Anti-inflammatory Effects of Aronia Extract on Rat Endotoxin-Induced Uveitis


PURPOSE. Aronia crude extract (ACE) with high levels of polyphenol compounds has been reported to have antioxidative effects in vitro and in vivo. In this study, attention was focused on the antioxidative effect of ACE. The purpose of the present study was to investigate the effect of ACE on endotoxin-induced uveitis (EIU) in rats. In addition, the endotoxin-induced expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 proteins was investigated in a mouse macrophage cell line (RAW 264.7) treated with ACE in vitro, to clarify the anti-inflammatory effect.

METHODS. EIU was induced in male Lewis rats by a footpad injection of lipopolysaccharide (LPS). Immediately after the LPS inoculation, 1, 10, or 100 mg ACE or 10 mg prednisolone was injected intravenously. After 24 hours, the aqueous humor was collected from both eyes, and the number of infiltrating cells, protein concentration, nitric oxide (NO), prostaglandin (PG)-E2, and TNF-α levels in the aqueous humor were determined. RAW 264.7 cells treated with various concentrations of ACE were incubated with 10 μg/mL LPS for 24 hours. Levels of NO, PGE2, and TNF-α were determined by an enzyme-linked immunosorbent assay. The expression of iNOS and COX-2 proteins was analyzed by Western blot analysis.

RESULTS. The number of inflammatory cells, the protein concentrations, and the levels of NO, PGE2, and TNF-α in the aqueous humor in the groups treated with ACE were significantly decreased in a dose-dependent manner. In addition, the anti-inflammatory effect of 100 mg ACE was as strong as that of 10 mg prednisolone. The anti-inflammatory action of ACE was stronger than that of either quercetin or anthocyanin administered alone. ACE also suppressed LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells in vitro in a dose-dependent manner.

CONCLUSIONS. The results suggest that ACE has a dose-dependent anti-ocular inflammatory effect that is due to the direct blocking of the expression of the iNOS and COX-2 enzymes and leads to the suppression of the production of NO, PGE2, and TNF-α. (Invest Ophthalmol Vis Sci. 2005;46:275–281) DOI:10.1167/iovs.04-0715

Berries are rich in phenolic compounds as well as many essential nutritional components, such as flavonoids and phenolic acids, which have a wide range of biological properties, including antioxidant and anticarcinogenic properties. Epidemiologic evidence suggests that high consumption of flavonoids may provide protection against coronary heart disease, stroke, and lung cancer. The high level of scavenging activity of berry extracts toward chemically generated reactive oxygen species has been described in several studies. Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry, because they retard oxidation degradation of lipids and thereby improve the quality and nutritional value of food. It has been reported that the fruits and leaves of berries such as the blackberry, raspberry, and strawberry contain potent scavengers of chemically generated reactive oxygen species.

The fruit of the aronia (Aronia melanocarpa), a shrub of the rosaceous family native to North America and Russia, has a dark purple peel and contains high levels of polyphenol compounds. Recently, it has been reported that aronia crude extract (ACE) has potent antioxidative effects in vitro and in vivo. However, there have been no reports on the effects of ACE on lipopolysaccharide (LPS)-induced inflammation in vivo or in vitro.

Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by an injection of LPS or lipoteichoic acid. In this model, LPS may directly activate the vascular endothelium, macrophages, and other cells. Cellular infiltration and protein extravasation in the anterior part of the eye reaches a maximum 20 to 24 hours after LPS treatment. In the vitreous and retina, cellular infiltration reaches a maximum 48 hours after LPS treatment. Exposure to exogenous bacterial toxins, such as LPS, stimulates cellular inflammatory responses and releases factors such as nitric oxide (NO) and prostaglandin (PG)-E2, cytokines, including tumor necrosis factor (TNF)-α, and eicosanoid mediators, which promote inflammatory responses. In particular, as suggested by the protective effects afforded by TNF-α-neutralizing antibodies, increased plasma TNF-α levels during endotoxemia and Gram-negative sepsis contributes to lethality.

Three types of nitric oxide synthase (NOS) isoforms have been identified in cells. Endothelium NOS and neural NOS are both constitutive NOS isoforms. The NO produced by constitutive NO acts to maintain normal vasoactivity in an active state of vasodilation through a Ca²⁺-dependent pathway and acts as a neurotransmitter in neuron signal transmission. NOS in macrophages and hepatocytes is an inducible (i)NOS isof orm, and its activation is Ca²⁺ independent. After exposure to...
endogenous and exogenous stimulators, iNOS is induced quantitatively in various cells, such as macrophages, smooth muscle cells, and hepatocytes, and triggers several disadvantageous cellular responses that can cause inflammation and stroke.26 The level of NO production induced by iNOS may reflect the degree of inflammation, and therefore we might be able to evaluate the effect of an anti-inflammatory drug by measuring NO levels. Recently, Ilieva et al.27 reported that Ginkgo biloba extract prevents the inflammation of EIU by suppressing generation of NO.

Arachidonic acid metabolites, such as PGE2, thromboxane B2, and leukotriene B4, have been implicated as major inflammatory mediators. Inhibition of cyclooxygenase (COX), and therefore prostaglandin production, is the common mechanism of action of nonsteroidal anti-inflammatory drugs.28 PGE2 is a major COX product at inflammatory sites, where it contributes to local blood flow increases, edema, and pain sensitization. As is now well appreciated, COX exists in two isoforms.29–31 In general terms, COX-1 is constitutive and is found in such places as the endothelium, stomach, and kidney, whereas COX-2 is induced by proinflammatory cytokines, by endotoxins in cells in vitro, and at inflammatory sites in vivo.

In the present study, we investigated the influence of ACE on EIU in rats. The anti-inflammatory potency of ACE in vivo was compared with that of prednisolone. In addition, to clarify the anti-inflammatory effect, we also investigated the expression of iNOS and COX-2 in a mouse macrophage cell line (RAW 264.7) treated with ACE in vitro.

**MATERIALS AND METHODS**

Quercetin was purchased from Wako Pure Chemical Industries Co., Ltd. (Tokyo, Japan). Anthocyanin (cyanidin-3-glucoside) was purchased from Funacoshi Co., Ltd. (Osaka, Japan).

**Preparation of ACE**

Aronia fruit (597.3 g), harvested in Hokkaido, Japan, was homogenized with 50% (wt/wt) ethanol. The homogenized sample was filtered under reduced pressure and extracted again with an equal volume of 50% (wt/wt) ethanol. The filtrate of the extract was concentrated and dried with a vacuum and a freeze-dryer, respectively, and 47.2 g of dried extract was obtained.

The composition of the extract was as follows: (1) sugars (54.2%): sorbitol 22.9%, glucose 14.5%, fructose 16.8%; (2) organic acid (18.0%): citric acid 2.4%, malic acid 14.8%, unkonomet 0.8%; (3) polyphenols (16.9%): anthocyanin (cyanidin-3-glucoside, cyaniding-3,5-diglucoside) 13.5%, quercetin 1.4%, unknown 2.0%; and (4) soluble fiber (10.8%).

**Animal Groups and EIU**

Eight-week-old male Lewis rats, weighing 180 to 220 g were used. EIU was induced by a footpad injection of 200 μg LPS (100 μg each footpad) from *Salmonella typhimurium* (Sigma-Aldrich, St. Louis, MO) that had been diluted in 0.1 mL phosphate-buffered saline (PBS, pH 7.4).

Rats were injected intravenously with 1, 10, or 100 mg ACE or 10 mg prednisolone diluted in 0.1 mL PBS containing 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Intravenous injections were administered immediately after LPS stimulation. It has been reported that quercetin52–55 and anthocyanin,54–56 which are contained in ACE, also have an anti-oxidizing effect. To compare the anti-inflammatory effect in the ACE-treated groups with that in the groups treated with the individual compounds, we used the same concentrations of anthocyanin and quercetin as is found in ACE, and the same experimental protocol as that for the ACE groups. For the LPS and the control groups, we used the same schedule as for the ACE groups, but with only PBS containing 0.1% DMSO administered intravenously.

Rats were killed, and the aqueous humor (15–20 μL/rat) was collected from both eyes by an anterior chamber puncture with a 30-gauge needle 24 hours after the LPS injection. Animals were handled and cared for according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Anterior Chamber Cell Count and Protein Concentration**

For cell counting, the aqueous humor sample was suspended in an equal amount of Türk stain solution, and the cells were counted with a hemocytometer under a light microscope. The number of cells per field (an 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) was used to determine the total protein concentration in the aqueous humor. The 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.

**Cell Culture and LPS Stimulation**

The RAW 264.7 mouse macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and 10% heat-inactivated fetal bovine serum (Invitrogen-Gibco, Grand Island, NY) and maintained at 37°C in a humidified incubator containing 5% CO2. RAW 264.7 cells were seeded onto a 24-well plate (5 × 104 cells/well) for the experiments. The cells treated with 1, 10, or 100 μg/mL ACE for 24 hours were stimulated with 10 μg/mL of LPS from *Salmonella typhimurium* for 24 hours, unless otherwise stated.

ACE was dissolved in 0.01% DMSO. For the control group, RAW cells were cultured with 0.01% DMSO alone.

**Determination of Nitrite Concentration in the Aqueous Humor**

NO was measured as its end product, nitrite, by using Griess reagent, as described elsewhere (Sigma-Aldrich).37 The culture supernatant (100 μL) was mixed with 100 μL of Griess reagent for 10 minutes, and the absorbance at 550 nm was measured in a microplate reader. The concentration of nitrite in the samples was determined from a sodium nitrate standard curve. The data represent the mean of eight determinations ± SD.

**Levels of TNF-α, PGE2, and Monocyte Chemoattractant Protein-1 in the Aqueous Humor**

We made slight modifications to the method of Ohta et al.38 The aqueous humor was collected and accurately diluted 10-fold with PBS (pH 7.4). ELISA kits (R&D Systems, Minneapolis, MN) were used to measure the levels of TNF-α, PGE2, and monocyte chemoattractant protein (MCP)-1 in the aqueous humor, according to the manufacturer’s instructions. The ELISA assay was performed in duplicate.

**Cell Viability**

For determination of cell viability, 50 mg/mL of methylthiazol diphenyl tetrazolium bromide (Sigma-Aldrich) was added to 1 mL of the cell suspension (5.3 × 10^4 cells/mL in 24-well plates) for 24 hours, and the MTT formazan formed was dissolved in acidic 2-propanol. Optical density was measured with a plate reader at 590 nm. The optical density of the MTT formazan formed by untreated cells was taken to be 100%.

**Western Blot Analysis**

Cells were washed with ice-cold PBS and then lysed in cold NP-40 lysis buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/mL each of leupeptin,
aprotinin, and pepstatin) for 10 minutes at 4°C. Plates were then scraped, and crude lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C. Aliquots of the cleared lysates were diluted with 2× SDS sample buffer, and SDS-polyacrylamide gel electrophoresis was performed. Protein expression was analyzed by Western blot analysis by the following standard procedures. The primary antibody (anti-NOS; Upstate Biotechnology, Lake Placid, NY) was developed with horse-radish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) and visualized by chemiluminescence (Amersham Biosciences).

**Effects of Different Batches of ACE on EIU**

To determine the reproducibility of the measured biological activity of different batches of the extract, we subjected two different batches to the same extraction methods and determined their effects on EIU determined with the same experimental schedule and methods.

**Statistical Analysis**

Data are expressed as the mean ± SD and were analyzed by analysis of variance (ANOVA). The Tukey-Kramer test was used to compare the two treatment groups. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Effect of ACE on EIU**

In the LPS group, the number of inflammatory cells that infiltrated the aqueous humor 24 hours after LPS treatment was 25.7 ± 7.1 × 10^3 cells/mL (mean ± SD, n = 8). The number of inflammatory cells in the groups treated with ACE was significantly lower than that in the LPS group, and the decrease was dose-dependent (Fig. 1A). The effect of the 100-mg dose of ACE on the number of cells in the aqueous humor was almost the same as that observed with the 10-mg prednisolone dose (Fig. 1A). Treatment with either quercetin or anthocyanin resulted in a mild reduction in the cell count. The effect of the 1-mg dose of ACE on the aqueous humor cell count was almost the same as that in the 100-mg quercetin or 100-mg anthocyanin groups. No infiltrating cells were detected in the aqueous humor from the control group (rats without LPS).

The protein concentration (Fig. 1B)—that is, the levels of NO (Fig. 1C), TNF-α (Fig. 1D), and PGE2 (Fig. 1E)—in the groups treated with ACE was significantly lower than that observed in the LPS group. The reduction in these parameters in the 100-mg ACE group was almost the same as that in the prednisolone group (Figs. 1B–E). The effect of the 1-mg dose of ACE on the aqueous humor cell count was almost the same as that in the 100-mg quercetin or 100-mg anthocyanin groups (Figs. 1B–D).

No MCP-1 was detected in the control group of rats, whereas in the LPS group, the MCP-1 level was 8.2 ± 1.7 pg/mL. The 100-mg ACE and prednisolone groups were found to have significantly reduced levels of MCP-1 in the aqueous humor (Fig. 1F). This influence on the MCP-1 concentrations was not seen during the quercetin and anthocyanin treatments in EIU.

**Effects of Different Batches of ACE on EIU**

When compared with the LPS group, the number of inflammatory cells in the aqueous humor in the ACE-treated group significantly decreased in a dose-dependent manner (Fig. 2, Batches A and B). In addition, in the ACE-treated group, protein concentrations and levels of NO, TNF-α, and PGE2 in the aqueous humor significantly decreased in a dose-dependent manner (data not shown).

**Expression of the iNOS and COX-2 Proteins**

To clarify the inhibitory action of ACE on LPS-induced NO and PGE2, the expression of the iNOS and COX-2 proteins was examined with an immunoblot method with anti-iNOS antibody and anti-COX-2. Expression of the iNOS protein was strongly detected in LPS-stimulated RAW cells (Fig. 3A, lane 2). Expression of the iNOS protein significantly decreased in a dose-dependent manner within the concentration range from 1 to 100 μg/mL ACE (Fig. 3A, lanes 3–5).

Although expression of COX-2 was as a 72-kDa protein was detected in normal cells (Fig. 3B, lane 1), there was strong expression in LPS-stimulated cells (Fig. 3B, lane 2). Expression of the COX-2 protein decreased in a dose-dependent manner within the concentration range from 1 to 100 μg/mL of ACE (Fig. 3B, lanes 3–5).

ACE did not decrease cell viability in RAW 264.7 cells when these cells were incubated for 24 hours with 100 μg/mL ACE alone (data not shown).

**DISCUSSION**

The results of this study indicate that ACE suppresses the development of EIU in a dose-dependent manner. In particular, the anti-ocular-inflammatory effect of 100 mg ACE was as strong as that observed with a 10-mg dose of prednisolone. The anti-ocular inflammation effect of ACE was stronger than that of quercetin or anthocyanin.

Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease. Ingestion of alcohol-free red wine or a phenolic compound mixture extracted from red wine has been shown to improve the antioxidant status of plasma in humans. Con'sumption of controlled diets high in fruits and vegetables has also been shown to increase the antioxidant capacity of plasma significantly, which cannot be explained by an increase in the plasma α-tocopherol concentration. Moreover, epidemiologic studies have found that there is a significant negative association between the intake of fruits and vegetables and heart disease mortality. Previous research has determined the antioxidant effects of some flavonoid compounds and has attempted to define the structural characteristics that contribute to scavenging activity. The phenolic acids present in berries are hydroxylated derivatives of benzoic acid and cinnamic acid.

Oxidative stress is known to be a major factor in triggering local inflammation and tissue damage during the inflammatory process, and ACE's antioxidant properties have been proposed to underlie its beneficial effects on inflammation. It is known that NO production, induced by bacterial LPS or cytokines, plays an important role in endotoxemia and inflammatory conditions. In a study we performed, our results suggested that carotenoid astaxanthin prevents the inflammation of EIU by suppressing NO generation. In addition, it has been reported that both quercetin and anthocyanin, which are contained in ACE, have antioxidant effects. Subsequently, quercetin and anthocyanin were shown to have an anti-inflammatory effect. However, the anti-ocular-inflammatory effect of ACE was found to be stronger than that of quercetin or anthocyanin administered individually. This suggests that the anti-ocular-inflammatory effect of ACE is not attributable to only one factor but is due to a complex series of anti-inflammatory factors within ACE.

To elucidate the anti-inflammatory mechanism of ACE, we focused our attention on ACE's antioxidant activity and measured the concentration of NO in the aqueous humor in vivo. Our results showed that ACE suppressed NO production in the aqueous humor and the expression of the iNOS enzyme in a
These results are in agreement with those of our in vivo experiment. In addition, our results indicated that the extract, even at a concentration of 100 μg/mL, did not change cell viability. Therefore, the inhibition of LPS-induced NO production by ACE is not the result of ACE's cytotoxicity in the cells.

Large amounts of NO production induced by bacterial LPS or cytokines have been reported to play a central role in endotoxemia and inflammatory conditions. Indeed, iNOS was localized in epithelial cells in the iris-ciliary body during the early stages of EIU. In human uveitis, increased NO levels have been detected in the aqueous humor in patients with Behçet's disease. Mandai et al. reported that suppression of iNOS activity by an iNOS inhibitor, N⁶-nitro-l-arginine, inhibits development of EIU. Else-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933230/)
where, Goureau et al. demonstrated the possibility of modulating this inflammatory disease by intraperitoneal injection of the iNOS inhibitor \textsuperscript{N}\textsuperscript{\texttrademark}-nitro-L-arginine methylester. In this study, ACE inhibited the development of EIU and suppressed LPS-induced iNOS expression in a dose-dependent manner. Therefore, the present results support their hypothesis.

TNF-\(\alpha\) is a pleiotropic cytokine produced principally by activated macrophages and monocytes, and it also has an important role in nonspecific resistance to various infectious agents. The results of the present study indicate that ACE can decrease the TNF-\(\alpha\) concentration in a dose-dependent manner in vivo. The level of the inhibition of TNF-\(\alpha\) corresponds to the decrease in NO production observed separately with the administration of ACE. Zhang et al. have reported that EIU in mice is exacerbated by decreasing levels of circulating proinflammatory cytokines, in particular TNF-\(\alpha\). In addition, Kasner et al. suggested that TNF-\(\alpha\) is not directly involved in the pathogenesis of EIU in mice and may protect against inflammatory processes in EIU. However, in human uveitis, increased blood levels of TNF-\(\alpha\) have been detected, particularly in patients with Behçet’s disease with uveoretinitis. Ohno et al. have reported that administering anti-TNF-\(\alpha\) chimeric monoclonal antibodies (infliximab) to patients with Behçet’s disease suppressed the frequency of ocular episodes. In the present study on EIU in rats, LPS significantly increased TNF-\(\alpha\) levels in the aqueous humor, and numerous studies have documented that LPS increases TNF-\(\alpha\) levels in the aqueous humor of rats with EIU. The different effects of TNF-\(\alpha\) on EIU may be due to differences in species (rat and mouse) or animal strain. However, the details have not been clarified and therefore should be further investigated.

The mechanism of the NO suppression of TNF synthesis is not known. A potential link may be PGE2. It has been reported that NO activates COX enzymes and thereby leads to a marked increase in PGE2 production. The suppressive effect of PGE2 on TNF synthesis through elevated AMP levels has been convincingly demonstrated. Our results are in agreement with the previous studies, as we found that ACE suppressed the levels of LPS-induced PGE2 and TNF-\(\alpha\) in a dose-dependent manner in vivo. In addition, our results indicated that the ACE decreased the expression of the COX-2 enzyme in a dose-dependent manner. Chang et al. reported that NF-\(\kappa\)B is involved in the induction of COX-2. The COX-2 gene, as well as those for iNOS and TNF-\(\alpha\), possess binding sites for several transcription factors, including NF-\(\kappa\)B. A possible mechanism for the inhibition of COX-2 expression by ACE is the suppression of NF-\(\kappa\)B activity, which may be enhanced by reactive oxygen species.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933230/)

**Figure 2.** Effect of different batches of ACE on EIU. The aqueous humor was collected 24 hours after LPS treatment. Each value represents the mean \pm SD of results in eight rats. *\(P < 0.05\) and **\(P < 0.01\) versus the LPS group.

The two isozymes of COX are encoded by two separate genes and exhibit distinct cell-specific patterns of expression, regulation, and subcellular localization. COX-2 is primarily responsible for increased PGE2 production during inflammation, and PGE2 is generally considered to be a proinflammatory agent. However, studies show that COX-2 may play an anti-inflammatory role in some situations. One of these studies has indicated that prostanooids may exert beneficial effects on retinal blood perfusion and that they may even act as neuroprotective agents. Recently, Shiratori et al. have suggested that \(\alpha\)-melanocyte-stimulating hormone, a neuroimmunomodulator, has an antioocular inflammatory effect by blocking COX-2 expression. In the results of this study, ACE suppressed the development of EIU and decreased LPS-induced COX-2 expression in vitro in a dose-dependent manner. Therefore, our findings agreed with the results of previous studies and suggested that blocking of COX-2 protein expression is one of the anti-inflammatory mechanisms of ACE.

The safety of ACE has not been thoroughly investigated. However, when the doses of extract used in the present study were administered intravenously, there were no marked changes in rat behavior or food and water consumption.

In summary, this study indicates that ACE has a dose-dependent anti-ocular-inflammatory effect on EIU. In particular, the effect of 100 mg ACE was as strong as that of 10 mg prednisolone. Possible mechanisms for this effect of ACE include suppression of NO, PGE2, and TNF-\(\alpha\) production through the direct inhibition of the expression of the iNOS and COX-2 enzymes. These findings suggest that ACE may be a promising agent for the treatment of ocular inflammation.

**References**


