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Prevention of Endotoxin-Induced Uveitis in Rats by Benfotiamine, a Lipophilic Analogue of Vitamin B1

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Abstract

Purpose—To study the amelioration of ocular inflammation in endotoxin-induced uveitis (EIU) in rats by benfotiamine, a lipid-soluble analogue of thiamine.

Methods—EIU in Lewis rats was induced by subcutaneous injection of lipopolysaccharide (LPS) followed by treatment with benfotiamine. The rats were killed 3 or 24 hours after LPS injection, eyes were enucleated, aqueous humor (AqH) was collected, and the number of infiltrating cells, protein concentration, and inflammatory marker levels were determined. Immunohistochemical analysis of eye sections was performed to determine the expression of inducible–nitric oxide synthase (iNOS), cyclooxygenase (Cox)-2, protein kinase C (PKC), and transcription factor NF- κ B.

Results—Infiltrating leukocytes, protein concentrations, and inflammatory cytokines and chemokines were significantly elevated in the AqH of EIU rats compared with control rats, and benfotiamine treatment suppressed these increases. Similarly increased expression of inflammatory markers iNOS and Cox-2 in ciliary body and retinal wall was also significantly inhibited by benfotiamine. The increased phosphorylation of PKC and the activation of NF- κ B in the ciliary body and in the retinal wall of EIU rat eyes were suppressed by benfotiamine.

Conclusions—These results suggest that benfotiamine suppresses oxidative stress–induced NF- κ B–dependent inflammatory signaling leading to uveitis. Therefore, benfotiamine could be used as a novel therapeutic agent for the treatment of ocular inflammation, especially uveitis. (*Invest Ophthalmol Vis Sci.*

Endotoxin-induced uveitis (EIU) in rodents, which mimics human uveitis, has been used to evaluate the therapeutic efficacy of drugs, especially those that could prevent or stop ocular inflammation.^{1–4} In EIU, lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component, is known to initiate a cascade of signaling reactions to activate redox-sensitive transcription factors and the expression of inflammatory cytokines, chemokines, and other inflammatory markers such as cyclooxygenase (Cox)-2 and inducible–nitric oxide synthase (iNOS).^{4–6} Increased expression of inflammatory markers results in the breakdown of the blood-ocular barrier and the infiltration of leukocytes in ocular tissues, which contributes to the development of EIU.⁵ This is similar to infection-induced uveitis in humans. Although autoimmune diseases and infections are considered the main causes of uveitis, in many patients the etiology of uveitis remains unknown.^{7,8} The endotoxin causes the breakdown of the blood-ocular barrier, resulting in the infiltration of inflammatory cells in the anterior chamber of the eye, increased protein concentrations, and increased levels of cytokines, chemokines, and nitric

oxides in aqueous humor (AqH) resulting in anterior uveitis. Even though EIU is generally considered to be a model of anterior uveitis, various recent studies have shown that it also involves inflammation at the posterior segment.^{5,9} This is evident from the recruitment of leukocytes that adhere to the retinal vasculature and infiltration to the vitreous cavity during EIU.^{5,9} Although inflammation resolves in a couple of days, hyperinflammation induces apoptosis and cell death in the inflammatory and resident ocular cells that could be sight threatening.¹⁰ Reactive oxygen species (ROS) are known to mediate the inflammatory signals of LPS and of various cytokines and chemokines, resulting in the activation of protein kinase C (PKC) isozymes and downstream signaling pathways that activate NF- κ B.¹⁰⁻¹³ In addition, LPS is known to directly activate the PKC/I κ B/NF- κ B cascade through the activation of a family of pattern-recognition receptors known as toll-like receptors (TLRs).^{14,15} Of the approximately 10 TLRs known thus far, TLR4 has been shown to act as a receptor for Gram-negative bacterial toxins such as LPS and to mediate LPS-induced inflammation in various cell types,^{16,17} and it has been suggested as a potential target in various diseases.¹⁸ Studies have also shown the expression of TLR4 in ocular tissues in response to LPS, which may activate the signaling pathways that lead to NF- κ B activation.^{19,20} LPS-induced activation of NF- κ B leads to increased expression of inflammatory cytokines, including TNF- α and IL-1 β , chemokines such as monocyte chemoattractant protein (MCP)-1, and other inflammatory proteins such as iNOS and Cox-2.^{4,5} Recent studies suggest a biphasic role of NF- κ B during inflammation. In the early phase it induces the expression of proinflammatory genes, and in the late phase it activates the expression of anti-inflammatory genes that help resolve inflammation.^{21,22} However, in recurrent inflammation such as uveitis, cellular homeostasis is disturbed and pharmacologic intervention becomes mandatory to tame the inflammation and to save patients from discomfort and the threat of vision loss. Immunosuppressants including steroids, used to control inflammation, come with many serious side effects, including the threat of glaucoma and cataract.²³

Although vitamins such as E and C have been shown to prevent ocular inflammation,^{24,25} the role of vitamin B1 and its derivatives, such as benfotiamine, in the prevention of uveitis has never been explored. Benfotiamine (*S*-benzoylthiamine-*O*-monophosphate) is a lipid-soluble analogue of vitamin B1 (thiamine) and has a unique open-ringed structure (Fig. 1A) that enables it to pass directly through the cell membrane, resulting in increased bioavailability.^{26,27} Even though thiamine and its analogue benfotiamine have been shown to prevent oxidative stress-induced pathologic conditions, benfotiamine, because of its permeability through the biological membranes, is several times more effective an antioxidant than thiamine. Indeed, recent studies have shown the efficacy of benfotiamine in alleviating diabetic nephropathy,²⁸ neuropathy,²⁹ retinopathy,³⁰ and other oxidative stress-induced pathologic conditions in various experimental models.³¹⁻³³ In a recent study,³⁰ benfotiamine has been shown to block the pathways that activate PKC through a cascade of kinases that activate NF- κ B. Further, benfotiamine has been found to prevent the activation of PKC and NF- κ B in the retina of subjects with diabetic retinopathy.³⁰ In the present study, we have shown that the treatment of EIU rats with benfotiamine diminishes the inflammatory cell infiltration in AqH, the production of inflammatory cytokines such as IFN- γ , IL-1 β , IL-4, and TNF- α and of chemokines such as MCP-1 and cytokine-induced neutrophil chemoattractant-2, and the activation of NF- κ B. These results suggest a possible therapeutic application of benfotiamine in ocular inflammation.

Materials and Methods

Chemicals

Benfotiamine was obtained from Vitaspace (New York, NY). Nitrite/nitrate, Cox-2, and PGE₂ assay kits were obtained from Cayman Chemical Inc. (Ann Arbor, MI). Rat TNF- α

ELISA kit was obtained from BD Biosciences (San Diego, CA). Carboxymethylcellulose and LPS from *Escherichia coli* (strain 0111:B4) were obtained from Sigma (St. Louis, MO). Antibodies against phospho-p65 (Ser536) were purchased from Cell Signaling (Danvers, MA), and iNOS, Cox-2, and phospho-PKC- β II pan (Ser660) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents used were of analytical grade.

Animal Groups and EIU

EIU was induced in rats as described previously.⁴ Briefly, 6-week-old male Lewis rats weighing approximately 150 g each were randomly divided into four groups ($n = 6$). EIU was induced by subcutaneous injection at two locations of *E. coli* LPS (200 μ g) dissolved in 100 μ L PBS (pH 7.4). Rats were fed benfotiamine dissolved in carboxymethylcellulose (1%, wt/vol) and given orally by gavages at a volume ratio of 4 mL/kg²⁹ 3 hours before and immediately after LPS injection. All animals were kept in the University of Texas Medical Branch Animal Care Center. All the animal studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Infiltrating Cells and Proteins in Aqueous Humor

Twenty-four hours after LPS injection, the rats were euthanatized, and AqH was collected immediately from eyes by anterior chamber puncture with a 30-gauge needle under the surgical microscope. For cell counting, AqH samples were suspended in an equal amount of Trypan-blue solution, and the cells were counted with a hemacytometer under a light microscope (Olympus Optical Ltd., Tokyo, Japan). The total protein concentration in the AqH samples was measured by Bio-Rad (Hercules, CA) protein assay kit. AqH samples were stored on ice until used; cell counts and total protein concentrations were measured on the day of sample collection.

Determination of Cytokines and Chemokines in Aqueous Humor

Cytokine and chemokine levels in the AqH were assessed with a commercially available rat cytokine antibody array system according to the manufacturer's (Ray Biotech, Inc., Norcross, GA) instructions. AqH from two rat eyes was pooled and diluted with an assay buffer supplied with the assay system, and equal amounts of AqH were used for the determination of inflammatory cytokines and chemokines. Densitometry analysis of the array was performed with a digital imaging system (Image Station; Eastman Kodak, Rochester, NY).

Histopathologic Evaluation

Rats were euthanatized at 3 or 24 hours after LPS injection, and their eyes were enucleated immediately and stored in 4% paraformaldehyde solution for 24 hours at 4°C. Then the eyes were washed twice in ice-cold PBS and kept in 70% alcohol at 4°C until embedded in paraffin. Sagittal sections (5 μ m) were cut and stained with hematoxylin and eosin. For histopathologic evaluation, the iris-ciliary body complex, anterior chamber, vitreous, and retina were observed under light microscope.

Immunohistochemical Studies

Paraffin sections were warmed at 60°C for 1 hour and deparaffinized in xylene, after which they were rehydrated by passing through 100%, 95%, 80%, and 70% ethanol and in deionized water. After peroxidase blocking with 3% H₂O₂, the sections were rinsed in PBS twice for 5 minutes each time and were incubated with blocking buffer (2% BSA, 0.1% Triton X-100, 2% normal rabbit IgG, 2% normal goat serum) overnight at 4°C. Sections were incubated with antibodies against iNOS, Cox-2, phospho-p65 antibodies (Ser536), and phospho-PKC- β II pan (Ser660) for 1 hour at room temperature, after which they were stained with a universal immunohistochemistry procedure (LSAB+System-HRP; DakoCytomation, Carpinteria, CA),

examined under bright-field light microscopy (EPI[b]-800; Nikon, Tokyo, Japan), and photographed with a Nikon camera fitted to the microscope.

Statistical Analysis

Data were expressed as mean $\pm$ SD. For analyzing EIU scores, the Kruskal-Wallis test was used for the overall group effect. Wilcoxon-Mann-Whitney tests were used for the pairwise comparisons across groups. Analyses were stratified by side (left and right). All computations were performed with the SAS system (SAS/STAT: User's Guide, Version 9; SAS Institute, Cary, NC). One-way ANOVA was used to compare inflammatory markers. $P < 0.05$ was considered statistically significant.

Results

Effect of Benfotiamine on EIU-Induced Leukocyte Infiltration and Protein Concentration in AqH

The pathologic symptoms of EIU in Lewis rat eyes injected with LPS and treated without or with benfotiamine were graded in blinded fashion with a slit lamp microscope to evaluate its efficacy. As shown in Figure 1B, at 24 hours after LPS injection, the clinical scores for the EIU rats were 3.0 ± 0.5 and were significantly ($P < 0.0008$; Wilcoxon-Mann-Whitney test) reduced to 1.3 ± 0.5 ($P < 0.005$) after benfotiamine treatment. We next examined leukocyte infiltration in the rat eye sections stained with hematoxylin and eosin. As shown in Figure 2A, enormous inflammatory cell infiltration was observed in EIU eye sections at the AqH and at the vitreous regions. In benfotiamine-treated EIU rat eyes, no significant infiltration of cells was observed. Further, we manually measured the number of infiltrated cells in the AqH by using a hemacytometer. As shown in the Figure 2B, approximately $95 \times 10^4/\text{mL}$ leukocytes infiltrated the EIU rat eye AqH, but none infiltrated the control rat eye AqH. In the benfotiamine-treated EIU rat eye, the number of leukocytes in AqH was significantly reduced (35×10^4 cells/mL). Control rats treated without or with benfotiamine alone did not show any infiltrated cells in the AqH or vitreous chamber. Next we measured total protein concentration in the AqH, which represents increased levels of inflammatory cytokines and chemokines. As shown in Figure 2C, an approximately 10-fold increase in protein levels was observed in the EIU rats, and benfotiamine prevented elevated protein concentration significantly (>60%). Thus, our results suggest that benfotiamine treatment could prevent the EIU-induced infiltration of inflammatory cells and the release of inflammatory proteins in the AqH.

Effect of Benfotiamine on Endotoxin-Induced Cytokine Release in AqH

Given that EIU is marked by the excessive release of inflammatory cytokines and chemokines that aggravate inflammation, we next measured the levels of various cytokines and chemokines in the AqH by an antibody array. As shown in Figure 3, we observed significantly enhanced secretion of cytokines such as IFN- γ (~45%), IL-1 β (~20%), IL-4 (77%), TNF- α (25%), and chemokines such as MCP-1 (~4-fold), β -nerve growth factor (NGF; 25%), and cytokine-induced neutrophil chemoattractant-2 (70%). In addition, LPS also increased the levels of vascular endothelial growth factor (20%) in the AqH of EIU rat eyes. However, benfotiamine significantly prevented the release of these inflammatory markers in the EIU rat eye AqH (Fig. 3).

Effect of Benfotiamine on EIU-Induced Inflammatory Markers in AqH

Because the inflammatory markers NO and PGE2 are implicated in inflammation during EIU, we examined immunohistochemically the expression of enzymes that synthesize these inflammatory markers (i.e., iNOS and Cox-2 enzymes, respectively) in various regions of eye. EIU rat eyes showed increased expression of iNOS and Cox-2 proteins in the iris-ciliary body

complex and neural retina (Fig. 4AI, 4AII), as indicated by increased staining pertaining to these antigens. Treatment with benfotiamine significantly prevented the expression of iNOS and Cox-2 proteins, indicating the inhibition of expression of these proteins by benfotiamine.

Effect of Benfotiamine on PKC and NF- κ B Activity in EIU Rat Eyes

The activation of redox-sensitive transcription factor NF- κ B during oxidative stress is a hallmark of inflammation during EIU, which transcribes various inflammatory marker genes, including those of interleukins, cytokines, chemokines, iNOS, and Cox-2. We, therefore, examined the effect of benfotiamine on the activation of NF- κ B during EIU in rat eyes. Fluorescence immunostaining of rat eye sections obtained after 3 hours of post-LPS challenge with antibodies against phospho-p65 (active subunit of NF- κ B) showed significant intensity for NF- κ B staining at the iris-ciliary body complex and at the retinal wall (Figs. 4BI, 4BII). The increase in NF- κ B staining observed after EIU was significantly prevented by treatment with benfotiamine. No significant NF- κ B staining was observed for control or benfotiamine-treated rat eyes.

Protein kinase C (PKC) isozymes, specifically PKC β II, have been shown to be the main activators of NF- κ B.³⁴ Hence, we next examined the effect of benfotiamine on LPS-induced PKC- β II activation in the rat eyes. As shown in Figures 4CI and 4CII, increased staining for phospho-PKC- β II was observed in the cell membrane of the ciliary bodies and the cells of the retina in EIU rat eyes, and treatment with benfotiamine prevented it. Control rat eyes showed no significant staining pertaining to phosphorylated PKC- β II. These results indicate that benfotiamine prevented endotoxin-induced ocular inflammation in rats by inhibiting the NF- κ B-mediated expression of inflammatory markers.

Discussion

Uveitis is an inflammatory disorder of the eye that has a lifetime cumulative incidence of approximately 4 per 1000 in the general population.³⁵ The inflammation generally resolves in a few weeks; however, in two thirds of patients it becomes recurrent.³⁶ The recurrent form of uveitis may result in other sight-threatening conditions, including cataract, glaucoma, and macular edema.³⁶ The treatment available for uveitis primarily includes corticosteroids, with suggested topical or systemic application. However, corticosteroids have severe side effects and may cause cataract, glaucoma, and susceptibility to microbial infections and may decrease patient quality of life.^{23,37} Indeed, many alternative approaches, including antioxidants³⁸⁻⁴⁰ and herbal extracts (such as aronia crude extract from *Aronia melanocarpa*³), carotenoids (such as fucoxanthin,⁴¹ astaxanthin,⁴² Ginkgo biloba,² and leutine,⁴³), and anticytokine therapies (such as anti-TNF) have been shown to prevent ocular inflammation in experimental animals.^{39,40}

We report for the first time that the vitamin B1 analogue benfotiamine prevents endotoxin-induced uveitis in rats. Benfotiamine is the most potent of the allithiamines, a group of lipid-soluble form of thiamine, also found in traces in roasted garlic and other herbs of the genus *Allium*.⁴⁴ The unique open-ringed structure of benfotiamine makes it pass directly through cell membranes, in contrast to thiamine salts, and readily reach several organs and tissues. Benfotiamine is absorbed more effectively in the intestine and reaches approximately 5-fold higher maximum plasma levels of thiamine.⁴⁴ The bioavailability is approximately 3.6-fold as high as that of thiamine hydrochloride and other lipophilic thiamine derivatives.²⁶ A recent randomized, double blind, placebo-controlled clinical study in Germany indicated that benfotiamine at a dose of 600 mg/d and higher had no side effects.⁴⁵ A number of studies have also revealed its potential to alleviate diabetic microangiopathy, neuropathy, and other oxidative stress-induced pathologic conditions in various experimental models.²⁸⁻³³ Vitamin B1 deficiency produces beriberi, and 70% of patients with beriberi have ocular abnormalities

such as dry eye, optic atrophy, and epithelial changes in conjunctiva. No studies are available that show the relationship between vitamin B1 deficiency and uveitis in humans. However, the relationship between vitamin B1 deficiency and bacterial infections has been observed in animal and human studies.^{46,47} In addition, thiamine deficiency produces fiber cell degeneration in mouse lenses.⁴⁸ No reports are available that show thiamine or benfotiamine supplementation prevents uveitis. Recently, benfotiamine has been shown to block three pathways, including the activation of PKC, that ultimately activate NF- κ B.³⁰ It normalizes protein kinase C (PKC) activity and prevents NF- κ B activation in the retina of patients with diabetes.³⁰ These results suggest that benfotiamine supplementation may be used as a potent antioxidant in treating oxidative stress-induced inflammatory disorders. Even though benfotiamine has been extensively used for ameliorating diabetic complications, the effect of benfotiamine supplementation has not been investigated in ocular inflammatory conditions. In this report, we show for the first time that oral supplementation of benfotiamine significantly prevented the development of EIU in Lewis rats. Recent studies have also indicated the use of vitamins and antioxidants in the prevention of uveitis. Cid et al.⁴⁹ have shown that vitamin E supplementation to the experimental lens-induced uveitis in Brown Norway rats prevents uveitis complications. However, 4-month oral supplementation of vitamin E to patients with uveitis-associated macular edema did not show any significant effect in preventing visual acuity.²⁴ Further, vitamin C has been shown to prevent retinal edema in an experimental uveitis model in guinea pigs.²⁵ In a randomized double-masked study in human patients with acute anterior uveitis, oral vitamins C and E as additional treatments did not reduce flare or cell measurement but significantly improved visual acuity.⁵⁰

Although various studies suggest that supplementation with vitamins E and C may improve the severity of uveitis complications in an experimental animal model of uveitis,^{24,25,49,50} the excessive use of these inhibitors causes increased toxicity. For example, vitamin C has been shown to increase the levels of lipid peroxidation-generated lipid aldehydes such as 4-oxynonenal and 4-hydroxynonenal, which cause cytotoxicity.⁵¹ However, lipophilic vitamins such as vitamin E and benfotiamine have been shown to be nontoxic at clinically relevant doses.^{45,50} Our results and those from other laboratories suggest that benfotiamine may prevent the activation of transcription factor NF- κ B,³⁰ suggesting that benfotiamine may prevent NF- κ B-dependent signaling events such as the transcription of inflammatory cytokines, chemokines, and other markers that cause inflammation. In uveitis large amounts of inflammatory cytokines and chemokines are secreted in the AqH and cause tissue damage. Our results show that benfotiamine significantly attenuated the expression of inflammatory cytokines, chemokines, and other proteins, such as Cox-2 and iNOS, which cause tissue damage and blood vessel dilation in EIU. These inflammatory markers have been shown to be under the transcriptional control of redox-sensitive transcription factor NF- κ B. Several studies have shown that the inhibitors of NF- κ B successfully prevented uveitis in rats. Furthermore, it has recently been shown that inhibition of PKC, which is known to inhibit the activation of NF- κ B, decreases the inflammatory cell migration and protection of ocular tissues during EIU.¹⁰ In our studies, we found that benfotiamine treatment inhibited the activation of PKC and NF- κ B, thereby preventing ocular inflammation and endotoxin-induced uveitis in rats. Our results are in concert with a recent study that implicates benfotiamine in blocking the activation of DAG, PKC, and NF- κ B in diabetic rats.³⁰ Although the exact mechanism regarding how benfotiamine might block the activation of these signaling molecules is not clear, studies suggest that during infection-induced oxidative stress, cell metabolism becomes hyperactivated and results in excessive use of glucose,⁵² which may lead to excessive ROS production that in turn could activate the signaling cascade that causes inflammation. Benfotiamine has been shown to regulate glucose metabolism by activating glycolytic enzyme transketolase, which diverts excess sugar to the pentose phosphate pathway, thereby inhibiting the overuse of glucose and the resultant excessive ROS production.⁵³ This antioxidant property of benfotiamine could prevent the activation of PKC and NF- κ B,³⁰ which in turn could block inflammation. Thus,

by preventing the activation of NF- κ B signals, benfotiamine could inhibit the synthesis and secretion of oxidative stress-induced inflammatory markers, which are responsible for increased ocular inflammation. In summary, our results suggest that the lipophilic vitamin B1 analogue benfotiamine prevents endotoxin-induced uveitis in rats and suggests the use of vitamin B1 supplementation as a novel therapeutic approach for preventing ocular inflammation.

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Figure 1.

Benfotiamine prevents LPS-induced clinical symptoms of EIU in rats. **(A)** Chemical structure of benfotiamine. **(B)** The pathologic score of EIU in Lewis rat eyes injected with LPS in the absence and presence of benfotiamine was determined at 3 and 24 hours with a slit lamp microscope. Results are given as mean $\pm$ SD ($n = 6$). # $P < 0.0008$ versus control (C). ** $P < 0.005$ versus EIU (Wilcoxon-Mann-Whitney test).

Figure 2.

Benfotiamine prevents EIU-induced inflammatory cell infiltration and protein concentration in AqH. **(A)** Histopathologic changes in the anterior chamber of EIU rat eyes in the absence and presence of benfotiamine. Serial sections of paraformaldehyde-fixed rat eyes were stained with hematoxylin and eosin and were observed under a light microscope. Magnification, 200 $\times$. **(B)** The inflammatory cells and **(C)** total protein concentration in the AqH were measured 24 hours after LPS injection by using trypan-blue exclusion cell counting and Bradford methods, respectively. Results are given as mean $\pm$ SD ($n = 6$). $\#P < 0.001$ versus control (C). $**P < 0.001$ versus EIU.

Figure 3.

Benfotiamine prevents EIU-induced inflammatory cytokines and chemokines in AqH. The AqH from EIU rats was used to measure secreted cytokines and chemokines by an antibody array system. Presented here are the percentage control values for individual cytokines, taking control as 100% ($n = 4$) after densitometry analysis. [#] $P < 0.001$ versus control (C). ^{*} $P < 0.001$ versus EIU. BEN, benfotiamine; EIU, endotoxin-induced uveitis.

Figure 4.

Benfotiamine prevents the expression of Cox-2 and iNOS and the activation of NF- κ B and PKC in EIU. (**AI, AII**) Serial sections of paraformaldehyde-fixed EIU rat eyes (24 hours) were immunostained with antibodies against (**AI**) Cox-2 and (**AII**) iNOS and were observed under a light microscope. (**BI, BII**) Serial sections of EIU rat eyes (3 hours) were immunostained with antibodies against phospho-p65 (Ser536). (**CI, CII**) Phospho-PKC β II (Ser660) followed by FITC-labeled secondary antibodies and mounted with fluorescence mounting medium with DAPI. Antibody staining intensity was observed under a fluorescence microscope for FITC and DAPI. A representative microphotograph is shown ($n = 4$). Magnification, 200 $\times$. I, iris; CB, ciliary body; R, retina; C, control; EIU, endotoxin-induced uveitis; Ben, benfotiamine.