MORPHOLOGICAL, FUNCTIONAL AND IMMUNOMODULATORY EFFECTS OF ROSUVASTATIN AND ASPIRIN IN CULTURED MICROGLIAL CELLS

Summary of Ph.D. Thesis

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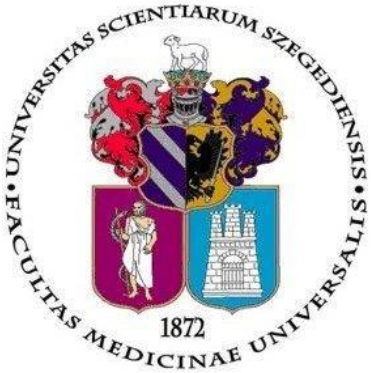

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I. **Kata D**, Gulya K (2014) Aspirin regulates the inflammatory responses of activated microglia. **9th FENS Forum of Neuroscience, Milan, Italy, FENS-0477; C112**

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INTRODUCTION

Roles of microglial cells in the central nervous system

Microglia is the principal immune cell in the central nervous system (CNS). Microglial cells are the most remarkable sensors that contribute to the homeostatis of the CNS. They play important roles in both physiological and pathophysiologycal conditions such as traumatic injury, stroke, ischemia or neurodegenerative diseases. Microglial cells are well known for their functions related to morphological features. In the healty brain micoglial cells have a ramified morphology. These cells have small soma with branching processes. This appearance is associated with the "resting" state. Upon challenges such as infection, ischemia, trauma, neurodegenerative diseases or altered brain homeostasis microglia transform from resting to activated state. The activation leads to rapid and profound changes in their morphology, functions and gene expression patterns. During the activation microglia reduce their cellular complexity by retracting their processes and turn into an amoeboid phenotype. Activated microglial cells are also capable of releasing different types of soluble factors with inflammatory and immunoregulatory effects. They are able to release chemoattractive factors which can guide immune cells to the CNS. Further, surface molecules for cell-cell and cell-matrix interactions such as complement receptors and major histocompatibility complex (MHC) molecules are also upregulated. Activated microglial cells also have increased proliferative and phagocytic activity. Although such anti-inflammatory mechanisms are essential in protecting the CNS, activated microglial cells can also be harmful to neurons. Microglial cells and inflammatory responses play crucial role in connection with multiple neurodegenerative diseases. In Alzheimer's disease (AD) brain microglial cells could be located in the centre of senile plaques. Amyloid-beta (Aβ) protein activate the production of proinflammatory cytokines and mediators such as IL-1β, IL-6, IL-8, TNF-α. Parkinson's disease can also be characterized by overactive immune response of the brain. Pharmacological regulation of microglial functions may play an important role in the prevention and during the progression of several neurodenegenrative diseases.

The mechanisms and effects of statins

Statins (3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors) are commonly used in the treatment of high blood cholesterol level. Their main biochemical effect is the reduction of the amount of low-density lipoprotein (LDL) cholesterol, but they can also increase the amount of high density lipoprotein (HDL) cholesterol and reduce triglyceride concentration. Statins act by blocking the cholesterol synthesis through the inhibition of the enzyme: inhibition of its first step also decreases the pools of intermediate metabolites in the pathway that have crucial metabolic functions. Through their effect of isoprenylated G-protein-mediated signalling, statins also regulate a wide rage of cellular functions. Apart from their therapeutic use in cardiovascular diseases, however, statins may also have beneficial effects in the CNS. Statins attenuate neuroinflammation and reduce senile plaque formation and inflammatory responses. They induce neuroprotection by promoting the expression of neurothophic factors such as BDNF, and also protect cells and tissues from oxidative damage by reducing the production of ROS. Of all the commercially available statins, rosuvastatin exhibits the greatest inhibitory effect on cholesterol biosynthesis. The strong anti-inflammatory and immunomodulatory effects of statins indicate that these drugs may have beneficial regulatory effects on microglial cells and may be able to modulate the strong neuroinflammatory reactions orchestrated by microglial cells.

The mechanisms and effects of aspirin

Aspirin is one of the most widely used drugs worldwide. It exerts analgesic, anti-inflammatory and antipyretic effets. It is extensively studied in the cardiovascular system. Its main action is the inhibition of cyclooxygenase (COX) activity. COX is the key enzyme in the conversion of arachidonic acid to prostaglandins and other lipid mediators which are involved in several physiological and pathological processes including inflammation. During the initial phase of the inflammation prostaglandins play crucial role as local mediators in the development of inflammatory conditions and promote the activation of proinflammatory cytokines. Aspirin is able to downregulate pro-inflammatory cytokines released by macrophages such as IL-1β, IL-6, IL-12, IFN-γ. COX-2 acetylation by aspirin results in a modulated enzymatic activity which initiates the generation of new substrates that can interact with lipoxygenase to genetare aspirin-triggered lipoxins. These lipoxins are anti-inflammatory mediators that inhibit pro-inflammatory cytokine release. In the brain both COX-1 and COX-2 are constitutively

expressed. COX-1 is an important player in several inflammatory actions in the CNS and upregulated in numerous models of neuroinflammation. In AD brain increased levels of COX-1, COX-2 and PGE_2 can be observed. COX-1 expressing microglial cells surround amyloid plaques in AD. COX-1 expressing microglial cells are located in the developing necrotic core after focal ischemia and COX-1 inhibition reduces the oxidative stress and neuronal injury after global ischemia. These observations suggest that COX expressing microglial cells play crucial roles under pathological situations and COX inhibition by aspirin might be beneficial.

SPECIFIC AIMS

Inflammation has a critical role in the initiation and progression of neurodegeneration. Microglial activation results in profound morphological, functional and gene expression changes that activate both pro- and anti-inflammatory mechanisms that in turn not only protect the nervous tissue but can elicit chronic inflammation that could lead to the development of neuropathological conditions. Drugs that alter the excessive microglial activation might therefore be useful for the prevention and treatment of several neurodegenerative diseases. Statins are used for the primary treatment of hypercholesterolemia albeit their anti-inflammatory effects are also noted. Although a few previous studies examined the effets of statins on the CNS, these experiments only used lipophilic statins such as simvastatin or atorvastatin. Rosuvastatin exhibits the greatest inhibitory effect on cholesterol biosynthesis but its effect on the CNS has not been examined perhaps because of its presumed hydrophylic nature. Since rosuvastatin could enter the brain through specific transporters we decided to investigate its effect on microglial cells. Aspirin is one of the most commonly used non-steroidal anti-inflammatory drugs. In spite of its widespread effects on immune cell functions, it has not been extensively studied in microglial cells so we decided to clarify its effects on CNS immune functions. We investigated the pleiotropic effects of rosuvastatin and aspirin on secondary microglial cultures derived from mixed primary cultures of 18-day-old embryonic (E18) rat forebrains under control (unchallenged) and bacterial lipopolysaccharide (LPS)-challenged conditions.

Our specific aims were:

1. To characterize the effects of rosuvastatin and aspirin on the morphological, including cytoskeletal and immunocytochemical features of microglial cells;
2. To characterize the effect of rosuvastatin on cell adhesion and proliferation of microglial cells;
3. To determine if rosuvastatin and aspirin alter microglial phagocytic properties;
4. To determine the effects of rosuvastatin and aspirin on microglial cytokine release including both proinflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines;
5. To characterize the effects of rosuvastatin and aspirin on the expression patterns of inflammation related genes in pure microglial cells.

MATERIALS AND METHODS

Preparation and maintenance of cell cultures

Pure microglial cells were isolated from mixed primary cortical cell cultures of rat embryos. The cells were plated in DMEM/10% FBS medium on a poly-L-lysine-coated culture flask (75 cm^2 , 12 x 10^6 cell/flask) and cultured for a number of days in vitro (DIV) at 37 °C in a humidified air atmosphere supplemented with 5% CO_2 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 x 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 were kept separate.

Cell culture treatments

On the fourth day of subcloning (subDIV4), DMEM was replaced and the expanded pure microglial cells were treated with either bacterial lipopolysaccharide (LPS; 20 ng/ml), rosuvastatin (1 μM final conc.) or aspirin (0.1 mM (low) and 1 mM (high) final conc.) alone, or with a combination of LPS + rosuvastatin, or LPS + aspirin, and the effects were compared in a variety of morphological and functional tests. LPS treatment served as an immunochallenge. Depending on the experiments, the treatments lasted for 6, 24, or 72 h at 37 ^oC.

Immunocytochemistry

Pure secondary microglial cultures treated with different treatment regimens were fixed on coverslips with 4% formaldehyde. After permeabilization and blocking of the nonspecific sites the cells on the coverslips were incubated overnight in a humidified chamber with rabbit anti-Iba1 polyclonal antibody (1:500). Then incubated with the Alexa Fluor 568 fluorochrome-conjugated goat anti-rabbit antibody (1:1,000), and the nuclei were stained with 0.5 μl/ml Hoechst 33258 dye. The coverslips were air-dried and mounted on microscope slides in Vectashield mounting medium.

Western blot analysis

For the Western blot analyses, 5-10 μg of protein was separated on a sodium dodecyl sulfate/polyacrylamide gel, transferred onto Hybond-ECL nitrocellulose membrane and incubated overnight with either a rabbit anti-Iba1 polyclonal antibody (1:1,000) or a mouse anti-GAPDH monoclonal antibody (clone GAPDH-71.1; 1:20,000). The membranes were incubated with the peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000) for Iba1 or with the peroxidase-conjugated rabbit anti-mouse secondary antibody (1:2,000) for GAPDH Western blots.

Quantitative determination of in vitro phagocytosis

The fluid-phase phagocytic capabilities of the control and variously treated pure microglial cell cultures were determined via the uptake of fluorescent microspheres (2 μm in diameter). At the end of the treatment period, 1 μl of a 2.5% aqueous suspension of fluorescent microspheres per ml was added to the culture and incubated for 60 min at 37 °C.

Quantitative determination of IL-1β, IL-10 and TNF-α

For ELISA assays, the supernatants were collected from the Petri dishes of each treatment and stored at -20 ^{o}C. Concentrations of IL-1β, IL-10 and TNF-α were measured with rat-specific ELISA kits.

Determination of cell adhesion and proliferation

To measure changes in cell adhesion, proliferation and cell viability, the ACEA Real-Time Cell Analysis (RTCA) system and 16-well E-Plates were used. This system measured the electrical impedance of the cells expressed as cell index in real-time. After equilibration the E-plate was loaded into the RTCA machine and the cell index was measured continuously for 60 h using the xCELLigence real-time cell analysis system. Cell indices at 24 h were

analyzed for comparison with cell proliferation data. To estimate the number of surviving/proliferating microglial cells, the cultures were collected and counted in a Burker chamber.

RNA isolation

Cells were washed with PBS, incubated in lysis buffer (RA1), then collected and mixed with 70% ethanol in RNase-free water. The mixture was transferred through columns and washed with 80% ethanol in diethylpyrocarbonate-treated water, and then with W2 wash buffer. Total RNA was eluted in RNase free-water. One µl RNase inhibitor was added to the samples.

RNA expression

Reverse transcription from 3 µg total RNA in 30 µl was performed with the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's protocol. cDNA was diluted to 80 µl with nuclease-free water. The instrumentation included the Bravo automatic liquid handling system for polymerase chain reaction assay preparation and a LightCycler 1536 System, or a Light Cycler Nano Instrument for cycling. The expression of 116 inflammation-related genes, together with that of 6 control genes, was measured with Universal Probe Library assays using intron-spanning gene-specific primers. Gene expression was analyzed by GraphPad Prism 6.

Image and statistical analysis

For the determination of microglial cell purity, Hoechst 33258-labelled cell nuclei that belonged to Iba1-immunopositive cells were counted on coverslip-cultured samples. For each culture, 50-100 randomly selected microscope fields were analyzed. For the measurement of area (μm^2), perimeter (µm) and transformation index (TI), Iba1-immunoreactive microglial cell images were converted into binary replicas by using thresholding procedures implemented by ImageJ and Adobe Photoshop CS5.1 software (Adobe Systems, Inc., San Jose, CA, USA). Microglial cell silhouettes were acquired by transforming the raw digital files of Iba1-immunoreactive cells recorded under fluorescent microscope light to binary files. The color cell images were transformed into their binary replicas (silhouettes) by using automatic thresholding procedures. After the values of cell perimeter (µm) and cell area (μm^2) had been determined, the TI reflecting the degree of process extension was determined according to the following formula: [perimeter of cell $(\mu m)]^2 /4\pi$ [cell area (μm^2)]. All statistical comparisons were made by using R 3.1.0 for

Windows. Results were analyzed with two-way ANOVA, and the Bonferroni correction was used to establish significance between groups. Values were presented as means ± SD; $p < 0.05$ was considered significant.

RESULTS

Morphological effects of rosuvastatin and aspirin

The morphological changes elicited by rosuvastatin and aspirin in unchallenged (control) and LPS-challenged pure microglia cultures were documented through the use of Iba1 immunocytochemistry, and quantitatively analyzed on binary silhouettes of individual microglial cells. Most of the unchallenged and untreated (control) microglia displayed ameboid morphology with TI < 3; they had predominantly ameboid shape, occasionally with small pseudopodia. LPS challenge decreased area, perimeter and TI values (by 48%, 65% and 78%, respectively) as compared to the controls. Rosuvastatin induced the formation of numerous microspikes. These slender cytoplasmic projections (filopodia) resulted in significantly increased perimeter and TI values of these cells; the average TI in this group increased about 10-fold, to above 19, as compared with the controls. Rosuvastatin treatment in LPS-challenged cells resulted in a significantly enlarged and more ramified cell form (TI > 7) with a much larger perimeter value as compared with their respective control values, indicating that rosuvastatin profoundly antagonized the morphological changes characteristic of LPS-induced microglial activation. In relation to the substantially increased size of the LPS-challenged and rosuvastatin-treated microglia, their Iba1 immunoreactivity was also significantly increased.

When LPS-challenged cells were treated with aspirin, significantly enlarged and more ramified cells were seen as compared to the values of the LPS-challenged cells indicating that aspirin was able to reverse the morphological changes induced by LPS-challenge. Both aspirin doses increased the TI values to the control level (low: 5.73 ± 2.70; high: 7.36 ± 3.48) and induced ramified morphology with thick processes and microspikes.

Rosuvastatin inhibits proliferation and cell adhesion

Rosuvastatin significantly inhibited cell proliferation in both unchallenged and LPS-challenged cultures, by 47.8% and 68.9%. Rosuvastatin also inhibited cell adhesion in both unchallenged (control) and LPS-challenged microglia.

Both rosuvastatin and aspirin reduce the phagocytic activity in activated microglia

Microglial function is inherently related to its phagocytic activity. In pure microglial cultures (subDIV4), the control (unchallenged and untreated) microglia exhibited a low level of fluid-phase phagocytosis, engulfing 2.62 ± 1.7 beads per cell during rosuvastatin treatment, and 3.63 ± 1.6 beads per cell during aspirin treatment. LPS challenge increased the phagocytic activity of the microglial cells significantly. On average, the LPS-challenged cells accumulated 25.39 ± 11.4 beads per cell. Neither rosuvastatin or aspirin alone affected phagocytosis appreciably, as the number of phagocytosed microbeads remained low in both cases. However, both rosuvastatin and aspirin inhibited phagocytosis drastically in LPS-challenged microglia. Rosuvastatin inhibited phagocytosis significantly, by nearly 80% (4.67 ± 3.9), and aspirin (0.1 mM or 1 mM) decreased phagocytosis dose-dependently by about 30% (9.20 ± 4.25) and 70% (3.86 ± 1.85 microbeads per cell), respectively, as compared to LPS treatment.

Both rosuvastatin and aspirin decrease proinflammatory and increase anti-inflammatory cytokine levels

Activated microglia are known to express several pro- and anti-inflammatory cytokines. The basal level for IL-1β in unchallenged (control) microglia was 15.00 ± 5.6 pg/ml. Rosuvastatin did not change this level significantly. LPS challenge significantly elevated the IL-1β level in the activated microglia. However, when added together with LPS, rosuvastatin significantly lowered this elevated IL-1β level, by about 45%. A similarly strong effect of rosuvastatin was demonstrated on the level of TNF-α. When rosuvastatin was co-administered to LPS-challenged cells for either 6 h or 24 h, it significantly inhibited the overproduction of TNF-α, by 39% and 40%, respectively. Rosuvastatin affected the production of IL-10, an anti-inflammatory cytokine. When rosuvastatin was co-administered with LPS, it dramatically boosted the IL-10 protein expression to about 750% of the basal level.

When the basal levels of the proinflammatory cytokines IL-1β and TNF-α and the anti-inflammatory cytokine IL-10 in unchallenged microglia were compared with the levels from aspirin-treated LPS-challenged cells, a regulatory pattern similar to that seen after rosuvastatin treatments emerged. Co-incubation of LPS and 1 mM aspirin significantly inhibited IL-1β level by about 50%, while 0.1 mM aspirin decreased it by about 16.5 %. A similarly strong effect of aspirin was demonstrated on the level of TNF-α. When aspirin

was co-administered to LPS-challenged cells for either 6 h or 24 h, it inhibited the production of TNF-α significantly in both doses: 1 mM aspirin decreased the TNF-α level by about 25% and 50%, and 0.1 mM aspirin inhibited its level only after 6 h by about 16%. Aspirin also affected the production of IL-10, an anti-inflammatory cytokine. LPS challenge increased the basal IL-10 level significantly. When aspirin was co-administered with LPS, the IL-10 protein expression was further increased as compared to the LPS challenged value.

These data indicate that both rosuvastatin and aspirin have strong overall anti-inflammatory effects, the result of a combination of a strong inhibition of proinflammatory cytokine production and a similarly strong activation of anti-inflammatory cytokine production, in LPS-challenged (activated) microglia.

Effects of rosuvastatin and aspirin on the expression of inflammation-related genes

When the profound morphological and functional effects of rosuvastatin and aspirin on the pro- and anti-inflammatory capabilities of the microglia became apparent, we set out to analyze the effects of these treatments on the expression of 116 inflammation-related genes in unchallenged and LPS-challenged pure microglial cells.

Treatment with 1 μM rosuvastatin in unchallenged and LPS-challenged microglia could induce either the upregulation or the downregulation of a number of genes. The genes upregulated by the LPS challenge included those coding for chemokine ligands such as *Cxcl1*, *Ccl2*, *Ccl4*, *Ccl5*, *Cxcl9*, *Ccl19*, *Ccl24*, and the *Il11* or *Il23r*. Rosuvastatin treatment in unchallenged cells affected fewer, but similarly important microglial genes such as the anti-inflammatory *Ccl5* and *Mbl2*, the latter being a crucial factor in the development of innate immunity. Rosuvastatin also downregulated the proinflammatory *Ccr1*. Some of the LPS-upregulated genes were inhibited by rosuvastatin, as seen in the case of *Ccl24*, where a substantial, 73.5% decrease in gene expression was observed, down from the 24.5-fold increase after LPS treatment to a 6.5-fold increase, or in the case of *Ccr1*, where a 49.5% decrease in gene expression was detected.

Treatments with 0.1 mM or 1 mM aspirin in unchallenged and LPS-challenged microglia could also induce either the upregulation or the downregulation of a number of genes. Similarly to rosuvastatin treatment the genes upregulated by the LPS challenge included those coding for chemokine ligands such as *Cxcl1*, *Ccl2*, *Ccl4*, *Ccl5*, *Cxcl5*, *Cxcl9*, and the *Il1β*, *Il6* or the nitric oxide synthase (*Nos2*). In unchallenged cells the genes

downregulated by aspirin included the proinflammatory *Il1rn*, *Il6* and *Ccr1*. Some of the LPS-upregulated genes were inhibited by aspirin, as seen in the case of the proinflammatory peptides *Ccr1*, *IL1-rn* or *Nos2*.

CONCLUSION

We carried out a complex quantitative investigation of the morphological, functional and gene expression characteristics of pure microglial cells of embryonic origin after either rosuvastatin or aspirin treatment in unstimulated or LPS-challenged cultures, and highlighted the complex beneficial effects of both drugs that make them excellent candidates for preventive neuroinflammatory therapy with well-balanced properties of enhanced anti-inflammatory and subdued proinflammatory effects. Both rosuvastatin and aspirin elicit strong responses to microglial functions in vitro. They inhibit the harmful pro-inflammatory signals and significantly enhance the beneficial anti-inflammatory actions after LPS challenge in pure microglial cells. Their pleiotropic beneficial effects include the robust inhibition of phagocytosis and the synthesis of proinflammatory cytokines combined with a very strong stimulation of anti-inflammatory cytokine production, and a beneficial differential expression of a number of inflammation-related genes. In connection with rosuvastatin we also observed some anti-mitogenic effect.

As activated microglia often damage neuronal tissues or cause chronic inflammation by excessive cytokine production combined with high level of phagocytosis, effective inhibition of proinflammatory actions by these drugs could be an important prophylactic therapy in preventing neuroinflammation and thus neurodegeneration.

In summary the main findings of this study are:

1. Quantitative morphology revealed that both rosuvastatin and aspirin promoted ramified microglial morphology with TI > 6, and both drugs were able to antagonize the morphological changes characteristic of LPS-induced microglial activation (TI < 2);
2. Rosuvastatin inhibited cell proliferation by 40-60%, and decreased the adhesion of microglial cells;
3. Both rosuvastatin and aspirin were able to reduce the LPS-induced robust phagocytic activity but they did not affect basal (physiological) phagocytosis;

4. Rosuvastatin and aspirin had strong overall anti-inflammatory effect as they inhibited the production of proinflammatory cytokines (IL-1β, TNF-α) and stimulated the anti-inflammatory IL-10 synthesis in activated microglial cells;
5. Analysis of the gene expression patterns revealed that both rosuvastatin and aspirin had various effects on a number of inflammation related genes in pure microglial cells. Out of the 116 inflammation related genes rosuvastatin significantly changed the expression pattern of 47 genes, while aspirin altered significantly the expression level of 30 genes. They inhibited several proinflammatory genes such as *Ccr1*, *Ccl24*, *IL-6* or *Nos2*, while they upredulated other genes such as the anti-inflammatory *Cxcl1*, *Ccl5* or *IL-10*.