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The effect of minocycline on indolamine 2, 3 dioxygenase expression and the levels of kynurenic acid and quinolinic acid in LPS-activated primary rat microglia

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ABSTRACT

Microglia are one of the most important neural cells in the central nervous system (CNS) which account for 10–15% of all cells found in the brain. A vast majority of studies indicate that microglia play a pivotal role in protection and damage of the CNS. It has been shown that microglia are mainly scavenger cells but also produce a barrage of factors that are involved in tissue repair and neural regeneration. Several lines of evidence indicate that unregulated activation of microglia in response to either endogenous or exogenous insults results in the production of toxic factors that propagate neuronal injury. Studies demonstrated that the activated microglia secret the excessive amounts of quinolinic acid (QA) and kynurenic acid (KYNA) which are highly toxic for the neuronal cells. In line with this, indolamine 2, 3 dioxygenase (IDO), an enzyme producing KYNA and QA has been shown to be elevated during the inflammation in microglia. In this study, we established primary microglial cell cultures obtained from cerebral cortices of 1-day neonatal Wistar rats. Minocycline (20–60 μM) or its vehicle was added to the culture media 60 min prior to 48 h incubation with lipopolysaccharide (LPS; 10 ng/mL). Using a specific process of adhesion and shaking of the cultured glial cells, a purified culture of approximately 94% enriched microglia was obtained and then, corroborated by immunocytochemistry (ICC). The cell viability after minocycline treatments was assessed using the MTT colorimetric assay. The expression of IDO was evaluated using qPCR. The levels of KYNA and QA were determined using enzyme-linked immunosorbent assay (ELISA). The results showed that minocycline significantly decreased the levels of both KYNA and QA in glia cells exposed to LPS. Moreover, minocycline decreased the expression of IDO in treated LPS-induced microglia. It seems that minocycline has a potent ability to oppress the inflammatory process via the decrease in production of IDO expression and the concentrations of KYNA and QA.

1. Introduction

Microglia, the brain-resident macrophages, are considered the first line of defense against either exogenous or endogenous insults and play a crucial role in the immune surveillance of the central nervous system (CNS) [13]. When the microglia become hyperactive or unregulated, they produce various proinflammatory cytokines such as interleukin-1 beta (IL-1 beta), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha) which remarkably intensify the process of tissue destruction followed by neurodegeneration [15]. Hence, inhibition of microglial activation may serve as a therapeutic strategy to control and manage of neuronal damage during inflammatory cascades [1]. Cultured microglia have been widely used for the study of physiological and biological events occurred in these types of cells. Lipopolysaccharide (LPS) provides an *in vitro* and *in vivo* models for researchers to activate macrophage-like cells resulting in initiation of producing different mediators such as nitic oxide, IDO expression, leukotrienes etc. [22]. Therefore, this model resembles phenomena taking place in neural injury, ischemia, Alzheimer' disease (AD), multiple sclerosis (MS) and other neuroinflammatory disorders [8]. IDO, an enzyme involved in conversion of L-tryptophan to N-formyl kynurenine which is immediately converted to KYNA and QA [21]. A large body of research indicates that IDO is a double-edged sword in immune system. While it has been shown that IDO possesses immunosuppressive properties paving the way for cancers cells to overexpress the IDO gene in order to escape from the immune system [17], its metabolites such as KYNA can pass

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the blood–brain barrier (BBB) and thus could act as neurotoxins, e.g., during systemic infection [28]. Interestingly, despite a neurotoxic effect of KYNA on neuronal cells, KYNA can downregulate interleukin-23 (IL-23) and interleukin-17 (IL-17) in dendritic cells (DCs) and Th17 cells through inhibiting GPCR35 [23].

A second generation semi-synthetic tetracycline antibiotic named "minocycline" has a broad spectrum the antibacterial [2]and antiviral [18]activities. In addition to its antimicrobial activity, minocycline has miraculously shown to confer a neuroprotective role in neuroinflammatory and neurodegenerative diseases of the CNS [25]. Minocycline is capable of crossing the BBB and exerting anti-inflammatory effects on activated microglia thereby suppression of interleukin-1 beta converting enzyme (ICE), cyclooxygenase-2 (COX-2) and prostaglandins (PGs) [4]. Due to paradoxical reports on the role of IDO enzyme and its metabolites in promotion/inhibition of inflammation, we decided to measure the levels of IDO at the level of gene expression and its metabolites by ELISA method to see the effect of minocycline on LPS-induced microglia.

2. Materials and methods

2.1. Preparation of primary murine microglia

To obtain the primary murine microglia from Wistar rat, eight 1-day old neonatal Wistar rats were purchased from animal house of university of Tehran. The neonates were anesthetized (ether) and then decapitated. After removal of the meninges, brains were minced and dissociated with 0.25% trypsin/0.5 mM EDTA. Dissociated cells were then transferred through nylon cell strainer with 70 μm pores (BD biosciences, Heidelberg, Germany). Cells were collected by centrifugation followed by resuspension in Dulbecco's modified Eagle's medium (DMEM) containing 0.584 g/l glutamine and 4.5 g/l glucose, sodium pyruvate and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and cultured in T-75 flask in 5% CO_2 at 37 °C. The culture medium was twice weekly changed. Once the glial cultures were completely confluent (nearly after 12–15 days) the cells were shaken at 230 rpm for three hours at 37 °C. Detached cells were centrifuged at 168 × *g* for 10 min. Cell pellets were re-suspended in warm DMEM 10:10:1 and plated at a density of 200,000 cells/cm^2. For experiments, microglial cells were re-suspended in RPMI medium supplemented with 2% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Immunocytochemistry using anti-Iba1

To test the purity of microglia in previous section, we stained them using a specific marker for microglia rabbit anti-rat/mouse Iba-1 (Abcam, Cambridge, UK). The procedure of staining was done in according to manufacturer's instructions. Briefly, glial cells were grown on glass coverslips previously coated with poly-L-lysine. The cells were then fixed with 4% formaldehyde for 10 min. Then, cells were gently rinsed 3 times for 5 min each with PBS, then blocked with PBST (PBS with 0.3% Triton-100) for 20 min. After that, glial cells were incubated for 1 h in dark with rabbit anti-rat Iba1antibody at a dilution of 1: 250, together with Hoechst to stain nucleus of the cells (Invitrogen H3569, dilution 1:5000). The cells were rinsed 3 times for 5 min each. Then, the procedure was followed by incubating cells with Alexa 568 (goat anti-rabbit, Life Tech Cat. No. A11036, dilution 1: 400) for 45 min. Before imaging, cells were mounted with Fluoromount-G (Southern-Biotech, Cat. No. 0100-01). Of note, GFAP-Cy3 (Sigma C9205) at a dilution of 1:500 was applied as negative control. The purity of the microglia was obtained 93%.

2.3. Cell culture treatment

Glial cells were pre-treated with 20 μmol/L, 40 μmol/L or 60 μmol/L minocycline (Sigma, St. Louis, MO, USA) 60 min at 37 °C and 5% CO_2. After that, cells were then stimulated with 10 ng/ml of LPS (Sigma, St. Louis, MO, USA) to induce inflammation for 16 h at 37 and 5% CO_2.

2.4. Cell viability assay

Cell viability was evaluated by the MTT (3-4-5-dimethylthiazole-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay. Cells were incubated with MTT (0.25 mg/ml) for 24 h and 48 h at 37 °C. The formazan crystals in the cells were solubilized with DMSO. The level of MTT formazan was determined by measuring its absorbance at 490 nm.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Glial cells of 3×10^4 cells/well were plated into 48-well microtiter plates. Minocycline and LPS were added to the culture media of the cells for 48 h. Then, the levels of QA and KYNA were measured using an ELISA kit in accordance to manufacturer's instructions (MyBioSource, San Diego, CA, USA).

2.6. Real-time PCR

Total RNA was extracted with Trizol (Gibco, Grand Island, NY, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using a reverse transcription system to make cDNA (TaKaRa, Japan). Approximately 20 μl of cDNA was yielded of which 1 μl was used for each real-time PCR analysis. The cDNA was then amplified by PCR with primers of IDO: sense primer 5′- GCATCA AGACCCGAAAGCAC-3′ and the antisense primer 5′- CACGAAGTCAC GCATCCTCT -3′ resulting in 92 nucleotides. As a reference gene, GAPDH was used to normalize for each cDNA sample the expression of IDO. The primers used for GAPDH were the following sense 5′-AGGT CGGTGTGAACGGATTTG-3′ and anti-sense 5′-TGTAGACCATGTAGTTG AGGTCA-3′. The conditions used for IDO gene expression were as 2 min hold at 50 °C and 2 min hold at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The quantification data were analyzed with the Rotor Gene software (Ver.5) to obtain relative IDO expression. PCR products were examined for the presence of by-products using the melting curve analysis provided by the software, and were run on a 2% agarose gel electrophoresis and visualized by UV illumination after ethidium bromide staining.

2.7. Statistical analysis

The comparison among groups was done and data were expressed as the means ± SD using ANOVA followed by Tukey's tests performed by GraphPad prism (GraphPad Software. San Diego, CA, USA). The comparison between two groups was carried out using Student's *t*-test or Mann-Whitney U test when variances are unequal.

3. Results

3.1. KYNA was decreased in response to minocycline treatment

The level of KYNA was elevated when the cells were treated with LPS ($p < .01$). Minocycline lowered the level of KYNA in cells exposed to LPS in doses of 20 μg/ml and 40 μg/ml in a dose dependent manner ($p < .05$). There was not any significant difference in concentrations of KYNA between cells treated with 40 μg/ml and 60 μg/ml minocycline ($p = .62$). The level of KYNA in all treatments has been depicted in Fig. 1A.

3.2. QA was decreased in cells treated with minocycline

Similar to KYNA, the concentration of QA was elevated in cells stimulated with LPS (Fig. 1B) ($p < .01$). It was shown that minocycline