Suppressive effects of astaxanthin against rat endotoxin-induced uveitis by inhibiting the NF-κB signaling pathway.

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Abstract

We investigated the effects of astxanthin (AST), a carotenoid, on endotoxin-induced uveitis (EIU), and measured the expression of inflammatory cytokines and chemokines in the presence or absence of AST over the course of the disease. EIU was induced in male Lewis rats by footpad injection of lipopolysaccharide (LPS). Immediately after the LPS inoculation, either 1, 10, or 100 mg/kg of AST were injected intravenously. Aqueous humor was collected at 6, 12 and 24 hours after LPS inoculation and the number of infiltrating cells in the anterior chamber were counted. In addition, we assayed the concentration of protein, nitric oxide (NO), tumor necrosis factor (TNF)-α and prostaglandin (PG) E2. Immunohistochemical staining with a monoclonal antibody against activated NF-κB was performed in order to evaluate the effects of AST on NF-κB activation. Rats injected with AST showed a significant decrease in the number of infiltrating cells in anterior chamber. Moreover, AST-treated rats with EIU showed significantly lower concentrations of protein, NO, TNF-α and PGE2 in the aqueous humor. Even the early stages of EIU were suppressed by injection of AST. The number of activated NF-κB-positive cells was lower in iris-ciliary bodies treated with 10 or 100

mg/kg AST at 3 hours after LPS injection. These results suggest that AST reduces ocular inflammation in eyes with EIU by downregulating proinflammatory factors and by inhibiting the NF-κB-dependent signaling pathway.

Introduction

Nuclear factor (NF)-κB is a transcription factor composed of heterodimers or homodimers of rel family proteins, such as Rel A (p65), RelB, cRel, p50 and p52 (Baeuerle and Baltimore, 1996). NF-κB is sequestered in the cytoplasm and is bound to inhibitory κB (IκB) proteins, such as IκBa, IκBb, IκBc, p105 and p100 (Beg et al., 1992).

Lipopolysaccharide (LPS), a major component of the outer membranes of Gram-negative bacteria, can trigger a variety of inflammatory reactions by binding to Toll-like receptor 4 (Pugin et al., 1994) (O'Neill and Dinarello, 2000). As with proinflamatory cytokines and stimuli that trigger cellular stress, LPS-triggered signaling results in the activation of NF-κB, which couples signal transduction to the expression of LPS-dependent genes (Baeuerle and Henkel, 1994) (Baldwin, 1996). Exposure to

outer bacterial toxins such as LPS stimulates cellular inflammatory responses, and releases some factors such as nitric oxide (NO) (Chen et al., 2001) (Boujedaini et al., 2001), prostaglandin E2 (PGE2) (Bellot et al., 1996) (Murakami et al., 2000), cytokines including tumor necrosis factor-α (TNF-α) (Tracey and Cerami, 1994), and eicosanoid mediators, which promote inflammatory responses. In particular, increased plasma TNF-α levels during endotoxemia and gram negative sepsis contributes to lethality as suggested by the protective effects afforded by TNF-α neutralizing antibodies(Tracey et al., 1987). 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 (Baeuerle and Henkel, 1994) (Baldwin, 1996) (Barnes and Karin, 1997) (May and Ghosh, 1998) (Collins et al., 1995). Reactive oxygen intermediates (ROIs) have also been proposed to mediate NF-κB activation induced by a variety of proinflammatory stimuli, including LPS, TNF-α and IL-1. Essentially all NF-κB activators induce generation of ROIs, and direct treatment of cells with exogenous pro-oxidants activates NF-κB (Schulze-Osthoff et al., 1995).

In the Lewis rat model of endotoxin-induced uveitis (EIU), the inflammation is more severe in the anterior segment than in the posterior segment of the eye (Herbort et al., 1990). Cellular inflammation in EIU starts 4 hours after injection of LPS, with maximum infiltration after 18-24 hours (Yang et al., 1996). During EIU, the infiltrating inflammatory cells produce various cytokines and chemokines. The cytokines IL-1β, TNF-α and IL-6, along with the chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-2, are considered to be particularly essential in the pathogenesis of EIU (de Vos et al., 1994b) (de Vos et al., 1994a) (Yoshida et al., 1994) (Mo et al., 1999). Additionally, one of the first responses in the eye to LPS injection is the generation of nitric oxide by iNOS (Goureau et al., 1995) (McMenamin and Crewe, 1997).

Antioxidants such as α-tocopheryl succinate (Neuzil et al., 2001) and probucol (Dichtl et al., 1999) inhibit NF-κB activity and block the expression of pro-inflammatory genes as well as production of NO and PGE2 (Pahan et al., 1998). Astaxanthin (AST) is a non-pro-vitamin A carotenoid that is abundant in fruits and vegetables; it is also present in marine animals. AST and AST-like products are

commonly indicated as antioxidants (Kurashige et al., 1990) and immune modulators (Bennedsen et al., 1999). One effect of AST is to scavenge reactive oxygen species (ROS) (Mortensen et al., 1997). We previously reported that AST showed a dose-dependent anti-inflammatory effect (Ohgami et al., 2003). Lee et al. reported astaxanthin inhibited the production of inflammatory mediators by blocking NF-κB activation in vitro (Lee et al., 2003). However, no study has proven that AST suppresses NF-κB in vivo. Our previously work only examined the presence of proinflammatory cytokines in aqueous humor at 24 hours after the induction of EIU in rats (Ohgami et al., 2003). In order to clinically utilize the antiinflammatory activity of AST, it is necessary to clarify which stage of inflammation AST targets. Therefore, we investigated the effects of AST on the chronological changes in EIU. Furthermore, we investigated the effects of AST treatment on the expression of NF-κB in the iris-ciliary body (ICB) with EIU.

Materials and Methods

Animals

Animal groups and EIU

Eight-week-old male Lewis rats were used. The rats weighed 180-220 g. EIU was induced by footpad injection of 200 μg of LPS (100 μg in each footpad) from *Salmonella typhimurium* (Sigma, St. Louis, MO, USA). LPS was diluted in 0.1 ml of phosphate buffered saline (pH 7.4, PBS).

Rats were injected intravenously with 1, 10 or 100 mg/kg of AST diluted in 0.1 ml of PBS containing 0.1% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). Intravenous injections were administered immediately after the LPS stimulations. For the LPS and control groups, we used the same schedule as for the AST-treated groups, with PBS containing 0.1% DMSO administered intravenously.

Animals were handled and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Aqueous humor samples

Rats were euthanized and the aqueous humor was collected immediately from both eyes by anterior chamber puncture using a 30-gauge needle under a surgical

microscope at 6, 12 and 24 hours after LPS injection.

Anterior chamber cell numbers and protein concentration

For cell counting, the aqueous humor sample was suspended in an equal amount of Türk stain solution, and cells were counted using a hemocytometer under a light microscope. The number of cells per field (equivalent of 0.1 ml) was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample.

A BCA protein assay reagent kit (Pierce, Rockford, IL, USA) was used to determine the total protein concentration in the aqueous humor. Aqueous humor samples were stored in ice water until testing, and cell counts and total protein concentrations were measured on the day of sample collection.

Determination of nitrite concentration in the aqueous humor

NO was measured as its end product, nitrite, using Griess reagent, as described elsewhere (Sigma, St. Louis, MO, USA) (Sewer et al., 1998). The culture supernatant

(100 μl) was mixed with 100 μl of Griess reagent for 10 min, and the absorbance at 550 nm was measured using a microplate reader. The concentration of nitrite in the samples was determined from a sodium nitrite standard curve. Data represent the mean of 8 determinations ± SD.

Levels of TNF-α and PGE2 in the aqueous humor

ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the levels of TNF-α and PGE2 in the aqueous humor according to the manufacturer's instructions. ELISA was carried out in duplicate.

Immunohistochemical study of NF-κB

In the rat EIU model, NF-κB has been shown to migrate to the nucleus of the ciliary body and iris cells three hours after LPS administration when inflammatory cells are not seen in the anterior chamber. Three hours after LPS injection, rats were anesthetized with pentobarbital sodium (30 mg/kg), and the eyes were fixed by intracardiac perfusion of 4% paraformaldehyde in 0.1 M PBS. The eyes were then

dehydrated and embedded in paraffin. Next, 5 μM sagittal sections were cut near the optic nerve head and stained with hematoxylin and eosin.

For immunohistochemical study of NF-κB, sections were cut from the same paraffin blocks followed by hematoxylin and eosin staining. Slides were rinsed twice in PBS and were incubated with normal goat serum followed by p65 antibody (Santa Cruz, c-20; dilution, 1:200). Binding of primary antisera was localized using Cy-3 conjugated goat anti-rabbit IgG (dilution, 1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Nuclei were then stained with PBS containing YO-PRO-1 (Molecular Probes, Eugene, OR, USA) for 5 minutes. Slides were examined by laser scanning confocal microscopy (MRC-1024; Bio-Rad, Richmond, CA, USA; and LSM 510; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Data values were expressed as means ± SD. Data were analyzed by analysis of variance (ANOVA). The Tukey-Kramer test was used for ad hoc comparison to compare the two treatment groups and p values of less than 0.05 were considered to be

statistically significant.

Results

Number of inflammatory cells and protein concentration in aqueous humor of treated EIU rats

Leukocytes, mainly polymorphonuclear cells, were found in the anterior chamber of the LPS group at 24 hours after LPS inoculation (Fig. 1b). However, with 100 mg/kg of AST, few inflammatory cells were seen in the anterior chamber and ICB (Fig. 1c).

In contrast to untreated rats with EIU, which exhibited increased numbers of inflammatory cells in the aqueous humor, treatment with 10 and 100 mg/kg of AST significantly suppressed the increase in inflammatory cells in the aqueous humor (Fig. 2a). At 24 hours after LPS injection, the LPS group had $132.8 \pm 14.2 \times 10^5$ cells/ml, whereas, treatment with 10 mg/kg AST had $37.7 \pm 5.9 \times 10^5$ cells/ml (Fig. 2a). In the LPS group, the protein concentration in the aqueous humor at 24 hours reached a peak (Fig. 2b). The protein concentration in the AST group tended to decrease in a

dose-dependent fashion at 6, 12 and 24 hours after LPS injection (Fig. 2b).

Levels of NO, TNF-ϙ and PGE2 in the aqueous humor

At 6 and 12 hours after LPS injection, the treatment with 10 or 100 mg/kg of AST significantly reduced NO levels when compared with the LPS group (Fig. 3a). The treatment with 1 mg/kg of AST produced only a mild reduction in NO concentration, and there was no significant difference from levels in the LPS group (Fig. 3a). In the LPS group, the TNF-α concentration in the aqueous humor after 24 hours reached maximum levels (Fig. 3b). Treatment with AST significantly reduced the TNF-α concentration when compared with the LPS group at 6, 12 and 24 hours after LPS injection (Fig. 3b). PGE2 concentration in the LPS group was markedly increased after 12 hours when compared with 6 hours (Fig. 3c). The PGE2 concentration in the AST groups tended to decrease in a dose-dependent fashion at 12 and 24 hours after LPS injection (Fig. 3c).

Suppression NF-κB activation in ICB 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. Active NF-κB-positive cells were increased in ICB at 3 hours after LPS injection (LPS group) (Fig. 4b). This activation in the ICB was not present at 24 hours after LPS inoculation (data not shown). In contrast, the numbers of activated NF-κB-positive cells were lower in ICB treated with 100 mg/kg AST (Fig. 4 c). Normal controls showed only background levels (Fig. 4 a).

To obtain a quantitative measure of NF-κB activity in the ICB, the active NF-κB-positive cells were counted (n=6). In the control group, no active NF-κB-positive cells were detected in the ICB. LPS injection resulted in a marked increase in the percentages of active NF-κB-positive cells in ICB at 3 hours (31.2% $\pm$ 7.8%). In the AST group, the percentages of active NF-κB-positive cells decreased in a dose-dependent fashion (Fig. 5).

Discussion

The present results show that AST reduced ocular inflammation, as indicated by reduced cellular infiltration and protein concentration in the anterior chamber. There was also suppression of proinflammatory cytokine and chemokine concentrations in the aqueous humor of endotoxin injected rats. The EIU rats displayed a progressive increased in protein and NO levels in the aqueous humor, with TNF-α , which reached maximum levels in the aqueous humor 24 hours after endotoxin injection. Because AST treatment suppressed endotoxin-mediated elevation of protein, NO and TNF-α in the aqueous humor, it is reasonable to conclude that AST suppression occurs early in the inflammatory response. This early influence on ocular inflammation must be what prevents the subsequent elevation of TNF-α and PGE2.

Both TNF-α and IL-1β activate and are activated by NF-κB. Thus, gene products that are regulated by NF-κB also cause the activation of NF-κB. NF-κB acts on proinflammatory cytokine genes, including TNF-α and IL-1β, chemokines (chemotactic cytokines that attract inflammatory cells to sites of inflammation), enzymes that generate mediators of inflammation, immune receptors, and adhesion

molecules that play a key role in the initial recruitment of leukocytes to sites of inflammation. The activation of NF-κB therefore leads to a coordinated increase in the expression of many genes whose products mediate the inflammatory loop and perpetuate local inflammatory responses. Our results show that AST reduced the ocular inflammation, as indicated by reduced cellular infiltration and protein concentration in the aqueous humor. We found that AST inhibited the in vivo activation of NF-κB in the ICB in EIU. Our study was the first to demonstrate that AST suppresses NF-κB in vivo.

Antioxidant enzymes, such as superoxide dismutase (Moreira et al., 2004) and catalase (Clark and Valente, 2004), and pharmacologic antioxidants like N-acetyl cysteine (Shao et al., 2004) (Woo et al., 2004), inhibit NF-κB-dependent gene expression, including expression of TNF-α (Sanlioglu et al., 2001). Therefore, reactive oxygen species-induced oxidative stress may play an important role in NF-κB activation and pro-inflammatory cytokine production in EIU. The anti-inflammatory effects of AST through its suppression of NF-κB activation may be based on its antioxidant activity. A number of anti-oxidants efficiently inhibit NF-κB activation induced by LPS in a number of cell systems (Ohta et al., 2002) (Baeuerle and Henkel, 1994) (Yamamoto

and Gaynor, 2001) (Schreck et al., 1992), especially in vivo (Nemeth et al., 1998) (Liu et al., 1999) (Lauzurica et al., 1999). These effects appear to be mediated by the powerful radical scavenger properties of the antioxidant, which apparently counteract the reactive oxygen intermediates signals generated by NF-κB activation (Schulze-Osthoff et al., 1995) (Schreck et al., 1992) (Munoz et al., 1996).

In summary, this study suggests that AST has a dose-dependent ocular anti-inflammatory effect, by the suppression of NO, PGE2, and TNF-α production, through blocking NF-κB signaling pathway. These results suggest that AST may be a promising agent for the treatment of ocular inflammation.

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Figure Legends

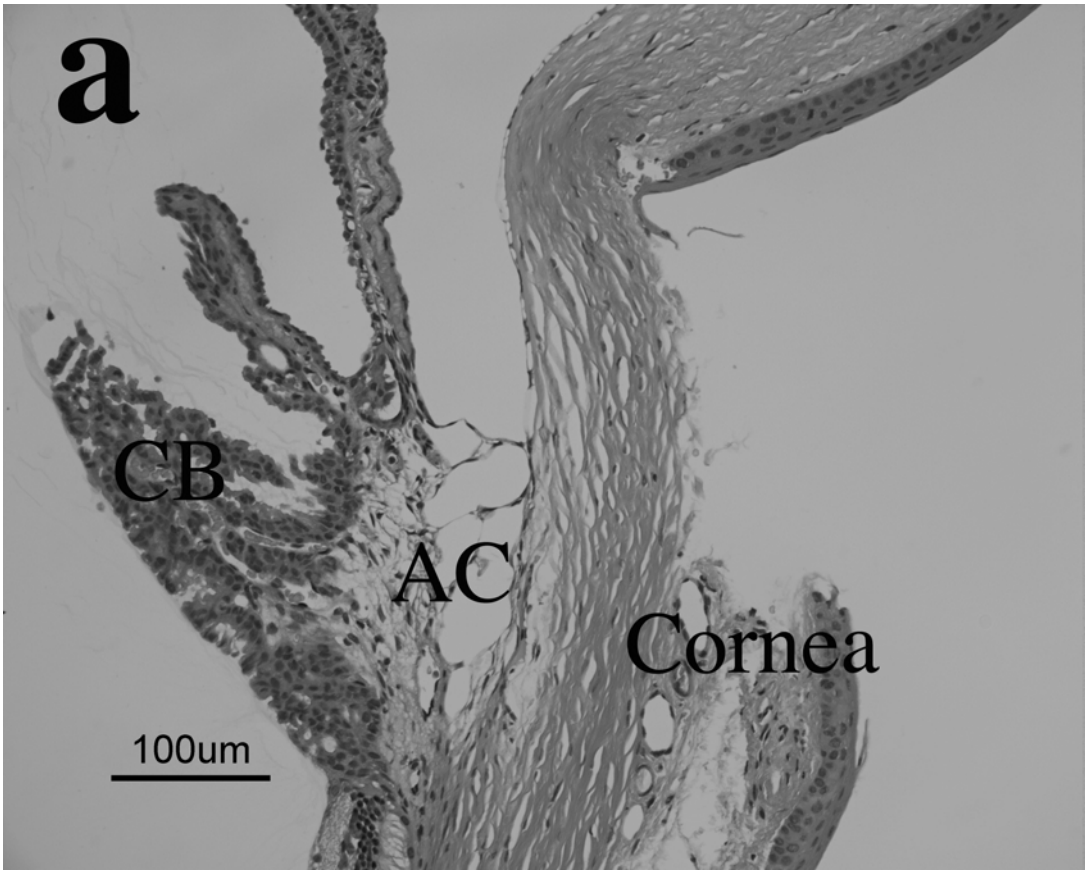
Fig. 1. Histologic changes in the anterior segment of the eye at 24 hours after LPS injection; Control (a), Untreated rats, LPS group (b), Rats treated with 100 mg/kg of AST(c), AC; anterior chamber, CB; ciliary body, HE staining; original magnification, Bars, 100 μm .

Fig. 2. Effect of AST on LPS-induced cell number (a) and protein concentration (b) in aqueous humor. The aqueous humor was collected at 6, 12 and 24 hours after LPS treatment. Each value represents the mean ± SD of 8 rats. Asterisks (*, **) indicate a significant difference from the LPS group at $p < 0.05$ and $p < 0.01$, respectively.

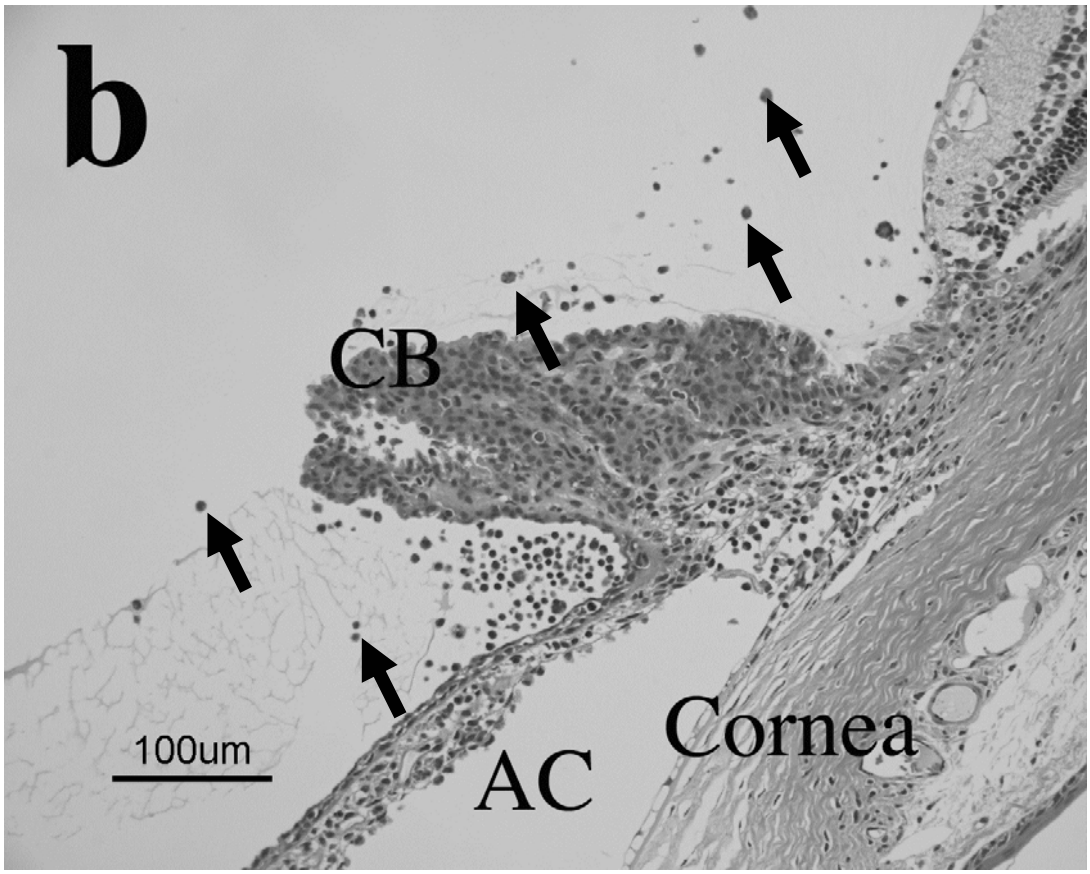
Fig. 3. Effect of AST on LPS-induced NO (a), TNF-α (b) and PGE2 in aqueous humor. The aqueous humor was collected at 6, 12 and 24 hours after LPS treatment and assayed using ELISA. Each value represents the mean ± SD of 8 rats. Asterisks (*, **) indicate a significant difference from the LPS group at $p < 0.05$ and $p < 0.01$, respectively.

Fig. 4. Photomicrographs of enface preparations of rat ICB immunostained with antibodies against NΦ−κB p65 (red). Dual-immunofluorescence labeling showed colocalization (red) in nuclei (green). Rats were injected with LPS without (b) or with 100 mg/kg (c) of AST. Eyes were enucleated 3 hours after LPS administration. x 200.

Fig. 5. Quantitative analysis of NF-κB-positive cells in the ICB at 3 hours after LPS injection. Each value represents the mean ± SD of 6 rats. Asterisks (**) indicate a significant difference from the LPS group at $p < 0.01$.



[Fig.1a Suzuki et al]



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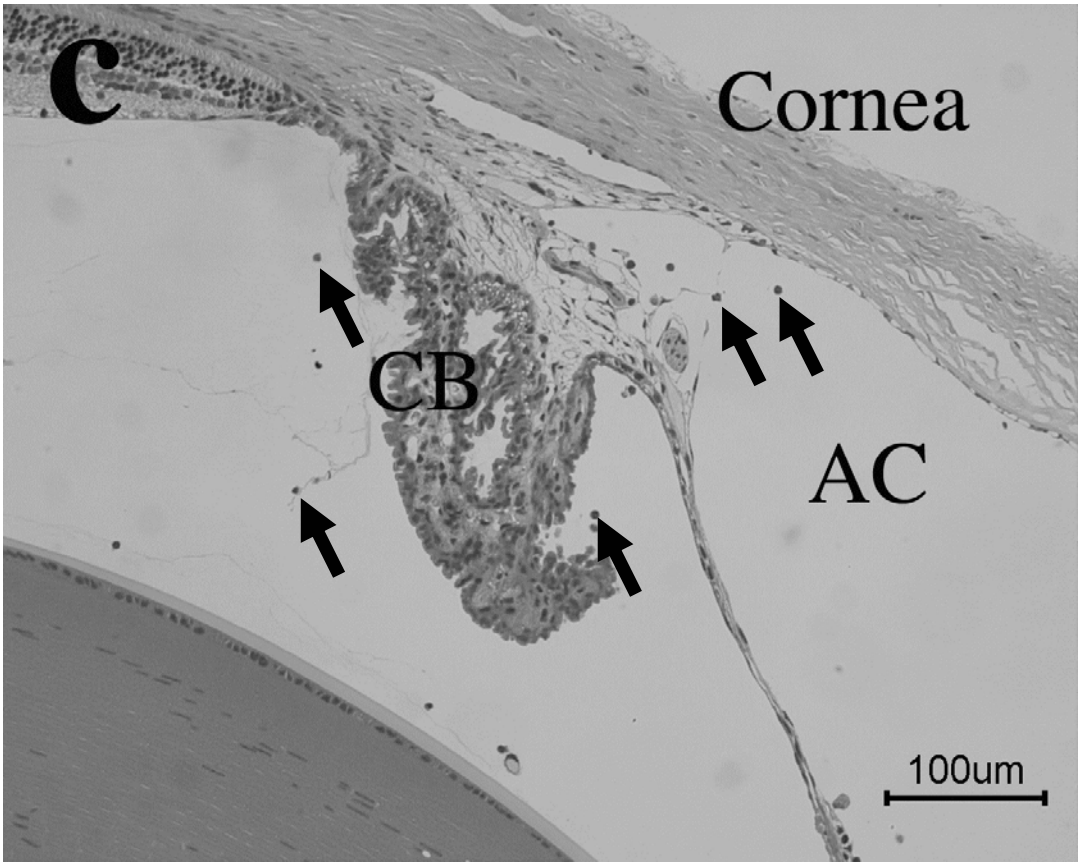
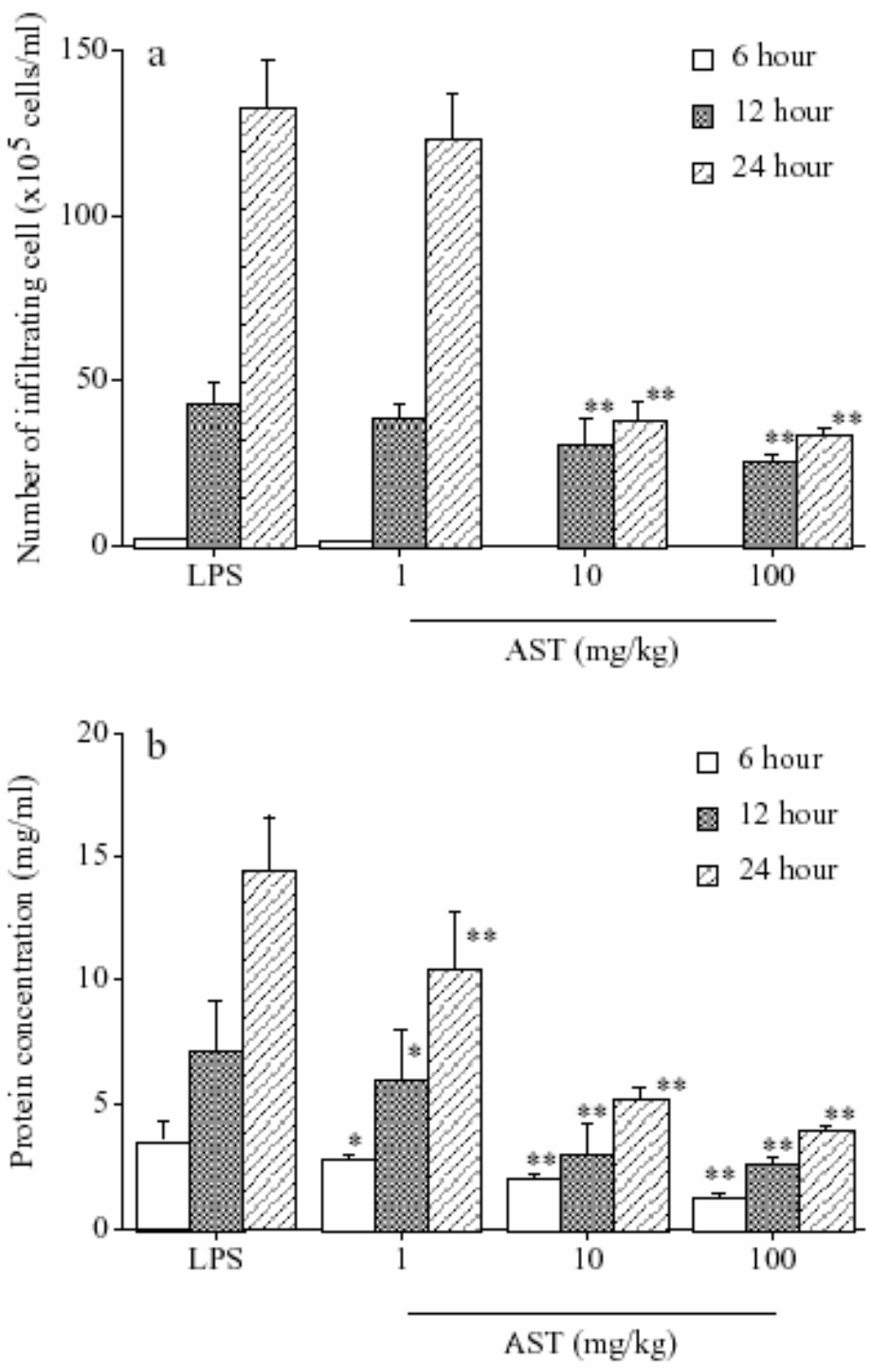
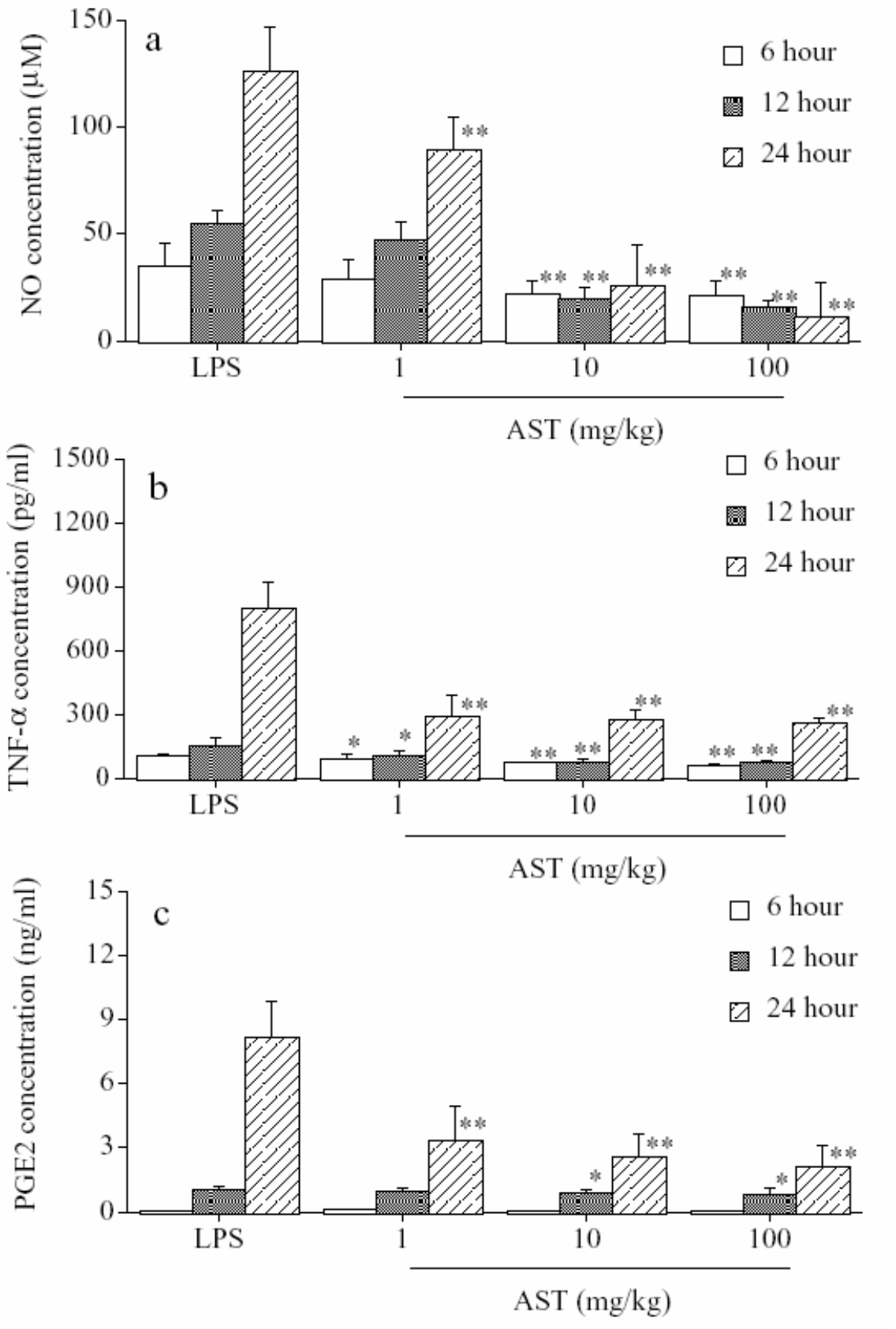


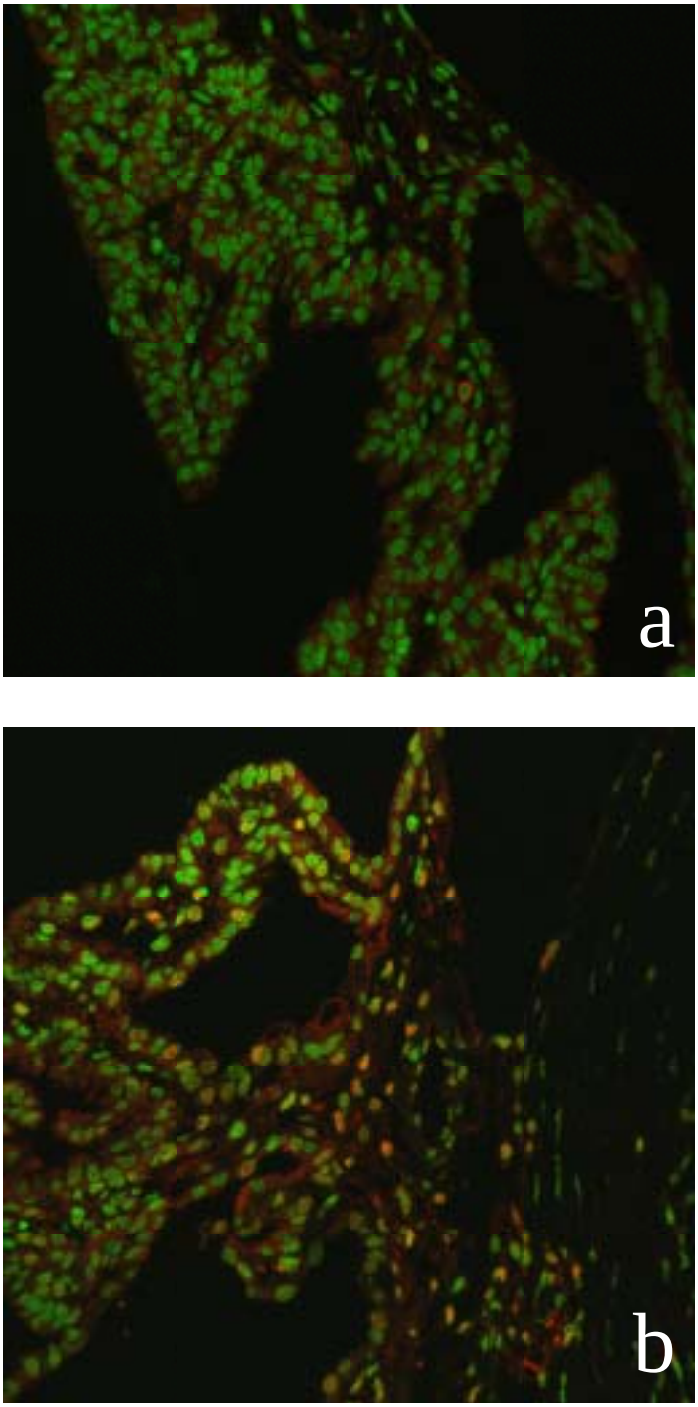
[Fig.1c Suzuki et al]



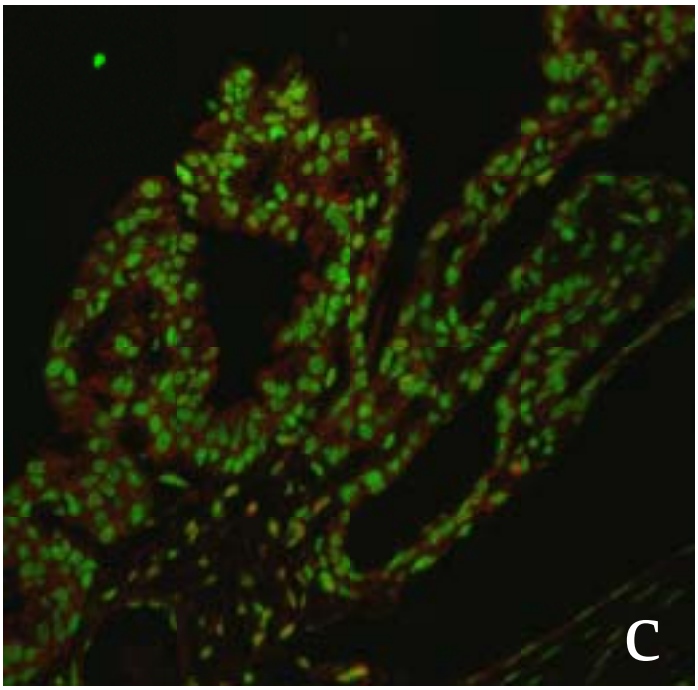
[Fig.2. Suzuki et al]



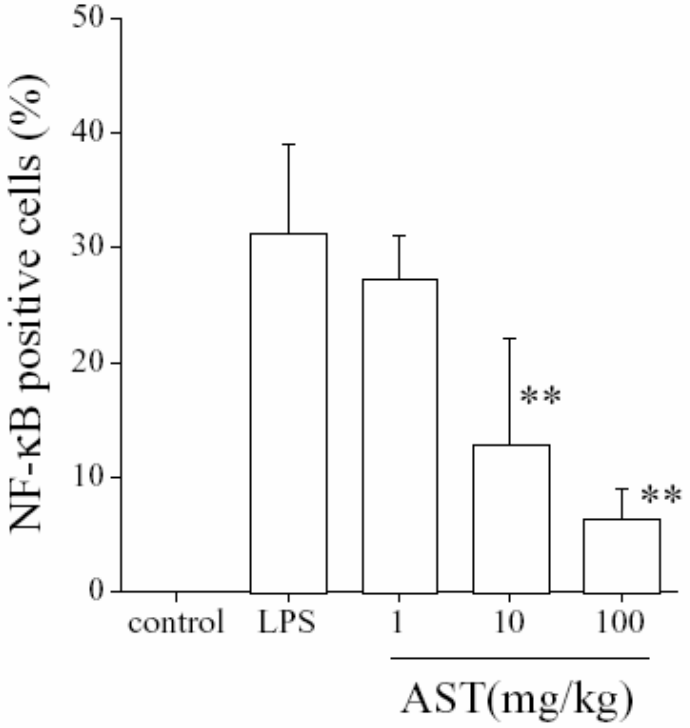
[Fig3. Suzuki et al]



[Fig.4a,b Suzuki et al]



[Fig.4c Suzuki et al]



[Fig.5 Suzuki et al]