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Inhibitory Action of Minocycline on Lipopolysaccharide-Induced Release of Nitric Oxide and Prostaglandin E2 in BV2 Microglial Cells

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Microglia are the major inflammatory cells in the central nervous system and become activated in response to brain injuries such as ischemia, trauma, and neurodegenerative diseases including Alzheimer's disease (AD). Moreover, activated microglia are known to release a variety of proinflammatory cytokines and oxidants such as nitric oxide (NO). Minocycline is a semi-synthetic second-generation tetracycline that exerts anti-inflammatory effects that are completely distinct form its antimicrobial action. In this study, the inhibitory effects of minocycline on NO and prostaglandin E_2 (PGE_2) release was examined in lipopolysaccharides (LPS)-challenged BV2 murine microglial cells. Further, effects of minocycline on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression levels were also determined. The results showed that minocycline significantly inhibited NO and PGE_2 production and iNOS and COX-2 expression in BV2 microglial cells. These findings suggest that minocycline should be evaluated as potential therapeutic agent for various pathological conditions due to the excessive activation of microglia.

Key words: Minocycline, Nitric oxide, PGE_2, iNOS, COX-2, Microglia

INTRODUCTION

Activated microglia participate in the pathogenesis of various neurological diseases through expressing of major histocompatibility complex (MHC) and adhesion molecules and releasing a host of soluble factors (Gebicke-Haerter, 2001; Milner and Campbell, 2003). A number of these factors, such as the glia-derived neurotrophic factor, are potentially beneficial to the survival of neurons (Salimi *et al.*, 2003). However, the majority of factors produced by activated microglia are pro-inflammatory and neurotoxic (Pocock and Liddle, 2001). These include the cytokines tumor necrosis factor-α (TNF-α) and interleukin-β (IL-1β), free radicals such as nitric oxide (NO) and superoxide, fatty acid metabolites such as eicosanoids, and quinolinic acid. Previous studies have demonstrated that excessive quantities of individual factors produced by activated microglia can be deleterious to neurons (Boje and Arora, 1992; Chao *et al.*, 1992; McGuire *et al.*, 2001). The involvement of microglial activation in the pathogenesis of several neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) was also suggested (Gebicke-Haerter, 2001). For instance, reactive microglia were found to colocalize with neuritic plaques in the cortical region of AD brains (Rogers *et al.*, 1988). In PD brains, large numbers of human leukocyte antigen (HLA-DR)-positive reactive microglia were found in the substantia nigra (SN), a region in which the degeneration of dopaminergic neurons was most prominent (McGeer *et al.*, 1988). Further, results from both *in vivo* and *in vitro* studies have established an association of microglial activation with the pathogenesis of other brain disorders including amyotrophic lateral sclerosis, multiple sclerosis, and prion-related diseases (Brown, 2001; Dickson *et al.*, 1993; Raine, 1994).

Minocycline is a second generation semi-synthetic antibiotic of the tetracycline family. It is absorbed rapidly and completely and has a superior penetration through the brain-blood barrier. Recently, several studies have reported that minocycline exerts anti-inflammatory effects completely

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distinct from its antimicrobial action (Gabler and Creamer, 1991; Ryan and Ashley, 1998). Minocycline has been shown to have neuroprotective effects in global brain ischemia (Arvin *et al.*, 2002), traumatic brain injury (Sanchez Mejia *et al.*, 2001), neuronal apoptosis induced by ionizing radiation (Tikka *et al.*, 2001), and 6-hydroxydopamine-induced nigral dopamine neuron degeneration (Du *et al.*, 2001; He *et al.*, 2001). However, the exact mechanism by which minocycline provides neuroprotection is not clear.

In the present study, given the possibility that microglial activation contributes to the pathogenesis of various brain disorders associated with excessive inflammatory response, the anti-inflammatory effects of minocycline on LPS-induced microglial activation were investigated in a BV2 microgial cell model. We demonstrated that minocycline significantly suppressed LPS-induced microglial activation by decreasing NO and PGE_2 release in a concentration-dependent manner. Furthermore, adiminstration of minocycline suppressed expression levels of iNOS and COX-2 in BV2 microglial cells.

MATERIALS AND METHODS

Materials

Minocycline and LPS were obtained from Sigma (St. Louis, MO). Anti-murine iNOS and anti-cyclooxygenase-2 (COX-2) antibodies were obtained from Transduction Laboratories (Lexington, KY). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated.

Cell culture

The immortalized murine BV2 cell line that exhibits phenotypic and functional properties of reactive microglial cells (Blasi *et al.,* 1990; Bocchini *et al.,* 1992) was obtained from M. McKinney (Mayo Clinic, Jacksonville, FL). The cells were grown and maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin and 10 U/mL penicillin at 37°C in a humidified incubator with 5% CO_2. All experiments were carried out on subconfluent cultures.

Measurement of nitrite release

Accumulated nitrite was measured in the cell supernatant by the Griess reaction (Green *et al.,* 1982). The conditions of cell culture and treatment were same with those in ELISA. In brief, 100 μL of Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well microtiter plate and absorbance was read at 540 nm using a plate reader. Sodium nitrite, diluted in culture media at concentrations ranging from 10 to 100 μM, was used to prepare a standard curve.

Measurement of prostaglandin E_2 (PGE_2)

BV2 were seeded in 96-well culture plates in DMEM containing 10% FBS. Cells were preincubated with minocycline prior to LPS treatment. Supernatant were harvested and centrifuged at 10,000 g for 10 min and levels of PGE_2 were measured by an enzyme-linked immunoassay (EIA) from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instructions.

Immunoblottings

Cells (1×10^6) were washed with PBS, collected, and centrifuged at 1,000 g for 5 min. For whole cell extract, 0.5 mL of RIPA buffer (1×PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added phenylmethylsulfonylfluoride (PMSF 0.4 mM) was added to the pellet, incubated for 10 min on ice, centrifuged at 1,000 g for 3 min at 4°C. Protein concentration from the supernatant was determined (Bio-Rad) and 30 μg of proteins were loaded for SDS-PAGE. Electrophoresis was performed and proteins were transferred from the gel to a nitrocellulose membrane. Membranes were blocked 1 h in TBS containing 0.1% Tween-20 and 5% dry milk, incubated overnight with primary antibodies that recognize iNOS (1:1,000, Transduction Laboratories) or COX-2 (1:1,000, Transduction Laboratories) and then horseradish peroxidase (HRP) conjugated secondary antibodies (1:2,000) for 2 h. Membranes were washed with TBS containing 0.1% Tween-20 and visualized with an ECL Plus Western blotting detection system (Amersham International, Little Calfont, U.K.). Immunoreactivities were quantified by Western blot imaging analysis using ImageQuant software from Molecular Dynamics, Inc (UK).

Statistics

Data were analyzed using the paired *t*-test, and values were considered significantly different when the two-tailed *P* value was <0.05. Results were expressed as mean± SEM values.

RESULTS

Effect of minocycline on NO production

The effects of minocycline on NO production in LPS-stimulated BV-2 cells were investigated. The cells were treated with LPS alone or with various concentrations of minocycline. When BV2 cells were stimulated with LPS (200 ng/mL) for 16 h, the accumulation of nitrite, a stable oxidized product of NO, was increased in the culture medium. This increase was significantly suppressed in a concentration-dependent manner by administration of minocycline whereas minocycline itself did not affect basal levels (Fig. 1). No cytotoxic effect of minocycline tested in this study was observed (data not shown).

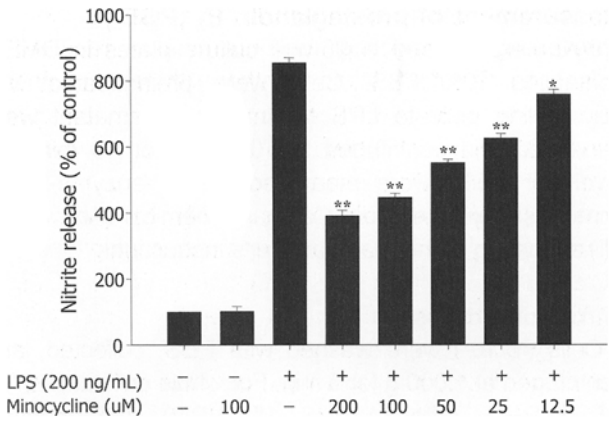

Fig. 1. Effect of minocycline on LPS-stimulated nitrite release in BV2 microglial cells. In the presence of LPS (200 ng/mL), the administration of minocycline significantly reduced LPS-induced release of nitrite in a concentration-dependent manner in BV2 microglial cells, whereas minocycline itself did not affect basal level of NO production. Basal levels of nitrite without LPS and minocycline were 3.5±0.1 μM. Data were presented as % of control value. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

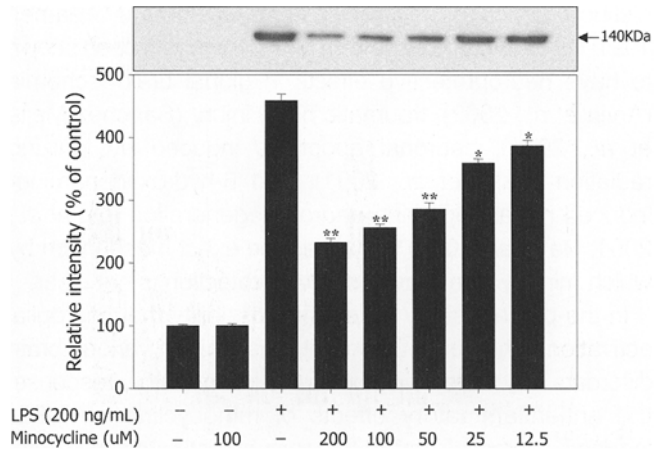

Fig. 2. Effect of minocycline on LPS-induced iNOS protein expression. Levels of iNOS protein were measured in LPS-stimulated cells using a monoclonal antibody raised against murine iNOS (Transduction Laboratories). Minocycline suppressed LPS-induced iNOS expression in a concentration-dependent manner. Top panel shows a representative immunoblot and bottom panel shows quantitative values of immunoblots. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

Effect of minocycline on iNOS expression

NO is produced by expression of iNOS gene in response to immune stimuli. To examine whether minocycline inhibits NO production via suppression of iNOS expression, the effect of minocycline on the levels of iNOS protein in LPS-stimulated BV2 cells was determined. Western blot analysis demonstrated that the level of iNOS protein was suppressed in a concentration-dependent manner by treatment with minocycline in LPS-stimulated cells (Fig. 2) whereas total protein levels determined by β-tubulin remained unchanged (data not shown). Minocycline also suppressed LPS-induced iNOS mRNA levels. However the suppression of iNOS mRNA expression was not concentration dependent (data not shown).

Effects of minocycline on PGE_2 production and COX-2 expression

It is known that PGE_2 is produced in activated microglia through the induction of the pro-inflammatory enzyme COX-2 (Egger *et al.*, 2003). When treated with LPS, BV2 cells produced robust amount of PGE_2 and this increase was inhibited by minocycline treatment in a concentration-dependent manner (Fig. 3). Further, COX-2 protein levels were also examined to determine the effect of minocycline on the expression of COX-2 protein. The result showed that minocycline significantly suppressed LPS-induced COX-2 expression in a concentration-dependent manner (Fig. 4). Minocycline itself did not affect the basal levels of PGE_2 production and COX-2 expression.

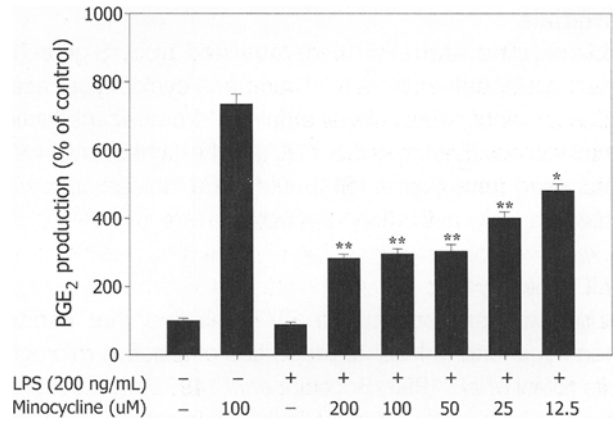

Fig. 3. Effect of minocycline on PGE_2 production. PGE_2 concentration was measured in the culture media of LPS-stimulated cells for 16 h by an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). In the presence of LPS (200 ng/mL), the administration of minocycline significantly reduced LPS-induced production of PGE_2 in a concentration-dependent manner in BV2 microglial cells, whereas minocycline itself did not affect the basal levels of PGE_2. Basal levels of PGE_2 without LPS and minocycline were 1.6±0.3 ng/mL. Data were presented as % of control value. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

DISCUSSION

Minocycline is a semisynthetic second-generation tetracycline that exerts anti-inflammatory effects independent of its antimicrobial action (Gabler and Creamer, 1991; Ryan and Ashley, 1998). Because minocycline has been

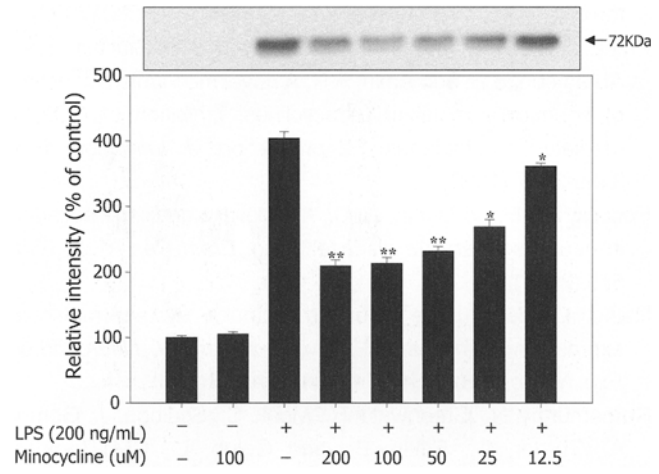

Fig. 4. Effects of minocycline on LPS-induced COX-2 protein expression. Levels of COX-2 protein were measured in LPS-stimulated cells for 16 h by Western blot analysis using a polyclonal antibody (Transduction Laboratories). Minocycline significantly suppressed LPS-induced COX-2 expression in a concentration-dependent manner. Top panel shows a representative immunoblot and bottom panel shows quantitative values of immunoblots. Data represent three independent experiments and were expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

previously shown to exert anti-inflammatory activity in macrophages and other immune cells in periphery (Davies *et al.,* 1996; Patel *et al.,* 1999), we hypothesized that minocycline may be also anti-inflammatory in CNS providing neuroprotection under the conditions where the inflammatory activation of microglia plays a pathogenic role in neuronal injuries. The present study demonstrated that minocycline exerted anti-inflammatory effects by suppressing the production of the inflammatory mediators such as NO and PGE_2 and further decreasing expression of responsible genes such as iNOS and COX-2, respectively, in LPS-challenged BV2 microglial cells.

Activated microglia have been described in several human chronic neurodegenerative diseases, including Alzheimer's disease (AD), AIDS dementia, and Parkinson's disease (PD) (Gao *et al.,* 2002; Nelson *et al.,* 2002). Many of the effects of activated microglial cells are mediated by their numerous secretory products such as NO and PGE_2 (Scali *et al.,* 2000). Peroxynitrite, formed by the reaction of NO and superoxide anion, is responsible for the major part of NO-induced neurotoxicity (Schulz *et al.,* 1995). COX-2 is the key enzyme in the formation of prostaglandins (PGs) from arachidonic acid and is expressed in activated microglial cells which appear to be an important source of PGs during inflammatory conditions (Bauer *et al.,* 1997). The temporal correlation between increased levels of PGs and various neuropathological processes has led to the hypothesis that PGs contribute to neurodegeneration (Shimizu and Wolfe, 1990). Thus, suppression of these mediators may be an effective therapeutic strategy for preventing of protecting from inflammatory reaction and diseases. Our data, taken together with previous findings by others, indicate that minocycline exerts a variety of pharmacological and biological effects independent of its antimicrobial activity, which include inhibition of matrix metalloproteinases (Uitto *et al.,* 1994), NOS expression (Amin *et al.*, 1996), tumor progression, angiogenesis (De Clerck *et al.*, 1994), and inflammation (Ramamurthy *et al.*, 1994). We speculate that the pleiotropic properties of minocycline are presumably due to its ability to target other multifunctional signaling molecules, such as NO and PGE_2.

In conclusion, our study demonstrated that minocycline inhibited the production of NO and PGE_2 in LPS-stimulated BV2 microglial cells and that these anti-inflammatory effects were achieved by suppression of iNOS and COX-2 expression. Given the fact that microglial activation contributes to pathogenesis of several disorders, minocycline may be a potential therapeutic agent for inflammatory brain diseases. However, further studies are necessary to determine the exact mechanism by which minocycline suppresses expression of iNOS and COX-2.

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