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The flavonoid rutin modulates microglial/macrophage activation to a CD150/CD206 M2 phenotype

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ABSTRACT

Rutin is a glycosylated flavonoid present in many fruits and plants that has been demonstrated to have anti-inflammatory and antioxidant properties. However, little is known about the mechanisms underlying microglial activation and its effects on the regulation of cytokines and chemokines associated with inflammatory responses in the central nervous system. In this study we examined the effect of rutin on resting or lipopolysaccharide (LPS)-stimulated microglia and characterized their modulation to an activated M1 phenotype or an alternatively activated M2 phenotype. Microglial cells were treated with rutin (1–100 μM); alternatively, microglial cells were stimulated with LPS and the cells were then treated with rutin (50 μM). The results revealed that rutin treatment was not toxic to microglial cells and induced a dose-dependent increase in microglial proliferation associated with changes in morphology after 24 h of treatment. Rutin also induced microglial activation characterized by an increase in OX-42 positive cells and a large proportion of cells with a CD150/CD206-positive M2 phenotype. Rutin also induced a decrease in the mRNA levels of TNF, IL1β, IL6 and iNOS, reduced the production of IL6, TNF, and nitric oxide, and increased production of the M2 regulatory cytokine IL10 and arginase. Rutin also significantly inhibited the LPS-induced expression of PTGS2, IL18 and TGFβ mRNA. These findings show that rutin has the ability to promote microglial proliferation and induces microglial polarization to the M2 profile when cells are stimulated with LPS. These results point this flavonoid as a possible alternative in the treatment or prevention of neurodegenerative disorders.

1. Introduction

Microglia, the resident immunocompetent cells in the central nervous system (CNS), are the first line of defense against any type of brain injury [1]. Acting as sentinels of the CNS, microglia constantly screen their microenvironment and display functional plasticity during activation, which involves changes in cell number, morphology, surface receptor expression, and the production of growth factors and cytokines [2]. The changes reflect altered activation states induced by signals arising from injured neurons and surrounding glia. Microglia has been shown to produce pro-inflammatory and anti-inflammatory cytokines. As macrophages, microglia may exhibit a classically activated M1 phenotype or an alternatively activated M2 phenotype [3]. However, upon stimulation of these cells, growth factors and chemokines are also produced [4]. In parallel with the production of proinflammatory cytokines, microglial neuroinflammation is commonly associated with the production of reactive oxygen species (ROS), nitric oxide dependent activation, and reactive nitrogen species (RNS) [5,6]. ROS are molecules containing oxygen that oxidize and react with

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vulnerable cell components, including proteins, nucleic acids and lipids. The brain is particularly vulnerable to excessive generation of ROS and RNS. In neural tissues, oxidative stress may result in the interruption of signaling and homeostasis. Microglia have been associated with the pathophysiology of age-related diseases, such as Parkinson's Disease (PD), Alzheimer's disease (AD) [4,6,7], excytotoxicity-induced neurodegeneration [8], glioma [9], and modulation of their response has been regarded as a therapeutic strategy [10,11]. Flavonoids are natural water-soluble chemical compounds of low molecular weight that comprise a class of secondary metabolites produced by plants. They may occur in free form (aglycone) or conjugated to sugars (glycosides). These compounds can be found in fruits, leaves, flowers, and seeds and are widely distributed in the plant kingdom, particularly in angiosperms [12]. Many studies have been demonstrated the antioxidant and anti-inflammatory properties of plant flavonoids [13], and their use as dietary supplements has been suggested [14–17]. In recent years, some studies have also demonstrated that flavonoids can sensitize the microglial response by characterizing the antioxidant and anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated microglia [18–21]. These anti-inflammatory and antioxidant properties have been attributed to rutin, a glycosylated flavonoid present in many fruits and plants [22]. More recently, the anti-inflammatory and antioxidant properties associated with the neuroprotective effects of plant extracts have been associated with the presence of rutin [23,24]. Rutin also exhibits the ability to inhibit beta-amyloid aggregation and cytotoxicity, inhibits the production of nitric oxide (NO) and pro-inflammatory cytokines and attenuates oxidative stress *in vitro* [25]. Rutin was also demonstrated to ameliorate damage associated with spinal cord injury [26]. In a previous study, we also characterized microglial activation and proliferation in primary astrocyte/microglia co-cultures treated with rutin [27]. However, little is known about mechanisms of microglial activation and its effects on the regulation of cytokines and chemokines associated with inflammatory responses in the CNS. In this study, we examined the effect of rutin in resting and lipopolysaccharide (LPS)-stimulated microglia and characterized modulation to an activated M1 phenotype or an alternatively activated M2 phenotype.

2. Materials and methods

2.1. Microglial cultures

Microglial cells were obtained from the cortex of Wistar newborn rats (0–2 days old) from the Animal Facility of the Federal University of Bahia (Salvador, Brazil) and performed according to Brazilian guidelines for production, maintenance and use of animals for teaching activities and scientific research and the local Ethical Committee for Animal Experimentation, protocol number (0272012, ICS - UFBA). Microglial isolation was performed according to the protocol established in the Guaza Lab at the Cajal Institute in Madrid [28]. In brief, after decapitation, the forebrains of newborn Wistar rats were dissociated mechanically and resuspended in DMEM supplemented with 10% Fetal bovine serum (FBS), 10% Serum equine (HS), 4 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured on poly-D-lysine (25 μg/mL) -coated flasks. Upon reaching confluence (7–10 days), adherent microglial cells were harvested by shaking at 165 rpm at 37 °C for 3 h. Isolated microglia were seeded into 96-, 24- or 6-well plates at a density of $3 \times 10^4/cm^2$, and experiments performed after 24 h. In all cases, the cells were cultured at 37 °C in 5% CO_2.

2.2. Treatments

The flavonoid rutin was extracted from *Dimorphandra mollis* seeds. For treatments, rutin was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) at a concentration of 100 mM and stored in the dark at −4 °C. Twenty-four hours after seeded, the microglia medium was changed, and rutin was added directly in the culture medium at a final concentration of 1, 10, 50 or 100 μM for 24 h. The negative control group was treated with DMSO diluted in culture medium at the highest equivalent volume used in the treated group (0.1%) and showed no significant effect on the analyzed parameters compared to cells that did not receive the diluent. We added 1 μg/mL Lipopolysaccharides from *Escherichia coli* (LPS) (Sigma Aldrich, USA) to the culture medium 24 h before treatments to generate the M1 microglial phenotype.

2.3. Cytotoxicity analysis

Cytotoxicity was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Sigma) test in microglial cells treated for 24 h with rutin (50 and 100 μM) and/or LPS (1 μg/mL) or in control conditions The experiments were performed in 96-well plates (TPP, Trasadingen, Switzerland). Cell viability was quantified by the conversion of yellow MTT to purple MTT formazan by the mitochondrial dehydrogenases of living cells (Hansen et al., 1989). Control and treated cells were incubated with MTT at a final concentration of 1 mg/mL for 2 h. Thereafter, cells were lysed with 20% (w/v) sodium dodecyl sulfate (SDS) and 50% (v/v) dimethyl formamide (DMF) (pH 4.7), and the plates were maintained at 37 °C overnight to dissolve the formazan crystals. The optical density of each sample was measured at 490 nm with a Bio-Rad 550PLUS spectrophotometer. Three independent experiments were performed with eight replicate wells for each analysis. The results are shown as the percent viability of the treated groups relative to the control, which was considered 100%.

2.4. Cell morphology and immunocytochemistry

Cell morphology and proliferation were first investigated 24 h after treatment with rutin (1, 10 and 50 μM), LPS (1 μg/mL) or the vehicle control (0.1% DMSO). Morphological changes and cell activation were studied by analysing the immunocytochemistry patterns for the proteins Ionized calcium binding molecule-1 (Iba-1) (for microglial morphology), OX42/CD11b (a marker of all activated microglia), CD68 (M1 phagocyte microglial marker), iNOS (M1 microglial marker), CD206 (M2A microglial marker) and CD150 (M2C microglial marker).

Microglia was seeded into 24-well plates (TPP, Trasadingen, Switzerland) with cover slips pretreated with poly-D-ornithine (10 μg/mL, Sigma-Aldrich). After treatments, the cultures were rinsed three times with PBS at pH 7.4 and fixed with 4% paraformaldehyde for 20 min at 0 °C. Excess paraformaldehyde was discarded, and the plates were allowed to dry at room temperature. Cells were then rehydrated with PBS and permeabilized with PBS-T, and the non-specific binding of antibody reagents was blocked by pre incubating the plates with 3% bovine serum albumin (BSA) in PBS. Next, the cultures were incubated with the following antibodies: rabbit polyclonal antibody against Iba 1 (1:200, Wako), rabbit polyclonal antibody against iNOS (1:50 in PBS, Abcam), mouse monoclonal antibody against OX-42/CD11b/c (1:200, Caltag), rat monoclonal antibody against CD150 (1:100, Bio-Rad), rat monoclonal antibody against CD206 (1:100, Bio-Rad), or rat monoclonal antibody against CD68 (1:100, Bio-Rad). All antibodies were diluted in PBS/BSA (1%) and kept in a humid chamber at 4 °C overnight. The next day, the cells were rinsed 3 times with PBS and