Doxycycline Attenuates Endotoxin-Induced Uveitis by Prostaglandin E2-EP4 Signaling

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Purpose. We explored the anti-inflammatory effects of doxycycline in experimental uveitis and the underlying mechanisms.

Methods. Rats with endotoxin-induced uveitis (EIU) received doxycycline (1.5 mg/kg) or the control vehicle via intraperitoneal injection. Clinical scores were graded under a slit lamp. Rat peritoneal macrophages were used in vitro to further explore the anti-inflammatory mechanisms of doxycycline. The levels of nitric oxide (NO), TNF-α, IL-1β, prostaglandin E2 (PGE2), cyclooxygenase (COX)-2, I kappa B-α (IxB-α), inducible nitric oxide synthase (iNOS), Akt, caspase-3, and nuclear factor-kappa B (NF-kB) were analyzed.

Results. Treatment with doxycycline dramatically reduced the clinical scores of EIU (P < 0.001), with significant decreases in inflammatory cell infiltration, protein concentrations, and the production of NO, TNF-α, and IL-1β in the aqueous humor (AqH). In vitro, doxycycline significantly inhibited the production of NO, IL-1β, and TNF-α in peritoneal macrophages by modulating the PI3K/Akt pathway. Importantly, we found that doxycycline significantly enhanced COX2 expression and PGE2 production both in vivo and in vitro. Furthermore, simultaneous injection of an EP4 antagonist and doxycycline significantly blocked the doxycycline-mediated inhibition of macrophages and the PI3K/Akt pathway in vitro. Furthermore, simultaneous injection of an EP4 antagonist and doxycycline significantly blocked the doxycycline-mediated attenuation of EIU.

Conclusions. Doxycycline can ameliorate EIU, and PGE2-EP4 signaling is essential for the anti-inflammatory effects of doxycycline in vitro and in vivo.

Keywords: uveitis, doxycycline, tetracyclines, inflammation, macrophage

Uveitis is an intraocular inflammatory disorder that is highly prevalent worldwide and can cause severe visual loss because of its recurrence and secondary complications, such as cataracts, glaucoma, and cystoid macular edema.1,2 In clinical practice, the conventional treatment for uveitis includes the use of corticosteroids and immunosuppressive agents to control the inflammatory process.1,2 However, long-term exposure to these drugs may result in potentially significant adverse effects.2,3 Furthermore, many patients are resistant to or cannot tolerate these agents.3 Therefore, novel safe and effective therapies are desirable.

Tetracyclines, a broad-spectrum antibiotic drug family that includes tetracycline, doxycycline, minocycline, and other derivative pharmaceuticals, exhibit an attractive variety of nonantibiotic properties. There are currently more than 200 ongoing clinical trials on the use of tetracyclines in various types of diseases.4 In particular, doxycycline is a long-acting, low-cost, semisynthetic tetracycline that has been used safely for decades in clinical settings. Recently, the anti-inflammatory and immunosuppressive properties of doxycycline have received increasing attention. Doxycycline has been reported to be useful for treating a number of inflammatory and/or immune diseases, including rheumatoid arthritis, rosacea, periodontitis, experimental autoimmune neuritis, and myocardial infarction.5-9 Therefore, doxycycline may represent a therapeutic option for the treatment of uveitis. However, the function of doxycycline in uveitis has not yet been explored. Moreover, the mechanisms by which doxycycline mediates anti-inflammatory responses remain elusive. Thus, in this study, we investigated the anti-inflammatory mechanisms of doxycycline and the therapeutic effect of doxycycline on endotoxin-induced uveitis (EIU).

Materials and Methods

Animals

Inbred male Wistar rats, 6 to 8 weeks old and weighing 160 to 180 g, were obtained from the Guangzhou Animal Testing Center and maintained in an air-conditioned room with a 12-hour light-dark cycle. The animals were provided access to food and water ad libitum until they were used for experiments. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University, and all procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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iovs.arvojournals.org | ISSN: 1552-5783

6686
Antibodies and Reagents

Doxycycline, prostanoid E2 (PGE2), EP1 to EP4 antagonists, and lipopolysaccharide (LPS; Escherichia coli 055:B5) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The following antibodies were used: anti-cyclooxygenase (COX)-2, anti-Akt, anti-phosphorylated Akt (p-Akt), anti-nuclear factor-kappa B (NF-κB) p65, anti-phosphorylated NF-κBp65 (p-NF-κBp65), anti-IκB antibody (IκB-α), anti-inducible nitric oxide synthase (iNOS), anti-β-actin, and a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA).

Induction of EIU and Drug Treatment Protocols

Endotoxin-induced uveitis was induced as previously described. Doxycycline (1.5 mg/kg, based on a previous study) was injected intraperitoneally in rats with EIU or the supernatants of cultured cells were measured via ELISA (eBioscience, San Diego, CA, USA). The activity of PI3K was determined using a PI3K activity ELISA: Pico kit (Echelon Biosciences, Inc., Salt Lake City, UT, USA). The concentrations of PGE2 in the supernatants or AqH were determined using ELISA kits (Cayman Chemical, Ann Arbor, MI, USA). The AqH from the eyes of two to three rats or supernatants were pooled, and 50 μL was used for a single assay. All measurements were performed in duplicate. The levels of NO in the supernatants or AqH were measured using the Griess reaction. Briefly, 50-μL aliquots of the samples were mixed with 0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide at room temperature for 10 minutes.

Quantification of Infiltrating Cells and Protein Concentrations in the Aqueous Humor

Aqueous humor (AqH, 20–25 μL/rat) was obtained from both eyes via anterior chamber puncture with a 30-gauge needle under a surgical microscope while the animal was under deep anesthesia induced through an intraperitoneal injection of a 10% chloral hydrate solution (0.4 mL/100 g) 24 hours after LPS injection. For cell counting, the AqH samples were suspended in Trypan blue solution (1:5), and the cells were counted under light microscopy using a hemocytometer. The concentration of total protein in AqH was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Histopathologic Examination

For histopathologic examination, the rats were killed 24 hours after LPS injection, and their intact eyes were immediately enucleated, stored in a 10% neutral buffered formalin solution for 24 to 48 hours at room temperature, dehydrated in a graded ethanol series, and embedded in paraffin. Then, 5-μm sagittal sections were cut near the optic nerve head and stained with hematoxylin and eosin (H&E).

Enzyme-Linked Immunosorbent Assay and NO Assay

Western Blotting

Real-Time PCR

Statistical Analysis

RESULTS

Doxycycline Treatment Attenuates EIU

To examine the therapeutic effects of doxycycline on EIU, doxycycline was injected intraperitoneally in rats with EIU following LPS injection. Lipopolysaccharide administration generated typical signs mimicking human uveitis, including ciliary congestion, iris blood vessel dilatation, pupil occlusion and fibrinous membrane formation. Clinical scores were...
evaluated at 24 hours after LPS injection. As shown in Figure 1A, doxycycline administered at 6 hours and 12 hours after LPS injection reduced the inflammatory scores of the EIU rats compared with the untreated group. However, the suppressive effects were not as significant as those observed in animals treated with doxycycline administered immediately after LPS injection. Therefore, we chose the immediate administration of doxycycline after LPS injection in the following studies. Representative images of ocular symptoms demonstrate that doxycycline treatment significantly attenuated iris blood vessel dilatation, pupil occlusion and fibrinous membrane formation (Fig. 1B). Lipopolysaccharide application induced a significant increase in the number of cells and the levels of protein in the AqH compared with control rats, and doxycycline administration significantly decreased the number of cells and protein levels in the AqH in treated rats (Figs. 1C, 1D).

**Doxycycline Suppresses Inflammation in EIU**

Next, we investigated the effects of doxycycline treatment on the inflammatory profiles in EIU. Histologic analyses revealed significantly decreased inflammatory cell numbers in the ICB in doxycycline-treated rats compared with control animals (Fig. 2A). Next, we determined the effects of doxycycline on the levels of local inflammatory mediators in the AqH using ELISA. The data are presented as the mean ± SD. **P < 0.01.

**Doxycycline Inhibits the Release of Proinflammatory Mediators by Macrophages In Vitro**

To explore the anti-inflammatory mechanism of doxycycline in EIU, rat peritoneal macrophages were isolated and used as an in vitro model. For this purpose, purified rat peritoneal macrophages were plated in six-well plates for 24 hours and subsequently stimulated with LPS (1 μg/mL) for another 16 hours in the presence or absence of doxycycline at increasing concentrations. The levels of TNF-α and IL-1β in the culture supernatants were measured via ELISA. The results showed that doxycycline treatment led to a dose-dependent inhibition of TNF-α and IL-1β release by peritoneal macrophages activated by LPS (Figs. 3A, 3B). Compared with 40 μM doxycycline, 80 μM doxycycline inhibited IgE release more effectively, but B-cell viability was affected (Fig. 3C). Therefore, we chose 40 mM doxycycline in the following experiments unless specifically indicated. Additionally, the mRNA expression of TNF-α and IL-1β in peritoneal macrophages was significantly reduced after doxycycline treatment compared with untreated peritoneal macrophages (Figs. 3E, 3F). Nitric oxide production and iNOS expression were determined via the Greiss reaction and Western blotting, respectively. As shown in Figures 3E and 3F, doxycycline treatment significantly suppressed the levels of LPS-induced NO release and iNOS expression.

**PI3K/Akt/1xB-α/NF-kB Signaling Is Involved in the Doxycycline-Mediated Inhibition of Macrophages**

Nuclear factor κB is a key heterodimeric transcription factor that is responsible for inflammatory and immune processes including uveitis.5,16 Thus, we asked whether NF-κB signaling is involved in the doxycycline-mediated inhibition of peritoneal macrophages. We found that stimulation of macrophages with LPS increased phosphorylated NF-κBp65 protein expression, whereas doxycycline treatment significantly inhibited phos-
phorylated NF-κBp65 expression in macrophages (Fig. 4A). IkB-α degradation exposes a nuclear localization signal that leads to the phosphorylation of NF-κB. Therefore, we also investigated whether doxycycline modulates NF-κB activity in peritoneal macrophages by inhibiting IkB-α degradation. As expected, doxycycline significantly inhibited IkB-α degradation in peritoneal macrophages (Fig. 4B). Because the PI3K/Akt pathway plays a critical role in macrophage activation and NF-κB activation, we next evaluated whether doxycycline could modulate the PI3K/Akt pathway in rat peritoneal macrophages. These results showed that although stimulation of macrophages with LPS resulted in increased Pi3K activity and p-Akt protein expression, treatment with doxycycline significantly reduced Pi3K activity and p-Akt expression in peritoneal macrophages (Figs. 4C, 4D).

**Prostaglandin E2-EP4 Signaling Is Responsible for the Doxycycline-Mediated Anti-Inflammatory Effects on Peritoneal Macrophages**

Our in vivo results showed that doxycycline reduced TNF-α, IL-1β, and NO production but increased PGE2 production and COX2 expression. Thus, we next asked whether COX2/PGE2 signaling was involved in the doxycycline-mediated anti-inflammatory effects on peritoneal macrophages. As shown in Figures 5A and 5B, doxycycline treatment also significantly increased COX2 expression and PGE2 production in peritoneal macrophages. Typically, PGE2 exerts its biological functions via four subtypes of prostaglandin E receptors, EP1 to EP4. Thus, to further confirm and elucidate the roles of COX2/PGE2 in the doxycycline-mediated suppression of inflammatory mediator release in peritoneal macrophages, antagonists of EP1 to EP4 were used. We observed that although the addition of an antagonist of EP1, EP2, or EP3 did not affect the anti-inflammatory effects of doxycycline on peritoneal macrophages, the addition of an EP4 antagonist significantly reversed the doxycycline-mediated inhibitory effects on peritoneal macrophages following LPS stimulation (Figs. 5C, 5D). Additionally, treatment with the EP4 antagonist led to an increase in inflammatory cytokine release in macrophages stimulated with LPS, but this increase was not as significant as that observed in macrophages treated with LPS and doxycycline (Figs. 5E, 5F). As shown in Figures 5G and 5H, EP4 blockade also significantly decreased the ability of doxycycline to inhibit the PI3k/Akt pathway. Together, these results indicate that PGE2-EP4 signaling plays important roles in the doxycycline-mediated inhibition of macrophages.

**Figure 2.** Doxycycline attenuates inflammation in EIU. (A) Representative images of H&E staining of ICB samples from rats in the indicated experimental group at 24 hours after LPS challenge (scale bars: 200 μm). (B–D) The levels of TNF-α, IL-1β, and NO in AqH were measured by ELISA and the Griess reaction (n = 6). (E–G) The levels of iNOS, (phosphorylated) NF-κBp65, and COX2 in the ICB were determined via Western blotting (n = 6). (H) The levels of PGE2 in AqH were measured by ELISA. The data are presented as the mean ± SD. **P < 0.01.
Our in vitro studies showed that PGE2-EP4 signaling plays a critical role in the doxycycline-mediated inhibition of peritoneal macrophages. Therefore, we next determined whether PGE2-EP4 signaling was also involved in the doxycycline-mediated inhibition of EIU. To this end, the EP4 antagonist (L-161,982, 1 mg/kg) and doxycycline were injected simultaneously following LPS administration. These results showed that treatment with the EP4 antagonist significantly blocked the doxycycline-mediated decrease in the clinical scores of EIU, cell numbers and protein levels in the AqH (Figs. 6A–C). As shown in Figures 6D through 6F, the EP4 antagonist also significantly decreased the inhibitory ability of doxycycline with regard to inflammatory mediator production. These findings suggest that the upregulation of COX2/PGE2-EP4 signaling might contribute, at least in part, to the doxycycline-mediated attenuation of EIU.

**DISCUSSION**

Endotoxin-induced uveitis is an established animal model of some types of uveitis in humans, particularly acute anterior uveitis.\(^{11}\) Endotoxin-induced uveitis is induced via the systemic injection of a single sublethal dose of LPS, and the maximum inflammatory response is achieved at 24 hours after LPS injection.\(^{10},^{11}\) In the present study, we investigated the anti-inflammatory effects and mechanisms of doxycycline in EIU. The results of this study demonstrated that doxycycline could significantly attenuate the ocular inflammatory response in EIU rats at 24 hours after LPS injection, with significant decreases in inflammatory cell infiltration and the production of NO, TNF-α, and IL-1β in the AqH. These findings suggest that doxycycline is capable of inhibiting EIU. Importantly, compared with the drugs currently used to treat uveitis, which
generate certain serious side effects and complications, doxycycline has been used relatively safely for decades in clinical settings with fewer side effects. Furthermore, combined therapy with tetracyclines and steroids has shown promising therapeutic effects in asthmatic patients. In addition, doxycycline has been used in ocular diseases, such as meibomian gland disease and rosacea, and recurrent corneal erosions. In a randomized, proof-of-concept clinical trial of participants with diabetic retinopathy who received oral doxycycline monohydrate, 50 mg, daily for 24 months was associated with improved frequency doubling the perimetry foveal sensitivity compared with placebo. Thus, doxycycline may be a potential therapeutic alternative for uveitis treatment.

The NF-kB proteins are a ubiquitously expressed family of transcription factors that are crucial for the synthesis and release of various inflammatory mediators. Most agents, including LPS, activate NF-kB through the phosphorylation or degradation (or both) of IκB-α. IκB-α degradation exposes a nuclear localization signal and leads to the activation of NF-κB. In EIU, LPS binds to Toll-like receptor 4 (TLR4) on macrophages and other cells to activate the IκB-α/NF-kB pathway. The subsequent increase in the release of inflammatory mediators contributes to the development of uveitis. In this study, our in vivo results showed that doxycycline inhibits the expression of phosphorylated NF-kBp65 protein. In vitro, using peritoneal macrophages, we found that doxycycline reduced the LPS-induced release of inflammatory mediators by modulating the IκB-α/NF-kB pathway. Furthermore, we demonstrated the inhibitory effects of doxycycline on the PI3K/Akt pathway, which is the upstream signaling pathway for NF-kB activation. These findings are concordant with previous studies suggesting that doxycycline may attenuate EIU by modulating the PI3K/Akt/NF-kB pathway.

Prostaglandin E2, a short-lived potent bioactive lipid mediator, is a major downstream product of arachidonic acid metabolism via the cyclooxygenase COX-2. Prostaglandin E2 exerts a wide variety of biological effects, including anti-inflammatory and proinflammatory effects, by binding to the prostaglandin E receptors, EP1 to EP4. In the current study, our results showed that doxycycline increased COX2 expression and PGE2 production in the eyes of rats with EIU. It was further confirmed in rat peritoneal macrophages that COX2/PGE2 signaling was upregulated following doxycycline treatment. Importantly, we found that the addition of an EP4 antagonist significantly reversed the doxycycline-mediated inhibitory effects on peritoneal macrophages. Even more importantly, simultaneous injection of the EP4 antagonist and doxycycline abolished the therapeutic effects of doxycycline on EIU. Collectively, these compelling findings suggest that PGE2-EP4 signaling contributes, at least in part, to the doxycycline-mediated attenuation of EIU. Although the anti-inflammatory function of doxycycline was discovered several decades ago, the underlying molecular mechanisms remain elusive. Thus, it is noteworthy that PGE2-EP4 signaling was implicated in the anti-inflammatory effects of doxycycline.

Based on the doxycycline dose of 1.5 mg/kg used in this study, this is equivalent to approximately 90 mg per day for human subjects (approximately 60 kg). This is a lower dose compared with the typical antimicrobial dose of doxycycline given to humans (200 mg per day, Food and Drug Administration). However, it is similar to a dose in previous studies that showed that the subantimicrobial dose of doxycycline (40–100 mg per day) provided significant benefit in the treatment of human patients.

Although the EIU model is a good model of auto-inflammatory uveitis, translation into clinical application should proceed with caution. The EIU model evaluates uveitis and response to treatment in a short period of time (24 hours), whereas uveitis in human patients is a chronic disease with
peaks and relapses of activity. Uveitis treatment requires long-term therapies. Although a small pilot study has shown that oral doxycycline monohydrate at 50 mg daily for 24 months is safe and improved the frequency of doubling perimetry foveal sensitivity in patients with diabetic retinopathy, further study is still needed to verify this in patients with uveitis.

In summary, our study is the first to demonstrate the therapeutic effects of doxycycline on experimental uveitis. Our results showed that doxycycline reduced inflammatory mediator release from peritoneal macrophages, possibly by modulating the PI3K/Akt/NF-κB pathway. Importantly, we further elucidated the anti-inflammatory mechanisms of doxycycline by demonstrating that PGE2-EP4 signaling is essential for doxycycline-mediated anti-inflammatory effects in vitro and in vivo.

Acknowledgments

Supported by the Natural Science Foundation of China (81271051 and 81300740). The authors alone are responsible for the content and the writing of the paper.

Disclosure: J. Huang, None; W. Su, None; X. Chen, None; X. Cheng, None; Y. Dai, None; L. Han, None; D. Liang, None

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