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Minocycline inhibits LPS-induced retinal microglia activation

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Abstract

Retinal neurodegenerative disease involves an inflammatory response in the retina characterized by an increase in inflammatory cytokines and activation of microglia. The degree of microglia activation may influence the extent of retinal injury following an inflammatory stimulus. Cytokines released by activated microglia regulate the influx of inflammatory cells to the damaged area. Thus, a therapeutic strategy to reduce cytokine expression in microglia would be neuroprotective. Minocycline, a semisynthetic tetracycline derivative, is known to protect rodent brain from ischemia and to inhibit microglial activation. In this study, we activated retinal microglia in culture with lipopolysaccharide (LPS) and attempted to determine whether minocycline could reduce the production of cytokines from activated microglia at both gene and protein levels. Changes in inflammatory cytokines, TNF-alpha and IL-1beta, were measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) in the presence or absence of LPS. We also measured the levels of nitric oxide (NO) by the nitrate reductase method under similar conditions. LPS treatment induced a significant upregulation of the mRNA and release of TNF-alpha, IL-1beta, and NO from retinal microglia. Minocycline inhibited these releases. Thus, minocycline might exert its antiinflammatory effect on microglia by inhibiting the expression and release of TNF-alpha, IL-1beta, and NO.

Keywords: Microglia; Retina; Neurodegenerative disease; LPS; IL-1beta; TNF-alpha; NO; Minocycline

1. Introduction

Diabetic retinopathy is one of the leading causes of blindness in adults of working-age. The causes of vascular and visual pathology in this condition are not yet fully understood. Much of the research effort has been focused on vascular changes. Significant involvement of neuronal and glial components has been implicated as well. Microglia, normally quiescent, become activated by diabetes (Rungger-Brandle et al., 2000; Zeng et al., 2000). Both the number and activity of microglial cells were elevated as reflected by the increase in OX-42, a microglial cell marker (Rungger-Brandle et al., 2000; Zeng et al., 2000). Retina is composed of four major classes of cells. Among them, microglia are the most difficult to study in vivo due to its migratory behavior and morphological transformation.

Cultured microglia have been used extensively to study microglial behavior. Lipopolysaccharide (LPS) is widely used to activate macrophage-like cells. Treating cell cultures with LPS is used widely as a model to simulate infection. Upon CNS injury, activated microglia release compounds such as glutamate, free radicals, proteases, cytokines, leukotrienes, and nitric oxide (NO) (Sayyah et al., 2003; Godbout et al., 2004; Kremlev et al., 2004). Thus, it is reasonable to support that hyperactivity of microglia in the CNS may be cytotoxic and may lead to subsequent injury including inflammation and neuronal degeneration. Based on studies using animal models, chemicals that modulate

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microglial activation have been proposed as candidate therapeutic agents in diseases such as ischemic brain injury (Yrjanheikki et al., 1999), traumatic brain, and spinal cord injuries (Giulian and Robertson, 1990; Holmin and Mathiesen, 1996).

Recently, several reports have indicated that minocycline, a second-generation semi-synthetic tetracycline with a broad-spectrum antimicrobial activity (Amin et al., 1996; Golub et al., 1998), effectively crosses the blood-brain barrier (Teng et al., 2004) and inhibits the activation of microglia as well as IL-1beta converting enzyme, COX-2 and PGE_2 production (Amin et al., 1996; Hughes et al., 2004; Kim et al., 2004). Minocycline has also been used in the management of acne (Eady et al., 1990) and periodontal disease (Van Steenberghe et al., 1993).

Microglial activation in retina has been detected in the early stages of retinal neurodegenerative diseases, such as diabetic retinopathy. The goal of this study, therefore, is to evaluate whether minocycline plays a role in modulating LPS-induced microglia with respect to the expression and release of IL-1beta, TNF-alpha, and NO in culture. Our results would provide an initial evaluation on the potential application of minocycline in the treatment of retinal neurodegenerative diseases.

2. Methods and materials

2.1. Primary retina microglia culture

Microglial cells were isolated from retinas of newborn Sprague–Dawley (SD) rats according to Roque and Caldwell (1993), with minor modifications. Briefly, retinas free of blood vessels were dissected from newborn (within 72 h) SD rat pups. Tissues were collected into 0.01 M PBS and washed two times with ice-cold 0.01 M PBS, then digested with 0.125% trypsin for 20 min before mechanical dissociation. Trypsin was then inactivated with DMEM/F12 (1:1) (Invitrogen, CA) plus 10% fetal bovine serum (FBS) (Hyclone, UT). Cells were collected by centrifugation, re-suspended in culture medium and plated onto T75 cell culture dishes (Corning, NY) at a density of 1×10^6 cells/mm^2. All cultures were maintained in a humidified CO_2 incubator (Sanyo, Japan) at 37 °C and 5% CO_2 and fed on the third day, then once every 4 days. After 2 weeks, microglia were harvested in culture media containing serum by shaking the flasks at 100 rpm for 1 h. The cell suspension was centrifuged and the detached cells were replated in DMEM/F12 (1:1) + 10% FBS at designated densities for various experiments described below. The purity of the microglial cultures was inspected by immunocytochemical staining and flow cytometric (FCM) analysis for CD11b, a microglial marker. The morphology of microglia in culture was carefully examined by phase contrast and fluorescent microscopy.

2.2. Activation of microglia by LPS

Microglia collected from culture flasks were seeded at a density of 3×10^5 cells/well onto 24-well tissue culture plate. One day after seeding, the culture wells were washed with PBS and 0.5 ml serum-free medium containing 3 ng/ml LPS (*Escherichia coli* OB4:1111; Sigma, MO) was added to each well for 24 h. From 1 to 6, 12, 24 h, 50 μl aliquots of incubation medium were taken and analyzed for IL-1beta, TNF-alpha, and NO. After 24 h, LPS containing medium was replaced with fresh medium with serum. The morphology of microglia was then examined.

2.3. Minocycline treatment

Each culture well was fed with medium containing 0 (control) or 60 μM minocycline (Sigma, MO), 1 h prior to the addition of 0 (control) or 3 ng/ml LPS. Aliquots of medium (50 μl) were taken directly from the well for the measurement of IL-1beta, TNF-alpha, and NO.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Bhat et al., 1998; Caivano and Cohen, 2000). Briefly, RT was performed using ThermoScript™ RT reagents (Invitrogen, CA). The sequences of primers for rat IL-1beta, TNF-alpha, iNOS and beta-actin used in this study are listed in Table 1. PCR amplification was performed using 25 (for IL-1beta), 28 (for TNF-alpha), or 28 (for iNOS) cycles at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 60 s, respectively. For beta-actin, amplification was performed using 25 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 60 s. PCR was completed by a final extension at 72 °C for 10 min. Equal volumes of reaction mixture from each sample were loaded onto 2% agarose gels. The gel images were digitally captured for analysis of intensity with Quantity One 1-D Analysis software (Bio-Rad, CA). Levels of IL-1beta, TNF-alpha, iNOS mRNA were normalized to that of beta-actin in the same sample.

Table 1
Primer sequences used in this study

	Sequence 5′	Sequence 3′	Size of fragment (bp) (reference)
IL-1beta	AAGCTCTCCACCTCAATGGACAG	CTCAAACTCCACTTTGGTCTTGA	260 (Caivano and Cohen, 2000)
TNF-alpha	CACGCTCTTCTGTCTACTGA	GGACTCCGTGATGTCTAAGT	568 (Bhat et al., 1998)
iNOS	CGTGTGCCTGCTGCCTTCCTGCTGT	GTAATCCTCAACCTGCTCCTCACTC	672 (Bhat et al., 1998)
Beta-actin	GCCACTGCCGCATCCTCTT	ATCGTACTCCTGCTTGCTGA	408

2.5. Cytokines determination

After exposure to LPS and minocycline, culture medium samples were collected and centrifuged. Aliquots of the incubation medium (50 μl) were used for measuring the levels of IL-1beta and TNF-alpha with an Enzyme-Linked Immunosorbent Assay Kit (R&D Systems, MN).

2.6. NO determination

NO was measured as NO metabolite (nitrates and nitrites) release using a Nitric Oxide Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. NO is chemically active and rapidly converts to NO_3^- and NO_2^- in vivo. This method uses nitrate reductase to reduce NO_3^- to NO_2^- specifically, and the content of NO_2^- is determined colorimetrically. This kit is sensitive, stable and simple to use, with a major advantage of measuring the total amount of NO_3^- and NO_2^- through nitrate reductase.

2.7. Statistics

All experiments were performed at least three times. Graphical representation of data was derived from combined results, rather than representative data from one single experiment. Data were presented as the arithmetic mean percentages of control ± standard errors of the mean (S.E.M.). Experimental data were analyzed with one-way analysis of variance (ANOVA) for statistically significant differences ($p < 0.05$).

3. Results

3.1. Cultured microglia from rat retina

Microglia were collected from 14-day primary mixed glial cell cultures (Fig. 1A) prepared from new-born SD rats with a 'shaking off' method. Cultures were used for LPS and minocycline treatment study 1 day after re-seeding. By this time, the microglia had recovered from the isolation process and acquired a normal morphology (Fig. 1B and G). Immunocytochemical studies showed that all cultured cells stained positively for CD11b (Fig. 1C, E and G), with staining localized to the cell membrane. FCM analysis of the cultured cells demonstrated that more than 96% of the cells stained positively for CD11b-PE (Fig. 1D). None of these cells showed positive staining for glial fibrillary acidic protein (GFAP), indicating that the isolated cells were microglia and were not contaminated with astrocytes or Müller cells (data not shown).

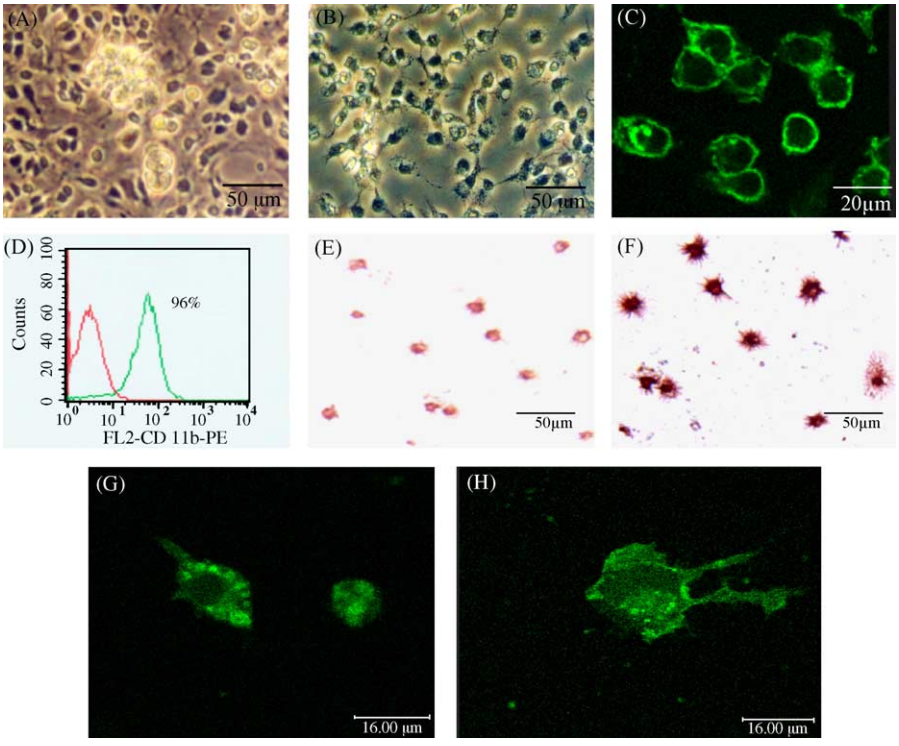

Fig. 1. Characterization of microglia in culture. (A) Mixed retinal cells prepared from newborn SD rats in culture for 14 days. Bar = 50 μm. (B) Isolated microglia from A in culture for 1 day. Bar = 50 μm. (C) Confocal microphotograph of microglia showing fluorescent staining of CD11b in cell the membrane. Bar = 20 μm. (D) FCM analysis of microglia purity with CD11b-PE. Red line: negative control; green line: CD11b-positive cells. (E) Untreated microglia immunostained for CD11b and counterstained with DAB. Bar = 50 μm. (F) Microglia treated with 3 ng/ml LPS for 24 h immunostained for CD11b and counterstained with DAB. (G) Confocal micrograph of untreated microglia immunostained with CD11b-FITC. Bar = 16 μm. (H) Microglia treated with 3 ng/ml LPS for 24 h immuno-stained with CD11b-FITC. Bar = 16 μm.

3.2. Effects of LPS on microglial morphology

Primary microglial cultures were treated with a range of concentrations (3×10^{-5} to 300 ng/ml) of LPS for 24 h. Culture supernatants were collected and assayed for TNF-alpha. LPS at 3 ng/ml was chosen to activate retinal microglia (data not shown). In control cultures (Fig. 1E), most microglia had a light brown stain (for CD11b) and did not posses many processes. LPS-treated microglia were ramified and darkly stained indicating activation (Fig. 1F). When microglia were stained with CD11b-FITC with (Fig. 1H) or without (Fig. 1G) LPS, the cell bodies of LPS-treated microglia appeared larger and bore long blunt ruffles with thin thread-like projections (Fig. 1H), whereas, the cell bodies of microglia without LPS treatment was smaller with fewer observable processes (Fig. 1G).

3.3. Effects of LPS on IL-1beta, TNF-alpha, and NO

Culture media were collected every hour for up to 6 h, then at 12 and 24 h after LPS treatment. The levels of IL-1beta in control culture media without LPS treatment did not change throughout the experimental period (Fig. 2, upper panel). In LPS-treated cultures, levels of IL-1beta in the culture media began to increase significantly from 2 to 3 h of LPS treatment and continued to increase throughout the 24 h period. The TNF-alpha content in medium was significantly increased at 1 h of LPS treatment. The increment of increase began to level off at 5 h (Fig. 2, middle panel).

The level of NO in the culture medium was determined by measuring the production of NO metabolites, nitrates and nitrites, in the presence of nitrate reductase. As shown in Fig. 2 (bottom panel), LPS-induced activation of microglia involved a significant time-dependent increase in NO level in the culture medium. The increment of NO levels became significant after 6 h of LPS treatment, and continued for 12 and 24 h.

3.4. Effects of minocycline on mRNA expression of IL-1beta, TNF-alpha, and iNOS

Microglia were treated with different concentrations of minocycline for 24 or 48 h, and cell viability was determined by [^{3}H] thymidine conversion. Minocycline at 20 to 140 μM did not induce obvious cell death in cultured microglia during the 24 h incubation. In cultures incubated for 48 h, minocycline dosage above 60 μM induced significant cell death. Thus, 60 μM minocycline was used to treat microglial cultures for 1 h before co-treatment with 3 ng/ml LPS for 6 h. RT-PCR was performed to detect IL-1beta, TNF-alpha, and iNOS mRNA levels. LPS treatment induced increases in mRNA levels of IL-1beta, TNF-alpha and iNOS in microglia (Fig. 3). Incubation of microglia with 60 μM minocycline alone for 6 h did not induce any detectable changes in IL-1beta, TNF-alpha and iNOS mRNA levels (data not shown); but the same concentration of minocycline reduced the LPS-induced IL-1beta, TNF-alpha, and iNOS gene expression (Fig. 3).

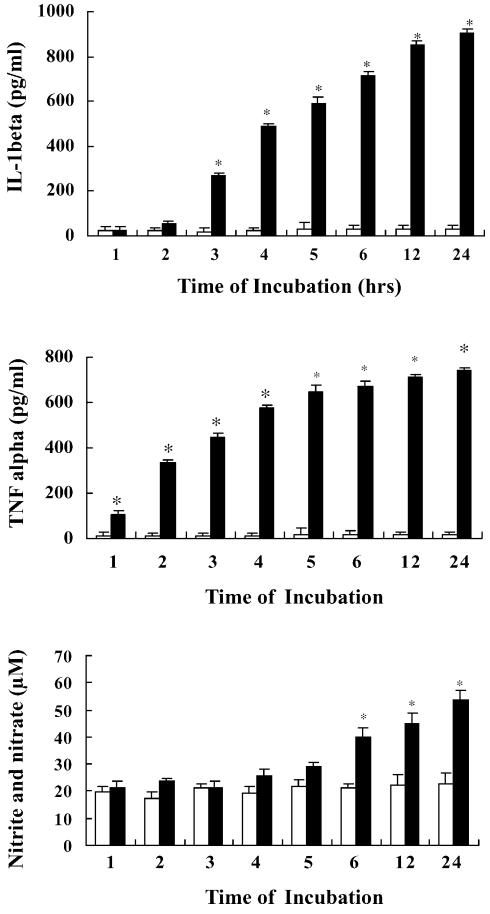

Fig. 2. Release of IL-1beta, TNF-alpha, NO from cultured retinal microglia with or without minocycline treatment and with LPS (3 ng/ml). Culture supernatants were collected at various time points and assayed for IL-1beta, TNF-alpha and NO. IL-1beta, TNF-alpha were examined with an ELISA Kit. NO formation was measure as its metabolites (nitrite and nitrate) in the medium. Data expressed are the mean ± S.E.M. of three individual experiments. $^{*}p < 0.05$ compared with the controls.

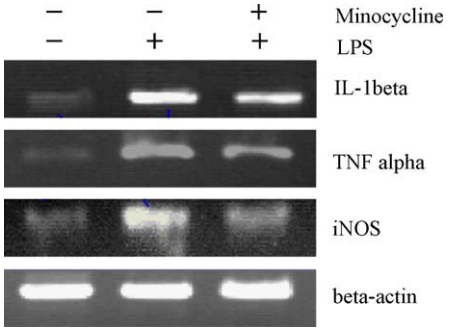

Fig. 3. Comparison of mRNA levels of IL-1beta, TNF-alpha, and iNOS in microglial cultures with or without 3 ng/ml LPS-treatment in the presence and absence of 60 μM minocycline pretreatment. Beta-actin was used as internal reference.

Table 2
Effects of minocycline on LPS-induced release of IL-1beta, TNF-alpha and NO

Groups	IL-1beta	TNF-alpha	NO
Control	97.71 ± 9.35	64.77 ± 8.71	21.76 ± 3.06
LPS	654.42 ± 41.40*	502.69 ± 11.49*	40.53 ± 5.79*
Minocycline	358.02 ± 15.81*	294.86 ± 28.27*	26.78 ± 2.81*

Retina microglia were pre-treated with 60 μM minocycline for 1 h followed by co-treatment with 3 ng/ml LPS for 6 h. Culture supernatants were collected and assayed for IL-1beta, TNF-alpha and NO. Data were mean ± S.E.M. of three individual experiments.
* $p < 0.05$ compared with the LPS-treated cultures.

3.5. Effects of minocycline on the release of IL-1beta, TNF-alpha and NO

The effects of minocycline on the LPS-induced release of IL-1beta, TNF-alpha, and NO to the culture medium were measured (Table 2) in microglia pre-treated with 60 μM minocycline for 1 h and then co-treated with 3 ng/ml LPS for 6 h. Culture media were collected and assayed for IL-1beta, TNF-alpha, and NO. Incubation of microglia with 60 μM minocycline alone for 6 h did not affect the level of IL-1beta, TNF-alpha, and NO in medium (data not shown). In the presence of LPS, the inhibitory effect of minocycline on the LPS-induced release of IL-1beta, TNF-alpha and NO was evident and significant (Table 2). Minocycline inhibited LPS-induced NO production by 73.26%, and by 53.25% and 47.46% inhibition for IL-1beta and TNF-alpha, respectively.

4. Discussion

Microglia are known for playing a key role in mediating inflammatory processes associated with various neurodegenerative diseases (Dehmer et al., 2000; Jamin et al., 2001). A prospective population-based cohort study of about 7000 individuals revealed that the relative risk of Alzheimer's disease decreases with prolonged use (more than 2 years) of nonsteroidal anti-inflammatory drugs (NSAIDs) (In't Veld et al., 2001). Potential participation of microglial activation is also reported in diabetic retinopathy (Rungger-Brandle et al., 2000; Zeng et al., 2000), glaucomatous optic nerve degeneration (Yuan and Neufeld, 2001), and retinal degeneration (Zeiss and Johnson, 2004). Thus, it is possible that the target of some antiinflammatory drugs may be microglia, which is believed to be the major immunocompetent cell type in the nervous system.

The use of primary culture of retina microglia offers the advantage of studying the response of microglia to controlled and designated treatments with more ease. More importantly, it provides a system to observe a pure population of microglia responding to treatment (for example, LPS). In our laboratory, primary culture of retinal microglia can achieve over 96% purity, as confirmed by immunocytochemistry and FCM determination.

LPS treatment has been used extensively in inflammatory studies (Carter and Dick, 2003; Maekawa et al., 2002). It has also been shown that LPS activates microglia and exerts neurocytotoxic effects in both in vitro and in vivo systems (Hughes et al., 2004; Nakamura et al., 1999). We observed that LPS treatment activates microglia. This was indicated by the morphological changes in microglia and by the dramatic increase in levels of CD11b, a microglial cell marker, in LPS-treated culture. LPS-treated microglia exhibited an increase in cytokines (IL-1beta, TNF-alpha) and NO levels, indicating that retinal microglia releases these factors when activated. Therefore, LPS-treated retinal microglia in culture provides a feasible model for studying the roles of microglia in retinal inflammation.

We have demonstrated that LPS induces the release of cytokines (TNF-alpha and IL-1beta) and NO into the culture medium. The release of these factors was time-dependent. Levels of TNF-alpha were elevated within the first 1 h of LPS treatment, the earliest among the three factors. IL-1beta release was elevated at 3 h while NO showed the slowest response (6 h after LPS treatment). LPS treatment also significantly elevated mRNA levels of IL-1beta, TNF-alpha, and iNOS, indicating that microglia in the retina respond to inflammation by upregulating gene and protein expression of IL-1beta, TNF-alpha and iNOS.

Minocycline has been used in the management of acne (Eady et al., 1990) and periodontal disease (Van Steenberghe et al., 1993). Being a second-generation semisynthetic tetracycline with broad-spectrum antimicrobial activity, minocycline has been shown to exert biological effects completely distinctive from tetracycline (Golub et al., 1998). The mechanism by which minocycline suppresses LPS-induced activation of microglia is not clearly understood. Minocycline has been shown to inhibit the induction of iNOS by increasing degradation of iNOS mRNA and by reducing the amount of iNOS protein (Yrjanheikki et al., 1999; Amin et al., 1996). Moreover, minocycline inhibits certain matrix metalloproteases (Power et al., 2003) as well as caspase-1 and caspase-3 (Chen et al., 2000; Zhu et al., 2002) by reducing their mRNA expression. Considering that caspase-1 and caspase-3, in particular, promote apoptotic cell death, and that inhibition of caspases protects neurons against low-dose irradiation (Tikka et al., 2001), it is possible that neuroprotection by minocycline is achieved, at least partially, by a reduction in caspase-induced apoptosis. These effects on cell death may also be partly due to the effects of minocycline on cytokine release, as observed in this study. Another possible mechanism is a direct interaction of minocycline with free radicals or reactive oxygen species, as minocycline have been shown to scavenge superoxides (Zhang et al., 2003) and peroxinitrite (Whiteman and Halliwell, 1997) in non-neuronal in vitro assays.

Zhang et al. (2004) observed that minocycline protected photoreceptors from light-induced loss through the inhibi-

tion of retinal microglial activation. However, Hughes et al. (2004) suggested that minocycline delayed photoreceptor death in a microglia-independent manner. This discrepancy may be due to the liposomal clodronate that could not penetrate the blood–retinal barrier, and thus, did not deplete the retinal-residing microglia. Other studies have shown that phagocytic cells during the early stages of inherited photoreceptor degeneration were derived from resident microglial cells and not from peripheral macrophages (Thanos, 1992; Roque et al., 1996). Further investigation is required to determine whether the minocycline induced delay of photoreceptor death is microglial dependent.

Minocycline has emerged as a potent inhibitor of microglial activation. Its potential as a drug is favored due to its easy absorption from the gastrointestinal tract with a half-life of 18 h. It is also highly lipid soluble and has a superior ability to penetrate the blood-brain and blood–retinal barriers (Teng et al., 2004; Hughes et al., 2004). Moreover, it has been used widely in clinics (Eady et al., 1990; Van Steenberghe et al., 1993) and has been considered to be relatively safe for humans in three clinical trials for rheumatoid arthritis (Stone et al., 2003). However, several studies have shown that minocycline either did not protect or was even detrimental in models of hypoxia-ischemia (Tsuji et al., 2004) and Huntington's disease (Diguet et al., 2003; Smith et al., 2003). Considering these contradictory data, the limitation of minocycline efficacy to a rather narrow window of concentration (Babicha and Tiptonb, 2002). Further, minocycline instability in aqueous solutions (Diguet et al., 2003) may possibly explain the contradictory clinical data on the health effects of this agent. Careful evaluation before clinical use in humans, exhibiting a variety of neurological disorders is therefore necessary.

In conclusion, minocycline may be a good candidate drug for retinal neurodegenerative disease. Further understanding of the mechanisms of minocycline action would provide important insights into potential therapeutic interventions for inflammation-related neurodegenerative diseases.

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