Inhibitory Effects of Pyrrolidine Dithiocarbamate on Endotoxin-Induced Uveitis in Lewis Rats

Kouichi Obta,1 Kohzo Nakayama,2 Toru Kurokawa,1 Takanobu Kikuchi,1 and Nagabisa Yoshimura1

PURPOSE. To determine the effect of pyrrolidine dithiocarbamate (PDTC), an antioxidant nuclear factor (NF)-κB inhibitor, on the ocular inflammation induced by lipopolysaccharide (LPS).

METHODS. Endotoxin-induced uveitis (EIU) was produced by a footpad injection of 200 μg LPS in male Lewis rats. PDTC (200 mg/kg) was injected intraperitoneally 30 minutes before the LPS administration. The number of infiltrating cells and protein concentration in the aqueous humor (AqH) was determined from the AqH collected at 24 hours. Immunohistochemical staining with a monoclonal antibody against activated NF-κB was performed to evaluate the effect of PDTC on NF-κB activation. Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNA expression in the iris-ciliary body (ICB) were determined by RNase protection assay (RPA). The levels of these cytokines and nitric oxide (NO) production were also determined.

RESULTS. The number of cells in the AqH was 1100 ± 254 cells/μL in rats injected with LPS and 90 ± 43 cells/μL in rats pretreated with PDTC (P < 0.001). The concentration of proteins was significantly lower in the AqH of rats pretreated with PDTC than in those without PDTC. The number of activated NF-κB-positive cells in the ICB was reduced by the PDTC treatment. The ICB at 6 hours after LPS injection exhibited increased expression of IL-1β, IL-6, and TNF-α mRNAs, which was decreased after PDTC pretreatment. PDTC also significantly diminished the levels of these cytokines and nitrite-nitrate in the AqH.

CONCLUSIONS. These results suggest that PDTC reduces ocular inflammation in eyes with EIU by downregulating proinflammatory cytokine expression and by inhibiting the NF-κB-dependent signaling pathway. (Invest Ophthalmol Vis Sci. 2002; 43:744–750)

Endotoxin-induced uveitis (EIU) is an animal model for acute uveitis in humans.1 This inflammatory response is characterized by leakage of proteins into the anterior chamber (AC) and subsides within 48 hours.2,3 These results suggest that PDTC reduces ocular inflammation in eyes with EIU by downregulating proinflammatory cytokine expression and by inhibiting the NF-κB-dependent signaling pathway. (Invest Ophthalmol Vis Sci. 2002; 43:744–750)

The exact mechanism producing the EIU has yet to be determined, although cytokines seem to play important roles.2-9 Cytokines are signaling proteins released by immune cells, endothelial cells, and other resident cells that serve as important mediators. Tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 are likely to be involved in EIU.2-9 Nitric oxide (NO)10-12 and adhesion molecules, such as E- and P-selectin,13,14 are also involved in the pathogenesis of EIU.

As with proinflammatory cytokines and many stimuli that trigger cellular stress, LPS-triggered signaling results in the activation of nuclear factor (NF)-κB, which couples signal transduction to the expression of LPS-dependent genes.14,15 A wide array of mediators related to inflammation, including TNF-α, IL-1, IL-6, and 8,16 inducible nitric oxide synthase (iNOS); endothelial adhesion molecules; the intercellular adhesion molecule (ICAM)-1; and the vascular cell adhesion molecule (VCAM)-1, require NF-κB activation for their expression in response to LPS.14,16 In addition, some of the mediators involved in septic shock, such as TNF-α and IL-1 which are activated through NF-κB, also activate NF-κB, thus promoting their own secretion and generating a positive loop that amplifies the cytokine cascade and the inflammatory response.14,16 Reactive oxygen intermediates (ROIs) have been proposed to mediate the NF-κB activation induced by a variety of proinflammatory stimuli, including LPS, TNF-α, and IL-1. In fact, essentially all NF-κB activators induce generation of ROIs, and direct treatment of cells with different exogenous pro-oxidants activates NF-κB.19

However, the use of exogenous antioxidants with radical scavenging properties has also suggested a role for ROIs in NF-κB activation. Different antioxidants, including pyrrolidine dithiocarbamate (PDTC), the glutathione precursor N-acetyl-cysteine (NAC), and the antioxidant enzyme thioredoxin have thus been reported to inhibit NF-κB in several cell systems.14,15,20 In addition, PDTC also protects animals from septic shock in vivo.21-23

The purpose of this study was twofold: first, to investigate the time course of the expression of NF-κB in the iris-ciliary body (ICB) of rats with ocular inflammation, and second, to examine the effect of PDTC on uveitis in rats. We show that PDTC inhibited the infiltration of cells and extravasation of proteins into the AC and also inhibited the local release of proinflammatory cytokines produced in the ICB. Our results also show that the in vivo suppression of NF-κB in the ICB was attenuated by the inflammation.

MATERIALS AND METHODS

Animals

Male Lewis rats (6–8 weeks old, 150–200 g) were obtained from a local animal supplier (Japan SLC, Hamamatsu, Japan). All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of EIU and PDTC Treatment

The rats were injected intraperitoneally with 50, 100, or 200 mg/kg PDTC in 1 mL pyrogen-free saline.22 Thirty minutes later, they were injected in one footpad with 200 μg LPS (Salmonella minnesota; Sigma, St. Louis, MO) in 0.1 mL sterile pyrogen-free saline.2

From the Departments of 1Ophthalmology and 2Anatomy, Shinshu University School of Medicine, Matsumoto, Japan.

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Corresponding author: Kouichi Obta, Department of Ophthalmology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; kohta@hsp.md.shinshu-u.ac.jp.

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Quantification of Infiltrating Cells and Protein Concentration in AqH

Aqueous humor (AqH) was collected from eyes using a 30-gauge needle and 20-μL micropipettes (Fisher Scientific, Pittsburgh, PA) by capillary attraction immediately after killing the animals 24 hours after LPS injection.23,24 The AqH was pooled in siliconized microcentrifuge tubes (Fisher Scientific), and before centrifugation, 2 μL AqH from one rat was stained with 0.4% trypan-blue solution, and the number of leukocytes was counted under phase-contrast microscopy. The total protein concentration in the AqH samples was measured using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL) and was expressed relative to a bovine albumin standard.

Histopathologic Evaluation

At 3, 6, and 24 hours after LPS injection, rats were deeply anesthetized by pentobarbital sodium (30 mg/kg), and the eyes were fixed by an intracardiac perfusion of 4% paraformaldehyde in 0.2 M phosphate buffer. The eyes were enucleated and after immersion in the same fixative for 12 hours, the eyes were dehydrated and embedded in paraffin. Then, 5-μm sagittal sections were cut near the optic nerve head and stained with hematoxylin and eosin (H-E).

Immunohistochemical Studies for NF-κB

Sections were cut from the same paraffin blocks followed by H-E staining. The sections were then treated with 3% hydrogen peroxide to block intrinsic peroxidase activities and incubated with 2% normal goat serum for 30 minutes. After they were rinsed, the sections were incubated overnight with a monoclonal antibody against the p65 subunit of activated NF-κB (Chemicon, Temecula, CA; 5 μg/mL) at 4 °C. This subunit contains the epitope that is available for binding only after IκB dissociation. Secondary anti-mouse antibody conjugated to fluorescein isothiocyanate (1:250, FITC; Dako, Glostrup, Denmark) was applied according to the manufacturer’s instructions. The working concentrations of antibodies were determined after applying various concentrations. The secondary antibody was applied for 60 minutes at room temperature in PBS containing 1% bovine serum albumin. To identify the NF-κB-positive nuclei, the specimens were stained with propidium iodide (PI; 20 μg/mL) for 10 minutes at room temperature. The sections stained without the primary antibody were used as a negative control.

Quantitative Analysis

The ICB was viewed and photographed with a scanning laser confocal microscope (model LSM 410; Zeiss, Oberkochen, Germany) using a green filter to detect FITC and a red filter for PI. The numbers of FITC-labeled NF-κB-positive cells and PI-stained cells were counted in six areas (90 × 90 μm). The cell counts were made by two masked examiners. Data are expressed as the number of cells per area at each time point, and the results are expressed as mean ± SEM.

Collection of the ICB

Eyes were enucleated from the rats at 6 hours after LPS injection. The eyes were cut into two pieces along the equator, and the posterior segment including the lens was discarded. The ICB was collected with fine forceps, and the RNA in the isolated ICB was extracted immediately.

RNA Preparation and RNase Protection Assay

To evaluate the inhibitory effect of PDTC on ocular tissues, total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method.25 ICBs were dissected, homogenized, and centrifuged to remove cellular debris. The RNA pellet obtained from four eyes was resuspended in nuclease-free water and processed together as a group. Detection and quantification of rat cytokine mRNAs were accomplished with a multiprobe RPA system (BD PharMingen; San Diego, CA) as recommended by the supplier. Briefly, a mixture of [32P]UTP-labeled antisense riboprobes was generated from a template set (rCK-1 Multi-Probe Template Set; BD PharMingen). This set contains anti-sense RNA probes that can hybridize with target rat mRNAs encoding IL-1α, -1β, -2, -3, -4, -5, -6, and -10 and TNF-β, TNF-α, and IFN-γ. In addition, two housekeeping gene products, L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were present. Four micrograms total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the 32P anti-sense riboprobe mixture. Nuclease-protected RNA fragments were purified by ethanol precipitation. After purification, the samples were resolved on 5% polyacrylamide sequencing gels. The gels were dried and subjected to autoradiography. Protected bands were observed after exposure of gels to x-ray film. Specific bands were identified by their individual migration pattern in comparison with the undigested probe, and the bands were analyzed on a bioimage analyzer (BAS-1500; Fuji, Tokyo, Japan).

Quantification of Cytokine Levels in AqH

The levels of IL-1β, IL-6, and TNF-α in the stored AqH obtained from rats with EIU were assayed with a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The AqH from the two eyes of a rat was diluted up to 50 μL and used for one assay. The data from four animals contributed to each time point. The ELISA assay was repeated once or twice. The lower detected level of each cytokine was 31.2 pg/mL.

Determination of NO Levels in AqH

NO synthesis was determined by the nitrite release using a spectrophotometric assay based on the Griess reaction. The total level of nitrate plus nitrite in the AqH was measured by using a nitrite-nitrate colorimetric assay kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s protocol. To measure the total products of nitric oxide, the conversion of nitrate to nitrite by nitrate reductase was required.

Statistical Analysis

Results are expressed as mean ± SEM and were analyzed statistically by using the one-way analysis of variance followed by the Scheffé post hoc test. P < 0.05 was considered to be statistically significant.

Results

Effect of PDTC on Cellular Infiltration and Protein Concentration in AqH

After conducting a preliminary dose-ranging study (data not shown), we used 200 mg/kg PDTC for the experiments. Leukocytes, mainly PMNs, were found in the AC of rats with EIU at 24 hours after LPS administration (Fig. 1B). PDTC-treated eyes showed a lower number of cellular infiltration (Fig. 1C); thus the number of infiltrating cells was 1100 ± 254 cells/μL (n = 12) in the untreated rats, and pretreatment with 200 mg/kg PDTC resulted in a significant reduction of the cell number to 90 ± 43 cells/μL (P < 0.001; n = 12, Fig. 2A). In contrast, no infiltrating cell was detected in AqH from either control or PDTC-treated rats without LPS (n = 8 and 4, respectively).

The protein concentration in AqH was 11.1 ± 0.9 mg/mL in rats 24 hours after LPS injection (n = 9), and pretreatment with PDTC reduced the protein concentration to 3.5 ± 0.6 mg/mL (P < 0.0001; n = 9, Fig. 2B). AqH from the normal control rats and from rats treated with only PDTC showed lower levels of PDTC (0.9 ± 0.1 mg/mL, n = 7; and 1.0 ± 0.7 mg/mL, n = 4, respectively).
PDTC Prevents the Activation of NF-κB in ICB of Eyes with EIU

Activated NF-κB-like immunoreactivity was studied in paraffin-embedded sections. To obtain information on the distribution of NF-κB activation, we used an antibody that recognized the p65 subunit epitope of NF-κB. Because this epitope is exposed only after degradation of the inhibitory protein I-κB, this antibody recognizes activated p65. Immunohistochemical examination for activated NF-κB demonstrated partial activation in ICB cells at 3 hours after LPS injection (Fig. 3A). More widespread activation of NF-κB was present at 6 hours after LPS injection (Fig. 3B). This activation in the ICB was not present at 24 hours, however (Fig. 5C). In contrast, the activation of NF-κB was suppressed by pretreatment with PDTC (Figs. 3D–F).

To obtain a quantitative measure of NF-κB activity in the ICB, the active NF-κB-positive cells were counted (n = 3). Normal control showed only background (1.1% ± 0.7%) activity. LPS injection resulted in a marked increase in the percent of active NF-κB-positive cells in ICB at 3 and 6 hours (by 43.4% ± 5.5% and 58.9% ± 3.8%, respectively; P < 0.01; Fig. 4). The percentages of activated NF-κB-positive cells were significantly lower in eyes of rats treated with PDTC (10.1% ± 1.5% at 3 hours, and 11.8% ± 1.2% at 6 hours; P < 0.001).

Suppression of mRNA Expression in ICB by PDTC

NF-κB is activated by LPS in different cell types and plays a key role in regulating the inducible gene expression of a number of proinflammatory mediators. We therefore studied the possible effect of PDTC on the LPS induction of these cytokines in ocular tissues. The effect of PDTC pretreatment on the expression of different cytokines implicated in ocular inflammation was investigated by an RNase protection assay (RPA), as described.23,24 Total RNA was extracted from ICB tissues obtained from eyes with EIU at 6 hours after LPS injection and an autoradiograph of a gel that covered the molecular size ranges of IL-1α, -1β, -2, -3, -4, -6, and -10 and TNF-β, TNF-α, IFN-γ mRNA transcripts and of the housekeeping genes L32 and GAPDH are shown in Figure 5A.23,25,26 Whereas tissues removed from normal eyes contained no remarkable inflammatory transcripts except the housekeeping genes, IL-6 mRNA was highly expressed in the ICB, and IL-1β and TNF-α mRNAs were also detected. A quantitative densitometric analysis of this gel is displayed in Figure 5B (n = 3). Compared with levels of L32 mRNA, the IL-1β, IL-6, and TNF-α mRNA levels in EIU were higher than in normal eyes. Treatment with PDTC reduced these expressions compared with the levels in eyes without PDTC treatment.

Effect of PDTC on IL-1β, IL-6, and TNF-α in AqH of Eyes with EIU

Because IL-1β, IL-6, and TNF-α mRNA levels increased in the ICB, we next studied the levels of these cytokines in the AqH of LPS-treated rats at 24 hours. As expected, upregulated levels of IL-1β, IL-6, and TNF-α were detected (n = 6, 144.5 ± 35.8 pg/mL; n = 6, 448 ± 99 pg/mL; and n = 6, 83.4 ± 32.1 pg/mL,
inhibition of the transcription of these cytokines. No cytokine was detected in AqH of normal eyes and without PDTC pretreatment. **cant statistical difference between the ICBs obtained from eyes with

respectively. No cytokine was detected in AqH of normal eyes

Downregulation of NO Production in AqH by PDTC

To test whether the inhibitory effect of PDTC on EIU is related to an effect on NO production, we measured the nitrite-nitrate levels (breakdown products of NO) in the AqH of rats treated and not treated with PDTC at 24 hours after LPS administration. High levels of nitrite were detected in the AqH of untreated rats (Fig. 7), and in contrast, PDTC inhibited the increase in nitrite levels in the treated animals significantly (n = 4, P < 0.05). The level of nitrite in normal control eyes was below the reliable range (12.5 μM).

Discussion

The activation of the transcription factor NF-κB was assessed by in situ immunohistochemical localization of the DNA-binding subunit p65 in eyes with LPS-induced ocular inflammation as observed in other inflammatory models induced by LPS.27,28 Our results clearly demonstrated that there was a significant upregulation of activated NF-κB in the ICB during the ocular inflammation. This is the first demonstration of p65 expression in eyes with uveitis induced by LPS.

NF-κB regulates a host of inflammatory and immune responses14,15,29 and cellular growth properties,30 by increasing the expression of specific cellular genes. These include genes encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition, such as members of the major histocompatibility complex (MHC), proteins involved in antigen presentation, and receptors required for neutrophil adhesion and migration.29

In EIU, LPS stimulates inflammatory cells to produce different cytokines, such as IL-1β, IL-6, IFN-γ, and TNF-α and chemokines.2–9 We analyzed cytokine gene expression in the ICB of rats with EIU by RPA. A marked upregulation was detected in the expression of IL-1β, IL-6, and TNF-α. Gene expressions of Th-1–associated cytokines, such as IL-2 and IFN-γ and Th-2–associated cytokines, such as IL-4 or -10, were not observed. Our findings that IL-1β, IL-6, and TNF-α mRNA were upregulated after LPS administration are in line with the results of previous in vivo studies.2,5,7,8 Although the contribution of TNF-α and IL-6 to EIU is pleiotropic,4,7,9,31 these cytokines may play important roles in the pathogenesis of EIU. Our data indicate that the expression was closely associated with the mRNA expression of proinflammatory cytokine–related NF-κB activation.

Our results show that PDTC reduced the ocular inflammation, as indicated by reduced cellular infiltration and protein concentration in the AqH. We found that PDTC inhibited the in vivo activation of NF-κB in the ICB of LPS-challenged rats. IL-1β, IL-6, and TNF-α mRNA gene expressions were also in-

inhibitory effect of PDTC on EIU

respectively; Figs. 6A–C). Pretreatment with PDTC significantly reduced the levels of these cytokines in the AqH (n = 6, 4.0 ± 2.7 pg/mL; n = 6, 26 ± 26 pg/mL; and n = 6, 13.1 ± 2.6 pg/mL, respectively; P < 0.01, P < 0.05, and P < 0.05, respectively). No cytokine was detected in AqH of normal eyes and PDTC-treated eyes. These data paralleled those showing an inhibition of the transcription of these cytokines.

Downregulation of NO Production in AqH by PDTC

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FIGURE 3. Photomicrographs of en face preparations of rat ICB after immunostaining with antibodies against NF-κB p65 (green). Dual-immunofluorescence labeling showed the colocalization (yellow) in nuclei (red). Rats were injected with LPS without (A–C) or with (D–F) PDTC pretreatment. Eyes were enucleated 3 hours (A, D), 6 hours (B, E), and 24 hours (C, F) after LPS administration. Increased nuclear and cytoplasmic NF-κB p65 staining was observed in the LPS-injected rats at 3 and 6 hours (A, B, respectively). PDTC pretreatment reduced NF-κB p65 immunoreactivity (D, F). Bars, 50 μm.

FIGURE 4. Quantitative analysis of NF-κB–positive cells in the ICB after LPS injection. Results are mean ± SEM (n = 3). Normal control showed only background (1.1% ± 0.7%) activity. There was a significant statistical difference between the ICBs obtained from eyes with and without PDTC pretreatment. ***P < 0.01, ****P < 0.001 by Scheffe post hoc test.
hibited by intraperitoneal PDTC pretreatment. PDTC-treated rats also showed lower IL-1β, IL-6, and TNF-α levels in the AqH. Our findings that IL-1β and IL-6 mRNAs in the ICB and their

products in the AqH were inhibited by PDTC are interesting, because PDTC interferes with the production of TNF-α, but not IL-1 or -6, in a model of LPS-induced shock.21,25 Our results clearly demonstrated that activation of NF-κB is associated with the ocular expression of IL-1β, IL-6, and TNF-α. The mechanism for the local inflammation in eyes stimulated with LPS may differ from that of the LPS-induced systemic response in shock or sepsis.

FIGURE 5. Effects of PDTC on LPS-induced cytokine gene expression in the ICB. RNA was extracted 6 hours after LPS injection for assessment of proinflammatory cytokine gene expression by an RNase protection assay. (A) Autoradiograph of the RNase protection assay. On the basis of the undigested marker probes’ migration patterns, specific bands were identified for each cytokine: lane M, undigested marker probe; lane 1, ICB from normal rats; lane 2, ICB from rats with LPS injection without PDTC pretreatment; and lane 3, ICB from rats with LPS injection with PDTC pretreatment. (B) Quantification of the results, with a bioimage analyzer. The intensity of each transcript relative to that of the L32 housekeeping gene is presented. This experiment is representative of two separate experiments (n = 3 per group of rats).

FIGURE 6. Effects of PDTC on cytokines in AqH at 24 hours after LPS administration. The levels of IL-1β (A), IL-6 (B), and TNF-α (C) were significantly inhibited by pretreatment with PDTC. Data are the mean ± SEM of two separate experiments (n = 3 rats in each group). No cytokine was detected in AqH from normal and PDTC-treated eyes. Significant difference: *P < 0.05 and **P < 0.01, by ANOVA.
Glucocorticoids, such as dexamethasone and prednisolone, are widely used for their anti-inflammatory and immunosuppressive properties. One possible mechanism underlying this activity is the inhibition of NF-κB activation. This inhibition is mediated by the induction of the iB inhibitor protein. Non-steroidal anti-inflammatory drugs, such as salicylate and aspirin, also inhibit NF-κB-dependent transcription. Yamamoto and Gaynor have reviewed a variety of therapeutic agents intended to block NF-κB activity. A number of antioxidants, including PDTC, efficiently inhibit NF-κB activation induced by LPS in a number of cell systems. These effects appear to be mediated by the powerful radical scavenger properties of this antioxidant, which apparently counteract the ROI signals generated by NF-κB activation.

Although the precise primary targets involved in the inhibition of NF-κB by PDTC has not yet been identified, in Hela cells, PDTC has been shown to block IκB phosphorylation, precluding the dissociation of NF-κB from IκB and subsequent NF-κB translocation from the nucleus in response to TNF-α. This suggests that IκB-α proteolysis is controlled by the cell redox status.

PDTC has been reported to prevent NF-κB activation and to reduce iNOS induction in LPS-treated rats. In contrast to these effects of NOS inhibitors, the targeting of NF-κB with agents such as PDTC may be a more effective strategy in the treatment of septic shock, because inhibition of NF-κB activation selectively prevented the increase in iNOS activity and iNOS-mediated NO production. TNF-α, IFN-γ, and IL-1β were also effective inducers of iNOS expression and were synergistic with LPS in mediating iNOS expression.

Our results show the in vivo relationship between NF-κB activation and the expression of inflammatory genes, the in vivo selectivity of PDTC in inhibiting NF-κB activation, and the effects on the inflammatory genes in the ICB and their products in AqH of inhibiting NF-κB activation by use of PDTC. We studied the expression of IL-1β, IL-6, and TNF-α because of their critical roles in the endotoxin-induced ocular inflammatory response.

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