting either in impairment of their immune functionalities or enhanced neurotoxic power. Moreover, these findings shed light on possible complexity of this effect, produced by simultaneous affection of the levels, distribution and function of the critical proteins within microglial membrane compartments.

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Introduction

A growing body of evidence suggests that dysregulated cholesterol metabolism may be involved in the pathogenesis of Alzheimer disease (AD)[1]. Inhibition of cholesterol synthesis by statins was shown to decrease amyloid-β levels and AD pathology in several animal models of AD [1,2], while high fat diets dramatically exacerbated AD-related pathology in mouse models [3,4]. The observations from rabbits suggest that hypercholesterolemia and heart disease accelerate brain ageing. Epidemiological studies show a good match with the animal experiments, as they confirmed positive correlations among three disease-related conditions – hypercholesterolemia, high incidence of coronary artery disease and the increased risk of AD [5]. Brain ageing as well as AD pathology have been documented to be associated with an increase of free cholesterol in the brain [6]. Moreover, products of both enzymatic and non-enzymatic oxidation of cholesterol (oxysterols) have also been linked to pathology of AD. Unlike unoxidized cholesterol, the oxysterols are able to cross the blood brain barrier and flow from circulation into brain and vice versa [7,8]. In addition, dysregulated expression of specific cholesterol hydroxylases may also account for elevation of oxysterols in the brain parenchyma [8].

However, in contrast to well-recognized consequences of hypercholesterolemia state on peripheral macrophages with regard to atherosclerosis development (involving accumulation of cholesterol-laden foam cells within atherosclerotic plaques [9]), only a few reports provide link between the neurodegenerative diseases and cholesterol-load derived pathophysiology of their brain counterpart, microglia [10].

Microglia are a type of glial cells that act as the main form of active immune defense in the central nervous system (CNS). Under normal conditions, the microglial cells are involved in a range of processes, including the initiation of apoptosis during neuronal development, expression of neurotrophins, control of synaptogenesis and synaptic activity [11–14]. Furthermore, the microglial cells seem to be able to recognize a wide range of extracellular material,

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[1] *Abbreviations used:* AD, Alzheimer disease; H_2DCF-DA, 2′,7′-dichloro-dihydrofluorescein diacetate; DF, detergent-free; DRMs, detergent-resistant microdomains (membranes); CHOL, cholesterol; EGF, epidermal growth factor; EPOX, cholesterol 5α,6α-epoxide; ERK, extracellular signal-regulated kinase, GAPDH, glyceraldehyde phosphate dehydrogenase; IL, interleukin; LPS, lipopolysaccharide; MCD, methyl-β-cyclodextrin; MEK, mitogen-activated protein kinase; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NF-κB, nuclear factor - κB; suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin; NBT, nitroblue tetrazolium; OGP, octyl-β-glucopyranoside; PMA, phorbol myristate acetate; PS, proteasome; TX, TritonX-100.

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take this material up, and degrade it. Hence, these cells are regarded as "house-keepers" cleaning up cellular debris and toxic substances in the CNS. However, activated microglia produce also a variety of proinflammatory and neurotoxic factors that are shown to induce and/or exacerbate neurodegeneration [15].

Recent studies have established contribution of toll-like receptors (TLRs) activation to neuropathologies such as ischemia and neurodegeneration [16]. Toll-like receptor-4 responds predominantly to lipopolysaccharide (LPS) from gram-negative bacteria through its co-receptor, myeloid differentiation protein-2 (MD-2) [17]. TLR-dependent microglia activation plays a crucial role in initiating host defence responses during CNS microbial infection. However, LPS also serves as one of the most common inflammogens, used to investigate the impact of inflammation on neuron death. Gao et al. [18] demonstrated that free radicals generated by LPS-activated microglia are the primary contributor to the degeneration of dopaminergic neurons in animal model of Parkinson disease. In addition, TLR4 interaction in microglia with endogenous ligands, such as amyloid β has been shown to be a critical mechanism in development of chronic neuroinflammation and neurotoxicity in AD [19].

Various studies provide evidence that cholesterol and its oxidized metabolites may affect expression and synthesis of the membrane proteins of various cell types, including neurons [20,21]. However, the role of cholesterol as one of the major building blocks of the human and animal plasma membrane, (critically influencing the membrane's permeability as well as functionalities of the embedded proteins) may be in these studies underestimated. Moreover, the alteration of cellular cholesterol levels may have critical impact primarily on the plasma membrane depositions within localized regions, called lipid rafts.

Lipid rafts or "detergent-resistant membranes" (DRMs), membrane microdomains that are enriched in cholesterol and sphingolipids, have been implicated in diverse physiologic mechanisms, such as signal transduction, trafficking and lipid transport [22]. The cell signaling function of DRMs is thought to arise from the ability of these microdomains to selectively retain or exclude special proteins, what is resulting in the formation of active multiprotein complexes. Because lipid rafts may be functionally altered by pathophysiologic changes in lipid metabolism [23], the analysis of DRMs-resident proteins may provide useful insights into signaling mechanisms that operate in normal state as well as in disease.

In the present work, the impacts of supplementation with cholesterol (CHOL) and (ep)oxy-cholesterol (EPOX, cholesterol 5α,6α-epoxide) on two membrane proteins, critically involved in promoting prooxidant and neurotoxic profile of microglia, the cytosolic NADPH-oxidase factor p47[phox] and class B scavenger receptor CD36, were analysed in murine BV-2 microglia cells. In addition to investigation of their membrane redistributions, the corresponding functional changes (assessed by oxidant-production-assay and phagocytosis assay) also were investigated. Furthermore, the membrane redistribution and corresponding membrane-region-specific activities of 26S proteasome, the protease critical for clearance of damaged or oxidized proteins and immune signalling in microglia cells, were explored as well. Finally, the alterations of microglia characteristics due to EPOX and CHOL exposure were confronted with their cytotoxic effects on co-cultured PC12 cells.

Material and methods

Materials

All reagents were of analytical grade or the highest purity available. Cholesterol, cholesterol 5α,6α-epoxide, methyl-β-cyclodextrin (MCD), filipin III, carboxylate-modified polystyrene yellow-green latex beads (1 μm), 2′,7′-dichloro-dihydro-fluorescein diacetate (H_2DCF-DA), nitroblue tetrazolium (NBT) and other chemicals were from Sigma (Germany) unless otherwise stated. Vybrant® Alexa Fluor® 488 Lipid Raft Labeling Kit and Alexa Fluor®546 Donkey Anti-Goat IgG (H + L) was from Molecular Probes (Eugene, OR). Rabbit polyclonal antibody to proteasome β type 5 subunit, rabbit polyclonal antibody to flotillin-1, and mouse monoclonal anti-GAPDH antibody were purchased from Abcam (Cambridge, UK). Santa Cruz Biotechnology provided goat polyclonal p47-phox antibody and rabbit polyclonal anti-CD36 antibody.

Cell culture and cholesterol treatment

The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia (Perugia, Italy; [24]). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (Biochrom), 2 mM L-glutamine and 1% P/S (100 U/ml penicillin, 100 μg/ml streptomycin, Biochrom AG) and maintained in 5% CO_2 at 37 °C. Cells were used for 20 passages at most. PC12 cells were a gift from Dr. V. Ergin (Department of Molecular Biology & Genetics, Faculty of Medicine, Gazi University, Ankara, Turkey). PC12 cells were cultured in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 1% P/S (100 U/ml penicillin, 100 μg/ml streptomycin) in 5% CO_2 at 37 °C.

The subconfluent layers of BV-2 were treated with cholesterol (CHOL, 20 μmol/l) and cholesterol 5α,6α-epoxide (epoxycholesterol, EPOX, 20 μmol/l) for 24 h, followed by additional 24 h-incubation with EPOX (20 μmol/l) and CHOL (20 μmol/l) with or without bacterial lipopolysaccharide (LPS, 10 μg/ml) from *Escherichia coli* (Enzo Life Sciences, GmbH, Germany). Alternatively, the cells were treated with the same concentrations of EPOX and CHOL during 48 h-period followed by stimulation with phorbol myristate acetate (PMA, 2 μmol/l, 2 h). In some experiments, following the LPS-stimulation or prior to stimulation with PMA, the cells were incubated with cholesterol chelating substance, methyl-β-cyclodextrin (MCD, 1 mmol/l, 10 mmol/l) in complete medium for 30 min at 37 °C. For co-culture conditions, PC-12 cells were added to BV-2 cells in a ratio 2:1 in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% P/S containing CHOL and EPOX with or without LPS.

Cell viability and morphology

Viability was evaluated by trypan blue staining (0.4% trypan blue in phosphate-buffered saline, PBS). Cellular morphology was examined in living cells using Leica DM IL equiped with Integrated Modulation Contrast. The cell size as well as other parameters were calculated using Image J (version 1.46r, NIH, http://rsb-web.nih.gov/ij/). The average parameter value for each treatment was calculated from at least 3 randomly selected images (minimum 150 cells in total). Viability of PC-12 cells was evaluated by MTT assay and acridine orange/ethidium bromide (AO/EB) staining as described before [27]. Briefly, the co-cultured cells in the wells were triturated. Ten microliters of cell suspension was mixed with 2 μl AO/EB (100 μg/ml:100 μg/ml) dye solution and examined by fluorescent microscopy. One hundred microliters of cell suspension was mixed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (10 μl, 5 mg/ml) and the solubilized formazan was evaluated spectrophotometrically at 570 nm.

Isolation of detergent-resistant domains (DRMs)

DRMs were isolated according to Adam et al. [25] with slight modifications. Ten to fifty million cells were used for each extraction method. At the end of incubations the cells were washed

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