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ROSUVASTATIN ENHANCES ANTI-INFLAMMATORY AND INHIBITS PRO-INFLAMMATORY FUNCTIONS IN CULTURED MICROGLIAL CELLS

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Abstract—Microglial activation results in profound morphological, functional and gene expression changes that affect the pro- and anti-inflammatory mechanisms of these cells. Although statins have beneficial effects on inflammation, they have not been thoroughly investigated for their ability to affect microglial functions. Therefore the effects of rosuvastatin, one of the most commonly prescribed drugs in cardiovascular therapy, either alone or in combination with bacterial lipopolysaccharide (LPS), were profiled in pure microglial cultures derived from the forebrains of 18-day-old rat embryos. To reveal the effects of rosuvastatin on a number of pro- and anti-inflammatory mechanisms, we performed morphometric, functional and gene expression studies relating to cell adhesion and proliferation, phagocytosis, pro- and anti-inflammatory cytokine (IL-1β, tumor necrosis factor α (TNF-α) and IL-10, respectively) production, and the expression of various inflammation-related genes, including those related to the above morphological parameters and cellular functions. We found that microglia could be an important therapeutic target of rosuvastatin. In unchallenged (control) microglia, rosuvastatin inhibited proliferation and cell adhesion, but promoted microspike formation and elevated the expression of certain anti-inflammatory genes (*Cxcl1*, *Ccl5*, *Mbl2*), while phagocytosis or pro- and anti-inflammatory cytokine production were unaffected. Moreover, rosuvastatin markedly inhibited microglial activation in LPS-challenged cells by affecting both their morphology and functions as it inhibited LPS-elicited phagocytosis and inhibited pro-inflammatory cytokine (IL-1β, TNF-α) production, concomitantly increasing the level of IL-10, an anti-inflammatory cytokine. Finally, rosuvastatin beneficially and differentially affected the expression of a number of inflammation-related genes in LPS-challenged cells by inhibiting numerous pro-inflammatory and stimulating several anti-inflammatory genes. Since the microglia could elicit pro-inflammatory responses leading to neurodegeneration, it is important to attenuate such mechanisms and promote anti-inflammatory properties, and develop prophylactic therapies. By beneficially regulating both pro- and anti-inflammatory microglial functions, rosuvastatin may be considered as a prophylactic agent in the prevention of inflammation-related neurological disorders.

Key words: anti-inflammation, gene expression, lipopolysaccharide, phagocytosis, pro-inflammation, rosuvastatin.

INTRODUCTION

Microglia, the main immune cells in the central nervous system (CNS), are derived from the monocyte/macrophage lineage (Ginhoux et al., 2010). They play important roles in both physiological and pathophysiological conditions such as traumatic injury, stroke, ischemia or neurodegenerative diseases (Kreutzberg, 1996). In response to activation, the microglia transform from a resting state to an activated form, during which profound morphological and functional changes take place, such as process retraction, proliferation, phagocytosis and cytokine expression (Gehrmann et al., 1995; Kreutzberg, 1996; Hanisch, 2002; Luo and Chen, 2012). Although such anti-inflammatory mechanisms are essential in protecting the CNS, activated microglial cells can also be harmful to neurons by eliciting neuroinflammation that could lead to neurodegeneration (Banati et al., 1993; Gehrmann et al., 1995; Gonzalez-Scarano and Baltuch, 1999; Streit, 2002; Graeber, 2010; Gresa-Arribas et al., 2012; Ghosh et al., 2013). In Alzheimer's disease (AD), for example, the microglia produce pro-inflammatory factors such as interleukin-1β (IL-1β) around the amyloid plaques, and these factors may themselves become important components of the AD pathology because of their ability to increase the expression of amyloid precursor protein (Cordle and Landreth, 2005; Ghosh et al., 2013).

Accumulating evidence indicates that a sequence of events contributes to the development and progression of AD, including oxidative stress, inflammation, and altered cholesterol metabolism (Gamba et al., 2015). Oxidative stress may be crucial in the development of

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Abbreviations: AD, Alzheimer's disease; Cd, cluster of differentiation; CNS, central nervous system; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle's Medium; E18, embryonic day 18; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); Iba1, ionized calcium-binding adaptor molecule 1; IL, interleukin; LPS, bacterial lipopolysaccharide; MS, multiple sclerosis; PBS, phosphate-buffered saline; RT, room temperature; S.D., standard deviation; subDIV, subcloned days *in vitro*; TBS, Tris-buffered saline; TI, transformation index; TNF-α, tumor necrosis factor α.

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neuroinflammation as oxidized cholesterol could act as a link connecting peripheral hypercholesterolemia to altered cholesterol metabolism in the brain (Gamba et al., 2015). Cholesterol modulates the processing of amyloid precursor protein and the production of β-amyloid peptides (Shobab et al., 2005), while removing cholesterol ameliorates the production these peptides in animal models (Bodovitz and Klein, 1996; Simons et al., 1998).

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are the agents of first choice for the treatment of high cholesterol levels (Taylor et al., 2013). Although their main effects are related to the lipid metabolism (inhibition of cholesterol synthesis, reduction of the levels of low-density lipoproteins and triglycerides, and stimulation of the expression of high-density lipoproteins), they also strongly modulate inflammatory cells around atherosclerotic plaques (Wierzbicki et al., 2003; Burg and Espenshade, 2011). Apart from their therapeutic use in cardiovascular diseases, statins may also have beneficial effects in the CNS (Zipp et al., 2007; van der Most et al., 2009; Famer et al., 2010) as animal studies have demonstrated that statins attenuate neuroinflammation (Zelcer et al., 2007) and reduce senile plaques and inflammatory responses (Kurata et al., 2012).

Interestingly, in spite of being an obvious target for statins, microglial cells have not been at the focus of statin research. There have only been a few studies to demonstrate that under *in vitro* circumstances the microglia respond to statins such as atorvastatin and simvastatin (Lindberg et al., 2005; Nakamichi et al., 2006). In the present study, we investigated the effects of rosuvastatin, the most widely used and arguably the most effective statin (Nissen et al., 2006; Nicholls et al., 2011), on cultured pure microglia cells derived from mixed cultures of 18-day-old embryonic (E18) rat forebrains under control (unstimulated) and bacterial lipopolysaccharide (LPS)-stimulated conditions (Nakamura et al., 1999; Lund et al., 2006; Gresa-Arribas et al., 2012). To reveal the effects of rosuvastatin on a number of pro- and anti-inflammatory mechanisms, we performed morphometric, functional and gene expression studies relating to cell adhesion and proliferation, phagocytic capability, pro- and anti-inflammatory cytokine (IL-1β, tumor necrosis factor α (TNF-α) and IL-10, respectively) production, and the expression of various inflammation-related genes, including those related to the above morphological parameters and cellular functions.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were carried out in strict compliance with the European Council Directive (86/609/EEC) and EC regulations (O.J. of EC No. L 358/1, 18/12/1986) regarding the care and use of laboratory animals for experimental procedures, and followed the relevant Hungarian and local legislation requirements. The experimental protocols were approved by the Institutional Animal Welfare Committee of the University of Szeged (I-74-II/2009/MÁB). The pregnant Sprague–Dawley rats (45 rats, 170–190 g) were kept under standard housing conditions and fed *ad libitum*.

Antibodies

For a thorough characterization of different microglial phenotypes developed *in vitro*, an antibody against ionized calcium-binding adaptor molecule 1 (Iba1), an intracellular actin- and Ca^{2+}-binding protein expressed in the CNS specifically in macrophages and microglia (Ahmed et al., 2007), was used in our immunocytochemical and Western blot analyses. The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as an internal control in Western blot experiments (Wu et al., 2012). Dilutions of primary and secondary antibodies, and also incubation times and blocking conditions for each antibody used were carefully tested for both immunocytochemistry and Western blot analysis. To detect the specificities of the secondary antisera, omission control experiments (staining without the primary antibody) were performed. In such cases, no fluorescent or Western blot signals were detected.

Cell cultures

Pure microglial cells were isolated from mixed primary cortical cell cultures of rat embryos of either sex by the method we described earlier (Szabo and Gulya, 2013). Sibling embryos obtained from the same pregnancy were processed for culturing together; each pregnancy was considered as an independent experiment. Briefly, 10–12 fetal rats (E18) under ether anesthesia were decapitated and the frontal lobe of the cerebral cortex was removed, minced with scissors, incubated in 9 ml Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, USA) containing 1 g/l D-glucose, 110 mg/l Na-pyruvate, 4 mM L-glutamine, 3.7 g/l $NaHCO_3$, 10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, 25 μg/ml amphotericin B and 0.25% trypsin for 10 min at 37 °C, and then centrifuged at 1000*g* for 10 min at room temperature (RT). The pellet was resuspended and washed twice in 5 ml DMEM containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and centrifuged for 10 min at 1000*g* at RT. The final pellet was resuspended in 2 ml DMEM/10% FBS, after which the cells were plated in the same medium on a poly-L-lysine-coated culture flask (75 cm^2, 12×10^6 cell/flask) and cultured at 37 °C in a humidified air atmosphere supplemented with 5% CO_2, in one or other of the following ways: (1) in poly-L-lysine-coated coverslips (15 × 15 mm; 2×10^5 cells/coverslip) for immunocytochemistry; (2) in poly-L-lysine-coated Petri dishes (60 mm × 15 mm; 4×10^5 cells/dish) for Western blot analyses and enzyme-linked immunosorbent assay (ELISA) studies; or (3) in a poly-L-lysine-coated culture flask (75 cm^2, 12×10^6 cells/flask) for the subsequent generation of pure microglial cell cultures.

Secondary microglial cells were subcloned from mixed primary cultures (DIV7) maintained in a poly-L-lysine-coated culture flask (75 cm^2, 12×10^6 cells/flask) by shaking the cultures at 100 rpm in a platform shaker for 30 min at 37 °C. Cultures from the same pregnancy

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